Journal of Chemical Ecology, Vol. 17, No. 1, 1991

CHEMOTYPES OF *Cyperus rotundus* IN PACIFIC RIM AND BASIN: DISTRIBUTION AND INHIBITORY ACTIVITIES OF THEIR ESSENTIAL OILS

KOICHIRO KOMAI,[1,2] CHUNG-SHIH TANG,[1,*] and ROY K. NISHIMOTO[3]

[1]*Department of Agricultural Biochemistry*
[3]*Department of Horticulture*
University of Hawaii
Honolulu, Hawaii 96822

(Received March 28, 1990; accepted August 6, 1990)

Abstract—Four major chemotypes of *Cyperus rotundus* L. (purple nutsedge) have been reported based on the composition of essential oils in mature tubers. Distribution of the H, M, K, and O type in countries of the Pacific Rim and Basin was investigated. In general, the H type dominates on the islands of Japan, and the O type has the widest range of distribution. The O type also dominates the Pacific Basin islands except for Hawaii, where the K-type is dominant. Inhibitory activity of the essential oils from *C. rotundus* tubers against the seedling growth of lettuce and oats was in the order of H > M > K > O. Seven major sesquiterpenes were isolated from the oils and their inhibitory activities determined. Results suggest that *C. rotundus* of different chemotypes may have different allelopathic activity in the crop–weed interaction.

Key Words—*Cyperus rotundus*, purple nutsedge, weeds, allelopathy, chemotype, essential oil, sesquiterpene.

INTRODUCTION

The geographical variation of *Cyperus rotundus* L. (purple nutsedge) has been studied by Komai et al. (1977) based on the sesquiterpene composition of essential oils from the mature tubers. Three chemotypes, i.e., H, M, and O type

*To whom correspondence should be addressed.
[2]On leave from the Department of Agricultural Biochemistry. Current address: Faculty of Agriculture, Kinki University, Nara, Japan.

0098-0331/91/0100-0001$06.50/0

were found in Asia (Komai and Ueki, 1981). Recently, we reported the presence of the K type, a new chemotype in the Hawaiian Islands (Komai and Tang, 1989). Major sesquiterpenes in each essential oil (%) are as follows:

H type: α-cyperone (38.6); β-selinene (18.5), and cyperol (7.4)
M type: α-cyperone (30.7), cyperotundone (19.4), β-selinene (17.8), cyperene (7.2), and cyperol (5.6)
K type: cyperene (28.7), cyperotundone (8.8), petchoulenyl acetate (8.0), and sugeonyl acetate (6.9).
O type: cyprene (30.8), and cyperotundone (13.1)

To obtain a more complete picture of the geographical distribution of these four chemotypes, additional clones were collected from countries and islands of the Pacific Rim and Basin and their chemotypes determined according to the established method (Komai and Tang, 1989). Inhibitory activities of the four essential oils against lettuce and oat seedling growth were compared. Seven major sesquiterpenes were isolated and their inhibitory activities determined. Results suggest that *C. rotundus* of different chemotypes may have different allelopathic activity against crops. The significance of these findings is discussed.

METHODS AND MATERIALS

Sample Preparation. Mature tubers of *Cyperus rotundus* L. were collected from various regions in the Pacific region countries. A total of 254 clones were obtained (Table 1). Essential oils were prepared from each sample by *n*-hexane extraction as soon as the sample was obtained (Komai and Tang, 1989). Prior experiments showed that the sesquiterpene composition was consistent in essential oils of the tubers within a given chemotype (Komai et al., 1977; Komai and Tang, 1989).

Individual sesquiterpenes were prepared by repeated silica gel column chromatography from the K-type tubers collected from Oahu, Hawaii, and M type from the Miyazaki Prefecture in Japan. Seven compounds, including α-cyperone, cyperotundone, cyperol, β-selinene, cyperene, sugeonol acetate, and patchoulene acetate were isolated and purified for bioassay.

Instrumental Analyses. GC of the essential oils was performed on an SP-7100 Gas Chromatograph (Spectral Physics) equipped with a flame ionization detector and a 20-m × 0.25-mm-ID, DB-5 fused silica capillary column (J&W Scientific). The oven temperature was programmed from 90 to 250°C at 4°C/min. For GC-MS analysis an HP-5730 gas chromatograph–mass spectrometer was used. Conditions for GC of the GC-MS were similar to those of the GC analysis. For the MS, 70 eV and 300 mA electron energy were used. The ion-

TABLE 1. DISTRIBUTION OF CHEMOTYPES OF *Cyperus rotundu* IN PACIFIC RIM AND BASIN

Chemotype	Distribution: country, region, or island (No. of clones analyzed)	
H type	Japan	Honshu (44); Shikoku (15); Kuyshu (17); Tanegashima Island (11); Yakushima Island (1); Amamioshima Island (12); Okinawa Island (6)
	U.S.A.	Black Sand Beach on the Island of Hawaii (5)
M type	Japan	Kuyshu (5); Tanegashima Island (2); Amamioshima Island (2); Okinawa (9)
	Taiwan (4)	
	S. China*	
	Hong Kong*	
	Vietnam*	
O type	Japan	Amamioshima Island (4); Okinawa (23)
	Taiwan (7)	
	Philippines (13)	
	Thailand (14)	
	Indonesia	N. Sumatera (1); S. Sumatera (1); E. Java (1); W. Java (1); S. Sulawesi (1)
	U.S.A.	Guam (3); Hawaii Island (1); Kauai Island (2); Maui Island (2); Oahu Island (3); Arizona (1); Mississippi (1); Alabama (1); S. California (4)
	Samoa (1)	
	Saipan (1)	
	Palau (1)	
	Cook Island (1)	
	Australia (2)	
	Tonga (4)	
K type	U.S.A.	S. California (1); Hawaii Island (3); Kauai Island (5); Maui Island (7); Oahu Island (9)
	Mexico (1)	

*Unpublished data, K. Komai.

source temperature was 200°C. Compounds were identified by comparison of GC retention times and mass spectra with those of the authentic compounds.

Inhibitory Activities. Inhibition of the lettuce (*Lactuca sativa* L. Anuenue) seedling growth was performed by transferring 20 pregerminated seeds to a Petri dish (diam. 6 cm) containing a Whatman No. 1 filter paper treated with the *n*-hexane extract of each chemotype (Table 2). For sesquiterpenes, 0.5, 1, or 5 mmol of the individual compound in hexane were applied (Table 3). Two milliliters of H_2O were added to moisten the filter paper after the solvent was evaporated. Hypocotyl and radicle length were measured four days after incu-

TABLE 2. EFFECTS OF ESSENTIAL OILS FROM *C. rotundus* TUBERS ON GROWTH OF LETTUCE SEEDLINGS[a]

	Concentration (ppm)	Chemotype			
		H	M	K	O
A	1000	11.9c	13.9c	15.7bc	15.7bc
	500	13.5c	16.5bc	18.1b	16.9b
	250	14.2c	16.0bc	21.9a	22.4a
B	1000	9.6d	10.2d	12.8c	13.6bc
	500	13.2c	15.8b	13.1bc	15.1b
	250	15.7b	17.0b	16.3b	20.8a

[a]A: radicle length in mm (control = 22.7 mm). B: hypocotyl length in mm (control = 20.3 mm). Means within the plant part (among chemotypes) followed by the same letter are not significantly different at the 0.05 level of probability as determined by Duncan's mean separation.

TABLE 3. EFFECTS OF SESQUITERPENES ISOLATED FROM *C. rotundus* TUBERS ON GROWTH OF LETTUCE SEEDLINGS[a]

	Treatment (mol × 10^4)	Sesquiterpene						
		I	II	III	IV	V	VI	VII
A	50	9.0d	9.8d	9.1d	13.7c	15.1bc	15.6bc	14.6c
	10	11.6cd	10.1d	11.2cd	19.1ab	14.5c	18.4ab	21.9a
	5	16.2bc	17.5b	17.3b	19.7ab	19.3ab	20.6a	20.5ab
B	50	3.4d	5.9cd	5.0d	10.1b	11.7b	8.6c	9.1c
	10	4.3d	7.8c	5.3d	12.6a	10.1b	12.2ab	10.3b
	5	10.4b	12.0b	12.7a	15.0a	14.1a	14.4a	14.3a

[a]A: radicle length in mm (control = 19.2 mm). B: hypocotyl length in mm (control = 15.8 mm). Sesquiterpene: I: α-cyperone; II: cyperotundone; III: cyperol; IV: β-selinene; V: cyperene; VI: sugeonol acetate; VII: patchoulene acetate. Means within the plant part (among sesquiterpenes) followed by the same latter are not significantly different at the 0.05 level of probability as determined by Duncan's mean separation.

bation and the inhibitory activity was expressed as percent growth of those of the control (i.e., with H_2O alone).

Methods for determining inhibition of oat (*Avena sativa* L.) seedlings (Tables 4 and 5) were similar to those of the lettuce except a 9-cm Petri dish was used and 4 ml of H_2O were added to the essential-oil-treated filter paper instead. The lengths of leaf sheath and radicle were measured after four days. All experiments were performed in the dark at 23°C in covered Petri dishes.

TABLE 4. EFFECTS OF ESSENTIAL OILS FROM *C. rotundus* TUBERS ON GROWTH OF OAT SEEDLINGS[a]

	Concentration (ppm)	Chemotype			
		H	M	K	O
A	1000	14.6cd	12.8d	16.6c	18.0c
	500	16.9c	15.8cd	12.9b	21.5b
	250	24.4b	23.5b	28.2a	30.7a
B	1000	8.0d	7.2d	12.6c	13.5bc
	500	8.9cd	11.4c	16.9b	16.8b
	250	17.2b	16.2b	23.2a	24.5a

[a] A: radicle length in mm (control = 30.5 mm). B: leaf sheath length in mm (control = 23.7 mm). Means within the plant part (among chemotypes) followed by the same letter are not significantly different at the 0.05 level of probability as determined by Duncan's mean separation.

TABLE 5. EFFECTS OF SESQUITERPENES ISOLATED FROM *C. rotundus* TUBERS ON GROWTH OF OAT SEEDLINGS[a]

	Treatment (mol × 10^4)	Sesquiterpene						
		I	II	III	IV	V	VI	VII
A	50	11.7d	14.2cd	13.9d	22.0d	22.0b	23.9b	21.7b
	10	17.4c	17.5c	20.1c	24.2b	24.2b	27.3ab	24.0b
	5	23.1b	24.6b	24.4b	30.0a	30.5a	31.9a	29.2a
B	50	7.4d	10.5cd	9.6cd	15.3b	13.7a	15.0b	14.3c
	10	8.1d	11.8c	11.7c	20.2b	19.5b	19.1b	19.0b
	5	16.8b	15.9b	17.7b	26.1a	24.9a	22.0a	21.9a

[a] A: radicle length in mm (control = 30.5 mm). B: leaf sheath length in mm (control = 23.7 mm). Sesquiterpene: I: α-cyperone; II: cyperotundone; III: cyperol; IV: β-selinene; V: cyperene; VI: sugeonol acetate; VII: patchoulene acetate. Means within the plant part (among sesquiterpenes) followed by the same letter are not significantly different at the 0.05 level of probability as determined by Duncan's mean separation.

RESULTS AND DISCUSSION

Distribution of Purple Nutsedge Chemotypes in Pacific Rim and Basin. Table 1 shows that of the 254 clones analyzed, the O type has the broadest distribution, ranging from the Asian to the American mainland. Although in most cases the sample size was too small to draw any conclusions, it nevertheless showed that the O type was the exclusive chemotype collected from Aus-

tralia, Indonesia, and other Pacific islands including Samoa, Saipan, Republic of Palau, Guam, Cook Island, and Tonga. The O type was also the only chemotype of clones collected from Arizona, Mississippi, and Alabama. The Hawaiian islands are an interesting exception, where the K type dominates the *C. rotundus* population. In addition, O-type clones found in Hawaii are higher in cyperotundone and lower cyperene content compared to those found elsewhere; it may be designated as the Hawaiian O type.

In a previous publication, we speculated that the K type might have evolved in the Hawaiian islands (Komai and Tang, 1989). In the present experiment, one K-type and four O-type clones were determined from a southern California collection, and the only clone collected from Medellin, Mexico, was also a K type. Thus, it is also possible that the K type has a central American origin. More data on clones from the American mainland are required to clarify this.

Of the eight clones collected from different locations in Indonesia, three had a GC profile unlike those of *C. rotundus*. A comparison of the GC profiles showed that they were *Cyperus tuberosus* (Komai, unpublished) and were misidentified as *C. rotundus*. This is hardly surprising since these two species are morphologically quite similar. The fact that GC profiles were able to differentiate the two suggests the usefulness of the present technique in the identification of *Cyperus* species, especially for those with little morphological distinction (Backer and Bakhuizen van der Brink, Jr., 1968).

The H type was found only in Japan in an earlier study (Komai and Ueki, 1981) and also represents ca. 2/3 of the Japanese *C. rotundus* population. The unexpected presence of the H type in the Black Sand Beach area on the island of Hawaii leads to the speculation that the localized distribution of H type in this tourist resort has been a recent introduction.

Inhibitory Activity of Essential Oils. The growth inhibitory activity on lettuce and oat seedlings by essential oils prepared from the four chemotypes of *C. rotundus* are shown in Table 2 and 4, respectively. The overall potency of inhibition was in the order of H > M > K > O type, although the difference between H and M type on oats was neglegible. At the lowest concentration tested (i.e., 250 ppm of essential oil in aqueous suspension), the growth of lettuce seedlings (radicle plus hypocotyl elongation) was reduced by 30, 23, and 11% of the control in H, M, and K type, correspondingly. The O type slightly enhanced lettuce seedling growth at 250 ppm, although it is not significantly different from the control at 5% level. At 500 and 1000 ppm, the O and K type were similar in inhibitory activities. Results on oat seedlings showed that at 250 ppm, the growth was reduced to 24, 28, and 5% by H, M, and K type, respectively. A slight enhancement was again observed in the O type at 250 ppm, and similar activities were recorded in the O and K type at 500 and 1000 ppm.

Inhibitory Activity of Individual Sesquiterpenes. Activities of the major

sesquiterpenes isolated from the nutsedge tubers on lettuce and oat seedling growth are shown in Table 3 and 5, respectively. Sesquiterpenes with ketone (i.e., α-cyperone and cyperotundone) or hydroxyl (i.e., cyperol) groups were more inhibitory than the acetates and hydrocarbons. For example, α-cyperone at the 5 mmol level was nearly twice as inhibitory as β-selinene, cyperene, sugeonol acetate, or pachoulene acetate in both lettuce and oats. Based on our previous findings (Komai and Tang, 1989), approximately half of the sesquiterpenes in the H and M type *C. rotundus* are ketones and alcohols, while the O and K type contain only ca. 10% ketones and no alcohols. These data correspond to the observed differences in inhibitory activities.

C. rotundus has been considered as the most serious weed in cultivated land in tropical and subtropical regions (Holm, 1971). In recent years, increasing evidence has supported the role of allelopathy in weed–crop interactions (Putnam and Tang, 1986). It is likely that in *C. rotundus*, with its relatively high sesquiterpene content, allelopathy also contributes to the interference on crop plants. Based on our results, it may be theorized that O and K type *C. rotundus* would be allelopathically less damaging to crop productivity than the H and M type.

While additional laboratory and field experiments are necessary to verify this concept, it is true that, by definition, the chemotype within species of a donor plant shall determine the chemical impact on its environment, which would lead to different levels of allelopathic activity. A recent study (Siriwardana, 1986) in Hawaii showed that the yield of six crops of fresh bean pods were not significantly reduced by *C. rotundus* infestation ranging from 390 to 1100 shoots/m^2 at three weeks. These results differ from those of William and Warren (1975), who reported an average of 41% yield reduction over two bean crops with similar *C. rotundus* densities. Both studies utilized similar row and plant spacing. It is possible that among many other variables in comparing two separate sets of experiments, chemotype played a role in the discrepancies observed.

Acknowledgments—The authors are grateful to the numerous collaborators in the countries of the Pacific Rim and Basin who provided samples in this study. Supported in part by the U.S. Department of Agriculture under CSRS Special Grant 90-34135-5184, managed by the Pacific Basin Advisory Group (PBAG). Journal Series 3470 of Hawaiian Institute of Tropical Agriculture and Human Resources.

REFERENCES

BACKER, C.A., and BAKHUIZEN VAN DER BRINK, JR., R.C. 1968. *Cyperus* L. in pp. 468–481, Flora of Java Vol. 3. Published under the auspices of the Rijksherbrium, Leyden, The Netherlands.

HOLM, L. 1971. The role of weeds in human affairs. *Weed Sci.* 19:485–490.

KOMAI, K., and TANG, C.S. 1989. A chemotype of *Cyperus rotundus* in Hawaii. *Phytochemistry* 28:1883–1886.

KOMAI, K., and UEKI, K. 1981. Geographical variation of essential oils in tubers of purple nutsedge. Pages 387–389. *in* Proceedings, 8th Asian Pacific Weed Science Society Conference, Nov. 22–29, 1981, Bangalore, India.

KOMAI, K., IWAMURA, J., and UEKI, K. 1977. Isolation, identification and physiological activities of sesquiterpenes in purple nutsedge. *Weed Res. (Japan)* 22:14.

PUTNAM, A., and TANG, C.S. (eds.) 1986. Allelopathy: State of the science, pp. 1–19, *in* A. Putnam and C.S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

SIRIWARDANA, T.D. 1986. Low rates of glyphosate for management of *Cyperus rotundus* L., PhD dissertation. University of Hawaii. 118 pp.

WILLIAM, R.D., and WARREN, G.F. 1975. Competition between purple nutsedge and vegetables. *Weed Sci.* 23:317–323.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

2,2′-OXO-1,1′-AZOBENZENE: SELECTIVE TOXICITY OF RYE (*Secale cereale* L.) ALLELOCHEMICALS TO WEED AND CROP SPECIES: II

WILLIAM R. CHASE, MURALEEDHARAN G. NAIR,* and ALAN R. PUTNAM[1]

Natural Products Chemistry Laboratory
Department of Horticulture
Michigan State University
East Lansing, Michigan 48824

(Received June 21, 1990; accepted August 9, 1990)

Abstract—Three allelochemicals from rye or its breakdown products were evaluated for activity on garden cress (*Lepidum sativum* L.), barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.], cucumber (*Cucumis sativus* L.), and snap bean (*Phaseolus vulgaris* L.). 2,4-Dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA), 2(3H)-benzoxazolinone (BOA), and 2,2′-oxo-1,1′-azobenzene (AZOB) were all applied singly at 50, 100, and 200 ppm and in two- and three-way combinations each at 50 and 100 ppm. AZOB at 100 and 200 ppm produced 38–49% more inhibition than DIBOA, while combinations of BOA/DIBOA, which contained AZOB at 100 ppm had 54–90% more inhibition when compared to DIBOA/BOA combinations. All combinations were slightly antagonistic to barnyardgrass, while several combinations caused a synergistic response to garden cress germination and growth. Cucumbers and snap beans exhibited both types of responses, depending on the allelochemical combination and application rate. The plant-produced benzoxazinones were more inhibitory to crops than weeds. Therefore, improved herbicidal selectivity would be expected if there were rapid transformation of the benzoxazinones to the microbially produced AZOB.

Key Words—Allelopathy, 2,4-dihydroxy-1,4(2H)-benzoxazine-3-one, DIBOA, 2(3H)-benzoxazolinone, BOA, 2,2′-oxo-1,1′-azobenzene, AZOB, synergism, antagonism.

*To whom correspondence should be addressed.
[1]Present address: 49823 Gallatin Road, Gallatin Gateway, Montana 59730.

0098-0331/91/0100-0009$06.50/0

INTRODUCTION

Rye (*Secale cereale* L.) is an annual cereal grain that has shown potential as a cover crop in a variety of cropping systems (Putnam and Defrank, 1983; Barnes and Putnam, 1986) because it has been found to reduce weed biomass, improve soil aggregation, reduce soil erosion, and enhance water penetration and retention (Blevins et al., 1971; Young, 1982). With the renewed interest in lowering energy inputs, reduced-tillage systems employing cover crops will undoubtedly be an important tool of the future. Maintenance of residues on the soil surface will be inherent to these reduced-tillage systems. Residues can interact with weeds or crops to potentially reduce the growth of the plants. This interference can arise from competition for essential plant growth requirements such as light, water, and nutrients (Knavel et al., 1977; Teasdale and Frank, 1983; Mascianica et al., 1986; Skarphol et al., 1987; Glenn and Welker, 1989) or from allelopathic chemicals released into the environment from decaying plant residues (Shilling et al., 1985; Barnes et al., 1987; Nair et al., 1990).

Although there have been numerous studies on toxicity of rye residues (Kimber, 1973; Chou and Patrick, 1976; Putnam and Defrank, 1983; Shilling et al., 1985; Barnes and Putnam, 1986; Barnes et al., 1987), none has offered a complete explanation of interference reported by these plant residues. There have been several attempts to isolate and identify the many compounds within rye showing biological activity. Virtanen et al. (1957) isolated a cyclic hydroxamic acid, 2(3H)-benzoxazoline (BOA), from 4-day-old rye seedlings and reported BOA as being responsible for the chemical resistance of rye to *Fusarium* infection. Tang et al. (1975) further quantified BOA present in rye and found eight times more BOA in the shoots than in the roots. Chou and Patrick (1976) identified nine compounds from decaying rye tissue that were inhibitory to lettuce seed germination. Zungica et al. (1983) evaluated rye for two hydroxamic acids, 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) and BOA, and found them commonly occurring in both cultivated and wild accessions. Shilling et al. (1985) isolated phenyllactic acid (PLA) and hydroxybutyric acid (HBA) from field-grown rye and found each allelochemical inhibited redroot pigweed (*Amaranthus retroflexus* L.), common lambsquarters (*Chenopodium album* L.), and common ragweed (*Ambrosia artemisiifolia* L.) growth at concentrations of 2 mM. Barnes et al. (1987) compared the activity of four rye compounds, PLA, HBA, DIBOA, and BOA, and reported DIBOA and BOA to be the most toxic to large crabgrass [*Digitaria sanguinalis* (L.) Scop.], barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.], proso millet (*Panicum miliaceum* L.), redroot pigweed, tomato (*Lycopersicon esculentum* Mill.), and lettuce (*Lactuca sativa* L.) root growth at concentrations of 67–250 ppm.

Nair et al. (1990) and Chase et al. (unpublished results) isolated a microbially produced 2,2′-oxo-1,1′-azobenzone (AZOB) from soil enriched with

BOA and showed that DIBOA, BOA, and AZOB were inhibitory to barnyardgrass and garden cress (*Lepidum sativum* L.) at concentrations of 67–250 ppm. In these tests, AZOB was more biologically active than BOA and DIBOA.

Although there have been numerous compounds isolated and identified from rye tissue, it is difficult to predict the active concentration of any one compound at any one time as it occurs in nature. It is possible that they might occur either alone or in numerous combinations under various environmental and plant conditions. Since the hydroxamic acids, BOA and DIBOA, and their AZOB breakdown product exhibited the most activity, the objective of this study was to evaluate these three known rye allelochemicals singly and in combinations for their activity on various weeds, cucumbers and snap beans.

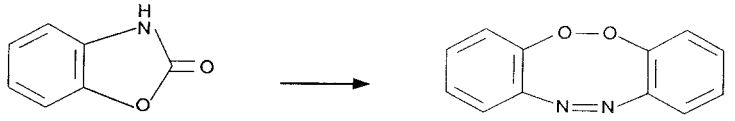
METHODS AND MATERIALS

Spray Reagents for TLC Detection. DIBOA (Figure 1) was detected on thin-layer plates with ferric chloride ($FeCl_3$) spray reactant consisting of 5% $FeCl_3$ in 95% ethanol, acidified with concentrated HCl. A spray reagent consisting of 1% ceric sulfate ($CeSO_4$) in concentrated H_2SO_4 was used to detect BOA (Figure 1).

Source of Allelochemicals. BOA was obtained from Aldrich Chemical company and AZOB was produced from BOA, using the procedure of Nair et al. (1990) (Scheme 1).

Extraction of DIBOA. Seedlings of rye (Wheeler, 11 days old, 2.2 kg) were homogenized in a Waring blender with distilled water (4.5 liters), and kept at room temperature (1 hr) to ensure enzymatic hydrolysis of the DIBOA glucoside to DIBOA. After filtering through cheesecloth, the filtrate was heated in a water bath until the temperature of the extract reached 70°C. It then was cooled immediately in an ice bath. Coagulated components were removed by vacuum filtration, using Whatman No. 1 filter paper. Filtrate was then lyophilized at 5°C and stored at −20°C until further separation.

Lyophilized rye extract (121.91 g) was stirred with HCl in MeOH (1.5 liters, pH 1.0) for 2 hr at room temperature. The mixture then was filtered through a sintered glass filter (fine, 4–5 Å) and the filtrate dried in vacuo. This crude residue was extracted with $CHCl_3$ (3 × 100 ml), and the organic extract



BOA AZOB

SCHEME 1. Transformation of benzoxazolinone to 2,2′-oxo-1,1′-azobenzene.

was evaporated to dryness. The oily residue (35.3 g) was redissolved in $CHCl_3$ and precipitated with hexane (500 ml) and filtered. This procedure was repeated twice and the combined $CHCl_3$–hexane extract was dried in vacuo (12.84 g). A TLC analysis of this crude product confirmed the presence of DIBOA.

Further purification of DIBOA from the above partially purified mixture was carried out by vacuum liquid chromatography. A slurry of column silica (40–60 mesh size, 260 g) in $CHCl_3$–MeOH (4 : 1) was packed under vacuum in a sintered glass filter (medium, 10–15 Å) and washed with $CHCl_3$–MeOH (4 : 1, 300 ml). The partially purified gum containing DIBOA (12.84 g) in $CHCl_3$–MeOH (4 : 1, 30 ml) was applied on the silica under vacuum and eluted with the same solvent system. Four fractions were collected in 250-ml portions and analyzed by TLC. Fractions 2 and 3, positive for DIBOA by $FeCl_3$ spray, were combined and evaporated to dryness (10.94 g). This was purified again by flash column chromatography (silica, $CHCl_3$–MeOH 4 : 1 v/v). The initial fraction (30 ml) was discarded and, upon TLC analysis, fractions 1–21 (each 5 ml) were found to be negative for DIBOA. Fractions 22–29 (each 10 ml), monitored by TLC with $FeCl_3$ spray, were found to contain the largest quantity of DIBOA. Removal of solvent at reduced pressure afforded a brown solid (2.43 g). Final purification of DIBOA was achieved by TLC (silica, $CHCl_3$–MeOH, 4 : 1) and the resulting product recrystallized from $CHCl_3$–MeOH mixtures, and gave pale yellow crystals of DIBOA (187 mg).

Bioassay. Activity of three known compounds associated with rye (DIBOA, BOA, and AZOB) were assayed on two weed indicators (garden cress and barnyardgrass) and two crops [cucumber (*Cucumus sativus* L.) and snap bean (*Phaseolus vulgarus* L.)]. Growth parameters recorded were seed germination and root and shoot lengths. All three compounds were applied alone at 50, 100, and 200 ppm and in two- and in three-way combinations, each applied at 50 and 100 ppm. The experimental design was a randomized complete block design with four replications.

Stock solutions (1 mg/ml methanol) of purified compounds were applied to filter paper (Whatman No. 1) that lined the Petri dishes. Pure methanol also was included as a control. After application of the compounds on the filter paper, the solvent was allowed to evaporate (2–3 hr) in the dark before seed placement. In the snap bean assays, the solvent was allowed to evaporate for an additional 6–8 hr prior to planting.

Weed seeds (15) were uniformly distributed on filter paper (4.25 cm) in Petri dishes (60 × 15 mm), after which distilled water (1.5 ml) was added. Garden cress and barnyardgrass seeds then were incubated for 72 and 96 hr, respectively. Flurry cucumber seeds (5) were uniformly distributed on filter paper (7 cm) in Petri dishes (100 × 15 mm), and then distilled water (2 ml) was added. Cucumbers then were incubated for 120 hr. Bush Blue Lake 47 snap bean seeds (4) were uniformly distributed on seed germination paper (5 ×

5 cm) in Magenta boxes (6 × 6 × 9 cm) after which distilled water (5 ml) was added. A cellulose sponge (3.0 × 3.8 × 6.2 cm) saturated with distilled water was suspended from the sides of the box before attaching the lids. Each container was individually sealed with parafilm (American Can Co.) and incubated for 216 hr.

Incubation of Bioassay. After placing the lids onto the containers, they were randomly placed on trays (36 × 57 cm) lined with damp brown paper toweling. Each tray held one to two blocks and was covered with a clear plastic bag before placement into the incubator at 26°C for the duration of the study.

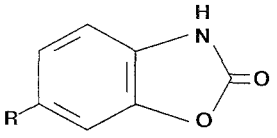
Statistical Methods. Data obtained in bioassays were subjected to analyses of variance and means were compared with the least significant difference (LSD) test. Tables represent the means from duplicate experiments. Analyses for synergism or antagonism of combination treatments were conducted according to Colby's methods (1967).

RESULTS AND DISCUSSION

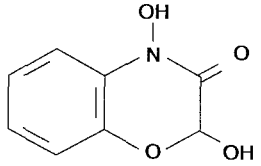
DIBOA Quantification. Since DIBOA was not available commercially, it was necessary to isolate it from field- or greenhouse-grown rye tissue. The modification of DIBOA extraction procedure gave a higher yield of DIBOA than previously reported (Nair et al., 1990). The major difference in the extraction step was the treatment of lyophilized extract with HCl in MeOH. Barnes and Putnam (1986) quantified hydroxamic acids by colorimetric methods and obtained a 27-fold increase of DIBOA in greenhouse-grown rye shoots over field grown. Our yields were lower than Barnes and Putnam (1987) but were fourfold higher than Nair et al. (1990). However, we did not process all the positive DIBOA-containing fractions. This DIBOA along with BOA and AZOB was used for evaluating allelopathic activity of these compounds under field and greenhouse conditions.

Relative Biological Activity of Allelochemicals. Of the three chemicals tested on barnyardgrass, AZOB was the most active, reducing both root and shoot growth at all three application rates (Table 1). Combinations of the compounds containing AZOB showed 41–67% more activity on roots and 20–33% more activity on shoots when compared to combinations of DIBOA/BOA at the same concentrations. The combinations showed slightly less activity when compared to expected values by Colby's methods. This would suggest that these compounds are antagonistic when they are applied together.

For garden cress, all parameters measured were inhibited by the application of AZOB (Tables 2 and 3). Germination was reduced by three- to fourfold by combinations containing AZOB/BOA each at 100 ppm. In addition, AZOB or BOA applied singly showed two- to fourfold more activity on both shoot and



BOA: R = H
MBOA: = OMe



DIBOA

FIG. 1. Benzoxazolinones and benzoxazinones isolated from rye or corn plants.

TABLE 1. BARNYARDGRASS ROOT AND SHOOT RESPONSE TO DIBOA, BOA, AND AZOB APPLIED SINGLY AND IN COMBINATION[a]

	% of control					
	Root			Shoot		
Compound	50 (ppm)	100 (ppm)	200 (ppm)	50 (ppm)	100 (ppm)	200 (ppm)
DIBOA	83	75	60	94	92	76
BOA	101	91	82	86	86	82
AZOB	50	28	38	79	65	79
DIBOA and BOA[b]	90 (84)	73 (68)		100 (81)	89 (79)	
DIBOA and AZOB	46 (42)	24 (21)		80 (71)	60 (60)	
BOA and AZOB	53 (50)	31 (26)		72 (68)	61 (56)	
DIBOA, BOA, and AZOB	52 (42)	26 (19)		67 (84)	61 (51)	
LSD (0.05)		13			14	

[a] ( ) = expected value: $E = D^1 \times B^1/100$. E = expected value, D^1 and B^1 are growth as a percent-of-control of allelochemicals.
[b] All combinations were each applied singly at the given rate.

root growth when compared to DIBOA. Shoot and root growth was severely inhibited with combinations of AZOB/BOA or AZOB alone at 100 ppm. In the above-mentioned combinations, the observed values were always less than the expected, suggesting synergism. The order of activity on garden cress was AZOB > BOA > DIBOA, which is in agreement with Nair et al. (1990).

TABLE 2. GARDEN CRESS GERMINATION RESPONSE TO DIBOA, BOA, AND AZOB APPLIED SINGLY AND IN COMBINATIONS[a]

Compound	Germination (% of control)		
	50 (ppm)	100 (ppm)	200 (ppm)
DIBOA	101	102	102
BOA	106	105	95
AZOB	100	94	97
DIBOA and BOA[b]	107 (107)	104 (107)	
DIBOA and AZOB	93 (101)	71 (96)	
BOA and AZOB	90 (106)	24 (96)	
DIBOA, BOA, and AZOB	92 (107)	17 (101)	
LSD (0.05)		10	

[a] ( ) = expected value calculated by $E = D^1 \times B^1/100$.
[b] All combinations were each applied singly at the given rate.

TABLE 3. GARDEN CRESS ROOT AND SHOOT RESPONSE TO DIBOA, BOA, AND AZOB APPLIED SINGLY AND IN COMBINATIONS[a]

Compound	% of control					
	Root			Shoot		
	50 (ppm)	100 (ppm)	200 (ppm)	50 (ppm)	100 (ppm)	200 (ppm)
DIBOA	77	90	65	100	86	83
BOA	82	60	52	74	61	47
AZOB	74	56	33	56	34	42
DIBOA and BOA[b]	98 (63)	77 (54)		86 (74)	64 (52)	
DIBOA and AZOB	93 (57)	35 (50)		71 (56)	19 (29)	
BOA and AZOB	50 (61)	13 (34)		34 (41)	6 (21)	
DIBOA, BOA, and AZOB	51 (47)	8 (30)		32 (41)	5 (18)	
LSD (0.05)		23			21	

[a] ( ) = expected value calculated by $E = D^1 \times B^1/100$.
[b] All combinations were each applied singly at the given rate.

For cucumbers, DIBOA was more inhibitory to both root and shoot growth than either BOA or AZOB when applied singly (Table 4). When applied singly at 50 ppm, none of the chemicals reduced root or shoot growth, but when concentrations were 100 ppm or greater, there were significant reductions in growth.

TABLE 4. CUCUMBER ROOT AND SHOOT RESPONSE TO DIBOA, BOA, AND AZOB APPLIED SINGLY AND IN COMBINATIONS[a]

	% of control					
	Root			Shoot		
Compound	50 (ppm)	100 (ppm)	200 (ppm)	50 (ppm)	100 (ppm)	200 (ppm)
DIBOA	91	55	31	98	66	30
BOA	87	76	56	83	52	47
AZOB	85	79	82	82	63	76
DIBOA and BOA[b]	53 (79)	39 (42)		41 (81)	43 (34)	
DIBOA and AZOB	76 (77)	51 (43)		86 (80)	61 (42)	
BOA and AZOB	79 (74)	70 (60)		61 (68)	59 (33)	
DIBOA, BOA, and AZOB	65 (67)	47 (53)		61 (67)	44 (21)	
LSD (0.05)		17			32	

[a] ( ) = expected value calculated by $E = D^1 \times B^1/100$.
[b] All combinations were each applied singly at the given rate.

Again, DIBOA, BOA, and all combinations were more active with increasing concentrations. The DIBOA/BOA combinations were 14–44% more inhibitory than the combinations of BOA/AZOB and appeared to be synergistic, while the BOA/AZOB combinations appeared to have antagonistic effects. This suggested that the most toxic compounds to cucumbers were ones found in the rye herbage and not the microbially produced AZOB. If rapid conversion of these compounds did occur, one could expect improved cucumber crop safety in the presence of decomposing plant material.

In the snap bean assay, there was more variation, perhaps due to seed variability or more intricate experimental design. Overall, shoot growth appeared more sensitive than roots, with DIBOA and BOA showing a rate response with increasing concentrations (Table 5). For combinations with 50 ppm of each compound, only slight synergistic activity was seen, while at 100 ppm, antagonism was evident. Applications of BOA at 100 and 200 ppm and combinations of BOA/AZOB showed the greatest inhibition. Both large-seeded dicot crops were more affected by DIBOA/BOA and BOA/AZOB combinations. From previous work (Putnam and Defrank, 1983; Skarphol et al., 1987) both cucumber and snap beans have been shown to respond favorably to cover crop residues. A possible explanation is that the large-seeded crops are located too deeply to be influenced by the residues, or, alternately, the concentrations of these chemicals are too low to affect the emergence of the crops. The effect seen on small-seeded weeds such as cress and barnyardgrass could come from rapid degradation of the benzoxazinones from the injured plant material and the more active

TABLE 5. SNAP BEAN ROOT AND SHOOT RESPONSE TO DIBOA, BOA, BOA, AND AZOB APPLIED SINGLY AND IN COMBINATIONS[a]

	% of control					
	Root			Shoot		
Compound	50 (ppm)	100 (ppm)	200 (ppm)	50 (ppm)	100 (ppm)	200 (ppm)
DIBOA	102	91	107	95	79	72
BOA	104	64	50	60	56	24
AZOB	108	83	94	85	66	68
DIBOA and BOA[b]	84 (106)	79 (54)		92 (57)	55 (44)	
DIBOA and AZOB	80 (110)	101 (76)		61 (81)	50 (52)	
BOA and AZOB	96 (112)	75 (53)		29 (51)	44 (37)	
DIBOA, DOA, and AZOB	80 (114)	66 (48)		74 (48)	51 (29)	
LSD (0.05)		32			38	

[a] ( ) = expected value calculated by $E = D^1 \times B^1/100$.
[b] All combination each were applied singly at the given rate.

microbially produced AZOB. Moreover, the smaller seeds are located closer to the soil surface where higher concentrations of these compounds might exist.

CONCLUSIONS

The benzoxazinones have been reported in cereal grains and corn plants (Virtanen et al., 1957). In addition, the recent isolation of the microbially produced AZOB may help to account for a portion of the 88% of inhibition by rye residues that Barnes and Putnam (1987) could not account for. These results show that both small-seeded species were strongly inhibited by AZOB, while garden cress was inhitited by BOA as well. These results agree with Barnes and Putnam (1987) and Nair et al. (1990), who reported BOA to be more active on dicot and small-seeded crop species. For monocots, there has been allelochemical activity reported with DIBOA. The finding that AZOB has herbicidal potential against several small-seeded species gives hope that microbially produced allelochemicals would be useful in weed control.

In general, when comparing the response of the different growth parameters, root and shoot inhibition were more sensitive indicators of allelopathic activity than germination inhibition. Only in the case of garden cress was germination a positive indicator of activity. Neither of the large-seeded crops nor barnyardgrass showed significant germination inhibition. This indicated that the

mode of action for barnyardgrass, cucumber, and snap bean was not on germination processes but rather on early plant development processes. This suggests the expected field response would be reduced plant growth rather than a reduction in number of plants.

Our results not only suggest that these compounds are selectively allelopathic, but indicate the relative quantities of these compounds are important in determining the expected outcome. Earlier bioassays with these compounds used only small-seeded species whereas ours compared larger-seeded crops. It appears that selectivity can be achieved based on seed size and seed placement. This same principle has allowed the selective use of synthetic herbicides for decades. However, the difficulty with allelochemicals is to predict or quantify what might be present at any particular time in the field.

REFERENCES

BARNES, J.P., and PUTNAM, A.R. 1986. Evidence for allelopathy by residues and aqueous extracts of rye (*Secale cereale* L.) *Weed Sci.* 34:384–390.

BARNES, J.P., and PUTNAM, A.R., 1987. Role of benzoxazinones in allelopathy by rye (*Secale cereale* L.). *J. Chem. Ecol.* 13(4):889–905.

BARNES, J.P., PUTNAM, A.R., BURKE, B.A., and AASEN, A.J. 1987. Isolation and characterization of allelochemicals in rye herbage. *Phytochemistry* 26(5):1385–1390.

BLEVINS, R.L., COOK, D., PHILLIPS, S.H., and PHILLIPS, R.E. 1971. Influence of no-tillage on soil moisture. *Agron. J.* 63:593–596.

CHOU, C.H., and PATRICK, Z.A. 1976. Identification and phytotoxic activity of compounds produced during decomposition of rye and corn residues in soil. *J. Chem. Ecol.* 2(3):369–387.

COLBY, S.R. 1967. Calculating synergistic and antagonistic responses of herbicide combinations. *Weeds* 15:20–22.

GLENN, D.M., and WELKER, W.V. 1989. Orchard soil management systems influence rainfall infiltration. *J. Am. Soc. Hortic. Sci.* 114(1):10–14.

KIMBER, R.W. 1973. II. The effect of time of rotting of straw from some grasses and legumes on the growth of wheat seedlings. *Plant Soil* 38:347–361.

KNAVEL, D.E., ELLIS, J., and MORRISON, J. 1977. The effect of tillage systems on the performance and elemental absorption by selected vegetable crops. *J. Am. Soc. Hortic. Sci.* 102(3):323–327.

MASCIANICA, M.P., WILSON, H.P., WALDEN, R.F., HINES, T.E., and BELLINDER, R.R. 1986. No-tillage snapbean growth in wheat stubble of varied height. *J. Am. Hortic. Soc.* 111(6):853–885.

NAIR, M.G., WHITENACK, C.J., and PUTNAM, A.R. 1990. 2,2′-oxo-1,1′-azobenzene. A microbially transformed allelochemical from 2,3-benzoxazoline: I. *J. Chem. Ecol.* 16(2):353–364.

PUTNAM, A.R., and DEFRANK, J. 1983. Use of phytotoxic plant residues for selective weed control. *Crop Protection* 2(2):173–181.

SHILLING, D.G., LIEBL, R.A., and WORSHAM, A.D. 1985. Rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) mulch: The suppression of certain broadleaved weeds and the isolation and identification of phytotoxins, pp., 243–271 *in* A.R. Putnam and C.S. Tang, (eds.). The Chemistry of Allelopathy. John Wiley & Sons, New York.

SKARPHOL, B.J., COREY, K.A., and MEISINGER, J.J. 1987. Response of snap beans to tillage and cover crop combinations. *J. Am. Soc. Hortic. Sci.* 112(6):936–941.

TANG, C.S., CHANG, S.H., HOO, D., and YANAGIHARA, K.H. 1975. Gas chromatographic determination of 2(3)-benzoxazolinones from cereal plants. *Phytochemistry* 4:2077–2079.

TEASDALE, J.R., and FRANK, J.R. 1983. Effect of row spacing on weed competition with snap beans (*Phaseolus vulgaris* L.). *Weed Sci.* 31:81–85.

VIRTANEN, A.I., HIETALA, P.K., and WAHLROOS, O. 1957. Antimicrobial substances in cereals and fodder plants. *Arch. Biochem. Biophys.* 69:486–500.

YOUNG, H.M., JR. 1982. No-Tillage Farming, 2nd ed. No-Til Farmer, Inc., Brookfield, Wisconsin. 202 pp.

ZUNGICA, G.E., AGRANDONA, V.H., NIEMEYER, H.M., and CORCUERA, L.J. 1983. Hydroxamic acid content in wild and cultivated Gramineae. *Phytochemistry* 22(12):2665–2668.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

COMPARISON OF FURANOCOUMARIN CONCENTRATIONS OF GREENHOUSE-GROWN *Ruta chalepensis* WITH OUTDOOR PLANTS LATER TRANSFERRED TO A GREENHOUSE

ALICJA M. ZOBEL

Department of Chemistry
Trent University
Peterborough, Ontario, Canada K9J 7B8

(Received February 26, 1990; accepted August 9, 1990)

Abstract—*Ruta chalepensis* contained concentrations of furanocoumarins 25–50% of those found in *R. graveolens* both in the whole leaf and on its surface. On the leaf surface of plants grown all year indoors in a greenhouse, they increased steadily between November 1 and December 14 on mature upper and lower leaves. New growth upper leaves on December 14 contained less than mature upper leaves. Plants transferred from outdoors to the greenhouse showed decreased concentrations after the first two weeks, followed by recovery both in the whole leaf and on the leaf surface. Proportions of xanthotoxin and bergapten to psoralen changed during the experiment. On the leaf surfaces and in the whole upper leaves of the indoor plants, the proportions were often similar, but in the transferred plants, in most cases, psoralen was less than bergapten or xanthotoxin in the upper leaves and markedly less in the lower leaves. Implications of these findings for possible effects of environmental changes on secondary plant metabolism are discussed.

Key Words—Furanocoumarins, *Ruta chalepensis*, photophytodermatitis, surface deposition, plant defense.

INTRODUCTION

Linear furanocoumarins, or psoralens, which are known to cause photophytodermatitis, are localized to some extent on the leaf surfaces of plants that elaborate them (Zobel and Brown, 1988b, 1989, 1990a,b). An outstanding example is *Ruta graveolens*, which has been noted since ancient times for inducing this dermatitis. We have found that, in extreme cases, over 55% of the total fur-

0098-0331/91/0100-0021$06.50/0

anocoumarins of the leaf, in micrograms per gram fresh weight, can be extracted from the surface (Zobel and Brown, 1989, 1990a).

Studies on *Heracleum lanatum* (Umbelliferae) have revealed significant changes during the growing season in the concentrations of the three predominant furanocoumarins in this species: psoralen, xanthotoxin, and bergapten, both on the leaf surface and in the whole leaves (Zobel and Brown, 1990b).

A hypothesis has been advanced that a function of these surface coumarins is to protect the plant against microbial attack, acting as a defense barrier (Zobel and Brown, 1989). Any such external barrier must be affected by changes in the plant's environment, as well as its developmental changes.

A number of workers have hypothesized about the role of these compounds in plants (Camm et al., 1976; Berenbaum, 1978; Berenbaum and Neal, 1985; Städler and Buser, 1984; Matern et al., 1988), and on their biological activity (Chakraborty et al., 1957; Murray et al., 1982; Towers, 1987; Nitao, 1988), especially under UV irradiation (Pathak and Fitzpatrick, 1959; Grekin and Epstein, 1981; Towers and Yamamoto, 1985; Towers, 1986). Some investigations have dealt with increased concentrations of furanocoumarins as a response to infection (Ashwood-Smith et al., 1985; Surico et al., 1987) and to other stresses (Beier and Oertli, 1983). Recently experiments have demonstrated changes in concentrations of furanocoumarins depending on the plant's developmental stage (Berenbaum and Zangerl, 1986) and on environmental and nutritional conditions (Zangerl and Berenbaum, 1987).

In order to ascertain to what extent such changes might occur in a plant in response to changes in its environment, I have examined *Ruta chalepensis*, comparing concentrations in plants grown outdoors that were subsequently transferred to a greenhouse with those of control plants grown in a greenhouse for the whole year.

METHODS AND MATERIALS

Four 2-year-old *R. chalepensis* subshrubs, each with about 20 long shoots each containing about 40 leaves, growing outdoors in the soil during the summer growing season (outdoor plants), were used. They were transferred to pots on October 1, 1988, before the autumn frosts, and allowed to remain outdoors for the rest of that month to let the plants recover from root damage. Four others grown throughout the year in a greenhouse (indoor plants) were used as controls. The first leaf samples for analysis were collected from both groups on October 1 as a control for how well the potted plants had recovered from transplanting, and the second at the beginning of the experiment on November 1 just before the outdoor plants were transferred to a greenhouse. The next three samples were collected at ca. two-week intervals (November 15 and 30, and

December 14). From each plant, four of the uppermost leaves of similar size, 2–4 cm long, were collected. These were not new growth, but small-sized, mature leaves. Separate samples of such leaves were collected for analysis from the indoor and outdoor plants, and, in each case, the lowest three undamaged green leaves were collected from the same plants for comparison purposes. In contrast to *R. graveolens*, there were no senescent yellowish leaves during the whole experiment.

The leaves of each sample were weighed and surface coumarins extracted by a previously described method (Zobel and Brown, 1988a, 1989). The furanocoumarins in the extracts were also purified and determined as described earlier (Thompson and Brown, 1984; Zobel and Brown, 1988a).

RESULTS

Transferring *R. chalepensis* plants to a greenhouse from outdoors resulted in changed concentrations of furanocoumarins both on the leaf surface and in the whole leaf, compared to the plants growing indoors throughout the year (Table 1).

The indoor plants exhibited physiological changes depending on the time of year and the photoperiod, without influence of other environmental condi-

TABLE 1. CONCENTRATIONS[a] OF TOTAL FURANOCOUMARINS IN WHOLE LEAF AND ON LEAF SURFACE OF *Ruta chalepensis*

Leaves	Collection date (1988)	Indoor plants			Outdoor plants		
		Σ P + X + B[b]		% on surface	Σ P + X + B		% on surface
		Whole leaf	On surface		Whole leaf	On surface	
Upper	Oct. 1	1050 ± 50	390 ± 20	37	1200 ± 60	480 ± 40	40
	Nov. 1	1030 ± 50	390 ± 30	38	1160 ± 60	500 ± 40	43
	Nov. 15	1455 ± 80	480 ± 30	33	850 ± 40	315 ± 30	37
	Nov. 30	900 ± 80	660 ± 50	73	1130 ± 50	610 ± 40	54
	Dec. 14[c]	630 ± 50	290 ± 30	47	710 ± 60	450 ± 30	63
Lower, green	Oct. 1	650 ± 50	320 ± 20	49	800 ± 40	200 ± 15	25
	Nov. 1	660 ± 50	300 ± 30	46	760 ± 60	170 ± 20	23
	Nov. 15	700 ± 40	340 ± 30	49	505 ± 40	170 ± 20	35
	Nov. 30	750 ± 50	400 ± 40	54	880 ± 80	350 ± 30	40
	Dec. 14	650 ± 50	360 ± 30	55	525 ± 30	260 ± 20	50

[a] μg/g fresh weight.
[b] P: psoralen, X: xanthotoxin, B: bergapten.
[c] New growth.

tions such as wind and rain. The outdoor plants (brought into the greenhouse November 1), in contrast, were affected by the transfer indoors, in response to their new environment. All but the December 14 leaves were mature, differing only in size and location on the plant. On December 14, as the upper leaves of both groups of plants were then new growth, the experiment was terminated, because, as shown below, the results obtained suggested a different physiology from that of the mature leaves.

Table 1 compares the sum of the concentrations (Σ values) of psoralen, xanthotoxin, and bergapten (P + X + B). On November 1 the concentrations were similar to those of October 1 in the same group of plants, but the outdoor plants had higher concentrations in the whole leaf than the indoor plants, both in the upper leaves (difference 130 μg/g) and in the lower leaves (100 μg/g), and on the surface of the upper leaves (110 μg/g). On the lower leaves, the indoor group had over 40% more than the outdoor (difference of 130 μg/g). Upper leaves of both groups contained much more total furanocoumarins than the lower leaves, with a difference of ca. 400 μg/g, corresponding to more than half of the lower leaves' concentration.

The indoor plants showed a steady increase in surface concentrations of the furanocoumarins on the mature leaves. The total concentrations increased until November 15 in the upper leaves, and until November 30 in the lower, followed by a decrease.

The lower leaves of the indoor plants had a stable percentage of furanocoumarins on the surface (46–55%), whereas the outdoor plants began with a low percentage (23% of the total), increasing steadily to 50% at the conclusion. The surface concentrations in the lower leaves of the indoor plants paralleled those of the whole leaf, but the upper leaves did not always follow this pattern, i.e., the 30% increase in the whole leaf concentration on November 15 was unaccompanied by any increase in the surface concentration, which showed a marked increase only after two weeks.

Changes were observed after the plants had been transferred into the greenhouse. On November 1 the outdoor plants contained 10% more furanocoumarins in the whole upper leaves and 20% more on the surface than the indoor. The lower leaves contained much smaller concentrations than the upper, both in the whole leaf and on the surface, with the percentage on the surface little more than half that of the upper leaves (23% vs. 43%). On November 15, the upper leaves of the outdoor plants showed decreased concentrations in the whole leaves and on the surface. The lower leaves showed decreased concentration in the whole leaves, but it was unchanged on the surface. They recovered in the next two weeks and made up or even exceeded the difference; this recovery was especially visible on the surface. For mature leaves, the highest percentages on the surface were 54% and 50%.

Ratio of Psoralen to Xanthotoxin and Bergapten. Table 2 shows the pro-

TABLE 2. RATIOS[a] OF CONCENTRATIONS OF FURANOCOUMARINS IN WHOLE LEAF AND ON LEAF SURFACE OF *Ruta chalepensis* PLANTS

		Indoor plants				Outdoor plants			
	Collection date	Whole leaf		Leaf surface		Whole leaf		Leaf surface	
Leaves	(1988)	X[b]	B[b]	X	B	X	B	X	B
Upper	Nov. 1	0.9	0.97	0.86	0.93	1.9	1.6	1.6	1.5
	Nov. 15	2.1	2.6	1.1	0.93	1.1	1.6	0.8	1.3
	Nov. 30	1.3	1.0	1.2	0.93	2.6	2.4	2.2	2.4
	Dec. 14[c]	0.9	0.70	0.91	0.76	2.6	1.9	2.6	2.2
Lower, green	Nov. 1	13.5	7.0	7.6	7.4	5.1	3.1	2.7	3.0
	Nov. 15	25.7	22.5	28.7	25.0	3.1	1.6	4.5	4.6
	Nov. 30	21.6	8.1	16.5	6.2	10.8	5.2	7.5	3.9
	Dec. 14	18.3	10.6	18.3	10.8	13.3	7.9	11.5	7.7

[a]Based on psoralen = 1.
[b]X: xanthotoxin, B: bergapten.
[c]New growth.

portions of the three furanocoumarins in the whole leaf and on the leaf surface, with the value for psoralen taken as unity. Four distinct groups of ratios are evident: (1) the upper leaves of the indoor plants, with similar concentrations of the three furanocoumarins (except November 15); (2) their lower leaves, showing a drastic diminution of psoralen (7 to 25 times); (3) upper leaves of the outdoor plants, having bergapten and xanthotoxin concentrations 1.1–2.6 times that of psoralen (except November 15); and (4) lower leaves of the outdoor plants, with bergapten and xanthotoxin concentrations ca. 1.6–13.3 times those of the upper leaves.

DISCUSSION

These observations have demonstrated that, after transfer to the greenhouse, the plants responded by increasing furanocoumarin production and steadily increasing extrusion to the surface. As well, new growth after this recovery contained a higher percentage on the surface than new growth of the indoor plants (63% vs. 47%); this interesting phenomenon needs more investigation before conclusions can be drawn.

The findings of the present experiment, and unpublished data from this laboratory on *Ruta graveolens* showing similar trends in response, but with concentration values at least twice as high, suggest that there are two parallel

physiological processes influencing furanocoumarin concentrations in the whole leaf and on its surface. The first process, genetically dependent, governs the levels of these compounds and their proportions according to the period of the year and is typical of the species; the second is a physiological and biochemical response to changes in environmental conditions. Further experiments are needed to compare *R. chalepensis* and *R. graveolens* to other species with respect to these phenomena, because if, as seems probable, furanocoumarins play a defense role in plants, their levels in the whole leaf and especially on its surface should show changes with varying environmental conditions. Measurements taken over a complete vegetative period on leaves of *Heracleum lanatum* showed changes in concentrations on the surface and in the whole leaves depending on their developmental stage and on the time within the vegetation period (Zobel and Brown, 1990b). Investigations are now in progress with similar measurements for both *Ruta* species.

Acknowledgments—The studies reported here were financially supported by operating grant A2487 from the Natural Sciences and Engineering Research Council of Canada to Prof. S.A. Brown.

REFERENCES

ASHWOOD-SMITH, M.J., CESKA, O., and CHAUDHARY, S.K. 1985. Mechanism of photosensitivity reactions to diseased celery. *Br. Med. J.* 290:1249.

BEIER, R.C., and OERTLI, E.H. 1983. Psoralen and other linear furanocoumarins as phytoalexins in celery (*Apium graveolens*). *Phytochemistry* 22:2595–2597.

BERENBAUM, M. 1978. Toxicity of a furanocoumarin to armyworms: A case of biosynthetic escape from insect herbivores. *Science* 201:532–534.

BERENBAUM, M., and NEAL, J.J. 1985. Synergism between myristicin and xanthotoxin, a naturally occurring plant toxicant. *J. Chem. Ecol.* 11:1349–1358.

BERENBAUM, M.R., and ZANGERL, A.R. 1986. Variation in seed furanocoumarin content within the wild parsnip (*Pastinaca sativa*). *Phytochemistry* 25:659–661.

CAMM, E.L., WAT, C.-K., and TOWERS, G.H.N. 1976. An assessment of the roles of furanocoumarins in *Heracleum lanatum*. *Can. J. Bot.* 54:2562–2566.

CHAKRABORTY, D.P., DAS GUPTA, A., and BOSE, P.H. 1957. On the antifungal action of some natural coumarins. *Ann. Biochem. Exp. Med.* 17:59–62.

GREKIN, D.A., and EPSTEIN, J.H. 1981. Psoralens and UV-A (PUV-A) and photocarcinogenesis. *Photochem. Photobiol.* 36:857–860.

MATERN, U., STRASSER, H., WENDORFF, H., and HAMERSKI, D. 1988. Coumarins and furanocoumarins, pp. 3–21, *in* I. Vasil and F. Constabel (eds.). Cell Culture and Somatic Cell Genetics of Plants, Vol. 5. Academic Press, New York.

MURRAY, R.D.H., MÉNDEZ, J., and BROWN, S.A. 1982. The Natural Coumarins: Occurrence, Chemistry and Biochemistry. Wiley, Chichester.

NITAO, J.K. 1988. Artificial defloration and furanocoumarin induction in *Pastinaca sativa* (Umbelliferae). *J. Chem. Ecol.* 14:1515–1522.

PATHAK, M.A., and FITZPATRICK, T.B. 1959. Bioassay of natural and synthetic furocoumarins (psoralens). *J. Invest. Dermatol.* 32:509–518.

STÄDLER, E., and BUSER, H.-R. 1984. Defense chemicals in the leaf surface wax synergistically stimulate oviposition by a phytophagous insect. *Experientia* 40:1157–1159.

SURICO, G., VARVANO, L., and SOLFRIZZO, M. 1987. Linear furanocoumarins accumulation in celery plants infected with *Erwinia carotovera* pathovar *carotovera*. *J. Agric. Food Chem.* 35:406–409.

THOMPSON, H.J., and BROWN, S.A. 1984. Separations of some coumarins of higher plants by liquid chromatography. *J. Chromatogr.* 314:323–336.

TOWERS, G.H.N. 1986. Induction of cross-links in viral DNA by naturally occurring photosensitizers. *Photochem. Photobiol.* 44:187–192.

TOWERS, G.H.N. 1987. Fungicidal activity of naturally occurring photosensitizers. *Am. Chem. Soc. Symp. Ser.* 339:231–240.

TOWERS, G.H.N., and YAMAMOTO, E. 1985. Interactions of cinnamic acid and its derivatives with light. *Ann. Proc. Phytochem. Soc. Eur.* 25:271–288.

ZANGERL, A.R., and BERENBAUM, M. 1987. Furanocoumarins in wild parsnip. Effects of photosynthetically active radiation, ultraviolet light and nutrients. *Ecology* 68:516–520.

ZOBEL, A.M., and BROWN, S.A. 1988a. Determination of furanocoumarins on the leaf surface of *Ruta graveolens* with an improved extraction technique. *J. Nat. Prod.* 51:941–946.

ZOBEL, A.M., and BROWN, S.A. 1988b. Furanocoumarins on plant surfaces. *Bulletin de Liaison no. 14 du Groupe Polyphenols. Compte-rendu des Journées Internationales d'Étude et de l'Assemblée Générale*, pp. 65–68.

ZOBEL, A.M., and BROWN, S.A. 1989. Histological localization of furanocoumarins in *Ruta graveolens*. *Can. J. Bot.* 67:915–921.

ZOBEL, A.M., and BROWN, S.A. 1990a. Dermatitis-inducing furanocoumarins on the leaf surfaces of rutaceous and umbelliferous plants. *J. Chem. Ecol.* 16:693–700.

ZOBEL, A.M., and BROWN, S.A. 1990b. Seasonal changes of furanocoumarin concentrations in leaves of *Heracleum lanatum*. *J. Chem. Ecol.* 16:1623–1634.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

EFFECTS OF MIXTURES OF FOUR PHENOLIC ACIDS ON LEAF AREA EXPANSION OF CUCUMBER SEEDLINGS GROWN IN PORTSMOUTH B_1 SOIL MATERIALS[1]

THOMAS M. GERIG[2,*] and UDO BLUM[3]

[2]*Department of Statistics*
[3]*Department of Botany*
North Carolina State University
Raleigh, North Carolina 27695

(Received June 19, 1990; accepted August 20, 1990)

Abstract—Cucumber seedlings growing in a 1 : 2 mixture of soil (Portsmouth B_1) and sand adjusted to pH 5.2 were treated every other day five times with 0, 0.0625, 0.125, 0.25, or 0.5 μmol/g soil of ferulic, caffeic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, sinapic, syringic, or vanillic acids. Treatments began when seedlings were 8 days old. The effects on mean absolute rates of leaf expansion were used to estimate the relative potencies of these phenolic acids to ferulic acid. Based on the results of this experiment, ferulic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids were chosen for further study. Materials and procedures were identical in the second study, but treatments consisted of mixtures of the four phenolic acids at concentration combinations designed to achieve 40% or 60% inhibition of absolute rates of leaf expansion. Using joint action analysis, a model describing the action of the phenolic acid mixtures was developed. A model involving only two factor terms was sufficient to describe the observed responses of cucumber leaf area to the phenolic acid mixtures. The action of *p*-hydroxybenzoic acid on absolute rates of leaf expansion was inhibited by the presence of the other three phenolic acids. No other antagonisms or synergisms existed among the four compounds.

Key Words—Allelopathy, phenolic acid mixtures, similar joint action analysis, mean absolute rates of leaf expansion, *Cucumis sativus*.

*To whom correspondence should be addressed.

[1]This research was partially supported by the North Carolina Agricultural Research Service, Raleigh, North Carolina 27695-7643 and by the US–Spain Joint Committee for Scientific and Technological Cooperation project CCA-8309/166.

0098-0331/91/0100-0029$06.50/0

INTRODUCTION

Soils contain complex mixtures of phenolic acids (Whitehead, 1964; Guenzi and McCalla, 1966; Whitehead et al., 1981, 1983; Kuiters and Denneman, 1987; Kuiters et al., 1987). Many of these phenolic acids, either individually or in mixtures, have been shown to have allelopathic activity in numerous bioassays (see for example Rice, 1984; Einhellig, 1987).

Concentrations of phenolic acids in soils are determined primarily by the rate of input (i.e., leaf leachates, root exudates, or decomposition and leaching of plant debris), absorption and adsorption by seeds and roots, fixation by soil components, leaching, and microbial utilization (Skujins, 1967; Haider and Martin, 1975; Huang et al., 1977; Dalton et al., 1983; Rice, 1984; Shann and Blum, 1987). That the rates of these processes are differentially affected by the environment suggests that the qualitative and quantitative content of phenolic acids in soil solutions is constantly changing. Understanding how various mixtures of phenolic acids affect plant growth, thus, becomes essential to the identification of the role of allelopathic interactions in managed and natural communities.

Predicting how phenolic acid mixtures of varying composition (i.e., numbers and concentrations) affect seed germination and plant growth in soil, however, is not simple, since effects of individual phenolic acids in mixtures may be synergistic, additive, or antagonistic (Rasmussen and Einhellig, 1977; Blum et al., 1984, 1985, 1989; Einhellig, 1987). Recently, the authors characterized the action of phenolic acid pairs on plant growth by use of joint action analysis (Gerig et al., 1989; Blum et al., 1989). In the current work, we extend this analysis to mixtures of four phenolic acids.

METHODS AND MATERIALS

General Aspects. Cucumber seeds (*Cucumis sativus* cv. Early Green Cluster; Wyatt Quarles Seed Company, Raleigh, North Carolina) were germinated in the dark at 28–30°C in trays containing sterile vermiculite and Hoagland's solution (Hoagland and Arnon, 1950). After 24 hr, the trays containing the seedlings were transferred to a light bank and exposed in a 12-hr light period (140 μEisteins/m^2/sec), followed by a 12-hr dark period. Seedlings were planted in 155-ml cups (one per cup) containing 150 g of a soil–sand mixture (1 : 2 by weight). Portsmouth B_1-horizon soil material (fine loamy, mixed, thermic Typic Umbraquaalts) was obtained from the NCSU agricultural research farm in Plymouth, North Carolina, sieved, air-dried, and adjusted to pH 5.2 with calcium hydroxide. River sand was rinsed with deionized water and then air-dried. The cups, which did not have drainage holes, were inserted through holes in a plywood sheet that protected everything below the soil surface from direct irra-

diance of the light banks (Blum and Dalton, 1985). Seedlings were grown at room temperature (21–30°C) with a 12-hr light period. Seedlings were supplied with 7 ml double-strength Hoagland's solution every other day. Sufficient distilled water was added daily to bring the weight of a given cup (160 g for cup and soil) and its seedling to approximately 190 g. Cup plus seedling weight was maintained above 170 g. For further details of the cup system, see Blum et al. (1987). Length and width measurements were taken for each leaf (excluding cotyledons) on day 8, first day of phenolic acid treatments, and day 18, the last day of the experiment.

Phenolic Acid Treatments. In the first study, seedlings were treated every other day (maximum of five treatments) with 0, 0.0625, 0.125, 0.25 or 0.5 μmol/g soil of ferulic (FER), caffeic (CAF), *p*-coumaric (PCO), *p*-hydroxybenzoic (POH), protocatechuic (PRO), sinapic (SIN), syringic (SYR), or vanillic (VAN) acid. Phenolic acid treatments were alternated with nutrient solution application. The pH of each solution was adjusted to pH 5.2 with NaOH.

Seedlings in the second study were treated every other day (five treatments, maximum) with all possible combinations of FER, PCO, POH or VAN. Concentration levels for individual phenolic acids and each phenolic acid in the two-, three-, or four-way mixtures were determined in the first study. The concentrations of individual phenolic acids and the phenolic acid mixtures were adjusted so that each provided approximately 40% or 60% inhibition. Concentrations ranged from 0.05 to 0.37 μmol/g for FER, 0.06–0.41 for PCO, 0.17–0.72 for POH, and 0.04–0.35 for VAN.

Experimental Design. The experimental designs for the seedling bioassays were as follows. In study 1, eight individual phenolic acids were each tested at five concentrations. In study 2, 31 combinations of phenolic acids were tested. In both studies, all treatments were replicated three times in complete blocks, with a complete set of treatments being randomly arranged under each of three light banks.

Data Analyses. Leaf areas were determined from length and width measurements of leaves and the following equation:

$$\text{Leaf area} = -1.457 + 0.00769LW$$

($P = 0.0001$, $R^2 = 0.98$, $N = 121$), where leaf area is in cm^2 and length (L) and width (W) values are in mm (Blum and Dalton, 1985). Mean absolute rates of leaf expansion (AGR) were calculated for each seedling by the equation (Radford, 1967):

$$\text{AGR} = \text{leaf area of seedling on day 18} - \text{leaf area of seedling on day 8}$$

Data were analyzed by nonlinear regression (Gallant, 1975) and joint action analysis (Gerig et al., 1989).

RESULTS AND DISCUSSION

Study 1: Effects of Individual Phenolic Acids. Data from the first study were analyzed to determine the potencies (on an equimolar basis) with respect to growth inhibition on cucumber seedlings of each of eight phenolic acids. FER was found to be the most potent and was used as the reference compound. The data were analyzed through use of a model closely related to the joint action model which predicted AGR by the function:

$$f(Z; \beta) = \begin{cases} \beta_1/(1 + \exp\{\beta_3[\ln(Z) - \beta_2]\}), & Z > 0 \\ \beta_1, & Z = 0 \end{cases} \quad (1)$$

where β is the vector of unknown parameters, Z, the effective concentration of the mixture, is defined by

$$Z = \text{cFER} + \beta_4\text{cCAF} + \beta_5\text{cPCO} + \beta_6\text{cPOH} + \beta_7\text{cPRO} + \beta_8\text{cSIN} + \beta_9\text{cSYR} + \beta_{10}\text{cVAN}$$

and where, for convenience, cFER, cCAF, $\cdots$, cVAN denote the concentration (μmol/g) of FER, CAF, $\cdots$, VAN acids in the mixture. In study 1, only one compound was present in a treatment. For that treatment, the other compounds had a concentration of zero.

The parameter β_1 measures the response under control conditions [i.e., $f(0; \beta) = \beta_1$]; β_2 is the value of ln (Z) required to achieve 50% inhibition, and β_3 controls the rate at which the curve descends as a function of Z.

The estimates of the parameters in the model, including β_4, β_5, $\cdots$, β_{10}, the potencies of each compound relative to FER, are shown in Table 1. Potencies are ordered from largest to smallest, and compounds that appear together in a grouping did not differ significantly at the 0.05 level of significance.

The compounds included in study 2 were selected from the eight used in the first. Compounds FER, PCO, VAN, and POH were chosen to include a range of potencies and phenolic acid types (i.e., corresponding benzoic and cinnamic acid derivatives). SIN was excluded because it reacted with the soil, turning it red.

To select concentrations for the constituents of each of the mixtures in study 2, combinations of the compounds FER, PCO, POH, and VAN were chosen so each would contribute the same amount to the effective concentration. Thus, from Table 1 we see, for combinations of PCO and POH (when cFER = cVAN = 0), that the effective concentration was:

$$Z = 0.67\text{cPCO} + 0.35\text{cPOH}$$

TABLE 1. PARAMETER ESTIMATES INCLUDING POTENCIES OF PHENOLIC ACIDS RELATIVE TO FERULIC ACID

Parameter	Estimated value	
β_1: Average of three control values (cm^2/11 days)	60.32	
β_2: Log(Z) for 50% inhibition	−1.089	
β_3: Rate parameter	1.108	
Phenolic acid	Relative potency	Compound groupings[a]
FER - ferulic acid - 4 hydroxy-3-methoxycinnamic	1.00	a
β_8: SIN - sinapic acid - 3,5 dimethoxy-4-hydroxycinnamic	0.70	a b
β_5: PCO - p-coumaric acid - p-hydroxycinnamic	0.67	b
β_{10}: VAN - vanillic acid - 4 hydroxy-3-methoxybenzoic	0.67	b
β_9: SYR - syringic acid - 3,5 dimethoxy-4-hydroxybenzoic	0.59	b c
β_4: CAF - caffeic acid - 3,4 dihydroxycinnamic	0.38	c d
β_6: POH - p-hydroxybenzoic acid - p-hydroxybenzoic	0.35	d
β_7: PRO - protocatechuic acid - 3,4 dihydroxybenzoic	0.13	e

[a] Potencies of compounds followed by the same letter do not differ at the 0.05 level of significance.

To obtain the approximate concentrations required to achieve 40% inhibition, we solved the equation:

$$f(Z; \hat{\beta}) = \hat{\beta}_1(1 - 0.4)$$

for Z, where $f(Z; \beta)$ is defined in (1) and is evaluated at the estimated parameter values, $\hat{\beta}$, given in Table 1. Thus, $Z = 0.485$ and the corresponding concentrations:

$$\text{cPCO} = (0.485/2)/0.67 = 0.36\ \mu\text{mol/g}$$

$$\text{cPOH} = (0.485/2)/0.35 = 0.69\ \mu\text{mol/g}$$

As expected, the realized inhibition rates for the mixtures differed from those predicted from study 1 since the model for the first ignored potential synergistic or antagonistic effects. Moreover, to increase ease of application, the actual concentrations used in study 2 differed slightly from those chosen by this process.

Study 2: Effects of Mixtures of Phenolic Acids. Joint action analysis was applied to the entire data set, allowing treatment combinations involving different concentrations of up to four phenolic acids. Table 2 lists AGR means and standard deviations by treatment group. The experimental design utilized a sequence of four nested models. The first, and most encompassing, included

TABLE 2. MEANS AND STANDARD DEVIATIONS BY TREATMENT FOR STUDY 2[a]

Concentration				AGR		Concentration				AGR	
FER	PCO	POH	VAN	Mean	SD	FER	PCO	POH	VAN	Mean	SD
0.00	0.00	0.00	0.00	53.70	7.38						
Single-component mixtures											
0.00	0.00	0.00	0.21	40.51	5.81	0.00	0.00	0.00	0.35	35.10	5.00
0.00	0.00	0.44	0.00	34.12	3.89	0.00	0.00	0.72	0.00	26.82	1.96
0.00	0.25	0.00	0.00	39.67	6.78	0.00	0.41	0.00	0.00	28.70	2.76
0.23	0.00	0.00	0.00	38.25	8.43	0.37	0.00	0.00	0.00	26.84	4.09
Two-component mixtures											
0.00	0.00	0.29	0.09	36.31	9.53	0.00	0.00	0.47	0.15	30.65	4.88
0.00	0.12	0.00	0.09	45.33	6.47	0.00	0.20	0.00	0.15	35.49	2.17
0.10	0.00	0.00	0.09	42.10	10.06	0.16	0.00	0.00	0.15	37.35	1.25
0.00	0.12	0.29	0.00	44.88	0.91	0.00	0.20	0.47	0.00	30.33	2.13
0.10	0.00	0.29	0.00	38.70	1.49	0.16	0.00	0.47	0.00	26.24	2.82
0.10	0.12	0.00	0.00	44.81	5.55	0.16	0.20	0.00	0.00	34.09	6.58
Three-component mixtures											
0.00	0.08	0.21	0.06	42.80	2.98	0.00	0.13	0.35	0.09	33.80	6.82
0.06	0.00	0.21	0.06	40.45	3.09	0.10	0.00	0.35	0.09	37.85	12.05
0.06	0.08	0.00	0.06	40.38	3.81	0.10	0.13	0.00	0.09	30.81	3.10
0.06	0.08	0.21	0.00	39.45	3.91	0.10	0.13	0.35	0.00	35.52	9.40
Four-component mixtures											
0.05	0.06	0.17	0.04	46.98	9.51	0.07	0.09	0.28	0.07	43.25	5.19

[a] FER = ferulic acid, PCO = *p*-coumaric acid, POH = *p*-hydroxybenzoic acid, VAN = vanillic acid; concentrations in μmol/g soil; AGR = mean absolute rate leaf expansion, cm^2/11 days.

all possible terms, including those with one, two, three, and four factors, where, for example, each (of six) two-factor term involved the concentrations of a pair of compounds. The second and third models included terms with up to three and two factors, respectively. The fourth model, which allowed for no antagonism or synergism and which described similar joint action (Gerig et al., 1989), contained only single-factor terms. Based on the analysis of the data, the model allowing for up to two-factor terms was chosen.

This chosen two-factor model for describing the joint action of four compounds is a generalization of the two-factor, joint action analysis model (Gerig et al., 1989). The function $f(Z; \beta)$, as specified in (1), gives the AGR expected when seedlings have been treated with a given mixture, where Z is the effective concentration of the mixture. If cFER, cPCO, cPOH, and cVAN are the concentration in μmol/g of FER, PCO, POH, and VAN acids, respectively, then Z for the mixture is defined as

$$Z = \text{cFER} + \beta_4\text{cPCO} + \beta_5\text{cPOH} + \beta_6\text{cVAN} + 2\beta_7(\beta_4\text{cFERcPCO})^{1/2} + 2\beta_8(\beta_5\text{cFERcPOH})^{1/2} + 2\beta_9(\beta_6\text{cFERcVAN})^{1/2} + 2\beta_{10}(\beta_4\beta_5\text{cPCOcPOH})^{1/2} + 2\beta_{11}(\beta_4\beta_6\text{cPCOcVAN})^{1/2} + 2\beta_{12}(\beta_5\beta_6\text{cPOHcVAN})^{1/2}$$

Parameters β_4, β_5, and β_6 are potencies for compounds PCO, POH, and VAN relative to that of compound FER. Parameters β_7, β_8, $\cdots$, β_{12} are the associated (pairwise) coefficients of synergy. When a coefficient of synergy is positive (negative), the effect of the corresponding pair of phenolic acids is greater (smaller) than expected under the similar joint action model.

The data were used to fit the parameters by nonlinear regression using the SAS procedure PROC NLIN (SAS Institute Inc., 1988). A summary of the results is given in Table 3. Table 3 is organized with the most encompassing (four-term) model represented at the top, followed by successive simplifications of the model. As would be expected, models that included more terms had smaller error sums of squares, and as higher order terms were removed from the model, the sums of squares increased. F tests compared the model associated with a given row with that associated with the row below. The following demonstrates the calculation of the test statistic for comparing the model that includes up to four-factor terms with that which includes up to three-factor terms:

$$F = [(2603.2 - 2499.2)/(19 - 18)]/[2499.2/77] = 3.20$$

From the P value of 0.078, we see that the model with terms up to four factors does not fit the data significantly better than that with terms up to three factors, nor does the model with terms up to three factors fit the data significantly better ($P = 0.259$) than that with terms up to two factors. However, there is a significant ($P = 0.001$) deterioration for the fit in the model with only single-factor

TABLE 3. SUMMARY RESULTS FOR JOINT ACTION ANALYSIS

Terms included in the model, up to	Degrees of freedom		Error sums of squares	Tests that deleted terms are zero:		
	Model	Error		F	df	P value
Four factors	19	77	2499.2	3.20	1,77	0.078
Three factors	18	78	2603.2	1.35	4,78	0.259
Two factors	14	82	2784.0	4.14	6,82	0.001
One factor (SJA)	8	88	3628.3			

terms as compared to that with up to two-factor terms. The decision to adopt the two-factor model was based on this analysis.

The detailed analysis of the data using the two-factor model is presented in Table 4. From the table we see that the dose (in μmol/g) required to achieve 50% inhibition is 0.40, 0.50, 0.65, and 0.50 for FER, PCO, POH, and VAN, respectively. These reflect the differing potencies of the compounds, FER being the most potent. There is an interesting and suggestive pattern for the coefficients of synergy values. Significant antagonism is demonstrated between POH and FER, between POH and PCO, and, marginally, between POH and VAN. Two hypotheses concerning the joint action of the compounds are consistent with this pattern: (A) that POH inhibits the uptake of FER, PCO, and VAN, or (B) that each of FER, PCO, and VAN inhibit the uptake the POH. The following alternate definition of effective concentration models the first hypothesis (Model A):

$$Z = Z_1 \exp(-\beta_7\beta_5 \text{cPOH}) + \beta_5 \text{cPOH}$$

where Z_1 = cFER + β_4cPCO + β_6cVAN. When β_7 is positive and cPOH increases, the multiplicative factor, exp $(-\beta_7\beta_5$cPOH), will damp the magnitude of the contribution of FER, PCO, and VAN to the effective concentration. For this reason, β_7 is referred to as a damping parameter.

The following alternate definition of effective concentration models the second hypothesis (model B):

$$Z = Z_1 + \beta_5 \text{cPOH} \exp(-\beta_7 Z_1)$$

TABLE 4. PARAMETER ESTIMATES FOR TWO-FACTOR MODEL

Parameter	Estimated value		Compounds FER	PCO	POH	VAN
β_1: Average of three control values (cm^2/11 days)	53.57					
β_2: Log(Z) for 50% inhibition μmol/g	−0.925		0.40	0.50	0.65	0.50
β_3: Rate parameter	1.515[a]					
Potencies relative to FER			1.00	0.79	0.61[a]	0.79
Coefficients of synergy			FER	PCO	POH	VAN
		FER		0.06	−0.20[a]	−0.09
		PCO			−0.32[a]	−0.01
		POH				−0.19

[a] Significant at the 0.05 level.

where Z_1 = cFER + β_4cPCO + β_6cVAN. When β_7 is positive and as Z_1 increases, the multiplicative factor, exp $(-\beta_7 Z_1)$, will damp the magnitude of the contribution of POH to the effective concentration.

Using nonlinear regression, these models were fit yielding the analyses in Tables 5 and 6. Table 7 summarizes and compares the analyses for two- and one-factor models along with models A and B. It can be seen in Tables 5 and 6 that in each case the damping parameter, β_7, is significantly greater than zero and that the estimates of other parameters are similar in value to those for the two factor model as listed in Table 4. Table 7 shows that the data fit models A and B about as well as they fit the two-factor model. It should be noted that the two-factor model and models A and B are not nested, so the F tests are only approximate. Thus, on the basis of this analysis there is no way to choose between the models A and B.

TABLE 5. FERULIC, *p*-COUMARIC, AND VANILLIC ACID UPTAKE INHIBITED BY *p*-HYDROXYBENZOIC ACID (MODEL A)

		FER	PCO	POH	VAN
β_1 Mean of three control values (cm^2/11 days)	53.88				
β_2 Log-dose 50% inhibition μmol/g	-0.867	0.42	0.51	0.58	0.53
β_3 Rate parameter	1.438[a]				
β_4-β_6 Potency relative to FER		1.00	0.82	0.72	0.80
β_7 Damping constant	17.38[a]				

[a] Different from zero at the 0.05 level of significance.

TABLE 6. *p*-HYDROXYBENZOIC ACID UPTAKE INHIBITED BY FERULIC, *p*-COUMARIC, AND VANILLIC ACID (MODEL B)

		FER	PCO	POH	VAN
β_1 Mean of three control values (cm^2/11 days)	53.78				
β_2 Log-dose 50% inhibition μmol/g	-0.917	0.40	0.50	0.64	0.48
β_3 Rate parameter	1.720[a]				
β_4-β_6 Potency relative to FER		1.00	0.80	0.62	0.83
β_7 Damping constant	4.07[a]				

[a] Different from zero at the 0.05 level of significance.

TABLE 7. MODELS A AND B VS. TWO-FACTOR MODEL

Terms included in the model, up to	Degrees of freedom model	Error sums of squares	Tests that deleted terms are zero:			
			F	*df*	*P* value	versus:
Two factors	14 82	2784.0	0.69	5,82	0.632	Model A
			2.19	5,82	0.063	Model B
Model A	9 87	2898.0	21.92	1,87	0.0000	Single factor
Model B	9 87	3155.0	13.05	1,87	0.0005	Single factor
One factor (SJA)	8 88	3628.3				

CONCLUSIONS

Based on similar ring substitutions, the cinnamic and benzoic acids can be paired as follows: CAF and PRO, FER and VAN, PCO and POH, and SIN and SYR, respectively. Although it was not possible to identify the roles, if any, of the various substitutions on the benzene ring, the data clearly show that the cinnamic acid derivatives were more inhibitory to cucumber seedlings than their corresponding benzoic acid derivatives. This is identical to what has been observed for cucumber seedlings growing in nutrient culture (Blum et al., 1985). Whether this difference in inhibition is due to differences in toxicity of the compounds, differences in uptake, or differences in the ability of cucumber seedlings to detoxify these types of compounds is not known. Shann and Blum (1987), however, noted that FER uptake by cucumber seedlings was 50–75% higher than that of POH in nutrient culture.

The joint action of the four chosen phenolic acids on the absolute rates of leaf expansion of cucumber seedlings was surprisingly easy to characterize. Their action apparently did not depend on complicated interactions involving three or four compounds. Characterization of the interactions of all possible two-compound combinations was sufficient. Significant antagonistic effects were noted only for POH and FER, POH and POC, and, marginally, for POH and VAN. Shann and Blum (1987) noted, using ^{14}C-labeled FER and POH, that the uptake of FER by cucumber seedlings was unaffected by the presence of POH, but that the uptake of POH was reduced 30% in the presence of FER. This, in combination with our modeling efforts here, suggests that the uptake of POH by cucumber seedlings may be affected by other phenolic acids and that model B describes the joint action of the compounds.

That individual components in a mixture are antagonistic in their action does not, however, negate that mixtures may have a greater impact on growth unless, of course, the compounds are highly antagonistic. The effects of all combinations (two- and thee-way) that did not include POH were additive.

Clearly, the demonstrated joint action of phenolic acids on cucumber seedlings growth in the Portsmouth B_1 soil system suggests that concentrations of individual phenolic acids in a mixture required for a given growth inhibition decline as the number of phenolic acids in mixture increase. In this laboratory study, using only four phenolic acids (in nature mixtures are much more complex), 10, 10, 24, and 7 μg/g soil of FER, PCO, POH, and VAN, respectively, were required to inhibit growth of cucumber seedlings 15%. Such concentrations of phenolic acids, depending on extraction procedures, have been found in both forest and agricultural soils (Guenzi and McCalla, 1966; Shindo et al., 1978; Katase, 1981; Whitehead et al., 1982; Kuiters et al., 1987).

REFERENCES

BLUM, U., and DALTON, B.R. 1985. Effects of ferulic acid, an allelopathic compound, on leaf expansion of cucumber seedlings grown in nutrient culture. *J. Chem. Ecol.* 11:279–301.

BLUM, U., DALTON, B.R., and RAWLINGS, J.O. 1984. Effects of ferulic acid and some of its microbial metabolic products on radicle growth of cucumber. *J. Chem. Ecol.* 10:1169–1191.

BLUM, U., DALTON, B.R., and SHANN, J.R. 1985. Effects of various mixtures of ferulic acid and some of its microbial metabolic products on cucumber leaf expansion and dry matter in nutrient culture. *J. Chem. Ecol.* 11:619–641.

BLUM, U., WEED, S.B., and DALTON, B.R. 1987. Influence of various soil factors on the effects of ferulic acid on leaf expansion of cucumber seedlings. *Plant Soil* 98:111–130.

BLUM, U., GERIG, T.M., and WEED, S.B. 1989. Effects of mixtures of phenolic acids on leaf area expansion of cucumber seedlings grown in different pH Portsmouth A_1 soil material. *J. Chem. Ecol.* 15:2413–2423.

DALTON, B.R., BLUM, U., and WEED, S.B. 1983. Allelopathic substances in ecosystems: Effectiveness of sterile soil components in altering recovery of ferulic acid. *J. Chem. Ecol.* 9:1185–1201.

EINHELLIG, F.A. 1987. Interactions among allelochemicals and other stress factors of the plant environment, pp. 343–357, *in* G.R. Walker (ed.). Allelochemicals: Role in Agriculture and Forestry. ACS Symposium Series 330. American Chemical Society. Washington, D.C.

GALLANT, A.R. 1975. Nonlinear Regression. *Am. Stat.* 29:73–81.

GERIG, T.M., BLUM, U., and MEIER, K. 1989. Statistical analysis of joint inhibitory action of similar compounds. *J. Chem. Ecol.* 15:2403–2412.

GUENZI, W.D., and MCCALLA, T.M. 1966. Phytotoxic substances extracted from soil. *Soil Sci. Soc. Am. Proc.* 30:214–216.

HAIDER, K., and MARTIN, J.P. 1975. Decomposition of specifically carbon-14 labeled benzoic and cinnamic acid derivatives in soil. *Soil. Sci. Soc. Am. Proc.* 29:657–662.

HOAGLAND, D.R., and ARNON, D.J. 1950. The water-culture method of growing plants without soil. *Calif. Agric. Exp. Stn. Circ.* 347.

HUANG, P.M., WANG, T.S.C., WANG, M.K., WU, M.H., and HSU, N.W. 1977. Retention of phenolic acids by noncrystalline hydroxy-aluminum and -iron compounds and clay minerals of soils. *Soil Sci.* 123:213–219.

KATASE, T. 1981. Distribution of different forms of *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids in forest soils. *Soil Sci. Plant Nutr.* 27:365–371.

KUITERS, A.T., and DENNEMAN, C.A.J. 1987. Water-soluble phenolic substances in soils under several coniferous and deciduous tree species. *Soil Biol. Biochem.* 19:765–769.

KUITERS, A.T., LAKEMAN, M.H., and MIDDLEKOOP, E. 1987. Phenolic substances in humuspodzol profile and their impact on some woodland herbs at low nutrient supply. *Acta. Bot. Neerl.* 36:261–270.

RADFORD, P.J. 1967. Growth analysis formulae—their use and abuse. *Crop. Sci.* 7:171–175.

RASMUSSEN, J.A., and EINHELLIG, F.A. 1977. Synergistic inhibitory effects of *p*-coumaric and ferulic acid on germination and growth of sorghum. *J. Chem. Ecol.* 3:197–205.

RICE, E.L. 1984. Alellopathy. Academic Press, Orlando.

SAS INSTITUTE, INC. 1988. SAS/STAT User's Guide Release 6.03 Edition. SAS Institute Inc., Cary, North Carolina. pp. 675–712.

SHANN, J., and BLUM, U. 1987. Uptake of ferulic and *p*-hydroxybenzoic acids by *Cucumis sativus*. *Phytochemistry* 26:2959–2964.

SHINDO, H., OHTA, S., and KUWATSUKA, S. 1978. Behavior of phenolic substances in the decaying process of plants IX. Distribution of phenolic acids in soils of paddy fields and forests. *Soil Sci. Plant. Nutr.* 24:233–243.

SKUJINS, J.J. 1967. Enzymes in soils, pp. 371–414, in A.D. McLaren and G.H. Peterson (eds.). Soil Biochemistry. Marcel Dekker, New York.

WHITEHEAD, D.C. 1964. Identification of *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids. *Nature* 202:417–418.

WHITEHEAD, D.C., DIBB, H., and HARTLEY, R.D. 1981. Extractant pH and the release of phenolic compounds from soils, plant roots and leaf litter. *Soil Biol. Biochem.* 13:343–348.

WHITEHEAD, D.C., DIBB, H., and HARTLEY, R.D. 1982. Phenolic compounds in soil as influenced by the growth of different plant species. *J. Appl. Ecol.* 19:579–588.

WHITEHEAD, D.C., DIBB, H., and HARTLEY, R.D. 1983. Bound phenolic compounds in water extracts of soils, plant roots and leaf litter. *Soil Biol. Biochem.* 15:133–136.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

MECHANISMS OF RESISTANCE IN WILD RICE *Oryza brachyantha* TO RICE LEAFFOLDER *Cnaphalocrocis medinalis* (GUENÉE) (LEPIDOPTERA: PYRALIDAE)

R. RAMACHANDRAN[1,2,*] and Z.R. KHAN[1]

International Rice Research Institute (IRRI)
P.O. Box 933
Manila, Philippines

(Received June 6, 1990; accepted August 8, 1990)

Abstract—A wild rice, *Oryza brachyantha*, was rated as highly resistant to rice leaffolder, *Cnaphalocrocis medinalis* (Guenée), while its F_1 hybrid from a cross with a high-yielding, leaffolder-susceptible rice, IR31917-45-3-2, was rated resistant in a standard screening test. In comparison with IR31917-45-3-2, *O. brachyantha* and the F_1 hybrid were unsuitable for oviposition, survival, and growth of *C. medinalis*. However, growth and survival of larvae in artificial diets containing lyophilized leaf powder of IR31917-45-3-2 and *O. brachyantha* were comparable. Studies on the feeding and settling preference of the larval stages suggested that the mechanism of resistance of *O. brachyantha* is that of antixenosis type. Olfactometer tests with first-instar larvae and electroantennogram responses of adults indicated a lack or low levels of volatile attractants in *O. brachyantha* and the F_1 hybrid in comparison with IR31917-45-3-2. Bioassays of sequential solvent extracts of these plants indicated that larval preference for IR31917-45-3-2 and nonpreference for *O. brachyantha* and the F_1 hybrid were due partly to chemical factors present in hexane and methylene chloride extractables. Closer arrangement of silica cells in the epidermal layer of *O. brachyantha* and a higher mandibular wear in larvae reared on *O. brachyantha* suggested that physical resistance due to silica may be an additional cause of resistance. It was hypothesized that the high levels of resistance observed in *O. brachyantha* may be due to an additive or synergistic action of the absence of attractants or feeding stimulants, the presence of deterrents, and the physical resistance offered by silica. The significance of these results to a successful wide hybridization

*To whom correspondence should be addressed.
[1]International Centre of Insect Physiology and Ecology, P. O. Box 30772, Nairobi, Kenya. Based on IRRI.
[2]Current address: Department of Entomology, University of Wisconsin, Madison, Wisconsin 53706.

0098-0331/91/0100-0041$06.50/0

program aimed at transferring resistance factors from wild rice to cultivated rice is discussed.

Key Words—Wild rice, *Oryza brachyantha*, rice, *Oryza sativa*, host plant resistance, antixenosis, attractants, deterrents, silica, rice leaffolder, *Cnaphalocrocis medinalis*, Lepidoptera Pyralidae.

INTRODUCTION

The rice leaffolder *Cnaphalocrocis medinalis* (Guenée) (Lepidoptera: Pyralidae) has emerged as a major pest of rice in tropical and subtropical Asia (Heinrichs et al., 1985a). The insect has increased in importance both in upland and lowland ricefields, particularly in areas where modern varieties are grown extensively (Mohandas, 1975; Litsinger et al., 1987). Several outbreaks of the pest have been reported in Bangladesh, China, Fiji, India, Japan, Korea, Malaysia, Nepal, Philippines, Sri Lanka, and Vietnam (Khan et al., 1988). Breeding for leaffolder resistance in rice is considered a practical and safe means of controlling this pest in Asia (Heinrichs et al., 1985a). However, the degree of resistance observed in cultivated rice is only of a moderate level.

Wild relatives of cultivated crop plants often possess disease and insect resistance. High levels of insect resistance have been recorded in wild relatives of tobacco (Heusing and Jones, 1988), potato (Boiteau and Singh, 1988), dry bean (Cardona et al., 1989), and rice (Heinrichs et al., 1985a,b). Several wild relatives of *Oryza sativa* also were found to be resistant to rice leaffolders (Heinrichs et al., 1985a; Khan et al., 1989), of which several accessions of *Oryza brachyantha* were identified as highly resistant (Heinrichs et al., 1985a; Medina and Tyron, 1986). Since *O. brachyantha* and *O. sativa* are of different genomes, conventional techniques cannot be adopted for breeding purposes. However, recent developments in breeding techniques (Brar and Khush, 1986) permit such hybridization and, at the International Rice Research Institute (IRRI), there is an active program aimed at introducing useful genes for insect and disease resistance through wide hybridization.

Resistance of crop plants to leaf-feeding insects has been correlated with physical factors (Coley, 1983; Feeny, 1970; Webster, 1975); the presence of feeding deterrents (Otieno et al., 1985; Reynolds et al., 1985) metabolic inhibitors (Smith, 1985), toxic chemicals (Lin et al., 1987), and volatile repellents (Khan et al., 1987; Liu et al., 1988); or the absence of kairomones (Rembold and Tober, 1985). Understanding the bases of resistance and the biological activity of allelochemicals involved can facilitate breeding programs aimed at transferring resistant factors to cultivated crops (Davis et al., 1989; Rembold and Tober, 1985). Such knowledge also will ensure that resistant varieties do not adversely affect other biological control agents regulating the pests (Kauff-

man and Kennedy, 1989). The present study was undertaken to understand the mechanisms and causes of leaffolder resistance in *O. brachyantha* (accession no. 100115) and an F_1 hybrid obtained by crossing *O. brachyantha* with a high-yielding rice parent, IR31917-45-3-2, using the embryo rescue technique (Jena and Khush, 1984).

METHODS AND MATERIALS

Plants and Insects. Thirty- to 40-day-old plants of *O. brachyantha* (IRRI accession no. 100115) and a high-yielding rice leaffolder-susceptible breeding line of *O. sativa*, IR31917-45-3-2, grown in an insect-proof screenhouse, were used in the study. An F_1 hybrid of *O. brachyantha* and IR31917-45-3-2 was propagated as clones from three plants. Forty-day-old IR36 and TKM6 plants were used as the standard susceptible and resistant checks, respectively, in some of the experiments. The rice leaffolder *C. medinalis* was reared on IR36 plants in a greenhouse following the rearing method developed at IRRI (Waldbauer and Marciano, 1979).

Evaluation of Resistance. To confirm their resistance, *O. brachyantha*, IR31917-45-3-2, and their F_1 hybrid, along with IR36 and TKM6 plants, were tested against *C. medinalis* following the procedure described by Khan et al. (1989). Forty-day-old test plants were infested at the rate of two first-instar larvae per tiller. The larvae were enclosed by Mylar cages (22 cm diam. and 60 cm high). Fifteen days after infestation, the cages were removed, and the extent of damage of each leaf was determined by the standard evaluation system (IRRI, 1988). Damage ratings were computed using the number of leaves in each damage grade, and damage ratings were computed. All entries were replicated six times.

Sequential Solvent Extraction of Plants. Lyophilized leaves of IR31917-45-3-2, *O. brachyantha*, and the F_1 hybrid were ground in a UDY mill with a 100-mesh screen. One hundred grams of powder from each test plant was extracted in series twice with 200 ml each of *n*-hexane, methylene chloride, methanol, and water at room temperature for 24 hr. Organic solvents in each extract were evaporated under reduced pressure and water extractables were lyophilized to dryness. All extractables were weighed and redissolved in their respective solvents at desired concentrations for bioassays.

Orientation of First-Instar Larvae. The orientational response of first-instar *C. medinalis* larvae to crushed leaves of *O. brachyantha*, IR31917-45-3-2, and the F_1 hybrid was studied in an olfactometer. The olfactometer consisted of a glass tube (1 cm diam. and 30 cm long) with a median hole (0.3 cm diam.). Two plungers (glass tubes of 0.7 cm diam. and 10 cm long) one holding the test material (0.5 g of crushed leaf or a 5 × 2.5-cm filter paper treated with

extracts of test plant) and the other holding a rolled paper towel of equal dimension (for tests with leaf) or a filter paper treated with solvent (for tests with extracts) were slid into either end of the tube and placed such that their ends were 3 cm away from the median hole. The whole arrangement rested on a Perspex box (30 × 30 × 5 cm) filled with water. The top of the Perspex box was covered with a filter paper of equal dimension. The Perspex box was fitted onto a lower box holding a red 25-W electric bulb. The experiment was conducted in a dark room with the red light from the bottom. The water in the Perspex box prevented the warming up of the surface of the box from the heat generated by the bulb below. The filter paper on the surface of the box ensured further diffusion of the red light.

Ten newly emerged first-instar larvae of *C. medinalis* were released into the tube with a camel's hair brush. Air was pushed through the plungers at the rate of 1 ml/min with a peristaltic pump (LKB Bromma 12000 Variaperpex). After 3 min of release, the distribution of the larvae on either side of the center was recorded. To facilitate the quantification of the distribution of the larvae, 0.5 cm on either side of the release hole was designated as the starting point. Larvae in this section were considered as not having oriented and were excluded from the analysis. The percentage of larvae on the side of the stimulus (crushed leaf or extract-treated filter paper) and on the control side (rolled paper towel or solvent-treated filter paper) were recorded. The experiment was replicated 10 times and treatments were randomized within each replication. All the replications used a new tube with the position of the control and stimulus plungers interchanged.

Orientation responses of first-instar larvae to 2.5 μl of 500, 1000, 2500, and 5000 ppm of hexane, and methylene chloride extractables of the test plants were studied. Methanol and water extractables were not used, as preliminary bioassays indicated the absence of any activity. After application of the extract to the filter paper, an evaporation time of 1 min was allowed before they were inserted into the arms of the olfactometer. No air was pushed through the plungers in experiments with plant extracts.

Settling Preference of First- and Third-Instar Larvae. First- and third-instar larvae of *C. medinalis* were provided a choice of leafcuts of two test plants. For first-instar larvae, one leafcut (3 cm long) of each of the two test plants was placed equidistant opposite the other in petri dishes lined with moist filter paper, with the adaxial surface up. Cut ends of the leaves were wrapped with moist cotton. Ten newly emerged larvae were released in the center of the Petri dish. The Petri dishes were covered with perforated aluminum foil and kept in darkness. After 5 hr, the number of larvae settled on leafcuts of each test plant was counted.

For third-instar larvae, 6-cm-long leafcuts from 40-day-old plants were presented for 6 hr to four larvae starved but water-satiated for 3 hr. Four leafcuts of two test plants were arranged alternately equidistant on a circle in 15-cm-diam. Petri dish arena, whose lid was lined with 12.5-cm-diam. wet filter paper disk. Larvae were released in each Petri dish, and after 5 hr the number of larvae that settled on the leafcuts of the test plants were counted. The area eaten on each leafcut also was measured. Experiments with the first and third instars were replicated 10 and six times, respectively.

Bioassays of the sequential solvent extracts consisted of leafcuts of IR36 plants treated with either hexane, methylene chloride, methanol, or water extractables (10 μl on a 3-cm leafcut for first instars or 20 μl on a 6-cm leafcut for third instar) and were presented with their respective solvent-treated control leafcuts to 10 first-instar larvae or four third-instar larvae as described above. Experiments with the first-instar larvae were conducted at 312, 625, 1250, 2500, and 5000 ppm of each extractable. For the third instars, hexane and methylene chloride extractables were tested at 500, 1000, 2500, and 5000 ppm and methanol and water extractables were tested at 2500 and 5000 ppm only. The number of first-instar larvae settled on the treated and control leafcuts 5 hr after infestation were counted. The difference in the percentage of larvae that settled on the treated and the control leafcuts were used as an index of settling preference of the first-instar larvae. The leaf area fed by the third-instar larvae on treated and control leafcuts was measured after 24 hr. The difference in the area fed between control and treated leaves at each extract concentration was used as an index of feeding preference of the third instars.

Dispersal of First-Instar Larvae. A 6-cm leafcut of *O. brachyantha*, IR31917-45-3-2, F_1 hybrid, and the resistant check TKM6 were placed individually inside a Petri dish (9 cm diam.) lined with a moist filter paper. Ten first-instar *C. medinalis* larvae were released on each leafcut. The Petri dishes were arranged in a randomized block design consisting of 10 replicates in the laboratory. At 10-min intervals, the number of larvae remaining on the leafcut was counted for 1 hr. Subsequently, similar observations were taken at hourly intervals up to 5 hr.

Solvent extracts were bioassayed using IR36 leafcuts treated with 20 μl (10 μl on the adaxial and abaxial surface of the leafcut) of 1000, 2500, and 5000 ppm of hexane and methylene chloride extact. Observations on the number of larvae that settled on the leafcuts were taken 1 and 5 hr after infestation.

Feeding and Growth on Excised Leaves. To determine the leaf area consumed and percent increase in body weight, *C. medinalis* larvae starved for 3 hr but water-satiated were weighed individually on a microbalance (Mettler, ME30) and then enclosed singly in a Petri dish (9 cm diam.) with two leafcuts

(6 cm long) of the test plants. After 24 hr, larvae were starved but water-satiated for 4 hr so that all the green matter was excreted out of the gut. Individual larval weights were recorded and the percent weight increase in 24 hr was calculated for each test plant. The leaf area consumed by an individual larva was recorded according to Khan et al., (1989). The experiment was replicated 10 times.

The antifeedant activity of sequential solvent extracts was determined by enclosing a single third-instar larva with two IR36 leafcuts (6 cm long) treated with 20 μl of 2500 or 5000 ppm of extractables for 24 hr. The experiment was replicated 10 times. The leaf area scraped by the larvae was measured at the end of the experiment.

Survival and Growth on Potted Plants and Artificial Diets. Potted plants of *O. brachyantha*, IR31917-45-3-2, F_1 hybrid, IR36 (susceptible check), and TKM6 (resistant check) covered with Mylar cages (60 cm high and 22 cm diam.) were arranged in a randomized complete block design and infested with first-instar larvae in batches of 20. Each pot had five plants consisting of 17–20 tillers. Each treatment had six replications. The number of larvae surviving was counted 15 days after infestation. Subsequently, the plants were checked every day for pupation. A growth index was calculated as the ratio of the percentage of larvae developing into pupae to the mean growth period in days. The higher the growth index, the more suitable the plant was for *C. medinalis* growth. One day after pupation, the pupae were sexed and weighed on a microbalance (Mettler, ME30).

To study the growth responses of larvae on artificial diet containing test plant material, intact leaves of IR31917-45-3-2 and *O. brachyantha* were lyophilized and ground in a UDY mill with a 100-mesh screen. The leafpowder was incorporated in the leaffolder artificial diet developed at IRRI (Khan, 1987) at a ratio of 90% diet and 10% leaf powder. No leaf powder was added to one set of diet (control). Ten grams of treated or control diet were placed in each plastic cup and covered with a snug-fitting lid. Five first-instar larvae were released into each cup, and the cups were kept inverted at 28°C and 60 $\pm$ 5% relative humidity. Fifty cups were maintained for each treatment and the control. Five days after infestation, the percent mortality in each cup was recorded and the density of larvae was reduced to two per cup. Fifteen days after infestation, 20 randomly selected larvae from each treatment were weighed individually. The remaining larvae were allowed to pupate, and their larval duration and pupal weights were recorded.

Oviposition. To determine the ovipositional preference of mated female moths, *O. brachyantha*, IR31917-45-3-2, and the F_1 hybrid were planted in a seed box (55 $\times$ 40 $\times$ 15 cm). The plants were spaced 25 cm from each other. The seed box was covered with a cage (55 $\times$ 40 $\times$ 80 cm) made of a wooden frame and nylon mesh. Five pairs of field-collected moths were released in the center of each cage. Cotton dipped in 25% honey solution was provided in the

center of the cage on a wooden stake. The experiment was conducted in an insectary at a mean temperature of 27 ± 4°C and was replicated 10 times. After 48 hr, the number of eggs laid on each plant was counted.

Moth ovipositional response was tested by releasing five pairs of 3-day-old mated females on individual potted plants of *O. brachyantha*, IR31917-45-3-2, and F_1 hybrid. The plants were covered by Mylar cages (22 cm diam. and 60 cm high). The experiment was replicated 10 times in an insectary at 27 ± 4°C. The number of eggs laid was counted 48 hr after release.

Electroantennogram Responses of Adults. Antennal preparation for measuring the EAG responses of moths was the same as that described by Ramachandran et al. (1990). The stimulus was applied from a Pasteur pipet containing 215 mg of crushed leaves of test plants. Electroantennogram responses of ten 4-day-old females and ten 2-day-old males to leaves of IR36, TKM6, IR31917-45-3-2, *O. brachyantha*, and the F_1 hybrid were recorded. Antennae were stimulated at 2-min intervals and stimulation with a test leaf was followed and preceded by purging with a standard (100 μg of 1-hexanol). Absolute EAG potentials were converted to relative responses to the standard following the method of Guerin and Visser (1980).

Mandible Wear Pattern of Larvae Reared on Test Plants. Ten first-instar larvae were caged on three 40-day-old potted plants of IR31917-45-3-2 and *O. brachyantha*. Larvae were collected from the plants after 7, 14, and 21 days and preserved in 70% ethanol. The mandibles of the larvae were dissected and mounted on slides. Measurements on the width (estimate of the absolute size of mandible) and length (measure of wear-related changes in the incisor length) (Raupp, 1985) were recorded under a microscope fitted with an ocular micrometer.

Since the size and stage of larvae reared for the same duration on IR31917-45-3-2 and *O. brachyantha* differed significantly at the three different sampling times, a comparison of mandible size across the different sampling times was not valid. The width and length of mandibles of different instars of the larvae may be linearly related. If width is an absolute measure of mandible size and length a measure of the wear on the mandibular incisors (Raupp, 1985), then the relationship between the width and length of larvae reared on the test plants would be different if wear on the mandibles due to feeding on these plants is different. We tested this hypothesis by examining the differences in the linear relationship between width and length of mandibles of larvae reared on IR31917-45-3-2 and *O. brachyantha*.

Silica Deposition Pattern and Content. Leaves of 40-day-old IR31917-45-3-2, *O. brachyantha*, and the F_1 hybrid plants were cut into 2-cm pieces and immersed in 70% ethanol for three days. The leaves were placed on glass slides with their adaxial surface facing down. With a razor blade, the tissue from the abaxial surface was scraped until only the dorsal epidermal layer remained. A

2-mm piece of leaf was removed and boiled for 1 min in 20 ml phenol containing five drops of 0.1% aqueous safranin. The leaf pieces then were mounted on glass slides using the safranin–phenol solution and examined under a microscope. A regular pattern of silicified cells was observed in all three tests plants. Rows of silica cells were arranged longitudinally on the epidermal layer. A set of three to five widely spaced single rows of silica cells (designated as single rows) was arranged in between two closely arranged rows (designated as double rows). The number of single rows in between double rows, the distance between two single rows, the distance between the two single rows in a double row, and the number of silicified cells in a microscopic field were counted from 10 randomly selected leaf pieces. Crude silica content was estimated from oven-dried leaf samples of the test plants by the gravimetric method described by Yoshida et al. (1976).

Statistical Analysis. All percentage values were transformed to arcsine/p before analysis. Back-transformed means and standard errors are presented. In other cases, nontransformed data were used for analysis. Details of the analysis are presented along with the tables or figures.

RESULTS

Plant Damage. After 15 days of infestation, the percentage of leaves damaged on the test plants was significantly different ($F_{4,25} = 47.69$, $P < 0.001$). The lowest damage was observed on *O. brachyantha*, followed by the F_1 hybrid. The percentage of damaged leaves in IR31917-45-3-2, IR36, and the standard resistant check TKM6 were not significantly different. Damage rating based on the number of leaves with each damage grade (IRRI, 1988) was also the lowest on *O. brachyantha* and the F_1 hybrid, followed by TKM6 (Table 1).

Sequential Solvent Extraction of Plants. Sequential solvent extractions of IR31917-45-3-2, *O. brachyantha*, and the F_1 hybrid yielded 1.3, 1.8, and 1.6% with hexane; 1.7, 2.1, and 2.3% with methylene chloride; 8.6, 9.5, and 11.6% with methanol; and 4.2, 4.2 and 4.7% with water.

Orientation of First-Instar Larvae. A significantly higher proportion of first-instar larvae oriented towards crushed leaves of IR31917-45-3-2 than to the control (paired t test, $P < 0.01$) (Table 2). However, no significant difference was observed in the number of larvae orienting to *O. brachyantha* or F_1 hybrid as compared to those orienting to the control (Table 2).

Hexane extractables of IR31917-45-3-2 attracted first-instar *C. medinalis* larvae at all concentrations tested (Table 3). A similar attraction to methylene chloride extractables of IR31917-45-3-2 was evident at 2500 and 5000 ppm. Attraction to hexane extractables of *O. brachyantha* was significant at 1000 ppm. At the same concentration, significant orientation of larvae away from the treated side was observed for those exposed to methylene chloride extractables.

TABLE 1. *C. medinalis* FEEDING DAMAGE TO IR31917-45-3-2, *O. brachyantha*, F_1 HYBRID OF IR31917-45-3-2 AND *O. brachyantha*, TKM6, AND IR36 RICE PLANTS[a]

Plant	Damaged leaves (%)	Damage rating
IR31917-45-3-2	87 ± 5a	8.7ab
O. brachyantha	16 ± 2c	0.3d
F_1 (IR31917-45-3-2 × *O. brachyantha*)	32 ± 6b	2.3c
TKM6 (resistant check)	68 ± 5a	7.3b
IR36 (susceptible check)	75 ± 4a	0.9a

[a] In a column, means followed by the same letter are not significantly different [$P = 0.05$; Duncan's (1951) multiple range test]. Average of six replications; each replication had 17–20, 40-day-old, tillers infested with 20 first-instar larvae. Damage rating was calculated following the standard evaluation system for rice (IRRI, 1988).

TABLE 2. ORIENTATION OF FIRST-INSTAR LARVAE OF *C. medinalis* TO CRUSHED LEAVES OF IR31917-45-3-2, *O. brachyantha*, AND THEIR F_1 HYBRID[a]

Plant	Percent larvae orienting to		Difference and its significance
	Crushed leaf	Control	
IR31917-45-3-2	71 ± 3.5	29 ± 3.5	42**
O. brachyantha	57 ± 3.7	43 ± 3.7	14ns
F_1 hybrid	58 ± 4	42 ± 4	16ns

[a] Mean of 10 replications. ns, not significant.
**, significant at $P < 0.01$. Paired t test (Zar, 1984).

Settling Preference of First- and Third-Instar Larvae. A significantly higher number of larvae settled on IR31917-45-3-2 and TKM6 leaves when these leaves were presented with *O. brachyantha* and F_1 hybrid leaves in two-choice tests. When a choice was given between *O. brachyantha* and the F_1 hybrid, a significantly higher proportion of larvae was recovered from the F_1 hybrid (Table 4).

Similarly, the number of third-instar larvae that settled on *O. brachyantha* was significantly lower when presented with IR31917-45-3-2, the F_1 hybrid, and TKM6 in two-choice test (Table 4). However, no significant difference was observed in the settling behavior of the third-instar larvae when the F_1 hybrid was presented with IR31917-45-3-2 or TKM6. Larval feeding was greater on IR31917-45-3-2, TKM6, and the F_1 hybrid when presented in a two-choice test with *O. brachyantha*, and was more on IR31917-45-3-2 when presented with

TABLE 3. ORIENTATION OF FIRST-INSTAR *C. medinalis* LARVAE TO HEXANE AND METHYLENE CHLORIDE EXTRACTABLES OF IR31917-45-3-2 AND *O. brachyantha*[a]

Concentration	% larvae orienting to IR31917-45-3-2	Control	Difference ± SEM and its significance	% larvae orienting to *O. brachyantha*	Control	Difference ± SEM and its significance
Hexane extractables						
5000	68	32	36 ± 7**	53	47	6 ± 3ns
2500	64	36	28 ± 6**	49	51	− 2 ± 9ns
1000	68	32	36 ± 5**	62	38	24 ± 7*
500	64	36	28 ± 6**	54	46	9 ± 6ns
Methylene chloride extractables						
5000	69	31	38 ± 11*	54	46	9 ± 14ns
2500	69	31	38 ± 8*	46	54	− 8 ± 13ns
1000	56	44	12 ± 6ns	42	58	−16 ± 5*
500	52	48	4 ± 6ns	49	51	− 2 ± 6ns

[a]2.5 μl of the extractables were applied on a 5 × 2.5-cm filter paper and the solvent allowed to evaporate for 1 min before conducting the test. *$P < 0.05$, **$P < 0.01$, ns = not significant, paired *t* test (Zar, 1984).

the F_1 hybrid (Figure 1). However, there was no significant difference in the area fed on the test leafcuts when the choice was between the F_1 hybrid and TKM6 (Figure 1).

First-instar larvae preferred to settle on the control leafcuts when presented with leafcuts treated with either 5000 ppm hexane extractables or 1250, 2500 or 5000 ppm methylene chloride extractables of *O. brachyantha* and F_1 hybrid in a choice test (Figure 2, $P < 0.001$, paired *t* test). At lower concentrations, no significant preference for either control or treated leaves was observed. However, at 625 and 312 ppm of hexane extractables of IR31917-45-3-2, a significant preference for treated leaves was evident (Figure 2; differences were significant at $P < 0.05$, paired *t* test). Similarly, there was a significant preference for leafcuts treated with 5000 ppm of methanol extractables and 312 ppm of water extractables of IR31917-45-3-2.

Third instars fed significantly more on the control leaves when presented with leaves treated with 500, 1000, 2500, and 5000 ppm of hexane or methylene chloride extractables of *O. brachyantha* in a choice test (Figure 3) ($P < 0.001$, paired *t* test). No significant differences were evident in tests with hexane and methylene chloride extractables of IR31917-45-3-2. The methanol and water extractables of *O. brachyantha* and IR31917-45-3-2 did not affect the feeding preference of the third-instar larvae.

TABLE 4. SETTLING RESPONSE OF FIRST-INSTAR AND THIRD-INSTAR LARVAE OF *C. medinalis* ON LEAFCUTS OF *O. brachyantha*, IR31917-45-3-2, F_1 HYBRID OF *O. brachyantha* AND IR31917-45-3-2, AND TKM6 IN TWO-CHOICE TESTS[a]

	First instar		Third instar	
Plant	Larvae that settled (%)	Difference[b]	Larvae that settled (%)	Difference[b]
IR31917-45-3-2	86 ± 3	83**	71 ± 8	58*
O. brachyantha	3 ± 6		13 ± 9	
IR31917-45-3-2	82 ± 6	72**	36 ± 6	-4^{ns}
F_1 hybrid	10 ± 4		42 ± 8	
F_1 hybrid	37 ± 7	30**	50 ± 0	42**
O. brachyantha	7 ± 4		8 ± 5	
TKM6	83 ± 8	83**	58 ± 11	50*
O. brachyantha	0 ± 0		8 ± 5	
TKM6	79 ± 4	69**	50 ± 7	13^{ns}
F_1 hybrid	10 ± 3		38 ± 6	

[a] Data are averages of ten replications.
[b] ns, not significant; *significant, $P < 0.05$; **$P < 0.01$, ns = not significant (paired t test, Zar, 1984).

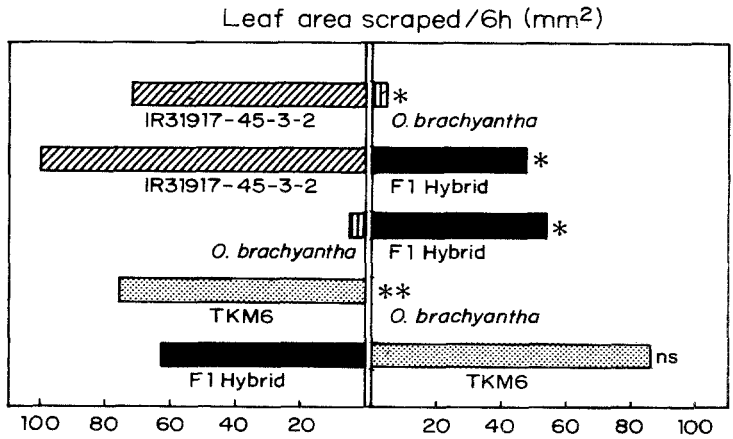


FIG. 1. Leaf area scraped (mm^2)/third instar *C. medinalis* larva/6 hr in choice tests. *Significant difference, paired t test, $P < 0.05$ (Zar, 1984).

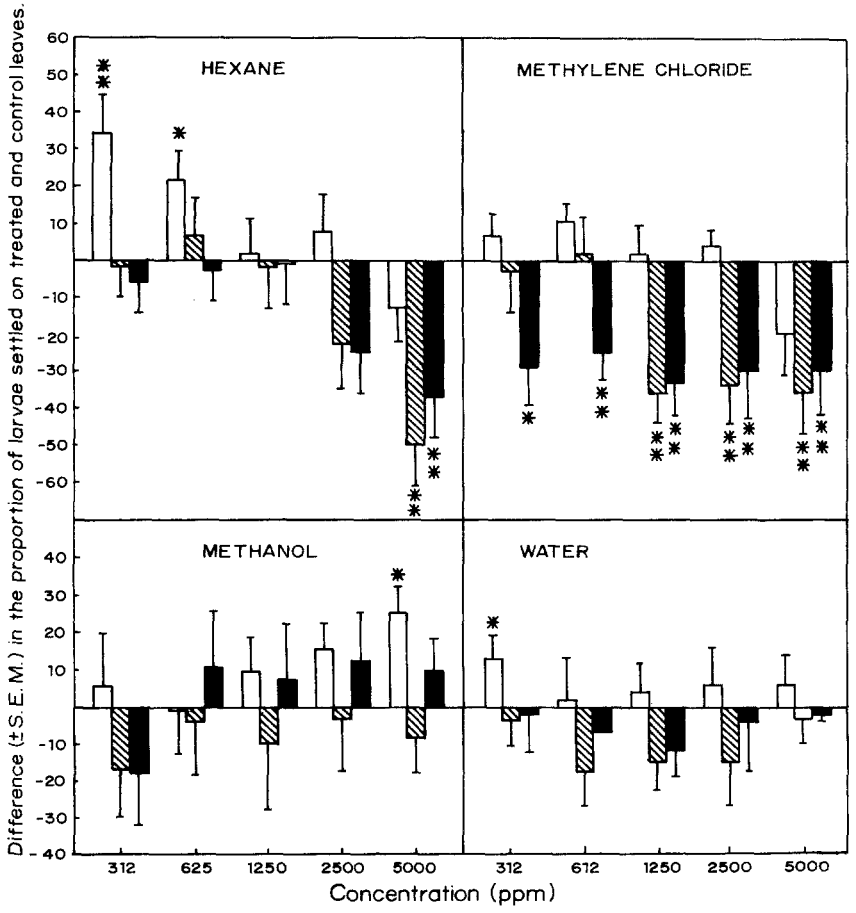


FIG. 2. Differences in the proportion of first-instar *C. medinalis* larvae settled on extract-treated and their respective solvent-treated control leafcuts [(number of larvae settled on treated leafcuts—number of larvae settled on control leafcuts/total number of larvae recovered) × 100]. Empty bars = extractables of IR31917-45-3-2; lined bars = extractables of *O. brachyantha*; filled bars = extractables of F_1 hybrid. Positive difference indicates a preference for treated leafcuts. *Significant at $P < 0.05$, **significant at $P < 0.001$, paired t test (Zar, 1984). Vertical lines indicate standard error of difference.

Dispersal of First-Instar Larvae. Ten minutes after release, significantly fewer larvae remained settled on *O. brachyantha* and the F_1 hybrid as compared with those on IR31917-45-3-2 and TKM6 (Figure 4). The rate of decrease in the number of larvae that remained settled on *O. brachyantha* and the F_1 hybrid leaves was higher than that for larvae on IR31917-45-3-2 and TKM6. At the

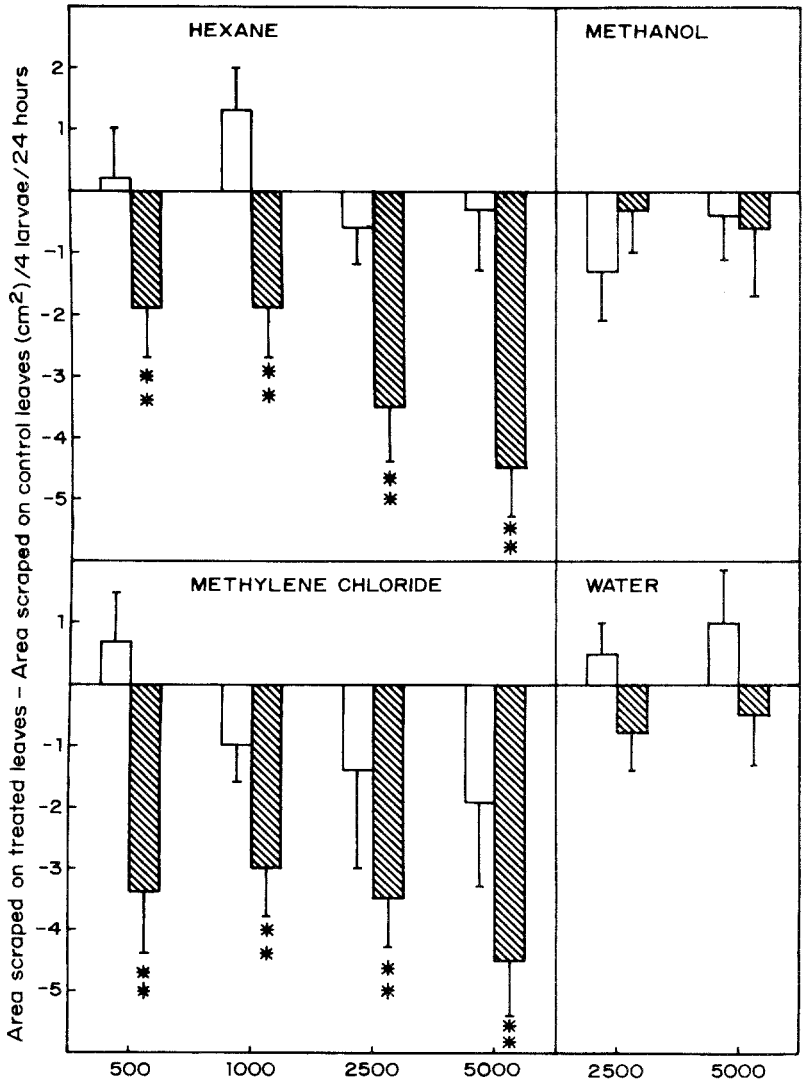


FIG. 3. Differences in the area scraped/4 third-instar *C. medinalis* larvae/24 hr between sequential solvent extractable-treated leafcuts and their respective solvent-treated leafcuts in choice tests. Empty bars = extractables of IR31917-45-3-2; lined bars = extractables of *O. brachyantha*. *Significant at $P < 0.05$, **significant at $P < 0.001$, paired t test, (Zar, 1984). Vertical bars indicate standard error of difference.

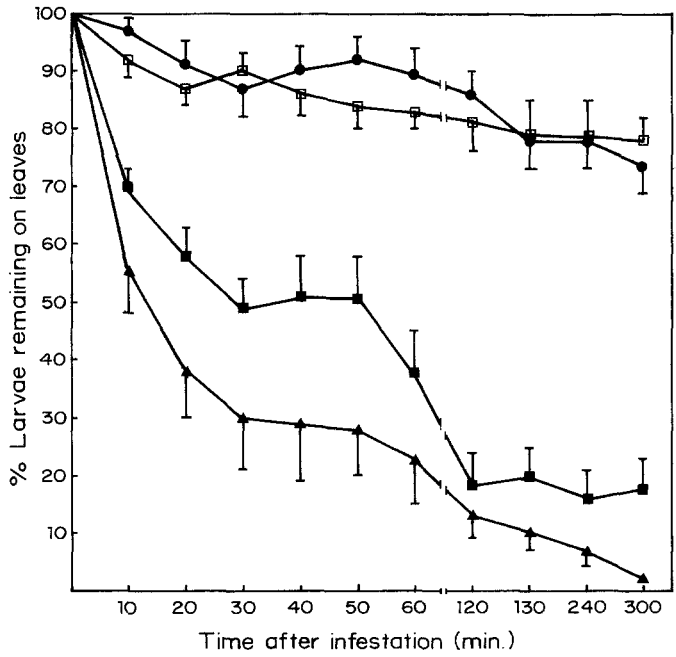


FIG. 4. Percentage of first-instar *C. medinalis* larvae settling on rice leaves (● = IR31917-45-3-2, □ = TKM6, ■ = F_1 hybrid, ▲ = *O. brachyantha*) from 0 to 300 min postinfestation. Vertical bars indicate standard error of means.

end of the experiment, only 15% and 1% of larvae remained settled on the F_1 hybrid and *O. brachyantha*, respectively.

In a similar bioassay with plant extracts, a significantly higher number of first-instar larvae remained settled on control leaves than on leaves treated with extractables of all three plants (Figure 5). A significantly higher proportion of larvae remained settled on leaves treated with hexane and methylene chloride extractables of IR31917-45-3-2 compared to leaves treated with the same solvent extractables of *O. brachyantha*. The proportion of larvae that remained settled on the leaves treated with extractables from the F_1 hybrid was intermediate to both its parents. Trends were similar at both 1-hr and 5-hr observation times (Figure 5).

Feeding and Growth on Excised Leaves. In no-choice tests, the area fed by the third-instar larvae in 24-hr was significantly lower on *O. brachyantha* than on other test plants ($F_{3,24} = 16.2$, $P < 0.01$) (Table 5). Larvae gained ca. 30% of their initial weight when fed for 24 hr on IR31917-45-3-2, IR36, or TKM6 leaves. Larval weight gain after feeding on the F_1 hybrid leaves was

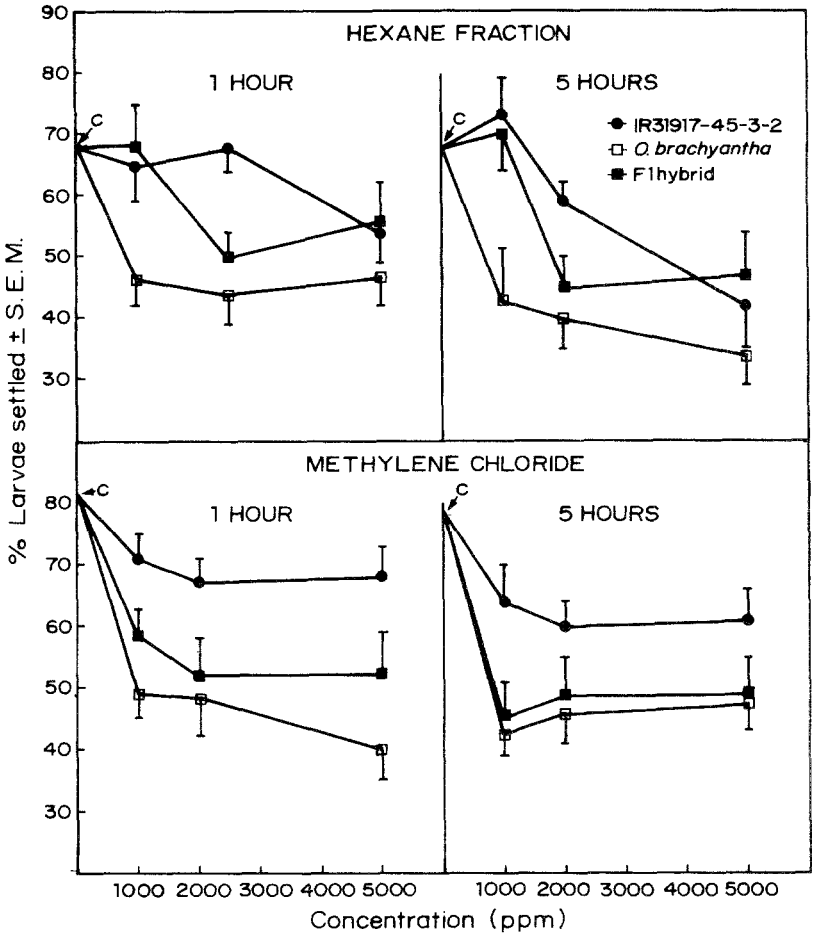


FIG. 5. Settling behavior of first-instar *C. medinalis* larvae to hexane and methylene chloride extractables of IR31917-45-3-2, *O. brachyantha*, and their F_1 hybrid in a no-choice test, c = solvent treated control.

significantly lower (21%). Weight gain was only 1% on *O. brachyantha* leaves (Table 5).

The area of leaf scraped in 24 hr by third-instar larvae on leaves treated with 5000 and 2500 ppm of hexane, methylene chloride, methanol, or water extracts of IR31917-45-3-2, *O. brachyantha*, and the F_1 hybrid did not differ significantly from that of their respective controls.

Survival and Growth on Potted Plants and Artificial Diets. Fifteen days after infestation, survival of larvae on IR31917-45-3-2, IR36, and TKM6 were

TABLE 5. PHYSIOLOGICAL RESPONSES OF *C. medinalis* LARVAE FED IR31917-45-3-2, *O. brachyantha*, F_1 HYBRID OF IR31917-45-3-2 AND *O. brachyantha*, IR36, AND TKM6 RICE PLANTS[a]

Plant	Leaf area scraped/larvae per 24 hr (cm^2)[b]	Weight gain/ larva per 24 hr[b] (%)	Larval survival 15 DAI[c]	Pupation (%)[c]	Larval period (days)[c]	Growth index[c,d]	Pupal weight (mg)[c]
IR31917-45-3-2	3.66 ± 0.9a	33.7 ± 6.4a	71 ± 3a	71 ± 3a	17.9 ± 0.8c	4.0a	20.9 ± 1.1a
O. brachyantha	0.54 ± 0.2b	1.3 ± 3.8b	12 ± 3c	0c			
F_1 (IR31917-45-3-2 × *O. brachyantha*)	2.84 ± 0.6a	21.1 ± 4ab	40 ± 6b	36 ± 5b	22.0 ± 0.9a	1.6b	14.8 ± 1.0c
TKM6 (resistant check)	3.74 ± 0.6a	34.8 ± 5.2a	62 ± 5a	62 ± 5a	22.0 ± 0.5a	2.8ab	17.6 ± 1.3b
IR26 (susceptible check)	3.8 ± 0.8a	36.4 ± 6.4a	70 ± 4a	70 ± 4a	19.4 ± 0.8b	3.6a	21.2 ± 1.1a

[a] In a column, means followed by the same letter are not significantly different [$P = 0.05$; Duncan's (1951) multiple range test].
[b] Average of ten replications.
[c] Average of six replications; each replication had 20 newly emerged larvae caged on 40-day-old plants.
[d] Growth index, percent larvae becoming pupae divided by mean development period.

similar and significantly higher than on *O. brachyantha* and the F_1 hybrid (Table 5). The larvae surviving on *O. brachyantha* were still in their second and third stadia, while those on IR31917-45-3-2, IR36, and TKM6 were already in their fourth and fifth stadia. All larvae that had survived up to 15 days pupated on IR31917-45-3-2, IR36, and TKM6, whereas no pupation occurred on *O. brachyantha* (Table 5). Heavier pupae were obtained from IR31917-45-3-2 and IR36 than from TKM6 and the F_1 hybrid. Larval duration was longer on TKM6 and the F_1 hybrid than on IR36 and IR31917-45-3-2 (Table 5).

Five days after infestation, the percentage of larvae that survived did not differ significantly between artificial diet without any leaf powder (control) and diet incorporated with leaf powder of IR31917-45-3-2 or *O. brachyantha* (Table 6). Subsequently, 96% of the larvae pupated in control diets while 77% and 69% pupated in IR31917-45-3-2 and *O. brachyantha* leaf powder-incorporated diets, respectively (Table 6). Incorporation of leaf powder significantly decreased the larval and pupal weights as compared with those on control diets (Table 6). Larval duration in the leaf powder-incorporated diets also was delayed significantly in comparison to larvae fed control diets. However, the performance of larvae in diets with IR31917-45-3-2 leaf powder was comparable to larvae fed diets with *O. brachyantha* leaf powder (Table 6).

Oviposition. Adult female *C. medinalis* preferred to oviposit on IR31917-45-3-2 plants in choice tests with *O. brachyantha* and the F_1 hybrid. Seventy-three percent of the eggs were laid on the IR31917-45-3-2, 24% on F_1 hybrid, and 5% on *O. brachyantha* (Table 7).

The ovipositional response of *C. medinalis* to the three test plants also differed significantly in a no-choice oviposition test ($F_{2,18} = 23.32$, $P < 0.01$).

TABLE 6. PERFORMANCE OF *C. medinalis* FED ARTIFICIAL DIET INCORPORATED WITH LEAF POWDER OF IR31917-45-3-2 AND *O. brachyantha*[a]

Parameter	Control	IR31917-45-3-2	*O. brachyantha*
Survival/cup after 5 days (%)[b]	50.7 ± 2.5a	50.6 ± 2.7a	51.4 ± 2.9a
Survival to pupation (%)[c]	96	76.5	69.10
Larval weight (mg)[d]	7.35 ± 0.98a	2.95 ± 0.96b	4.33 ± 1.0ab
Larval duration (days)	25 ± 0.7a	29.1 ± 0.8b	30.3 ± 0.7b
Pupal weights (mg)	22.9 ± 0.5a	20.7 ± 0.5b	19.2 ± 0.5b

[a] Freeze-dried leaf powder was incorporated in a leaffolder artificial diet (Khan, 1987). Means in a row not followed by the same letter are significantly different, Duncan's (1951) multiple range test, $P < 0.05$.

[b] On the sixth day, the density/cup was reduced to two.

[c] Survival to pupation was calculated on the basis of the proportion of all caterpillars that pupated after the sixth day.

[d] Weight of 22 randomly selected larvae, 15 days after infestation.

The number of eggs laid on IR31917-45-3-2 was significantly more than that laid on the F_1 hybrid and *O. brachyantha* (Table 7).

Electroantennogram Responses of Adults. The EAG responses of both the male and female moths of *C. medinalis* to crushed leaves of IR36, TKM6, and IR31917-45-3-2 were similar and significantly higher than the EAG response to leaves of *O. brachyantha* (Table 8). The response to leaves of the F_1 hybrid was intermediate to the responses to IR31917-45-3-2 and *O. brachyantha* (Table 8).

Mandible Wear Pattern of Larvae Reared on Test Plants. Within both rices tested, there was a significant linear relationship between mandible width and length when data for 7-, 14-, and 21-day sampling dates were combined (Figure 6; linear regression significant, $P < 0.01$). The elevation of the regression line for larvae reared on IR31917-45-3-2 was significantly higher than that for larvae reared on *O. brachyantha* (Figure 6, $F_{1,103} = 16.96$, $P < 0.001$). This indicates that, for a given width of mandible, the length was greater in larvae reared

TABLE 7. OVIPOSITION OF *C. medinalis* ON IR31917-45-32, *O. brachyantha*, AND THEIR F_1 HYBRID IN CHOICE AND NO-CHOICE TESTS[a]

	No-choice (No. of eggs/5 females/2 days)	Choice (% eggs laid)
IR31917-45-3-2	170 ± 20a	73 ± 5a
O. brachyantha	40 ± 4c	5 ± 2c
F_1 hybrid	83 ± 9b	22 ± 5b

[a] In a column, means followed by the same letter are not significantly different [$P < 0.05$, Duncan's (1951) multiple range test].

TABLE 8. EAG RESPONSES OF *C. medinalis* TO CRUSHED LEAVES[a] OF RICE PLANTS

Rice plant	Relative EAG responses[b] ± SEM	
	Female	Male
IR36	79.5 ± 4.0 a	83.8 ± 1.6 a
TKM6	80.3 ± 4.4 a	88.1 ± 8.7 a
IR31917-45-3-2	81.3 ± 4.3 a	94.6 ± 3.7 a
Oryza brachyantha	65.3 ± 3.6 b	69.6 ± 3.5 b
F_1 hybrid	70.2 ± 3.8 ab	80.5 ± 1.8 ab

[a] 215 mg of crushed rice leaves were used as a stimulus source. Means in a column not followed by the same letter are significantly different, Duncan's (1951) multiple range test, $P < 0.05$.
[b] Response relative to 10 μl of 10 mg/ml solution of 1-hexanol in heptane.

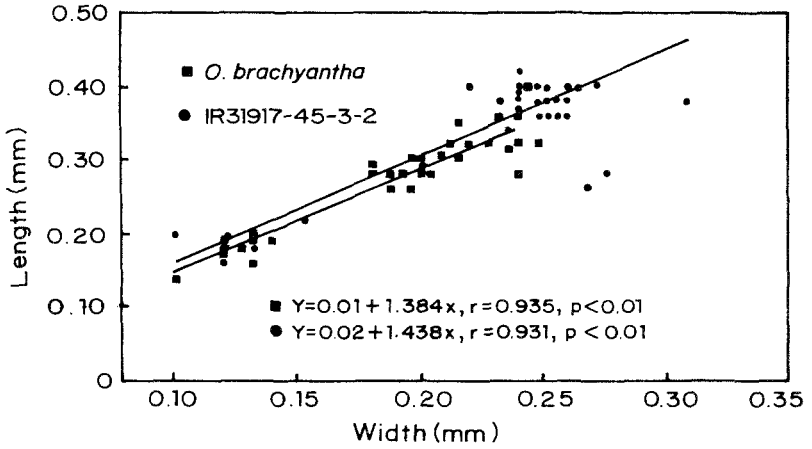


FIG. 6. Relationship between mandible width and mandible length of *C. medinalis* larvae reared on IR31917-45-3-2 and *O. brachyantha*.

on IR31917-45-3-2 than on *O. brachyantha*, suggesting a higher mandibular wear in larvae feeding on the latter plants.

Silica Deposition Pattern and Content. IR31917-45-3-2, *O. brachyantha*, and the F_1 hybrid did not differ significantly in the number of single rows of silica cells deposited between two double rows. The distance between two adjacent single rows of silica cells was smaller in *O. brachyantha* and the F_1 hybrid (Table 9) as compared with IR31917-45-3-2. The two rows in a double row of silica cells were more closely placed in *O. brachyantha* as compared with IR31917-45-3-2; in the F_1 hybrid, this value was intermediate. *O. brachyantha* leaves had a significantly higher number of silica cells per unit area than IR31917-45-3-2 or the F_1 hybrid (Table 9). The silica content of the leaves of the three test plants did not differ significantly.

DISCUSSION

The suitability of plants as hosts to insects is determined by an array of interactions between insect responses to various plant characteristics (Saxena, 1969). The main categories of behavioral and physiological responses during insect establishment on plants are orientation, settling, feeding, metabolism of ingested food, growth, adult survival, egg production, and oviposition (Saxena, 1969). A plant's resistance to insects could be due to the disruption of one or more of the physiological and behavioral responses of the insect to the plant characteristics (Khan and Saxena, 1985). Depending on whether the plant char-

TABLE 9. SILICA DEPOSITION PATTERN IN EPIDERMAL LAYER OF ADAXIAL SURFACE AND SILICA CONTENT OF IR31917-45-3-2, *O. brachyantha* AND THEIR F_1 HYBRID LEAVES[a]

Parameter	IR31917-45-3-2	*O. brachyantha*	F_1 hybrid
Number of single rows of silica cells in between double rows	4.1 ± 0.3a	4.6 ± 0.2a	4.2 ± 0.2a
Distance between adjacent rows of silica cells in double rows (mm)	0.0535 ± 0.005a	0.0412 ± 0.001b	0.0041 ± 0.003b
Distance between adjacent single rows	0.1718 ± 0.006a	0.1162 ± 0.005c	0.1426 ± 0.008b
Number of silica cells/unit area	72 ± 5.0a	86 ± 4b	70 ± 5a
Silica content (%)	4.73 ± 0.30a	4.48 ± 0.14a	5.21 ± 0.67a

[a] Means in a row followed by the same letter are significantly different. Duncan's (1951) multiple range test, $P < 0.05$.

acteristics affect the insect's behavior or physiology, the modalities of resistance of a plant could be classified as antixenosis or antibiosis (Painter, 1951; Wiseman, 1985).

Our results indicated that the resistance of *O. brachyantha* to *C. medinalis* is due to strong nonpreference. Nonpreference of adults was expressed in the lower number of eggs laid on *O. brachyantha* in both choice and no-choice tests. In the larval stages, nonpreference was expressed in the small proportion of first- and third-instar larvae settling on *O. brachyantha* when presented with the other test plants in choice tests. In no-choice experiments, a large proportion of larvae crawled off the leaves of *O. brachyantha*. A similar phenomenon was observed in larvae of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), which crawled off a resistant corn line Antigua 2D-118 (Wiseman et al., 1983). These effects against adult oviposition and first-instar settling also were observed in the F_1 hybrid but the magnitude was less than that in *O. brachyantha*.

The low survival and reduced growth rate of larvae on potted plants of *O. brachyantha* as compared with other rice plants may indicate high levels of antibiosis in this wild rice. However, the absence of significant differences in the growth and survival of larvae in artificial diets incorporated with the leaf powder of *O. brachyantha* and IR31917-45-3-2 suggests that mortality of larvae on the wild rice may have been due to reduced food intake and consequent starvation. Low levels of feeding by third-instar larvae on excised leaves of *O. brachyantha* as compared with those on the rice plants further support this conclusion. The growth responses of larval stages fed artificial diets containing

leafpowder of resistant plants have been considered an accurate indicator of antibiotic properties of resistant plants (Wilson et al., 1984; Wiseman, 1985). Incorporation of leaf powder of TKM6, a resistant rice variety, significantly reduced the growth and pupal weights of *C. medinalis* as compared with other test plants, suggesting that antibiosis is one of the mechanisms of resistance in this variety (Khan et al., 1989).

Our studies on the orientation of the first-instar *C. medinalis* larvae and electroantennogram responses of adult moths to leaves of the test plants suggested that susceptible IR31917-45-3-2 may contain volatile chemicals that are attractant to both larvae and adults. The neutral reaction of first-instar larvae and the lower EAG response of adult moths to volatiles from crushed leaves of *O. brachyantha* and the F_1 hybrid indicate either a lack or low levels of these chemicals in these plants. The presence of volatile chemicals that are attractant or repellent and their relative proportion in soybeans were attributed as one of the factors that confer resistance (Liu et al., 1988). Differential attraction of *Heliothis armigera* (Hubner) to resistant and susceptible pigeonpea cultivars has been related to differences in the relative concentation of volatile compounds in their aromagrams (Rembold and Tober, 1985). Headspace analysis of the volatile profiles of strawberry cultivars during periods of resistance and susceptibility to *Tetranychus urticae* Koch suggested that causes of resistance may be due to a buildup of volatile salicylates accompanied by accumulation of nonvolatile phenols (Hamilton-Kemp et al., 1988). The olfactory sensitivity of the rice leaffolders to several plant volatiles is known (Ramachandran et al., 1990). Identification of the volatile chemicals emanating from the plants in this study would enable us to determine empirically the exact role of these chemicals in the resistance of *O. brachyantha*.

The susceptibility of IR31917-45-3-2 and the resistance of *O. brachyantha* and the F_1 hybrid were due partly to allelochemicals, extractable with hexane and methylene chloride, as indicated by the results of bioassays with the solvent extracts of these plants. Results of olfactometer tests and settling preference assays with first-instar larvae suggested that one of the causes of susceptibility of IR31917-45-3-2 might be the presence of attractants or feeding stimulants in these fractions. The settling preference of first-instar larvae in choice and no-choice tests and the feeding preference of third instars in choice tests suggest the presence of deterrent chemicals in the hexane and methylene chloride extractables of *O. brachyantha* and the F_1 hybrid plants. However, these deterrents did not have any antifeedant effect on the third-instar larvae, probably because of the low biological activity of these deterrent chemicals.

Bernays and Chamberlain (1982) and Raubenheimer and Simpson (1990) found that deterrent effects of tannic acid to *Locusta migratoria* (L.) were observed only in a choice test and not in no-choice feeding trials. Raubenheimer and Simpson (1990) hypothesized that a feeding deterrent either raises the

threshold for initiation of insect feeding, so that feeding begins only when the inhibitory feedback from the gut and blood is lower, or lowers the threshold for meal termination so that the insect stops feeding at a lower gut volume. Both these mechanisms may result in reduced consumption by an insect. However, if the thresholds for initiation and termination of feeding are altered equally, then the patterns of feeding would be unaffected by the feeding deterrent, resulting in similar quantities of consumption in controls and treatments (Raubenheimer and Simpson, 1990). This also may explain why the feeding deterrents in the hexane and methylene chloride fractions of *O. brachyantha* did not show any antifeedant properties against the third-instar larvae of *C. medinalis*.

The loss of adverse effects of *O. brachyantha* on the biology of *C. medinalis* when *O. brachyantha* was incorporated into artificial diets and the absence of antifeedant effects of the sequential solvent extracts prompted us to examine the possibility of additional physical causes of resistance in *O. brachyantha*. The pattern of silica deposition on the epidermal layer of rice leaves and its wearing effect on the mandibles of the larval stages has been considered one of the factors that contributed to the rice plant's resistance to leaffolders (Hanifa et al., 1974). The difference in the elevation of regression lines between the width (a measure of the absolute size of the jaw) and the length (a measure of the wear on the incisor) of the mandibles of larvae reared on *O. brachyantha* and IR31917-45-3-2 suggests that mandible wear of larvae feeding on wild rice may have been higher. More closely placed rows of silica cells in the leaves of *O. brachyantha* as compared with those in IR31917-45-3-2 may have conferred some level of physical resistance to feeding by *C. medinalis* larvae on this wild rice.

The high degree of resistance of *O. brachyantha* to leaffolders appears to be the result of additive or synergistic action from a lack of volatile attractants, the absence of less volatile attractants and/or feeding stimulants, the presence of feeding deterrents, and physical resistance offered by the closer arrangement of silica cells. The probability of transferring leaffolder resistance to cultivated rice by wide hybridization with *O. brachyantha* seems fair, considering the multiplicity of resistance factors involved. However, the degree of resistance obtained may not be very high, if our hypothesis—that resistance in *O. brachyantha* is the result of joint action of the resistance factors involved—is correct. Wide hybridization to improve the degree of resistance in some cultivars (i.e., choosing a rice parent that already has one or more of the resistance factors) may be a more successful strategy.

Acknowledgments—The paper reports collaborative research done by the International Centre of Insect Physiology and Ecology, Nairobi, Kenya, and the International Rice Research Institute, Manila, Philippines. We thank Dr. G.S. Khush and Dr. K.K. Jena, IRRI, Department of Plant Breeding, Genetics, and Biochemistry for providing the F_1 hybrid plants.

REFERENCES

BERNAYS, E.A., and CHAMBERLAIN, D.J. 1982. The significance of dietary tannin for locusts and grasshoppers. *J. Nat. Hist.* 16:261–266.

BOITEAU, G., and SINGH, R.P. 1988. Resistance to the Greenhouse whitefly, *Trialeurodes vaporarium* (Westwood) (Homoptera: Aleyrodidae), in a clone of the wild potato *Solanum berthaultii* Hawkes. *Ann. Entomol. Soc. Am.* 81:428–431.

BRAR, D.A. and KHUSH, G.S. 1986. Wide hybridization and chromosome manipulation in cereals. *In* D.A. Evans, W.R. Sharp, and P.V. Ammirato (eds.). Handbook of Plant Cell Culture. Macmillan, New York. 221–263.

CARDONA, C., CARNEM, E.P. KORNEGAY, J., VALOR, J., and SERRANO, M. 1989. Antibiosis effects of wild dry bean accessions on the Mexican bean weevil and the bean weevil (Coleoptera: Bruchidae). *J. Econ. Entomol.* 82:310–315.

COLEY, P.D. 1983. Herbivory and defensive characteristics of tree species in a lowland tropical forest. *Ecol. Monogr.* 53:209–233.

DAVIS, F.M., NG SEN-SEONG, and WILLIAMS, W.P. 1989. Mechanisms of resistance in corn to leaf feeding by Southwestern corn borer and European corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 82:919–922.

DUNCAN, D.B. 1951. A significant test for differences between ranked treatments in an analysis of variance. *Va. J. Sci.* 2:171–189.

FEENY, P.P. 1970. Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* 51:565–581.

GUERIN, P.M., and VISSER, J.H. 1980. Electroantennogram responses of the carrot fly, *Psila rosae* to volatile plant components. *Physiol. Entomol.* 5:111–119.

HAMILTON-KEMP, T.R., ANDERSEN, R.A., RODRIGUES, J.G., LOUGHRIN, J.H., and PATTERSON, C.G., 1988. Strawberry foliage headspace vapor components at periods of susceptibility and resistance to *Tetranychus urticae* Koch. *J. Chem. Ecol.* 14:789–796.

HANIFA, A.M., SUBRAMIANIAM, T.R., and PONNAIYA, B.W.X. 1974. Role of silicon in resistance to the leaf roller, *Cnaphalocrocis medinalis* Guenée in rice. *Indian J. Exp. Biol.* 12:463–465.

HEINRICHS, E.A., CAMANAG, E., and A. ROMENA. 1985a. Evaluation of rice cultivars for resistance to *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 78:274–278.

HEINRICHS, E.A., VIAJANTE, V.D., and A. ROMENA. 1985b. Resistance of wild rices, *Oryza* spp. to the whorl maggot *Hydrellia philippina* Ferino (Diptera: Ephydridae). *Environ. Entomol.* 14:404–407.

HEUSING, J., and JONES, D. 1988. Antibiosis to the tobacco hornworm (*Manduca sexta*) in wild species of *Nicotiana*. *Entomol. Exp. Appl.* 47:267–274.

IRRI (INTERNATIONAL RICE RESEARCH INSTITUTE). 1988. Standard evaluation system for rice. The International Rice Testing Programme, P.O. Box 933, Manila, Philippines.

JENA, K.K., and KHUSH, G.S. 1984. Embryo rescue of interspecific hybids and its scope in rice improvement. *Int. Rice Genet. Newsl.* 1:133–134.

KAUFFMAN, W.C., and KENNEDY, G.G. 1989. Toxicity of allelochemicals from wild insect-resistant tomato *Lycopersicon hirsutum* f. *glabratum* to *Campoletis sonorensis*, a parasitoid of *Heliothis zea*. *J. Chem. Ecol.* 7:2051–2060.

KHAN, Z.R. 1987. Artificial diet for rearing rice leaffolder (LF). *Int. Rice Res. Newsl.* 12(6): 30–31.

KHAN, Z.R., and SAXENA, R.C. 1985. Behavioral and physiological responses of *Sogatella furcifera* (Homoptera: Delphacidae) to selected resistant and susceptible rice cultivars. *J. Econ. Entomol.* 78:1280–1286.

KHAN, Z.R., CIEPIELA, A., and NORRIS, D.M. 1987. Behavioral and physiological response of

cabbage looper, *Trichoplusia ni* (Hubner) to steam distillates from resistant versus susceptible soybean plants. *J. Chem. Ecol.* 13:1903–1915.

KHAN, Z.R., BARRION, A.T., LITSINGER, J.A., CASTILLA, N.P., and JOSHI, R.C. 1988. A bibliography of rice leaffolders (Lepidoptera: Pyralidae). *Insect Sci. Appl.* 9:129–174.

KHAN, Z.R., RUEDA, B.P., and CABALLERO, P. 1989. Behavioral and physiological responses of rice leaffolder, *Cnaphalocrocis medinalis* to selected wild rices. *Entomol Exp. Appl.* 52:7–13.

LIN, S.Y.H., TRUMBLE, J.T., and KUMAMOTO, J. 1987. Activity of volatile compounds in glandular trichomes of *Lycopersicon* species against two insect herbivores. *J. Chem. Ecol.* 7:707–716.

LITSINGER, J.A., BARRION, A.T., and SOEKARNA, D. 1987. Upland rice insect pests: Their ecology, importance, and control. IRRI Research Paper Series No. 123. International Rice Research Institute, Los Banos, Laguna, Philippines.

LIU, S.H., NORRIS, D.M., and MARTI, E. 1988. Behavioral responses of female adult *Trichoplusia ni* to volatiles from soybeans versus a preferred host, lima bean. *Entomol. Exp. Appl.* 49:99–109.

MEDINA, E.B., and TYRON, E.H. 1986. Resistance of selected *Oryza sativa* and *O. brachyantha* cultivars to the rice leaffolder. *Int. Rice Res. Newsl.* 11(6):10–11.

MOHANDAS, N. 1975. Studies on the chemical control and insect-plant relationship of the rice leafroller, *Cnaphalocrocis medinalis* Guenée (Pyraustidae: Lepidoptera). PhD thesis. Kerala Agricultural University, Kerala, India.

OTIENO, D.A., HASSANALI, A., and NJOROGE, P.W. 1985. Chemical basis of TVu 946 stem resistance to *Maruca testulalis* (Geyer). *Insect. Sci. Appl.* 6:259–262.

PAINTER, R.H., 1951. Insect Resistance in Crop Plants. Macmillan, New York.

RAMACHANDRAN, R., KHAN, Z.R., CABALLERO, P., and JULIANO, B.O. 1990. Olfactory sensitivity of two sympatric species of rice leaffolders (Pyralidae: Lepidoptera) to plant volatiles. *J. Chem. Ecol.* 16:2647–2666.

RAUBENHEIMER, D., and SIMPSON, S.J. 1990. The effects of simultaneous variation in protein, digestible carbohydrate and tannic acid on the feeding behavior of larval *Locusta migratoria* (L.) and *Schistocerca gregaria* (Forskal). I. Short-term studies. *Physiol. Entomol.* 15:219–233.

RAUPP, M.J. 1985. Effects of leaf toughness on mandibular wear of leaf beetle, *Plagiodera versicolora. Ecol. Entomol.* 10:73–79.

REMBOLD, H., and TOBER, H. 1985. Kairomones as pigeonpea resistance factors against *Heliothis armigera. Insect Sci. Appl.* 6:249–252.

REYNOLDS, G.W., SMITH, C.M., and KESTER, K.M. 1985. Reductions in consumption, utilization, and growth rate of soybean looper (Lepidoptera: Noctuidae) larvae fed foliage of soybean genotype PI 227687. *J. Econ. Entomol.* 77:1371–1375.

SAXENA, K.N. 1969. Patterns of insect plant relationships determining susceptibility or resistance of different plants to an insect. *Entomol. Exp. Appl.* 12:751–776.

SMITH, C.M. 1985. Expression, mechanisms and chemistry of resistance in soybean, *Glycine max* L. (Merr.) to the soybean looper, *Pseudoplusia includens* (Walker). *Insect Sci. Appl.* 6:243–248.

WALDBAUER, G.P., and MARCIANO, A.P. 1979. Mass rearing of the rice leaffolder, *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae) under greenhouse conditions. *J. Entomol. Res.* 3:1–8.

WEBSTER, J.A. 1975. Association of plant hairs and insect resistance: an annotated bibliography. Agricultural Research Service Miscellaneous Publication 1297. United States Department of Agriculture, Washington, D.C.

WILSON, R.L., WISEMAN, B.R., and WIDSTROM, N.W. 1984. Growth response of corn earworm (Lepidoptera: Noctudeae) larvae on meridic diets containing fresh and lyophilized corn silk. *J. Econ. Entomol.* 77:1159–1162.

WISEMAN, B.R. 1985. Types and mechanism of host plant resistance to insect attack. *Insect Sci. Appl.* 6:239–242.

WISEMAN, B.R., DAVIS, F.M., and WILLIAMS, W.P. 1983. Fall armyworn: Larval density and movement as an indication of nonpreference in resistant corn line. *Prot. Ecol.* 5:135–141.

YOSHIDA, S., FORNO, D.A., COCK, J.H., and COMEZ, K.A. 1976. Laboratory manual for physiological studies of rice. Third edition. The International Rice Research Institute, Los Banos, Laguna, Philippines.

ZAR, J.H. 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

FEEDING RESPONSES OF *Chilo partellus* (SWINHOE) (LEPIDOPTERA: PYRALIDAE) LARVAE TO SORGHUM PLANT PHENOLICS AND THEIR ANALOGS

BALDWYN TORTO,[1] AHMED HASSANALI,* KAILASH N. SAXENA, and SAGARY NOKOE

The International Centre of Insect Physiology and Ecology (ICIPE), P.O. Box 30772, Nairobi, Kenya

(Received May 9, 1990; accepted August 20, 1990)

Abstract—Phagostimulatory responses of third-instar larvae of *Chilo Partellus* to phenolic components identified in an ethyl acetate extract of the leaf whorls of 3-week-old plants of *Sorghum bicolor* cultivar IS 18363 were studied in no-choice bioassays. The major components in the extract were identified as 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid, with 4-hydroxy-3-methoxycinnamic acid, 3,4-dihydroxycinnamic acid, and 4-hydroxycinnamic acid present in minor amounts. All but 4-hydroxycinnamic acid were stimulatory at the doses tested. 4-Hydroxybenzaldehyde was more stimulatory than other potential biogenetic analogs. Hydroxybenzoic acids generally elicited greater feeding response than cinnamic acids, and the pattern of oxygen substitution in the benzene ring was related to bioactivity.

Key Words—*Chilo partellus*, Lepidoptera, Pyralidae, sorghum, feeding response, phenolics, phagostimulant, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 4-hydroxy-3-methoxycinnamic acid, 3,4-dihydroxycinnamic acid, 4-hydroxycinnamic acid.

INTRODUCTION

The variation in palatabilty of different cultivars of a host plant to insect pests has been attributed in a number of studies to quantitative or qualitative differences in the content of their allelochemics (Sogawa and Pathak, 1970; Hedin

*To whom correspondence should be addressed.
[1]Present address: Department of Chemistry, University of Maine, Aubert Hall, Orono, Maine 04469.

0098-0331/91/0100-0067$06.50/0

et al., 1977; Robinson et al., 1978; Woodhead, 1982; Haskins and Gorz, 1985; Otieno et al., 1985). Previous studies on different cultivars of *Sorghum bicolor* have shown that qualitative differences in the chemical composition of surface wax play a significant role in determining the level of damage caused by the nymphs of *Locusta migratoria* (Woodhead, 1983). Specific surface wax chemicals also have been implicated in the differential establishment of young larvae of *Chilo partellus* on the leaf whorls of certain sorghum varieties (Woodhead and Taneja, 1987).

We have recently shown that feeding by third-instar *C. partellus* larvae on sorghum is mediated by surface and tissue chemicals of different polarities extractable sequentially with hexane, ethyl acetate, and methanol (Torto et al., 1990). As part of an attempt to identify the stimuli involved in the feeding process of this insect and to elucidate the allelochemical basis of the palatibility of different cultivars of host plants, we have carried out a detailed fractionation and analysis of an ethyl acetate extract of a preferred sorghum cultivar IS 18363, in order to identify the phagostimulatory constituents in the extract. In this paper, we report the effects of phenolic constituents identified in the extract and some of their analogs on the feeding of the third-instar larvae of the insect.

METHODS AND MATERIALS

Insects. Freshly molted third-instar larvae of *C. partellus* were obtained from stock cultures maintained on an artificial diet at the ICIPE insectary (Ochieng et al. 1985). Third-instar larvae feed first on the leaves of the host plant before gradually boring into the stem. This stage was selected for bioassay owing to the longer-term objective of elucidating the basis for the change in larval feeding sites.

Extraction and Fractionation. Freshly cut, undamaged whorls of 3-week-old sorghum plants were extracted by dipping them first in hexane (15 min) and then in ethyl acetate (15 min) followed by soaking in methanol (24 hr). All extracts were concentrated to dryness in vacuo.

A sample of the ethyl acetate concentrate was redissolved in ethyl acetate and extracted with water (Figure 1). The combined aqueous phase was freeze-dried and the organic phase concentrated to dryness in vacuo. In bioassays, both phases stimulated larval feeding. Preliminary HPLC analyses of the two partition phases showed that phenolic components in the extract were present predominantly in the organic phase, while the aqueous phase contained components in common with those in the methanol extract of the whorls (Figure 2). The two major phenolic components in the organic phase were separated by preparative HPLC on a Varian 5000 liquid chromatograph, equipped with a Varian MCH-10 reverse phase column, 50 cm $\times$ 8 mm. The column was eluted with

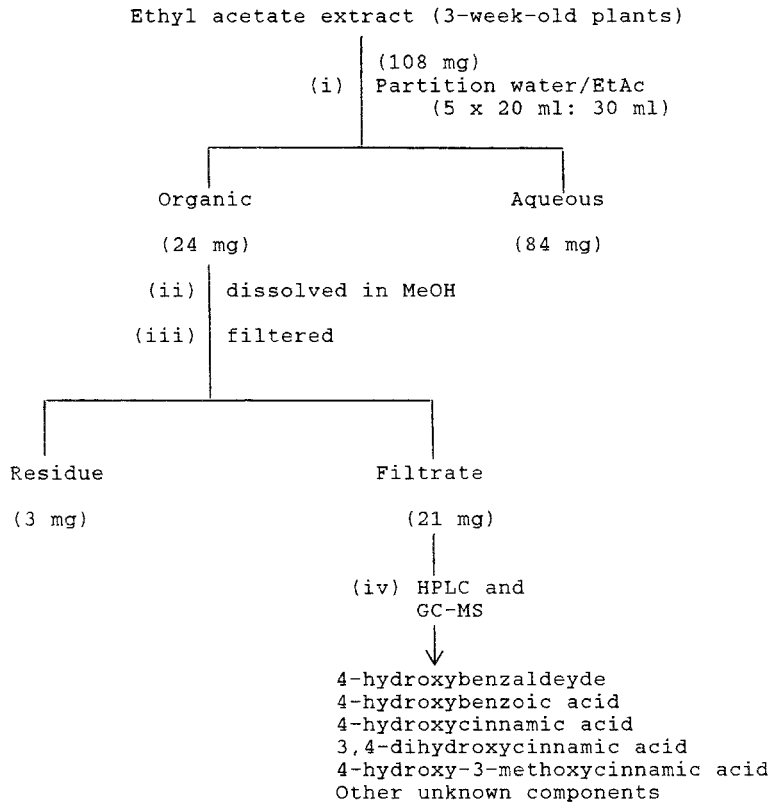


FIG. 1. Flow diagram for the isolation of *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde from ethyl acetate extract of the leaf whorls of the 3-week-old plants of sorghum IS 18363.

20% aqueous methanol at a flow rate of 3.0 ml/min at 25°C and the eluate monitored at 240 nm. The fractions corresponding to the two major components were pooled, concentrated, and identified by mass spectrometry and coinjection with authentic samples.

Acetylation of Organic Phase. Since the preparative HPLC separation of the organic phase yielded only the two major phenolic components, the minor components present in this phase were identified as acetyl derivatives. Acetylation of the organic phase with acetic anhydride in dry pyridine at 0°C for 24 hr, gave a mixture of acetates that were identified by GC-MS.

Bioassays for Feeding Activity. The no-choice feeding tests were conducted in small glass vials (23 × 27 mm) with tight porous caps. A wad of moist cottonwool placed inside the cap maintained a high humidity. The cottonwool was covered with a fine-wire mesh that fitted tightly inside the cap.

TABLE 1. MEAN WEIGHT OF DISK CONSUMED (mg) BY THREE THIRD-INSTAR LARVAE ON CELLULOSE ACETATE DISKS TREATED WITH PHENOLIC CONSTITUENTS OF SORGHUM AND THEIR ANALOGS

Phenolic group	Compound	Mean wt (mg) consumed[a]
4-derivatives	4-hydroxybenzaldehyde	0.11099 a
	4-methoxybenzyl alcohol	0.09817 b
	4-hydroxybenzoic acid	0.06625 c
	4-methoxybenzoic acid	0.06603 c
	4-hydroxybenzyl alcohol	0.05540 d
	4-methoxybenzaldehyde	0.02537 e
	4-hydroxycinnamic acid	inactive
Dihydroxy derivatives	3,4-dihydroxybenzoic acid	0.15801 a
	3,4-dihydroxycinnamic acid	0.03630 b
Hydroxymethoxy derivatives	4-hydroxy-3-methoxybenzoic acid	0.09172 a
	4-hydroxy-3-methoxycinnamic acid	0.03409 b
Other compounds	chlorogenic acid	0.07342 a
	quinic acid	0.04092 b

[a] Means with the same letter in a phenolic group are not significantly different ($P < 0.05$) Duncan's multiple-range test.

Test samples in solvents were applied topically to both sides of cellulose acetate disks (12 mm diameter, 7.902 ± 0.348 mg, Cole-Parmer Co., Chicago, Illinois) and were dried in a stream of warm air (Doss and Shanks, 1986). Each test disk then was dampened with 15 μl of double-distilled water and placed in the vial. Control disks dipped into solvent and air dried were similarly treated with double-distilled water. Three third-instar larvae, starved for 24 hr prior to testing, were placed into each vial. All the feeding tests were conducted for 24 hr in the dark at 78 ± 2% relative humidity and 29 ± 2°C. Test and control disks were weighed several times to a constant weight before and after the assay on a Cahn 21 milligram balance to ±0.001 mg.

The following were assayed: (1) organic and aqueous partition phases of the ethyl acetate extract; (2) purified synthetic samples of 4-hydroxybenzoic acid and 4-hydroxybenzaldehyde, the two major phenolic components of the ethyl acetate extract; (3) purified samples of some selected analogs of the two compounds comprising 4-hydroxybenzyl alcohol, 4-methoxybenzaldehyde, 4-methoxybenzoic acid, and 4-methoxybenzyl alcohol; (4), purified synthetic samples of 4-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid, and 4-hydroxy-3-methoxycinnamic acid, the three minor components of the ethyl acetate extract, and their analogs comprising 4-hydroxy-3-methoxybenzoic acid, 3,4-dihydroxybenzoic acid and 2, 5-dihydroxybenzoic acid; and (5) chlorogenic

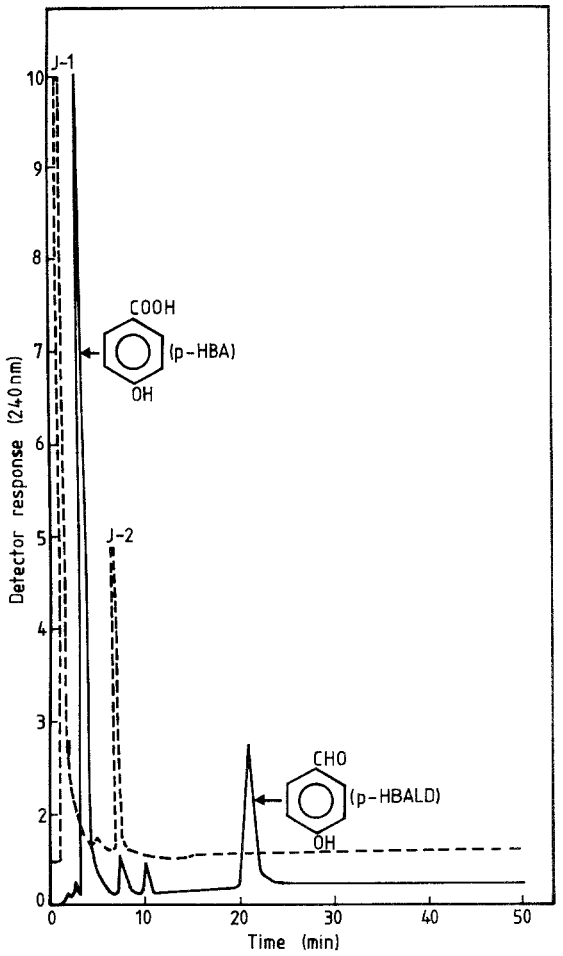


FIG. 2. Elution profiles of the organic (———) and aqueous (– – – –) partition phases of ethyl acetate extracts of leaf whorls of the 3-week-old plants of IS 18363 on a Zorbax C-18 column (25 × 4.6 mm).

acid, a relatively common constituent of plants (Sondheimer, 1964), and its degradative product, quinic acid. Compounds were obtained from Aldrich Chemical. Co. (Milwaukee, Wisconsin), purified by recrystallization, and the purity of each was checked by HPLC and/or GC. All compounds were tested at 10, 25, 50, 150, 250, 350, and 450 μg/disk. Each test was replicated 15 times.

The relative feeding response was calculated for each dose by dividing the mean weight of treated disks consumed by that of the control disks consumed.

The means were subjected to analysis of variance for differences due to phenolic compounds and doses. Overall means were separated using Duncan's multiple-range test. Dose–response plots for the phenolic compounds were derived from the equation: $y = ax^b c^x$ using the least-squares procedure on the logarithmic form: $\ln y = \ln a + x \ln c + b \ln x$, where y = relative feeding response, x = dose (μmol/disk), and a, b, and c are constants that determine the nature and slope of the plot.

Determination of Extent of Oxidation of 4-Hydroxybenzaldehyde during Bioassays. Since aldehydes are rapidly oxidized on exposure to air, an attempt was made to determine the extent of oxidation of 4-hydroxybenzaldehyde during the assay period. 4-Hydroxybenzaldehyde was dissolved in methanol to give 1 mg/ml solution. Ten cellulose acetate disks each were treated with 200 μg of 4-hydroxybenzaldehyde and then dried in a stream of warm air. Prior to the assay the disks were moistened with 15 μl of double-distilled water. Five of the disks were extracted immediately with 1 ml of methanol, and a 10-μl aliquot of each extract was analyzed by reverse-phase HPLC. The remaining five disks in assay containers were maintained at the conditions of the bioassay, i.e., 78 $\pm$ 2% relative humidity, temperature 29 $\pm$ 2°C for 24 hr. The disks then were extracted similarly with methanol and analyzed. The mean peak areas corresponding to 4-hydroxybenzaldehyde in the chromatograms after 0 and 24 hr of the assay were calculated and compared. Peaks representing the absorptions of 4-hydroxybenzioc acid also were checked for in the chromatograms.

RESULTS

Both the aqueous and organic partition phases of the ethyl acetate extract of IS 18363 elicited feeding responses from *C. partellus* larvae. The organic phase components were found to be mainly phenolic (Figure 3). The major compounds were 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid, identified by mass spectral analysis of samples isolated from a micropreparative HPLC column. The minor components were 4-hydroxy-3-methoxycinnamic acid, 3, 4-dihydroxycinnamic acid, and 4-hydroxycinnamic acid, identified as their acetyl derivatives by comparison of their GC-MS data with those of authentic samples and also confirmed by coinjection with authentic samples on an HPLC reverse-phase column. Studies of the aqueous components will be reported elsewhere.

All the phenolic constituents, with the exception of 4-hydroxycinnamic acid, elicited feeding responses from third-instar *C. partellus* larvae (Figures 4 and 5; Table 1). The dose–response curves of the phenols followed roughly the same pattern, reaching an optimum and then dropping. Analyses of variance indicated significant differences in feeding responses due to the two sets of treat-

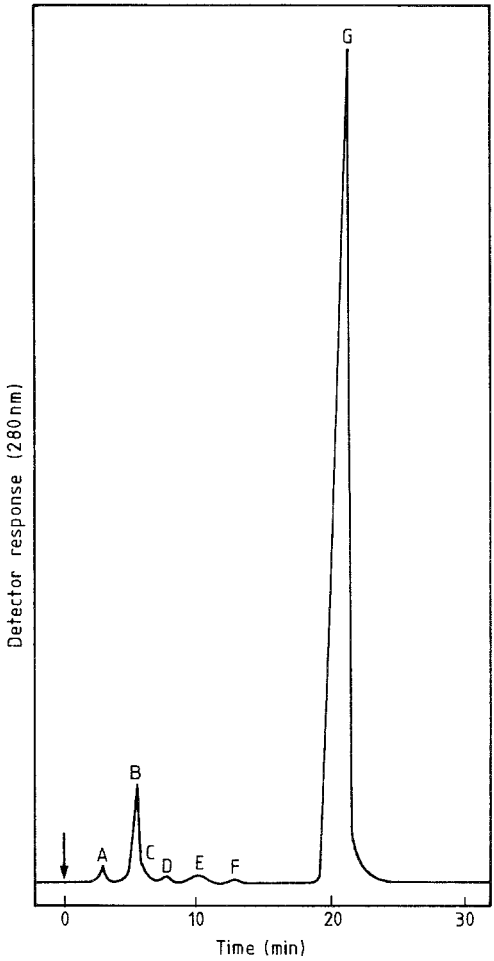


FIG. 3. Elution HPLC profile of the ethyl acetate extract of the leaf whorls of the 3-week-old plants of IS 18363 on a Zorbax C-18 column (25 cm × 4.6 mm). (A) unknown; (B) 4-hydroxybenzoic acid; (C) 3,4-dihydroxycinnamic acid; (D) unknown; (E) 4-hydroxy-cinnamic acid; (F) 4-hydroxy-3-methoxycinnamic acid; (G) 4-hydroxybenzaldehyde.

ment effects: dose ($F = 96.6$, $P < 0.0001$) and phenolic material ($F = 195$, $P < 0.0001$). There were also significant interactions between these effects, although Duncan's multiple-range procedure was able to separate the means computed from feeding rates over the whole dose range of the bioassays (Table 1).

4-Hydroxybenzaldehyde was the most stimulatory of the compounds. There

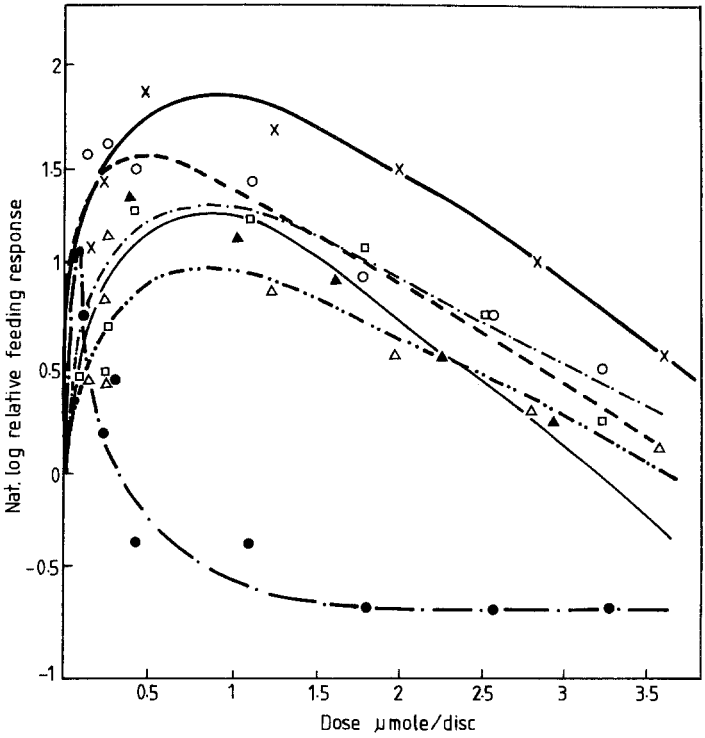


FIG. 4. Dose-response relationships for 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, and analogs. The relationships, derived by the least-squares procedure ($r^2 = 0.8405 - 0.9815$), represent plots of ln y versus x in the equation: $\ln y = \ln a + x \ln c + b \ln x$: ——— 4-hydroxybenzaldehyde (x); ----- 4-hydroxybenzoic acid (○); —··—·· 4-hydroxybenzyl alcohol (△); —·—·—· 4-methoxybenzyl alcohol (□); ——— 4-methoxybenzoic acid (▲); ——·——· 4-methoxybenzaldehyde (●).

was no significant oxidation of the aldehyde during the bioassay period; therefore the observed activity was essentially due to the aldehyde. Oxidation of the aldehyde to the corresponding acid and its reduction to the alcohol caused a significant drop in activity (Table 1). In contrast, a comparison of the activities of the methoxy derivatives of these compounds showed the opposite trend, the alcohol being most stimulatory and the aldehyde least.

For the cinnamic acids, 4-hydroxycinnamic acid was nonstimulatory at all the doses tested, but 4-hydroxy-3-methoxycinnamic acid and 3, 4-dihydroxycinnamic acid gave comparable feeding rates (Figure 5).

Comparison of hydroxybenzoic acid and its analogs with the cinnamic acids

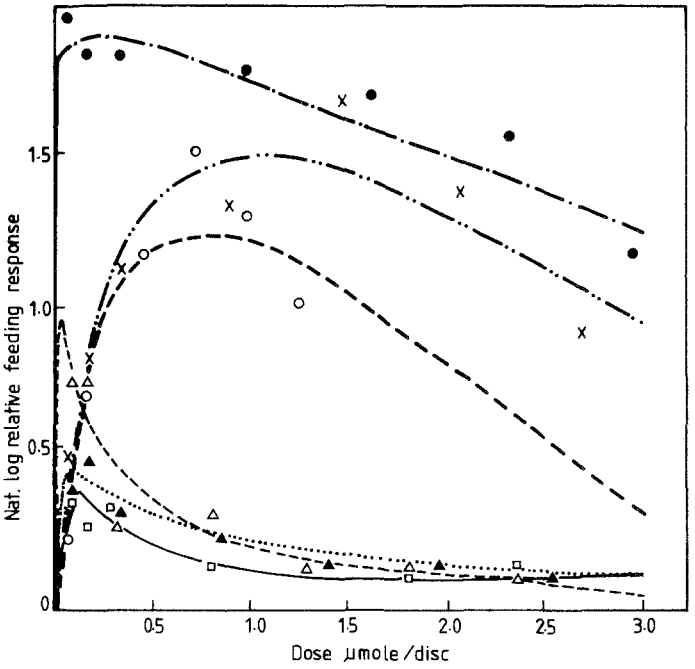


FIG. 5. Dose–response relationships for selected oxygenated benzoic and cinnamic acids and derivatives. The relationships, derived by the least-squares procedure ($r^2 = 0.8508 - 0.9069$), represents plots of ln y versus x in the equation: $\ln y = \ln a + x \ln c + b \ln x$; —·— 3,4-dihydroxybenzoic acid (●); —··— 4-hydroxy-3-methoxybenzoic acid (x); --- chlorogenic acid (○); ----- quinic acid (△); ····· 3,4-dihydroxycinnamic acid (▲); ——— 4-hydroxy-3-methoxycinnamic acid (□).

showed that the activities of the former differed significantly from those of the latter (Table 1). Hydroxybenzoic acids were generally more stimulatory than their corresponding cinnamic acids. Thus 4-hydroxy-3-methoxybenzoic acid was more stimulatory than 4-hydroxy-3-methoxycinnamic acid; 3,4-dihydroxybenzoic acid was more stimulatory than 3,4-dihydroxycinnamic acid; and whereas 4-hydroxybenzoic acid was a strong stimulant, 4-hydroxycinnamic acid was not stimulatory.

Of interest was the complete lack of response to 2,5-dihydroxybenzoic acid, a known phenolic constituent of sorghum seedlings (Woodhead and Cooper-Driver, 1979) but not encountered in the varieties studied in the present work. On the other hand, chlorogenic acid, which has not been reported as a

constituent of sorghum, and its degradative product, quinic acid, were both stimulatory.

DISCUSSION

Two groups of compounds are present in the ethyl acetate extracts of whole undamaged whorls of IS 18363, a cultivar of *S. bicolor* preferred by *C. partellus* larvae: phenolics and more polar, but not yet identified, components (Figure 2). The major phenolic feeding stimulants identified were 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid. Interestingly, 4-hydroxybenzaldehyde has been shown previously to deter feeding by the locust *L. migratoria* and the aphid *Schizaphis graminum* (Woodhead and Bernays, 1978; Dreyer et al., 1981; Woodhead, 1982). On the other hand, Fisk (1990) found that a phenolic extract from sorghum was stimulatory to the homopteran *Peregrinus maidis*, and Baker et al. (1986) showed that 4-hydroxybenzaldehyde and some of its derivatives are strong feeding stimulants for the elm bark beetle *Scolytus multistriatus*. A range of allelochemics now has been demonstrated to play different and often opposite semiochemical roles in different insects (Janzen, 1979; Matsuda and Senbo, 1986; Norris, 1986; Whitman, 1988), reflecting differing evolutionary responses of these herbivores to defensive chemicals elaborated by plants.

The dose effect on the stimulatory activity of the phenolic compounds bioassayed in the present study is noteworthy. Of particular interest is the dose–response relationship of 4-methoxybenzaldehyde (Figure 4), which is stimulatory at very low doses but rapidly becomes a deterrent at higher doses. This pattern is probably replicated by some of the other compounds at sufficiently high doses and suggests that caution needs to be exercised in interpreting feeding response data based on a limited dose range that diverges appreciably from the concentrations of the allelochemics in natural systems.

Comparison of the stimulatory effects of benzoic and cinnamic acids shows that activity is dependent upon the nature and type of substitution in these compounds. Cinnamic acids were weaker feeding stimulants than the corresponding benzoic acids. The variation in the stimulatory activity of cinnamic acids suggests that the extra oxygen at the 3-position in the benzene ring may be crucial for the activity of this class of phenolic compounds. This would account for the significant stimulatory activity of 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid but lack of activity by 4-hydroxycinnamic acid. Conversely, all three corresponding hydroxybenzoic acids, including 4-hydroxybenzoic acid, demonstrated significant activities. The complete lack of feeding response to 2,5-dihydroxybenzoic acid indicates that the substitution patterns on the benzene ring for this class of compounds are important for their bioactivity to *C. partellus*.

Chlorogenic acid, a common plant constituent (Sondheimer, 1964), but so far not reported to be present in sorghum, previously has been found to play various roles in host plant selection by insects. Found in potato leaves, it is phagostimulatory to the Colorado potato beetle, *Leptinotarsa decemlineata* (Hsiao and Fraenkel, 1968). It also has been reported to be stimulatory to some leaf beetles such as *Gastrophysa atrocyanea* but deterrent to others such as *Lochmaeae capreae cibrata* (Matsuda and Senbo, 1986). Other workers have observed that the compound served as a growth factor for certain insects, e.g., silkworm larvae (*Bombyx mori*) (Kato and Yamada, 1968) and the potato aphid, *Macrosiphum euphorbiae* (Chawla et al., 1974). In the present study, it stimulated feeding by larval *C. partellus* and was a better stimulant than its degradative products, quinic and 3,4-dihydroxycinnamic acids (Figure 5; Table 1).

In summation, our results suggest that although structural requirements for activity within each phenolic group tested are relatively specific, the insect appears to perceive and respond to a relatively broad range of plant phenols, albeit to varying levels of sensitivity. The implications of these findings are not clear. At the chemoreception level, it may indicate the presence of several sets of receptors with different degrees of specialization for different structural types. Since larval *C. partellus* is an oligophagous insect that feeds on sorghum, maize, and some wild grasses, it is perhaps important that it should be able to recognize the different profiles of allelochemics it may encounter within its host range. Single-cell electrophysiological studies and identification and quantification of phenolic feeding allelochemics from different hosts might shed some light on these questions. In addition, the precise roles of these phenols in the feeding process and the way they interact with the polar and nonpolar groups of phagostimulatory compounds need detailed elucidation before a complete picture of factors governing host palatability to this insect can emerge.

Acknowledgments—We thank Dr. P.G. McDowell for help with GC-MS analysis. The study was carried out with support to B.T. from the German Academic Exchange Program (DAAD) and the ICIPE for which we are very grateful.

REFERENCE

BAKER, J.E., RAINEY, D.P., NORRIS, D.M., and STRONG, F.M. 1986. *p*-Hydroxybenzaldehyde and other related phenolics as feeding stimulants for the smaller European elm bark beetle. *For. Sci.* 14:91–95.

CHAWLA, S.S., PERRON, J.M., and CLOUTIER, M. 1974. Effects of different growth factors on the potato aphid, *Macrosiphum euphorbiae* (Aphididae: Homoptera), fed on artificial diet. *Can. Entomol.* 106:273–280.

DOSS, R.P., and SHANKS, C.H. 1986. Use of membrane filters as substrate in insect feeding bioassays. *Bull. Entomol. Soc. Am.* 32:248–249.

DREYER, D.L., REESE, J.C., and JONES, K.C. 1981. Aphid feeding deterrents in sorghum. Bioassay, isolation and characterisation. *J. Chem. Ecol.* 7:273–284.

FISK, J. 1980. Effects of HCN, phenolic acids and related compounds in *Sorghum bicolor* on the feeding behavior of the planthopper *Peregrinus maidis*. *Entomol. Exp. Appl.* 27:211–222.

HASKINS, F.A., and GORZ, H.J. 1985. Dhurrin and *p*-hydroxybenzaldehyde in seedlings of various sorghum species. *Phytochemistry* 24:597–598.

HEDIN, P.A., JENKINS, J.N., and MAXWELL, F.G. 1977. Behavioral and development factors affecting host-plant resistance to insects, pp. 231–275, *in* P.A. Hedin (ed.). Host Plant Resistance to Pests. ACS Symposium series No. 62, American Chemical Society, Washington, D.C.

HSIAO, T.H., and FRAENKEL, G. 1968. Isolation of phagostimulative substances from the host plant of the Colorado potato beetle. *Ann. Entomol. Soc. Am.* 61:476–484.

JANZEN, D.H. 1979. New horizons in the biology of plant defenses, pp. 331–350, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores. Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

KATO, M., and YAMADA, H. 1968. Silkworm requires 3, 4-dihydroxybenzene structure of chlorogenic acid as a growth factor. *Life Sci.* 5:717–722.

MATSUDA, K., and SENBO, S. 1986. Chlorogenic acid as a feeding deterrent for Salicaceae-feeding leaf beetle, *Lochmaeae capreae cribrata* (Coleoptera: Chrysomelidae) and other species of leaf beetles. *Appl. Entomol. Zool.* 21:411–416.

NORRIS, D.M. (ed.). 1986. Antifeeding compounds, pp. 96–146, *in* Sterol Biosynthesis, Inhibitors and Antifeeding Compounds. Springer-Verlag, Berlin.

OCHIENG, R.S., ONYANGO, F.O., and BUNGU, M.D.O. 1985. Improvement of techniques for mass culture of *Chilo Partellus* (Swinhoe). *Insect Sci. Appl.* 6:425–428.

OTIENO, D.A., HASSANALI, A., and NJOROGE, P.W. 1985. Chemical basis of TVu 946 stem resistance to *Maruca testulalis* (Geyer). *Insect Sci. Appl.* 6:259–262.

ROBINSON, J.F., KLUN, J.A., and BRINDLEY, T.A. 1978. European corn borer: A nonpreference mechanism of leaf feeding resistance and its relationship to 1,4-benzoxazin-3-one concentration in dent corn tissue. *J. Econ. Entomol.* 71:461–465.

SOGAWA, K., and Pathak, M.D. 1970. Mechanisms of brown planthopper resistance to Mudgo variety of rice (Hemiptera: Delphacidae). *Appl. Entomol. Zool.* 5:145–158.

SONDHEIMER, E. 1964. Chlorogenic acid and related depsides. *Bot. Rev.* 30:667–712.

TORTO, B., HASSANALI, A., and SAXENA, K.N. 1990. Chemical aspects of *Chilo partellus* feeding on certain sorghum cultivars. *Insect Sci. Appl.* In press.

WHITMAN D.W. 1988. Allelochemical interactions among plants, herbivores, and their predators, pp. 11–64, *in* P. Barbosa and D. Letourneau (eds.). Novel Aspects of Insect-Plant Interactions. John Wiley & Sons, New York.

WOODHEAD, S. 1982. Leaf surface chemicals of seedling sorghum resistance to *Locusta migratoria*, pp. 375–376, *in* J.H. Visser and A.K. Minks (eds.) Proceedings, 5th International Symposium on Insect-Plant Relationships. Pudoc, Wageningen.

WOODHEAD, S. 1983. Surface chemistry of *Sorghum bicolor* and its importance in feeding by *Locusta migratoria*. *Physiol. Entomol.* 8:345–352.

WOODHEAD, S., and BERNAYS, E.A. 1978. The chemical basis of resistance of *Sorghum bicolor* to attack by Locusta migratoria. *Entomol. Exp. Appl.* 24:123–144.

WOODHEAD, S., and COOPER-DRIVER, G. 1979. Phenolic acids and resistance to attack in *Sorghum bicolor*. *Biochem. Sys. Ecol.* 7:309–310.

WOODHEAD, S., and TANEJA, S.L. 1987. The importance of the behavior of young larvae in sorghum resistance to *Chilo partellus*. *Entomol. Exp. Appl.* 45:47–54.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

EFFECT OF EXPERIENCE WITH PINE (*Pituophis melanoleucus*) AND KING (*Lampropeltis getulus*) SNAKE ODORS ON Y-MAZE BEHAVIOR OF PINE SNAKE HATCHLINGS

JOANNA BURGER,[1,*] WILLIAM BOARMAN,[1] LYNN KURZAVA,[1] and MICHAEL GOCHFELD[2]

[1]*Department of Biological Sciences*
Rutgers University
Piscataway, New Jersey 08855

[2]*Environmental and Community Medicine*
UMDNJ-Robert Wood Johnson Medical School
Piscataway, New Jersey 08854

(Received July 3, 1990; accepted August 20, 1990)

Abstract—The abilities of hatchling pine snakes (*Pituophis melanoleucus*) and king snakes (*Lampropeltis getulus*) to discriminate the chemical trails of pine and king snakes was investigated in Y-maze experiments. Pine snakes were housed for 17 days either with shavings impregnated with pine snake odor, king snake odor, or no odor to test for the effect of experience on choice. Both pine and king snake hatchlings entered the arm with the pine snake odor and did not enter the arm with the king snake odor. The data support the hypothesis that hatchlings of both species can distinguish conspecific odors from other odors and that our manipulation of previous experience was without effect for pine snake hatchlings.

Key Words—Chemical cues, pine snakes, king snakes, *Pituophis melanoleucus*, *Lampropeltis getulus*, experience.

INTRODUCTION

Following the trail of conspecifics or prey has been demonstrated in controlled laboratory experiments for snakes (Brown and Maclean, 1983; Chiszar et al., 1986; Ford, 1982; Ford and O'Bleness, 1986; Ford and Schofield, 1984; Gehl-

*To whom correspondence should be addressed.

0098-0331/91/0100-0079$06.50/0

bach et al., 1971; Heller and Halpern, 1981; Burger, 1989) and for lizards (Cooper and Vitt, 1986a). Reptiles respond to odors on applicators by increased activity or increased tongue flicks (Cooper and Vitt, 1984, 1985, 1986b; Cooper et al., 1986). These studies suggest the use of chemical-detecting abilities in several important contexts including recognition of prey (Burghardt, 1973; Chiszar et al., 1986), detection of conspecifics, (Cooper and Vitt, 1984), discrimination of male from female conspecifics (Cooper and Vitt, 1984), discrimination of conspecific and closely related syntopic congeners (Cooper and Vitt, 1986c), trailing conspecifics to find hibernacula (Brown and Maclean, 1983; Burger, 1989) or mates (Ford and O'Bleness, 1986), and differentiation of ophiophagous from nonophiophagous snakes (Weldon and Burghardt, 1979; Weldon, 1982; Cooper, 1990). For a further review, see Burghardt (1980) and Von Achen and Rakestraw (1984).

Snakes can distinguish conspecific from heterospecific odor trails (Ford and O'Bleness, 1986). Further, pine snake hatchlings avoid the odor trail of their predator, king snakes, and approach the odor trail of pine snakes (Burger, 1989). The influence of experience on scent-trailing by snakes has not been investigated. Since lizards or snakes are housed in cages, they have been exposed to odors of their own conspecifics for some time. Thus, previous experiments with chemoreception have not determined if the snakes were merely selecting a familiar odor and avoiding an unfamiliar one. In this paper we report experiments designed to examine discrimination by hatchling pine (*Pituophis m. melanoleucus*) and king (*Lampropeltis getulus*) snakes exposed to odors of adult pine and king snakes and to examine the role of previous experience on discrimination in pine snakes. King snakes prey on other species of snakes (Fitch, 1963; Ditmars, 1935); therefore, pine snakes should avoid the odor of king snakes and select the pine snake odor in Y-maze tests. King snake hatchlings may avoid the odor of conspecifics as they too may be eaten by adult king snakes. However, king snake odor would normally be the familiar odor for hatchling king snakes.

METHODS AND MATERIALS

Under appropriate state permits, pine snake eggs from 12 clutches were collected from the Pine Barrens of southern New Jersey (Ocean, Cumberland, and Monmouth counties). Eggs ($N = 78$) incubated at 28°C were hatched and maintained in the laboratory in 1988. Eggs ($N = 16$) from two female king snakes also were hatched in the laboratory. Snakes were maintained individually at 28°C in plastic cages (30 × 15 × 9 cm) containing pine shavings or paper. Snakes were given the opportunity to drink water. All snakes were tested when 17–20 days old, after they had shed but before they were fed. King snakes

were maintained in cages with paper not previously exposed to snake odors until testing.

Pine snake hatchlings from each clutch were divided randomly in three groups, and each group was assigned to one home cage shavings group. The three groups were: (1) shavings from a pine snake cage, (2) shavings from a king snake cage, and (3) clean shavings (control). Shavings for the experimental treatment groups were obtained by placing clean shavings 1.5 cm thick in the bottom of the cage of an adult pine or king snake and leaving them. Every five days the shavings in the adult pine (hereafter called pine snake shavings) and king (hereafter called king snake shavings) snake cages were changed, and the odorous shavings placed in zip-lock bags in a freezer. These shavings were then placed 1.5 cm thick in the bottom of the approximate pine snake hatchling cages from the time of hatching until testing.

There were four experiments: (1) pine snake hatchlings tested with a Y-maze choice of king snake versus pine shavings; (2) king snake hatchlings tested with a Y-maze choice of king snake versus pine snake shavings; (3) pine snake hatchlings tested with king snake and pine snake shavings from the freezer; and (4) pine snake hatchlings tested with king and pine snake shavings from the home cages of the pine snake hatchlings (after they had lived there for several days). This set of experiments was designed to test whether pine and king snakes distinguished odor from pine and king snake adults; whether previous experience affected pine snake hatchling choice; and whether pine snakes could distinguish (as measured by tongue flicks) the pine snake from king snake shavings and the used (in pine snake home cages) pine snake shavings from used king snake shavings.

To determine whether pine snakes could make directional responses to chemical trails, Y-maze experiments were conducted. The base arm of the Y maze was 1 m long and 15 cm wide, with 15 cm high wooden sides. At the end of the base arm were two side arms (experimental arms, 45° angle from the initial arm) having the same dimensions as the base arm. Clean shavings were placed in the base arm, and king snake shavings and pine snake shavings were each placed over paper in different experimental arms. The experimental shavings with the paper were changed after each trial, and the location of the adult-scented shavings was reversed between each trial.

In the Y-maze experiments each snake was introduced into the base arm facing the opening with its head at the entrance and allowed to move freely up the arm to the intersection, where it usually moved into one of the experimental arms. The trial ended when the snake left the Y maze through the experimental arms. The time each snake was in the base and experimental arm, time at the Y junction, and the number of tongue flicks when the snake was in each location were recorded. An assistant released the snake into the maze. Each snake was tested only once.

Following the Y-maze experiments, pine snakes were placed in a base arm of similar size containing either adult pine snake or adult king snake shavings, and we counted the number of tongue flicks per 15 sec when the snake was moving over the surface (after Cooper and Vitt, 1986d). The hatchling was first tested on the odor of one species and then on the other. The order of presentation was switched between each hatchling tested.

After this experiment, pine snakes were tested in the same apparatus using tongue flicks as a measure of response, but the shavings used were removed from all home cages and mixed by type (all pine snake shavings or all king snake shavings). These shavings, therefore, had been initially moved over by either an adult king snake or adult pine snake and subsequently moved over by pine snake hatchlings. This experiment was designed to determine if pine snake hatchlings were still able to distinguish the two adult odors on the shavings after several days of use by other hatchlings.

Chi-square tests were used to determine the significance of the response frequency in the Y-maze experiments, and Kruskal-Wallis tests were used to determine differences in central tendencies of time and tongue flicks in the experiments. Given are means $\pm$ one standard deviation in the text.

RESULTS

Most snakes moved rapidly in the base arm, remained motionless for a few seconds at the Y junction, explored the Y junction for a few more seconds, and then moved rapidly down one arm.

Pine snake hatchlings in the Y-maze experiment entered the side arm containing the pine snake shavings at significantly greater than chance frequency, regardless of the home cage shavings they had experienced (Table 1). Although more pine snakes that lived with king snake shavings entered the arm containing the king snake shavings than did those under the other rearing conditions, the differences were not significant. Six of the 10 snakes that entered the king snake arm backed up several times before moving to the end of the arm. Further, eight snakes that ultimately chose the pine snake side first started down the king snake side and backed up to the Y junction before going down the pine snake side.

Pine snake hatchlings behaved similarly in the base arm regardless of their ultimate choice (Table 2). Further, they behaved similarly at the Y junction, largely because, regardless of final choice, some snakes first started down both sides. However, hatchlings entering the king snake side moved more slowly and made significantly more tongue flicks than those entering the pine snake arm.

TABLE 1. RESPONSE OF PINE AND KING SNAKES IN Y-MAZE CHOICE TEST AND TONGUE FLICKS PER 15 SEC (MEAN ± 1 SD) FOR PINE SNAKES WHEN MOVING SLOWLY ON SHAVINGS

	Number of snakes	Arm entered: Pine snake shavings	Arm entered: King snake shavings	$\chi^2(P)$
Pine snake choice test				
Pine snake shavings	26	24	2	18.60 (0.001)
King snake shavings	26	20	6	7.52 (0.01)
No odor shavings	26	24	2	18.60 (0.001)
King snake choice test				
(no home cage shavings)	14	12	2	7.14 (0.01)
Pine snake tongue flicks				
Test shavings	78	18.2 ± 3.2	22.1 ± 3.9	5.96 (0.01)
Home cage shavings	27	19.3 ± 2.8	24.0 ± 2.1	4.35 (0.01)

King snake hatchlings in the Y-maze experiment also entered the arm containing the pine snake odor in a significantly greater number of trials than entered the arm containing the king snake odor (Table 1). Although sample sizes were small, king snakes emitted slightly more tongue flicks when in the king snake arm than when in the pine snake arm (Table 2), but sample sizes were too small to allow statistical evaluation.

Pine snake hatchlings emitted more tongue flicks per 15 sec in response to the king snake shavings than in response to the pine snake shavings (Table 1). In this experiment the shavings used were from the cages of adult king and adult pine snakes that were similar to those used in the Y-maze choice experiments. We then tested the hatchling pine snakes with shavings from the home cages of the hatchlings to detect whether there was still adult king and adult pine snake odor that the hatchlings could distinguish. These cage shavings had pine snake hatchlings moving on them for five days in addition to originally being in the cages of either an adult king or an adult pine snake. Pine snake hatchlings again gave significantly more tongue flicks to the king snake shavings compared to the pine snake shavings.

Previously it has been shown that pine snake hatchlings prefer to enter the arm with a conspecific when given a choice of conspecific versus no odor; they avoid king snake odor when given a choice of king snake versus no odor (Burger, 1989). This experiment was designed to test the effect of previous experience with the odors of king and pine snakes.

TABLE 2. TIME SPENT AND NUMBER OF TONGUE FLICKS EMITTED BY SNAKES IN Y MAZE WHEN GIVEN A CHOICE OF KING OR PINE SNAKE ODOR[a]

	Choice		
	King snake shavings	Pine snake shavings	$\chi^2(P)$
Pine snakes			
Number of snakes	10	68	
Base arm			
Time (sec)	9.0 ± 5.4	10.2 ± 13.3	0.51 (NS)
Tongue flicks	18.2 ± 3.6	17.6 ± 4.3	0.24 (NS)
Choice site			
Time motionless (sec)	17.4 ± 32.0	20.2 ± 47.3	0.73 (NS)
Flicks	4.5 ± 4.0	3.3 ± 2.2	0.20 (NS)
Explore			
Time (sec)	29.1 ± 26.1	38.1 ± 69.1	1.24 (NS)
Flicks	19.8 ± 5.6	17.1 ± 5.1	1.09 (NS)
Experimental arm			
Time (sec)	52.2 ± 47.6	18.0 ± 26.6	8.67 (0.003)
Flicks	22.2 ± 4.8	17.5 ± 3.5	8.09 (0.003)
King snakes[a]			
Number of snakes	2	14	
Base arm			
Time (sec)	12.0 ± 6.0	14.2 ± 8.0	NS
Tongue flicks	16.0 ± 2.0	15.7 ± 0.2	NS
Choice site			
Time motionless (sec)		0.3 ± 0.4	
Flicks		0	
Explore			
Time (sec)	15.0 ± 0	14.1 ± 17.5	NS
Flicks (sec)		13.5 ± 6.0	
Experimental arm			
Time	17.5 ± 2.5	15.1 ± 7.1	NS
Flicks	19.5 ± 0.5	15.7 ± 2.5	NS

[a] Statistical test not performed because of small sample sizes. Given are means ± 1 SD for the choice they made. Tongue flicks are per 15 sec NS = not significiant.

DISCUSSION

This set of experiments, in conjunction with previous experiments (Burger, 1989), shows that: (1) Both pine and king snake hatchlings select the pine snake arm more frequently than the king snake arm. (2) Pine snake hatchlings prefer the pine snake shavings even when they are reared since hatching on shavings with a king snake odor. Thus, pine snakes are not merely responding to familiar

odors (in some cases, their own), but are responding to the specific odors. (3) Pine snake hatchlings distinguish between the odors by giving more tongue flicks to the king snake shavings compared to the pine snake shavings. (4) Pine snake hatchlings can distinguish king snake odor from pine snake odor even though the shavings have been in cages of hatchling pine snakes for several days. This is the first study to demonstrate differential selection of chemical odors that were over five days old, and it demonstrates a preference for pine snake odors regardless of previous experience.

Given the previous tests (Burger, 1989), the present experiment shows that hatchling pine and king snakes select and follow the shavings containing the chemical odor of an adult pine snake and avoid the shavings containing the chemical odor of an adult king snake. Pine and king snake hatchlings emerge from their nests in late August and early September and find hibernacula in the next few weeks (Burger et al., 1988). New Jersey pine snakes hibernate in underground burrows that they dig themselves, although elsewhere gopher snakes (*Pituophis m. deserticola*) hibernate in mammal burrows with other species of snakes (Brown et al., 1974; Parker and Brown, 1973; Burger et al., 1988).

Pine snakes in New Jersey hibernate in dens with up to 25 pine snakes and three king snakes (Burger et al., 1988), thus it would be adaptive for both species to be able to follow very weak trails of pine snake. A naive hatchling wandering about the pine barrens may come across chemical trails of only a few pine snakes. In the present Y-maze experiment the chemical odor on the shavings was five days old, yet both pine and king snakes chose the pine snake arm and avoided the king snake arm. Thus the odor was sufficiently strong to be assessed five days later. Further, both king and pine snake hatchlings distinguished five-day-old odors of adult pine and king snake as measured by tongue flick rates.

King snakes prey on other species of snakes (Fitch, 1963; Ditmars, 1935), including pine snakes in the pine barrens. Presumably, they would also prey on conspecifics. Indeed, after our experiments, one of the king snake hatchlings ate one of the other king snake hatchlings. Thus hatchlings of both species have an adaptive reason for avoiding king snake trails. Our results with tongue flicks of pine snake hatchlings corroborate findings of a greater tongue flicking response by squamates to potential predators. Naive garter snakes (*Thamnophis elegans*) (Weldon, 1982) and adult skinks (*Eumeces laticeps*) (Cooper, 1990) emitted significantly more tongue flicks to swabs rubbed against the skin of king snakes than to those from nonophiophagous snakes or blank swabs.

One objective of the study was to determine the role of prior experience in the choice experiments. Hatchling pine snakes caged for 17 days with shavings with odors of either pine or king snake showed similar responses to the choice test; all preferred the pine snake odor. Thus prior experience with a

familiar odor did not alter their choice. Moreover, we found that pine snake hatchlings were able to distinguish the pine snake from the king snake odor even though the shavings had been in cages of hatchling pine snakes for several days.

Acknowledgments—We thank R.T. Zappalorti for help while collecting the pine and king snake eggs.

REFERENCES

BROWN, W.S., and MACLEAN, F.M. 1983. Conspecific scent-trailing by newborn timber rattlesnakes, *Crotalus horridus*. *Herpetology* 39:430–436.

BROWN, W.S., PARKER, W.S., and ELDER, J.A. 1974. Thermal and spatial relationships of two species of coleubrid snakes during hibernation. *Herpetology* 30:32–38.

BURGER, J. 1989. Following of conspecific and avoidance of predator chemical cues by pine snakes (*Pituophis melanoleucus*). *J. Chem. Ecol.* 15:799–806.

BURGER, J., ZAPPALORTI, R.T., GOCHFELD, M., BOARMAN, W.I., CAFFREY, M., DOIG, V., GARBER, S.D., LAURO, B., MIKOVSKY, M. SAFINA, C., and SALIVA, J. 1988. Hibernacula and summer den sites of pine snakes (*Pituophis melanoleucus*) in the New Jersey Pine Barrens. *J. Herpetol.* 22:425–433.

BURGHARDT, G.M. 1973. Chemical release of prey attack: Extension to naive newly hatched lizards, *Eumeces fasciatus*. *Copeia* 1973:178–181.

BURGHARDT, G.M. 1980. Behavioral and stimulus correlates of vomeronasal functioning in reptiles: Feeding, grouping, sex, and tongue use, pp. 275–301, *in* D. Muller-Shwarze, R.M. Silverstein (eds.). Chemical Signals in Vertebrates and Aquatic Invertebrates. Plenum Press, New York.

CHISZAR, D., RADCLIFF, C., BOYD, R., RADCLIFF, A., YUN, H., SMITH, H.M., BOYER, R., ATKINS, B., and FEILER, F. 1986. Trailing behavior in cottonmouths (*Agkistrodon piscivorus*). *J. Herpetol.* 20:269–272.

COOPER, W.E. 1990. Chemical detection of predators by a lizard, the broad-headed skink (*Euneces laticeps*). *J. Exp. Zool.*

COOPER, W.E., and VITT, L.J. 1984. Detection of conspecific odors by the female broad-headed skink, *Eumeces laticeps*. *J. Exp. Zool.* 229:49–54.

COOPER, W.E., and VITT, L.J. 1985. Responses of the skinks, *Eumeces fasciatus* and *E. laticeps*, to airborne conspecific odors: Further appraisal. *J. Herpetol.* 19:481–486.

COOPER, W.E., and VITT, L.J. 1986a. Tracking of female conspecific odor trails by male broad-headed skinks (*Eumeces laticeps*). *Ethology* 71:242–248.

COOPER, W.E., and VITT, L.J. 1986b. Interspecific odor discrimination by a lizard (*Eumeces laticeps*). *Anim. Behav.* 34:367–376.

COOPER, W.E., and VITT, L.J. 1986c. Interspecific odor discriminations among syntopic congeners in scincid lizards (*Genus Eumeces*). *Behavior* 97:1–9.

COOPER, W.E., and VITT, L.J. 1986d. Thermal dependence of tongue-flicking and comments on use of tongue flicking as an index of squamate behavior. *Ethology* 71:177–186.

COOPER, W.E., GARSTKA, W.R., and VITT, L.J. 1986. Female sex pheromone in the lizard *Eumeces laticeps*. *Herpetology* 42:361–366.

DITMARS, R.I. 1935. Serpents of the Northeastern States. New York Zoological Society, New York. 40 pp.

FITCH, H.S. 1963. Natural history of the racer *Coluber constrictor*. *Univ. Kans. Publ.* 15:351–468.

FORD, N.B. 1982. Species specificity of sex pheromone trials of sympatric and allopatric garter snakes (*Thamnophis*). *Copeia* 1982:10–13.

FORD, N.B., and OBLENESS, M.L. 1986. Species and sexual specificity of pheromone trails of the garter snake, *Thamnophis marcianus*. *J. Herpetol.* 20:259–262.

FORD, N.B., and SCHOFIELD, C.W. 1984. Species specificity of sex pheromone trails in the plains garter snake, *Thamnophis radix*. *Herpetologica* 40:51–55.

GEHLBACK, F.R., WATKINS, J., and KROLL, J. 1971. Pheromone trail following studies of typhlopid, leptotyphlopid, and colubrid snakes. *Behavior* 40:282–294.

HELLER, S., and HALPERN, M. 1981. Laboratory observations on conspecific and congeneric scent trailing in garter snakes (*Thamnophis*). *Behav. Neur. Biol.* 33:372–377.

PARKER, W.S., and BROWN, W.S. 1973. Species composition and population changes in two complexes of snake hibernacula in northern Utah. *Herpetology* 29:319–326.

VON ACHEN, P.H., and RAKESTRAW, J.L. 1984. The role of chemoreception in the prey selection of neonate reptiles, *in* R.A. Seigel, L. E. Hunt, J. L. Knight, L. Malaret, and N. L. Zuschlag (eds.). Vertebrate Ecology and Systematics—A Tribute to Harry S. Fitch. Museum of Natural History, University Kansas, Lawrence, Kansas.

WELDON, P.J. 1982. Responses to ophiophagous snakes by snakes of the genus *Thamnophis*. *Copeia* 1982:788–794.

WELDON, P.J., and BURGHARDT, G.M. 1979. The ophiophage defensive response in crotaline snakes: Extension to new taxa. *J. Chem. Ecol.* 5:141–151.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

LABORATORY AND FIELD TESTS WITH THE SYNTHETIC SEX PHEROMONE OF THREE *Matsucoccus*[1] PINE BAST SCALES

B.E. HIBBARD,[2] G.N. LANIER,[2,5] S.C. PARKS,[3] Y.T. QI,[4] F.X. WEBSTER[2,*] and R.M. SILVERSTEIN[2]

[2]*State University of New York*
College of Environmental Science and Forestry
Syracuse, New York 13210

[3]*Department of Forestry, College of Agriculture*
Chonnam National University
Yongbong-dong, Kwangju 500-757
Republic of Korea

[4]*Shanghai Institute of Entomology*
Academia Sinica
Shanghai, China

(Received May 10, 1990; accepted August 20, 1990)

Abstract—Matsunone, (2*E*,4*E*)-4,6,10,12-tetramethyl-2,4-tridecadien-7-one, is the primary sex pheromone of *Matsucoccus resinosae*, *M. thunbergianae*, and *M. matsumurae*. The synthetic compound was tested in the laboratory and in field tests with all three species, and significantly more males responded to synthetic matsuone than to controls. The attraction of synthetic matsuone to males in field tests was not significantly different from that of virgin females at the same matsuone release rate. An analog of matsuone previously shown to be biologically active was also tested in the laboratory with *M. resinosae* and *M. thunbergianae*, and field tested with *M. resinosae*. The active analog required a dose approximately 100 times greater than matsuone to yield maximum laboratory bioassay response, and in field tests, attraction was not significantly different from controls at doses at which matsuone was significantly attractive.

Key Words—*Matsucoccus resinosae*, *M. thunbergianae*, *M. matsumurae*, Coccoidea, Margarodidae, scale insects, pine bast scales, sex pheromone, (2*E*,4*E*)-4,6,10,12-tetramethyl-2,4-tridecadien-7-one, matsuone.

*To whom correspondence should be addressed.
[1]Homoptera: Coccoidea: Margarodidae.
[5]Deceased.

INTRODUCTION

Pine bast scales in the genus *Matsucoccus* Cockerell are major pests of pine in France (Carle, 1968, 1974), Israel (Mendel et al., 1988), Korea (Park, 1988), China (Li et al., 1980), and the United States (Bean and Godwin, 1955; McClure, 1976, 1983b). Studies on the chemical communication of these insects began with Doane's (1966) report of a female-produced sex attractant for *Matsucoccus resinosae* Bean and Godwin. Sex pheromones have since been documented in *M. matsumurae* (Kuwana) (Qi et al., 1983), *M. thunbergianae* Millar and Park (Park et al., 1986), *M. massonianae* Young et Hu (Young and Qi, 1983), and *M. josephi* Bodenheimer and Harpaz (Sternlicht et al., 1983). Although a taxonomic revision has not been formally proposed, *M. matsumurae* and *M. resinosae* are almost certainly the same species (McClure, 1983a; Young et al., 1984).

Park et al. (1986) reported the isolation of the sex pheromone of *M. resinosae* and the demonstration of its cross-attractiveness with *M. thunbergianae*. Spectral data available to Lanier et al. (1989) reduced the number of possible structures for this compound to four: **1, 2, 3** and **4** (Figure 1). On the basis of strong circumstantial evidence, Lanier et al. (1989) identified the pheromone of *M. resinosae*, *M. matsumurae*, and *M. thunbergianae* as (2*E*,4*E*)-4,6,10,12-tetramethyl-2,4-tridecadien-7-one (**1,** Figure 1), and gave it the trivial name matsuone. Although we were successful in synthesizing two analogs of this structure (**3** and **4,** Figure 1) and demonstrating biological activity with one of them (**3**), the compound we proposed as the true pheromone (**1**) in Lanier et al. (1989) was not available for testing. We recently synthesized matsuone (**1**) (see Figure 1) and its geometric isomer (2*E*,4*Z*)-4,6,11,12-tetramethyl-2,4-tridecadien-7-one (**2**) (Webster et al., 1990). We report here laboratory and field tests of three species of *Matsucoccus* pine bast scales with the two new synthetic compounds (**1** and **2**) and those analogs (**3** and **4**) tested by Lanier et al. (1989).

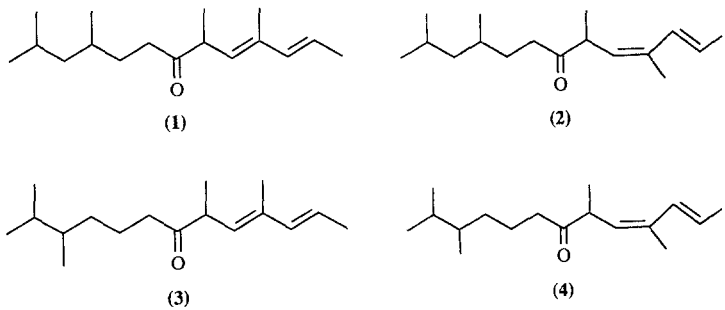


FIG. 1. Chemical structures of matsuone and the three analogs tested.

METHODS AND MATERIALS

Field Sites and Insect Sources. Field experiments were conducted in Dutchess County, New York, U.S.A., from June 7 to September 15, 1989; in Tonggang County, Chonnam Province, Korea, from April 5 to 7, 1989; and the suburbs of Wu Xi City, China, from April 25 to 27, 1989 for *M. resinosae*, *M. thunbergianae*, and *M. matsumurae*, respectively. Infested branches of *Pinus resinosa* Ait., *P. thunbergiana* Franco, and *P. tabulaeformis* Carr. containing cocoons of *M. resinosae*, *M. thunbergianae*, and *M. matsumurae*, respectively, were placed in emergence cages similar to those described by Lanier et al. (1989). Males emerging from the cocoons were tested in laboratory behavioral bioassays.

Laboratory Bioassays. The bioassay procedures used for *M. resinosae* and *M. thunbergianae* were those of Park et al. (1986) and Lanier et al. (1989). First, the test compound in 1 μl hexane in a micropipet was deposited just inside the tip of a medicine dropper. Excess hexane was expelled by depressing the bulb of the medicine dropper 20 times, and the dropper was positioned about 8 mm to one side of a pedestrian male. Puffs of air were delivered to the antennae of the pedestrian male by gently pressing the bulb at 1.5-sec intervals. Relative attraction was measured as the number of sides of a 4-cm-equilateral triangle along which a male would follow the retreating dropper tip. The highest response rating, three, indicated that the male followed the dropper for all three sides of the triangle, and the lowest response rating, zero, indicated no attraction. Incomplete following of the dropper along a side of the triangle was designated by the fraction of the side completed. For *M. thunbergianae*, males showing the response score of three to 2×10^{-3} female equivalents (see Lanier et al., 1989) were tested, while with *M. resinosae*, the positive control was a live virgin female placed in the dropper tip.

Bioassays with *M. matsumurae* in China used the method of Qi et al. (1983) and Lanier et al. (1989). Two filter paper disks of 1 cm diameter, each containing one of two materials or one material and a solvent blank, were placed into a 7-cm-diameter Petri dish. After the solvent had evaporated, 10 males were introduced and the Petri dish was covered. The behavior of males in the dish was observed, and the number of males on each of the disks was recorded after 10 min.

Field Experiments. In Korea, the field experiments were performed in a *P. thunbergiana* forest with trees approximately 8 m tall. White plastic cards (10 $\times$ 14 cm) coated with Tanglefoot were affixed to horizontal bamboo poles 0.5 and 1.5 m above the ground. Traps were set in open areas of 15–40 m diam. and 7–20 m away from the trees. Treatments were assigned randomly on the poles so that each pole contained one replicate with different treatments spaced 1 m apart on the pole. Unbaited traps were placed in the field at 6 pm, April

5, 1989. Treatments of 222 μg matsuone in 60 μl benzene on rubber GC septa (Supelco Inc., Bellefonte, Pennsylvania), 60 μl benzene on rubber GC septa, and 20 newly emerging females in a 2 × 2 × 0.5-cm fine net cage were replicated 10 times: five each at 0.5 and 1.5 m above the ground. Emerging females used as baits were collected between 8:00 and 9:30 AM, April 6. Baits were attached with pins to the lower portion of the sticky traps between 9:30 and 10:00 AM, April 6. Those males that were caught on untreated traps in the early morning were removed as the baits were set. Counts of these traps were then made between 12:00 and 1:00 PM on both April 6 and 7 by removing the males from the traps during counting.

In China, the field trapping experiments were conducted in a suburban forest of *P. tabulaeformis*. Three replications of the following six treatments were tested: 1, 10, and 40 μg matsuone on filter paper, one and five newly emerged females, and a benzene control. The trap, a piece of sticky cardboard (28 × 23 cm) folded in the middle at a 60° angle, was similar to that described by Lanier et al. (1989). Three 1-cm-diam. holes were cut into each flap, and a roll of filter paper (5 × 0.8 cm) that served as the pheromone dispenser was hung inside the trap. Traps were hung from pine branches at heights between 1.5 and 2 m. Distance between traps was greater than 20 m.

In New York, traps placed 2.44 m high were positioned between 1 and 2 m from the nearest branches and were at least 1.5 m from each other within a small planting of *P. resinosae*. Most of the trees were widely spaced, so that the crown was visible to the ground. Since *M. resinosae* usually occurs in the crowns, this site was ideal for setting traps at reasonable heights. Tree height at the site ranged from 1.5 m to 20 m. Dewill elm bark beetle traps (Dewill Inc., Elmhurst, Illinois) were cut into thirds (sticky surface of 20 × 48 cm), formed into cylinders 15 cm in diameter with the sticky surface facing outward, and stapled around a vertical pole 2.44 m in height. The baits were protected from the sun by fastening them with wire approximately 2 cm within inverted plastic film canisters (3 cm diam. × 4.8 cm). The bait apparatus then was stapled to the pole so that it was situated inside the sticky cylinder. Trials with live females as a treatment were set up between 8:00 and 9:30 AM, and results were recorded after 1:00 PM. Trials without live females were set up between 1:30 and 5:30 PM, and results were recorded after several days.

The first field experiment in New York tested the relative attraction of 25 μg matsuone (**1**), 250 μg matsuone, 25 μg **2**, 250 μg **2**, and a hexane control. Rubber GC septa served as the pheromone dispenser. Five replications of this experiment were set up on June 8 and dismantled on June 15. Ten more replications were set up on June 20 and dismantled on June 25.

We next compared known quantities of matsuone with attraction to live adult females. Six replicates of 20 live females each (collected between 6:30 and 8:30 AM on August 15, 1989) were placed in fine netting and added to the

traps along with baits by 9:30 AM. Four doses of matsuone were chosen and replicated 10 times: 10, 50, 100, and 250 μg. Trap catch was recorded between 1:00 and 2:00 PM the same day. The pheromone dispenser for this experiment was a cotton dental wick (Healthco International, Boston, Massachusetts). The last experiment reported herein tested 200 μg each of matsuone (**1**), a hexane control, and **2, 3,** and **4** on rubber GC septa. The experiment was set up September 15 and dismantled September 25.

Release Rate Determination. The relative release rates of matsuone from rubber septa, dental cotton wicks, and filter paper were determined in the laboratory. Matsuone was added to the dispensers, and they were immediately placed in a 250-ml Erlenmeyer flask that was protected from UV radiation with aluminum foil. Each of the dispensers was tested with 250 μg of matsuone, and filter paper was also tested at 40 μg because this dose was effective for trapping males in China. There were four replicates of each dispenser at each dose tested. House vacuum pulled room air through activated carbon (6–14 mesh), past the dispensers, and through 0.6 g Porapak Q, on which matsuone was collected. Flow through the flask was held constant at 100 ml/min. Temperature was held constant at 20 $\pm$ 2°C. Volatiles were recovered from the Porapak with 4 ml of pentane. Extractions were made after 24, 48, and 72 hr for each dispenser, and also at 168 hr for rubber septa. The concentration of matsuone in the samples was determined with an HP 5890 gas chromatograph fitted with a 50-m methyl silicon capillary column (DB-1, Supelco, Inc., Bellefonte, Pennsylvania) with 1 ng/μl *n*-octadecane as an internal standard.

Statistical Analysis. The statistical package SAS (SAS Institute, 1985) was used for data analysis. All field data were analyzed with one-way or two-way analysis of variance (ANOVA), then by Duncan's (1955) multiple-range test when significant F values were found in the ANOVA. The trap-catch data were subject to log $(X + 1)$ or square root $(X + 0.5)$ transformations as needed to meet the assumption of equal variance (Winer, 1971). Nonparametric procedures were required for analysis of laboratory bioassays with *M. resinosae* and *M. thunbergianae*. A rank transformation followed by the procedures above served this purpose (Conover and Iman, 1981; SAS Institute, 1985, p. 19).

RESULTS

Laboratory Bioassays. In bioassays with *M. thunbergianae* in Korea, responses of pedestrian males to all doses of matsuone tested except the lowest dose, 0.04 pg, were significantly higher ($P < 0.05$) than the control (Table 1). Maximum responses to matsuone were elicited by doses of 4.0 pg to 0.40 ng, while the dose required for maximum response to **3** was about 0.50 ng. Differences in attraction between the highest dose of **3** (0.50 ng) and doses of mat-

TABLE 1. MEAN RESPONSES IN LABORATORY BIOASSAYS OF *Matsucoccus thunbergianae* MALES TO DILUTIONS OF MATSUONE AND ANALOG **3**[a]

Material	Quantity (pg)	No. tested	Mean response ± SE
Hexane		24	0.06 ± 0.03 E
Matsuone	4.0×10^{-2}	24	0.21 ± 0.07 DE
	4.0×10^{-1}	24	1.08 ± 0.23 C
	4.0×10^{0}	24	2.88 ± 0.09 A
	4.0×10^{1}	24	2.73 ± 0.15 A
	4.0×10^{2}	24	2.79 ± 0.11 A
	4.0×10^{3}	24	2.04 ± 0.24 B
Analog **3**	5.0×10^{-1}	24	0.15 ± 0.05 E
	5.0×10^{0}	24	0.42 ± 0.10 D
	5.0×10^{1}	24	1.98 ± 0.22 B
	5.0×10^{2}	24	2.69 ± 0.15 A

[a] Although the raw data are shown, statistical analysis was performed on rank transformed data. Significant differences are indicated by different letters after the mean.

suone from 4.00 pg to 0.40 ng were not significant.

In bioassays with *M. resinosae* in the United States, responses to doses between 50.0 pg to 5.0 ng of matsuone, and between 5.0 ng and 50.0 ng of **3** were not significantly different from the positive control of a live female (Table 2). All doses of **2** and **4** elicited lower ($P < 0.05$) responses than a live female. All doses of matsuone and doses of 50.0 pg, 5.0 ng, and 50.0 ng of **2** gave greater responses than the hexane control. No dosage of **4** gave response significantly greater ($P < 0.05$) than the hexane control. For **2** and **3,** responses to all doses from 50.0 pg to 50.0 ng were higher ($P < 0.05$) than to the hexane control.

Laboratory bioassays in China utilizing the Petri dish bioassay indicate that *M. matsumurae* are significantly attracted to matsuone (Table 3). At all doses tested except the lowest dose (10 ng), more ($P < 0.01$) males were attracted to filter paper disks containing matsuone than to disks with benzene alone. Increasing the dosage of matsuone increased the number of males on the matsuone disk. In a choice test comparing 0.20 μg matsuone with 5 female equivalents (see Lanier et al., 1989) of crude extract, there was no significant difference between the number of males on the two filter paper disks. When comparing 0.20 μg matsuone in choice tests with 8.0 μg **3,** significantly more males were found on the filter paper disk with 0.2 μg of matsuone.

Field Tests. In field tests with *M. thunbergianae* in Korea, significantly more males were caught in traps baited with 222 μg matsuone 0.5 m from the ground than all other traps (Figure 2). Traps baited with 20 live females placed

TABLE 2. MEAN RESPONSES IN LABORATORY BIOASSAYS OF *Matsucoccus resinosae* MALES TO DILUTIONS OF MATSUONE, MATSUONE ANALOGS, AND LIVE FEMALES[a]

Material	Quantity (pg)	No. tested	Mean response
Live females	1 female	42	3.00 ± 0.00 A
Hexane		45	0.10 ± 0.04 HI
Matsuone	5.0×10^{-1}	5	1.70 ± 0.52 CDE
	5.0×10^{0}	24	1.53 ± 0.21 CDE
	5.0×10^{1}	32	2.57 ± 0.16 ABC
	5.0×10^{2}	14	2.71 ± 0.19 AB
	5.0×10^{3}	12	2.71 ± 0.19 AB
	5.0×10^{4}	5	1.60 ± 0.51 CD
Analog **2**	5.0×10^{-1}	9	0.00 ± 0.00 I
	5.0×10^{0}	8	0.44 ± 0.19 GHI
	5.0×10^{1}	8	0.95 ± 0.32 FG
	5.0×10^{2}	8	0.69 ± 0.19 FGH
	5.0×10^{3}	6	1.92 ± 0.52 BCD
	5.0×10^{4}	5	0.67 ± 0.11 EFG
Analog **3**	5.0×10^{-1}	5	0.00 ± 0.00 I
	5.0×10^{0}	5	0.50 ± 0.34 GHI
	5.0×10^{1}	20	1.43 ± 0.00 DEF
	5.0×10^{2}	15	1.85 ± 0.19 CDE
	5.0×10^{3}	9	2.78 ± 0.06 AB
	5.0×10^{4}	7	2.25 ± 0.00 ABC
Analog **4**	5.0×10^{-1}	5	0.00 ± 0.00 I
	5.0×10^{0}	8	0.50 ± 0.44 GHI
	5.0×10^{1}	15	0.00 ± 0.26 I
	5.0×10^{2}	15	0.23 ± 0.36 HI
	5.0×10^{3}	8	0.06 ± 0.00 I
	5.0×10^{4}	8	0.00 ± 0.31 I

[a] Although the raw data are shown, statistical analysis was performed on rank transformed data. Significant differences are indicated by different letters after the mean.

at 0.5 m also caught more ($P < 0.05$) males than controls, but there was no significant difference between any treatments placed at 1.5 m.

In China, traps baited with 10 and 40 μg of matsuone caught significantly more male *M. matsumurae* than control traps, traps with 1 female, and traps with 1 μg of matsuone (Figure 3). Differences between traps with 10 and 40 μg of matsuone and traps with five females were not significant, nor were differences between traps with 1 μg of matsuone and control traps. Traps with one or five females caught significantly more males than control traps.

In the United States, significantly more ($P < 0.05$) male *M. resinosae* were caught on traps baited with 250 μg of matsuone than those baited with 25

TABLE 3. MEAN RESPONSES OF *M. matsumurae* MALES IN CHOICE TESTS BETWEEN MATERIAL 1 AND MATERIAL 2[a]

Material 1 (ng matsuone)	Material 2	Mean response (material 1 − material 2)
10	Benzene	2.00
20	Benzene	3.67*
50	Benzene	4.67*
100	Benzene	6.67*
200	Benzene	7.67*
400	Benzene	8.33*
800	Benzene	9.33*
1300	Benzene	9.67*
200	5 female equivalents	1.67*
200	8000 ng analog **3**	5.33*

[a] Significant differences are indicated by different letters after the mean.
* = significantly different from zero ($P < 0.01$).

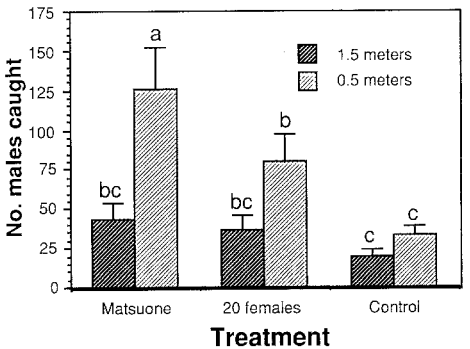


FIG. 2. Number of male *M. thunbergianae* caught in traps baited with 222 μg matsuone on rubber septa, 20 live females, and a solvent control. Although raw data are shown, statistical analysis was performed on log $(X + 1)$ -transformed data. Bars topped by different lowercase letters are significantly different ($P < 0.05$) as indicated by Duncan's (1955) numerical multiple-range test. Standard error bars are indicated.

μg matsuone, 25 μg **2**, 250 μg **2**, or a hexane control (Figure 4); no other significant differences were found. In dose–response tests using cotton dental wicks as the pheromone dispenser, there was no significant difference between traps baited with 100 and 250 μg matsuone, and there was no difference between control traps and traps with 20 live females (we cannot explain this last result).

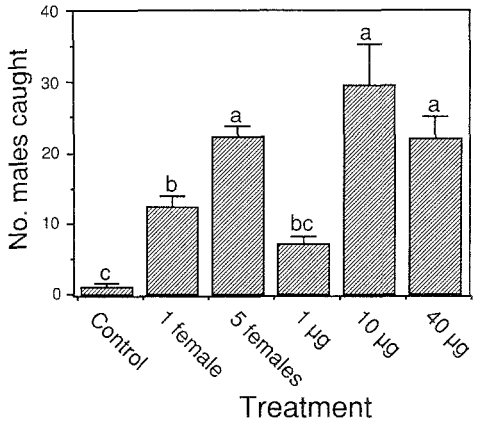


FIG. 3. Number of male *M. matsumurae* caught in traps baited with matsuone on filter paper or live females. Bars topped by different lowercase letters are significantly different ($P < 0.05$) as indicated by Duncan's (1955) numerical multiple-range test. Standard error bars are indicated.

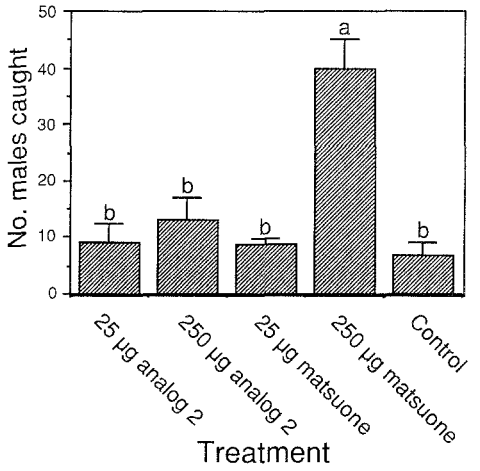


FIG. 4. Number of male *M. resinosae* caught in traps baited with matsuone, **2**, or solvent on rubber septa. Although raw data are shown, statistical analysis was performed on square root ($X + 0.5$) -transformed data. Bars topped by different lowercase letters are significantly different ($P < 0.05$) as indicated by Duncan's (1955) numerical multiple-range test. Standard error bars are indicated.

All other differences were significant (Figure 5), including the difference between male catches on traps baited with 10 μg matsuone and control traps. A regression of the dose-response curve was significant ($P < 0.05$) with an R^2 of 50.9%. In a similar experiment in which all analogs were tested, more males ($P < 0.05$) were caught on traps baited with matsuone than traps baited with any analog. There was no significant difference between catches on traps baited with **2, 3, 4,** and the hexane control (Figure 6).

Release Rates. The release rate of matsuone from filter paper rolls and dental cotton wicks was significantly greater than that from rubber GC septa (Supelco, Inc., Bellefonte, Pennsylvania) for each of the first three days (Table 4). Significantly more matsuone was released from cotton wicks than filter paper on day 2, but there was no significant difference on days 1 or 3. The release rate of 40 μg matsuone from filter paper was higher than that of 250 μg matsuone from rubber septa on day 1, but this difference was not significant on day 2 or day 3. The average release rate from rubber septa from days 4–7 was equivalent to that released from rubber septa on day 2.

DISCUSSION

Park (1988) placed newly emerged *M. thunbergianae* females in a device designed to collect the female sex pheromone on filter paper. The filter paper was extracted with hexane, and 5×10^{-3} female hour equivalents (FHE) yielded

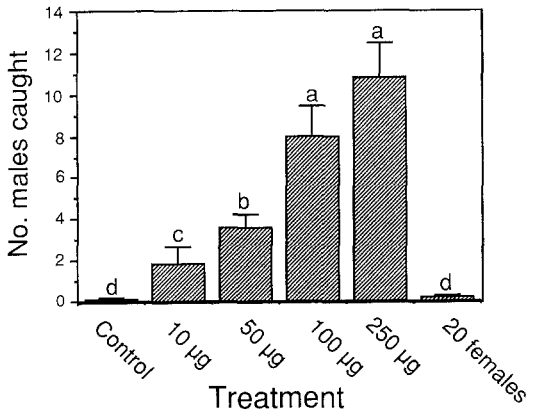


FIG. 5. Number of male *M. resinosae* caught in traps baited with live females or different doses of matsuone on cotton dental wicks. Although raw data are shown, statistical analysis was performed on log $(X + 1)$ -transformed data. Bars topped by different lowercase letters are significantly different ($P < 0.05$) as indicated by Duncan's (1955) numerical multiple-range test. Standard error bars are indicated.

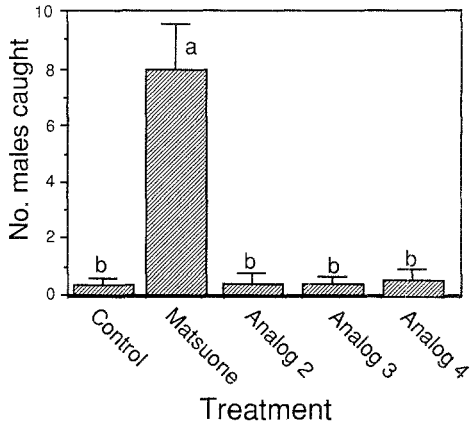


FIG. 6. Number of male *M. resinosae* caught in traps baited with 200 μg of matsuone and three analogs on rubber septa. Although raw data are shown, statistical analysis was performed on log $(X + 1)$ data. Bars topped by different lowercase letters are significantly different $(P < 0.05)$ as indicated by Duncan's (1955) numerical multiple-range test. Standard error bars are indicated.

TABLE 4. RELEASE RATE OF MATSUONE FROM PHEROMONE DISPENSERS AS MEASURED IN THE LABORATORY

Dispenser type	Load rate (μg)	Release rate (μg/day ± SE)			
		Day 1	Day 2	Day 3	Days 4–7
Rubber septa	250	1.50 ± 0.60ef	0.41 ± 0.03fg	0.12 ± 0.02g	0.41 ± 0.09e
Cotton wick	250	12.52 ± 2.84ab	8.93 ± 0.29bc	1.98 ± 0.29e	
Filter paper	250	17.03 ± 1.68a	3.95 ± 0.80d	1.06 ± 0.19ef	
Filter paper	40	6.15 ± 0.96d	1.09 ± 0.90ef	0.25 ± 0.10fg	

an average response of 2.2 in the same bioassay as used in the present study (5×10^{-2} FHE gave an average of 3.0). This response is equivalent to the response of *M. thunbergianae* males to 4.0 pg of matsuone and *M. resinosae* males to 50.0 pg matsuone reported herein. If we assume that all of the sex pheromone was collected on the filter paper in Park (1988) and that matsuone is the only active compound, we can estimate the production of matsuone (based on the level of response) to be in the range of 0.8 ng/hr for *M. thunbergianae* and 10 ng/hr for *M. matsumurae* and *M. resinosae*.

Because we determined the release rate of matsuone from the dispensers utilized in the present study (Table 4), we can compare the field trap catch of

synthetic matsuone to that of natural matsuone. The field tests in China were conducted for three days, so if we use the release rate of 40 μg matsuone from filter paper from day 2 as an average, approximately 54 ng/hr of synthetic matsuone were released (Table 4). *M. matsumurae* females produce approximately 10 ng/hr, so the five females tested would have produced approximately 50 ng/hr of natural matsuone (a conservative estimate given our assumptions). The number of male *M. matsumurae* caught on traps baited with five females and those baited with 40 μg matsuone on filter paper were not significantly different. In experiments in Korea with *M. thunbergianae*, septa were loaded with matsuone the afternoon before being applied to the traps, and the experiments were set up for two days, so it is again appropriate to use the release rate data from day 2. The release rate of matsuone from rubber GC septa on day 2 was approximately 17 ng/hr (Table 4), while the calculated release rate of 20 female *M. thunbergianae* would be approximately 16 ng/hr. Both traps baited with 20 females and traps baited with 222 μg synthetic matsuone on rubber GC septa caught significantly more males than control traps at 0.5 m in height. Traps baited with synthetic matsuone also caught significantly more males than traps baited with 20 females at 0.5 m (Figure 2), but this difference in trap catch was not significant when data from both heights (0.5 and 1.5 m) are pooled. If our calculation of the release rate of matsuone from these *Matsucoccus* spp. is correct, it appears that matsuone can account for all of the attraction to female-baited traps.

Demonstrating biological activity of the synthetic compound is crucial to verifying the identification of a pheromone. We have shown that synthetic matsuone is significantly attractive to three species of pine bast scales in laboratory and field tests, and we have shown that matsuone can account for all of the attraction of live females in the field. We have corroborated the conclusion of Lanier et al. (1989) that (2*E*,4*E*)-4,6,10,12-tetramethyl-2,4-tridecadien-7-one is the major sex pheromone of *M. resinosae*, *M. thunbergianae*, and *M. matsumurae*. Lanier et al. (1989) were correct in their prediction that matsuone would show much higher activity than its analogs. In laboratory bioassays with both *M. resinosae* and *M. thunbergianae*, the dose of **2** (the most active analog) required to produce a full response was approximately 100 times greater than that required for matsuone. In field trials with *M. resinosae* with 200 μg of all analogs present on rubber septa, only traps baited with matsuone caught significantly more males than the control.

The final identification step is also the first step in the utilization of semiochemicals in integrated pest management. Synthetic pheromones have been produced for a number of other Coccoidea and have been successfully incorporated into management programs (for detection and monitoring) for the citrus mealybug, *Planococcus citri* (Risso) (Ortu and Delrio, 1982); the citrophilous mealybug, *P. calceolariae* Mask. (Rotundo et al., 1979); the San Jose scale,

Quadraspidiotus perniciosus (Comstock) (Angerilli and Logan, 1986; Pfeiffer 1985); and the California red scale, *Aonidiella aurantii* (Maskell) (Ervin et al., 1985; Moreno and Kennett, 1981, 1985; Sternlicht et al., 1981). Sex pheromones have even been used for mating disruption for the California red scale (Bar-Zakay et al., 1989). Synthetic matsuone produced by Webster et al. (1990) trapped significantly more male *M. resinosae*, *M. thunbergianae*, and *M. matsumurae* than control traps in the present experiments. The results from the present paper indicate that synthetic matsuone may be a valuable component in the management programs of *M. resinosae*, *M. thunbergianae*, and *M. matsumurae*.

Acknowledgments—We thank Aijun Zhang for synthesizing the matsuone and **2** used in this study, Mr. Ray Vaughan from the Department of Environmental Conservation at Stonykill for help in locating field sites in New York, Mr. Randy Randazzo from the Sprout Lake Camp and Mr. Dick Conkline for cooperation in using their land for field sites in New York. We also thank Mr. Conkline for permission to down red pine trees for laboratory work. Mr. Hyung-Sun Kim from the Chonnam Forest Experiment Station assisted in the Korean field tests. Funding was provided in part by a grant from the National Science Foundation and the Department of Entomology, Forestry Research Institute of Korea.

REFERENCES

ANGERILLI, N.P.D., and LOGAN, D.M. 1986. The use of pheromone and barrier traps to monitor for San Jose scale (Homoptera: Diaspididae) phenology in the Okanagan Valley of British Columbia. *Can. Entomol.* 118:767–774.

BAR-ZAKAY, I., PELEG, B.A., and HEFETZ, A. 1989. Mating disruption of the California red scale *Aonidiella aurantii* (Homoptera: Diaspididae). *Hassadeh* 70:1228–1231.

BEAN, J.L., and GODWIN, P.A. 1955. Description and bionomics of a new red pine scale, *Matsucoccus resinosae*. *For. Sci.* 1:164–197.

CARLE, P. 1968. Méthode d'obtention massive des pontes de *Matsucoccus feytaudi* Duc. (Coccoidea, Margarodidae) par piégeage des femelles. *Ann. Sci. For.* 25:57–68.

CARLE, P. 1974. Le dépérissement du pin mésogéen en Provence. *Ann. Sci. For.* (Paris) 31:1–26.

CONOVER, W.J., and IMAN, R.L. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. *Am. Stat.* 35:124–129.

DOANE, C.C. 1966. Evidence for a sex attractant in females of the red pine scale. *J. Econ. Entomol.* 59:1539–1540.

DUNCAN, D.B. 1955. Multiple range and multiple *F* tests. *Biometrics* 11:1–42.

ERVIN, R.T., MORENO, D.D., BARITELLE, J.L., and GARDENER, P.D. 1985. Pheromone monitoring is cost effective. *Calif. Agric.*, Sept.-Oct. 17–18.

LANIER, G.N., QI, Y., WEST, J.R., PARK, S.C., WEBSTER, F.X., and SILVERSTEIN, R.M. 1989. Identification of the sex pheromone of three *Matsucoccus* pine bast scales. *J. Chem. Ecol.* 15:1645–1659.

LI, G., AHOQNG, L., HAN, R., LIU, X., and XIA, R. 1980. A study on the pine stem coccid *Matsucoccus matsumurae* Kuwara. *Rep. Inst. For. Lianoing Prov.* 9:1–27.

MCCLURE, M.S. 1976. Colonization and establishment of the red pine scale, *Matsucoccus resinosae* (Homoptera: Margarodidae) in a Connecticut plantation. *Environ. Entomol.* 5:943–947.

MCCLURE, M.S. 1983a. Temperature and host availability affect the distribution of Matsucoccus

matsumurae (Kuwana) (Homoptera: Margarodidae) in Asia and North America. *Ann. Entomol. Soc. Am.* 76:761–765.

MCCLURE, M.S. 1983b. Population dynamics of pernicious parasite; density dependent vitality of the red pine scale. *Ecology* 64:710–718.

MENDEL, Z., SHAPHIR, N., MADAR, Z., GOLAN, Y., SCHPETER, E., and ZEHAVI, A. 1988. The effect of habitat and age of the Aleppo pine plantation on damage caused by *Matsucoccus josephi*. *Hassadeh* 68:2203–2207.

MORENO, D.S., and KENNETT, C.E. 1981. Monitoring insect pest populations by trapping in California citrus orchards. *Proc. Int. Soc. Citriculture* 2:684–689.

MORENO, D.S., and KENNETT, C.E. 1985. Predictive year-end California red scale (Homoptera: Diaspididae) orange fruit infestations based on catches of males in the San Joaquin Valley. *J. Econ. Entomol.* 78:1–9.

ORTU, S., and DELRIO, G. 1982. Field trials with synthetic sexual pheromone of *Planococcus citri* (Rigso). *Redia* 65:341–354.

PARK, S.C. 1988. Biology and pheromone-mediated behavior of *Matsucoccus thunbergianae* in Korea with reference to *M. resinosae* in the United States ((Homoptera: Coccoidea: Margarodidae). PhD thesis. State University of New York, Syracuse, New York.

PARK, S.C., WEST, J.R., ABRAHAMSON, L.P., LANIER, G.N., and SILVERSTEIN, R.M. 1986. Cross-attraction between two species of *Matsucoccus*. Extraction, bioassay, and isolation of the sex pheromone. *J. Chem. Ecol.* 12:609–617.

PFEIFFER, D.G. 1985. Pheromone trapping of males and prediction of crawler emergence for San Jose scale (Homoptera: Diaspididae) in Virginia apple orchards. *J. Entomol. Sci.* 20:351–353.

QI, Y., YANG, M., and ZHAO, F. 1983. A preliminary study of the sex pheromone of the Japanese pine bast scale *Matsucoccus matsumurae* (Kuwana) (in Chinese, English summary). *Contrib. Shanghai Inst. Entomol.* 3:21–28.

ROTUNDO, G., TREMBLAY, E., and GIACOMETII, R. 1979. Final results of mass captures of the citrophilous mealybug males (*Pseudococcus calceolariae* Mask.) (Homoptera: Pseudococcidae) in a citrus grove. *Bull. Lab. Entomol. Agric. Portici* 30:266–274.

SAS Institute. 1985. SAS User's Guide: Statistics. *SAS Institute*, Cary, North Carolina.

STERNLICHT, M., BAR-ZAKAY, I., SZIVOS, Y., and DUNKELBLUM, E. 1981. Prediction of infestation by and control of the California red scale, *Aonidiella aurantii* (Maskell), through use of its pheromone. *Proc. Int. Soc. Citriculture* 2:695–700.

STERNLICHT, M., DUNKELBLUM, E., and GIESELMANN, M.J. 1983. Management of scale insects through utilization of their pheromone. Bet Dagan, Israel (unpublished).

WEBSTER, F.X., ZHANG, A., PARK, S.K., and PRESTWICH, G.D. 1990. Synthesis of matsuone, the sex pheromone of the red pine scale. *J. Org. Chem.* In press.

WINER, B.M. 1971. Statistical Principles in Experimental Design, 2nd ed. McGraw-Hill, New York.

YOUNG, B., and QI, Y. 1983. Application of sex pheromone to the identification of pine bast scales (Margarodidae, Coccoidea). *Contrib. Shanghai Inst. Entomol.* 3:273–278. (Chinese, English summary).

YOUNG, B., MILLER, D.R., and MCCLURE, M.S. 1984. Attractivity of th female sex pheromone of Chinese *Matsucoccus matsumurae* (Kuwana) to males of *M. matsumurae* in Japan and to males of *M. resinosae* Bean and Godwin in the United States (Margarodidae, Coccoidea, Homoptera). *Contrib. Shanghae Inst. Entomol.* 4:1–20.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

ALKYL SUBSTITUTION IN TERMINAL CHAIN OF (*Z*)-5-DECENYL ACETATE, A PHEROMONE COMPONENT OF TURNIP MOTH, *Agrotis segetum*.[1] SYNTHESIS, SINGLE-SENSILLUM RECORDINGS, AND STRUCTURE–ACTIVITY RELATIONSHIPS

STIG JÖNSSON,[2] TOMMY LILJEFORS,[2,*] and BILL S. HANSSON[3]

[2]*Department of Organic Chemistry 3*
Chemical Center, University of Lund
P.O. Box 124, S-221 00 Lund, Sweden
[3]*Department of Animal Ecology*
Ecology Building, University of Lund
S-223 62 Lund, Sweden

(Received May 23, 1990; accepted August 20, 1990)

Abstract—Structure–activity relationships for 6-, 7-, 8-, and 9-alkyl substituted analogs of (*Z*)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*, have been studied by electrophysiological single-sensillum recordings, and interpreted in terms of a receptor–interaction model. The compounds were prepared by alkenyl cuprate reactions with α,β-unsaturated carbonyl derivatives or alkyl halides. The electrophysiological results indicate steric repulsive interactions between the alkyl groups and the receptor in all the positions studied. This demonstrates a high complementarity between the receptor and the terminal alkyl chain.

Key Words—Structure–activity, single-sensillum recordings, receptor interaction, pheromone, (*Z*)-5-decenyl acetate, alkenyl cuprate, alkyl substitution, *Agrotis segetum*, Lepidotera, Noctuidae.

INTRODUCTION

(*Z*)-5-Decenyl acetate (**1**) is a pheromone component of the turnip moth, *Agrotis segetum* (Bestmann et al., 1978; Arn et al., 1980; Löfstedt et al., 1982). In previously reported structure–activity studies on analogs of compound **1**, we

*To whom correspondence should be addressed.
[1]Schiff., Lepidoptera: Noctuidae.

0098-0331/91/0100-0103$06.50/0

have developed a receptor-interaction model based on the analysis of single-cell data and molecular mechanics calculations. With this model a strong correlation was established between measured single-cell activities and calculated conformational energies required by the analogs to structurally mimic a particular conformation of the natural pheromone component (Liljefors et al., 1985; Bengtsson et al., 1987). This conformation is shown in Figure 1. The model has been applied successfully to chain-elongated (Liljefors et al., 1985, 1987), chain-shortened (Bengtsson et al., 1990), and dienic analogs (Bengtsson et al., 1987), and to configurational isomers (Liljefors et al., 1987), and is furthermore supported by studies on conformationally constrained cyclohexene analogs of **1** (Bengtsson, 1988).

The results of these investigations have provided information about the dimensions and 3D shape of the receptor cavity tuned to (*Z*)-5-decenyl acetate. In particular, our studies on chain-shortened analogs indicate that the terminal alkyl chain interacts with a highly complementary hydrophobic "pocket" approximately extending over the two methylene units of **1** closest to the terminal methyl group (Bengtsson et al., 1990).

In the present study, branched alkyl analogs **2–10** (Figure 2) of compound **1** were synthesized and employed to further characterize the nature of the interaction between the substrate and its receptor, and the degree of complementarity between the terminal alkyl chain and the receptor. These analogs are monomethyl substituted in the 9-, 8-, 7-, and 6-positions (compounds **2–5**) and geminally dimethyl substituted in the 9-, 8-, and 7-positions (compounds **6–8**). The series also includes analogs with ethyl substitution in the 8-position (**9**) and propyl substitution in the 7-position (**10**). The receptor responses to the structural variations were determined by the electrophysiological single-sensillum (single-cell) technique.

Three major effects on the activity may be anticipated for alkyl-substituted analogs of the natural pheromone component **1.** Alkyl substitution increases the lipophilicity of the substrate and the affinity for a lipophilic (hydrophobic) receptor site should increase. Secondly, steric repulsive interactions between

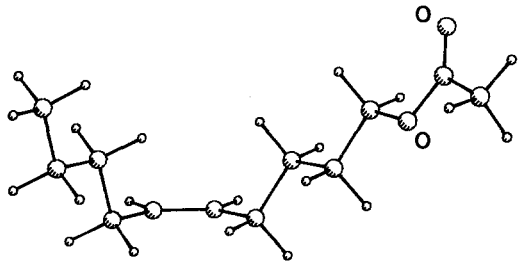


FIG. 1. The deduced biologically active conformation of (*Z*)-5-decenyl acetate.

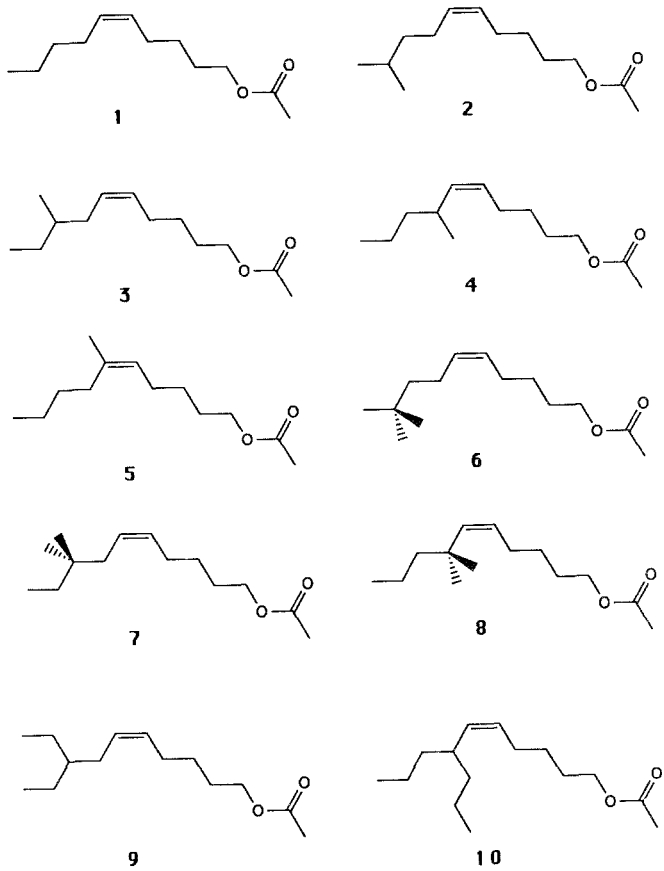


FIG. 2. Compounds studied.

the alkyl substituent and the receptor may cause a decrease or even complete elimination of the activity. Finally, the energy required by an alkyl substituted analog to acquire the biologically active conformation may be high due to steric repulsive interactions caused by the alkyl substituent. This, of course, decreases the activity. In the present study we have attempted to separate the latter two effects by calculating the required energy by the molecular mechanics method using the conformation shown in Figure 1 as a template for the biologically active conformation.

Structure–activity relationships for alkyl-branched pheromone analogs of the monoene acetate type have been reported previously by Priesner et al. (1977), Bestmann et al. (1979, 1980), and Schwarz et al. (1989). The extensive studies performed by Priesner et al. and Bestmann et al. on monoalkyl-substi-

tuted pheromone components of various species of Noctuidae, Tortricidae, and Cochylidae are based exclusively on electroantennographic (EAG) data, while the study by Schwarz et al. (1989) employs flight tunnel and male sex stimulation assays. Such data cannot be directly interpreted in terms of interactions with a single receptor. Only single-cell data are unambiguous in this respect. To our knowledge no single-cell study of alkyl-branched pheromone component analogs has been reported previously.

METHODS AND MATERIALS

All reactions of air- and water-sensitive materials were performed under a nitrogen atmosphere in oven-dried glassware. Anhydrous diethyl ether (ether) and tetrahydrofuran (THF) were distilled from dark-blue solutions of sodium benzophenone radical anion. *N,N'*-Dimethyl-*N,N'*-propylene urea (DMPU), purchased from Fluka AB, was distilled under vacuum from calcium hydride prior to use. The dimethyl sulfide complex of cuprous bromide was prepared by the method of House et al. (1975). The crude pheromone products were purified by flash chromatography with TLC silica gel 60H and petroleum ether–ethyl acetate (20:1) as eluent. Flash chromatographed products were further purified by preparative GLC using a 6-m × 4-mm 15% OV-351 column and/or by argentation chromatography (Houx et al., 1974). Analytical GLC was performed with a Varian 3400 capillary GLC fitted with a DB-Wax 30-m column or a OV-240-OH 25-m column, which is able to separate geometrical isomers. The purity of the isolated final products was >99%. No sample contained any detectable amount (<0.05%) of the natural pheromone component. [^{1}H]-and [^{13}C]NMR spectra on final products were recorded on a Varian XL-300 spectrometer. High resolution mass spectra were recorded on a Jeol JMS-SX 102 spectrometer. The procedures employed in the preparation of the pheromone analogs were modifications of methods described by Alexakis et al. (1980), and Gardette et al. (1985). The synthetic scheme is shown in Figure 3.

Preparation of Alkyl Iodides. The iodides in Figure 3 were prepared (unless otherwise mentioned) from the corresponding commercially available alcohols. The alcohol (0.1 mol) was dissolved in ether (40 ml) and pyridine (1.1 ml) was added. The mixture was cooled with an ice bath and PBr_3(3.8 ml, 0.04 mol) was slowly added. It then was stirred at room temperature for 6 hr and refluxed overnight. The mixture was cooled, poured into ice-water, and extracted with ether. The extracts were washed with $NaHCO_3$, dried, and the solvent evaporated. The crude bromide was added to a solution of NaI (22 g, 0.15 mol) in anhydrous acetone (100 ml). A white precipitate was formed immediately or slowly depending on the presence or position of the alkyl substituent. The mixture was stirred for 2–24 hr (monitored by GLC), and pentane (100 ml) was

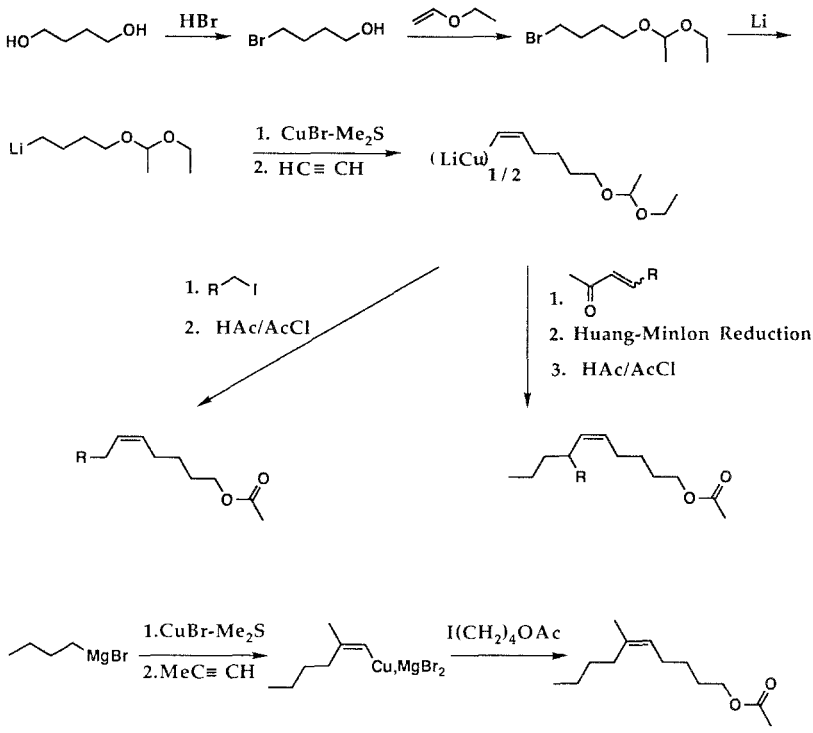


FIG. 3. Scheme for the preparation of compounds **2–10.**

added. After filtration and evaporation, the residue was taken up in another 100 ml of pentane and again filtered. Careful evaporation of the solvent afforded the iodide as a pale yellow oil (60–70% yield). The iodides were homogeneous by NMR and were used without further purification.

Preparation of 4-(1-Ethoxyethoxy)-Butyl Lithium. 4-Bromo-1-butanol was prepared according to the method of Kang et al. (1985). 1,4-Butanediol (44.6 ml, 0.5 mol) and 48% HBr (62.5 ml, 0.55 mol) in benzene (800 ml) were heated at reflux for 5 hr, while the formed water was trapped using a Dean-Stark separator. The solvent was removed under reduced pressure and the residue dissolved in anhydrous CH_2Cl_2 (200 ml). The mixture was cooled on ice to $<18°C$, then ethyl vinyl ether (48 ml, 0.5 mol) was added dropwise during 45 min, keeping the solution temperature between 10 and 16°C. The colorless solution was stirred for an additional hour at room temperature. The solution then was washed with 5% Na_2CO_3 (50 ml), dried with anhydrous Na_2CO_3 + $MgSO_4$, and the solvent evaporated. The product was distilled in carefully base-washed glassware to give 45 g (40% yield, bp 58–68°C/0.7 mm Hg) of

4-(1-ethoxyethoxy)-butyl bromide with a purity >96% (GLC). δ_H (300 MHz) 1.0–1.3 (6H, m, CH_3), 1.4–2.1 (4H, m, CH_2), 3.2–3.7 (6H, m, CH_2Br, CH_2O), 4.7 (1H, q, OCHO).

To freshly cut small chips of lithium in ether (20 ml), a solution of 4-(1-ethoxyethoxy)-butyl bromide (22.5 g, 0.1 mol) in anhydrous ether (60 ml) was added dropwise at −15°C during 1 hr. After the addition was complete, the solution was stirred for 1 hr at 0°C. The greyish turbid solution was then transferred to a 100-ml graduated glass cylinder, carefully dried, and purged with argon. The cylinder was stored at −15°C, allowing cloudy material to precipitate. After 1–2 days, the clear pale yellow solution was titrated by the method of Watson and Eastham (1967) and normally found to be 0.7–0.8 N (yield 70–80%). The solution of the organolithium reagent was stable for at least two months at this temperature.

Preparation of Lithium Bis[(Z)-6-(1-ethoxyethoxy)-hex-1-enyl]cuprate. This compound was prepared by carbocupration of acetylene (Gardette et al., 1985). To a well-stirred suspension of CuBr–Me_2S complex (2.88 g, 14 mmol) in ether (50 ml) at −50°C, 25 mmol of the ethereal solution of 4-(1-ethoxyethoxy)-butyl lithium was slowly transferred by using a double-tipped needle. The solution was warmed to −40°C and stirred for 30 min at this temperature. The almost clear colorless solution was cooled to −50°C, and acetylene (0.6 l, 25 mmol) was bubbled slowly into the reaction mixture. Stirring of the resulting dark green solution was maintained for 30 min at −25°C.

(Z)-5-Decenyl Acetate (1). This was prepared as previously described (Olsson et al., 1983).

9-Methyl-(Z)-5-decenyl Acetate (2). To a stirred solution of lithium bis[(*Z*)-6-(1-ethoxyethoxy)-hex-1-enyl]cuprate (12 mmol) at −30°C, DMPU (6 ml) in THF (40 ml), 3-methylbutyl iodide (4.7 g, 24 mmol) in 10 ml THF and triethyl phosphite (7.5 ml, 25 mmol) were successively added. The mixture was slowly allowed to reach room temperature and stirred overnight. It was then cooled to −20°C and quenched with 5 N HCl (40 ml). After stirring for 15 min with air bubbling into the mixture (which helps the work-up procedure), hexane (50 ml) was added and the precipitate filtered off. The aqueous layer was extracted once with ether (50 ml) and the combined organic extracts were washed once with 17% NH_3 (25 ml), then with saturated NH_4OH solution (25 ml), and finally dried over $MgSO_4$. The solvents were removed, and the residue was treated with glacial acetic acid (15 ml) and acetyl chloride (1.5 ml). The mixture was stirred at 30–40°C overnight and then poured onto ice (50 ml) in saturated sodium chloride solution. The acetate was extracted three times with pentane (25 ml); the organic phase was washed with $NaHCO_3$ solution and dried over $MgSO_4$. After evaporation of the solvent in vacuo, the crude product was purified by flash chromatography to give 2.3 g (45%) of **2** δ_H (300 MHz) 0.88 (d, 6H, J = 6.6 Hz, CH_3C), 1.18–1.26 (m, 2H, CH_3CCH_2), 1.36–1.46

(m, 2H=CCCH$_2$), 1.49–1.69 (m, 3H CH$_3$C**H**, CH$_2$CO), 1.98–2.10 (m, 4H, =CCH$_2$), 2.05 (s, 3H, COCH$_3$), 4.06 (t, 2H, CH$_2$O), 5.27–5.42 (m, 2H, J = 10.9 Hz, CH=CH). δ_C (75.4 MHz) 21.0, 22.5, 25.1, 26.0, 26.7, 27.6, 28.2, 38.9, 64.5, 128.9, 130.7, 171.2. MS (high resolution) for $C_{13}H_{24}O_2$ calcd 212.1776, found 212.1772.

8-Methyl-(Z)-5-decenyl Acetate (3). This was prepared from 2-methylbutyl iodide (4.7 g, 24 mmol) in the same manner as for **2** to give 2.7 g (53%). δ_H (300 MHz) 0.85 (d, 3H, J = 6.6 Hz, C**H**$_3$CH), 0.87 (t, 3H, C**H**$_3$CH$_2$), 1.05–1.22 (m, 1H, CH), 1.28–1.46 (m, 4H, =CCCH$_2$, CH$_3$C**H**$_2$), 1.58–1.68 (m, 2H, CH$_2$CO), 1.82–1.90 (m, 1H, =CCH$_2$) 1.97–2.09 (m, 3H, =CCH$_2$), 2.04 (s, 3H, COCH$_3$), 4.06 (t, 2H, CH$_2$OC), 5.36–5.40 (m, 2H, CH=CH). δ_C (75.4 MHz) 11.5, 19.1, 21.0, 26.0, 26.8, 28.2, 29.2, 34.2, 35.1, 64.5, 129.2, 129.7, 171.2. MS (high resolution) for $C_{13}H_{24}O_2$ calcd 212.1776, found 212.1788.

7-Methyl-(Z)-5-decenyl Acetate (4). 3-Penten-2-one was prepared by aldol condensation between acetone and acetaldehyde according to Stetter (1976). The product was distilled in the presence of 1 g of iodine crystals, giving a two-phase distillate in a temperature range of 100–132°C at atmospheric pressure. The formed water was separated off, the product dried with Na_2SO_4 and redistilled to give 22.7 g (15% yield, a major component, 55%, and a minor one, 40%, by GLC) of the product, bp. 119–122°C; lit. 122°C (Weast, 1971). [^{1}H]NMR in $CDCl_3$ showed three sharp signals at 1.8, 2.1, and 2.2 ppm, along with two separated multiplets at 5.8–6.2 and 6.5–7.1 ppm, which indicated a 3-penten-2-one/mesityl oxide mixture. To a stirred solution of 25 mmol of lithium bis[(Z)-6-(1-ethoxyethoxy)-hex-1-enyl]cuprate at −70°C, the freshly prepared α,β-unsaturated compound (2.4 ml) was added (Alexakis et al., 1980). After 10 min, the mixture was warmed to −10°C during 1 hr. The mixture was then hydrolyzed with saturated NH_4Cl (50 ml), hexane (50 ml) was added, the precipitate was filtered off, and the organic phase dried over $MgSO_4$. The yellow residue, 10 g, was added to a solution of ethylene glycol (45 ml) and KOH (8 g) at 90°C followed by 85% hydrazine hydrate (6 ml). The reaction mixture was heated at reflux temperature (145°C) for 1 hr, the volatile components were then allowed to distill off, increasing the temperature to 205°C. After 3 hr at this temperature, the mixture was cooled to ca. 80°C, poured into ice water (100 ml), and extracted with pentane (3 × 100 ml). The combined extracts were washed with brine, dried with $MgSO_4$, and concentrated in vacuo to give 4.4 g of a pale yellow oil as residue. The oil was acetylated in the usual manner, as described for compound **2**, followed by flash chromatography, yielding 1.1 g (41%) of **4**, 84% pure by GLC. The by-products were easily removed in the final purification. δ_H (300 MHz) 0.86 (t, 3H, C**H**$_3$CH$_2$), 0.91 (d, 3H, J = 6.7 Hz, C**H**$_3$CH), 1.12–1.31 (m, 4H, CH$_3$C**H**$_2$, CH$_3$CC**H**$_2$), 1.34–1.45 (m, 2H, =CCCH$_2$), 1.58–1.68 (m, 2H, CH$_2$CO), 2.00–2.09 (m, 2H, =CCH$_2$), 2.03

(s, 3H, $COCH_3$), 2.33–2.48 (m, 1H, CHCH=), 4.06 (t, 2H, CH_2O), 5.12 (m, 1H, J = 10.19 Hz, CHCH=), 5.26 (m, 1H, J = 10.9 Hz, =$CHCH_2$). δ_C (75.4 MHz) 14.2, 20.6, 21.0, 21.3, 26.2, 27.0, 28.2, 31.4, 39.8, 64.5, 127.5, 137.0, 171.2. MS (high resolution) for $C_{13}H_{24}O_2$ calculated 212.1776, found 212.1793.

6-Methyl-(Z)-5-decenyl Acetate (5). To an ice-cooled mixture of pyridine (50 ml) and acetic anhydride (30.6 g, 0.3 mol), crude 4-bromo-1-butanol (prepared from 0.2 mol of 1,4-butandiol and HBr, as described in the preparation of 4-(1-ethoxyethoxy)-butyl lithium) was added dropwise. The mixture was stirred for 4 hr at room temperature, water (200 ml) and ether (300 ml) were added, and the organic phase was washed with 1 M HCl (100 ml) and $NaHCO_3$ (100 ml). The mixture then was dried with $MgSO_4$, evaporated, and distilled to give 25.8 g (66% yield) of pure acetate bp 98–106°C /16 mm Hg). δ_H 1.5–2.1 (m, 4H, CH_2), 2.0 (s, 3H, $COCH_3$), 3.5 (t, 2H, CH_2Br), 4.1 (t, 2H, CH_2O). Butylmagnesium bromide was prepared from butyl bromide and magnesium turnings in ether and titrated. Propyne was prepared according to the method described by Jäger (1977). To a colorless suspension of CuBr–Me_2S complex (6.1 g, 30 mmol), ether (40 ml), and dimethyl sulfide (30 ml) at −45°C, the ethereal solution of butyl magnesium bromide (30 mmol) was added. After 2 hr of stirring at −45°C, propyne (0.71, 30 mmol) was bubbled into the yellow-orange suspension over a period of 10 min. The resulting greenish mixture was allowed to warm up to −25°C and stirred for 2 hr. DMPU (7.3 ml) in 30 ml of THF was added, followed by the 4-iodo-1-butyl acetate (7.3 g, 30 mmol) in 10 ml THF, and finally triethyl phosphite (7.7 ml). The mixture was then allowed to warm to room temperature (2 hr) and stirred for 8 hr. Hydrolysis with 3 M HCl (50 ml) at −20°C and filtration with subsequent washings, as described for compound **2,** followed by acetylation, afforded 14.6 g of crude product. Purification by flash chromatography gave 2.2 g (35% yield) of the trisubstituted olefin, along with small amounts of disubstituted olefin (which was carefully removed in the final purification). δ_H (300 MHz) 0.90 (t, 3H, CH_3CH_2), 1.23–1.42 (m, 6H, CH_3CH_2, CH_3CCH_2, $=CCCH_2$), 1.57–1.64 (m, 2H, CH_2CO), 1.65–1.67 (m, 3H, CH_3C=), 1.94–2.05 (m, 4H, $=CCH_2$), 2.02 (s, 3H, $COCH_3$), 4.06 (t, 2H, CH_2O), 5.06–5.11 (t, 1H, =$CHCH_2$). δ_C (75.4 MHz) 14.0, 21.0, 22.7, 23.4, 26.3, 27.3, 28.3, 30.3, 31.5, 64.5, 124.4, 136.1, 171.2. MS (high resolution) for $C_{13}H_{24}O_2$ calcd 212.1776, found 212.1782.

9,9-Dimethyl-(Z)-5-decenyl Acetate (6). Through the same procedure as described for **2,** but with 3,3-dimethylbutyl iodide (5.1 g, 24 mmol) in place of 3-methylbutyl iodide, 2.8 g (55%) of **6** was obtained. δ_H (300 MHz) 0.89 (s, 9H, CH_3C), 1.18–1.24 (m, 2H, CH_3CCH_2), 1.36–1.46 (m, 2H, $=CCCH_2$), 1.59–1.69 (m, 2H, CH_2CO), 1.93–2.10 (m, 4H, $=CCH_2$), 2.04 (s, 3H, $COCH_3$), 4.06 (t, 2H, CH_2O), 5.26-5.43 (m, 2H, J = 10.8 Hz, CH=CH). δ_C

(75.4 MHz) 21.0, 22.0, 26.0, 26.6, 28.2, 29.3, 30.4, 44.1, 64.5, 128.6, 131.2, 171.2. MS (high resolution) for $C_{14}H_{26}O_2$ calcd 226.1933, found 226.1964.

8,8-Dimethyl-(Z)-5-decenyl Acetate (7). 2,2-Dimethylbutanol was prepared from the Grignard reagent of 2-methyl-2-bromobutane and paraformaldehyde, bp 130–140°C/ 760 mm Hg. δ_H 0.8–1.0 (9H, s+t, CH_3), 1.1–1.4 (2H, m, CCH_2C), 3.3 (2H, s, CH_2O). The alcohol (26.4 mmol) was converted into the iodide by refluxing with triphenyl phosphite (6.9 ml, 26.4 mmol) and methyl iodide (2.3 ml, 37.0 mmol) for 24 hr. The resulting 2,2-dimethyl-1-butyl iodide was distilled off, and the fraction between 98 and 105°C /100 mm Hg was collected (2.8 g, 50% yield). The iodide was treated with lithium bis[(*Z*)-6-(1-ethoxyethoxy)-hex-1-enyl]cuprate (8 mmol) as described above for compound **2,** to give 0.2 g (7% yield) of compound **7**. δ_H (300 MHz) 0.81 (t, 3H, CH_3CH_2), 0.82 (s, 6H, CH_3C), 1.19–1.27 (q, 2H,CH_3CH_2), 1.35–1.46 (m, 2H, $=CCCH_2$), 1.59–1.68 (m, 2H, CH_2CO), 1.89–1.91 (m, 2H, $CH_3CCH_2C=$), 2.01-2.09 (m, 2H, $=CCH_2$), 2.04 (s, 3H, $COCH_3$), 4.06 (t, 2H, CH_2O), 5.40–5.44 (m, 2H, CH=CH). δ_C (75.4 MHz) 8.5, 21.0, 26.0, 26.4, 26.8, 28.3, 33.6, 34.1, 38.8, 64.5, 127.0, 130.5, 171.2 MS (high resolution) for $C_{14}H_{26}O_2$ calcd 226.1933, found 226.1937.

7,7-Dimethyl-(Z)-5-decenyl Acetate (8). This compound was prepared as described for **4** from mesityl oxide instead of 3-penten-2-one (2.2 ml, 19 mmol), affording 1.6 g (37%) of the product. δ_H (300 MHz) 0.88 (t, 3H, CH_3CH_2), 1.07 (s, 6H, $CH_3CC=$), 1.20–1.34 (m, 4H, CH_3CH_2, CH_3CCH_2), 1.36–1.46 (m, 2H, $=CCCH_2$), 1.60–1.70 (m, 2H, CH_2CO), 2.04 (s, 3H, $COCH_3$), 2.14–2.21 (m, 2H, $=CCH_2$), 4.07 (t, 2H, CH_2O), 5.11–5.25 (m, 2H, J = 12.1 Hz, CH=CH). δ_C (75.4 MHz) 14.9, 18.1, 21.0, 26.6, 27.9, 28.3, 29.0, 36.4, 46.7, 64.5, 128.6, 139.2, 171.2. MS (high resolution) for $C_{14}H_{26}O_2$ calcd 226.1933, found 226.1917.

8-Ethyl-(Z)-5-decenyl acetate (9). This compound was prepared from 2-ethylbutyl iodide (5.1 g, 24 mmol), following the standard procedure described above for **2**, to give 2.0 g (40%). δ_H (300 MHz) 0.85 (t, 6H, J = 7.1 Hz, CH_3CH_2), 1.18–1.34 (m, 4H, CH_3CH_2), 1.36–1.46 (m, 2H, $=CCCH_2$), 1.55–1.59 (m, 1H, CH_3CCH), 1.59–1.69 (m, 2H, CH_2CO), 1.96–2.10 (m, 4H, $=CCH_2$), 2.05 (s, 3H, $COCH_3$), 4.06 (t, 2H, CH_2O), 5.36–5.39 (m, 2H, CH=CH). δ_C (75.4 MHz) 11.1, 21.0, 25.4, 26.0, 26.9, 28.25, 30.5, 41.2, 64.5, 129.2, 129.8, 171.2. MS (high resolution) for $C_{14}H_{26}O_2$ calcd 226.1933, found 226.1943.

7-Propyl-(Z)-5-decenyl Acetate (10). 3-Hepten-2-one was prepared in analogy with the preparation of 3-penten-2-one, described above for compound **4**. The distillate, in the dehydration step, was obtained in a temperature range of 58–120°C /200 mm Hg and redistilled (bp 54–60°C /17 mm Hg), affording

28.8 g (26% yield, major component, 65% by GLC) of 3-hepten-2-one; lit. 62°C /15 mm Hg (Weast, 1971). δ_H 0.8–1.1 (3H, t, CH_3), 1.2–1.9 (4H, m, CH_2), 2.2 (3H, s, CH_3), 5.8–7.1 (2H, m, CH=CH). The conjugate addition was run with 19 mmol of lithium bis[(*Z*)-6-(1-ethoxyethoxy)-hex-1-enyl]cuprate, as described for analog **4**, to yield 3.6 g of crude product and after purification by flash chromatography 1.4 g (31%) of the product (80% pure by GLC). The by-products were easily removed in the final purification. δ_H (300 Mz) 0.85 (t, 6H, CH_3CH_2), 1.13–1.42 (m, 10H, CH_3CH_2, CH_3CCH_2, $=CCCH_2$), 1.58–1.68 (m, 2H, CH_2CO), 1.98–2.08 (m, 2H, $=CHCH_2$), 2.03 (s, 3H, $COCH_3$), 2.20–2.35 (m, 1H, CHC=), 4.06 (t, 2H, CH_2O), 5.12 (m, 1H, J = 10.9 Hz, CHCH=), 5.26 (m, 1H, J = 10.9 Hz, $=CHCH_2$). δ_C (75.4 MHz) 14.3, 20.5, 21.0, 26.2, 27.2, 28.3, 36.8, 38.2, 64.5, 128.7, 135.7, 171.2. MS (high resolution) for $C_{15}H_{28}O_2$ calcd 240.2089, found 240.2079.

Electrophysiology. Biological activities were determined by the single-sensillum technique (Kaissling, 1974). Olfactory receptor cells specifically tuned to (*Z*)-5-decenyl acetate (**1**) are present in antennal sensilla type SW1 of *Agrotis segetum* and are readily accessible for single-cell recordings (Hallberg, 1981; Löfstedt et al., 1982, van der Pers and Löfstedt, 1983). The method used was modified according to van der Pers and Den Otter (1978) and has been described previously (Liljefors et al., 1987; Bengtsson et al., 1990). Dose–response curves were constructed using five different stimulus concentrations. The concentration for the pheromone component **1** and the analogs ranged from 10^{-4} μg to 1 μg and $10^{-3}$$\mu$g to 100 μg, respectively, in decadic steps. For each stimulus concentration, 10 replicates were recorded and the mean value of the number of action potentials generated during 1 sec from the onset of stimulation was used in the construction of the dose–response curves. Errors are expressed as standard errors of mean (SEM). The relative activity of each compound is expressed as the reciprocal of the relative quantities required to elicit the same response from the receptor cell, as the natural pheromone component elicited.

Corrections for differences in volatility have been made using relative vapor pressures, as previously described (Liljefors et al., 1985; Bengtsson et al., 1990). The correction factors for compounds **2–8** are based on vapor pressure data for saturated hydrocarbons (pentane–tridecane) and their methyl-substituted analogs (Weast, 1971). The correction factors used are 2.2 for the methyl analogs **2–5** and 4.6 for dimethyl analogs **6–8.** The correction factor for compound **9** is 7.1.

Molecular Mechanics Calculations. Energy-minimized geometries and conformational energies were calculated using the molecular mechanics program MM2(85) (Burkert and Allinger, 1982). Starting structures for the energy-minimization program were obtained from the molecular modeling system MIMIC (Liljefors, 1983, von der Lieth et al., 1984). Van der Waals volumes

and surfaces were calculated and plotted by the program system SYBYL (Tripos Associates, Inc., 6548 Clayton Road, St. Louis, Missouri 63117).

RESULTS

Chemicals. The method used for the preparation of compounds **2–10** is based on the carbocupration of acetylene. This synthetic method affords products of very high (>99.9%) *Z* purity and is well suited for the synthesis of insect sex pheromones. It is also preparatively versatile, since both α- and ω-substituted analogs (with respect to the double bond) are obtained with the same synthetic intermediates. The 7-alkyl-substituted analogs were thus obtained by conjugate addition of (*Z*)-alkenyl cuprate to α,β-unsaturated ketones with subsequent Huang-Minlon reduction, while the 8- and 9-substituted analogs were obtained by alkylation of (*Z*)-alkenyl cuprate with alkyl halides (Figure 3). The trisubstituted olefin analog **5** was obtained in a similar way by carbocupration of propyne followed by alkylation. As pointed out by Gardette et al. (1985), a decisive factor for a successful preparation of the organocuprate reagent is the quality of the cuprous salt. The best results are obtained with the dimethyl sulfide complex of cuprous bromide. The choice of the protective group for the hydroxy function in the preparation of the lithium reagent is equally decisive (Gardette et al., 1985). Only a hydroxy function protected as an ethoxyethyl ether gives acceptable results.

Receptor Cell Responses. The results of the electrophysiological single-cell activity determinations for compounds **1–10** are shown in Figure 4. The

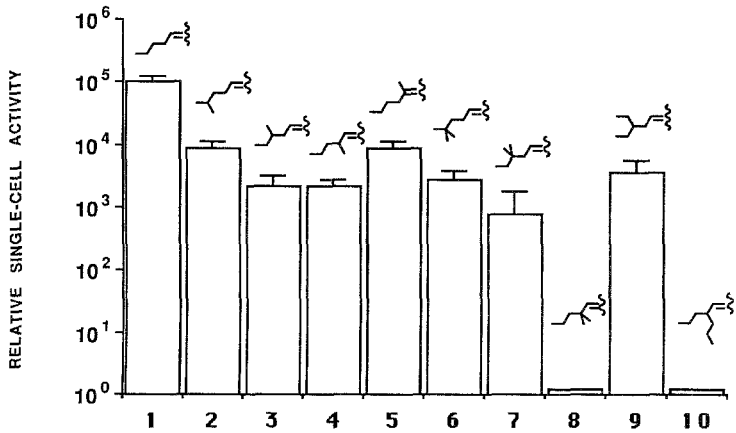


FIG. 4. Relative single-cell activities (+SEM) for compounds **1–10.**

data include corrections for differences in volatility (see Methods and Materials).

All analogs display reduced activity compared to the natural pheromone component **1.** Mono-methyl substitution in the 9- or 6-position (compounds **2** and **5,** respectively) reduces the activity by a factor of 10. This is the smallest activity decrease in the present series of analogs. A corresponding substitution in the 8- or 7-position (compounds **3** and **4,** respectively) results in a somewhat larger drop of the activity, by a factor of about 45 compared to the natural substrate **1.** These results are in qualitative agreement with those obtained by Priesner et al. (1977) in their EAG screenings of monomethylated pheromone components for several species of Noctuidae and Tortricidae. For analogs of (*Z*)-7-dodecenyl acetate, (*Z*)-9-tetradecenyl acetate, and (*Z*)-11-hexadecenyl acetate with the same terminal alkyl chain as compounds **2–4,** they report activity reductions by a factor of 10–30, largely irrespective of the position of the methyl group.

The 8-ethyl analog (compound **9**), displays an activity loss of about a factor of 30, close to the activity observed for the 8-methyl analog **3.** The 7-propyl compound **10** was found to be essentially inactive.

Geminal dimethyl substitution in the 9-, 8- or 7-positions (compounds **6–8**) results in a successive diminution of the activity. Compounds **6** and **7** are about 40 and 95 times less active than compound **1,** while compound **8** is essentially inactive. The activity decrease on the introduction of a second methyl group in the 9- or 8-position is essentially identical. However, a second methyl substitutent in the 7-position eliminates all receptor activity.

Conformational Energies. Conformational energies were calculated by the molecular mechanics method to evaluate the energies required by each of compounds **2–10** to attain their biologically active conformations, according to our model (Liljefors et al., 1987). These calculations were performed by first calculating the global energy minimum for each of compounds **2–10.** This was done by energy minimizations of all staggered conformations of the terminal alkyl chain for the cisoid and transoid conformers with respect to the vinylic bonds, while the acetate-substituted chain segment was kept in an all-anti conformation.

In a second series of calculations, energy-minimization was performed for the biologically active conformations of each of these compounds according to our model, which requires mimicking the positions of the acetate group, the double bond, and the terminal methyl group of the natural substrate in the conformation shown in Figure 1. This requires the main chain of the terminal alkyl part to adopt an all-anti conformation. An alkyl group in the 7-, 8-, 9-position may then adopt two different directions denoted A and B in Figure 5. In the B direction, the alkyl group is oriented towards the acetate substituted alkyl chain (''inwards'') and may suffer from steric repulsive interactions with this chain.

In direction A, an alkyl group is directed away from the other chain (''outwards''). For compound **2** the A and B directions correspond to different rotamers and the 9-methyl group may change its direction by rotation about the C8—C9 bond. In compounds **3** and **4** the methyl substituent in the A and B positions corresponds to different enantiomers. Compounds **9** and **10** are nonchiral, and the alkyl substituent may be oriented in the A or B directions through rotations about carbon–carbon single bonds.

The energy differences between the two sets of calculations correspond to the energies required by each of compounds **2–10** to acquired the biologically active conformation according to our model (Liljefors et al., 1987).

The results of these calculations are shown in Table 1. The most pronounced steric interactions are found for 8- and 7-alkyl substituents in direction B (compounds **3, 4, 7, 9,** and **10**). This is due to repulsive interactions between

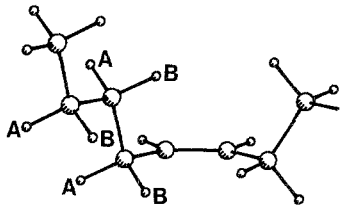


FIG. 5. Possible alkyl group directions in the biologically active conformation of compounds **2–10.**

TABLE 1. CALCULATED CONFORMATIONAL ENERGY DIFFERENCES (kcal/mol) BETWEEN BIOLOGICALLY ACTIVE CONFORMATION OF EACH OF COMPOUNDS **1–10** AND THEIR LOWEST ENERGY CONFORMATIONS[a]

Compound	A	A + B	B
1		0.0[b]	
2	0.3		0.1
3	0.7		1.8
4	0.4		2.0
5		0.2[b]	
6		0.7	
7		1.9	
8		0.8	
9	1.3		3.4
10	0.5		1.7

[a] A and B denote alkyl group directions according to Figure 5.
[b] the A/B designation is not applicable in this case

the alkyl substituent and the acetate-substituted alkyl chain in the cisoid conformation. An 8-ethyl substituent in direction A results in a conformational energy of 1.3 kcal/mol. All other substitutions result in small conformational energies, 0.1–0.8 kcal/mol. Figure 6 illustrates the difference in conformation between the global energy minimum and the biologically active conformation for compound **7** and the energy required to attain this conformation. Note that the conformation of the global energy minimum has to be rearranged with respect to the C7–C8 and C8–C9 bonds in order to mimic the structure of the natural pheromone component shown in Figure 1, as required by our model.

DISCUSSION

In previous structure–activity work on analogs of compound **1**, we found that a conformational energy increase of 1.7 kcal/mol for the analog to acquire its biologically active conformation corresponds to an activity decrease of a factor of 10 (Bengtsson et al., 1987). This finding may be employed in the present study to investigate if a decrease in the observed activity may be due to

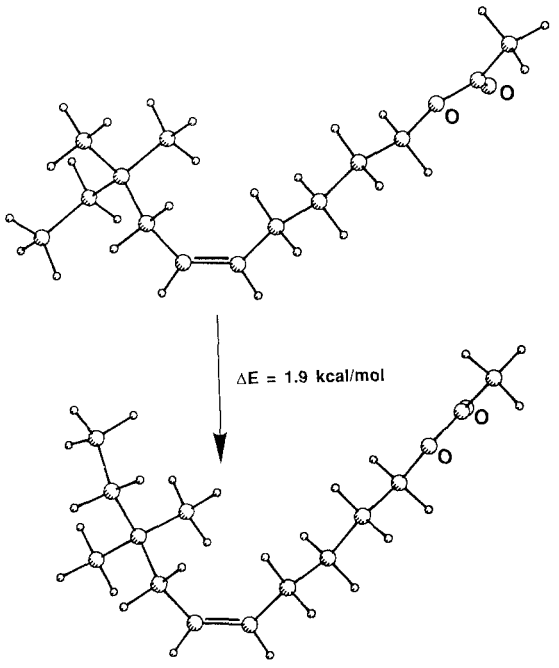


FIG. 6. Global energy minimum (top) and biologically active (bottom) conformations of compound **7**.

a conformational energy penalty or to steric repulsive interactions with the receptor.

A methyl substitutent in the 6-position, compound **5**, decreases the electrophysiological activity by a factor of 10 compared to the natural substrate **1** (Figure 4). The conformational energy required by compound **5** to mimic the natural component is calculated to be very small, 0.2 kcal/mol (Table 1). Thus, the observed activity decrease most probably is due to repulsive steric interactions between the methyl substitutent and the receptor. The van der Waals volume of compound **1** is illustrated in Figure 7. This volume represents the smallest cavity that can accommodate the natural pheromone component in its deduced biologically active conformation. The extra volume required by compound **5** is shown in Figure 8a. The surface shown represents the difference in volume between compound **5** and compound **1.** It is clear that a bulk of this size in the indicated area is not tolerated by the receptor, demonstrating a close fit between the receptor and the natural substrate in this area of the receptor cavity.

The 9-methyl compound **2** is nonchiral, and a methyl group may be oriented in either direction A or B (Figure 5) through rotation about the C8–C9 bond. The bulk produced in these directions by a methyl group is illustrated in Figure 8b, which shows the difference in the van der Waals volumes of the 9,9-dimethyl substituted compound **6** and compound **1.** If one of these directions is sterically unhindered, compound **2** may place its methyl group in the unhindered direction, and since the calculated conformational energy is very low (Table 1), the activity should be comparable to the natural component **1.**

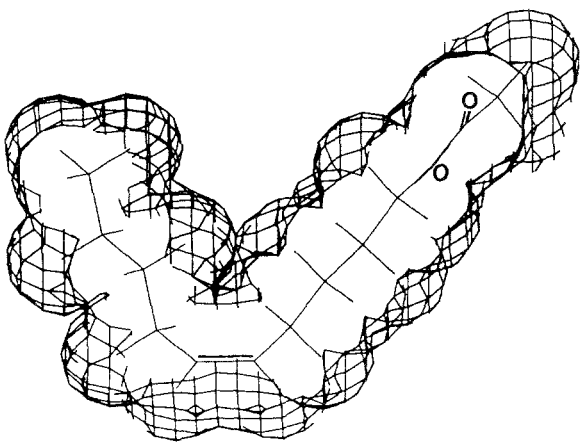


FIG. 7. Van der Waals surface of compound **1** in its deduced biologically active conformation.

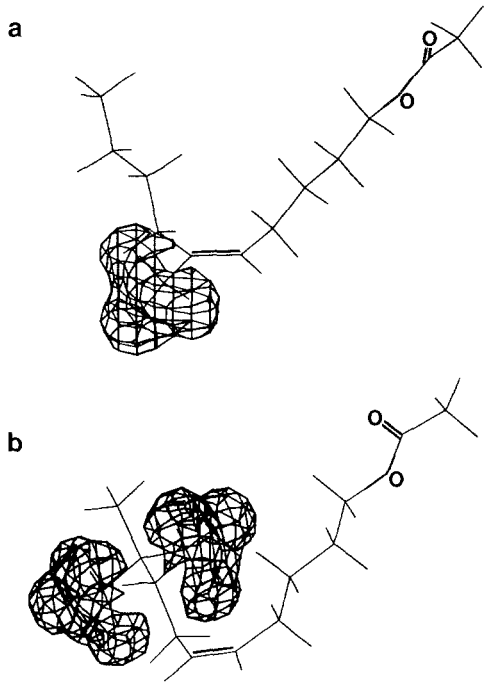


FIG. 8. The difference between the van der Waals volumes of (a) compounds **5** and **1** and (b) compounds **6** and **1.**

However, the observed activity for **2** is a factor of 10 lower than that of **1.** This strongly indicates that a methyl group in either direction A or B in Figure 5 causes repulsive steric interactions with the receptor. This is supported by the further decrease in activity of the geminally dimethyl substituted compound **6** by a factor of 5, in spite of the higher lipophilicity of this compound (Figure 4). Thus, the receptor displays high complementarity with respect to the natural substrate on both sides in the vicinity of the terminal methyl group.

A methyl substituent in the 8-position (compound **3**) decreases the electrophysiological activity by a factor of 45. Compound **3** is chiral and is tested as its racemate. In terms of our receptor interaction model, this means that half the molecules have the 8-methyl group in direction A (Figure 5) and the other half in direction B. The A direction results in a small calculated conformational energy, 0.7 kcal/mol, to acquire the biologically active conformation, while the B direction gives a somewhat higher energy, 1.7 kcal/mol (Table 1). The racemate should then display an activity decrease by less than a factor of 10 due to the conformational energy penalty. If both directions A and B are without steric hindrance at the receptor and if the difference in lipophilicity between com-

pound **3** and **1** is taken into account, the activity of **3** should be of the same magnitude as that for compound **1.** As this is not the case, our conclusion is that the receptor "wall" destructively interferes with a methyl group in the area about the 8-position as well. This also is indicated by the low activity of the dimethyl-substituted compound **7.** However, the question of whether the steric hindrance is caused by a methyl group in the A or B direction (or both) cannot be answered on the basis of the present data for compounds **3** and **7.** A study of the pure enantiomers of compounds **3** may give more information on this problem.

An ethyl substituent in the 8-position (compound **9**) decreases the electrophysiological activity by a factor of 30. In this case our result is somewhat different from that obtained by Prienser et al. (1977) in the EAG study mentioned above. In their study, an ethyl group in the β-position, with respect to the double bond, causes a drop of the activity by a factor of 100–300, which is the largest decrease they found for alkyl-branched analog of pheromone components with the same terminal alkyl part as **1.**

Compound **9** is nonchiral and displays the highest calculated conformational energies in the present series of molecules (Table 1). The calculated conformational energy for the B direction (Table 1 and Figure 5), in conjunction with the higher lipophilicity of compound **9** in comparison with compound **1,** is compatible with the observed activity decrease according to the relationship between conformational energy and electrophysiological activity discussed above. However, a combination of steric hindrance by the receptor in the A direction and the calculated conformational energy penalty, 1.3 kcal/mol (Table 1) for an ethyl group in this orientation, is also compatible with the observed activity.

Alkyl substitution in the 7-position (compounds **4, 8,** and **10**) causes drastic variations in the activity, depending on the number of substituents and alkyl size. One methyl group (compound **4**) decreases the activity by a factor of 45, while two methyl groups in this position result in an essentially inactive compound (**8**). As the calculated conformational energy penalty for compound **8** is only 0.8 kcal/mol (Table 1), this clearly indicates that an alkyl substituent in at least one of the directions A and B destructively interacts with the receptor in a severe way. An investigation of the pure enantiomers of the chiral compound **4** is necessary for further information on the exact nature of this interaction. Bestmann et al. (1980) studied the EAG response of the enantiomers of the 13-ethyl substituted analog of (*Z*)-11-tetradecenyl acetate, a pheromone component of *Ostrinia nubilalis* and *Argyrotaenia velutinana*. The *R* and *S* enantiomers were found to be 10 and 1000 times less active, respectively, than the natural pheromone component. This demonstrates that pheromone receptors tuned to achiral monoene acetates are capable of differentiating between enantiomers of alkyl substituted analogs.

The 7-propyl compound **10** is essentially inactive in our study. This is in sharp contrast to the very high EAG activities observed by Priesner et al. (1977) for α-propyl analogs corresponding to compound **10.** They report activity reductions by only a factor of 3–30. No corrections for differences in volatilities were made in their work. If such corrections are made, these α-propyl analogs are in fact at least as active as the natural pheromone component. Our calculated small conformational energy penalties for compound **10** (Table 1), and the lack of single-cell response to this compound, indicate that the bulk of the propyl group can not be accommodated by the (*Z*)-5-decenyl acetate receptor of *Agrotis segetum.*

CONCLUSIONS

The results of the structure–activity analysis of alkyl-substituted analogs of the natural pheromone component **1** strongly indicate a high complementarity between the receptor and the terminal alkyl part of its natural substrate. This confirms our previous conclusions based on studies of chain-shortened analogs (Bengtsson et al., 1990). The introduction of bulky groups in the terminal alkyl part of the substrate substantially reduces the electrophysiological response. In particular, the introduction of a propyl group, or geminal dimethyl substitution, in the α-position with respect to the double bond results in essentially inactive compounds. The inactivity of the α-propyl compound is in sharp contrast to previously reported EAG results for homologous pheromone components of other noctuid species.

Acknowledgments—This work was financially supported by the Swedish Natural Science Council, which is gratefully acknowledged. We thank Dr. Ingrid Pettersson for the calculations of the van der Waals surfaces and Dr. Robert E. Carter for linguistic criticism.

REFERENCES

ALEXAKIS, A., CAHIEZ, G., and NORMANT, J.F. 1980. Vinyl-copper derivatives XI. Reactivity of (*Z*)-alkenyl cuprates towards various electrophiles. Application to the synthesis of some natural products. *Tetrahedron* 36:1961–1969.

ARN, H., STÄDLER, E., RAUSCHER, S., BUSER, H.R., MUSTAPARTA, H., ESBJERG, P., PHILIPSEN, H., ZETHNER, O., STRUBLE, D.L., and BUES, R. 1980. Multicomponent sex pheromone in *Agrotis segetum*: Preliminary analysis and field evaluation. *Z. Naturforsch.*35c:986–989.

BENGTSSON, M. 1988. Structure–activity relationships for analogues of (*Z*)-5-decenyl acetate, a sex pheromone component of the turnip moth, *Agrotis segetum.* Synthesis and conformational analysis. PhD thesis. University of Lund, Sweden.

BENGTSSON, M., LILJEFORS, T., and HANSSON, B.S., 1987. Dienic analogues of (*Z*)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*: Synthesis, conformational analysis and structure-activity relationships. *Bioorg. Chem.* 15:409–422.

BENGTSSON, M., LILJEFORS, T., HANSSON, B.S., LÖFSTEDT, C., and COPAJA, S.V. 1990. Structure-activity relationships for chain-shortened analogs of (*Z*)-5-decenyl acetate a pheromone component of the turnip moth, *Agroties segetum*. *J. Chem. Ecol.*16:667–684.

BESTMANN, H.J., VOSTROWSKY, O., KOSCHATZKY, K.H., PLATZ, H., BROSCHE, T., KANTARDJIEW, I., RHEINWALD, M., and KNAUF, W. 1978. (*Z*)-5-Decenyl acetate, ein Sexuallockstoff für Männchen der Saateule *Agrotis segetum* (Lepidoptera). *Angew. Chem.* 90:815–816.

BESTMANN, H.J., RÖSEL, P., and VOSTROWSKY, O. 1979. Pheromones, XXV. Alkylverzweigte analoge von Lepidopterenpheromonen. *Liebigs Ann. Chem.* 1189–1204.

BESTMANN, H.J., HIRSCH, H.L., PLATZ, H., RHEINWALD, M. and VOSTROWSKY, O. 1980. Differenzierung chiraler Pheromon-Analoga durch Chemorezeptoren. *Angew. Chem.* 92:492–493.

BURKERT, U., and ALLINGER, N.L. 1982. Molecular Mechanics. American Chemical Society, Washington D.C.

GARDETTE, M., ALEXAKIS, A., and NORMANT, J.F. 1985. Carbocupration of alkynes by organocopper reagents bearing a protected hydroxy or thiol function. *Tetrahedron* 41:5887–5889.

HALLBERG, E., 1981. Fine-structural characteristics of the antennal sensilla of *Agrotis segetum* (Insecta: Lepidoptera). *Cell Tissue Res.* 218:209–218.

HOUSE, H.O., CHU, C.Y., WILKINS, J.M., and UMEN, M.J. 1975. The chemistry of carbanions. XXVII. A convenient precursor for the generation of lithium organocuprates. *J. Org. Chem.* 40:1460–1469.

HOUX, N.W.H., VOERMAN, S., and JONGEN, W.M.F. 1974. Purification and analysis of synthetic insect sex attractants by liquid chromatography on a silver-loaded resin. *J. Chromatogr.* 96:25–32.

JÄGER, V. 1977. Acetylene aus anderen Acetylenen und Acetylen-Derivaten durch Substitutionsreaktionen, pp. 408–677 in E. Müller (ed.). Methoden der organischen Chemie/(Houben-Weyl), Vol. 2a. Georg Thieme Verlag, Stuttgart.

KAISSLING, K.-E. 1974. Sensory transduction in insect olfactory receptors, pp. 243–273, in L. Jaenicke (ed.). Biochemistry of Sensory Functions. Springer-Verlag, Berlin.

KANG, S.K., KIM, W.S., and MOON, B.H. 1985. An effective method for the preparation of ω-bromoalkanols from α,ω-diols. *Synthesis* 1161–1162.

LILJEFORS, T. 1983. MOLBUILD—an interactive computer graphics interface to molecular mechanics. *J. Mol. Graphics* 1:111–117.

LILJEFORS, T., THELIN, B., VAN DER PERS, J.N.C., and LÖFSTEDT, C. 1985. Chain-elongated analogs of a pheromone component of the turnip moth, *Agrotis segetum*. A structure–activity study using molecular mechanics. *J. Chem. Soc. Perkin Trans.* 2:1957–1962.

LILJEFORS, T., BENGTSSON, M., and HANSSON, B.S., 1987. Effects of double-bond configuration on interaction between a moth sex pheromone component and its receptor: A receptor–interaction model based on molecular mechanics. *J. Chem. Ecol.* 13:2023–2040.

LÖFSTEDT, C., VAN DER PERS, J.N.C., LÖFQUIST, J., LANNE, B.S., APPELGREN, M., BERGSTRÖM, G., and THELIN, B. 1982. Sex pheromone components of the turnip moth, *Agrotis segetum*: Chemical identification, electrophysiological evaluation and behavioral activity. *J. Chem. Ecol.* 8:1305–1321.

OLSSON, A.-M., JÖNSSON, J.-Å, THELIN, B., and LILJEFORS, T. 1983. Determination of the vapor pressures of moth sex pheromone components by a gas chromatographic method *J. Chem. Ecol.* 9:375–385.

PRIESNER, E., BESTMANN, H.J., VOSTROWSKY, O., and RÖSEL, P. 1977. Sensory efficacy of alkyl-branched pheromone analogues in noctuid and tortricid Lepidoptera. *Z. Naturforsch.* 32c:979–991.

SCHWARZ, M., KLUN, J.A., FRITZ, G.L., UEBEL, E.C., and RAINA, A.K. 1989. European corn borer sex pheromone: Structure–activity relationships. *J. Chem. Ecol.* 15:601–617.

STETTER, H. 1976. Aldolkondensation von Aldehyden mit Ketonen, pp. 1457–1482, in E. Müller

(ed.). Methoden der organischen Chemie/(Houben-Weyl), Vol. II/2b. Georg Thieme Verlag, Stuttgart.

VAN DER PERS, J.N.C., and DEN OTTER, C.J. 1978. Single cell responses from olfactory receptor of small ermine moths (Lepidoptera: Yponomeutidae) to sex attractants. *J. Insect Physiol.* 24:337–343.

VAN DER PERS, J.N.C., and LÖFSTEDT. C. 1983. Continuous single sensillum recording as a detection method for moth pheromone components in the effluent of a gas chromatograph. *Physiol. Entomol.* 8:203–211.

VON DER LIETH, C.W., CARTER, R. E., DOLATA, D.P., and LILJEFORS, T. 1984. RINGS—a general program to build ring systems. *J. Mol. Graphics* 2:117–123.

WATSON, S.C., and EASTHAM, J.F., 1967. Colored indicators for simple direct titration of magnesium and lithium reagents. *J. Organomet. Chem.* 9:165–168.

WEAST, R.C. (ed.). 1971. Handbook of Chemistry and Physics, 52nd ed. Chemical Rubber Publishing Company, Cleveland, Ohio.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

METHODS AND PITFALLS OF EXTRACTING CONDENSED TANNINS AND OTHER PHENOLICS FROM PLANTS: INSIGHTS FROM INVESTIGATIONS ON *Eucalyptus* LEAVES

STEVEN J. CORK* and ANDREW K. KROCKENBERGER[1]

CSIRO
Division of Wildlife & Ecology
PO Box 84
Lyneham, ACT 2601, Australia

(Received May 25, 1990; accepted August 21, 1990)

Abstract—Optimal conditions for extraction of tannins and other phenolics from tree foliage and their subsequent storage rarely have been investigated. We investigated methods of drying leaves, optimal solvents, and the effects of light and temperature on the extractability and stability of condensed tannins (proanthocyanidins) and total phenolics from leaves of *Eucalyptus* trees. Aqueous acetone was a better solvent than aqueous methanol for condensed tannins and total phenolics, but condensed tannins were less stable in aqueous acetone than aqueous methanol. Stability of condensed tannins also was decreased substantially by room temperature versus 4°C and by exposure to indirect sunlight, although the assay for total phenolics was unaffected. For quantitative estimation of condensed tannins, extraction with 50% acetone was better than methods of direct analysis of leaf tissue. The highest estimates of total condensed tannins were obtained by exhaustive extraction with 50% acetone followed by direct analysis of the residue. Lyophilization of fresh leaf increased yield of condensed tannin (although usually by less than 10%). Lyophilization and subsequent storage of extracts had little effect on assays for condensed tannins or total phenolics.

Key Words—Tannin extraction, plant phenolics, *Eucalyptus*, browse analysis.

INTRODUCTION

Much attention has been paid to chemical analysis of tannins and other phenolics in plant foliage because of their postulated ecological importance in plant–plant and plant–animal interactions. Methods for chemical and biochemical

*To whom correspondence should be addressed.
[1] Present address: School of Biological Sciences, University of Sydney, N.S.W. 2006, Australia.

0098-0331/91/0100-0123$06.50/0

analysis of tannins have been reviewed in detail (Mole and Waterman, 1987a,b; Hagerman and Butler, 1989). However, methods of preparing leaf samples for chemical analysis, which can introduce greater errors than the analyses themselves, have received little critical attention apart from a recent paper by Hagerman (1988).

Frequently it is inconvenient to analyze fresh leaf tissue and some form of drying is needed. It is well established that air- or oven-drying, especially at temperatures above 70°C, decreases yield of tannins and other phenolics (Bate-Smith, 1975; Price et al., 1979; Gartlan et al., 1980; Lindroth and Pajutee, 1987; Hagerman, 1988). Lyophilization (freeze-drying) is the most promising compromise and is adopted uncritically by many workers. However, the few studies that have investigated the effects of lyophilization on yield of phenolics have shown that some loss, no loss, or some enhancement of tannin extractability is possible (Martin and Martin, 1983; Price et al., 1979; Hagerman, 1988).

Debate about the best solvent for tannins in plant tissues has been based on a limited number of studies of few plant species, and conclusions are not always consistent with one another. Aqueous methanol (boiling or at room temperature), acidic methanol, and aqueous acetone have been recommended by various authors. Usually one solvent is recommended without comparison with alternatives or without measurement or estimation of the extent of extraction achieved. The suitability of methanol as an extractant for hydrolyzable tannins has been questioned (Haslam et al., 1961; Swain, 1979), and its ability to extract condensed tannins from some plants also is low (Bate-Smith, 1973, 1975; Foo and Porter, 1980). Many workers now use aqueous acetone for extraction of tannins, although it does not give high recoveries with all plants (Martin and Martin, 1984; Stafford and Cheng, 1980). The presence of water in organic extractants can both increase extraction of phenolics and enhance their breakdown after extraction (Swain, 1979; Lindroth and Pajutee, 1987). The choice of water content usually appears to be arbitrary between 0% and 50% and to follow precedents set by other workers analyzing other plant species.

Extraction time has been studied even less rigorously. Times from a few minutes to one or more days have been used. Short extraction times aim to minimize chemical degradation of extracted tannins, and long times to maximize extraction, but seldom is the extent of extraction or degree of degradation quantified. Similarly, few published methodologies make any recommendations about exposure to light, which might affect the stability of phenolics.

We investigated the best pretreatment, solvent, time, and temperature for extraction of tannins and other phenolics from the foliage of *Eucalyptus* trees, as a preliminary to a survey of tannins in eucalypt forests in southeast Australia. The foliage of eucalypts contains a wide range of phenolic compounds, including condensed and hydrolyzable tannins and nontannin phenolics (Hillis, 1966;

Fox and Macauley, 1977; Cork, 1984; Cork and Pahl, 1984). We observed several trends that probably have general applicability, have been reported seldom or never before in the literature, and could substantially alter conclusions from chemical analyses of tannins in plants unless taken into account.

METHODS AND MATERIALS

Samples and Their Collection. We collected mature leaves from low branches of *Eucalyptus* spp. trees growing in the grounds of CSIRO Division of Wildlife and Ecology in Canberra. The following codes are used throughout this paper to designate the trees that were sampled: EMAN1 and EMAN2—two individuals of *Eucalyptus mannifera*; EMEL1 and EMEL2—*E. melliodora*; EVIM—*E. viminalis*; EPAUC—*E. pauciflora*; EHYB—unidentified hybrid eucalypt. The leaves were immediately frozen and ground in liquid nitrogen in a small coffee-grinder or by mortar and pestle. A portion of this ground, undried leaf was stored at −20°C and another portion was lyophilized first.

During all of the tests described below, the dry matter content of samples (including those lyophilized) was determined by oven-drying a subsample to constant mass at 70°C. Total phenolics in acetone or methanol extracts (details of extraction procedure are given below) were analyzed by the Folin-Ciocalteu method (Singleton and Rossi, 1965). Condensed tannins (a subset of total phenolics) in acetone or methanol extracts, solid residues remaining after extraction, and unextracted solid samples were analyzed by the HCl–butanol (proanthocyanidin) method (Reed et al., 1982; Mole and Waterman, 1987a). The HCl–butanol reagent used in all of these analyses (Mole and Waterman, 1987a) contained a transition-metal salt ($FeSO_4 \cdot 7H_2O$) to ensure consistency of proanthocyanidin yield (Porter et al., 1986). Correction was made for interference from chlorophyll by running unheated reagent + sample blanks (Swain and Hillis, 1959). The extent of interference was small compared with the tannin concentrations, so it was deemed unnecessary to take more comprehensive precautions, such as suggested by Watterson and Butler (1983). Due to the difficulty of obtaining suitable standards for phenolic analyses (Hagerman and Butler, 1989; Mole et al., 1989), we expressed all values relative to quebracho tannin (a condensed tannin). The quebracho tannin was purified (Asquith and Butler, 1985) from crude material supplied by A. E. Hagerman (Hagerman and Butler, 1989). Quebracho clearly produced relatively more color than eucalypt total phenolics in the Folin-Ciocalteu assay and/or less color than eucalypt condensed tannin in the HCl–butanol assay, resulting in estimates of condensed tannin often being greater than total phenolics. This did not, however, affect any of the comparisons made or conclusions drawn. We did not analyze for hydrolyzable tannins because a suitable method was not available, although one has come to our attention since (Inoue and Hagerman, 1988).

Investigation of Extraction Conditions (Experiment 1). We observed in preliminary experiments that when either acetone- or methanol-based aqueous solvents were used at room temperature with exposure to indirect sunlight (normal daytime room light), the concentration of condensed tannins (although not of total phenolics) in the extract fell rather than rose after about 2–5 hr. This suggested instability due to light and/or temperature. Therefore, experiment 1 was set up to investigate the stability of leaf extracts (in 50% and 70% acetone, and 50% methanol for reasons given below) under three storage conditions: (1) unprotected from indirect sunlight (on laboratory bench) at room temperature; (2) in a dark cupboard at room temperature; and (3) in the dark at 4°C. The extracts were sampled initially and after three days.

Investigation of Solvents (Experiment 2). In this experiment we compared methanol-based with acetone-based solvents because these are most often recommended. Preliminary investigations showed that anhydrous acetone or methanol extracted less than 50% of the total phenolics and condensed tannins that aqueous mixtures extracted, so no further investigation of anhydrous solvents was made. We had found previously that 50% methanol was superior to higher proportions of methanol in water for extracting phenolics from eucalypts. Fifty percent methanol was compared with 50% and 70% acetone in the experiments reported here.

Samples were extracted (0.02 g wet leaf per 5 ml solvent) in test tubes at 4°C in a sonicator (to minimize extraction time) for three successive 30-min periods. Previous tests had shown that negligible amounts of condensed tannins or total phenolics were removed by further extraction. After each period, the tubes were centrifuged (3000 rpm, 15 min, at 4°C) and the supernatant was removed, sampled, and replaced with fresh solvent. As an alternative to sonication, we also tested continuous agitation on a mechanical shaker at the maximum speed possible without loss of solvent by splashing and found it to give identical results (unpublished data).

Comparison of Extraction with Analysis of Whole Sample (Experiment 3). Because some authors (Bate-Smith, 1973, 1975; McArthur, 1988) have suggested that higher yields of condensed tannin can be obtained by analyzing whole tissue rather than extracts, we compared five alternative ways of estimating the total condensed tannin present in leaf samples: (A) leaf samples were extracted exhaustively in 50% acetone, as above; (B) samples were extracted exhaustively as in (A) and condensed tannins were also measured in the remaining solid residue; (C) condensed tannin was measured directly in unextracted, undried, ground leaf tissue; (D) analysis was performed as in (C) after pretreatment with boiling methanol (1 ml to 20 mg sample) for 5 min (Bate-Smith, 1973); and (E) analysis was performed as in (C) after sonication in 50% acetone (1 ml to 20 mg sample) for 30 min. Subsamples for all five treatments were taken from the same sample at the same time and processed simultaneously.

Effects of Methods of Drying (Experiment 4). With leaf samples from the same trees as used in the experiments described above, but sampled two months later, we tested the effects of three drying treatments on extraction and measurement of condensed tannins and total phenolics. Leaves were sampled and immediately ground in liquid nitrogen, then subsamples were analyzed after: (1) no drying; (2) lyophilization; or (3) oven drying at 70°C for 24 hr. We also investigated the feasibility of lyophilizing 50% acetone extracts for storage: subsamples were analyzed either immediately or after lyophilization, storage for up to a week, and reconstitution with water.

Statistical Analysis. Treatment means were compared using a two-factor analysis of variance (Zar, 1984) with eucalypt species as one factor and the treatment as the other. Arcsine transformation (Zar, 1984) was applied to percentages before analysis.

RESULTS

Experiment 1. Both light and temperature had pronounced effects on the stability of condensed tannins in leaf extracts (Table 1). Storage at 4°C in the dark, regardless of solvent, resulted in only small reductions in measurable condensed tannin, but room temperature and exposure to indirect sunlight caused much greater reductions. In most cases, the effects of light and temperature were less pronounced for 50% methanol than for the two concentrations of aqueous acetone. There was a statistical trend towards higher recovery of total phenolics after storage at room temperature in the dark than after the other two storage treatments, but the magnitude of this difference seems biologically insignificant (Table 1).

Experiment 2. As expected, all three solvents removed very little additional condensed tannin or total phenolics after 2 × 30-min sonications (Table 2). Aqueous acetone extracted considerably more condensed tannin and total phenolics than did aqueous methanol. Fifty percent acetone consistently extracted more condensed tannins than did 70% acetone and extracted as much or more total phenolics (Table 2).

Experiment 3. Of the five extraction–analysis treatments compared in experiment 3, exhaustive extraction with 50% acetone followed by analysis of the residue (treatment B) usually gave the highest estimate of total condensed tannins (Table 3). Direct analysis of ground leaf consistently gave a lower estimate of condensed tannin than did extraction alone. Pretreatment of the ground leaf with either boiling butanol or 50% acetone and sonication before adding the reagent improved the yield of condensed tannin but still gave an estimate at best 105% of treatment B and at worst 62% (Table 3). In all of these direct analyses the leaf tissue was colored red after boiling in HCl–butanol, indicating

TABLE 1. EFFECT OF LIGHT, TEMPERATURE, AND SOLVENT ON STABILITY OF CONDENSED TANNINS (CT) AND TOTAL PHENOLICS (TP) (EXPERIMENT 1)[a]

Species code	Solvent	Treatment					
		4°C/dark		Room temp./ dark		Room temp./ sunlight	
		CT	TP	CT	TP	CT	TP
EMAN2	50% Ac	96	97	83	99	63	94
	70% Ac	101	100	87	108	60	101
	50% Me	106	96	91	94	102	94
EMEL1	50% Ac	91	92	77	96	39	90
	70% Ac	99	96	83	102	35	96
	50% Me	103	94	87	98	83	92
EVIM	50% Ac	63	96	40	100	31	96
	70% Ac	107	98	93	104	61	102
	50% Me	70	94	53	97	65	95
EPAUC	50% Ac	85	98	69	104	42	99
	70% Ac	104	101	80	109	59	104
	50% Me	109	96	90	101	96	98
Means	50% Ac	84[b]	96[b]	67	100	44	95
	70% Ac	103[b]	99[b]	86	106	54	101
	50% Me	97[b]	95	80	98	86	95

[a] Extracts were analyzed initially and then after storage for three days under the conditions indicated. Data are means of duplicate determinations and are expressed as percentage of the initial concentration.
[b] Significant treatment effect ($P < 0.005$) within row.

that not all tannin was extracted and measured. This problem appeared to be less pronounced for treatment B, which was concluded to give the best possible estimate of total condensed tannins.

Experiment 4. Oven-drying greatly reduced the amounts of condensed tannin and total phenolics extractable by solvents and the amount of condensed tannin detectable in the residue remaining after extraction with 50% acetone (Table 4). Lyophilization caused a consistent increase in extractability and measurability of condensed tannin and total phenolics from leaf, although this increase was small in all but one case (Table 4). Lyophilization and reconstitution of 50% acetone extracts had no consistent effect on measurements (Table 4).

DISCUSSION

To interpret the role of tannins and other phenolics in plant–plant or plant–animal interactions, it is important that at least the total amount present in plant samples can be estimated confidently and preferably that quantitative extracts

TABLE 2. EFFECTS OF SOLVENT ON EXTRACTION OF CONDENSED TANNINS (CT) AND TOTAL PHENOLICS (TP) FROM EUCALYPT FOLIAGE (EXPERIMENT 2)[a]

Species code	Solvent	1st extraction		2nd extraction		3rd extraction		Totals	
		CT	TP	CT	TP	CT	TP	CT	TP
EMAN1	50% Ac	564	194	69	26	4	8	637	228
	70% Ac	527	181	82	25	0	6	609	212
	50% Me	487	163	97	29	13	11	597	203
EMAN2	50% Ac	468	202	85	34	13	9	566	245
	70% Ac	428	184	119	48	16	9	563	241
	50% Me	416	181	53	25	15	8	484	214
EMEL1	50% Ac	248	97	102	42	17	9	367	148
	70% Ac	269	116	43	18	14	7	326	141
	50% Me	233	98	48	20	15	7	296	125
EPAUC	50% Ac	165	90	31	18	6	7	202	115
	70% Ac	149	85	27	20	13	13	189	118
	50% Me	117	63	15	11	10	8	142	82
Means[b]	50% Ac							443	184
	70% Ac							421	178
	50% Me							379	157

[a] Extraction was in the dark, at 4°C, in a sonicator, for three successive 30-min periods. All data are expressed as quebracho equivalents (mg/g dry leaf). Values are means of duplicate determinations.

[b] Significant solvent effects ($P < 0.005$) on total yield of both condensed tannins and total phenolics.

can be obtained for chemical and biochemical analyses (Mole and Waterman, 1987a,b; Hagerman, 1988). The present study questions some untested notions prevalent in the literature about how best to estimate total condensed tannins, adds to the meagre data on the relative effectiveness of various solvents for phenolics in plant tissues, and focuses on some major errors that can occur if extractions are not performed, or extracts are not subsequently stored, under appropriate conditions.

Bate-Smith (1975) concluded that condensed tannins (proanthocyanidins) seldom are completely extractable from plants. He suggested direct analysis of unextracted leaf to estimate total condensed tannin. McArthur (1988) applied this approach to analysis of eucalypt foliage where it appeared that oven-drying had seriously reduced extractability of phenolics. Reed et al. (1982) analyzed residual phenolics in isolated cell-wall fractions of shrub foliage. However, direct analysis of plant tissue rarely has been compared with other ways of estimating total condensed tannins when fresh samples are used. We found that, for eucalypts, summing the results of analysis of the 50% acetone extracts and of the leaf residues gave 20–40% higher estimates of total condensed tannin

TABLE 3. EFFECTS OF SOLVENT EXTRACTION VERSUS DIRECT ANALYSIS OF LEAF TISSUE ON YIELD OF CONDENSED TANNINS (EXPERIMENT 3)[a]

	Percent of estimated total CT measured by				
Species code	(A) Exhaustive extraction	(B) Solvent + residue	(C) Whole sample	(D) Boiling methanol	(E) Presonication
EMAN1	95	100	73	87	86
EMAN2	96	100	66	80	75
EMEL1	95	100	79	81	105
EMEL2	89	100	67	76	105
EPAUC	86	100	62	66	62
EHYB	81	100	67	70	83
Means[b]	90	100	69	77	86

[a] Five treatments were employed: A, exhaustive extraction in 50% acetone as in Table 2; B, extraction as in A followed by direct analysis of condensed tannin remaining in the extracted residue; C, direct analysis of leaf tissue; D, analysis of leaf tissue after pre-treatment with boiling methanol for 5 min; and E, analysis of leaf tissue after sonication in 50% acetone, at 4°C for 30 min. Values are expressed as percentage of treatment B, which in all but two instances gave the highest estimate of total condensed tannins. Data are means of duplicate determinations on extracts and quadruplicate determinations on leaf tissues and residues.

[b] Significant treatment effect ($P < 0.005$).

than direct analysis of unextracted leaf and that extraction in 50% acetone alone also is superior to the direct analysis. Bate-Smith (1973, 1975) recognized the need to pretreat some leaf samples for tissue analysis, but we found that pretreatments gave only small improvements in most cases. A major benefit of combining use of solvent with analysis of the residue is that an estimate of the extractability of tannins (and other phenolics if a total phenolic analysis of the residue can be performed) is obtained. Extractability of tannins might have at least as much physiological and/or ecological significance for herbivores as the total amount present (Mole and Waterman, 1987a).

Very few studies that have included analysis of phenolics have stipulated extraction at low temperatures (e.g., Lindroth and Pajutee, 1987; Inoue and Hagerman, 1988), although boiling during extraction has been discouraged (Foo and Porter, 1980; Hagerman, 1988). We know of no studies that have emphasized protection from light during extraction. We presume that many workers perform their extractions without these precautions, as we have previously. In the present study, instability of condensed tannins caused by sunlight and room temperature was evident and substantial within 2–5 hr of the start of extraction. Therefore, it probably causes significant underestimates of extracted tannin even in short extraction periods. Inclusion of water in solvents is another source of

TABLE 4. EFFECTS OF THREE DRYING TREATMENTS (NO DRYING, FREEZE-DRYING, AND OVEN DRYING AT 70°C) ON EXTRACTION OF TOTAL PHENOLICS (TP) AND CONDENSED TANNINS (CT) IN 50% ACETONE, AND EFFECTS OF TWO SUBSEQUENT TREATMENTS OF 50% ACETONE EXTRACT (IMMEDIATE ANALYSIS OR ANALYSIS AFTER FREEZE-DRYING AND RECONSTITUTION WITH WATER) (EXPERIMENT 4)[a]

Species code	Extract treatment	Fresh leaf			Freeze-dried			Oven-dried		
		TP	CT	%Ex	TP	CT	%Ex	TP	CT	%Ex
EMAN1	Immed.	175	434	89	186	475	91	165	383	87
	Reconst.	184	477		195	488		172	393	
EMEL1	Immed.	148	358	90	152	382	87	146	362	86
	Reconst.	157	355		157	355		153	349	
EVIM	Immed.	166	26	80	163	29	81	138	14	69
	Reconst.	169	28		169	25		137	13	
EPAU	Immed.	82	187	73	134	307	90	73	120	58
	Reconst.	88	181		88	289		73	110	
EHYB	Immed.	142	289	81	151	314	81	93	161	60
	Reconst.	148	247		154	253		95	136	
Means	Immed.[b]	143	258	83	157	301	86	123	208	72
	Reconst.[c]	149	258		153	282		126	201	

[a] Also indicated (%Ex) is the percentage of total condensed tannin (estimated as in treatment B, Table 3) extracted by 50% acetone after the three drying treatments. All data are quebracho equivalents (mg/g dry leaf).
[b] Significant effect of drying treatment ($P < 0.05$) for TP, CT and %Ex.
[c] No significant effects of extract treatment ($P > 0.05$).

(hydrolytic) degradation of phenolics (Lindroth and Pajutee, 1987), but there is no alternative if quantitative estimates of phenolics are needed, because extraction usually is poor in anhydrous solvents. It appears from Table 1 that the structure of *Eucalyptus* spp. condensed tannins is altered in an aqueous solvent enough to reduce their reactivity with the HCl–butanol reagent but not sufficiently to reduce the intensity of color production on reaction with the Folin-Ciocalteu (total phenolics) reagent. This might be due to light-catalyzed polymerization (oxidative coupling) of condensed tannin chains that would not affect the phenolic hydroxyls with which the Folin-Ciocalteu reagent reacts (P. G. Waterman, personal communication). Consequently, if extraction and/or storage conditions are inappropriate, analysis of total phenolics might indicate stability but biochemical tests that rely on the reactivity of tannins with proteins or other substrates (Mole and Waterman, 1987b) could be seriously in error.

The optimal choice between acetone- and methanol-based solvents probably varies with the plant species under study (Hagerman, 1988), but some generalizations are possible. Extractability in methanol decreases with the size

of the tannin polymer (Goldstein and Swain, 1963; Foo and Porter, 1980), whereas no such trend is apparent for acetone (Jones et al., 1976; Foo and Porter, 1980). Acetone appears to break or prevent tannin–protein associations, whereas methanol does not (Foo and Porter, 1980; Hagerman and Robbins, 1987). Hagerman (1988) found aqueous acetone was superior to aqueous methanol for extracting tannins (mixtures of condensed and hydrolyzable) from several plant species, but the extent of superiority varied seasonally. We conclude that aqueous acetone should be the choice of solvent for extracting phenolics from eucalypts also, because it consistently extracted more condensed tannin and total phenolics than did aqueous methanol in the species we examined. This conclusion is strengthened by the observations that eucalypts contain high concentrations of hydrolyzable as well as condensed tannins (Hillis, 1966; Fox and Macauley, 1977; Cork et al., 1983; Cork and Pahl, 1984) and the aromatic ester (depside) bonds in hydrolyzable tannins are unstable in aqueous alcohols (see Hagerman, 1988).

Oven-drying of eucalypt foliage is reported to reduce the yield of total phenolics and condensed tannins by 12–100% (Cork et al., 1981; McArthur, 1988), and the reduction observed in the present study was 0–45% (Table 4). Reductions in extractability of tannins as a result of oven-drying have been reported for leaves of hickory, oak, and maple (Hagerman, 1988) and 20 African rain-forest species (Gartlan et al., 1980). Sun-drying produces similar reductions to oven-drying (Gartlan et al., 1980). In all but one of the eucalypt species investigated here, lyophilization increased extractability of condensed tannins and total phenolics by 2–10% (Table 4), which, depending on the reason for analysis, could be an error to be wary of. In one case (EPAU) lyophilization increased extractability from 73% to 90% (Table 4). Hagerman (1988) also concluded that lyophilization can affect extractability of tannins from foliage and that the effect can vary seasonally. The different effects of lyophilization and fresh analysis can be partly, but not fully, compensated for by analysis of residues as well as extracts to give an estimate of extractability and total tannin (Table 4).

Although the present study has concerned members of only one genus of plants, the phenolic chemistry of this genus is diverse (Hillis, 1966) and the species sampled were each from a separate infrageneric series (Chippendale, 1988). Therefore, the potential problems and pitfalls identified are likely to apply to many other plants. Inappropriate extraction conditions, especially lack of attention to the effects of light and temperature and the efficiency of the solvent in use, can make comparisons between plant species dubious and interpretation of physiological interactions between plants and animals very difficult. Therefore, we urge chemical ecologists analyzing plants for tannins and other phenolics to consider preparation and extraction conditions carefully, and we hope that this paper will assist.

Acknowledgments—We thank Ms. Mandy Yialeloglou for skilled technical assistance in the performance and development of the techniques used here. We also thank Dr. A. Hagerman and Dr. C. McArthur of Miami University, Ohio, U.S.A., for helpful advice on analytical techniques. However, we are solely responsible for the final choice and implementation of techniques. Financial support for this work was provided by a grant (8889/06) from the Australian National Parks and Wildlife Service.

REFERENCES

ASQUITH, T.N., and BUTLER, L.G. 1985. Use of dye-labeled protein as spectrophotometric assay for protein precipitants such as tannin. *J. Chem. Ecol.* 11:1535–1544.

BATE-SMITH, E.C. 1973. Tannins of herbaceous leguminoseae. *Phytochemistry* 12:1809–1812.

BATE-SMITH, E.C. 1975. Phytochemistry of proanthocyanidins. *Phytochemistry* 14:1107–1113.

CHIPPENDALE, G.M. 1988. *Eucalyptus, Angophora* (Myrtaceae), Flora of Australia, Vol. 19. Australian Government Publishing Service, Canberra.

CORK, S.J. 1984. Utilization of *Eucalyptus* foliage by arboreal marsupials. *Proc. Natr. Soc. Aust.* 9:88–97.

CORK, S.J., and PAHL, L. 1984. The possible influence of nutritional factors on diet and habitat selection by the ringtail possum (*Pseudocheirus peregrinus*), pp. 269–276, *in* A.P. Smith and I.D. Hume (eds.). Possums and Gliders. Australian Mammal Society, Sydney.

CORK, S.J., HUME, I.D., and DAWSON, T.J. 1983. Digestion and metabolism of a natural foliar diet (*Eucalyptus punctata*) by an arboreal marsupial, the koala (*Phascolarctos cinereus*). *J. Comp. Physiol.* 153:181–190.

FOO, L.Y., and PORTER, L.J. 1980. The phytochemistry of proanthocyanidin polymers. *Phytochemistry* 19:1747–1754.

FOX, L.R., and MACAULEY, B.J. 1977. Insect grazing on *Eucalyptus* in response to variation in leaf tannins and nitrogen. *Oecologia* 29:145–162.

GARTLAN, J.S., MCKEY, D.B., WATERMAN, P.G., MBI, C.N., and STRUHSAKER, T.T. 1980. A comparative study of the phytochemistry of two African rain forests. *Biochem. Syst. Ecol.* 8:401–422.

GOLDSTEIN, J.L., and SWAIN, T. 1963. Changes in tannins in ripening fruits. *Phytochemistry* 2:371–383.

HAGERMAN, A.E. 1988. Extraction of tannin from fresh and preserved leaves. *J. Chem. Ecol.* 14:453–461.

HAGERMAN, A.E., and BUTLER, L.G. 1989. Choosing appropriate methods and standards for assaying tannin. *J. Chem. Ecol.* 15:1795–1810.

HAGERMAN, A.E., and ROBBINS, C.T. 1987. Implications of soluble tannin–protein complexes for tannin analysis and plant defense mechanisms. *J. Chem. Ecol.* 13:1243–1259.

HASLAM, E., HAWORTH, R.D., MILLS, S.D., ROGERS, H.J., ARMITAGE, R., and SEARLE, T. 1961. Gallotannins. Part II. Some esters and depsides of gallic acid. *J. Chem. Soc.* 1961:1836–1842.

HILLIS, W.E. 1966. Variation in polyphenol composition within species of *Eucalyptus* L'Herit. *Phytochemistry* 5:541–556.

INOUE, K.H., and HAGERMAN, A.E. 1988. Determination of gallotannin with rhodanine. *Anal. Biochem.* 169:363–369.

JONES, W.T., BROADHURST, R.B., and LYTTLETON, J.W. 1976. The condensed tannins of pasture legume species. *Phytochemistry* 15:1407–1409.

LINDROTH, R.L., and PAJUTEE, M.S. 1987. Chemical analysis of phenolic glycosides: Art, facts, and artifacts. *Oecologia* 74:144–148.

MARTIN, J.S., and MARTIN, M.M. 1983. Tannin assays in ecological studies. Precipitation of ribulose-1,5-biphosphate carboxylase/oxygenase by tannic acid, quebracho, and oak foliage extracts. *J. Chem. Ecol.* 9:285–294.

MARTIN, M.M., and MARTIN, J.S. 1984. Surfactants: Their role in preventing the precipitation of proteins by tannins in insect guts. *Oecologia* 61:342–345.

MCARTHUR, C. 1988. Influences of tannins on digestion of eucalypt foliage in common ringtail possums (*Pseudocheirus peregrinus*) and an analysis of some chemical assays in the presence of condensed tannins. PhD thesis. Zoology Department, Monash University, Melbourne.

MOLE, S., and WATERMAN, P.G. 1987a. A critical analysis of techniques for measuring tannins in ecological studies. I. Techniques for chemically defining tannins. *Oecologia* 72:137–147.

MOLE, S., and WATERMAN, P.G. 1987b. A critical analysis of techniques for measuring tannins in ecological studies. II. Techniques for biochemically defining tannins. *Oecologia* 72:148–156.

MOLE, S., BUTLER, L.G., HAGERMAN, A.E., and WATERMAN, P.G. 1989. Ecological tannin assays: A critique. *Oecologia* 78:93–96.

PORTER, L.J., HRSTICH, L.N., and CHAN, B.G. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* 25:223–230.

PRICE, M.L., STROMBERG, A.M., and BUTLER, L.G. 1979. Tannin content as a function of grain maturity and drying conditions in several varieties of *Sorghum bicolor* (L.) Moench. *J. Agric. Food Chem.* 27:1270–1274.

REED, J.D., MCDOWELL, R.E., VAN SOEST, P.J., and HORVATH, P.J. 1982. Condensed tannins: A factor limiting the use of cassava forage. *J. Sci. Food Agric.* 33:213–220.

SINGLETON, V.L., and ROSSI, J.A. 1965. Colorimetry of total phenolics and phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16:144–158.

STAFFORD, H.A., and CHENG, T.-Y. 1980. The procyanidins of douglas fir seedlings, callus and cell suspension cultures derived from cotyledons. *Phytochemistry* 19:131–135.

SWAIN, T. 1979. Tannins and lignins, pp. 657–682, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

SWAIN, T., and HILLIS, W.E. 1959. The phenolic constituents of *Prunus domestica* I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* 10:63–68.

WATTERSON, J.J., and BUTLER, L.G. 1983. Occurrence of an unusual leucoanthocyanidin and absence of proanthocyanidins in sorghum leaves. *J. Agric. Food Chem.* 31:41–45.

ZAR, J.H. 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

TONGUE-FLICKING AND BITING IN RESPONSE TO CHEMICAL FOOD STIMULI BY AN IGUANID LIZARD (*Dipsosaurus dorsalis*) HAVING SEALED VOMERONASAL DUCTS: VOMEROLFACTION MAY MEDIATE THESE BEHAVIORAL RESPONSES

WILLIAM E. COOPER, JR.[1,*] and ALLISON C. ALBERTS[2]

[1]*Department of Biology*
Auburn University at Montgomery
Montgomery, Alabama 36117

[2]*Department of Biology*
University of California at San Diego
La Jolla, California 92993

(Received June 25, 1990; accepted August 21, 1990)

Abstract—In the iguanid lizard *Dipsosaurus dorsalis*, chemical food stimuli were discriminated from other odorants by vomerolfaction. This was demonstrated in a 2 × 3 experiment in which groups of lizards with sealed vomeronasal ducts or sham-sealed vomeronasal ducts responded to carrot chemical stimuli, cologne, and distilled water presented on cotton-tipped applicators. Abilities to detect and discriminate food chemicals were abolished in lizards having sealed vomeronasal ducts. For tongue-flick attack score and number of lizards biting, the sham-sealed group responded more strongly to carrot stimuli than to the control stimuli, but the group having sealed ducts did not. Lizards having sham-sealed ducts responded more strongly to carrot stimuli than did lizards having sealed ducts; responses by the two groups of lizards to control stimuli did not differ. Tongue-flicking occurred when the vomeronasal system detected a chemical stimulus from either carrot or cologne. Biting occurred only when the vomeronasal organ detected food stimuli (from carrot). Most duct-sealed lizards opened their mouths, some repeatedly. Mouth-opening thus occurs when the vomeronasal organ does not detect chemicals. It may be an attempt to stimulate or prime the vomeronasal organ or to dislodge the sealant.

Key Words—Vomeronasal system, vomerolfaction, feeding, food vomodor, Reptilia, Lacertilia, Iguanidae, *Dipsosaurus dorsalis*.

*To whom correspondence should be addressed.

0098-0331/91/0100-0135$06.50/0

INTRODUCTION

Squamate reptiles use chemical senses in diverse contexts, including detection of prey and predators, aggregation, identification and scent-trailing of conspecifics, and determination of sex, reproductive condition, and degree of relatedness of conspecifics (e.g., Burghardt, 1970; Duvall, 1979; Chiszar and Scudder, 1980; Garstka and Crews, 1981; Ford, 1982; Heller and Halpern, 1982; Weldon, 1982; Simon, 1983; Cooper and Vitt, 1984a,b, 1986a,b; Cooper et al., 1986; Thoen et al., 1986; Werner et al., 1987; Dial et al., 1989). Three major chemical senses could participate in these discriminations: vomerolfaction, olfaction, and taste (Cooper and Burghardt, 1990b).

Squamates chemically sample the environment by tongue-flicking. Chemicals gathered by the tongue eventually reach the vomeronasal organs via the vomeronasal ducts, one of which opens at the roof of the mouth on each side of the midline. These findings on chemical access to the vomeronasal organ hold for garter snakes (Halpern and Kubie, 1983) and a single lizard of the family Scincidae (Halpern and Kubie, 1980; Graves and Halpern, 1989). Because the relatively nonvolatile materials sampled by the tongue do not appear to reach the main olfactory epithelium (Graves and Halpern, 1989), chemical discriminations requiring lingual sampling of such nonvolatiles must be made by other chemical senses. The most likely candidates are taste and vomerolfaction (Cooper and Burghardt, 1990b), but some other chemical sense could be involved. For example, the trigeminal, facial, glossopharyngeal, and terminal nerves might support chemical sensitivity.

There is little indication that olfaction is necessary for some of the discriminations noted above (Burghardt and Hess, 1968; Burghardt and Pruitt, 1975; Kubie et al., 1978; Halpern and Frumin, 1979; Burghardt, 1980; Heller and Halpern, 1982), but it might be important for responses to airborne odors (e.g., Duvall et al., 1980). Another function of olfaction may be to activate chemical sampling by the tongue (Burghardt, 1970; Cowles and Phelan, 1958; Duvall, 1981; Simon and Moakley, 1985).

Gustation is virtually unstudied in squamates (Burghardt, 1970). In snakes taste cannot be a factor in lingually mediated chemical discriminations because the tongues of snakes lack taste buds (Morgans and Heidt, 1978). Lingual taste buds are present in most lizard families (Schwenk, 1985); preliminary examination suggests that they may be most abundant in iguanids and perhaps next in skinks (Schwenk, personal communication). Despite the presence of taste buds, gustation was not sufficient to allow normal lingual responses to novel environments or normal prey consumption by a scincid lizard (Graves and Halpern, 1990).

Several chemical discriminations by garter snakes require a functional vomeronasal system (Kubie et al., 1978; Halpern and Frumin, 1979) but may

be performed normally after olfactory nerve sections. The skink *C. ocellatus* responds normally to food only when the vomeronasal system is functional (Graves and Halpern, 1990). These findings suggest that the vomeronasal system may mediate important chemical discriminations, especially in feeding and pheromonal communication, in diverse squamates.

We examined the effect of blocking the vomeronasal ducts on responses to chemical food stimuli by the desert iguana, *Dipsosaurus dorsalis*. The desert iguana is the only iguanid species known to discriminate prey odors by tongue-flicking (Krekorian, 1989; Cooper and Alberts, 1990). It also can detect conspecific semiochemicals (Alberts, 1989; but see Pederson, 1988). This species has an unspecialized iguanid tongue having widely distributed taste buds (Schwenk, 1985). Reliance on the vomeronasal system for discrimination of food odors in such a species would suggest its widespread importance in lizards having tongues more specialized for chemical sampling.

METHODS AND MATERIALS

Desert iguanas were captured in Riverside County, California. They were housed for over a year at the University of California at San Diego, where they were used in studies of pheromonal communication and prey odor discrimination. They then were shipped to Auburn University at Montgomery, Alabama, where they were allowed to acclimate to laboratory conditions for over 45 days before the present experiment was conducted. Each lizard was housed individually in a 50 × 30 × 26-cm glass terrarium equipped with an undercage heater and a 75-W heat lamp. Each cage contained a 6-cm-deep layer of sand, a food bowl, and a ceramic shelter. The lizards were fed to satiation. The diet consisted of fresh vegetables, usually a mixture of grated carrots, turnips, yellow squash, zucchini, spinach, and broccoli. They also were fed crickets about once a week. A water bowl was not provided because the lizards remained fully hydrated from water obtained in food.

To determine whether access to the vomeronasal organ is necessary for prey odor discrimination, responses of two groups of desert iguanas to chemical food stimuli and two control substances were tested. The experimental group was composed of eight lizards that had the vomeronasal ducts sealed to prevent chemicals sampled by the tongue from reaching the vomeronasal epithelium; the control group originally consisted of eight lizards subjected to a sham sealing procedure. Under cold anesthesia, both vomeronasal ducts of lizards in the experimental group were sealed with surgical tissue adhesive (Three Bond of America, Inc., Torrance, California) as described by Graves and Halpern (1989, 1990). Lizards in the control group were treated identically except that the tissue adhesive was placed lateral to the openings of the vomeronasal ducts to

avoid sealing them. After the adhesive was dried with the help of an electric fan blowing air across the anesthetized lizards, the lizards were allowed to warm to room temperature before being returned to their home cages. All subjects recovered from the procedure quickly. They appeared to be normally active during the experiments and responded to the experimental situation as did untreated lizards except that behaviors related to chemosensory responses were affected.

In a randomized blocks (repeated measures) design, each lizard responded to chemical stimuli derived from carrot, to cologne, and to distilled water. Carrot was selected because it is a favored food in the laboratory and because the lizards respond strongly to its chemical cues (Cooper and Alberts, 1990). Cologne (Mennen Skin Bracer, Spice Scent) served as a control for responses to a substance having a readily detectable odor lacking import as food, predator odor, or as a social signal. Water was an odorless control for responses to the experimental situation. The stimuli were presented on cotton-tipped applicators that were prepared as follows: Initially, the cotton tip was dipped in distilled water; cologne was added by dipping the moistened applicator in cologne; carrot stimuli were imparted to the cotton by rubbing the moistened swab on the surface of a freshly cut carrot.

The stimuli were presented in an incompletely counterbalanced sequence, one per day, beginning two days after the sealing procedure on May 24, 1989. In the original design, all possible sequences were represented as evenly as possible, with remaining sequences selected randomly. However, two of the sham-sealed subjects could not be used because one repeatedly fled after the sham procedure and another appeared to be sick. The lizards were fed the day before the sealing procedure, but were not fed again until the experiment was over. Because the counterbalancing assured that almost equal numbers of lizards were tested with each stimulus at each of the three trials, hunger had no differential effect on results.

A trial was begun by slowly approaching a lizard's cage, removing its top, and placing the cotton swab 2 cm anterior to the lizard's snout. Tongue-flicks directed to the applicator were recorded for the ensuing 60 sec if the lizard did not bite the applicator. If it did bite, the trial was terminated and number of tongue-flicks before biting and latency to biting were recorded. The response variable analyzed was the tongue-flick attack score (TFAS), which combines tongue-flicking and latency to bite to give an overall index of feeding response strength (Burghardt, 1967, 1970; Cooper and Burghardt, 1990a). If a lizard did not bite, its TFAS was the number of tongue-flicks in 60 sec. If it did bite, TFAS was (60 sec minus latency to bite in seconds) plus a base unit, the base unit being the maximum number of tongue-flicks given by that lizard in response to any of the stimuli (Cooper and Burghardt, 1990a). The base unit selected assures that a predatory bite is weighted at least as heavily as the highest number

of tongue-flicks, which reflects our assumption that a bite is a stronger food-related response than any number of tongue-flicks.

Nonparametric analysis of experimental results was conducted as follows: Friedman two-way analysis of variance (Siegel, 1956) was used within each group, followed by individual comparisons according to procedures described by Hollander and Wolfe (1973); between-group comparisons were made for each condition by Mann-Whitney U tests (Siegel, 1956). Assessment of experimental effects for TFAS by a 2×3 factorial ANOVA for an experiment having repeated measures on one factor and unequal cell frequencies (Winer, 1962) had been planned, but could not be used as the primary analysis due to nonnormality. This analysis and a similar 2×2 ANOVA restricted to carrot and cologne stimuli were used to obtain approximate significance levels for interaction effects. The assumption of homogeneity of variance was met by testing for heterogeneity with Hartley's F_{max} and removing any significant heterogeneity by a logarithmic transformation [$\log_{10}$ (TFAS + 1)]. All tests were two-tailed with $\alpha = 0.05$.

RESULTS

All lizards in the control group tongue-flicked in response to all stimuli except for two that did not tongue-flick in response to distilled water. In contrast, individuals in the experimental group rarely tongue-flicked. Only three of eight tongue-flicked, one in all three conditions, one only to carrot, and another only to cologne. However, six of the lizards having sealed ducts opened and closed their mouths during trials, four of them in cologne trials. All three that tongue-flicked also opened and closed their mouths without tongue-flicking in at least one condition. Thus, they were capable of tongue-flicking, but usually did not. The experimental procedure produced no apparent restraint on lingual movements.

Because so few duct-sealed lizards tongue-flicked, the assumption of normality required for parametric ANOVA was violated, requiring nonparametric analysis that did not allow examination of interaction terms for tongue-flick data (Table 1). For the experimental group, tongue-flicks did not vary significantly over stimuli ($X_r^2 = 0.44$, $df = 2$, $P > 0.10$). However, numbers of tongue-flicks did differ significantly among conditions for the control group ($X_r^2 = 6.33$, $df = 2$, $P < 0.05$). Individual comparisons revealed that cologne and carrot induced significantly more tongue-flicks than water ($P < 0.05$ for cologne, $P = 0.05$ for water). Numbers of tongue-flicks by control lizards did not differ for carrot and cologne ($P > 0.10$). The control group tongue-flicked significantly more times than did the experimental group in response to carrot stimuli ($U = 4$; $N = 6, 8$; $P = 0.004$; one-tailed) and cologne ($U = 7.5$; $N =$

TABLE 1. NUMBERS OF TONGUE-FLICKS AND TONGUE-FLICK ATTACK SCORES (TFAS) BY EXPERIMENTAL AND CONTROL GROUPS OF DESERT IGUANAS RESPONDING TO CARROT CHEMICAL STIMULI, COLOGNE, AND DISTILLED WATER

	Tongue-flicks			TFAS		
	Carrot	Cologne	Water	Carrot	Cologne	Water
Vomeronasal ducts sealed						
Mean	0.88	1.13	0.50	0.88	1.13	0.50
SE	0.74	0.99	0.50	0.74	0.99	0.50
Range	0–6	0–8	0–4	0–6	0–8	0–4
Vomeronasal ducts open						
Mean	4.33	5.50	1.00	49.50	5.50	1.00
SE	1.05	1.67	0.37	10.52	1.67	0.37
Range	3–8	1–12	0–2	5–69	1–12	0–2

6, 8; $P = 0.02$; one-tailed). Tongue-flicks did not differ between groups for distilled water ($U = 23$; $N = 6, 8$; $P > 0.10$).

Biting occurred only in the carrot condition and only control lizards bit (five of six, Figure 1). Thus, significantly more control lizards bit in response to carrot stimuli than to the other stimuli ($X_r^2 = 7.5$, $df = 2$, $P < 0.025$). Carrot stimuli induced biting by significantly more lizards than did the other stimuli ($P < 0.05$ each). Within the carrot stimulus condition (and overall), significantly more control lizards bit applicators than did experimental lizards ($U = 4$; $N = 6, 8$; $P = 0.04$, one-tailed).

The analysis of TFAS data (Table 1) was conducted nonparametrically because less than half the duct-sealed lizards tongue-flicked, violating the assumption of normality required for parametric ANOVA. Responses by the experimental group did not differ among odor conditions ($X_r^2 = 0.44$, $df = 2$, $P > 0.10$). TFAS did vary significantly among conditions in the control group ($X_r^2 = 9.25$, $df = 2$, $P < 0.01$), being significantly greater for carrot than for cologne ($P < 0.01$) or water ($P < 0.01$) and for cologne than for water ($P < 0.05$). The control group had a significantly higher TFAS than the experimental group in response to carrot stimuli ($U = 2$; $N = 6, 8$; $P = 0.001$, one-tailed). TFAS did not differ between groups for the other stimuli (cologne: $U = 15$; $N = 6, 8$; $P > 0.10$; water: $U = 18$; $N = 6, 8$; $P > 0.10$).

Because these nonparametric tests do not directly examine interaction between treatment and odor condition, ANOVAs were conducted to determine the approximate significance of the interaction term. Because variances were heterogeneous ($F_{max} = 754.24$; $df = 3,5$; $P < 0.001$), the data were logarithmically transformed. Variances were homogeneous for the transformed data (F_{max}

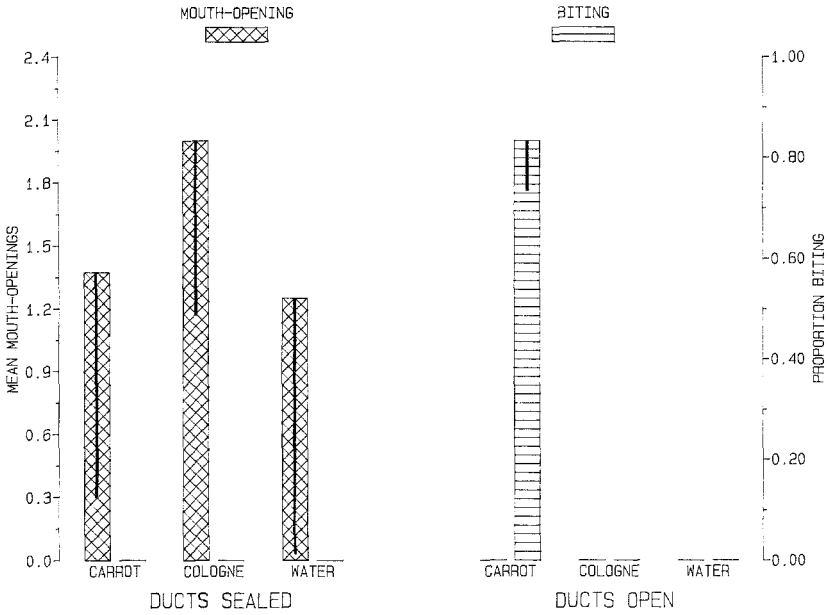


FIG. 1. Mean number of mouth-openings and proportion of lizards biting in response to chemical stimuli from carrot, cologne, and distilled water in groups having sealed and patent vomeronasal ducts. Dark lines represent 1.0 standard error of the mean for mouth-opening and 1.0 standard error of a proportion for biting.

= 3.59; $df = 3,5$; $P > 0.05$). The main treatment and odor effects for the 2 × 3 ANOVA were significant ($F = 112.15$; $df = 1, 12$; $P < 0.01$ and $F = 16.688$; $df = 2, 24$; $P < 0.001$, respectively). The interaction term was also significant ($F = 14.91$; $df = 2, 24$; $P < 0.001$), indicating that relative responsiveness differed among stimuli in the control group and the duct-sealed group. When the trials with water were eliminated from the analysis, a 2 × 2 ANOVA yielded similar results, with both main effects (treatment $F = 77.93$; $df = 1, 12$; $P < 0.001$; odor stimulus $F = 13.44$; $df = 1, 12$; $P < 0.005$) and the interaction ($F = 35.40$; $df = 1, 12$; $P < 0.001$) highly significant.

Only lizards with sealed vomeronasal ducts exhibited mouth-opening (Figure 1). The number of lizards showing this behavior in one or more conditions was significantly greater in the experimental group than in the control group (Fisher $P < 0.01$, one-tailed). However, this difference was not significant for any one condition. Furthermore, the frequency of mouth opening did not differ among conditions in the experimental group ($X_r^2 = 0.06$, $df = 2$, $P > 0.10$; carrot: SE = 1.1, range = 0–9; cologne: SE = 0.8, range = 0–6; water: SE

= 1.2, range = 0–8). Numbers of individuals opening the mouth at least once were three each for the carrot stimuli and water and four for cologne.

DISCUSSION

Patent vomeronasal ducts are necessary for desert iguanas to respond to food odors with normal rates of tongue-flicking and attack. This conclusion is supported by the findings that (1) lizards having sealed vomeronasal ducts tongue-flicked at lower rates than those having open ducts in response to odorous stimuli; (2) only those having open, unobstructed ducts showed higher tongue-flicking rates to odorous stimuli than to water; (3) individuals having patent vomeronasal ducts bit only applicators bearing carrot stimuli; and (4) individuals having patent ducts bit more frequently in response to carrot stimuli than did those having sealed ducts, which bit no applicators.

Failure by lizards having sealed vomeronasal ducts to respond to chemical food stimuli is best indicated by the TFAS data. That lizards having sealed ducts did not respond differentially among stimuli whereas lizards having patent ducts did so, with the greatest responses elicited by the food stimuli, suggests that the vomeronasal system is required for such discrimination. Three additional results in aggregate demonstrate that sealing the ducts induces a deficit in responsiveness to a chemical food stimulus. First, TFAS for lizards having patent vomeronasal ducts was significantly greater in response to carrot stimuli than for lizards having sealed ducts. Second, TFAS did not differ within the experimental group for cologne and carrot stimuli. Finally, the interaction between treatment and stimulus condition shows a specific elevation of TFAS in response to carrot stimuli by lizards having patent ducts, but not by lizards having sealed ducts.

Desert iguanas discriminate food from other chemicals (Cooper and Alberts, 1990; control group in this study), but those having sealed vomeronasal ducts do not by our measures. Vomerolfaction may be needed for food odor discrimination, but desert iguanas might discriminate chemical food stimuli without vomerolfactory cues, yet not respond by biting or by increased tongue-flicking. Vomeronasal feedback may provide an intrinsic reward for maintaining elevated tongue-flicking (Halpern, 1987). If vomodors (Cooper and Burghardt, 1990b) stimulate tongue-flicking, the apparent lack of discrimination could actually be a motivational deficit. However, because tongue-flicking is intimately associated with discrimination of chemical food stimuli by squamates (e.g., Burghardt, 1970), it is likely that in the absence of vomerolfaction, squamates are simply unable to recognize chemical food stimuli. Taste, olfaction, and other putative chemical senses were not affected by the experimental procedure. These senses, therefore, are not sufficient to elicit biting or elevated

tongue-flicking, at least for the food tested and in the absence of visual cues typical of food. The duct-sealing procedure clearly affected responsiveness (as indicated by tongue-flicking) to cologne, which presumptively was readily detected via olfaction. Presumably, the animals having sealed ducts detected cologne normally via olfaction, but exhibited a reduction in tongue-flicking because the tongue-flicking did not result in delivery of chemical stimuli to the vomeronasal organs.

Results on tongue-flicking are concordant with those for TFAS, except that tongue-flicking provides no evidence for discrimination of food stimuli by the control group. Several lizards bit applicators bearing carrot stimuli, some at short latency. Presumably, they did not tongue-flick more to carrot stimuli because they had less time than in other tests (Cooper and Vitt, 1980; Cooper and Burghardt, 1990a). Thus, the discrepancy does not indicate failure to discriminate.

Mouth-opening occurs in diverse squamates during tests of responses to odors on applicators (Cooper, unpublished observations), but frequent mouth-opening has not been reported previously during such tests. In desert iguanas, some openings were similar to yawning; others were more rapid and were repeated several times. That mouth-opening is restricted to individuals having blocked ducts suggests that it may indicate discomfort or have some chemosensory significance, as proposed for rattlesnakes (Graves and Duvall, 1983, 1985). It could be an attempt to stimulate the vomeronasal organ or clear the vomeronasal ducts. Our strong impression is that the observed results reflect the inability of experimental lizards to deliver chemical samples to the vomeronasal organs. Nevertheless, if mouth-opening does indicate discomfort, the possibility remains that the effect of duct closure is not specific to the vomeronasal sense.

The importance of vomerolfaction in feeding behavior has been confirmed in garter snakes (Halpern and Frumin, 1979) and very strongly suggested in a scincid lizard (Graves and Halpern, 1990) and an iguanid lizard (this paper). Iguanids and skinks have among the least specialized of lizard tongues. Their tongues are relatively rounded, fleshy, and lack the deep forking of snakes and some other lizards. One might expect that such tongues would be less efficient in transferring molecules to the vomeronasal ducts than would deeply forked, elongated tongues having fine tines. Although vomeronasal stimulation is possible after removal of the tip of the tongue (Halpern and Kubie, 1980) and strike-induced chemosensory searching is possible in the absence of the entire tongue (Carr et al., 1982), numerous studies show that tongue-flicking is the major means of collecting chemicals for transport to the vomeronasal organ (e.g., Wilde, 1938; Burghardt, 1980).

The greatest reliance on tongue-flicking during foraging occurs in families of lizards that are active foragers. Members of actively foraging families rely

on prey odors to detect prey whereas lizards that are ambush foragers do not (Cooper, 1989a,b, 1990b). Lingual elongation and tine development are greatest in families of active foragers (see McDowell, 1972). The desert iguana is an actively (but not intensively) foraging herbivore in a family consisting largely of insectivorous ambush foragers. Although it can discriminate chemical food stimuli from other chemical stimuli, it has a typically generalized iguanid tongue that is not elongate and lacks tines. If prey odor discrimination depends on vomerolfaction in such a lizard, it may well do so in other squamates having tongues more specialized for chemical sampling. Thus, despite a lack of evidence for lizard and snake families beyond the three mentioned, it can be hypothesized that vomerolfaction is the primary sense allowing prey odor discrimination in all of the major squamate families capable of it.

Acknowledgments—This work was partially supported by a grant-in-aid from Auburn University at Montgomery, Alabama, by a training grant to the University of California at San Diego from the National Institutes of Health, and by our respective Departments of Biology. We thank Brent Graves for discussion of the technique used to seal the vomeronasal ducts and for providing the sealant. We are also grateful to Mimi Halpern for making detailed useful comments on the manuscript and for encouraging cautious interpretation of the findings. Anonymous reviewers also made insightful comments that allowed material improvements.

REFERENCES

ALBERTS, A.C. 1989. Ultraviolet visual sensitivity in desert iguanas: Implications for pheromone detection. *Anim. Behav.* 37:129–137.

BURGHARDT, G.M. 1967. Chemical-cue preferences of inexperienced snakes: Comparative aspects. *Science* 157:718–721.

BURGHARDT, G.M. 1970. Chemical perception in reptiles, pp. 241–308, *in* J.W. Johnston, D.G. Moulton, and A. Turk (eds.). Advances in Chemoreception, Vol. 1. Communication by Chemical Signals. Appleton-Century-Crofts, New York.

BURGHARDT, G.M. 1980. Behavioral and stimulus correlates of vomeronasal functioning in reptiles: Feeding, grouping, sex, and tongue use, pp. 275–301, *in* D. Muller-Schwarze and R.M. Silverstein (eds.). Chemical Signals: Vertebrates and Aquatic Invertebrates. Plenum Press, New York.

BURGHARDT, G.M., and HESS, E.H. 1968. Factors influencing the chemical release of prey attack in newborn snakes. *J. Comp. Physiol. Psychol.* 66:289–295.

BURGHARDT, G.M., and PRUITT, C.H. 1975. Role of the tongue and senses in feeding of naive and experienced garter snakes. *Physiol. Behav.* 14:185–194.

CARR, J., MAXION, R., SHARPS, M., WEISS, D., O'CONNELL, B., and CHISZAR, D. 1982. Predatory behavior in a congenitally alingual Russell's viper (*Vipera russelli*). 1. Strike-induced chemosensory searching. *Bull. Md. Herpol. Soc.* 18:196–204.

CHISZAR, D., and SCUDDER, K.M. 1980. Chemosensory searching by rattlesnakes during predatory episodes, pp. 125–139, *in* D. Muller-Schwarze and R.M. Silverstein (eds.). Chemical Signals: Vertebrates and Aquatic Invertebrates. Plenum Press, New York.

COOPER, W.E., JR. 1989a. Absence of prey odor discrimination by iguanid and agamid lizards in applicator tests. *Copeia* 1989:472–478.

COOPER, W.E., JR. 1989b. Prey odor discrimination in varanoid lizards: Responses of gila monsters (*Heloderma suspectum*) and savannah monitors (*Varanus exanthematicus*) to chemical stimuli presented on cotton swabs. *Ethology* 81:250–258.

COOPER, W.E., JR. 1990a. Prey odor detection by teiid and lacertid lizards and its relationship to foraging mode in lizard families. *Copeia* 1990:237–242.

COOPER, W.E., JR. 1990b. Prey odor discrimination by anguid lizards. *Herpetologica*. 46:183–190.

COOPER, W.E., JR., and ALBERTS, A.C. 1990. Responses to chemical food stimuli by an herbivorous actively foraging lizard, *Dipsosaurus dorsalis*. *Herpetologica*. 46:259–266.

COOPER, W.E., JR., and BURGHARDT, G.M. 1990a. A comparative analysis of scoring methods for chemical discrimination of prey chemicals by squamate reptiles. *J. Chem. Ecol.* 16:45–65.

COOPER, W.E., JR., and BURGHARDT, G.M. 1990b. Vomerolfaction and vomodor. *J. Chem. Ecol.* 16:103–105.

COOPER, W.E., JR., and VITT, L.J. 1984a. Conspecific odor detection by the male broad-headed skink, *Eumeces laticeps*: Effects of sex and site of odor source and of male reproductive condition. *J. Exp. Zool.* 230:199–209.

COOPER, W.E., and VITT, L.J. 1984b. Detection of conspecific odors by the female broad-headed skink, *Eumeces laticeps*. *J. Exp. Zool.* 229:49–54.

COOPER, W.E., JR., and VITT, L.J. 1986a. Interspecific odour discrimination by a lizard (*Eumeces laticeps*). *Anim. Behav.* 34:367–376.

COOPER, W.E., JR., and VITT, L.J. 1986b. Tracking of female conspecific odor trails by male broad-headed skinks (*Eumeces laticeps*). *Ethology* 71:242–248.

COOPER, W.E., JR., and VITT, L.J. 1989. Prey odor discrimination by the broad-headed skink (*Eumeces laticeps*). *J. Exp. Zool.* 249:11–16.

COOPER, W.E., JR., GARSTKA, W.R., and VITT, L.J. 1986. Female sex pheromone in the lizard *Eumeces laticeps*. *Herpetologica* 42:361–366.

COWLES, R.B., and PHELAN, R.L. 1958. Olfaction in rattlesnakes. *Copeia* 1958: 77–83.

DIAL, B.E., WELDON, P.J., and CURTIS, B. 1989. Predator-prey signals: Chemosensory identification of snake predators by gekkonid lizards and its ecological consequences. *J. Herpetol.* 23:224–229.

DUVALL, D. 1979. Western fence lizard (*Sceloporus occidentalis*) chemical signals. 1. Conspecific discriminations and release of a species-typical visual display. *J. Exp. Zool.* 210:321–326.

DUVALL, D. 1981. Western fence lizard (*Sceloporus occidentalis*) chemical signals. II. A replication with naturally breeding adults and a test of the Cowles and Phelan hypothesis of rattlesnake olfaction. *J. Exp. Zool.* 218:351–361.

DUVALL, D., HERSKOWITZ, R., and TRUPIANO-DUVALL, J. 1980. Responses of five-lined skinks (*Eumeces fasciatus*) and ground skinks (*Scincella lateralis*) to conspecific and interspecific chemical cues. *J. Herpetol.* 14:21–127.

FORD, N.B. 1982. Species-specificity of sex pheromone trails of sympatric and allopatric garter snakes (*Thamnophis*). *Copeia* 1982:10–13.

GARSTKA, W.R., and CREWS, D. 1981. Female sex pheromone in the skin and circulation of a garter snake. *Science* 214:681–683.

GRAVES, B.M., and DUVALL, D. 1983. Occurrence and function of prairie rattlesnake mouth gaping in a non-feeding context. *J. Exp. Zool.* 227:471–474.

GRAVES, B.M., and DUVALL, D. 1985. Mouth gaping and head shaking by prairie rattlesnakes are associated with vomeronasal organ olfaction. *Copeia* 1985:496–497.

GRAVES, B.M., and HALPERN, M. 1989. Chemical access to the vomeronasal organs of the lizard *Chalcides ocellatus*. *J. Exp. Zool.* 249:150–157.

GRAVES, B.M., and HALPERN, M. 1990. Vomeronasal organ chemoreception in tongue-flicking,

exploratory, and feeding behaviour of the lizard, *Chalcides ocellatus*. *Anim. Behav.* 39:692–698.

HALPERN, M. 1987. The organization and function of the vomeronasal system. *Annu. Rev. Neurosci.* 10:325–362.

HALPERN, M., and FRUMIN, N. 1979. Roles of the vomeronasal and olfactory systems in prey attack and feeding in adult garter snakes. *Physiol. Behav.* 22:1183–1189.

HALPERN, M., and KUBIE, J.L. 1980. Chemical access to the vomeronasal organs of garter snakes. *Physiol. Behav.* 24:367–371.

HALPERN, M., and KUBIE, J.L. 1983. Snake tongue flicking behavior: clues to vomeronasal system functioning, pp. 45–72, *in* D. Muller-Schwarze and R.M. Silverstein (eds.). Chemical Signals in Vertebrates 3. Plenum Press, New York.

HELLER, S.B., and HALPERN, M. 1982. Laboratory observations of aggregative behavior of garter snakes, *Thamnophis sirtalis*: Roles of visual, olfactory, and vomeronasal senses. *J. Comp. Physiol. Psychol.* 96:984–999.

HOLLANDER, A., and WOLFE, D.A. 1973. Nonparametric Statistical Methods. John Wiley, New York.

KREKORIAN, C.O. 1989. Field and laboratory observations on chemoreception in the desert iguana, *Dipsosaurus dorsalis*. *J. Herpetol.* 23:267–273.

KUBIE, J.L., VAGVOLGYI, A., and HALPERN, M. 1978. Roles of vomeronasal and olfactory systems in courtship behavior of male garter snakes. *J. Comp. Physiol. Psychol.* 92:627–641.

MCDOWELL, S.B. 1972. The evolution of the tongue of snakes, and its bearing on snake origins, pp. 191–273, *in* T. Dobzhansky, M.K. Hecht and W.C. Steere (eds.). Evolutionary Biology, Vol. 6. Appleton-Century-Crofts, New York.

MORGANS, L.F., and HEIDT, G.A. 1978. Comparative tongue histology and scanning electron microscopy of the diamondback water snake (*Natrix rhombifera*) and black rat snake (*Elaphe obsoleta*) (Reptilia, Serpentes, Colubridae). *J. Herpetol.* 12:275–280.

PEDERSON, J.M. 1988. Laboratory observations on the function of tongue extrusion in the desert iguana (*Dipsosaurus dorsalis*). *J. Comp. Psychol.* 102:193–196.

SCHWENK, K. 1985. Occurrence, distribution and functional significance of taste buds in lizards. *Copeia* 1985:91–101.

SIEGEL, S. 1956. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York.

SIMON, C.A. 1983. A review of lizard chemoreception, pp. 119–133, *in* R.B. Huey, E.R. Pianka, and T.W. Schoener (eds.). Lizard Ecology: Studies of a Model Organism. Harvard University Press, Cambridge, Massachusetts.

SIMON, C.A., and MOAKLEY, G.P. 1985. Chemoreception in *Sceloporus jarrovi*: Does olfaction activate the vomeronasal system? *Copeia* 1985:239–242.

THOEN, C., BAUWENS, D., and VERHEYEN, R. 1986. Chemoreceptive and behavioral responses of the common lizard *Lacerta vivipara* to snake chemical deposits. *Anim. Behav.* 34:1805–1813.

WELDON, P.J. 1982. Responses to ophiophagous snakes by snakes of the genus *Thamnophis*. *Copeia* 1982:788–794.

WERNER, D., BAKER, E.M., GONZALES, E. DEL C., and SOSA, I.R. 1987. Kinship recognition and grouping in hatchling green iguanas. *Behav. Ecol. Sociobiol.* 21:83–89.

WILDE, W.S. 1938. The role of Jacobson's organs in the feeding reaction of the common garter snake, *Thamnophis sirtalis sirtalis* (Linn.). *J. Exp. Zool.* 77:445–465.

WINER, B.J. 1962. Statistical Principles in Experimental Design. McGraw-Hill, New York.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

RELEASE OF ALLELOCHEMICAL AGENTS FROM LITTER, THROUGHFALL, AND TOPSOIL IN PLANTATIONS OF *Eucalyptus globulus* LABILL IN SPAIN

A. MOLINA,[1] M.J. REIGOSA,[2,*] and A. CARBALLEIRA

[1]*Cátedra de Ecología*
Facultad de Biología
Universidad de Santiago de Compostela
Santiago de Compostela, Spain
[2]*Fisiología Vegetal*
Dep. Recursos Naturales y Medio Ambiente
Facultad de Ciencias de Vigo. Universidad de Vigo
Aptdo. 874. 36200 Vigo, Spain

(Received June 21, 1990; accepted August 28, 1990)

Abstract—Natural leachates of *Eucalyptus globulus* (throughfall, stemflow, and soil percolates) were collected daily during rainy spells in the vegetative period (February–July), and their effects on the germination and radicle growth of *Lactuca sativa* were measured. Concurrently, the effects of *L. sativa* of topsoil and leachates from decaying litter were determined. The results suggest that toxic allelochemicals released by *Eucalyptus globulus* may influence the composition and structure of the understory of the plantation and that this effect is attributable mainly to the decomposition products of decaying litter rather than to aerial leachates. The soil may neutralize or dilute allelopathic agents, at least below the top few cms.

Key Words—*Eucalyptus globulus*, allelopathy, *Lactuca sativa, natural leachates, soil, litter.*

INTRODUCTION

E. globulus is an exotic species of economic importance in Galicia (northwest Spain). It often has been implicated in the degradation of the environment where it has been planted. However, the ecological mechanisms controlling the struc-

*To whom correspondence should be addressed.

0098-0331/91/0100-0147$06.50/0

ture and diversity of a woodland understory are numerous and their individual effects difficult to distinguish. Any degradation produced by *E. globulus* is likely to be due to a group of related stress factors acting synergistically, rather than to a single well-defined cause.

Several authors (Bellot, 1949, 1966; Castroviejo, 1973; Alvarez and Malvar, 1979) have reported that planting *E. globulus* over heath decreases the number of species in the understory as compared with the original heath vegetation. Bara et al. (1985) found that plantations of *E. globulus* were no lower in species richness than *Pinus pinaster*, *Castanea sativa*, or *Quercus robur* plantations, but cover was less and the distribution of life forms different, with a scarcity of hemicryptophytes and geophytes and a preponderance of tall herbaceous species such as *Pteridium aquilinum* over heathland species such as heather and *Ulex* spp. They suggest that the kind of vegetation beneath *E. globulus* depends on water use (Diaz Fierros et al., 1982), on the accumulation and chemical composition of residues (which might give rise to allelopathic phenomena), and on competition for nutrients in poorer soils, and have observed that *E. globulus* increased the concentration of exchangeable Al, K, and Mg. Bara (1970), had found previously that the C:N ratio and acidification tended to increase in 5- to 12-year-old *E. globulus* plantations, whereas Guitian (1963) concluded that *E. globulus* extracts mobilized more Fe and Al than those from *Q. robur*, thus contributing to the degradation of the surrounding area.

The concurrence of plant species may involve both competition for nutrients (competition *sensu strictu*) and allelopathic phenomena in which toxic organic substances are released into the environment by certain species. The importance of the latter kind of process in forest ecology, especially in the case of *E. globulus* stands, has been emphasized by several researchers (Rice, 1974). Arias (1982) found *E. globulus* leaves to contain water-soluble and volatile compounds capable of inhibiting the germination and growth of grassland species; Baker (1966) reported that volatile compounds released by *E. globulus* suppressed germination and hypocotyl growth in *Castanea sativa* seedlings but did not affect *E. globulus* seedlings. In California *E. globulus* plantations, Del Moral and Muller (1969) have shown the importance of fog-drip in the transport and deposition of foliar metabolites and the potential influence of these metabolites on the diversity and structure of the understory community. The latter authors ruled out the possibility that conditions of illumination, nutrient availability, or soil humidity might suffice to explain the exclusion of grassland species (soil humidity may even be increased by nocturnal condensation beneath the canopy) and pointed out that in Australia, where *Eucalyptus* is native, there are usually few bushes and fewer grasses in the vicinity of eucalyptus trees.

Other species of the genus *Eucalyptus* containing soluble and volatile compounds that suppress the growth of grassland species include *E. camaldulensis* (Del Moral and Muller, 1970), *E. microtheca* (Al-Mousawi and Al-Naib, 1975,

1976; Al-Naib and Al-Mousawi, 1976), and *E. citriodora* (Nishimura et al., 1984). Del Moral et al. (1978) concluded that foliar leachates of *E. baxteri* can prevent the growth of certain heathland shrubs beneath its canopy in its native habitat and that this is not due to competition. Inhibition of test plants such as *E. viminalis* by foliar leachates, radicle exudates, litter, and soil extracts was also observed in their laboratory. On the other hand, Willis (1980) found that artificial leachates of green and fallen dead leaves of *E. regnans* only produced inhibition at high concentrations.

Chemical analysis of leaves and leachates of *E. globulus* has revealed a large number of water-soluble compounds of proven phytotoxic potential, including the phenolic compounds ellagic, gallic, caffeic, gentisic, *p*-coumaric, and chlorogenic acids and the terpenoids cineole, camphorol, and α-pinene (Hillis, 1966a,b; Del Moral and Muller 1969; Guenther, 1950). Terpenoids can be phytotoxic at concentrations as low as 1–3 μM (Asplund, 1968), and their effective involvement in allelopathic aggression by *Eucalyptus* spp. was noted by Putnam (1983). Trenbath and Fox (1976) reported that *E. bicostata* leachates did not contain the terpenoids found in artificial leaf extracts and suggested that their release into the environment might be brought about by the action of leaf-chewing insects.

The results discussed above show the allelopathic potential of eucalpytus trees but do not prove conclusively that allelopathic phenomena actually occur in plantations. Harper (1977) has emphasized that the techniques traditionally used for detecting allelopathic processes do not guarantee the validity of results under field conditions, and Stowe and Bong-Seop (1984) have maintained that hitherto research on allelopathy has been very extensive but insufficiently intensive. In order to evaluate the importance of allelopathy in a given situation and to distinguish between laboratory artifacts and genuine plant–plant interactions, it is necessary to establish a relationship between naturally released products and observed symptoms of phytotoxicity and to understand the dynamics of the substances involved.

In this paper we have assessed the phytotoxic potential of natural *E. globulus* leachates (stemflow, throughfall, and soil percolates) together with the capacity of the soil for controlling the phytotoxicity of decaying *E. globulus* litter.

METHODS AND MATERIALS

Description of the Site. The source of material was a 23-year-old *E. globulus* plantation located 250 m above sea level near Santiago de Compostela, Spain (42°50′N, 8°27′W). The plantation faces 218°N with a slope of 26 : 100. The density of the trees is 1289/ha, the average height is 28 m, and the average

diameter 1.3 m above ground is 22.6 cm. According to the FAO classification, the soil is a Humic Andosol, although local variations in depth produce areas of Humic Cambisol with Andic properties. The litter layer is deep and has an average depth of 35 dm, is rich in humic acids, and lies over a reddish brown transition layer and weathered amphibolite bedrock. The characteristics of the topsoil (0–20 cm) are listed in Table 1.

The average annual temperature of the area is 12.9°C, the average temperature during the coldest month is 5.2°C and the average annual precipitation is 1288 mm, of which only 137 mm fall during the three summer months. According to Papadakis' classification, the area has a mild maritime thermal regime and a humic humidity regime (Carballeira et al., 1983). The meteorological data for the period of the study (Figure 1) were obtained from the nearest weather station (C.S.I.C., Santiago de Compostela) and the rainfall sequence adjusted to the data obtained from pluviometers located in the proximity of the plantation.

Leachates, etc., were obtained from a 30 × 80-m plot lying parallel to the slope and containing 58 trees whose distribution by diameter is shown in Table 2.

Collection of Material. Foliar leachates were collected in 10 randomly placed PVC pluviometers 1 m deep and 20 cm in diameter. A layer of glass wool at the bottom of each prevented splashing and retained organic debris that might otherwise have contaminated the leachate.

Stemflow was taken from 13 randomly chosen trees over 25 cm in diameter by means of runnels encircling the bole at least three times.

Soil percolates were collected by means of 17 flat PVC lysimeters 22.5 cm wide, 50 cm long, and 15 cm deep, which were pushed horizontally into the soil without damage to its natural structure.

TABLE 1. CHEMICAL CHARACTERIZATION OF ANDOSOL USED

				Exchangeable bases ($AcONH_4$, pH 7) (meq/100 g)				pH	
Organic	C (%)	N (%)	C/N	Na	K	Ca	Mg	H_2O	ClK
	8.83	0.76	12	0.50	0.37	0.94	0.43	4.60	4.40

Extractable Al (ClK) (meq/100 g)	Extractable acidity (Cl_2Ba-TEA, pH 8.2) (meq/100 g)	Oxalate dithionite extractable	
		Fe_2O_3 (%)	Al_2O_3 (%)
0.83	38.92	4.22	5.47

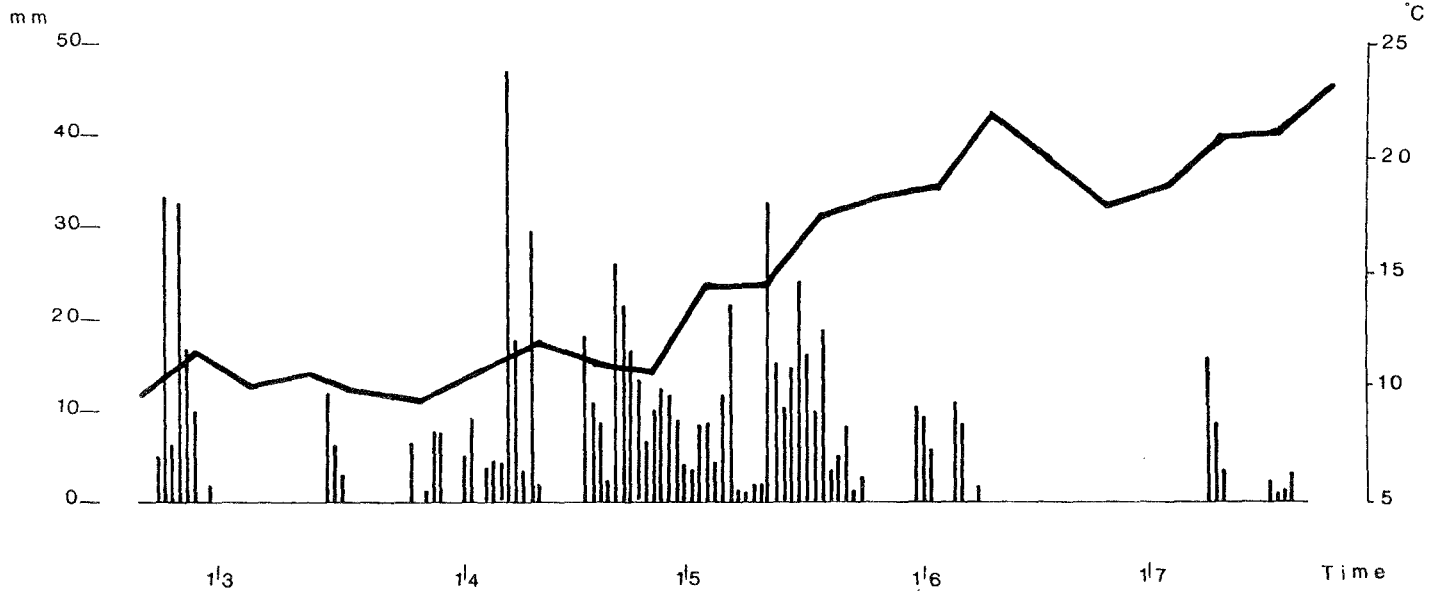


FIG. 1. Meteorological data for the period covered by the study.

TABLE 2. DIAMETER AND HEIGHT OF *E. globulus* TREES

Basal diameter at 1.3 m (cm)	Height min–max(m)	Trees %
4.6–15	7–29.5	37
15–25	8.5–31.5	18
25–35	25–48.5	26
35–45.9	39.5–49.5	19

All the above leachates were accumulated over 24-hr periods (9 AM to 9 AM) in opaque plastic bottles, which, like the collectors (pluviometers, etc.), were washed with distilled water before each collection period. After the amount collected each day had been measured at the site, samples were rapidly taken back to the laboratory for assay.

Leachates from decaying litter were obtained by collecting all the eucalyptus material that had fallen in the course of seven days onto randomly located 1-m squares (leaves, bark, twigs, etc.). Thus the material obtained was from natural abscission; Attiwill et al. (1978) pointed out that the composition of "artificial litter" obtained by manual abscission differs from "natural litter." The litter collected was thoroughly mixed to obtain homogeneous samples, and three samples were taken to determine dry weight at 80°C. Thirty-five 30-g samples then were placed in nylon litter bags of 2 mm mesh. Bocock (1964), MacCauley (1975), and Gloaguen and Touffet (1980) found no significant differences between rates of decomposition in bags with mesh sizes of 0.5–2.0 and 2.5–5.0 cm. On April 25, the bags were placed in groups of seven at five randomly chosen points on the site, and one bag was removed from each group after 1, 15, 30, 60 and 90 days.

Fifteen soil samples of the top 5 cm were taken at random using an auger 5 cm in diameter. Organic material and stones were removed and the sample was homogenized before assay.

Bioassays. Ideally, the effect of the leachates collected would be tested on species that are abundant in the vicinity of the plantation but not present in its understory. However, since all such species germinate and grow slowly and since it was found that the leachates lose most of their biological activity within 96–112 hr, such bioassays were not feasible. To measure the relative phytotoxic efficiency of the various natural release mechanisms investigated, we therefore used *Lactuca sativa* (var. Great Lakes), a fast-growing species commonly used in bioassays due to its well-known sensitivity to most phytotoxic substances and plant growth regulators.

Bioassays of throughfall, stemflow, soil percolates, and litter leachates (see

below) consisted of sowing 50 lettuce seeds on 3MM Whatman paper in each of five Petri dishes 10 cm in diameter and wetting them with 7.5 ml of the leachate being assayed (controls were wetted with water collected in the gauges placed outside the plantation). In bioassays of soil, the seeds were sown on 3MM Whatman paper laid over 30 g of soil at field capacity. After incubation in the dark for 48 hr at 24°C and 80% relative humidity, germination rates and radicle lengths were recorded and the statistical significance of the results was calculated by means of analysis of variance (Vesereau, 1968).

Litter leachates were prepared by saturating the collected material with distilled water, allowing it to soak for 24 hr in the dark at 15°C, and then washing with a distilled-water spray until 50 ml of extract per 100 g dry wt had been obtained, a dilution of 1:1. By further dilution with distilled water, 1:2, 1:10, and 1:50 (v/v) extracts were prepared.

In all assays, the pH lay within the range of acidities at which germination and root elongation of *L. sativa* are not affected (Table 3).

RESULTS AND DISCUSSION

Figure 2 shows that the only date on which the biological activity of foliar leachates differed from that of water from the control pluviometers at the $d = 0.01$ level was May 30. Radicle growth was significantly depressed by these leachates, in which leaf exudate had accumulated on the eucalyptus trees during six days without rain (see Figure 1). Although there had been 30 days without rain prior to rainfall on July 9, the amount of rain that fell on this occasion, and the manner in which it fell, apparently produced greater dilution of the exudates than on May 30.

Germination was significantly depressed (by over 65%) by samples of stemflow collected on March 14 and radicle growth was significantly reduced (by over 66%) by samples collected on July 9 (Figure 3). These dates marked

TABLE 3. AVERAGE VALUES AND VARIATION OF pH IN BIOASSAY EXTRACTS

	Min	Mean	Max
Rain water (control)	4.3	5.1	5.8
Foliar leachates	3.6	4.0	5.4
Cortical leachates	3.7	3.9	4.6
Edaphic percolates	3.7	4.1	4.7
Soil (pH in water 1:1 v/v)	3.5	4.6	4.9
Litter leachates	3.8	4.5	5.0

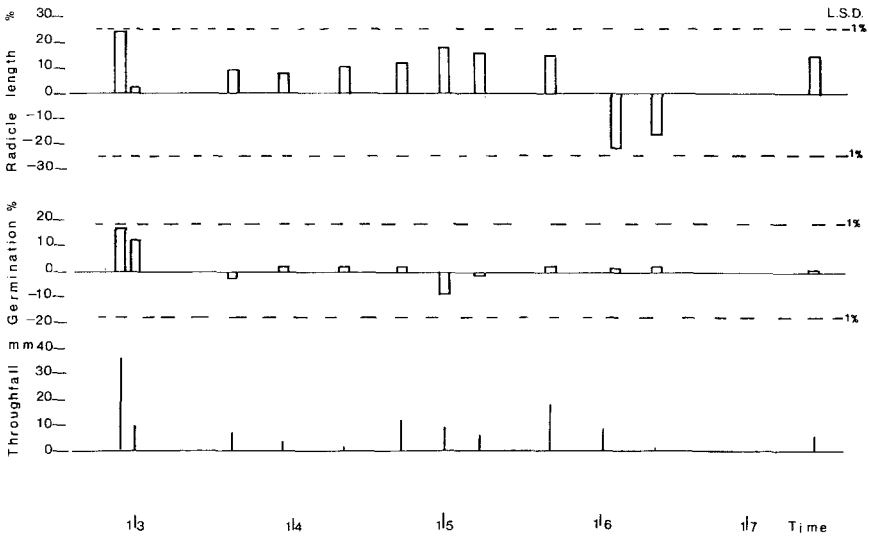


FIG. 2. Effects of natural foliar leachates from *E. globulus* on seed germination and linear growth of radicles of *Lactuca sativa*. (Results expressed as % of control values. Dashed line indicates LSD from control at 1% level of significance.)

the ends of the longest exudate accumulation periods (15 and 31 days, respectively).

The above results suggest that aerial leachates are not a significant source of allelochemical agents unless a set of fairly infrequent conditions coincide. Toxins must accumulate (Figure 4) during a long rainless period without leaching, and their allelopathic effects also depend on the quantity of rain falling, the duration of the rainfall, and on the phenological state of the plant (Mitchell, 1968; Tukey, 1969; Turner and Quarterman, 1975; Squires and Trollope, 1978). Figure 4 shows the height of the electrolyte concentration in the leachates after light rainfall.

Soil percolates (Figure 5) exhibited relatively little biological activity when compared with that of topsoil (Figure 6). Germination was unaffected, and radicle growth was only inhibited by soil leachates collected on April 18 and July 7, when the concentration of allelochemicals was high in the first leachate after long periods without rain.

Topsoil (Figure 6) had no significant effect on germination levels, but severely reduced radicle growth (up to 70% reduction, compared to controls). The three inhibition maxima coincide with the ends of the longest rainless periods of exudate accumulation (31, 10, and 8 days, ending on July 7, June 16, and May 30, respectively). Phytotoxicity increased notably as the summer

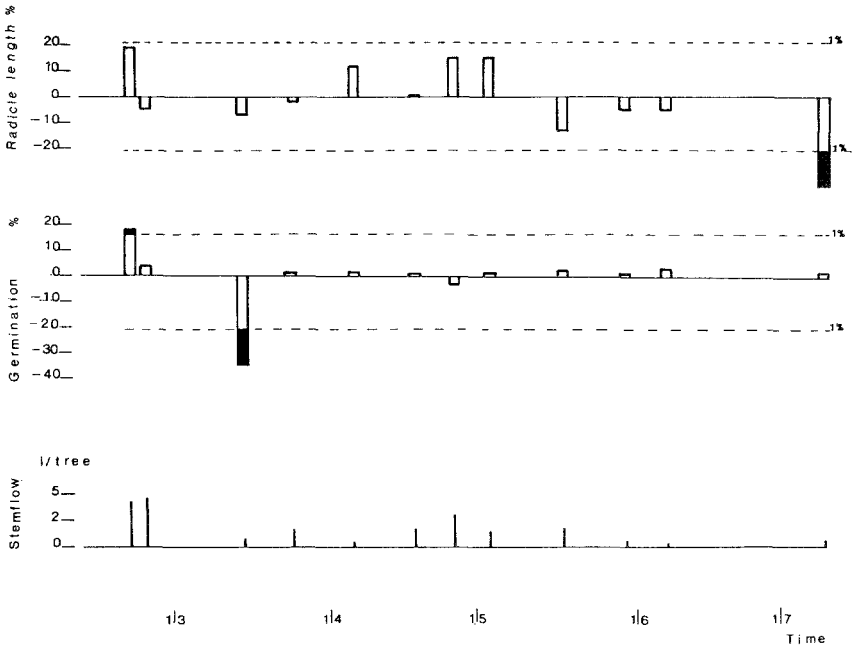


FIG. 3. Effects of natural cortical leachates from *E. globulus* on seed germination and linear growth of radicles of *Lactuca sativa*. (Results expressed as % of control values. Dashed line indicates LSD from control at 1% level of significance.)

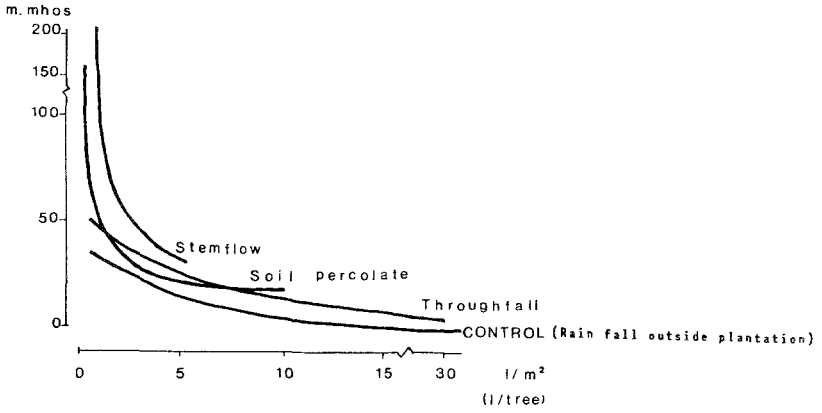


FIG. 4. Relationship between conductivity and leachate concentrations.

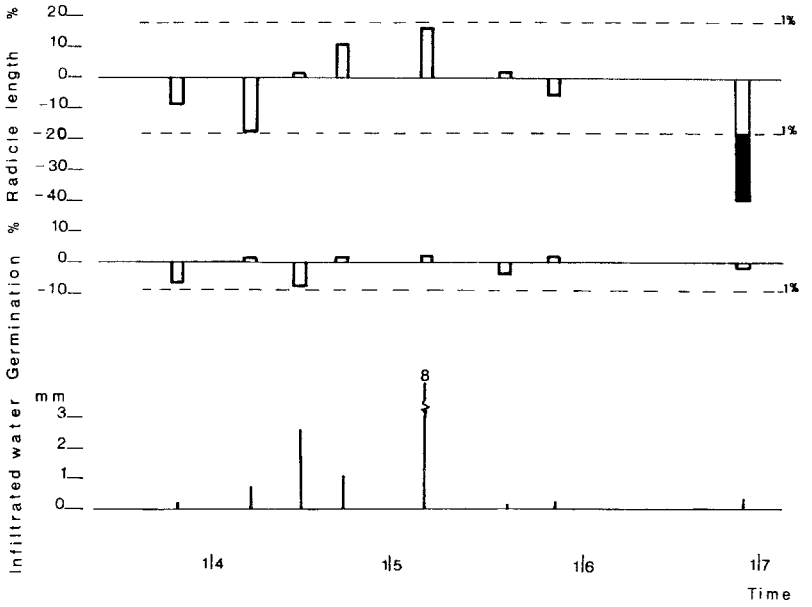


FIG. 5. Effects of natural topsoil percolates from *E. globulus* on seed germination and linear growth of radicles of *Lactuca sativa*. (Results expressed as % of control values. Dashed line indicates LSD from control at 1% level of significance.)

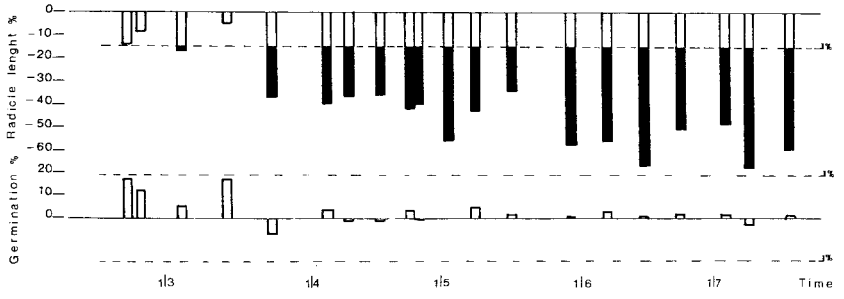


FIG. 6. Effects of soil (0–5 cm) from *E. globulus* on seed germination and linear growth of radicles of *Lactuca sativa*. (Results expressed as % of control values. Dashed line indicates LSD from control at 1% level of significance).

progressed, possibly due to variation in the quantity or nature of decomposing litter (Figure 8 below), acceleration of decomposition and toxin release due to higher temperatures (Molina et al., 1984), or changes in the phenology of the trees, the activity of soil microorganisms or water and redox potentials.

Figure 7 shows the germination and radicle growth bioassay data analyzed

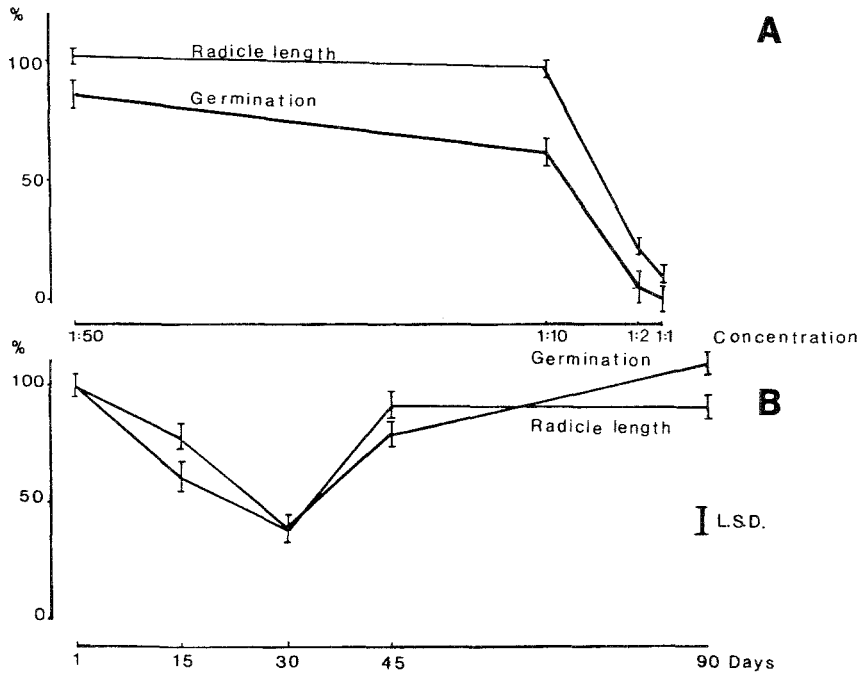


FIG. 7. Effects of natural litter leachates from *E. globulus* on seed germination and linear growth of radicles of *Lactuca sativa*. Split-plot analysis for leachates concentration (A) and time (B). (Results expressed as % of control values. Dashed line indicates LSD from control at 1% level of significance).

by a two-way analysis of variance, which showed no interaction. LSDs were calculated in two one-way analyses of variance. Germination was depressed by all the leachates prepared, but radicle growth was significantly decreased only by leachates 1 : 1 and 1 : 2. The maximum reductions in both germination levels and radicle growth were about 50%. Maximum toxicity was exhibited between 15 and 45 days after abscission, and leachates 1 : 1 and 1 : 2 still caused severe inhibition after 90 days, even though toxins should have leached out of the litter on site during this period. Leachates 1 : 10 and 1 : 50 depressed radicle growth up to day 30 and germination up to day 60. In assessing the significance of these results, it should be borne in mind that the total quantity of litter falling during the period covered by the study was well over 400 dry wt/m^2 (Figure 8), making the concentrations of the extracts bioassayed well within the concentration range that might be expected under natural conditions. *Eucalyptus* trees are peculiar in shedding litter continually, so that the release of toxic leachates from decaying litter occurs year-round. Most litter falls just before

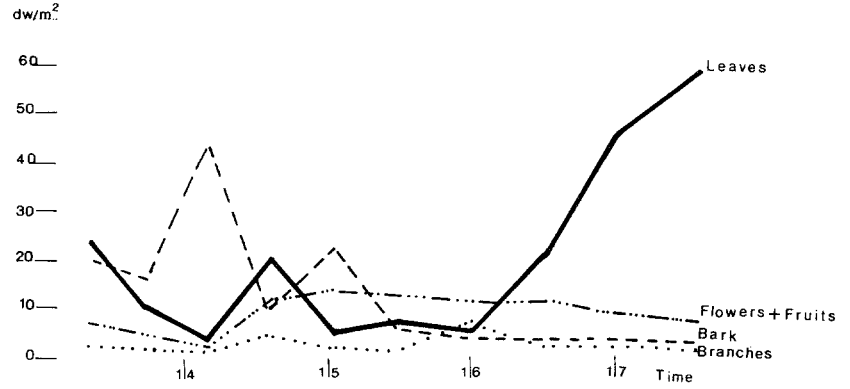


FIG. 8. Litter fall components of *E. globulus* over the study period.

the summer (Reichle, 1981), not in autumn as in the case of native species such as *Quercus robur*, thus circumventing the defensive strategies of native understory species.

The above results indicate that the most important mechanisms by which eucalyptus toxins are released into the soil are through the decomposition of fallen litter. Some seemingly contradictory findings should be noted, however. First, in the present study the high inhibitory activity of both litter leachates and topsoil were washed away by rainfall, whereas the contrary was observed by Becker and Drapier (1985) in *Abies alba* Mill. plantations. Second, litter leachates are more toxic than soil or soil percolates (although the loss of toxicity by the percolates may have been due merely to their already being diluted when collected). Third, and most strikingly, while litter leachates mainly depress germination, topsoil reduces radicle growth.

The processes in the soil to which allelochemicals released by plants are subjected are complex and largely unknown and may result in either synergistic or antagonistic effects (Rice, 1974; Kaminsky, 1981). Possible interactions include the breakdown of active compounds after absorption by soil colloids and the induced release from colloids of growth promotors, which might compensate for the inhibition caused by the eucalyptus toxins. Such promotors might proceed either from other species in the community or from microbial synthesis. Without a doubt, the allelopathic effects of *E. globulus* on the decomposition and structure of its own understory can only be fully elucidated by means of a thorough biochemical study of the soil, including its microflora and mycorrhizae. The interaction between direct and indirect allelopathic processes, and between these processes and water availability, pathogenic agents, the perturbation of nutrient uptake and ionic toxicity must all be considered. The present results, nevertheless, demonstrate that the chief mechanisms for the release of

allelochemicals by *E. globulus* is the decomposition of litter and that the toxicity of the litter is especially effective during the delicate establishment stage of the target species. This would explain the paucity of the understory, when compared with nearby groups of trees of other species, and might also affect the balance among different life forms (Bara et al., 1985).

REFERENCES

AL-MOUSAWI, A.H., and AL-NAIB, F.A.G. 1975. Allelopathic effects of *Eucalyptus microtheca. F. Mull. J. Univ. Kuwait. (Science)* 2:59–66.

AL-MOUSAWI, A.H., and AL-NAIB, F.A.G. 1976. Volatile growth inhibitors produced by *Eucalyptus microtheca. Bull. Bro. Res. Center. (Baghdad)*. 7:17–23.

AL-NAIB, F.A.G., and AL-MOUSAWI, A.H. 1976. Allelopathic effects of *Eucalyptus microtheca.* Identification and characterization of the phenolic compounds. *J. Univ. Kuwait (Science)* 3:83–88.

ALVAREZ, R., and MALVAR, M.E. 1979. Estudio comparativo de una lignosa de *Castanea sativa* Millar con lignosa de *Eucalyptus globulus* Labill. *Trabajos Compostelanos de Biología* VII. pp. 1–15.

ARIAS, A.M. 1982. Estudio del potencial alelopático de *Eucalyptus globulus* Labill. Tes. Lic. Facultad de Biología. Universidad de Santiago de Compostela.

ASPLUND, R.O. 1968. Monoterpenes; relationships between structure and inhibition of germination. *Phytochemistry* 7:1995–1997.

ATTIWILL, P.M., GUTHRIE, H.B., and LEUNING, R. 1978. Nutrient cycling in a *Eucalyptus oblique* (L'Herit) forest. I. Litter production and nutrient return. *Aust. J. Bot.* 26:71–79.

BAKER, H.G. 1966. Volatile growth inhibitors produced by *Eucalyptus globulus. Madroño* 18:207–210.

BARA, T.S. 1970. Estudio sobre el *Eucalyptus globulus. Comun. I.F.I.E.* 67:43.

BARA, T.S., RIGUERO, A.R., GIL, S.M.C., MANSILLA, V.P., and ALONSO, M.S. 1985. Efectos ecológicos del *Eucalyptus globulus* en Galicia. (Estudio comparativo con *Pinus pinaster* y *Quercus robur*). *Monografías I.N.I.A.* 50:1–381.

BECKER, M., and DRAPIER, J. 1985. Rôle de l'allélopathie dans les difficultés de régénération du sapin (*Abies alba* Mill). *Acta Oecol. Oecol. Plant.* 20:31–40.

BELLOT, F. 1949. Las comunidades de *Pinus pinaster* en el occidente de Galicia. *An. Edaf. Fis. Veg.* 8:75–119.

BELLOT, F. 1966. Vegetación de Galicia. *An. Inst. Bot. Cav.* 24:1–306.

BOCOCK, K.L. 1964. Changes in the amount of dry matter, nitrogen, carbon and energy in decomposing woodland leaf litter in relation to the activities of the soil fauna. *J. Ecol.* 52:273–284.

CARBALLEIRA, A., DEVESA, C., RETUERTO, R., SANTILLAN, E., and UCIEDA, F. 1983. Bioclimatología de Galicia. Publicaciones Fundación Pedro Barrié de la Maza. A Coruña., Spain. 391 pp.

CASTROVIEJO, S. 1973. El área suroccidental de los brezales gallegos. *An. Inst. Bot. Cav.* 30:197–213.

DEL MORAL, R., and MULLER, C.H. 1969. Fog drip: A mechanism of toxin transport from *Eucalyptus globulus. Bull Torrey Bot. Club* 96:467–475.

DEL MORAL, R., and MULLER, C.H. 1970. The allelopathic effects of *Eucalyptus camaldulensis. Am. Midland Nat.* 83:254–283.

DEL MORAL, R., WILLIS, E.J., and ASHTON, D.H. 1978. Suppression of coastal heath vegetation by *Eucalyptus baxteri. Aust. J. Bot.* 26:203–219.

DIAZ-FIERROS, D.V.F., CALVO, R.A., and PAZ, A.G. 1982. As especies forestais e os solos de Galicia. *Cuad. Ciencias Agrarias, Publ. Sem. Estudos Galegos.* 166 pp.

GLOAGUEN, J.C., and TOUFFET, J. 1980. Vitesse de décomposition et évolution minérale des litiéres sous climat atlantique. I. Le Hêtre et quelques coniféres. *Acta Oecol. Oecol. Plant.* 15:3–26.

GUENTHER, E. 1950. The Essential Oils, Vo. 4. D. Van Nostrand, New York. p. 75.

GUITIAN, F. 1963. Acción de las hojas de algunas de las especies vegetales en la movilización del hierro y arcilla del suelo. *Trab. Jardín Bot. Univ. Santiago de Compostela* 9:31–39.

HARPER, J.L. 1977. Population Biology of Plants. Academic Press, London.

HILLIS, W.E. 1966a. Variations in polyphenol composition within species of *Eucalyptus* L'Herit. *Phytochemistry* 5:541–556.

HILLIS, W.E. 1966b. Polyphenols in the leaves of *Eucalyptus* L'Herit: A chemotaxanomic survey. Introduction and study of the series *Globulus. Phytochemistry* 5:1075–1090.

KAMINSKY, R. 1981. The microbial origin of the allelopathic potential of *Adenostoma fasciulatum*, H.S.A. *Ecol. Monogr.* 5:365–382.

MACCAULEY, B.J. 1975. Biodegradation of litter in *Eucalyptus pauciflora* communities. I. Techniques for comparing the effects of fungi and insects. *Soil Biol. Biochem.* 7:341–344.

MITCHELL, C.A. 1968. Detection of carbohydrates leached from aboveground plant parts. MS thesis. Cornell University, Ithaca, New York.

MOLINA, A, REIGOSA, and M. J. CARBALLEIRA, A. 1984. Efectos alelopáticos durante la descomposición de residuos de *Eucalyptus globulus* Labill. *Cuad. Ciencieas Agraris, Publ Sem. Estudios Galegos* 5:117–131

NISHIMURA H., NAKAMURA, T. MIZUTANI, J. TAKEMICHI, N., and JUNYA, M. 1984. Allelopathic effects of *p*-menthane-3,8-diols in *Eucalyptus citriodora. Phytochemistry* 23:2777–2779.

PUTNAM, A.R. 1983. Allelopathic chemicals. *Chem. Eng. News* April (4):34–45.

REICHLE, D. E. 1981. Dynamic Properties of Forest Ecosystems. I.B.P. 23. Cambridge University Press, New York. 683 pp.

RICE, E. 1974. Allelopathy. Academic Press, New York. 353 pp.

SQUIRES, V.R., and Trollope, W.S.W. 1978. Allelopathy in the Karoo Shrub *Chrysocoma tennuifolia. S. Afr. Sci.* 75:85–89.

STOWE, L.G., and BONG-SEOP, K. 1984. The role of toxins in plant-plant interactions, pp. 707–741, *in* Handbook of Natural Toxins, Vol. 1. Plant and Fungal Toxins. (eds). Dekker, New York.

TRENBATH, B.R., and FOX, L.R. 1976. Insect frass and leaves from *Eucalyptus bicostata* as germination inhibitors. *Aust. Seed Sci. News Lett.* 2:34–39.

TUKEY, H.B., Jr. 1969. Implications of allelopathy in agricultural plant science. *Bot. Rev.* 35:1–16.

TURNER, H.B., and QUARTERMAN, E. 1975. Allelochemic effects of *Petalostemon gattingeri* on the distribution of *Arenaria patula* in cedar glades. *Ecology* 56:924–932.

VESEREAU, A. 1968. Methods statistiques in biologie et agronomie. Ed. Bailliere, Paris.

WILLIS, E.J. 1980. Allelopathy and its role in forest of *Eucalyptus regnans* F. Mull. PhD thesis. University of Melbourne, Australia.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

CHEMICAL DETECTION OF CONSPECIFICS IN THE CRAYFISH *Procambarus clarkii:* ROLE OF ANTENNULES

JIN W. OH and DAVID W. DUNHAM*

Department of Zoology
University of Toronto
Toronto, Ontario, Canada M5S 1A1

(Received November 10, 1989; accepted September 4, 1990)

Abstract—Tests with female crayfish (*Procambarus clarkii*) confirm that conspecifics can be detected chemically. The subjects were more attentive in the presence of water from a stranger's tank than in the presence of water from their own tank. Animals with intact antennules performed this discrimination best, but animals with one intact inner ramus and one intact outer ramus also clearly responded. Conspecific chemical discrimination after loss of both inner or both outer rami was weak (trial 2) or absent (trial 1). The relevance of this outcome to previous conflicting reports on the use of antennules in sex and species discrimination in crayfish is discussed.

Key Words—*Procambarus clarkii*, Crustacea, Decapoda, crayfish, antennule, chemical detection, chemical communication.

INTRODUCTION

There has been some interest in the ability of crayfish to detect other crayfish chemically. Reports in the literature address two kinds of discrimination. The first of these is the ability to discriminate conspecifics on the basis of sex. This ability has been demonstrated in *Orconectes virilis* by Hazlett (1985a), and in *Procambarus clarkii* by Ameyaw-Akumfi and Hazlett (1975) and Ameyaw-Akumfi (1976). Since the ablation of both the inner and outer rami, or of only the inner rami, of the antennules resulted in the loss of this ability in *Procambarus clarkii*, these authors implicated the inner rami in the relevant chemical transduction. Response to conspecifics of the opposite sex in the crayfish *Orco-*

*To whom correspondence should be addressed.

0098-0331/91/0100-0161$06.50/0

nectes propinquus was shown by Tierney et al. (1984) to occur only when the outer antennular rami were intact. Ablation of the outer rami resulted in the loss of ability to respond, while ablation of the inner rami did not affect this ability.

The second kind of discrimination investigated in the literature involves the ability of the test animal to distinguish itself from another conspecific. This type of discrimination has been studied in *Procambarus clarkii* by Little (1975), Itagaki and Thorp (1981), Thorp (1984), and Rose (1984), and in other species of crayfish by Little (1976), Thorp and Ammerman (1978), Tierney and Dunham (1982, 1984), Tierney et al. (1984), and Hazlett (1985b, 1989).

Rose (1986) reported chemical detection of non-self conspecifics, by intact *Procambarus clarkii* but showed equivocal results for discrimination of self from other stimuli.

We report here the results of a study of self versus stranger chemical discrimination by female *Procambarus clarkii*. We examine the role of both rami of the antennules in performing this discrimination.

METHODS AND MATERIALS

The subjects, mature, nongravid, intermolt female *Procambarus clarkii*, were obtained from a supplier in Louisiana. They were maintained on a light cycle of 12 hr dark–12 hr light, at 18–20°C, and fed raw fish daily. They were held in aerated, individual glass jars containing 2.5 liters of distilled water and a gravel substrate for five days before testing.

Antennule ablation was performed after anesthesia by immersion in ice water, and the animals were given at least 24 hr to recover from this procedure before testing. Stimulus water was obtained by gently scooping out 50 ml from a jar that had held one crayfish for five days, 1 hr before testing. Stimulus water came from a different crayfish for each trial.

A test consisted of first placing a crayfish and 2 liters of its own jar water in a 20 × 36 × 22 cm (high) aquarium. After 15 min, the stimulus water was placed in a 50-ml burette and adjusted to drip onto an airstone in the aquarium at a rate of about 1 drop every 2.5 sec. The experiments were performed at night, when the animals were most active (2100 to 0200 hr).

The behaviors of the animals were video-monitored for 1 hr from an adjacent room, using an infrared-sensitive camera. Both the order of the treatments (intact, inner ablates, other ablates, cross ablates), and the order of individual animals used in each test, were randomized. In trial 1, 10 different animals were used in each of the four tests (a total of 40 crayfish), and each animal was tested once with self water and once with stranger water. In trial 2, different animals were similarly tested, but only with stranger water. Trial 2 was a replicate of the stranger water test in trial 1, conducted with a new set of animals. The procedure followed was similar in most respects to that used by Rose (1986).

RESULTS AND DISCUSSION

Behaviors exhibited by the test subjects were resting, in which the subject neither moved or displayed; locomotion; and attention to the stimulus source. Attention included touching the airstone, climbing on the airstone, and chela displays of meral extension and meral spread (Bruski and Dunham, 1987).

The median attention scores from trial 1 for the four test groups of 10 crayfish each are shown in Figure 1. The raw scores for attention during the self and stranger water tests were compared, using the Wilcoxon signed-rank test (Castellan and Siegel, 1988), at the 1% significance level. The intact animals were more attentive in the presence of water from another crayfish than in the presence of their own water ($P < 0.001$), as were the cross ablates ($P < 0.003$). Both the inner ablates and the outer ablates showed no difference in attention between the two tests ($P > 0.50$ and $P > 0.28$, respectively).

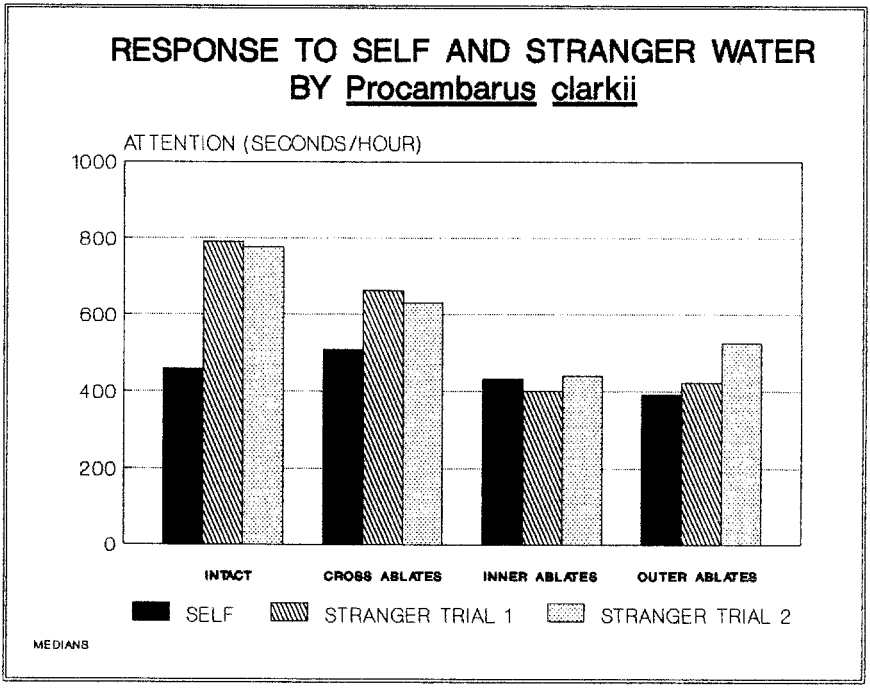


FIG. 1. Median attention scores from trial 1 of four groups ($N = 10$) of female crayfish (*Procambarus clarkii*) tested individually with water from their own home tank or a stranger's home tank. Cross ablates had one inner and one outer antennule ramus intact; inner ablates had only two outer rami; outer ablates had only two inner rami. Medial attention scores from trial 2, a replicate of the stranger water test in trial 1, with different animals, are shown also. Significance values are given in the text.

Attention scores from trial 1 also were compared across treatment groups, using the Wilcoxon-Mann-Whitney test (Castellan and Siegel, 1988), at the 1% significance level. The scores in the presence of self water were marginally greater (significance at the 5% level) for the cross ablates than the inner ablates ($P < 0.02$) or the outer ablates ($P < 0.03$). Otherwise, the scores in the presence of self water did not differ across groups. The attention scores in the presence of stranger water did not differ significantly between the inner ablates and the outer ablates ($P > 0.42$). The scores of intact animals in the presence of stranger water were significantly greater than those of the cross ablates ($P < 0.006$), and the scores of the latter were significantly greater than those of the inner ablates ($P < 0.0007$) or the outer ablates ($P < 0.001$).

Attention scores from trial 2 were compared across treatment groups using the Wilcoxon-Mann-Whitney test (Castellan and Siegel, 1988). Trial 2 scores also were compared with scores from the equivalent treatment groups in trial 1. The attention scores in the presence of stranger water did not differ significantly between the inner and the outer ablates ($P > 0.192$). The scores of intact animals in the presence of stranger water were significantly greater than those of the cross ($P < 0.007$), inner ($P < 0.0001$), and the outer ablates ($P < 0.0002$), but there was only a marginal difference (significant at the 5% level) between the cross ablates and the inner ($P < 0.05$) and the outer ablates ($P < 0.022$). The attention scores from trial 2, when compared with scores from the equivalent treatment groups in trial 1, showed no significant difference in all four cases ($P > 0.456$; $P > 0.100$; $P > 0.341$, and $P > 0.106$, respectively).

The attention-promoting effect of water from another isosexual conspecific shown here is consistent with the results of Rose (1986). What is surprising is the inability of animals with intact outer antennule rami to discriminate self from stranger water. The minimal requirement for this discrimination in our tests was one intact inner ramus and one intact outer ramus. Ameyaw-Akumfi and Hazlett (1975) reported that chemical sex discrimination in *Procambarus clarkii* was impaired when either both rami, or only the inner antennule rami were ablated. They did not report a test with only the outer rami ablated. However, Tierney et al. (1984), showed in *Orconectes propinquus* that response to conspecifics was only possible with intact outer rami and that the inner rami were not required (see also Gleeson, 1980).

Two hypotheses suggest themselves in considering these differences: (1) Discrimination of conspecifics in crayfish may be a fundamentally different process from stranger detection, regardless of the species involved. Different chemicals, different sensilla, or different CNS processing may be involved. (2) Alternatively, these two species (and genera) may differ in their means of transducing socially relevant chemicals, regardless of the particular discrimination being made. Further experiments are needed to define precisely the anatomical basis of semiochemical responses.

Tierney et al. (1986) presented indirect but persuasive evidence that one of two setal types, found on the outer, but not on the inner antennule ramus, was the sensillum where this kind of chemical transduction took place. We have examined *Procambarus clarkii* antennules under the light microscope and scanning electron microscope. We found that aesthetascs resembling those described and illustrated by Tierney et al. (1986) for the antennules of *Orconectes propinquus*, were present in *Procambarus clarkii*. The distribution of the aesthetascs on only the outer ramus of the antennule also resembled that of *Orconectes propinquus* (Oh and Dunham, unpublished data). Although we have no direct knowledge of the way in which the rami effect semiochemical transduction, there is no gross anatomical difference that suggests a different physical arrangement of sensilla.

Acknowledgments—This study was supported by an operating grant from the Natural Sciences and Engineering Research Council of Canada to D.W.D. We acknowledge helpful comments on the manuscript by two anonymous referees.

REFERENCES

AMEYAW-AKUMFI, C.E. 1976. Some aspects of breeding biology of crayfish. PhD thesis. University of Michigan. 252 pp.

AMEYAW-AKUMFI, C.E., and HAZLETT, B.A. 1975. Sex recognition in the crayfish *Procambarus clarkii. Science* 190:1225–1226.

BRUSKI, C.A., and DUNHAM, D.W. 1987. The importance of vision in agonistic communication of the crayfish *Orconectes rusticus*. I: An analysis of bout dynamics. *Behaviour* 103:83–107.

CASTELLAN, N.J., JR., and SIEGEL, S. 1988. Nonparametric Statistics for the Behavioral Sciences, 2nd ed. McGraw-Hill, New York.

GLEESON, R.A. 1980. Pheromone communication in the reproductive behavior of the blue crab *Callinectes sapidus. Marine Behav. Physiol.* 7:119–134.

HAZLETT, B.A. 1985a. Chemical detection of sex and condition in the crayfish *Orconectes virilis. J. Chem. Ecol.* 11(2):181–189.

HAZLETT, B.A. 1985b. Disturbance pheromones in the crayfish *Orconectes virilis. J. Chem. Ecol.* 11(12):1695–1711.

HAZLETT, B.A. 1989. Additional sources of disturbance pheromone affecting the crayfish *Orconectes virilis. J. Chem. Ecol.* 15(1):381–385.

ITAGAKI, H., and THORP, J.H. 1981. Laboratory experiments to determine if crayfish can communicate chemically in a flow-through system. *J. Chem. Ecol.* 7(1):115–126.

LITTLE, E.E. 1975. Chemical communication in maternal behavior of crayfish. *Nature* 255:400–401.

LITTLE, E.E. 1976. Ontogeny of maternal behavior and brood pheromone in crayfish. *J. Comp. Physiol.* 112:133–142.

ROSE, R.D. 1984. Chemical communication in crayfish: Physiological ecology, realism and experimental design. *J. Chem. Ecol.* 10(8): 1289–1291.

ROSE, R.D. 1986. Chemical detection of self and conspecifics by crayfish. *J. Chem. Ecol.* 12(1):271–276.

THORP, J.H. 1984. Theory and practice in crayfish communication studies. *J. Chem. Ecol.* 10(8):1283–1287.

THORP, J.H., and AMMERMAN, K.S. 1978. Chemical communication and agonism in the crayfish *Procambarus acutus acutus. Am. Midl. Nat.* 100(2):471–474.

TIERNEY, A.J., and DUNHAM, D.W. 1982. Chemical communication in the reproductive isolation of the crayfish *Orconectes propinquus* and *Orconectes virilis* (Decapoda, Cambaridae). *J. Crust. Biol.* 2:544–548.

TIERNEY, A.J., and DUNHAM, D.W. 1984. Behavioral mechanisms of reproductive isolation in crayfishes of the genus *Orconectes. Am. Midl. Nat.* 111(2):304–310.

TIERNEY, A.J., THOMPSON, C.S., and DUNHAM, D.W. 1984. Site of pheromone reception in the crayfish *Orconectes propinquus* (Decapoda, Cambaridae). *J. Crust. Biol.* 4(4):554–559.

TIERNEY, A.J., THOMPSON, C.S., and DUNHAM, D.W. 1986. Fine structure of aesthetasc chemoreceptors in the crayfish *Orconectes propinquus. Can. J. Zool.* 64:392–399.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

DIFFERENTIAL ALLELOCHEMICAL DETOXIFICATION MECHANISM IN TISSUE CULTURES OF *Antennaria microphylla* and *Euphorbia esula*

MARY ELLEN HOGAN and GARY D. MANNERS*

U.S. Department of Agriculture
Agricultural Research Service
Western Regional Research Center
800 Buchanan Street
Albany, California 94710

(Received July 23, 1990; accepted September 4, 1990)

Abstract—Callus and suspension cultures of *Antennaria microphylla* (small everlasting) and the noxious weed *Euphorbia esula* (leafy spurge) can glucosylate benzene-1,4-diol (hydroquinone) to the corresponding monoglucoside, arbutin. HPLC analysis of extracts from callus tissue corroborates the presence of hydroquinone in the cells of small everlasting. Constitutive levels of a UDPG-dependent glucosyltransferase were detected in cell-free extracts of this tissue. Although this detoxification enzyme was induced in leafy spurge suspension culture cells grown in the presence of hydroquinone, the activity was six-fold lower than that measured in small everlasting. Differential ability to detoxify hydroquinone provides a basis for the observed allelopathic interaction between small everlasting and leafy spurge.

Key Words—Allelopathy, *Antennaria microphylla*, small everlasting, *Euphorbia esula*, leafy spurge, tissue culture, hydroquinone, arbutin, glucosyltransferase, biotransformation.

INTRODUCTION

Environmental concerns about herbicide use have stimulated the search for alternative weed-control strategies. The natural phenomenon of allelopathy offers a potential new methodology to supplement conventional weed control programs. The use of allelopathy for weed control relies upon the species-specific responses of a target weed to chronic, sublethal doses of an allelochemical,

*To whom correspondence should be addressed.

0098-0331/91/0100-0167$06.50/0

available as an exudate or leachate from a neighboring plant or supplied as an exogenous formulation. Many allelopathic, noncompetitive plants have been examined for potential endogenous phytotoxins. These investigations have focused on the isolation, characterization, and bioassay of putative allelochemicals to identify major allelochemical sources and to specify structure–activity relationships. This basic chemical and biological research is vital to corroborate the existence of an allelopathic interaction. The exploitation of allelopathy for weed control, however, may not be fully utilized until the underlying biochemical mechanisms are defined.

Euphorbia esula (leafy spurge) and the low-growing, noncompetitive forb *Antennaria microphylla* (small everlasting) grow in close association in rangeland habitats. The reduction of leafy spurge growth in these stands has been attributed to natural chemical phytotoxins (allelochemicals) produced by small everlasting. Hydroquinone, a simple phenol that is strongly phytotoxic to leafy spurge, is biosynthesized by small everlasting (Manners and Galitz, 1985). We have reported recently the biotransformation of hydroquinone to its nonphytotoxic monoglucoside, arbutin, in callus and suspension cultures of small everlasting and leafy spurge (Hogan and Manners, 1990). Differences in the ability of the two species to detoxify hydroquinone could be a prominent factor in the observed dominance of small everlasting over leafy spurge. We now report the isolation of uridine diphosphoglucose (UDPG) -dependent hydroquinone glucosyltransferase activity from cell-free extracts of small everlasting and leafy spurge tissue cultures. These in vitro systems exhibit species-specific differences in the efficiency of enzyme-mediated glucoside formation.

METHODS AND MATERIALS

Tissue Cultures. Suspension and callus cultures of leafy spurge and small everlasting were grown as previously described (Hogan and Manners, 1990). Test hydroquinone solutions were prepared in absolute methanol and introduced aseptically into flasks of leafy spurge suspension culture cells at a final methanol concentration of 1% or less (Davis et al., 1978). HPLC analysis of media extracts showed that all the hydroquinone was absorbed by the cells within 24 hr (data not shown).

Barley Roots. Seeds of *Hordeum vulgare* cv. Larker were grown in vermiculite in 2-liter beakers (dark, 27°C) and watered biweekly with 100 ml of distilled water or 500 μM hydroquinone. Seven-day-old seedlings were harvested and washed free of vermiculite, and the root tissue was separated, frozen in liquid nitrogen, pulverized in a mortar and pestle and stored frozen until used.

Enzyme Extraction and Assay. All procedures were carried out at 4°C, unless otherwise stated. Callus tissue or cell suspensions of small everlasting

and leafy spurge were frozen with liquid nitrogen, pulverized with a mortar and pestle, and stored frozen until used. These homogenates and those from barley roots were extracted in a Bead Beater Cell Disrupter (Biospec Products, Bartlesville, Oklahoma) with 50% (w/v) 0.1-mm glass beads and the following cold extraction buffer: 0.1 M Tris MES (pH 7.0) containing 1 mM dithiothreitol and 2 mg/ml protease inhibitor. The homogenate was filtered through Miracloth, stirred for 5 min with 5% (w/v) Dowex 1X2 (Cl^- form) and filtered through another layer of Miracloth. The filtrate was centrifuged for 20 min at 15,000 rpm. The resulting supernatant was applied to a DEAE–Sephacel column (1.2 × 5 cm) and washed with five column volumes of elution buffer. Proteins were eluted from the column using a step gradient of KCl (8 ml each of 100, 250, and 500 mM KCl) in the elution buffer at a flow rate of 40 ml/hr and a fraction volume of 2 ml each. Protein concentrations of various fractions were determined with Coomassie blue by the method of Bradford (1976). Fractions containing glucosyltransferase activity were assayed in reaction mixtures containing UDPG (0.8 μmol), hydroquinone or arbutin (0.8 μmol), cysteine (0.2 μmol), Tris MES at pH 7.0 (40 μmol), and the enzyme preparation (90 μl) in a total volume of 200 μl (Tabata et al., 1976). These reaction mixtures were incubated under nitrogen (37°C, 1 hr) and analyzed for biotransformation products by HPLC analysis (Waters carbohydrate analysis column, 3.9 × 300 mm; acetonitrile–water, 65:35 v/v; 1 ml/min; UV detection at 280 nm, 10 μl injection). Retention times under these conditions were as follows: hydroquinone, 3.18 min; arbutin, 3.80 min; *p*-hydroxyphenyl-β-gentiobioside, 4.76 min. The specific activity of the enzyme was expressed in nkats (nanomoles of arbutin produced per hour per milligram protein).

Synthesis of p-Hydroxyphenyl-β-gentiobioside. This compound was synthesized from arbutin according to the method of Takiura et al. (1974), and the purity of the synthetic product was established by HPLC. Physical properties were in close agreement with reported values and yield was consistent with that reported.

RESULTS

Typical results from DEAE–Sephacel ion-exchange chromatography of enzyme extracts are shown in Figure 1. In all experiments, fractions exhibiting enzyme activity in subsequent assays were eluted in buffer containing 250 mM KCl (fractions 4–8). These fractions lost glucosylating activity within 24 hr when stored at 4°C or when frozen (−20°C). Acetone powder preparations did not possess enzyme activity; however, fresh plant or cell culture samples could be ground in liquid nitrogen and stored in the freezer without significant loss of enzyme activity over several months. This apparent lability of phenol gluco-

sylating enzymes has been reported previously in the literature (Kleinhofs et al. 1967) and has hampered present attempts at purification.

Enzyme assays with cell-free extracts of untreated small everlasting callus tissue confirmed the presence of constitutive levels of a UDPG-dependent glucosytransferase capable of biotransforming hydroquinone to arbutin (Table 1). No constitutive levels of this enzyme were measured in leafy spurge, but activity was substrate-induced when suspension culture cells of this species were fed 500 μM hydroquinone before extraction. The specific activity of the enzyme preparation from small everlasting callus was six-fold greater than in prepara-

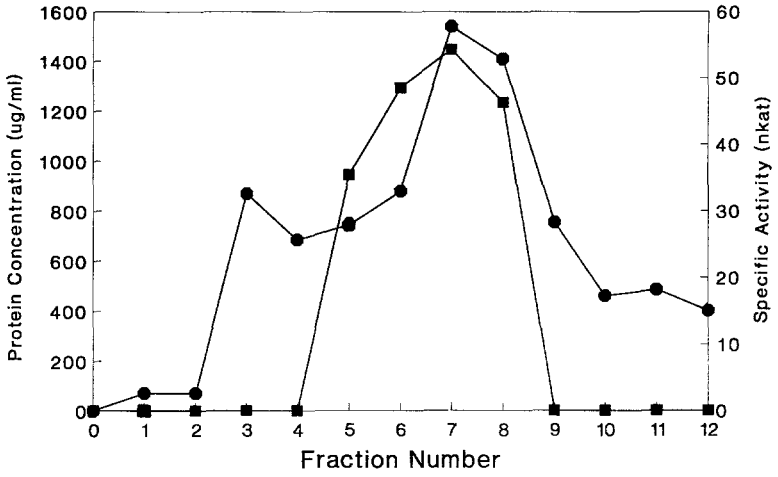


FIG. 1. Elution profile of glucosyltransferase activity from DEAE-Sephacel ion-exchange column. Protein was eluted with a stepwise gradient of KCl (0–500 mM) in extraction buffer. Protein concentration, – ● –; specific activity –■–; nkat = nanomoles of arbutin produced per hour per milligram protein.

TABLE 1. SPECIFIC GLUCOSYLTRANSFERASE ACTIVITY IN CELL-FREE EXTRACTS

Species	Pretreatment	Specific activity (nkat)[a]
Barley		35.4
Barley	500 μM hydroquinone	39.5
Small everlasting		35.6
Leafy spurge		0.0
Leafy spurge	500 μM hydroquinone	5.4

[a] Data are the average of at least three representative experiments. nkat = nanomoles of arbutin formed per hour per milligram protein.

tions obtained from treated leafy spurge and comparable to levels in barley roots, a control species in which hydroquinone-glucosylating activity has been previously reported (Glass and Bohm, 1971).

When arbutin was used as substrate for enzyme preparations from all three species, the unaltered monoglucoside was recovered from the assay mixture at the starting concentration. No diglucoside (i.e., *p*-hydroxyphenyl-β-gentiobioside) was formed (Table 2), suggesting that the enzyme activity measured in these assays is substrate-specific. Evidence for the existence of a second substrate-specific transferase capable of glucosylating arbutin to *p*-hydroxyphenyl-β-gentiobioside has been presented by other workers (Yamaha and Cardini, 1960b).

UDPG was the required high-energy glucose donor for hydroquinone biotransformation. Arbutin production via reverse β-glucosidase activity did not occur in the absence of UDPG. Cysteine also was shown to be an absolute cofactor for measurable glucosyltranseferase activity but EDTA was without effect and consequently was dropped from subsequent assays. β-Gluconolactone, a known inhibitor of β-glucosidase activity (Anderson and Hough, 1960), had no effect on hydroquinone glucosylation by cell-free extracts of all three species. UDPG-dependent glucosyltransferases have been reported to have freely reversible activity in vivo (Anderson and Hough, 1960); however, β-glucosidase activity was not detected in enzyme preparations from small everlasting, leafy spurge, or barley by using the described protocol, as evidenced by the lack of arbutin hydrolysis to hydroquinone. In vitro biotransformation

TABLE 2. REQUIREMENTS FOR GLUCOSYLTRANSFERASE ACTIVITY IN CELL-FREE EXTRACTS

Assay components	Cofactors	Activity (nkat)[a]
Enzyme extract + hydroquinone[b]		−
	+ UDPG	+
	+ UDPG, cysteine	++
	+ UDPG, EDTA	++
	+ UDPG, cysteine, EDTA	++
	+ UDPG, β-gluconolactone	+
	+ UDPG, β-gluconolactone, cysteine	++
Enzyme extract + arbutin[b]		−
	+ UDPG	−
	+ UDPG, cysteine	−
	+ UDPG, cysteine, β-gluconolactone	−

[a] − = no activity, + = measurable activity, ++ = maximal activity.
[b] Extracts obtained from all three species.

reactions with commercial β-glucosidase resulted in both *p*-hydroxyphenyl-β-gentiobioside and arbutin hydrolysis to hydroquinone, with or without added UDPG. Further purification of the enzyme fraction from these species will be required to detect and quantify reverse hydroquinone glucosyltransferase activity.

DISCUSSION

The detection of hydroquinone in extracts of small everlasting callus and the observed toxicity of this compound to leafy spurge (in both callus and suspension culture) indicates the participation of this phytotoxin in the allelopathic interaction between small everlasting and leafy spurge (Hogan and Manners, 1990). Previous work has suggested that the mechanism of this interaction is based on differential metabolism of hydroquinone (Manners and Galitz, 1985; Manners, 1987).

The absorption of hydroquinone from the medium by tissue cultures of both species suggested that the cells detoxified hydroquinone via glucosylation to form arbutin (Hogan and Manners, 1990). Most angiosperms possess the ability to glucosylate the phenolic hydroxyl groups of exogenously applied compounds (Pridham, 1964), and there have been numerous reports of enzyme-mediated phenol glucosylation reactions, both in whole plants (Pridham and Saltmarsh, 1963; Miles and Hagen, 1968) and plant tissue cultures (Tabata et al., 1976, 1988; Ushiyama and Furuya, 1989; Suzuki et al., 1987). Our results show leafy spurge to be considerably less efficient in the detoxification of hydroquinone than small everlasting.

Phenolic compounds are rarely present in unconjugated form in vivo (Kleinhofs et al., 1967), and therefore the hydroquinone pool size should remain fairly low. If hydroquinone uptake in these species is limited only by diffusion rates, as has been reported for barley (Glass and Bohm, 1971), then hydroquinone pool sizes should be regulated by the kinetics of its biotransformation enzymes. As was shown in the present study, hydroquinone and arbutin pool sizes could be directly correlated with glucosyltransferase activity; however, the situation in vivo would be more complex. Reversible glucosyltransferase activity (β-glucosidase activity) may hydrolyze arbutin to hydroquinone, and/or a second enzyme could utilize arbutin preferentially as the substrate for glucosylation to *p*-hydroxyphenyl-β-gentiobioside (Anderson and Hough, 1960). The latter reaction has been confirmed to occur via a substrate specific enzyme in cell-free wheat germ extracts (Yamaha and Cardini, 1960a,b). Tabata et al. (1976) observed a continual decrease in arbutin pool size during the stationary growth phase of *Datura innoxia* suspension culture cells, which they interpreted as the metabolism of arbutin to other compounds.

Continuous exposure of leafy spurge to physiological concentrations of hydroquinone in the field over a long period of time could produce chronic inhibition of growth. Differences in the kinetics of the hydroquinone glucosyltransferase enzyme as well as in the regulation of related enzymes (e.g., β-glucosidase, and other glucosyltransferases) may explain the differential toxicity of hydroquinone in the two species at sublethal (chronic) levels. Continued work is needed to define the biochemical factors affecting the relative pool sizes of hydroquinone and its glucosides in vivo. This problem is complex, involving examination of the regulation schemes for several different enzymes.

Knowledge of the biochemical basis for differential sensitivity to allelochemicals will facilitate the use of natural phytotoxic interactions in weed management and in the development of new herbicides. For example, an increased understanding of the regulation of enzymes responsible for xenobiotic conjugation could provide the basis for an alternative weed control strategy. Inhibitors or other exogenously applied compounds may shift the equilibrium of an enzyme-mediated conjugation reaction. Enhanced expression of latent genes encoding these detoxifying enzymes could be used to protect crops and reduce competition from susceptible weed species.

Acknowledgments—The authors wish to express their appreciation to Dr. David Davis of the USDA/Bioscience Research Laboratory in Fargo, North Dakota, for providing the leafy spurge suspension culture cells and for many helpful discussions. The technical assistance of Deborah Mark is gratefully acknowledged. This work was funded in part by USDA/CSRS grant 88-383000-3626.

REFERENCES

ANDERSON, J.D. and HOUGH, L. 1960. The reaction of carbohydrases with phenolic glucosides. *Biochem. J.* 77:564–567.

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

DAVIS, D.G., WERGIN, W.P., and DUSBABEK, K.E. 1978. Effects of organic solvents on growth and ultrastructure of plant cell suspensions. *Pestic. Biochem. Physiol.* 8:84–87.

GLASS, A.D.M., and BOHM, B.A. 1971. The uptake of simple phenols by barley roots. *Planta* 100:93–105.

HOGAN, M.E., and MANNERS, G.D. 1990. Allelopathy of small everlasting (*Antennaria microphylla*) to leafy spurge (*Euphorbia esula*) in tissue culture. *J. Chem. Ecol.*16(3):931–939.

KLEINHOFS, A., HASKINS, F.A. and GORZ, H.J. 1967. Trans-*o*-hydroxycinnamic acid glucosylation in cell-free extracts of *Melilotus alba. Phytochemistry* 6:1313–1318.

MANNERS, G.D. 1987. The role of phytochemistry in attacking the leafy spurge (*Euphorbia esula*) problem, pp. 228–237, in G.R. Waller (ed.). Allelochemicals: Role in Agriculture and Forestry. ACS Symposium Series 330. American Chemical Society, Washington, DC.

MANNERS, G.D., and GALITZ, D.S. 1985. Allelopathy of small everlasting (*Antennaria microphylla*): Identification of constituents phytotoxic to leafy spurge (*Euphorbia esula*). *Weed Sci.* 34:8–12.

MILES, C.D., and HAGEN, C.W. 1968. The differentiation of pigmentation in flower parts, IV. Flavanoid elaborating enzymes from petals of *Impatiens balsamina. Plant Physiol.* 43:1347–1354.

PRIDHAM, J.B. 1964. The phenol glucosylatin reaction in the plant kingdom. *Phytochemistry* 3:493–497.

PRIDHAM, J.B., and SALTMARSH, M.J. 1963. The biosynthesis of phenolic glucosides in plants. *Biochem. J.* 87:218–224.

SUZUKI, T., YOSHIOKA, T., TABATA, M., and FUJITA, Y. 1987. Potential of *Datura innoxia* cell suspension cultures for glucosylating hydroquinone. *Plant Cell Rep.* 6:275–278.

TABATA, M., IKEDA, F., HIRAOKA, N., and KONOSHIMA, M. 1976. Glucosylation of phenolic compounds by *Datura innoxia* suspension cultures. *Phytochemistry* 15:1225–1229.

TABATA, M., UMETANI, Y., OOYA, M., and TANAKA, S. 1988. Glucosylation of phenolic compounds by plant cell cultures. *Phytochemistry* 27(3):809–813.

TAKIURA, K., YAMAMOTO, M., MIYAJI, Y., TAKAI, H., HONDA, S., and YUKI, H. 1974. Studies of oligosaccharides. XV. Syntheses of hydroquinone glycosides of gentio oligosaccharides. *Chem. Pharmacol. Bull.* 22(10):2451–2454.

USHIYAMA, M., and FURUYA, T. 1989. Glucosylation of phenolic compounds by root culture of *Panax ginseng. Phytochemistry* 28(11):3009–3013.

YAMAHA, T., and CARDINI, C.E. 1960a. The biosynthesis of plant glycosides. I. Monoglucosides. *Biochim. Biophys. Acta* 86:127.

YAMAHA, T., and CARDINI, C.E. 1960b. The biosynthesis of plant glycosides. II. Gentiobiosides. *Biochim. Biophys. Acta* 86:133–137.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

IDENTIFICATION OF SEX PHEROMONE OF TOMATO PINWORM, *Keiferia lycopersicella* (WALS.)[1]

R.E. CHARLTON,[2,*] J.A. WYMAN,[3,5] J.R. McLAUGHLIN,[4] J.-W. DU,[2,6] and W.L. ROELOFS[2]

[2]*Department of Entomology,*
Cornell University
New York State Agricultural Experiment Station
Geneva, New York 14456

[3]*Department of Entomology*
University of California
Riverside, California 92521

[4]*Insect Attractants, Behavior, and Basic Biology Research Laboratory*
Agricultural Research Service, USDA
Gainesville, Florida 32604

(Received July 9, 1990; accepted September 6, 1990)

Abstract—A sex pheromone produced by female *Keiferia lycopersicella* (Walsingham) was isolated and identified as (*E*)-4-tridecenyl acetate, based on chemical analyses, electroantennogram assays, and field trapping in California and Florida. Males were captured equally well in traps baited with (*E*)-4-tridecenyl acetate alone or a variety of (*Z*)- and (*E*)-4-tridecenyl acetate blends, although the *Z* isomer was not detected in extracts of female glands.

Key Words—Sex pheromone, *Keiferia lycopersicella*, tomato pinworm, Lepidoptera, Gelechiidae, (*E*)-4-tridecenyl acetate.

INTRODUCTION

The tomato pinworm (TPW), *Keiferia lycopersicella* (Walsingham), is a major pest of tomatoes in North and Central America. TPW larvae are leaf rollers and also burrow into fruit, and as a result they are effectively sheltered from most

[*]To whom correspondence should be addressed.
[1]Lepidoptera: Gelechiidae.
[5]Present address: Department of Entomology, University of Wisconsin, Madison, Wisconsin 53706.
[6]Present address: Shanghai Institute of Entomology, Academia Sinica, Shanghai, P.R.C.

0098-0331/91/0100-0175$06.50/0

insecticides. There is thus considerable impetus to develop other control strategies, such as the use of the sex pheromone to directly suppress populations via mating disruption (Jiménez et al., 1988; Jenkins et al., 1990) or as a monitoring tool in IPM programs.

McLaughlin et al. (1979) were the first to demonstrate that female TPW emit a pheromone that can attract males in the laboratory and the field. We report here the isolation and identification of that pheromone as (*E*)-4-tridecenyl acetate and document its effectiveness as a trap lure for conspecific males in the field.

METHODS AND MATERIALS

Insects were shipped as pupae from Riverside, California, and Gainesville, Florida. The emerging adults were used directly for experiments or to establish a colony to provide additional adults. The insects were reared (Schuster and Burton, 1982) to fourth instar on tomato foliage in the greenhouse at 28 ± 4°C and then were transferred to environmental chambers held at 27 ± 1°C and on a 16L:8D photoperiod. The pupae were segregated by sex, and adults were separated daily.

Preparative glass GLC columns were packed with 3% OV-101 (methyl silicone) on 100–120 mesh Gas Chrom Q (2 m × 2 mm ID), and 10% XF-1150 (50% cyanoethyl methyl silicone) on 100–120 mesh Chromosorb W-AW-DMCS (3 m × 2 mm ID). The capillary columns used included a glass 7-m × 0.3-mm-ID Carbowax 20 M, and fused silica 30-m × 0.25-mm-ID Supelcowax 10 (Supelco) and 50-m × 0.25-mm-ID Silar 10C (Quadrex) columns.

Extracts were obtained by extruding and excising the ovipositors, including the pheromone gland, of 2- to 4-day-old females during early scotophase; glands were extracted in redistilled Skelly B (mainly hexanes) or *n*-heptane. The crude extract was concentrated using a nitrogen stream and an aliquot was injected onto the XF-1150 or OV-101 column. Fractions (generally 1 min) were collected from the columns using chilled 30-cm glass capillary tubes. Active fractions were identified by recording electroantennograms (EAGs) from excised antennae of 1- to 4-day-old males as described in Roelofs (1984). Antennae also were screened with a series of synthetic positional isomers of 12-, 13-, and 14-carbon *Z* and *E* monounsaturated acetates and alcohols, using a 10-μg loading on filter paper for each compound.

The isolated compound was further characterized by microozonolysis and hydrolysis with ethanolic NaOH followed by acetylation with acetyl chloride (Bjostad et al., 1980), as well as by capillary GLC analysis, and GLC-mass spectrometry. Microozonolysis was carried out by dissolving samples in 50 μl of carbon disulfide in 3-mm × 3-cm glass tubes chilled in a Dry Ice/acetone

bath. A stream of ozone was bubbled through the solution for 20 sec. This solution then was analyzed immediately by capillary GLC, and the products compared with ozonized standards. A Hewlett-Packard 5880 gas chromatograph equipped with a splitless injector and a flame ionization detector was used for the GLC analyses. By chromatographing a series of synthetic tridecenyl acetate standards on polar and very polar capillary columns, we were able to separate and identify all of the geometric and positional isomers (Heath et al., 1980). Comparison of the retention time of the pheromone with those of the standards allowed confirmation of the position and geometry of its double bond. Chemical ionization mass spectra were obtained with a Hewlett-Packard 5985 mass spectrometer interfaced with a Supelcowax 10 capillary column with isobutane as the reagent gas. Electron impact mass spectra were obtained with a Hewlett-Packard 5970 series Mass Selective Detector coupled with a 30-m Supelcowax 10 capillary column using helium as the carrier.

The (*E*)-4-tridecenyl acetate (*E*4–13 : OAc), (*Z*)-4-tridecenyl acetate (*Z*4–13 : OAc), (*E*)-8-tridecenyl acetate (*E*8–13 : OAc) and (*E*, *E*)-4,7-tridecadienyl acetate (*E*4, *E*7–13 : OAc) used in field tests were purchased from Farchan Chemical Co. GLC analyses of these compounds on XF-1150 and OV-101 revealed that they were >99% pure with no detectable amounts of other positional or geometric isomers.

Trapping studies were conducted in tomato fields in California and Florida using Pherocon 1C (Zoecon Corp., Palo Alto, California) traps baited with rubber septa (rubber stoppers, sleeve type, 5 × 9 mm, A.H. Thomas Co.) loaded with test chemicals dissolved in dichloromethane or four caged virgin female moths. Traps were deployed in a randomized complete block design with treatments replicated three to five times and replicates separated by at least 50 m. Traps were hung on stakes at plant canopy height (0.3–1.8 m), and separated from one another by 3–15 m. Every 3–10 days, trapped males were counted and the traps were rerandomized within blocks. Data were transformed to $(X + 0.5)^{1/2}$ prior to analysis of variance and means were separated using the Ryan-Einot-Gabriel-Welsh multiple range test (SAS Institute, 1985) to control the experiment-wise type-I error rate (Day and Quinn, 1989).

RESULTS

Following fractionation of the crude extract on XF-1150 (160°C), the largest peak and most of the EAG activity was found in the 6- to 8-min fraction; by comparison, saturated 13 : OAc, and 14 : OAc standards eluted at 6.7 min and 9.4 min, respectively. On OV-101 (150°C), the highest activity was localized in the 5- to 6-min fraction; under the same conditions, 12 : OAc eluted at 4.3 min and 13 : OAc at 6.1 min. Thus, on polar and nonpolar columns, the

compound had retention times characteristic of an unsaturated 13-carbon acetate. Injection of this purified active fraction on the Carbowax 20 M capillary column revealed that only one major peak was present. Upon coinjection with a series of synthetic positional isomers of *Z* and *E* tridecenyl acetates on Supelcowax 10 and Silar 10C capillary columns, the retention time of the active peak was found to match that of synthetic *E*4–13 : OAc exactly.

Saponification and reacetylation of the active fraction verified that the compound was an acetate. When the active fraction was hydrolyzed and the reaction products were injected onto the OV-101 and XF-1150 columns, all fractions were inactive by EAG. On both columns the retention time of the major peak now conformed to that of a monounsaturated alcohol. Following collection of the alcohol peak from OV-101, the fraction was acetylated and injected onto the OV-101 and XF-1150 columns, and activity was restored in the original 5- to 6-min and 6- to 8-min fractions, respectively.

The Δ4 position of the double bond in the EAG-active compound was determined by microozonolysis. This procedure produced two peaks on the Supelcowax-10 capillary column whose retention times were identical to those of the 4-oxobutyl acetate and nonanal generated from ozonolysis of authentic *E*4–13 : OAc.

Isobutane CI mass spectra of the active compound established that its molecular weight was 240 with diagnostic ions at 241 (M + 1) and 181 (M + 1 − 60, indicating the loss of acetic acid) again consistent with our interpretation that the compound was a monounsaturated 13-carbon acetate. The EI spectrum was identical in all respects to that of synthetic *E*4–13 : OAc; the parent ion (240) was not observed in the EI spectrum (Figure 1A).

Standardized EAG responses from the synthetic compound series showed that the highest depolarizations were elicited by the monounsaturated 13-carbon acetates, followed by the 12-carbon and the 14-carbon acetates. The alcohols were mostly inactive. Within the 13-carbon acetate series, the best antennal responses were obtained for *E*4–13 : OAc; *E*8–13 : OAc gave only slightly less activity, with *Z*4, *Z*5, and *E*7 compounds also giving good responses.

Despite the high EAG respones obtained from the latter four compounds, capillary GLC chromatograms of gland rinses showed only one major peak corresponding to *E*4–13 : OAc with no evidence (detection limits > 0.1% of *E*4–13 : OAc) of *Z*4–13 : OAc or other monounsaturated acetates (Figure 1B). The mean titer of *E*4–13 : OAc in females whose glands were extracted at lights-off on the day of emergence (age 2–24 hr) was 10.1 ng/female (based on two samples of 10 and 20 pooled glands).

The first field test compared male captures in response to different loadings of *E*4–13 : OAc, as well as to the highly EAG-active *E*8–13 : OAc alone or in combination with *E*4–13 : OAc. Also tested was another compound, *E*4, *E*7–13 : OAc, which was discovered to be an attractant for TPW males during the

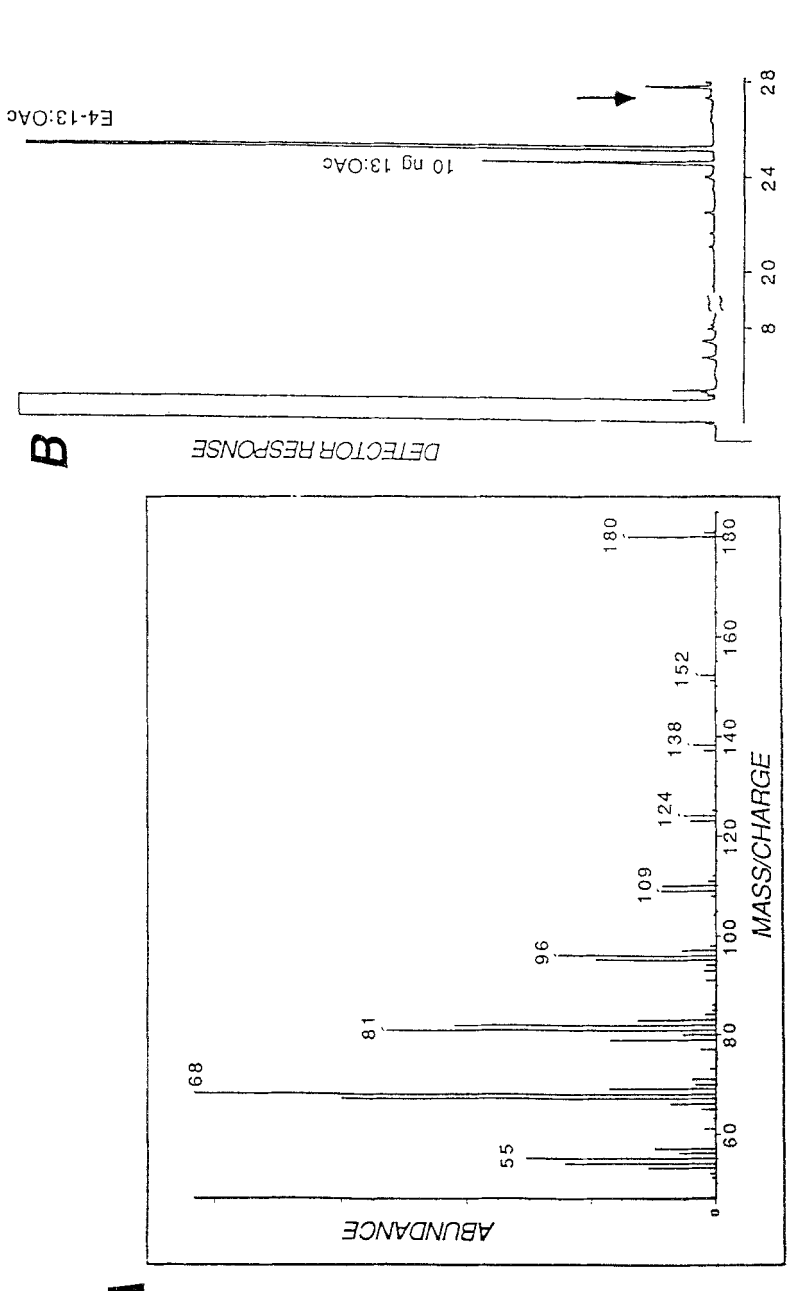


FIG. 1. (A) Electron impact mass spectrum of (*E*)-4-tridecenyl acetate from female gland extract. (B) Chromatogram of *K. lycopersicella* pheromone gland extract (pooled sample of 10 glands) with 10 ng 13:OAc added as internal standard. Arrow indicates retention time of tetradecanyl acetate. All monounsaturated acetates eluted between 13:OAc and 14:OAc under these conditions (30-m × 0.25-mm-ID Supelcowax 10; Temperature program: 80°C (2 min), then 4°C/min to 220°C (10 min).

course of field screening tests for *Phthorimaea operculella* (Zeller) attractants (G. Kennedy, personal communication). The 100- and 300-μg doses of *E*4–13:OAc captured the highest numbers of males but the 1-mg dose captured significantly fewer males (Table 1). These lures retained their potency over the entire 46 days they were deployed in the field. The 100-μg loading generally caught the highest number of males, but the relative effectiveness of the 300-μg and 1-mg dosages appeared to increase as the lures aged, probably due to declining emission rates.

*E*8–13:OAc and *E*4,*E*7–13:OAc by themselves were ineffective as trap lures (Table 1). When varying amounts of *E*4,*E*7–13:OAc were added to *E*4–13:OAc, it significantly depressed trap catch in a dose-dependent manner over the test as a whole and caused a reduction in captures during all but the last trapping interval (36–46 days); nevertheless, these traps still captured significantly more males than unbaited traps (Table 1). When *E*8–13:OAc was added to *E*4–13:OAc, the results were more erratic: total trap catch was significantly lower for both loadings, but during several of the trapping intervals, the low

TABLE 1. TOMATO PINWORM MALES CAUGHT IN PHEROCON 1C TRAPS BAITED WITH (*E*)-4-TRIDECENYL ACETATE AND OTHER COMPOUNDS AT CHULA VISTA AND IRVINE, CALIFORNIA, 1976[a]

Trap lure	Moths/trap/night during trapping interval (lure age)				Mean (mean total capture/trap)	
	8/26–9/6 (0–12)	9/7–9/19 (12–25)	9/20–9/30 (25–36)	10/1–10/10 (36–46)		
100 μg *E*4–13:OAc	13.9[a]	18.1[a]	19.5[a]	29.1[a]	19.7[a]	(907.6)
300 μg *E*4–13:OAc	9.0[ab]	14.6[ab]	18.0[a]	31.8[a]	17.7[ab]	(812.8)
1 mg *E*4–13:OAc	3.9[c]	4.5[d]	7.9[b]	29.4[a]	10.6[c]	(486.8)
300 μg *E*4,*E*7–13:OAc	0.2[d]	0.3[e]	0.1[d]	0.4[c]	0.3[e]	(11.8)
300 μg *E*8–13:OAc	0.9[d]	1.9[e]	1.4[c]	9.1[b]	3.1[d]	(141.4)
300 μg *E*4–13:OAc + 45 μg *E*8–13:OAc	9.8[a]	9.9[bc]	17.9[a]	29.5[a]	15.9[b]	(732.2)
300 μg *E*4–13:OAc + 300 μg *E*8–13:OAc	4.4[bc]	7.9[cd]	11.5[b]	24.5[a]	11.5[c]	(527.4)
300 μg *E*4–13:OAc + 15 μg *E*4,*E*7–13:OAc	4.6[bc]	7.2[cd]	8.6[b]	32.9[a]	12.4[c]	(571.6)
300 μg *E*4–13:OAc + 45 μg *E*4,*E*7–13:OAc	1.1[d]	0.8[e]	1.2[c]	18.0[b]	2.9[d]	(135.0)
Unbaited	0.5[d]	0.3[e]	0.2[d]	0.6[c]	0.4[e]	(16.4)

[a] Five replicates per treatment. Means in a column followed by the same letter are not significantly different according to Ryan-Einot-Gabriel-Welsh multiple range test ($P \geq 0.05$). All treatments dispensed from rubber septa except 1 mg loading of *E*4–13:OAc, which was released from polyethylene caps.

dosage captured as many males as *E*4–13:OAc alone (Table 1). The lack of captures in the *E*4,*E*7–13:OAc-baited traps was inconsistent with the results from the *P. operculella* test; possibly, the baits used in the earlier study were contaminated with small amounts of *E*4–13:OAc.

The second set of trapping experiments compared different ratios of *Z*- and *E*4–13:OAc. In both California and Florida, TPW males responded equally well to a wide range of *Z*:*E* ratios with optimal catch occuring to the 3% *Z* blend (Table 2). *Z*4–13:OAc alone captured significantly fewer males than any of the *E*:*Z* blends. Nonetheless, this compound had some intrinsic activity since it captured more males than did unbaited traps. In California, the best synthetic baits caught as many males as did virgin females (Table 2); females were not included in the results of the Florida test due to insecticide-induced mortality.

DISCUSSION

The evidence presented here indicates that the sex pheromone of the tomato pinworm is (*E*)-4-tridecenyl acetate. This compound apparently is unique among identified lepidopteran sex pheromones (Arn et al., 1986). Only a few moth species have been found to use 13-carbon acetates as pheromones (Baker et al., 1985) or attractants (Voerman and Heerebout, 1978; Willemse et al., 1987). Furthermore, pheromone components with unsaturation in the 4-position have

TABLE 2. TOMATO PINWORM MALES CAPTURED [MEAN/TRAP (SE)] IN TRAPS BAITED WITH VARIOUS BLENDS OF (*E*)- AND (*Z*)-4-TRIDECENYL ACETATE IN FLORIDA AND CALIFORNIA TOMATO FIELDS[a]

Trap lure	Homstead, Florida 6/3–6/21, 1977	Chula Vista, Irvine, California 11/2–11/12, 1976
100 μg *E*4–13:OAc	69.0[a] (4.9)	245.5[a] (104.0)
+3 μg *Z*4–13:OAc	85.0[a] (9.9)	293.5[a] (66.8)
+ 10 μg *Z*4–13:OAc	62.3[a] (7.3)	185.3[a] (50.7)
+ 30 μg *Z*4–13:OAc	67.0[a] (4.2)	216.3[a] (95.5)
+ 100 μg *Z*4–13:OAc	75.3[a] (4.1)	205.0[a] (80.4)
100 μg *Z*4–13:OAc	19.0[b] (5.8)	87.5[b] (30.3)
30 μg *E*4–13:OAc + 100 μg *Z*4–13:OAc	49.7[a] (11.8)	228.5[a] (49.3)
Unbaited	1.7[c] (0.3)	3.3[c] (1.3)
Virgin females		231.3[a] (75.9)

[a] Three and four replicates for Florida and California tests, respectively. Means in a column followed by the same letter are not significantly different according to Ryan-Einot-Gabriel-Welsh multiple range test ($P \geq 0.05$).

been encountered from only a handful of species belonging to three lepidopteran families: Saturniidae (Bestmann, 1987), Gracillariidae (Beevor et al., 1986), and Gelechiidae. In the latter family, the only other identified Δ4 13-carbon acetate pheromone is the blend of di- and triunsaturated acetates, (*E*, *Z*)-4,7-tridecadienyl acetate and (*E*, *Z*, *Z*)-4,7,10-tridecatrienyl acetate, which are used by *P. operculella* (Roelofs et al., 1975; Persoons et al., 1976). Willemse et al. (1987) found that (*Z*)-4-tridecenyl acetate was an attractant for males of another gelechiid, *Sophronia semicostella* (Hübner), but it is not known whether this compound is actually produced by the females. The pheromones of almost all other gelechiids are even-carbon-numbered chain acetates ranging from 10 to 16 carbons in length with one or two double bonds at odd-numbered carbon positions.

Our results show that 100 μg of pure *E*4–13 : OAc is an effective trap lure for monitoring *K. lycopersicella* populations. Curiously, even though *Z*4–13 : OAc was not found in extracts of female glands, traps baited with a broad range of *Z*4/*E*4–13 : OAc blends captured as many males as those baited with pure *E*4–13 : OAc. The *Z*4–13 : OAc is clearly not a behavioral antagonist, and therefore it should not be necessary to use geometrically pure *E*4–13 : OAc in field tests. In fact, a 96 : 4 *E* : *Z* mixture currently is generally used for monitoring traps and disruptant formulations (Jenkins et al., 1990). Optimal trap catch was obtained with pheromone dosages of 100 or 300 μg whereas 1 mg inhibited male attraction. These results are in agreement with those reported by Wyman (1979), who found that hollow-fiber dispensers emitting pheromone at lower rates captured significantly more males than did ones releasing high amounts. The TPW pheromone now is used widely to monitor infestations of this pest and to time insecticide treatment programs (Van Steenwyk et al., 1983). Moreover, it has been registered by the EPA, and commercial controlled-release formulations of the pheromone already provide effective control of TPW populations through mating disruption (Jiménez et al., 1988; Jenkins et al., 1990).

Acknowledgments—We thank Kathy Poole, Marlene Campbell, Frances Wadhams, and Robert Rutter for maintaining the insect cultures, and Henry Nakakahara and Anthony Antonio for assistance with field experiments.

REFERENCES

ARN, H., TOTH, M., and PRIESNER, E. 1986. List of Sex Pheromones of Lepidoptera and Related Attractants. International Organization for Biological Control, Paris.

BAKER, R., HERBERT, R.H., NEEQUAYE, N.N., and RAO, K.N. 1985. Sex pheromone of tobacco stem borer, *Scrobipalpa heliopa* (Lower) (Lepidoptera: Gelechiidae). *J. Chem. Ecol.* 11:989–998.

BEEVOR, P.S., CORK, A., HALL, D.R., NESBITT, B.F., DAY, R.K., and MUMFORD, J.D. 1986. Components of female sex pheromone of cocoa pod borer moth, *Conopomorpha cramerella*. *J. Chem. Ecol.* 12:1–23.

BESTMANN, H.J, ATTYGALE, A.B., BROSCHE, T., ERLER, J., PLATZ, H., SCHWARZ, J., VOSTROWSKY, O., WU, C.-H., KAISSLING, K.E., and CHEN, T.-M. 1987. Identification of three sex pheromone components of the female saturniid moth *Antheraea pernyi* (Lepidoptera: Saturniidae). *Z. Naturforsch.* 42C:631–636.

BJOSTAD, L.B., TASCHENBERG, E.F., and ROELOFS, W.L. 1980. Sex pheromone of the chokecherry leafroller moth, *Sparganothis directana*. *J. Chem. Ecol.* 6:487–498.

DAY, R.W., and QUINN, G.P. 1989. Comparison of treatments after an analysis of variance in ecology. *Ecol. Monogr.* 59:433–463.

HEATH, R.R., BURNSED, G.E., TUMLINSON, J.H., and DOOLITTLE, R.E. 1980. Separation of a series of positional and geometrical isomers of olefinic aliphatic primary alcohols and acetates by capillary gas chromatography. *J. Chromatogr.* 189:199–208.

JENKINS, J.W., DOANE, C.C., SCHUSTER, D.J., MCLUGHLIN, J.R., and JIMÉNEZ, M. 1990. Development and commercial application of sex pheromone for control of the tomato pinworm. pp. 269–280, *in* R.L. Ridgeway, R. M. Silverstein, and M.N. Inscoe (eds.). Behavior-Modifying Chemicals for Insect Management: Applications of Pheromones and Other Attractants. Marcel Dekker, New York.

JIMÉNEZ, M.J., TOSCANO, N.C., FLAHERTY, D.L., ILIC, P., ZALOM, F.G., and KIDO, K. 1988. Controlling pinworm by mating disruption. *Calif. Agric.* 42(6):10–12.

MCLAUGHLIN, J.R., ANTONIO, A.Q., POE, S.L., and MINNICK, D.R. 1979. Sex pheromone biology of the adult tomato pinworm, *Keiferia lycopersicella* (Walsingham). *Fla. Entomol.* 62:34–41.

PERSOON, C.J., VOERMAN, S., VERWIEL, P.E.J., RITTER, F.J., NOOYEN, W.J., and MINKS, A.K. 1976. Sex pheromone of the potato tuberworm moth, *Phthorimaea operculella*: Isolation, identification, and field evaluation. *Entomol. Exp. Appl.* 20:289–300.

ROELOFS, W.L. 1984. Electroantennogram assays: rapid and convenient screening procedures for pheromones. pp. 131–159, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, New York.

ROELOFS, W.L., KOCHANSKY, J.P., CARDE, R.T., KENNEDY, G.G., HENRICK, C.A., LABOVITZ, J.N., and CORBIN, V.L. 1975. Sex pheromone of the potato tuberworm moth, *Phthorimaea operculella*. *Life Sci.* 17:699–706.

SAS INSTITUTE 1985. SAS Users Guide: Statistics. Version 5 (ed.). SAS Institute, Cary, North Carolina.

SCHUSTER, D.J., and BURTON, R.L. 1982. Rearing the tomato pinworm (Lepidoptera: Gelichiidae) in the laboratory. *J. Ecol. Entomol.* 75:1164–1165.

VAN STEENWYK, R.A., OATMAN, E.R., and WYMAN, J.A. 1983. Density treatment level for tomato pinworm (Lepidoptera: Gelechiidae) based on pheromone trap catches. *J. Econ. Entomol.* 76:440–445.

VOERMAN, S., and HERREBOUT, W.M. 1978. A sex attractant for the leaf miner moth *Lithocolletis corylifoliella* and its influence on that of *L. blancardella* (Lep., Gracillariidae). *Entomol. Exp. Appl.* 23:96–98.

WILLEMSE, L.P.M., BOOIJ, C.J.H., and VOERMAN, S. 1987. New sex attractants for male Lepidoptera (Coleophoridae, Gelichiidae, Momphidae, Oecophoridae, and Yponomeutidae) found by field screeing in the Netherlands. *J. Appl. Entomol.* 103:508–515.

WYMAN, J.A. 1979. Effect of trap design and sex attractant release rates on tomato pinworm catches. *J. Econ. Entomol.* 72:865–868.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

FEEDING-STIMULANT ACTIVITY OF ALGAL GLYCEROLIPIDS FOR MARINE HERBIVOROUS GASTROPODS[1]

K. SAKATA,[2,*] K. KATO,[3] Y. IWASE,[3] H. OKADA,[3] K. INA,[3] and Y. MACHIGUCHI[4]

[2]*Research Laboratory of Marine Biological Science*
Faculty of Agriculture, Shizuoka University
Mochimune, Shizuoka 421-01, Japan

[3]*Department of Agricultural Chemistry*
Shizuoka University, 836 Ohya
Shizuoka 422, Japan

[4]*The Hokkaido Regional Fisheries Research Laboratory*
Katsurakoi, Kushiro 085, Hokkaido, Japan

(Received June 28, 1990; accepted September 10, 1990)

Abstract—Phagostimulant activity of glycerolipids such as digalactosyldiacylglycerol (DGDG), 6-sulfoquinovosyldiacylglycerol (SQDG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), etc., have been examined using the Avicel plate method for three kinds of marine herbivorous gastropods, the abalone *Haliotis discus*, the turban shell *Turbo cornutus*, and the topshell *Omphalius pfeifferi*. DGDG showed strong activity for all the test animals. SQDG was much less active than the other glycerolipids for abalone. The turban shell and the top shell responded more or less sensitively to all tested glycerolipids at doses of 10–20 μg/sample zone.

Key Words—Feeding-stimulants, phagostimulants, herbivorous gastropods, abalone, *Haliotis discus, Turbo cornutus*, DGDG, PC, SQDG, glycerolipids.

INTRODUCTION

In the course of our chemical studies on marine herbivorous gastropods, we have established a simple and reliable bioassay, the Avicel plate method, as a feeding stimulant assay for abalone, *Haliotis discus* (Sakata et al., 1984), turban

[*] To whom correspondence should be addressed.

[1] Chemical Studies on Phagostimulants for Marine Gastropods. Part VIII. For Part VII, see Sakata et al. (1988a).

0098-0331/91/0100-0185$06.50/0

shell, *Turbo cornutus*, and top shell, *Omphalius pfeifferi* (Sakata et al., 1988a). Several glycerolipids such as digalactosyldiacylglycerol (DGDG), 1,2-diacylglyceryl-4′-*O*-(*N*,*N*,*N*-trimethyl)-homoserine (DGTH), 6-sulfoquinovosyldiacylglycerol (SQDG), phosphatidylcholine (PC), etc., have been isolated as potent feeding stimulants for marine herbivorous gastropods from algal methanol extracts (Sakata et al., 1984, 1986, 1988a, b; Sakata and Ina, 1983, 1985).

In a preliminary experiment (Sakata et al., 1985), abalone were found to respond to 10–20 μg of DGDG, PC, etc., in a sample zone on an Avicel plate, suggesting that these glycerolipids are responsible for feeding behavior.

In this paper we describe results of feeding-stimulant assays with five kinds of glycerolipids: DGDG, DGTH, and SQDG (from the green alga *Ulva pertusa*; Sakata et al., 1988b), and PC and phosphatidylethanolamine (PE) (from a soybean lecithin; Sakata et al., 1989). Feeding responses of three taxonomically closely related gastropod species, *Haliotis discus*, *Turbo cornutus*, and *Omphalius pfeifferi*, are compared. DGDG showed strong activity for all test animals. Abalone responded more sensitively and selectively to the tested glycerolipids except for PE than the other herbivorous gastropods.

METHODS AND MATERIALS

Test Aquarium. A test aquarium (36 × 35 × 15 cm) was made of a polyvinyl chloride plate (5 mm thick) colored gray and separated into three compartments (12 × 35 × 15 cm) (Figure 1). This aquarium was set in a preformed

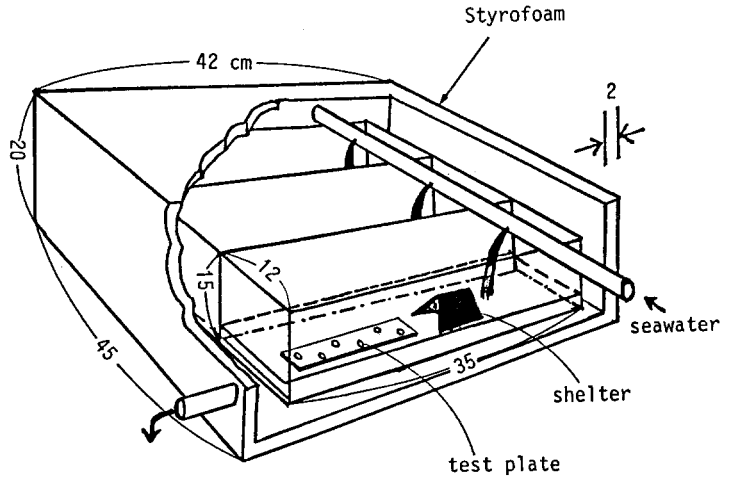


FIG. 1. A diagramatic view of an experimental aquarium.

box of polystyrol (Figure 1). Water temperature strongly influenced responses of test animals. At water temperature lower than 18°C, turban and top shells stopped feeding. Assay temperatures were maintained at 20–21°C, the optimum feeding temperature. Water depth was maintained at 5 cm with a drain. Seawater was circulated through a 2-mm-diameter hole in the water circulation tube at about 250 ml/min (Figure 1).

Test Animals. Abalone, *Haliotis discus* (1.5–2 years old), turban shell, *Turbo cornutus* (1.5 years old), and top shell, *Omphalius pfeifferi* (age unknown) were used. Abalone and turban shells were supplied by the Shizuoka and Toyama Prefecture Fish Farming Centers, respectively. Adult top shells were from Izu (Shizuoka Prefecture) and were purchased at a city market. Fifteen specimens of the abalone (shell length, 25–30 mm) and 20 specimens of the turban shell (opercula diameter, 10 mm) were kept in each compartment of the test aquarium. Turban shells used here were smaller than those used in earlier studies. The smaller-sized animals gave more reproducible results. Seven specimens of the top shell (opercula diameter, 6–8 mm) were also kept in each compartment. The air-dried brown alga, *Laminaria angustata* var. *longissima* ("nagakonbu" in Japanese) was fed test animals once every a few days as maintenance food.

Assay Method for Feeding-Stimulant Activity. This assay method is slightly modified from the previous one (Sakata and Ina, 1983; Sakata et al., 1984, 1988a). Test animals were starved for a day before the assay. Test glycerolipids were applied on the sample zones (23 mm diameter for abalone and top shell and 16 mm diameter for turban shell, whose area was half of the former, because the size of the turban shell was approximately half that of the other test animals) drawn on an Avicel plate. The Avicel plate (5 or 10 × 20 cm with 0.25 mm thickness) was made in the same way as previously reported (Sakata et al., 1984). Each glycerolipid solution was prepared with methanol at 1.0–4.0 mg/ml and contained an antioxidant, butylated hydroxytoluene (BHT, 2,6-di-*t*-butyl-4-methylphenol, ca. 3 μg/ml) to be able to apply each sample solution within 20 μl/sample zone. The test plates were set at the bottom of the test aquarium 2–3 hr after sunset. Circulation of seawater was maintained during the assay.

The next morning, test plates were scored for feeding activity. The assay was repeated at least 10 times at each dose for each sample. Typical assay results for the turban shell *T. cornutus*, and scoring of feeding activity are shown in Figure 2.

GLC Analysis of Fatty Acid Methyl Esters Obtained from Five Kinds of Glycerolipids. Each glycerolipid (2 mg) in methanol (2 ml) was extracted with 2 drops of methanolic N KOH at room temperature for 2 hr. The reaction mixture was treated with hexane (2 ml × 3). The hexane extract was subjected to GLC analysis, which was carried out on a Hitachi gas chromatograph model

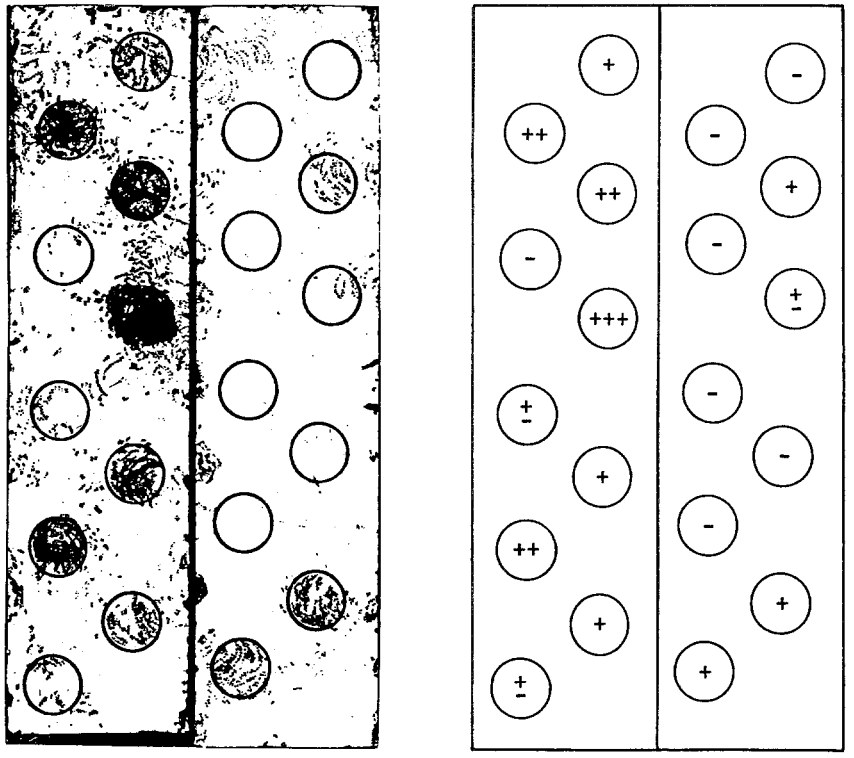


FIG. 2. Typical assay results for the turban shell, *Turbo cornutus* and judgment of the feeding activity. The feeding stimulant activity of each sample was judged in same way as previously reported (Sakata et al., 1984) for the abalone *Haliotis discus*. +++: Avicel in the sample zone and also around the sample zone has been bitten off. ++: almost all the Avicel in the sample zone has been bitten off. +: clear difference of traces are observed between inside the sample zone and outside. ±: a few more traces are observed inside the sample zone than outside. -: no or nearly the same number of traces are left inside the sample zone as those outside.

263-30 (FID) under the following conditions: PEG-HT packed column, ID 3 mm × 2 mm × 2 m; oven temperature: 210°C; carrier gas: N_2, 30 ml/min; injection and detection temperature: 240°C. Analytical data are shown in Table 1.

Recovery of Glycerolipids from Sample Zones. A pair of test plates was prepared with sample zones (3.3 cm in diameter) to which 200 μg of each glycerolipid was applied. One plate was set in an aquarium under the same conditions as in the bioassay, removed after 12 hr, and dried. Each sample zone was scraped off and extracted with methanol (500 μl). The other plate was loaded with the sample, and each zone extracted after evaporation of the sol-

vent. Benzyl alcohol (20 μg) was added to each methanol extract as an internal reference. The sample solution was analyzed by HPLC under the following conditions: column, Asahipak GC-310 porous polymer (MeOH, ID 7.6 × 500 mm); flow rate, 1 ml/min; detector, UV 210 nm. Analytical data are shown in Table 2.

TABLE 1. FATTY ACID COMPOSITION OF GLYCEROLIPIDS ISOLATED AS FEEDING STIMULANTS FOR MARINE HERBIVOROUS GASTROPODS

Fatty acid	DGDG[a]	DGTH[a]	SQDG[a]	PC[b]	PE[b]
$C_{16:0}$	27.5[c]	27.3	40.3	15.4	20.6
:1(9)		2.4	1.2		
:2(9,12)	4.3				
:3(8,11,14)	9.5				
:4(4,8,11,14)	5.6				
$C_{18:0}$					
:1(9)	5.0	13.2	17.1	17.0	10.5
:2(7,10)	15.1	7.2	3.5	62.3	62.8
:3(9,12,15)	32.0	10.2	37.9	5.3	6.1
:4(6,9,12,15)		18.5			
$C_{20:3(11,14,17)}$					
:4(8,11,14,17)		2.4			
:5(5,8,11,14,17)		2.1			
$C_{22:5(7,10,13,16,19)}$		11.8			
Unknown	1.0	4.9			

[a] Isolated from the green alga *Ulva pertusa*.
[b] Isolated from a soybean lecithin.
[c] Given as areal percentage of the peak of each fatty acid methyl ester on GLC.

TABLE 2. RECOVERY OF EACH GLYCEROLIPID FROM SAMPLE ZONE ON AN AVICEL PLATE

	DGDG[a]	DGTH[a]	SQDG[a]	PC[b]	PE[b]
Recovery A[c] (%)	87	94	100	96	90
Recovery B[c] (%)	77	87	98	70	80

[a] Isolated from *Ulva pertusa*.
[b] Isolated from a soybean lecithin.
[c] Recovery of each sample from a sample zone. A: just after application; B: after leaving in an aquarium for 12 hr.

RESULTS AND DISCUSSION

Fatty acids of the glycerolipids were mixtures of C_{16-22} and mainly C_{18} fatty acids (Table 3). Average molecular weight of each glycerolipid calculated from the GLC analysis data was as follows: DGDG, 914; DGTH, 757, SQDG, 843, PC, 774 and PE, 729, respectively. The molecular weight of SQDG was calculated as a sodium salt. Structures of these glycerolipids are shown in Figure 3, and the prominent differences were in polar groups. As shown in Table 2, recovery of each glycerolipid from each sample zone determined by HPLC was consistent and between 70 and 98%. Activity of each glycerolipid is shown in minimum sample amount (μg)/sample zone necessary to show + activity and to show ++ activity (definition, see Fig. 2) at a frequency of more than 80% in all trials.

Table 3 shows the activities of five glycerolipids (DGDG, DGTH, SQDG, PC, and PE) for the three closely related herbivorous gastropod species. DGDG showed strong activity for all test animals. A dose of 40 μg/sample zone, which was used as a positive control throughout this experiment, is enough to show strong activity at the surprisingly high reproducibility of 96.4% in 136 trials. This may suggest strong feeding attractant and phagostimulant activity of DGDG, because DGDG was desorbed from the Avicel plate slightly faster than the other glycerolipids tested (Table 2).

SQDG was much less active than the other glycerolipids for abalone. In the case of DGDG and DGTH, larger amounts of the compound are more potent than stimulants. SQDG showed no significant difference in the activity at doses

TABLE 3. FEEDING-STIMULANT ACTIVITY OF GLYCEROLIPIDS

	Haliotis discus		*Turbo cornutus*[a]		*Omphalius pfeifferi*	
Glycerolipids	+[b]	++[c]	+	++	+	++
DGDG[d]	5	30	10–5	30	10–5	30
DGTH[d]	10	30	20	80	20–10	50
SQDG[d]	50[e]	>300	20–10	40	10	50
PC[f]	5	40	10	50	20	80
PE[f]	>200	>200	20–10	50	20	40

[a] The area of a sample zone on the Avicel plate is half size of that used for the other test animals.
[b] Minimum sample amount (μg)/sample zone necessary to show + activity.
[c] The value expresses enough sample amount (μg)/sample zone to show ++ activity.
[d] Isolated from the green alga *Ulva pertusa*.
[e] With poor reproducibility.
[f] Isolated from a soybean lecithin.

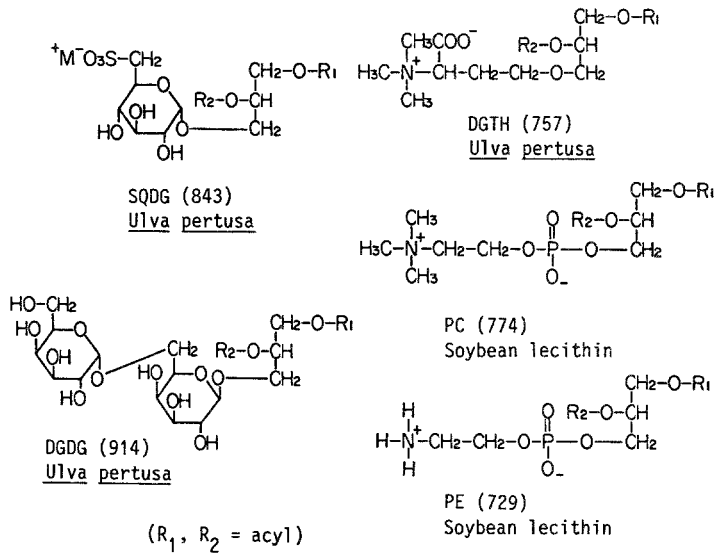


FIG. 3. Structures of feeding stimulants for marine herbivorous gastropods and their origin.

of 50, 100, and 300 μg/sample zone, respectively (Figure 4). Although the reason for this is not clear, this dose–response may suggest that SQDG causes saturation of its receptors at very low levels.

PE showed ++ activity at 50 and 40 μg for the turban shell and the top shell, respectively but had no activity for abalone even at a dose of 200 μg/zone, in spite of its structural similarity to PC which showed ++ activity at a dose of 40 μg for abalone.

Although these test animals belong to the Prosobranchia and are taxonomically closely related species, each glycerolipid showed different activity for each species.

For abalone, DGDG, DGTH, and PC showed ++ activity at doses of 40, 40, and 30 μg/sample zone, respectively, but PE showed no activity. SQDG showed dose-independent activity. Because abalone responded to the lowest doses, we considered that the chemical receptor of abalone is more selective and sensitive than that of the other test animals.

The turban shell and the top shell responded to all the tested glycerolipids, but the selectivity was not as sensitive as that of abalone.

Sea urchins also have been reported to show feeding preference to some particular species of algae (Vadas, 1977; Hay et al., 1986; Machiguchi, 1987). Recently we found that this unique bioassay method is also applicable to the sea urchin, *Strongylocentrotus intermedius* (''ezobafun'uni'' in Japanese), and

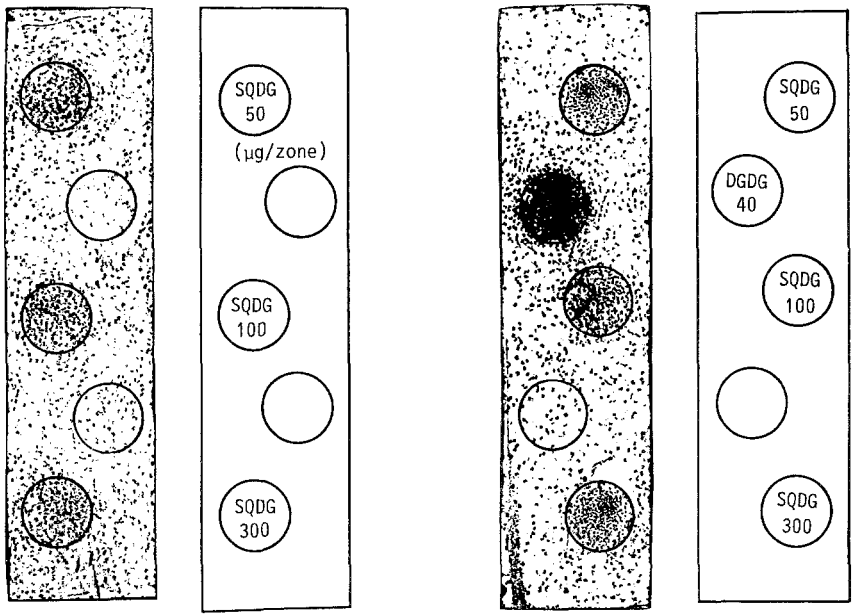


FIG. 4. Typical assay results of SQDG for the abalone, *Haliotis discus*.

glycerolipids such as PC, SQDG, etc., were effective feeding stimulants not only for the marine herbivorous gastropods but also for the sea urchin (Sakata et al., 1989).

Glycerolipids such as DGDG and PC are potent feeding stimulants for marine herbivorous gastropods and sea urchin. It is likely that glycerolipids occurring widely in the plant kingdom have evolved a role as feeding stimulants. Many biological studies, however, clearly show some algae are not preferred by these herbivores (Uki et al., 1986; Vadas, 1977; Hay et al. 1986). These observations suggest the presence of feeding inhibitors in those algae. Recently we have succeeded in establishing a bioassay method to screen algal feeding inhibitors (Sakata et al., 1990) by modifying the Avicel plate method. Application of this assay procedure to algal extracts resulted in isolation of feeding inhibitors and will be published elsewhere.

Acknowledgments—The authors wish to thank Shizuoka and Toyama Prefecture Fish Farming Centers for their generous gifts of the baby abalones and the young turban shells, respectively.

REFERENCES

HAY, M.E., LEE, R.R., and GUIEB, R.A. 1986. Food preference and chemotaxis in the sea urchin *Arbacia punctulata* (Lamarck) Philippi. *J. Exp. Mar. Biol. Ecol.* 96:147–153.

MACHIGUCHI, Y. 1987. Feeding behavior of sea urchin *Strongylocentrotus intermedius* observed in Y-shaped chamber. *Bull. Hokkaido Reg. Fish. Res. Lab.*, No. 51, pp. 33–37.

SAKATA, K., and INA, K. 1983. Digalactosyldiacylglycerols isolated from a brown alga as effective phagostimulants for a young abalone. *Agric. Biol. Chem.* 47:2957–2960.

SAKATA, K., and INA, K. 1985. Digalactosyldiacylglycerols and phosphatidylcholines isolated from a brown alga as effective phagostimulants for a young abalone. *Bull. Jpn. Soc. Sci. Fish.* 51:659–665.

SAKATA, K., ITOH, T., and INA, K. 1984. A new bioassay method for a young abalone, *Haliotis discus* Reeve. *Agric. Biol. Chem.* 48:425–429.

SAKATA, K., TSUGE, M., and INA, K. 1986. A simple bioassay for feeding-stimulants for the young seahare *Aplysia juliana. Mar. Biol.* 91:509–511.

SAKATA, K., SAKURA, T., KAMIYA, Y., and INA, K. 1988a. A simple feeding stimulant assay for marine herbivorous gastropods, the turban shell *Turbo cornutus* and the top shell *Omphalius pfeifferi. Nippon Suisan Gakkaishi* 54:1715–1718.

SAKATA, K., SAKURA, T., and INA, K. 1988b. Algal phagostimulants for marine herbivorous gastropods. *J. Chem. Ecol.* 14:1405–1416.

SAKATA, K., KATO, K., INA, K., and MACHIGUCHI, Y. 1989. Glycerolipids as potent feeding stimulants for the sea urchin, *Strongylocentrotus intermedius. Agric. Biol. Chem.* 53:1457–1459.

SAKATA, K., IWASE, Y., KATO, K., INA, K., and MACHIGUCHI, Y. 1990. A simple feeding inhibitor assay for marine herbivorous gastropods and the sea urchin *Strongylocentrotus intermedius* and its application to an unpalatable algal extract. *Nippon Suisan Gakkaishi.* In press.

UKI, N., SUGIURA, M., and WATANABE, T. 1986. Dietary value of seaweeds occurring on the pacific coast of Tohoku for growth of the abalone *Haliotis discus hannai. Bull. Jpn. Soc. Sci. Fish.* 52:257–266.

VADAS, R.L. 1977. Preferential feeding: An optimization strategy in sea urchins. *Ecol. Monogr.* 47:337–371.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

DUAL ROLE OF PYRROLIZIDINE ALKALOIDS IN NECTAR

ALAN R. MASTERS

Department of Zoology, University of Florida
Gainesville, Florida 32611

(Received July 16, 1990; accepted September 10, 1990)

Abstract—Pyrrolizidine alkaloids occur in several plant families, attracting ithomiine and danaine butterflies that specialize on the flowers. I show that pyrrolizidine alkaloids in artificial nectar also inhibit at least one butterfly, *Agraulis vanillae* (Nymphalidae: Heliconiinae), a more general forager. Inhibition was demonstrated in two ways: quantity of nectar consumed and number of artificial flowers visited. The amount of sucrose solution consumed by individual butterflies was measured using a microcapillary tube. Number of flower visits was determined using an array of artificial orange and yellow flowers. *A. vanillae* drank less sucrose solution with monocrotaline, a pyrrolizidine alkaloid, than without. When monocrotaline was placed into yellow flowers, *A. vanillae* learned to visit predominantly orange flowers. Evolutionarily, pyrrolizidine alkaloids in nectar may represent an adaptation to exclude butterflies. Ithomiines and danaines, seeking these compounds in larval food plants, were not excluded. Alternatively, ithomiines and danaines overcame the nectar defense. In either case, the plant effectively increased floral constancy by inhibiting generalist butterflies and attracting specialists.

Key Words—Pyrrolizidine alkaloids, pollination, nectar constituents, Ithomiinae, Danainae, Heliconiinae, Lepidoptera, Nymphalidae, agraulis vanillae.

INTRODUCTION

Secondary compounds are common in nectar (Baker and Baker, 1975). Whether their placement is incidental (Mothes, 1966) or naturally selected (Fraenkel, 1959; McKey, 1974), their presence in nectar may currently enhance plant fitness. Some nonsugar nectar constituents inhibit nectar consumption and flower visits by nectar robbers or poor pollinators (Janzen, 1979; Rhoades and Bergdahl, 1981; Stephenson, 1981, 1982). Moreover, secondary compounds in nec-

0098-0331/91/0100-0195$06.50/0

tar could enhance fitness by attracting more efficient pollinators (Baker and Baker, 1982).

Pyrrolizidine alkaloids are secondary compounds found in the Compositae, Boraginaceae, Fabaceae, and Apocynaceae (Bull et al., 1968; Mattocks, 1986). Ithomiine (Nymphalidae: Ithomiinae) and danaine (Nymphalidae: Danainae) butterflies are attracted to pyrrolizidine alkaloids (Masters, 1968; Pliske, 1976). These butterflies collect such alkaloids for mate attraction and defense (Edgar, 1982; Vasconcellos-Neto and Lewinsohn, 1984; Brown, 1984, 1985; Boppré 1978, 1986). Pyrrolizidine alkaloids may be acquired from dried plant parts, plant wounds (Edgar and Culvenor, 1975; Boppré, 1983), and floral nectar (Pliske, 1975a; Masters, 1990). While consuming nectar, ithomiines and danaines contact anthers and carry pollen to conspecifics (Pliske, 1975b).

Plants increase fitness when pollinators confine their visits to flowers of fewer species (Heinrich, 1976). Receipt or delivery of foreign pollen provides no benefit for the plant but incurs nectar and pollen costs (Rhoades and Bergdahl, 1981). Pyrrolizidine alkaloids increase flower constancy by attracting lepidopterans that seek flowers with these compounds in their nectars (Pliske, 1975b). Pyrrolizidine alkaloids may enhance flower constancy further by inhibiting other, more generalist visitors.

Here, I show that monocrotaline, a pyrrolizidine alkaloid, inhibits (1) amount of nectar consumed and (2) number of visits to artificial flowers by a generalist butterfly forager, *Agraulis vanillae* (Nymphalidae: Heliconiinae). In examining these two forms of inhibition, I provide the first evidence that a secondary nectar constituent attractive to some flower visitors plays a dual role of inhibiting another.

METHODS AND MATERIALS

Agraulis vanillae occurs from the United States (Opler and Krizek, 1984) to South America and the West Indies (De Vries, 1987). It is common in Gainesville, Florida, from June to November where it inhabits open areas, using *Passiflora incarnata* (Passifloraceae) as its principal larval food plant (May, 1986; Gannon, 1986). *A. vanillae* feeds on nectar from a variety of flowers, including "bee flowers" (Baker and Baker, 1975) such as shepard's needle *Bidens alba*, (Asteraceae) (May, 1986). *A. vanillae* is palatable to blue jays, *Cyanocitta cristata)*, silverbeak tanagers, *Rhamphocelus carbo magnirostratus*, and parson's tanagers, *Tachyphonus rufus* (Brower and Brower, 1964; Brower, 1984), and to the spider, *Nephila clavipes* (personal observation).

Two experiments tested the influence of monocrotaline (99%, Aldrich Chemical Co., 1984), a pyrrolizidine alkaloid isolated from *Crotalaria* sp. (Fabaceae) (Bull et al., 1968; Mattocks, 1986), on the foraging behavior of *A.*

vanillae. Both experiments were performed in autumn 1984 and summer 1985 in Gainesville, Florida.

Experiment 1. Effect of Monocrotaline on Consumption of Artificial Nectar by A. vanillae. Nectaries generally contain greater than 0.1 μg alkaloid/μg plant tissue (Brown, 1984); however, concentrations of pyrrolizidine alkaloids in natural nectars have not been reported. Consequently, a logarithmic scale of monocrotaline concentration from 0.001 μg/ml to 1.0 μg/ml was used in an attempt to cover ecologically relevant nectar concentrations.

Twenty *A. vanillae* males, captured as adults, were fed 30% sucrose solution (by weight) to satiation on both the day of capture and two days later. Four, six, eight, and 10 days after capture, *A. vanillae* were fed 30% sucrose solution and one concentration of monocrotaline in 30% sucrose solution. Individuals were not fed on days 1, 3, 5, 7, 9, and 11. Feedings were ended on day 12 with a pure sucrose solution.

Two groups were created to separate effects of presentation order and concentration. A group of 10 individuals were offered an increasing series of 0.001, 0.01, 0.1, and 1.0 μg/μl monocrotaline. The remaining individuals were offered monocrotaline in decreasing concentrations.

A calibrated 100-μl microcapillary tube was used to measure nectar consumption by *A. vanillae* (May, 1985). Butterflies remained in a 30 × 30 × 30-cm styrofoam chamber with a Plexiglas front and maintained at 28°C using a heat lamp. All feedings took place between 1000 and 1400 hr. The proboscis was inserted into a capillary tube containing 30% sucrose solution with or without 1 μg/μl monocrotaline. This initiated drinking without restraint. To assure satiation, the proboscis was reinserted once voluntarily removed. After exposure to monocrotaline, butterflies were offered 30% sucrose solution to satiation. Butterflies were held in glassine envelopes at 23°C between feedings with 12 hr light and 12 hr dark.

Experiment 2. Discrimination and Avoidance of Artificial Flowers with Monocrotaline. To determine if plants with pyrrolizidine alkaloid nectars inhibit visits by *A. vanillae*, flower preference experiments were performed, using artificial flowers in two flight cages (2.5 × 2.5 × 4 m) receiving direct sunlight.

"Flowers" were 1.8-cm-diameter disks cut from painted sheets of 0.5-cm-thick pressed foam mounting board. Into each disk was inserted a 1-cm section of hollow glass pipe, 0.3 cm diameter, so that an open end was flush with the painted surface of the flower. The other end of the glass tube was sealed with silicon aquarium sealant. Artificial flowers were arrayed on a base of 60 × 60 cm plywood atop 49 evenly spaced dowels 15 cm high and 0.8 cm wide.

Results from experiment 1 suggested that prior experience with monocrotaline enhanced the inhibitory effect of monocrotaline on subsequent nectar consumption by *A. vanillae* (see Results). Therefore, flower choice tests were begun by feeding freshly emerged male *A. vanillae* from microcapillary tubes: 5 μl of

30% sucrose solution on day 1, and 30% sucrose solution + 0.001 μg/μl monocrotaline on day 2, the concentration of monocrotaline being the lowest showing a negative effect on nectar consumption from Experiment 1.

Individuals were released to empty flight cages for two days. *A. vanillae* were individually exposed to 24 yellow and 24 orange flowers arranged randomly by color (the center dowel remained flowerless). All flowers of a given color contained 0.5 μl of the same artificial nectar type and were refilled after nectar and had been removed. Refilling did not seem to inhibit further butterfly foraging in any way.

Each foraging bout began by placing *A. vanillae* on an orange flower and inserting its proboscis into the well until the butterfly began to drink. Once released, the butterfly would continue drinking and then either fly up to the top of the cage or would continue foraging by flying to and feeding on different flowers in the array. An initial foraging bout of 15 flowers (during which time the butterfly continuously visited flowers) was recorded for each individual, and then a subsequent foraging bout of 15 flowers. Fifteen consecutive flowers were observed because the frequency of continuous foraging on 16 or more flowers was rarely observed. Two foraging bouts per individual were followed because preliminary trials suggested the possibility that learning may be important. Between 30 and 60 min elapsed between the initial foraging bout and the subsequent one.

To detect a possible innate color preference, 15 *A. vanillae* foraged on yellow and orange flowers when both contained 30% sucrose solution. Such a "control" group allowed comparison of *A. vanillae* behavior in the absence or presence of alkaloids. To determine if pyrrolizidine alkaloids inhibit flower visits, a "treatment" group of 15 different *A. vanillae* were exposed to yellow flowers containing 0.5 μl of 0.001 μg/μl monocrotaline in 30% sucrose nectar and orange flowers with sucrose solution.

RESULTS

Monocrotaline is not immediately toxic to *A. vanillae*. *A. vanillae* showed no adverse effects while drinking monocrotaline or visiting flowers containing them. Instead, butterflies avoided exposure by recoiling the proboscis, flying from feeders, or quickly leaving artificial flowers containing monocrotaline. The death of one individual in experiment 1 occurred before exposure to monocrotaline.

Experiment 1. Monocrotaline Inhibits Nectar Uptake. Monocrotaline in sucrose solution inhibited drinking by *A. vanillae*. Figure 1a depicts increasing concentrations of monocrotaline: Friedman one-way ANOVA for seven related samples, six degrees of freedom, nine cases, chi-square corrected for ties =

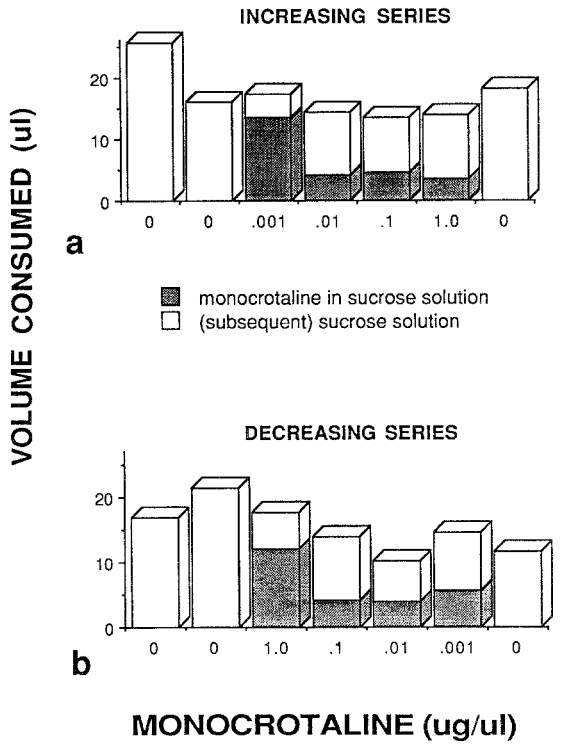


FIG. 1. (a and b) Mean amount of nectar taken for 10 wild-caught male *Agraulis vanillae* offered a diet of sucrose (0), followed by trials of sucrose solution with varying concentrations of monocrotaline ($\mu g/\mu l$), a pyrrolizidine alkaloid. Both increasing and decreasing concentrations show an initial decrease the first day of exposure to monocrotaline followed by a subsequent decrease upon the second exposure. Order was more important than concentration in determining amount taken (see text). Subsequent sucrose solution consumed showed that butterflies were still hungry, and consumed nearly equal volumes as those on days when monocrotaline was not offered.

36.156, $P < 0.001$; Figure 1b shows a decreasing series: Friedman one-way ANOVA for seven related samples, six degrees of freedom, 10 cases, chi-square corrected for ties = 39.497, $P < 0.001$. Sucrose uptake on the first two days and last day of presentation was variable but always exceeded 10 μl, in contrast to monocrotaline in sucrose solution, which never exceeded 10 μl after the first day of exposure (Figure 1). First exposure to monocrotaline inhibited nectar compared to the previous day (Wilcoxin matched-pairs sign ranks test, Figure 1a for increasing concentrations, $N = 9$, $P < 0.05$; Figure 1b for decreasing concentration $N = 10$, $P < 0.01$). However, the second day of monocrotaline exposure showed a further decrease in uptake rate, regardless of concentration.

Thus, there seems to be an effect of initial exposure that strengthens the degree of inhibition demonstrated later. There was a weak or no correlation between monocrotaline concentration and nectar consumption, however. (Simple parametric regression: increasing series $N = 63$, $r^2 = 0.154$, $P = 0.002$; decreasing series $N = 70$, $r^2 = 0.001$, $P = 0.8252$).

After rejecting the monocrotaline nectar twice, butterflies drank sucrose solution readily. The total amount of artificial nectar consumed per day (monocrotaline in sucrose solution + untainted sucrose solution offered immediately after) was the same or slightly less than days when only sucrose was offered (Figure 1a for total solution consumed in an increasing series, Friedman one-way ANOVA for seven related samples, six degrees of freedom, nine cases, chi-square corrected for ties = 13.751, $P = 0.0326$; Figure 1b for total solution consumed in a decreasing series, Friedman one-way ANOVA for seven related samples, six degrees of freedom, 10 cases, chi-square corrected for ties = 12.033, $P = 0.0612$).

Experiment 2. Monocrotaline Inhibits Flower Visits. When no monocrotaline was present, *A. vanillae* showed an innate preference for yellow flowers (Figure 2, open circles), choosing yellow over orange flowers 2.7 : 1 (based on totals, excluding the first visit when individuals were placed on an orange flower). The presence of monocrotaline in yellow flowers altered this preference, as the treatment group of *A. vanillae* preferred orange flowers, 6.5 : 1 over yellow flowers (Figure 2, closed circles; control and treatment groups are significantly different, Kolmogorov-Smirnov test on cumulative frequency distributions, $P < 0.05$).

The initial foraging bout of 15 flowers shows both control and treatment groups initially favor yellow flowers (Figure 3). However, the latter group gradually avoided yellow flowers, which contained monocrotaline (closed circles, Figure 3). This suggests multiple visits were required before *A. vanillae* learned to avoid flowers containing monocrotaline. Later, short-term memory allowed *A. vanillae* to avoid yellow flowers in subsequent foraging (closed circles, Figure 2).

DISCUSSION

Pollination efficiency may be enhanced by reducing pollen waste and foreign pollen loads associated with low flower constancy (Grant, 1950; Heinrich, 1976; Faegri and Van der Pijl, 1979). Floral constancy is enhanced in orchids (Orchidaceae) by secondary compounds that attract specialist bees (Faegri and Van der Pijl, 1979). Conversely, iridoid glycosides of *Catalpa speciosa* increase floral constancy by inhibiting nectar thieves but no legitimate pollinators (Stephenson, 1981, 1982). Pyrrolizidine alkaloids in nectar have been shown to be

FIG. 2. Artificial flower choice by a control (open circles) and treatment group (closed circles) of 15 freshly emerged male *A. vanillae* in a flight cage. Control butterflies were offered a random array of yellow and orange flowers, each with 0.5 μl sucrose solution. Treatment butterflies had a choice between orange flowers with sucrose solution and yellow flowers with a sucrose solution containing monocrotaline, a pyrrolizidine alkaloid. Proportion of yellow flowers corresponds to the percentage of 15 males that drank from a yellow flower. Males that did not visit a yellow flower chose to visit an orange flower instead. All males foraged 15 visits depicted here as a sequence of visits numbered 1 through 15. This bout was scored after an initial bout of 15 flower visits (Figure 3). Members of each group were first placed on an orange flower, shown here with a flower character.

butterfly attractants (Pliske, 1975a; Boppré, 1986), suggesting the ecological significance of pyrrolizidine alkaloids in nectar may be, at least in part, more effective pollination. This study indicates that a second role of pyrrolizidine alkaloids in nectar is to further increase floral constancy by inhibiting more generalized butterfly foragers, like *A. vanillae*.

Nitrogen is generally limiting in plants (Robinson, 1968; Jones, 1972), and nectar, with its nitrogen-containing alkaloids, is likely to be carried away by foragers. Evolutionarily, the placement of pyrrolizidine alkaloids in nectar may be incidental (Mothes, 1966) or the result of active secretion (McKey, 1974), but its continued presence is either maladaptive or is maintained by some selective advantage.

Baker and Baker (1975) proposed that alkaloids are maintained in nectar to inhibit butterfly visitors. They examined a large number of plants and found that all species with nectar alkaloids were principally pollinated by bees. In addition, not one of 46 species visited regularly by lepidopterans contained

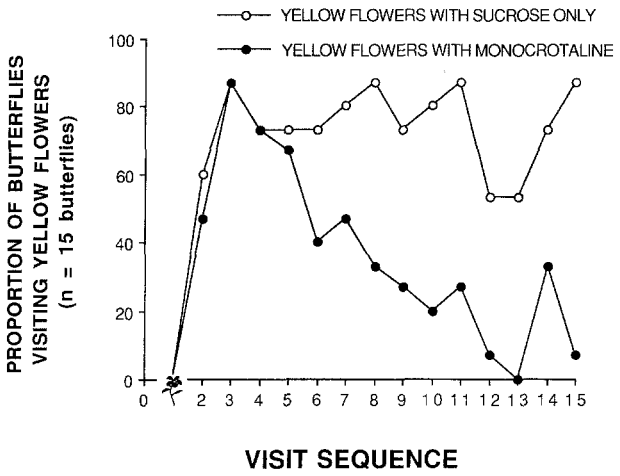


FIG. 3. Initial foraging bouts by the control and treatment groups of 15 freshly emerged *A. vanillae* males exposed to artificial flowers from Figure 2. Initial preferences for both groups was predominantly yellow flowers. However, butterflies exposed to monocrotaline in yellow flowers (closed circles) gradually stopped visiting such flowers, until orange flowers dominated their choices.

alkaloids. They suggested alkaloids in nectar inhibit butterflies without restricting pollination by bees, which tend to exhibit greater floral constancy than butterflies.

My findings support the hypothesis of the Bakers, showing that an alkaloid in artificial nectar inhibits at least one species of butterfly. Additional support for this hypothesis is not strong, at least in the case of pyrrolizidine alkaloids. Pyrrolizidine alkaloids have been found in honey (Denzier et al., 1977; Culvenor et al., 1981), and temperate *Senecio* spp. that contain pyrrolizidine alkaloids (Mattocks, 1986) are principally visited by bumblebees (*Bombus bifarius*) (Schmitt, 1980). However, Pliske (1975a, b) reports few or no visits by bees to flowers with pyrrolizidine alkaloids in the tropics. In addition, both bees and butterflies transfer pollen between *Senecio*, and while bees visit more flowers, butterflies are more likely to elicit outcrossing (Schmitt, 1980).

In the case of pyrrolizidine alkaloids, perhaps the Bakers' hypothesis should be extended to include specialist butterflies. The initial placement of pyrrolizidine alkaloids in floral nectar may have inhibited most butterflies, but not all. Ithomiines and danaines, already seeking pyrrolizidine alkaloids in larval food plants (Edgar, 1975, 1982, 1984; Drummond, 1981), may have readily specialized on such flowers. Alternatively, danaines and ithomiines may have broken through the defense and eventually sequestered the compounds for their own use (Ehrlich and Raven, 1964; Brower, 1969, 1970, 1984; Edgar, 1982;

Edgar et al., 1974). This may have further increased the plants' fitness if ithomiines and danaines became specialist foragers on such nectar at the same time.

The difference in proportion of bee visits between tropical and temperate plant species with pyrrolizidine alkaloids may underscore the role of a chemical that at once attracts and inhibits pollinators. In the temperate zone, where ithomiines never occur and danaines are represented by one species (*Danaus plexippus*), it is possible that bees are neither inhibited nor attracted, while most butterflies are inhibited (as the Bakers suggest). However, in the tropics, where ithomiines and danaines are more common, these specialist butterflies are attracted to flowers with pyrrolizidine alkaloids. This may increase the total number of visits per flower as butterflies that have fulfilled energetic needs continue to forage for chemicals (Boppré, 1986). Bees may choose to forage where competition for nectar is less intense. Conversely, their absence at pyrrolizidine alkaloid-containing flowers may be explained if their numbers are swamped by the increased visits by butterfiles. In the temperate zone, then, pyrrolizidine alkaloides serve to increase floral constancy only by inhibition of generalist butterflies. In the tropics, pyrrolizidine alkaloids also attract, further increasing the likelihood of pollination through increased flower visits.

Acknowledgments—The assistance of P.G. May with advice and use of materials is greatly appreciated. I thank also K.L. Masters, L.P. Brower, T.C. Emmel, A.A. Mejía, B.A. Drummond, and an anonymous reviewer for criticisms on the manuscript. This project was supported by the Department of Zoology, Univeristy of Florida.

REFERENCES

BAKER, H.G., and Baker, I. 1975. Studies of nectar-constitution and pollinator-plant coevolution, pp. 100–140, *in* L.E. Gilbert and P.H. Raven (eds.). Coevolution of Plants and Animals. University of Texas Press, Austin, Texas.

BAKER, H.G., and BAKER, I. 1982. Chemical constituent of nectar in relation to pollination mechanisms and phylogeny, pp. 131–171, *in* M.H. Nitecki (ed.). Biochemical Aspects of Evolutionary Biology. University of Chicago Press, Chicago, Illinois.

BOPPRÉ, M. 1978. Chemical communication, plant relationships, and mimicry in the evolution of danaid butterflies. *Entomol. Exp. Appl.* 24:264–277.

BOPPRÉ, M. 1983. Leaf-scratching—a specialized behaviour of danaine butterflies for gathering secondary plant substances. *Oecologia* 59:414–416.

BOPPRÉ, M. 1986. Insects pharmacophagously utilizing defensive plant chemicals (pyrrolizidine alkaloids). *Naturwissenschaften* 73:17–26.

BROWER, L.P. 1969. Ecological chemistry. *Sci. Am.* 220:22–29.

BROWER, L.P. 1970. Plant poisons in a terrestrial food chain and implications for mimicry theory. *Proc. Ann. Biol. Coll. Oregon State Univ.* 29:69–82.

BROWER, L.P. 1984. Chemical defense in butterflies, pp. 107–134, *in* R.I. Vane-Wright and P.R. Ackery (eds.). The Biology of Butterflies. Academic Press, London.

BROWER, L.P., and BROWER, J.V.Z. 1964. Birds, butterflies, and plant poisons: a study in ecological chemistry. *Zool. N.Y.* 49:137–159.

BROWN, K.S., Jr. 1984. Adult-obtained pyrrolizidine alkaloids defend ithomiine butterflies against a spider predator. *Nature* 309:707–709.

BROWN, K.S., Jr. 1985. Chemical ecology of dehydropyrrolizidine alkaloids in adult Ithomiinae (Lepidoptera, Nymphalidae). *Rev. Brasil. Biol.* 44:435–460.

BULL, L.B., CULVENOR, C.C.J., and DICK, A.T. 1968. The Pyrrolizidine Alkaloids. North-Holland, Amsterdam.

CULVENOR, C.C.J., EDGAR, J.A., and SMITH, L.W., 1981. Pyrrolizidine alkaloids in honey from *Echium plantagineum* L. *J. Agric. Food Chem.* 29:958–960.

DENZIER, M.L., THOMSON, P.A., BURGETT, D.M., and ISAACSON, D.J. 1977. Pyrrolizidine alkaloids: Their occurrence in honey from tansy ragwort (*Senecio jacobaea* L.). *Science* 195:497–499.

DE VRIES, P.J. 1987. The Butterflies of Costa Rica and Their Natural History: Papilionidae, Pieridae, Numphalidae. Princeton University Press, Princeton, New Jersey.

DRUMMOND, B.A. 1981. Ecological chemistry, animal behavior, and plant systematics. *Solanaceae Newsl.* 2:59–67.

EDGAR, J.A. 1975. Danainae (Lep.) and 1,2-dehydropyrrolizidine alkaloid-containing plants—with reference to observations made in the New Hebrides. *Phil. Trans. R. Soc. London Ser. B* 272:467–476.

EDGAR, J.A. 1982. Pyrrolizidine alkaloids sequestered by Solomon Island Danaine butterflies. The feeding preferences of the Danainae and Ithomiinae. *J. Zool. (London)* 196:385–399.

EDGAR, J.A. 1984. Parsonsieae: ancestral larval foodplants of the Danainae and Ithomiinae. *Sym. R. Entomol. Soc.* 11:91–93.

EDGAR, J.A., and CULVENOR, C.C.J. 1975. Pyrrolizidine alkaloids in *Parsonsia* species (family Apocynaceae) which attract danaid butterflies. *Experentia* 31:393–394.

EDGAR, J.A., CULVENOR, C.C.J., and PLISKE, T.E. 1974. Coevolution of danaid butterflies with host plants. *Nature* 250:646–648.

EHRLICH, P.R., and RAVEN, P.H. 1964. Butterflies and plants: A study in coevolution. *Evolution* 18:586–608.

FAEGRI, F., and VAN DER PIJL, I. 1979. The Principles of Pollination Ecology, 3rd ed. Pergamon Press, New York.

FRAENKEL, G.S. 1959. The raison d'etre of secondary plant substances. *Science* 129:1466–1470.

GANNON, A.T. 1986. Oviposition site selection by the gulf fritillary *Agraulis vanillae nigrior* (Michenor). Masters thesis. University of Florida, Gainesville, Florida.

GRANT, V. 1950. The flower constancy of bumblebees. *Bot. Rev.* 16:379–398.

HEINRICH, B. 1976. The role of energetics in bumblebee interrelationships, pp. 141–158, *in* L.E. Gilbert and P.H. Raven (eds.). Coevolution of Animals and Plants. University of Texas Press, Austin, Texas.

JANZEN, D.H. 1979. Why don't ants visit flowers? *Biotropica* 9:252.

JONES, D.A. 1972 Cyanogenic glycosides and their function, pp. 103–124, *in* J.B. Harborne (ed.). Phytochemical Ecology. Academic Press, New York.

MASTERS, A.R. 1990. Pyrrolizidine alkaloids in artificial nectar protect adult ithomiine butterflies from a spider predator. *Biotropica*. 22:298–304.

MASTERS, J.H. 1968. Collecting Ithomiidae with heliotrope. *J. Lepidop. Soc.* 22:108–109.

MATTOCKS, A.R. 1986. Chemistry and Toxicology of Pyrrolizidine Alkaloids. Academic Press, London.

MAY, P.G. 1985. A simple method for measuring nectar extraction rate in butterflies. *J. Lepidop. Soc.* 39:53–55.

MAY, P.G. 1986. Foraging selectivity in adult butterflies: Morphological, ecological, and physiological factors affecting flower choice. PhD dissertation. University of Florida, Gainesville, Florida.

McKey, D. 1974. Adaptive patterns in alkaloid physiology. *Am. Nat.* 108:305–320.

Mothes, K. 1966. Zur Problematik der metabolischen Exkretionen bei Pflanzen. *Naturwissenschaften* 53:317–323.

Opler, P.A., and Krizek, G.O. 1984. Butterflies East of the Great Plains. Johns Hopkins University Press, Baltimore, Maryland.

Pliske, T.E. 1975a. Attraction of Lepidoptera to plants containing pyrrolizidine alkaloids. *Environ. Entomol.* 4:455–473.

Pliske, T.E. 1975b. Pollination of pyrrolizidine alkaloid-containing plants by male Lepidoptera. *Environ. Entomol.* 4:474–479.

Pliske, T.E. 1976. The chemical basis of attraction of ithomiine butterflies to plants containing pyrrolizidine alkaloids. *J. Chem. Ecol.* 2:255–262.

Rhoades, D.F., and Bergdahl, J.C. 1981. Adaptive significance of toxic nectar. *Am. Nat.* 117:798–803.

Robinson, T. 1968. The Biochemistry of Alkaloids. Springer-Verlag, New York.

Schmitt, J. 1980. Pollinator foraging behavior and gene dispersal in *Senecio* (Compositae). *Evolution* 34:934–943.

Stephenson, A.G. 1981. Toxic nectar deters nectar thieves of *Catalpa speciosa*. *Am. Midl. Nat.* 105:381–383.

Stephenson, A.G. 1982. Iridoid glycosides in the nectar of *Catalpa speciosa* are unpalatable to nectar thieves. *J. Chem. Ecol.* 8:1025–1034.

Vasconcellos-Neto, J., and T.M. Lewinsohn. 1984. Discrimination and release of unpalatable butterflies by *Nephila clavipes*, a neotropical orb-weaving spider. *Ecol. Entomol.* 9:337–344.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

ADAPTIVE SIGNIFICANCE OF FURANOCOUMARIN DIVERSITY IN *Pastinaca sativa* (APIACEAE)

MAY R. BERENBAUM,[1,*] JAMES K. NITAO,[2] and ARTHUR R. ZANGERL[1]

[1]*Department of Entomology*
University of Illinois
320 Morrill Hall
505 S. Goodwin
Urbana, Illinois 61801

[2]*Department of Entomology*
243 Natural Sciences Building
Michigan State University
East Lansing, Michigan 48824

(Received March 26, 1990; accepted September 10, 1990)

Abstract—Fruits of *Pastinaca sativa* (Apiaceae), the edible parsnip, contain six different furanocoumarins that are differentially capable of ultraviolet-mediated cross-linkage of DNA and inhibition of DNA transcription. Individually, none of the other furanocoumarins present in parsnip seeds is as toxic as the photosensitizer xanthotoxin. Nevertheless, the natural mixture of compounds is toxicologically more effective against *Heliothis zea* (Lepidoptera: Noctuidae), both in the presence and absence of UV light, than is an equimolar amount of xanthotoxin. The difference in toxicity diminishes with increasing light levels. Thus, a series of structurally related natural products can display toxicity lacking in individual compounds and may represent an adaptive compromise to varying environmental conditions.

Key Words—*Heliothis zea*, corn earworm, Lepidoptera, Noctuidae, *Pastinaca sativa*, wild parsnip, Apiaceae, furanocoumarins, defense, xanthotoxin, photoactivation, bioassay.

INTRODUCTION

All plant species produce a variety of endogenous compounds. The abundance of chemical diversity is comprehensible in the context that compounds of different structures and modes of action can interact additively or synergistically

*To whom correspondence should be addressed.

0098-0331/91/0100-0207$06.50/0

(McKey, 1979; Berenbaum, 1985). Yet many plants display not only a diversity of biosynthetically unrelated chemical classes but also a diversity of compounds within a single chemical class. This within-class structural diversity of toxicologically similar compounds is puzzling in that such diversity necessitates the maintenance of biosynthetic pathways that may exact metabolic costs to the plant (McKey, 1979; Chew and Rodman, 1979). We therefore designed an experiment to determine whether a mixture of biosynthetically related secondary chemicals in a single plant species is toxicologically more effective against a generalized insect herbivore than is an equivalent amount of any single chemical of that structural type.

We chose as the focus for our study *Pastinaca sativa* (Apiaceae), the edible parsnip. This species contains a series of furanocoumarins, which are benz-2-pyrone derivatives with a furan ring at the 6,7 or 7,8 positions. Several of the furanocoumarins in parsnip leaves and seeds are phototoxic to a variety of organisms, including insects (Murray et al., 1982). Of the five furanocoumarins known to occur in the fruits of parsnip (Berenbaum et al., 1984), xanthotoxin is the most photoactive according to a number of criteria (Table 1). The linear furanocoumarins xanthotoxin and bergapten are capable in vitro of appreciable ultraviolet-catalyzed cross-linkage of DNA and subsequent interference with

TABLE 1. PHOTOACTIVITY OF NATURALLY OCCURRING FURANOCOUMARINS[a]

Furanocoumarin	DNA binding[b]	DNA cross-links[c]	Relative RNA[d]	Erythemal response[e]	Singlet oxygen[f]	Phototoxicity[h]
Psoralen (L)[g]	1.2	2.0	100	5	3.3–7.6	
Xanthotoxin (L)*	1.0	1.0	6	15	1.0	10
Bergapten (L)*	0.6	0.5–0.9	5	50	0.4–0.5	33
Angelicin (A)*	0.4	0.2	13		0	
Imperatorin (L)*						33
Isobergapten (A)				250		
Isopimpinellin (L)*				>2000		>100[i]

[a] * denotes furanocoumarins in fruits of *Pastinaca sativa*.
[b] Total photobinding of furanocoumarins to calf thymus DNA at 365 nm irradiation and normalized to xanthotoxin as unity (from Grossweiner, 1984).
[c] Cross-linking of calf thymus DNA by furanocoumarins at 365 nm irradiation normalized to xanthotoxin (from Grossweiner, 1984).
[d] Relative photoreactivity with rRNA normalized to psoralen (100) (from Musajo et al., 1974).
[e] Minimum topical dose to effect erythemal response in human skin, μg/6.45 cm^2 (from Pathak and Fitzpatrick, 1959).
[f] Singlet oxygen production normalized to xanthotoxin (from Grossweiner, 1984).
[g] L = linear furanocoumarin; A = angular furanocoumarin.
[h] Minimum subcutaneous injection (μg) required to elicit a severe photoreaction in 1- to 3-day-old chicks (from Ivie, 1978).
[i] Blistering observed at concentration of 100 μg.

transcription and replication. Angular furanocoumarins, such as sphondin and angelicin, are not known to form cross-links (although angelicin can form monoadducts and cause mutation and cell death; Ashwood-Smith et al., 1980); the remaining linear furanocoumarins are substantially less active in vitro as photosensitizers (Table 1).

METHODS AND MATERIALS

The furanocoumarin composition of ripe seeds of parsnip (Hollow Crown Improved; Stokes, Buffalo, New York) was quantified according to Berenbaum et al. (1984). The total furanocoumarin concentration in fruits was approximately 35.5% xanthotoxin, 31.8% imperatorin, 18.1% bergapten, 8.5% sphondin, 5.6% isopimpinellin, and 0.5% angelicin. Although reported as present in roots and buds, psoralen was not found in this fruit extract and was absent from fruits of several other varieties of edible parsnip and of wild parsnip (Berenbaum et al., 1984). Thus, 53.6% of the total furanocoumarin content (xanthotoxin and bergapten) consists of strongly photoactive furanocoumarins. We compared the toxicological efficacy of the Hollow Crown extract with equimolar amounts of a single photosensitizing constituent, xanthotoxin, against *Heliothis zea* (Lepidoptera: Noctuidae) (corn earworm), a highly polyphagous herbivore with over 100 recorded host plants (Kogan et al., 1978). *H. zea* feeds primarily on the fruits or seeds of most of its hosts and thus is an appropriate species for use in bioassays of toxicity of seed extracts.

To 50 g fresh weight of semidefined artificial diet (Nitao and Berenbaum, 1988), we added 138.84 mg furanocoumarin extract of parsnip (39 ml of an ether solution of 3.56 mg/ml); to a second 50-g aliquot of diet we stirred in 39 ml of ether containing 3.20 mg xanthotoxin/ml ether (Sigma, St. Louis, Missouri). Xanthotoxin is a known photosensitizer, the toxicity of which to insects is demonstrably dependent upon ultraviolet light (Berenbaum, 1978). The resulting concentration of xanthotoxin in the diet was equimolar (5.8×10^{-4} M) to that of the diet containing the furanocoumarin mixture. A 50-g aliquot of control diet was prepared in a similar manner but with 39 ml of ether only. The diet was kept liquid at 60°C and vigorously stirred until all of the ether was driven off.

We elected to use an extract of furanocoumarins from plant tissue, rather than reconstitute the observed mixture with pure compounds, largely for one reason—by using an extract, we hoped to include those furanocoumarins present in concentrations below our ability to detect and quantify them. Despite the fact that HPLC analysis of the extract failed to reveal the presence of nonfuranocoumarin constituents, the possibility does exist that other compounds possessing biological activity were present in the extract and, hence, in the diets.

Therefore, conclusions drawn from this study must be tempered by the possibility that unknown phytochemical constituents may contribute to treatment differences.

After the diets cooled and solidified, cores were punched out and placed in 0.7-ml Kimax test tubes (6 × 50 mm). One neonate *H. zea* larva was placed in each tube, and the tubes were plugged with cotton-tipped applicator sticks. To approximate the solar spectrum, the light source consisted of a 40-W Sylvania cool white fluorescent bulb and a 40-W Westinghouse FS 40 fluorescent sun lamp. The ultraviolet light dosage was varied by adjusting the distance between the light source and the larvae. Caterpillars were placed 85 cm ("high UV") and 100 cm ("low UV") away from the lights. Because light diminishes as the square of the distance, these distances constitute a 40% difference in UV intensity. Kimax glass test tubes screen out wavelengths below 280 nm, which are not present in the solar spectrum. A third light treatment was designed to protect larvae from ultraviolet light by means of a Kodak Wratten 2E filter, which eliminates all wavelengths of 400 nm or less.

Larvae in all three diet types were placed in all three light treatments; diet treatments at each UV level had two replicates with 15 larvae per replicate. Lights were automatically switched on for 16 hr each day and a constant temperature of 26°C was maintained throughout the assay period. First-instar mortality was recorded daily for six days, twice the average time for all control larvae to molt to second instar. Arcsine-transformed data were analyzed by multivariate repeated-measures analysis. The dependent variable was the proportion of initial larvae surviving each day. As such, survival is a within-subject repeated measure reflecting changes with time. UV level and furanocoumarin content are between-subject main effects.

To assess differences among parsnip furanocoumarins as larval feeding deterrents, as opposed to outright toxins, furanocoumarins were incorporated into artificial diet at the same concentrations and in the same manner as in the previous experiment. After evaporation of the ether, diets were poured into four-compartment 100 × 15-mm Falcon "X plate" Petri dishes. Three grams of diet were spread uniformly into each of two opposing compartments; the remaining two compartments were left empty. Three treatments of seven replicates were prepared in a no-choice bioassay; dishes contained either control diet, xanthotoxin diet, or mixed-furanocoumarin diet. After the diets cooled and solidified, 30 neonate *H. zea* were placed onto the diets in each dish. The dishes were covered and placed in the dark in a random arrangement. Larvae were free to move on or off the diet within the dishes, and the number of larvae on each diet was recorded at 2, 4, 8, 12 and 20 hr. Some larvae could not be located during the course of the assay. Two factors may explain the disappearance of larvae—very small neonate larvae may have escaped or larvae may have consumed one another (*H. zea* larvae are highly cannibalistic, particularly when their diet is less suitable). To avoid ambiguity in interpretation of plates in

which several larvae disappeared and to maintain balanced replicate sizes, we dropped the plate with the highest absentee rate in each of the three treatments (mixed, nine absent; xanthotoxin, seven absent; control, three absent). No more than three larvae disappeared from any of the remaining plates. The number of larvae observed off the diet was assumed to reflect the relative palatability of the diets. Arcsine-transformed proportions of larvae off diet each hour were treated as within-subject repeated measures. Data were analyzed in a multivariate repeated-measures design with treatment as the main effect.

RESULTS

Survivorship was significantly reduced by dietary additions of either xanthotoxin or the mixture of furanocoumarins ($P < 0.001$ for treatment effects in comparisons of xanthotoxin and mixture with control diet) (Figure 1). However, the pattern of mortality on xanthotoxin diets was consistently different from that on the diets containing the natural mixture of furanocoumarins in parsnip. Overall, mortality was significantly higher in the mixture than in the xanthotoxin treatment (Table 2). UV light exposure alone also had a significant negative effect on survivorship. In the absence of ultraviolet light, mortality on xanthotoxin diet was not significantly different from that on control diet ($P > 0.05$, t test); in contrast, nearly 50% of the larvae on the mixed-furanocoumarin diet died by the end of the six-day bioassay ($P < 0.001$, t test). Although, as the amount of ultraviolet light increased, the difference in overall mortality between the two furanocoumarin diets decreased from 43% (no UV) to 30% (low UV) to 17% (high UV), no significant UV by treatment interaction was found, indicating that differences between furanocoumarin diets were not influenced by UV level.

In the deterrency test, the number of larvae off diets containing either xanthotoxin or the mix of furanocoumarins was significantly greater than the number off diets without added chemicals (Figure 2), demonstrating that both furanocoumarin treatments are deterrent to *H. zea*. However, contrary to expectations, the diet containing xanthotoxin was effectively more deterrent at all time intervals than the diet containing the mix of furanocoumarins (Table 3). The lack of a significant interaction between furanocoumarin treatments and time suggests that the relative deterrency of the two diets did not change over the course of the experiment.

DISCUSSION

Assuming that effects of trace amounts of nonfuranocoumarin contaminants in the parsnip extract were absent or minimal, the pronounced toxicity of the natural mixture of furanocoumarins in the absence of UV suggests that a

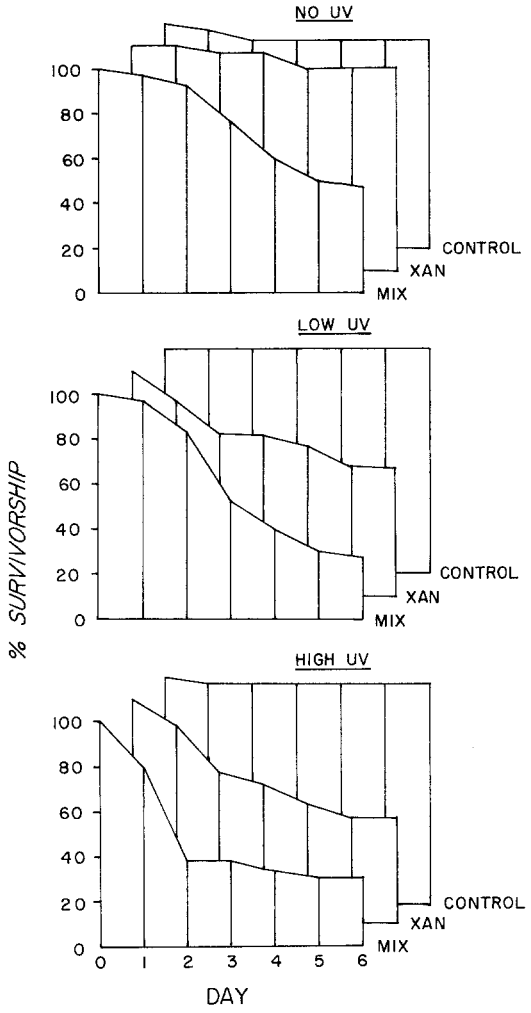


FIG. 1. Percent survivorship of *Heliothis zea* larvae on diets containing no furanocoumarins (control), one furanocoumarin (xanthotoxin), or a mixture of six furanocoumarins (mix) at different UV light intensities.

mechanism other than phototoxicity is involved. Detrimental effects of furanocoumarins unrelated to photoactivation have been observed in previous studies and were attributed to the fact that furanocoumarins are behavioral deterrents as well as postingestive toxins (Yajima et al., 1977; Berenbaum, 1978). In our experiments, however, xanthotoxin proved more deterrent than the mixture in

TABLE 2. MULTIVARIATE REPEATED-MEASURES ANALYSIS OF SURVIVORSHIP OF *Heliothis zea* LARVAE FED DIETS CONTAINING XANTHOTOXIN OR MIX OF FURANOCOUMARINS

Source	*df*	MS	*F*	*P*
Within-subject effects[a]				
UV	2	113.66	14.04	0.0055
XAN	1	123.86	15.30	0.0079
UV × XAN	2	2.61	0.32	0.7364
ERROR	6	8.09		
Between-subject effects[a]				
TIME				n.s.
TIME × UV				n.s.
TIME × XAN				n.s.
TIME × UV × XAN				n.s.

[a] Effects were judged significant at the 0.05 level only if the significance level of 0.05 was not exceeded for any of the following multivariate tests: Wilk's lambda, Pillai's trace, Hotelling-Lawley trace or Roy's greatest root.

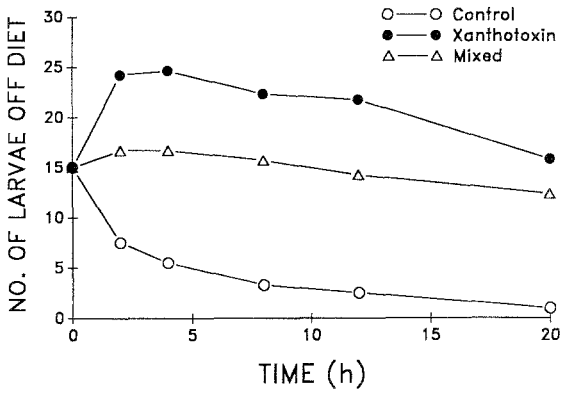


FIG. 2. Mean number of *Heliothis zea* larvae found off diets containing no furanocoumarins (control), one furanocoumarin (xanthotoxin), or a mixture of six furanocoumarins (mixed) as a function of time.

the absence of UV. The differential mortality of the two furanocoumarin diets, then, cannot be attributed to behavioral effects, such as deterrency, alone.

Physiological effects of furanocoumarins independent of UV-mediated photoaddition to DNA have previously been observed (Murray et al., 1982; Grossweiner, 1984; Caffieri, 1988) and may explain the enhanced toxicity of the furanocoumarin mix. The nature of the mode of action of the nonphotosen-

TABLE 3. MULTIVARIATE REPEATED MEASURES ANALYSIS OF PROPORTION OF *Heliothis zea* LARVAE OBSERVED OFF DIET DURING EACH HOUR OF OBSERVATION[a]

Source	*df*	MS	*F*	*P*
Between-subject effects				
TRT	1	2015.03	151.17	0.0001
ERROR	10	13.33		
Within-subject effects[b]				
TIME				*
TRT × TIME				*

[a] Treatment (TRT) consisted of diets containing either xanthotoxin or a mix of furanocoumarins.
[b] Effects with asterisks were judged significant at the 0.05 level if the significance level of 0.05 was not exceeded for any of the following multivariate tests: Wilk's lambda, Pillai's trace, Hotelling-Lawley trace, or Roy's greatest root.

sitizing insecticidal furanocoumarins is open to speculation. Several coumarins that do not cross-link DNA are nonetheless effective frame-shift mutagens in the absence of UV light (Ashwood-Smith et al., 1980). As a plant of roadsides, waste places, and disturbed areas, *P. sativa* is occasionally subjected to shading conditions (Zangerl and Berenbaum, 1990) under which photosensitizing furanocoumarins would be less effective. Under these circumstances, furanocoumarins whose primary mode of action is UV-independent can maintain the toxicological efficacy of the mixture in shaded environments. Evidence in support of such an adaptive role for a mixture of leaf furancoumarins in wild parsnip was obtained in a shading experiment in the greenhouse. Parsnip plants experiencing a 20% reduction in incident light decreased the overall quantity of leaf furanocoumarins by 38% but increased the proportion of nonphototoxic furanocoumarins from 39% to 51% (Zangerl and Berenbaum, 1987). The great structural diversity of furanocoumarins within the Apiaceae may well represent an adaptive compromise in defensive chemistry in the face of varying environmental conditions.

Acknowledgments—This work was supported in part by NSF 88-18205.

REFERENCES

ASHWOOD-SMITH, M.J., POULTON, G.A., BARKER, M., and MILDENBERGER, M. 1980. 5-Methoxypsoralen, an ingredient in several suntan preparations, has lethal, mutagenic and clastogenic properties. *Nature* 285:407–410.

BERENBAUM, M. 1978. Toxicity of a furanocoumarin to armyworms: A case of biosynthetic escape from insect herbivores. *Science* 201:532–534.

BERENBAUM, M. 1985. Brementown revisited: Interactions among allelochemicals in plants. *Recent Adv. Phytochem.* 19:139–169.

BERENBAUM, M.R., ZANGERL, A.R., and NITAO, J.K. 1984. Furanocoumarins in seeds of wild and cultivated parsnip. *Phytochemistry* 23:1809–1810.

CAFFIERI, S., DAGA, A., VEDALDI, D., and DALL'ACQUA, F. 1988. Photoaddition of angelicin to linolenic acid methylester. *J. Photochem. Photobiol., B. Biol.* 2:515–521.

CHEW, F.S., and RODMAN, J.E. 1979. Plant resources for chemical defense, pp. 271–308, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

GROSSWEINER, L.I. 1984. Mechanisms of photosensitization by furocoumarins, pp. 47–54, *in* Photobiologic, Toxicologic and Pharmacologic Aspects of Psoralens. National Cancer Institute Monograph 66. U.S. Department of Health and Human Services, Bethesda, Maryland.

IVIE, G.W. 1978. Linear furocoumarins (Psoralens) from the seed of Texas *Ammi majus* L (Bishop's weed). *J. Agric. Food Chem.* 26:1394–1403.

IVIE, G.W., HOLT, D.L., IVEY, M.C. 1981. Natural toxicants in human foods: Psoralens in raw and cooked parsnip root. *Science* 213:909–910.

KOGAN, J., SELL, D.K., STINNER, R.E., BRADLEY, J.R., and KOGAN, M. 1978. V. A Bibliography of *Heliothis zea* (Boddie) and *H. virescens* (F.) (Lepidoptera: Noctuidae). INTSOY series number 17. International Agricultural Publications. Urbana, Illinois.

MCKEY, D. 1979. The distribution of secondary compounds within plants, pp. 56–133, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores, Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

MURRAY, R.D.H., MENDEZ, J., and BROWN, S.A. 1982. The Natural Coumarins. John Wiley & Sons, Chichester, England.

MUSAJO, L., RODIGHIERO, G., CAPORALE, G., DALL'ACQUA, F., MARCIANA, S., BORDIN, F., BACCICHETTI, F., and BEVILACQUA, R. 1974. Photoreactions between skin-photosensitizing furocoumarins and nucleic acids, pp. 369–387, *in* T.B. Fitzpatrick, M.A. Pathak, L.C. Harber, M. Seiji, and A. Kukita (eds.). Sunlight and Man: Normal and Abnormal Photobiologic Reactions. University of Tokyo Press, Tokyo.

NITAO, J.K., and ZANGERL, A.R. 1987. Floral development and chemical defense allocation in wild parsnip (*Pastinaca sativa*). *Ecology* 68:521–529.

NITAO, J.K., and BERENBAUM, M.R. 1988. Laboratory rearing of the parsnip webworm, *Depressaria pastinacella* (Lepidoptera: Oecophoridea). *Ann. Entomol. Soc. Am.* 81:485–487.

PATHAK, M.A., and FITZPATRICK, T.B. 1959. Relationship of molecular configuration to the activity of furocoumarins which increase the cutaneous responses following long wave ultraviolet radiation. *J. Invest. Dermatol.* 32:255–264.

YAJIMA, T., KATO, N., and MUNAKATTA, R. 1977. Isolation of insect antifeeding principles in *Orixa japonica. Thunb. Ag. Biol. Chem.* 41:1263–1268.

ZANGERL, A.R., and BERENBAUM, M.R. 1987. Furanocoumarins in wild parsnip: Effects of photosynthetically active radiation, ultraviolet light, and nutrients. *Ecology* 68:516–520.

ZANGERL, A.R., and BERENBAUM, M.R. 1990. Furanocoumarin induction in wild parsnip: Genetics and populational variation. *Ecology.* 71:1933–1940.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

PLANT DEFENSES: CHLOROGENIC ACID AND POLYPHENOL OXIDASE ENHANCE TOXICITY OF *Bacillus thuringiensis* SUBSP. *kurstaki* TO *Heliothis zea*

C.T. LUDLUM,[1] GARY W. FELTON,[2] and SEAN S. DUFFEY[2,*]

Department of Entomology
University of California at Davis
Davis, California 95616

(Received March 14, 1990; accepted September 12, 1990)

Abstract—Two chemicals implicated in resistance of the tomato plant, chlorogenic acid and polyphenol oxidase, are known to form orthoquinones in damaged plant tissue. Orthoquinones have been reported to alkylate $-NH_2$ and $-SH$ groups of proteins and amino acids, altering solubility, digestibility, and, for some pathogenic viruses, infectivity. Here we explore effects of quinone alkylation on toxicity of an important microbial insecticide, *Bacillus thuringiensis* subsp. *kurstaki* (BTk), to larval *Heliothis zea*. BTk incubated with these phytochemicals and fed to larvae was more toxic than untreated BTk. Similar but less dramatic results arose when BTk was incubated with polyphenol oxidase alone. Digestibility experiments suggest that alkylation enhanced the solubilization and/or proteolysis of crystal protein in vivo. Implications of our results for compatibility of BTk with host-plant resistance and biological control are discussed.

Key Words—*Bacillus thuringiensis* subsp. *kurstaki*, chlorogenic acid, *Heliothis zea*, *Lepidoptera*, *Noctuidae*, host-plant resistance, *Lycopersicon esculentum*, polyphenol oxidase, tomato.

INTRODUCTION

Widespread concern over the use of broad-spectrum insecticides has enhanced the appeal of host-plant resistance (HPR) and microbial insectides as environmentally sound tactics for insect control. Although simultaneous use of these

* To whom correspondence should be addressed.
[1] Current address: Campbell Institute for Research and Technology, 28065 Co. Rd. 104, Davis, California 95616.
[2] Current address: Department of Entomology, University of Arkansas, Fayetteville, Arkansas 72701.

0098-0331/91/0100-0217$06.50/0

"biorational" tactics (Ferguson and Alford, 1985; Couch and Ross, 1980) is assumed to optimize plant defenses against herbivory (Kogan, 1986; Adkisson and Dyck, 1980; Bergman and Tingey, 1979), other workers have reported that compatibility among biological control agents may be the exception rather than the rule (Berenbaum, 1988; Shepard and Dahlman, 1988; Price et al., 1980). Most evidence of incompatibility has been obtained through studies of predators and parasitoids as affected by host-plant chemicals (Campbell and Duffey, 1979; Duffey and Bloem, 1986; Duffey et al., 1985; Price, 1986; Shephard and Dahlman, 1988).

Despite recognition that entomopathogens and other microorganisms are also influenced by the host plant (Berenbaum, 1988; Shephard and Dahlman, 1988; Barbosa and Saunders, 1985; Jones, 1984), few interactions between HPR and entomopathogens have been explored at a causative level. Many studies have shown that unspecified factors in plant foliage alter the severity of insect disease (Hare and Andreadis, 1983; Merdan et al., 1975; Stubblebine and Langenheim, 1977; Kunimi and Aruga, 1974) and that crude extracts of foliage can inhibit the growth of entomopathogens in vitro (Smirnoff and Hutchinson, 1965; Morris, 1974; Masymiuk, 1970). Fewer studies have demonstrated that specific plant natural products alter the course of disease in insects. For example, caffeic acid and other plant-derived phenolics in the gut of *Bombyx mori* were converted to compounds active against *Streptococcus faecalis* (Iizuka et al., 1974; Koike et al., 1979). Chlorophyll-A from mulberry was shown to bind to protein in the gut of *B. mori*, forming an antiviral complex (Hayashiya, 1978). Nicotine inhibited colony formation by *Bacillus thuringiensis* and rendered it less toxic to *Manduca sexta* (Krischik et al., 1988). The phenolics rutin and chlorogenic acid suppressed infectivity of two baculoviruses and lengthened the survival time of virally infected noctuids (Felton et al., 1987). Clearly, more biological and biochemical information is needed to predict the usefulness of insect control through simultaneous use of chemically based HPR and microbial insecticides. Modern trends in genetic enhancement of plant resistance, e.g., the transformation of crop plants to express delta-endotoxin of *B. thuringiensis* (MacIntosh et al., 1990; Gasser and Fraley, 1989; Meeusen and Warren, 1989; Adang et al., 1987; Barton et al., 1987; Vaeck et al., 1987), increases the urgency of this need.

A portion of our work on chemical bases of resistance of the tomato, *Lycopersicon esculentum*, to the noctuids *Heliothis zea* and *Spodoptera exigua*, has involved compatibility among biological control agents (Felton and Duffey, 1990; Bloem et al., 1989; Bloem and Duffey, 1990a, b; Felton et al., 1987; Duffey et al., 1985; Duffey and Bloem, 1986). We have focused particularly on the role of oxidative enzymes and phenolics in antinutritive bases of resistance against these insects. This resistance is based on the ability of oxidative enzymes such as polyphenol oxidases and peroxidase to activate certain *o*-dih-

ydroxyphenolics (e.g., chlorogenic acid) to *o*-quinones (e.g., chlorogenoquinone), which in turn alkylate dietary protein and reduce its nutritiousness to the insect (Felton et al., 1989b). Recently we showed this resistance to be incompatible with the action of a *Heliothis*-specific, singly embedded nuclear polyhedrosis virus (HzSNPV) against *H. zea* (Felton and Duffey, 1990). The incompatibility arose because alkylation by chlorogenoquinone altered proteinaceous occlusion bodies chemically and reduced the infectivity of virions.

Considering that *B. thuringiensis* subsp. *kurstaki* (BTk) is a recommended control measure for noctuid larvae on tomato (Anon., 1985) and that the active agent in BTk is a protein whose amino acids are susceptible to aklylation (Schnepf et al., 1985; Chestukhina et al., 1982; Pierpoint, 1983; Hurrell et al., 1982; Huber et al., 1981), we reasoned that alkylation from chlorogenoquinone production may alter the efficacy of BTk as it did with HzSNPV. Here we report that alkylation of BTk enhances its toxicity to *H. zea*. The relevance of our results to conventional and modern HPR is discussed.

METHODS AND MATERIALS

Insects

Eggs of *Heliothis zea* were obtained weekly from the Bioenvironmental Insect Control Laboratory, USDA (Stoneville, Mississippi). To disinfect eggs and free them from cloth on which they were oviposited, cloths were agitated 3 min in a 1% bleach solution and rinsed at least 4 min with distilled water. The eggs were sprinkled over multicellular rearing units placed in plastic trays of soy–wheat germ diet (Hartley et al., 1982). Larvae were reared under a 16:8 light–dark photoperiod until completion of their molt to the desired instar, when they were removed from the unit for use in assays.

Bacillus thuringiensis

A stock culture of *B. thuringiensis* subsp. *kurstaki* (BTk) (var. HD-1) was acquired from Prof. P. Baumann (University of California, Davis). Cells transferred from this and daughter cultures were grown in tryptone–yeast–glucose (TYG) medium containing the following per liter: 25 ml 1 M KH_2/Na_2HPO_4, pH 6.8; 10 ml of solution containing (per liter): 20 g $MgSO_4 \cdot 7H_2O$ and 1 g $CaCl_2$; 2 g D-glucose; 5.85 mg $MnCl_2 \cdot 4H_2O$; 5 ml 20% NH_4Cl; 2.5 g yeast extract (Difco), and 2.5 g tryptone (Difco). Solid medium contained TYG plus 20 g/liter agar, and stock cultures were grown on agar slants for three to four days and stored at 4°C. To obtain BTk for experimental use, cells were grown in liquid TYG medium at 30°C aerated by rotation. After complete sporulation, cultures were centrifuged 10 min at 27,500*g* in a Sorvall RC-5B centrifuge.

Spore-crystal pellets were washed once in 1 M NaCl to remove adsorbed proteases and once in 25 mM KH_2/Na_2HPO_4, pH 6.8. Pellets were then frozen, lyophilized, pooled, and stored at −20°C. The resulting preparation contained spores, crystals, and bacterial debris and is henceforth described as "whole" BTk. Its toxicity to *H. zea* was equivalent to that of a commercially available preparation of BTk, Dipel 2X (Abbott Laboratories, North Chicago, Illinois), containing 32,000 IU of potency per milligram. Unless specifically noted otherwise, all BTk used here was whole.

Intensification of Crystal Activity in BTk

Creation of Gradients. To create a preparation of BTk with an intensified crystal component the method of Sharpe et al. (1975) for separating crystals from spores was used. Linear gradients of 50–90% Renografin-76 (Squibb Diagnostics, Lamirada, California) in double-distilled water were created with an Isco gradient former (model 570) connected by tubing to two 250-ml flasks. Fifty-milliliter gradients were prepared one at a time.

Preparation and Centrifugation of Sample. Whole BTk (about 100 mg) was added to 2 ml Tris HCl, pH 8, containing 0.2% Tween-80 (Sigma, St. Louis, Missouri). The suspension was disaggregated by sonication at 175 W for 3 min. Samples (300 μl each) were layered onto the gradients, placed in a Sorvall HS-4 swinging-bucket rotor, and centrifuged 2 hr at 9400*g*. After centrifugation, bands were collected with a nonsterile Pasteur pipet. The fractions were checked microscopically for the presence of crystals. Fractions containing crystals were pooled, washed twice with double-distilled water, lyophilized, and stored at −20°C. The resulting preparation consisted predominantly of crystals but contained spores as well (ca. 1.6×10^8 spores/mg crystal preparation).

Bioassays

Bioassays involved either larvae placed on treated, synthetic diet or larvae administered treatments per os. All bioassays were replicated at least three times with 24–96 larvae/treatment. For dietary bioassays, synthetic diet at 45°C (ca. 1 ml) was poured into each well of 24-well polypropylene culture dishes (Falcon) and cooled to solidify the diet. Treatments were mixed, stirred mechanically at room temperature for 1.5 hr, and applied in 100-μl aliquots to the diet surface in each well. Stirring continued during application to keep the BTk suspended. Treated dishes were dried in warm (ca. 30°C) air and shaken by hand at least once a minute to ensure even coverage of the diet surface. One third-instar larva then was placed in each well. After infestation, dishes were sandwiched between glass plates. The assembly of dishes and plates was sealed in a polyethylene bag to prevent desiccation of the diet. Dishes were held five

days at 28°C in a 16:8 light–dark photoperiod, after which larvae were prodded gently with a probe. Any larva not responding to this stimulus was classified as dead.

To test the effects of alkylated crystal by injections per os, fifth-instar larvae were first anesthetized by placing them on ice ca. 15 min prior to injection. A 10-μl Hamilton syringe with a blunted needle (gauge 26S) was used to administer 10 μl of treatment to each larva. The needle was inserted approximately halfway into the digestive tract to inhibit regurgitation after administration of material; insects that regurgitated were discarded. Treated larvae were held without diet in 25-well translucent plastic trays (Bio-Serv, Frenchtown, New Jersey) covered with glass plates. Mortality was assessed 24 hr after injection.

Dietary bioassays involved a range of concentrations of phytochemicals and of BTk that included known or estimated levels of these substances in field-grown, BTk-treated tomato plants. We considered: (1) recommended rates for spray application of Dipel 2X, (2) approximate grams of foliage per unit area of canopy, (3) average moles chlorogenic acid per gram of fresh foliage, and (4) average polyphenol oxidase activity per gram of fresh foliage. Values in (2), (3) and (4) were taken from studies of field-grown plants (Felton et al., 1989b); the value obtained for (4) was translated to an appropriate quantity of commercially obtained polyphenol oxidase based on oxidase activity per milligram of enzyme stated by the vendor. We thus estimated a ratio of BTk, chlorogenic acid, and polyphenol oxidase per unit area of tomato as 1:100:2. Absolute concentrations of chlorogenic acid and polyphenol oxidase followed this ratio, so that the high concentration of BTk required to achieve meaningful mortality in *H. zea* required correspondingly high concentrations of phytochemicals. In the case of chlorogenic acid, the highest concentrations used in bioassays exceeded those in field-grown plants (Felton et al., 1989b).

Effects of Chlorogenic Acid and Polyphenol Oxidase in Vivo

Dietary bioassays were used to address the following questions about chlorogenic acid (CHA), polyphenol oxidase (PPO), and BTk: (1) Do CHA and PPO alone alter the toxicity of BTk to *H. zea*? (2) Do combinations of CHA and PPO alter the toxicity of BTk to *H. zea*? To answer the first question, CHA (Sigma Chemical Co. St. Louis, Missouri) was dissolved in 0.1 M KH_2/Na_2HPO_4 buffer, pH 7 (sodium phosphate buffer) at a concentration of 30 mg/ml. The pH of the solution was readjusted to 7.0 with 2 N NaOH, and the solution was diluted serially with buffer to yield concentrations of 5–30 mg CHA/ml. BTk was added to dilutions at 0.24 mg/ml. A suspension of BTk in buffer (0.24 mg BTk/ml) acted as a control. Bioassays of PPO and BTk involved mushroom tyrosinase (Sigma) with an activity of 2200 units CHA oxidase activity/mg enzyme; a unit is defined as $\Delta 0.001$ OD_{470}/min/g. The range of PPO

tested was 0.05–1.2 mg/ml buffer. Again, BTk in buffer was the control treatment. In both cases treatments were stirred, applied to diet, infested, and monitored as described.

To determine if combinations of CHA and PPO alter the toxicity of BTk to *H. zea*, a solution of CHA in buffer (24 mg/ml) was prepared, diluted, and mixed with BTk (0.24 mg/ml) as described. Next, a known amount of PPO was dissolved in an aliquot of cold buffer. Enzyme solution was added to each CHA–BTk mixture to create a ratio of milligrams PPO to milligrams CHA per milliliter of 1 : 50. The bioassay was then performed as described.

Because foliar levels of PPO can vary substantially within and among plants, another series of bioassays tested the effect on BTk toxicity when varying levels of PPO were incubated with a fixed concentration of CHA. PPO was accordingly added to suspensions of BTk in solubilized CHA to produce a final ratio of PPO to CHA of 1 : 10, 1 : 50, 1 : 100, and 1 : 200 (0.06–1.17 mg PPO/ml). BTk and CHA were kept constant at 0.24 mg/ml and 12.5 mg/ml, respectively.

Feeding Rate

To account for the possibility that CHA and/or PPO act as phagostimulants to *H. zea*, bioassays were performed in which CHA and PPO, singly or together, were combined with BTk in 0.1 M $KH_2/Na_2/HPO_4$ buffer to yield the following concentrations: 24.0 mg CHA/ml, 0.48 mg PPO/ml, and 0.24 mg BTk/ml. The treatments (CHA + PPO + BTk, CHA + BTk, and PPO + BTk) were mixed and incubated as previously. Amaranth (Sigma), a water-soluble dye that is nontoxic to larvae and does not cross the gut barrier after ingestion, was added to each treatment after incubation (5% w/v). A solution of amaranth in buffer also was prepared as a control. After treatments were applied to diet, fifth-instar larvae of *H. zea* starved 24 hr were placed on the diet.

Eighteen hours later, the gut of each larva was removed according to the method of Broadway and Duffey (1986) with modifications. A hemostat was used to grasp the anesthetized insect behind the head while the last two abdominal segments were removed. The gut was pulled with forceps onto absorbent paper where it was left briefly to drain of hemolymph before transferral to a 15-ml polycarbonate centrifuge tube containing 1.0 ml 1.0% Triton X-100 (Sigma). Frass produced by a larva during the 24-hr feeding period also was added to that tube. Contents of tubes were macerated 10 sec with a Tekmar Tissumizer and centrifuged 8 min at 17,000 rpm. To assess the presence of dye in the extract, aliquots of each supernatant were read at 522.5 nm (Windholz et al., 1983). Ten tubes were prepared for each treatment, and the experiment was replicated three times.

Radioisotope Experiments

Preparation of Isotope. Tritiated CHA ([^{3}H]CHA) was isolated from a mixture of tritiated rutin and CHA (Research Products International Corp., Gif-sur-Yvette, France) according to Isman and Duffey (1983). Specific activity of [^{3}H]CHA was 153 mCi/mmol CHA.

Alkylation and Counting of BTk. To determine whether CHA and PPO alkylate BTk under conditions established in bioassay incubations, preparations of BTk (whole and crystals) were incubated with these compounds and labeled CHA as for dietary bioassays. This experiment was replicated three times. The method of Felton and Duffey (1990) for alkylation of polyhedral protein was followed with the following modifications. Using 0.1 M $KH_2/Na_2/HPO_4$ buffer at pH 7, one suspension each of whole BTk and BTk crystal was prepared, as was one 25-ml solution of CHA (25.4 mg/ml, adjusted to pH 7) and one of PPO (0.5 mg/ml). Three 8-ml aliquots of the CHA solution and two 8-ml aliquots of buffer were poured. Various combinations of 1×10^6 dpm [^{3}H]CHA, BTk, and PPO were added to these aliquots to create five treatments: BTk crystal only; crystal plus PPO (5 mg); crystal plus CHA (250 mg); crystal plus CHA (250 mg) and PPO (5 mg); and whole BTk plus CHA (250 mg) and PPO (5 mg). Volumes in all treatments were adjusted to 10 ml. Specific activity of [^{3}H]CHA in the last three treatments was 1.4×10^{-3} mCi/mmol CHA. All treatments were incubated and the pellets isolated, dialyzed, and lyophilized as described by Felton and Duffey (1990).

To achieve complete hydrolysis of BTk before scintillation counting, samples were placed in 3-ml reaction vials (Pierce) with 250 μl 12.4 N HCl. The vials were sealed with Teflon caps and heated 1 hr at 50°C. An equal volume of 12.4 N NaOH neutralized the solution and was followed by 250 μl 30% H_2O_2 as a bleaching agent. Samples were transferred to scintillation vials, mixed with aqueous scintillation cocktail (Amersham Corp., Arlington Heights, Illinois), allowed to stand for 12 hr to minimize quenching, and then counted 5 min by the external standard ratios method. Appropriate background controls of hydrolyzing, neutralizing, and bleaching agents added to scintillation cocktail were also run.

Digestion of Alkylated BTk in Vitro

To examine whether alkylated BTk was more susceptible than unalkylated BTk to digestion in the insect gut, experiments were performed to digest BTk in vitro, to bioassay the digest, and to examine digestion products via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Alkylation of Crystal. A 30-mg batch of BTk crystal was alkylated by incubating it with CHA and PPO in sodium phosphate buffer as described, except

that a ratio (in milligrams) of BTk, CHA, and PPO of 1:75:1.5 was used. A second 30-mg batch was incubated in buffer as a control. Both treatments were dialyzed as described, lyophilized, and stored at 4°C. They will be referred to henceforth as "alkylated crystal" and "unalkylated crystal," respectively.

Isolation of Proteases. Guts of 25 early fifth-instar *H. zea* were pooled in 3 ml chilled 0.09% NaCl containing 0.02 M $CaCl_2$. After maceration and centrifugation of the guts, supernatant was filtered over a 0.45-μm nylon membrane and checked for tryptic and chymotryptic activities (Broadway and Duffey, 1986). These were established, via toluenesulfonyl-L-arginine methyl ester and benzoyl-L-tyrosine ethyl ester (Sigma), respectively, as 7.4 and 2.3 Δ0.001 OD_{max}/μl gut juice/min.

Digestion. Alkylated and unalkylated crystal was divided into three portions of approximately 10 mg and solubilized in 500 μl 0.04 M Tris, pH 8.1, containing 0.02 M $CaCl_2$. An aliquot of gut juice was added to each tube, and tube volumes were adjusted with buffer to 2.5 ml. Unalkylated crystal was similarly divided into three batches, suspended in buffer, and treated with enzyme. As controls, buffer and the same volume of enzyme used in other treatments were added to an additional three tubes. Tubes were inverted several times and placed in a shaking bath at 25°C. Preliminary tests showed that BTk crystal suspended in the buffer used for this digestion remained intact (unpublished data).

Protein Assays. Treatments were monitored at 0, 60, and 120 min to assess the progress of digestion. We first measured amines via 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Fields, 1972), then arginine and, to a lesser extent, other basic and aromatic residues of protein via Biorad dye reagent diluted fivefold (Compton and Jones, 1985; Bradford, 1976). After final monitoring at 120 min, treatments were divided rapidly into aliquots, frozen, and stored at −80°C for the next experiment.

Bioassays of Digests. To determine whether changes detected in treatments during digestion were accompanied by changes in larval mortality, individual frozen aliquots of digests were thawed at 4°C and administered per os to fifth-instar *H. zea*. As noted above, mortality was checked after 24 hr. This experiment was replicated by using larvae from six separate rearings.

SDS-PAGE. To ascertain whether alkylation of BTk alters the size of fragments normally produced during proteolysis of BTk crystal protein, digests from alkylated and unalkylated crystal were submitted to SDS-PAGE. One aliquot each of digested, alkylated BTk and of digested, unalkylated crystal was thawed at 4°C, transferred to 1-ml microfuge tubes, and centrifuged 5 min at 2000*g*. The supernatants were withdrawn and submitted to discontinuous SDS-PAGE in gels containing 12.5% (w/v) acrylamide and 0.1% SDS after Laemmli (1970). Molecular-weight standards were obtained from Sigma. Sample preparation and protocols for electrophoresis followed methods of Laemmli (1970). Gels were

stained for general protein using (1) a standard method with Coomassie brilliant blue R and (2) the silver-staining method with reagents and protocol provided by Biorad (Richmond, California).

RESULTS

Dietary Bioassays

Effects of CHA and PPO in Vivo. Placing BTk under alkylative conditions more than doubled its toxicity to third-instar *H. zea* as the concentrations of CHA and PPO rose. The increase in toxicity was highly significant (Figure 1) (least-squares regression, $F_{1,23} = 38.07$, $P < 0.001$). Mortality of *H. zea* also was enhanced when PPO combined with a fixed concentration of CHA rose from 0.06 mg PPO/ml to 1.17 mg PPO/ml (Figure 2) (least-squares regression, $F_{1,11} = 9.57$, $P < 0.025$). Polyphenol oxidase alone enhanced toxicity of BTk as well (Figure 3) (least-squares regression, $F_{1,16} = 11.24$, $P < 0.005$), but CHA alone did not (data not shown; (least squares regression, $F_{1,19} = 1.01$, $P > 0.3$).

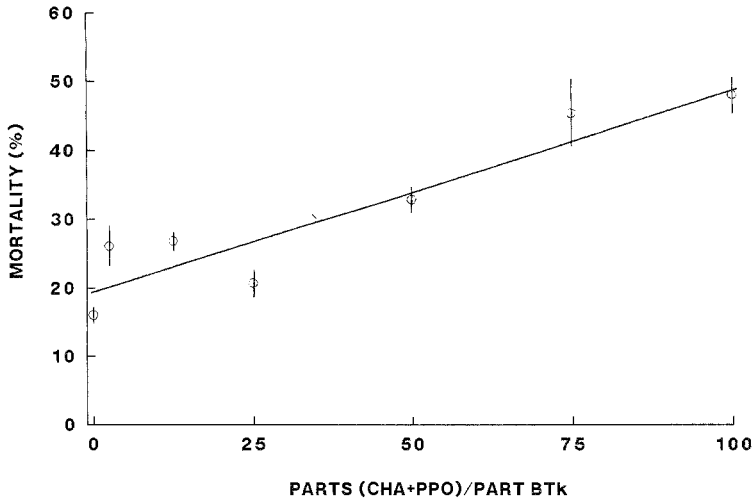


FIG. 1. Larval mortality in dietary bioassays of BTk treated with chlorogenic acid (CHA) and polyphenol oxidase (PPO). [BTk] = 0.24 mg/ml, [CHA] = 0–24 mg/ml, [PPO] = 0–0.48 mg/ml. Ratio of parts CHA to part PPO was constant at 50 : 1 while the ratio of parts CHA to part BTk varied (0 : 1–100 : 1). Each point represents mean mortality in three to five replicates containing 24–120 insects per treatment. Regression equation: $Y = 19.6 + 0.287X$; $r^3 = 0.623$. The slope is significantly greater than zero ($P < 0.001$).

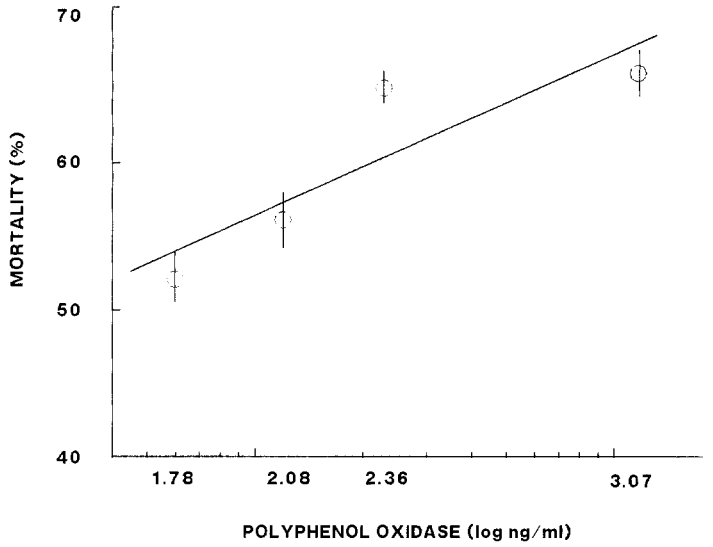


FIG. 2. Larval mortality in dietary bioassays of BTk with fixed CHA and increasing levels of PPO. [BTk] = 0.24 mg/ml, [CHA] = 12.5 mg/ml, [PPO] = 0.06–1.17 mg/ml (60–1170 ng/ml). Concentrations of PPO were log-transformed. Each point represents mean mortality (±SEM) in three replicates containing 72–96 insects per treatment. Regression equation: $Y = 4.534 (\log X) + 65.721$; $r^2 = 0.785$. The slope is significantly different from zero ($P < 0.025$).

Feeding Rate. The major influence on feeding rate of *H. zea* exposed to phytochemicals and BTk was BTk rather than CHA and/or PPO (Figure 4). The feeding rate of insects fed BTk, phytochemicals, and amaranth appeared several times lower than that of insects fed amaranth only. This difference was highly significant (t test, $P < 0.001$). Differences among BTk treatments were slight and not statistically significant except for treatments containing (1) BTk, CHA, and PPO and (2) BTk and PPO. Both (1) and (2) differed from each other but not from the other treatments containing BTk (two-sample test, $P > 0.05$). The low feeding rate in BTk treatments confirmed BT-induced feeding cessation noted here and elsewhere (Dulmage and Martinez, 1973; Retnakaran et al., 1983; Fast and Regniere, 1984; Alford and Holmes, 1986). No insect died in any treatment.

Radioisotope Experiments

BTk placed under alkylative conditions bound labeled CHA. In two of three experiments, crystal bound more CHA than did whole BTk (Table 1), suggesting that chlorogenoquinone interacted primarily with the crystal in this

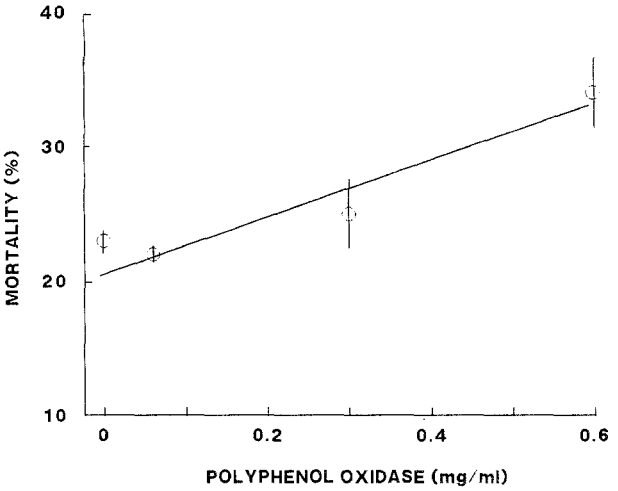


FIG. 3. Larval mortality in bioassays of BTk with PPO. [BTk] = 0.24 mg/ml, [PPO] = 0–0.06 mg/ml. Each point represents the mean mortality in two to four replicates containing 20–72 insects per treatment. Regression equation: $Y = 21.8 + 16.5X$; $r^2 = 0.413$. The slope is significantly different from zero ($P < 0.01$).

preparation. (The variation in binding evident in Table 1 may have been caused by clumping of spores in BTk preparations.) Radioactivity persisted in BTk despite prolonged dialysis against 8M urea. The binding therefore was deemed covalent, since urea is known to disrupt noncovalent interactions between protein and phenolics (Pierpoint, 1983).

Digestion of Alkylated BTk in Vitro

Protein Assays. Alkylated BTk was digested at a faster rate than unalkylated BTk (Figures 5 and 6). When TNBS reagent was used (Figure 5), the measured rate of increase in amines was the same for alkylated and unalkylated treatments between 0 min and 60 min but was greater thereafter for the alkylated treatment. The enzyme control gave lower absorbance throughout than either of the BTk treatments. Absorbance of the alkylated treatment was significantly higher at 0 min (t test, $P < 0.001$) than that from either the unalkylated or control treatment.

Except that readings of all treatments differed significantly from each other at 0 min (alkylated > unalkylated > enzyme control, $P < 0.05$), similar results were obtained with Biorad dye reagent (Figure 6) as with TNBS. Again, absorbance was consistently higher in the alkylated treatment than in the unalkylated or control treatment. Values increased for both BTk treatments but not for the enzyme control. At each reading, absorbance of alkylated and unalkylated BTk

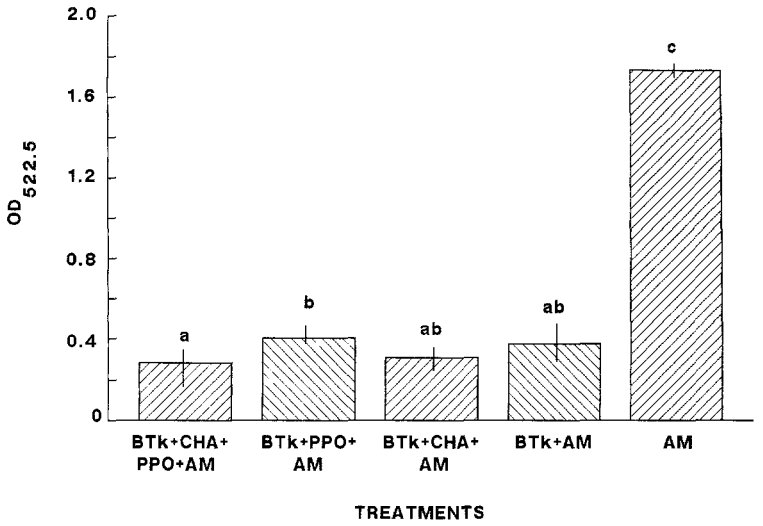


FIG. 4. Feeding rate of *H. zea* on treatments containing BTk and phytochemicals, as shown by absorbance of extracts of guts and frass. CHA = chlorogenic acid, PPO = polyphenol oxidase, AM = amaranth. [BTk] = 0.24 mg/ml, [CHA] = 24 mg/ml, [PPO] = 0.48 mg/ml, [AM] = 50 mg/ml. Each bar represents mean absorbance (±95% CI) of 10 extracts, each extract from one larva, replicated three times. Bars topped by different letters are significantly different.

TABLE 1. CHLOROGENIC ACID (CHA) BOUND TO BTk AFTER INCUBATION WITH TRITIATED CHA, UNLABELED CHA, AND POLYPHENOL OXIDASE (PPO) IN 0.01 M SODIUM PHOSPHATE BUFFER

Treatment[a]	Bound CHA (nmol CHA/mg BTk)		
	Exp. 1	Exp. 2	Exp. 3
Crystal + CHA + [^{3}H]CHA + PPO	49.1	4.3	10.0
Whole BTk + CHA + [^{3}H]CHA + PPO	35.0	13.7	7.5

[a] [BTk] = 5 mg crystal or whole BTk; [^{3}H]CHA = tritiated CHA at 1×10^6 dpm/incubation; CHA = 250 mg/incubation; and PPO = mushroom tyrosinase at 5 mg/incubation. Volume of each incubation = 15 ml.

differed significantly ($P < 0.02$). Absorbance from the control did not change between 0 and 60 min ($P > 0.3$) and dropped thereafter.

Bioassays of Digests. Alkylated BTk, which proved more digestible in vitro than unalkylated BTk, also proved more toxic in vivo (data not shown).

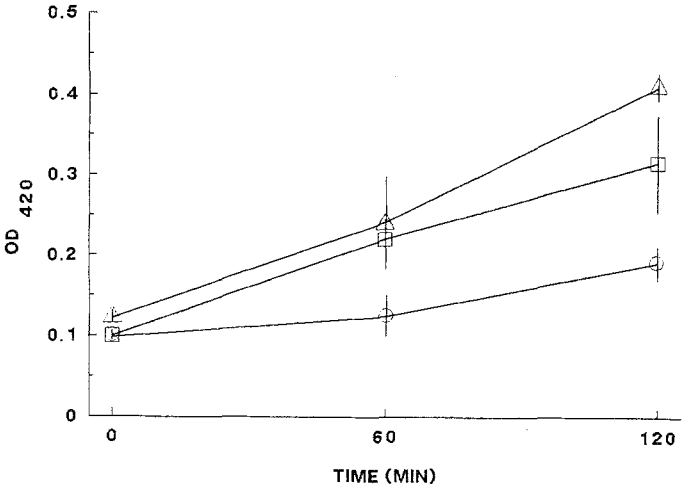


FIG. 5. In vitro digestion of BTk crystal by *Heliothis* proteases as measured by TNBS. △ = alkylated crystal; □ = unalkylated crystal; ○ = enzyme control. Each point is the mean (±95% CI) of six readings.

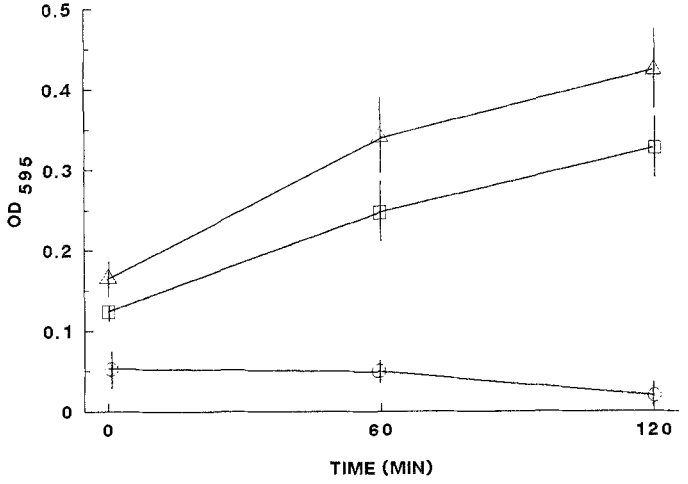


FIG. 6. In vitro digestion of BTk crystal by *Heliothis* proteases as measured by Biorad dye reagent. △ = alkylated crystal; □ = unalkylated crystal; ○ = enzyme control. Each point is the mean (±95% CI) of six readings.

The difference in mortality caused by alkylated and unalkylated BTk was significant (Wilcoxon matched-pairs signed-ranks test, $P < 0.05$).

SDS-PAGE. A complex banding pattern appeared when digests were submitted to SDS-PAGE. However, despite differences observed in the preceding experiments, no corresponding differences in banding by digests of alkylated and unalkylated material were apparent (data not shown).

DISCUSSION

Many factors are known to alter the insecticidal and bacterial activity of BTk. Abiotic factors (moisture, high temperature, and ultraviolet radiation) degrade BT in field applications (Beckwith and Stelzer, 1987; Leong et al., 1980; Ignoffo et al., 1974), while chemicals (pyrethroids, adjuvants, thuringiensin, organophosphates, and other synthetic insecticides) can diminish or enhance it (Bell and Romine, 1986; Moar et al., 1986; Salama et al., 1984; Mohamed et al., 1983; Faust and Bulla, 1982; Pfrimmer, 1979; Jaques and Morris, 1981; Bull et al., 1979). Plant natural products also influence the toxicity of BT. Soy protease inhibitors enhanced the insecticidal activity of BT when combined with BT, as did a protease inhibitor expressed with BTk insecticidal protein in genetically transformed tobacco (MacIntosh et al., 1990). Nicotine enhanced survivorship of *Manduca sexta* exposed to BTk (Krischik et al., 1988); the nonprotein amino acid L-canavanine had the opposite effect (Felton and Dahlman, 1984). However, chemical factors that change the toxicity of BTk often do so by influencing the insect and not the pathogen (Berenbaum, 1988; Krischik et al., 1988; Felton and Dahlman, 1984). Our paper presents evidence that two plant natural products, CHA and PPO, can alter pathenogenicity by acting directly on an entomopathogen.

Plant phenolics are known for their ability to form reactive *o*-quinones in the presence of oxidative enzymes like PPO, laccase, and peroxidase (Matheis and Whitaker, 1984; Pierpoint, 1983; van Sumere et al., 1975). Orthoquinones attack nucleophilic substituents of proteins, e.g., $-SH$ and $-NH_2$ groups, alkylating the proteins and changing them chemically (Barbeau and Kinsella, 1983, 1985; Matheis and Whitaker, 1984; Pierpoint, 1983; Blouin et al., 1982; Hurrell et al., 1982; van Sumere et al., 1975). Orthoquinones also may be formed by the monophenolase action of PPO on tyrosyl residues of proteins (Matheis and Whitaker, 1984), e.g., the toxic protein of BTk found predominantly in crystals but also in spore coats (Chestukhina et al., 1982; Huber et al., 1981; Tyrell et al., 1981; Bulla et al., 1980). These *o*-quinones cross-link with each other or with nucleophilic substituents of proteins (Matheis and Whitaker, 1984). In our study, *o*-quinone formation by the action of PPO on CHA (Figures 1 and 2) and of PPO on BTk (Figure 3) appears to have enhanced

toxicity of BTk protein to *H. zea*. Moreover, BTk was alkylated by chlorogenoquinone (Table 1), an expected result since crystal protein of BTk contains alkylatable amino acids such as cysteine, lysine, and methionine (Schnepf et al., 1985; Chestukhina et al., 1982; Huber et al., 1981; Tyrell et al., 1981; Bulla et al., 1980). How could alkylation have increased the toxicity of BTk? Three possibilities exist: (1) alkylation of the crystal protein altered interactions between the larval midgut and delta-endotoxin (Hofmann et al., 1988a,b; Bulla et al., 1980) or other events following hydrolysis of the native protein in vivo; (2) alkylation cross-linked the crystal protein, causing an unusually toxic molecule to be released by hydrolysis; or (c) alkylation sped the release of toxic fragments by enhancing solubilization and/or proteolytic cleavage of the crystal protein.

The first possibility, that alkylation influenced postproteolytic phenomena in BTk toxification, exceeded the scope of this study and requires further examination. The second possibility, that an unusually toxic peptide was formed by protein cross-linking, is unlikely. Chlorogenoquinone bound to BTk (Table 1) but did not cause protein cross-linking detectable by SDS-PAGE. Indeed, alkylation apparently failed to alter the gel retention time of any peptide released during digestion of BTk in vitro.

Inasmuch as alkylation enhanced the enzymatic hydrolysis of BTk (Figures 5 and 6), it is more plausible that alkylation improved solubilization and/or proteolysis of the crystal protein in vivo. Reductive alkylation of casein enhanced the solubility of this protein (Sen et al., 1981) while HzSNPV occlusion bodies (OBs) exposed to chlorogenoquinone proved less soluble than untreated OBs (Felton and Duffey, 1990). Proteins often are more digestible after they have interacted noncovalently with phenolics. As examples, noncovalent binding by CHA to fraction-1 protein from spinach rendered the protein more susceptible to trypsin hydrolysis (Barbeau and Kinsella, 1985), and tannins accelerated the enzymatic hydrolysis of bovine serum albumin after complexing with this protein (Mole and Waterman, 1985). Both these studies of protein–phenolic binding suggested strongly that complexing changed protein conformation and exposed new sites to proteolytic attack (Barbeau and Kinsella, 1985; Mole and Waterman, 1985). Protein appears to undergo conformational changes after covalent interactions with phenolics as well (Sen et al., 1981; Puigserver et al., 1979), although this protein–phenolic interaction often suppresses digestibility in the relatively few proteins studied (Hurrell et al., 1982; Puigserver et al., 1979; Duffey and Felton, 1989; Pierpoint, 1983; Pierpoint et al., 1977).

We propose that alkylation caused conformational changes in the toxic protein of BTk and that these changes enhanced digestibility and/or solubility of the protein. Consistent with the foregoing studies, it is plausible that conformational changes exposed more peptide bonds to proteolysis than were

accessible in unalkylated protein, speeding the release of toxic fragments. An acceleration in the release of proteinaceous products is evident in Figures 5 and 6. Moreover, bioassays of BTk digests show that the material produced at a faster rate (i.e., from alkylated BTk) was toxic to *H. zea*.

The chemical mechanism by which CHA and PPO accentuate the toxicity of BTk will require further clarification. However, we were able to eliminate three factors that could account for our results. The first was that stress imposed on *H. zea* by ingestion of CHA and PPO predisposed the insect to BTk toxification. However, *H. zea* exposed to both phytochemicals grew as well as insects not so exposed (data not shown). The second possibility was that either phytochemical alone could enhance the toxicity of BTk. This was true in the case of PPO only (Figure 3). The monophenolase activity of tyrosinase on tyrosyl resides in BTk protein accounts for this effect (Matheis and Whitaker, 1984), but enhancement was more pronounced when PPO acted on CHA (cf. percent mortality at highest x value, Figures 1 and 3). Finally, we excluded the possibility that both phytochemicals caused *H. zea* to ingest more BTk by acting as phagostimulants. Figure 4 illustrates that this is decidedly not the case.

Here we have provided further evidence that plant natural products can influence the efficacy of entomopathogens. To our knowledge, this is the first report that chemical alteration of protein in the entomopathogenic spore and toxin potentiates BTk. Although reports of inactivation by plant natural products substantially outnumber reports of potentiation (Shepard and Dahlman, 1988), most changes in the toxicity of entomopathogens are ascribed to effects of plant natural products on test herbivores (Berenbaum, 1988). It remains to be seen whether CHA and PPO in the plant have the same effect on BTk as occurred in our laboratory or whether they can enhance the potency of BT toxin expressed in transgenic plants (see especially MacIntosh et al., 1990, but also Meeusen and Warren, 1989; Barton et al., 1987; Adang et al., 1987; Vaeck et al., 1987). If so, we suspect that enhanced phenotypic expression of these phytochemicals would prove a mixed blessing: improved efficacy of BTk on the one hand, but simultaneously a threat to other forms of biological control and of HPR. We have mentioned the study in which chlorogenoquinone alkylated viral occlusion bodies (OBs), inhibiting their solubility and digestibility in vitro and reducing the infectivity of virions in vivo (Felton and Duffey, 1990). Chlorogenoquinone also may inactivate tomato and soy protease inhibitors (Felton et al., 1989a), implicated in resistance of these plants to insects (Ryan, 1979; Broadway et al., 1986).

Acknowledgments—This research was supported by USDA competitive grant 87-CRC-1-2371 awarded to S.S.D. and by a grant from O. Gass awarded to C.T.L. Critical reviews of an earlier draft by H. Kaya, S. Maeda, and L. Harshman were most useful.

REFERENCES

ADANG, M.J., FIROOZABADY, E. KLEIN, J., DEBOER, D., SEKAR, V., KEMP, J.D., MURRAY, E., ROCHELEAU, T.A., RASHKA, K., STAFFELD, G., STOCK, C., SUTTON, D., and MERLO, D.J. 1987. Expression of a *Bacillus thuringiensis* insecticidal crystal protein gene in tobacco plants, pp. 345-353, *in* C.J. Arntzen and C.A. Ryan (eds.). Molecular Strategies for Crop Protection. UCLA Symposia on Molecular and Cellular Biology, Vol. 48. Alan R. Liss, New York.

ADKISSON, P.L. and DYCK, V.A. 1980. Resistant varieties in pest management systems, pp. 233-251, *in* F.G. Maxwell and P.R. Jennings (eds.). Breeding Plants Resistant to Insects. John Wiley & Sons, New York.

ALFORD, A.R., and HOLMES, J.A. 1986. Sublethal effects of carbaryl, aminocarb, fenitrothion, and *Bacillus thuringiensis* on the development and fecundity of the spruce budworm (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 79:31-34.

ANONYMOUS. 1985. Integrated Pest Management for Tomatoes. University of California Statewide Integrated Pest Management Project. Publication 3274.

BARBEAU, W.E., and KINSELLA, J.E. 1983. Factors affecting the binding of chlorogenic acid to fraction 1 leaf protein. *J. Agric. Food Chem.* 31:993-998.

BARBEAU, W.E., and KINSELLA, J.E. 1985. Effects of free and bound chlorogenic acid on the in vitro digestibility of ribulose bisphosphate carboxylase from spinach. *J. Food Sci.* 50:1083-1087.

BARBOSA, P., and SAUNDERS, J.A. 1985. Plant allelochemicals: linkages between herbivores and their natural enemies, pp. 107-137, *in* G.A. Cooper-Driver and E.E. Conn (eds.). Chemically Mediated Interactions between Plants and Other Organisms. Plenum Press, New York.

BARTON, K.A., WHITELEY, H.R., and YANG, N.-S. 1987. *Bacillus thuringiensis* delta-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. *Plant Physiol.* 85:1103-1109.

BECKWITH, R.C., and STELZER, M.J. 1987. Persistence of *Bacillus thuringiensis* in two formulations applied by helicopter against the western spruce budworm (Lepidoptera: Tortricidae) in north central Oregon. *J. Econ. Entomol.* 80:204-207.

BELL, M.R., and ROMINE, C.L. 1986. *Heliothis virescens* and *H. zea* (Lepidoptera: Noctuidae) dosage effects of feeding mixtures of *Bacillus thuringiensis* and a nuclear polyhedrosis virus on mortality and growth. *Environ. Entomol.* 15:1161-1165.

BERENBAUM, M.R. 1988. Allelochemicals in insect-microbe-plant interactions: Agents provocateurs in the coevolutionary arms race, pp. 97-123, *in* P. Barbosa and D.K. Letourneau (eds.). Novel Aspects of Inset-Plant Interactions. Wiley-Interscience, New York.

BERGMAN, J.M., and TINGEY, W.M. 1979. Aspects of interaction between plant genotypes and biological control. *Bull. Entomol. Soc. Am.* 25:275-279.

BLOEM, K.A., and DUFFEY, S.S. 1990a. Effect of protein type and quantity on growth and development of larval *Heliothis zea* and *Spodoptera exigua* and the endoparasitoid *Hyposoter exiguae. Entomol. Exp. Appl.* 54:141-148.

BLOEM, K.A., and DUFFEY, S.S. 1990b. Interactive effect of protein and rutin on larval *Heliothis zea* and the endoparasitoid *Hyposoter exiguae. Entomol. Exp. Appl.* 54:149-160.

BLOEM, K.A., KELLEY., K.C. and DUFFEY, S.S. 1989. Differential effect of tomatine and its alleviation by cholesterol on larval growth and efficiency of food utilization in *Heliothis zea* and *Spodoptera exiguae. J. Chem. Ecol.* 15:387-398.

BLOUIN, F.A., ZARINS, Z.M., and CHERRY, J.P. 1982. Discoloration of proteins by binding with phenolic compounds, pp. 67-91, *in* J.P. Cherry (ed.). Food Protein Deterioration, Mechanisms and Functionality. ACS Symposium Series 206. American Chemical Society, Washington, D.C.

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* 72:248–254.

BROADWAY, R.M., and DUFFEY, S.S. 1986. The effect of dietary protein on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. *J. Insect Physiol.* 32:673–680.

BROADWAY, R.M., DUFFEY, S.S., PEARCE, G., and RYAN, C.A. 1986. Plant proteinase inhibitors: A defense against herbivorous insects? *Entomol. Exp. Appl.* 41:33–38.

BULL, D.L., HOUSE, V.S., ABLES, J.R., and MORRISON, R.K. 1979. Selective methods for managing insect pests of cotton. *J. Econ. Entomol.* 72:841–845.

BULLA, L.A., JR., BECHTEL, D.B., KRAMER, K.J., SHETHNA, Y.I., ARONSON, A.I., and FITZ-JAMES, P.C. 1980. Ultrastructure, physiology, and biochemistry of *Bacillus thuringiensis*. *CRC Crit. Rev. Microbiol.* 8:147–204.

CAMPBELL, B.C., and DUFFEY, S.S. 1979. Tomatine and parasitic wasps: potential incompatibility of plant antibiosis with biological control. *Science* 205:700–702.

CHESTUKHINA, G.G., KOSTINA, L.I., MIKHAILOVA, A.L., TYURIN, S.A., KLEPIKOVA, F.S., and STEPANOV, V.M. 1982. The main features of *Bacillus thuringiensis* delta-endotoxin molecular structure. *Arch. Microbiol.* 132:159–162.

COMPTON, S.J., and JONES, C.G. 1985. Mechanisms of dye response and interference in the Bradford protein assay. *Anal. Biochem.* 151:369–374.

COUCH, T.L., and ROSS, D.A. 1980. Production and utilization of *Bacillus thuringiensis*. *Biotech. Bioeng.* 22:1297–1304.

DUFFEY, S.S., and BLOEM, K.A. 1986. Plant defense–herbivore–parasite interactions and biological control, pp. 135–183, *in* M. Kogan (ed.). Ecological Theory and Pest Management Practice. Wiley-Interscience, New York.

DUFFEY, S.S., and FELTON, G.W. 1990. Role of plant enzymes in resistance to insects, pp. 289–313, *in* J.R. Whitaker and P.E. Sonnet (eds.). Enzymes in Agriculture. ACS Symposium Series 389. American Chemical Society, Washington, D.C.

DUFFEY, S.S., BLOEM, K.A. and CAMPBELL, B.C. 1985. Consequences of sequestration of plant natural products in plant–insect–parasitoid interactions, pp. 31–60, *in* D.J. Beothel and R.D. Eikenbary (eds.). Interactions of Plant Resistance and Parasitoids and Predators of Insects. Ellis Horwood Ltd., Chichester, U.K.

DULMAGE, H.T., and MARTINEZ, E. 1973. The effect of continuous exposure to low concentrations of the delta-endotoxins of *Bacillus thuringiensis* on the development of the tobacco budworm *Heliothis virescens*. *J. Invert. Pathol.* 22:14–22.

FAST, P.G., and REGNIERE, J. 1984. Effect of exposure time to *Bacillus thuringiensis* on mortality and recovery of the spruce budworm (Lepidoptera: Tortricidae). *Can. Entomol.* 116:123–130.

FAUST, R.M., and BULLA, L.A., JR., 1982. Bacteria and their toxins as insecticides, pp. 75–208, *in* E. Kurstak (ed.). Microbial and Viral pesticides. Marcel Kekker, New York.

FELTON, G.W. and DAHLMAN, D.L. 1984. Allelochemical induced stress: Effects of L-canavanine on the pathogenicity of *Bacillus thuringiensis* in *Manduca sexta*. *J. Invert. Pathol.* 44:187–191.

FELTON, G.W., and DUFFEY, S.S. 1990. Inactivation of a baculovirus by quinones formed in insect-damaged plant tissues. *J. Chem. Ecol.* 16:1221–1236.

FELTON, G.W., DUFFEY, S.S., VAIL, P.V., KAYA, H.K. and MANNING, J. 1987. Interaction of nuclear polyhedrosis virus with catechols: Potential incompatibility for host-plant resistance against noctuid larvae. *J. Chem. Ecol.* 13:947–957.

FELTON, G.W., BROADWAY, R.M., and DUFFEY, S.S. 1989a. Inactivation of protease inhibitor activity by plant-derived quinones: Complications for host-plant resistance against noctuid herbivores. *J. Insect Physiol.* 35:981–990.

FELTON, G.W., DONATO, K., DEL VECCHIO, R.J. and DUFFEY, S.S. 1989b. Activation of plant polyphenol oxidases by insect feeding reduces the nutritive quality of foliage for noctuid herbivores. *J. Chem. Ecol.* 15:2667–2694.

FERGUSON, M.P., and ALFORD, H.G. (eds.). 1985. Microbial/biorational pesticide registration: proceedings of a seminar. Cooperative Extension, University of California. Division of Agriculture and Natural Resources. Special Publication 3318.

FIELDS, R. 1972. The rapid determination of amino groups with TNBS. *Methods Enzymol.* 25B:464–468.

GASSER, C.S., and FRALEY, R.T. 1989. Genetically engineering plants for crop improvement. *Science* 244:1293–1299.

HARE, J.D. and ANDREADIS, T.G. 1983. Variation in the susceptibility of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) when reared on different host plants to the fungal pathogen, *Beauveria bassiana* in the field and laboratory. *Environ. Entomol.* 12:1891–1896.

HARTLEY, G.G., KING, E.G., BREWER, F.D., and GANTT, C.W. 1982. Rearing of the *Heliothis* sterile hybrid with a multicellular larval rearing container and pupal harvesting. *J. Econ. Entomol.* 75:7–10.

HAYASHIYA, K. 1978. Red fluorescent protein in the digestive juice of the silkworm larvae fed on host-plant mulberry leaves. *Entomol. Exp. Appl.* 24:228–236.

HOFMANN, C., LUETHY, P., HUETTER, R., and PLISKA, V. 1988a. Binding of the delta endoxotin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (*Piersis brassicae*). *Eur. J. Biochem.* 173:85–91.

HOFMANN, C., VANDERBRUGGEN, H., HOEFTE, H., VAN RIE, J., JANSENS, S., and VAN MELLAERT, H. 1988b. Specificity of *Bacillus thuringiensis* delta-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. *Proc. Natl. Acad. Sci. U.S.A.*

HUBER, H.E., LUETHY, P., EBERSOLD, H.-R., and CORDIER, J.-L. 1981. The subunits of the parasporal crystal of *Bacillus thuringiensis:* Size, linkage and toxicity. *Arch. Microbiol.* 129:14–18.

HURRELL, R.F., CUQ, J.L., and FINOT, P.A. 1982. Protein–polyphenol reactions. 1. Nutritional and metabolic consequences of the reaction between oxidized caffeic acid and the lysine residues of casein. *Br. J. Nutr.* 47:191–211.

IGNOFFO, C.M., HOSTETTER, D.L., and PINNELL, R.E. 1974. Stability of *Bacillus thuringiensis* and *Baculovirus heliothis* on soybean foliage. *Environ. Entomol.* 3:117–119.

IIZUKA, L., KOIKE, S., and MITZUTANI, J. 1974. Antibacterial substances in feces of silkworm larvae reared on mulberry leaves. *Agric. Biol. Chem.* 38:1549–1550.

ISMAN, M.B., and DUFFEY, S.S. 1983. Pharmacokinetics of chlorogenic acid and rutin in larvae of *Heliothis zea. J. Insect Physiol.* 29:295–300.

JAQUES, R.P., and MORRIS, O.N. 1981. Compatibility of pathogens with other methods of pest control and with different crops, pp. 695–716, *in* H.D. Burges (ed.). Microbial Control of Pests and Plant Diseases 1970–1980. Academic Press, London.

JONES, C.G. 1984. Microorganisms as mediators of plant resource exploitation by insect herbivores, pp. 53–99, *in* P.W. Price, C.N. Slobodchikoff, and W.S. Gaud (eds.). A New Ecology: Novel Approaches to Interactive Systems. Wiley-Interscience, New York.

KOGAN, M. 1986. Plant defense strategies and host-plant resistance, pp. 83–134, *in* M. Kogan (ed.). Ecological Theory and Integrated Pest Management. Wiley-Interscience, New York.

KOIKE, S., IIZUKA, T., and MIZUTANI, J. 1979. Determination of caffeic acid in the digestive juice of silkworm larvae and its antibacterial activity against the pathogenic *Streptococcus faecalis* AD-4. *Agric. Biol. Chem.* 43:1727–1731.

KRISCHIK, V.A., BARBOSA, P. and REICHELDERFER, C.F. 1988. Three trophic level interactions:

allelochemicals, *Manduca sexta* (L.), and *Bacillus thuringiensis* var. *kurstaki* Berliner. *Environ. Entomol.* 17:476–482.

KUNIMI, Y., and ARUGA, H. 1974. Susceptibility to infection with nuclear and cytoplasmic polyhedrosis virus of the fall webworm, *Hyphantria cunea* Drury reared in several artificial diets. *Japan J. Appl. Entomol. Zool.* 18:1–4.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.

LEONG, K.L.H., CANO, R.J., and KUBINSKI, A.M. 1980. Factors affecting *Bacillus thuringiensis* total field persistence. *Environ. Entomol.* 9:593–599.

MACINTOSH, S.C., KISHORE, G.M., PERLAK, F.J., MARRONE, P.G., STONE, P.G., STONE, T.B., SIMS, S.R., and FUCHS, R.L. 1990. Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. *J. Agric. Food Chem.* 38:1145–1152.

MASYMIUK, B. 1970. Occurrence and nature of antibacterial substances in plants affecting *Bacillus thuringiensis* and other entomopathogeneous bacteria. *J. Invert. Pathol.* 15:345–371.

MATHEIS, G., and WHITAKER, J.R. 1984. Modification of proteins by PPO and peroxidase and their products. *J. Food Biochem.* 8:137–162.

MEEUSEN, R.L., and WARREN, G. 1989. Insect control with genetically engineered crops. *Ann. Rev. Entomol.* 34:373–381.

MERDAN, A., ABDEL-RAHMAN, H., and SOLIMAN, A. 1975. On the influence of host plants on insect resistance to bacterial diseases. *Z. Angew. Entomol.* 78:280–285.

MOAR, W.J., OSBRINK, W.L.A., and TRUMBLE, J.T. 1986. Potentiation of *Bacillus thuringiensis* var. *kurstaki* with thuringiensin on beet armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 79:1443–1446.

MOHAMED, A.I., YOUNG, S.Y., and YEARIAN, W.C. 1983. Susceptibility of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) larvae to microbial agent-chemical pesticide mixtures on cotton foliage. *Environ. Entomol.* 12:1403–1405.

MOLE, S., and WATERMAN, P.G. 1985. Stimulatory effects of tannins and cholic acid on tryptic hydrolysis of proteins: Ecological implications. *J. Chem. Ecol.* 11:1323–1332.

MORRIS, O.N. 1974. Inhibitory effects of foliage extracts of some forest trees on commercial *Bacillus thuringiensis*. *Can. Entomol.* 104:1357–1361.

PFRIMMER, T.R. 1979. *Heliothis* ssp.: Control on cotton with pyrethroids, carbamates, organophosphates, and biological insecticides. *J. Econ. Entomol.* 72:592–598.

PIERPOINT, W.S. 1983. Reaction of phenolic compounds with proteins, and their relevance to the production of leaf protein, pp. 235–267, *in* L. Telek and H.D. Graham (eds.). Leaf Protein Concentrates. AVI Publishing, Westport, Connecticut.

PIERPOINT, W.S., IRELAND, R.J., and CARPENTER, J.M. 1977. Modification of proteins during the oxidation of leaf phenols. Reaction of potato virus X with chlorogenoquinone. *Phytochemistry* 16:29–34.

PRICE, P.W. 1986. Ecological aspects of host plant resistance and biological control: Interactions among these trophic levels, pp. 11–30, *in* D.J. Boethel and R.D. Eikenbary (eds.). Interactions of Plant Resistance and Parasitoids and Predators of Insects. Ellis Horwood Ltd., Chichester, U.K.

PRICE, P.W., BOUTON, C.E., GROSS, P., MCPHERON, B.A., THOMPSON, J.N., and WEIS, A.E. 1980. Interactions among three trophic levels: Influence of plants on interactions between insect herbivores and natural enemies. *Ann. Rev. Ecol. Syst.* 11:41–65.

PUIGSERVER, A.J., SEN, L.C., GONZALES-FLORES, E., FEENEY, R.E., and WHITAKER, J.R. 1979. Covalent attachment of amino acids to casein. 1. Chemical modification and rates of in vitro enzymatic hydrolysis of derivatives. *J. Agric. Food Chem.* 27:1098–1104.

RETNAKARAN, A., LAUZON, H., and FAST, P. 1983. *Bacillus thuringiensis* induced anorexia in the spruce budworm, *Christoneura fumiferana*. *Entomol. Exp. Appl.* 34:233–239.

RYAN, C.A. 1979. Proteinase inhibitors, pp. 599–618, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores, Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

SALAMA, H.S., FODA, M.S., and SHARABY, A. 1984. Novel biochemical avenues for enhancing *Bacillus thuringiensis* endotoxin potency against *Spodoptera littoralis* (Lep.: Noctuidae). *Entomophaga* 29:171–178.

SCHNEPF, H.E., WONG, H.C., and WHITELEY, H.R. 1985. The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. *J. Biol. Chem.* 260:6264–6272.

SEN, L.C., LEE, H.S., FEENEY, R.E., and WHITAKER, J.R. 1981. In vitro digestibility and functional properties of chemically modified casein. *J. Agric. Food Chem.* 29:348–354.

SHAPE, E.S., NICKERSON, K.W., BULLA, L.A., JR., and ARONSON, J.A. 1975. Separation of spores and parasporal crystals of *Bacillus thuringiensis* in gradients of certain X-ray contrasting agents. *Appl. Microbiol.* 30:1052–1053.

SHEPARD, M., and DAHLMAN, D.L. 1988. Plant-induced stresses as factors in natural enemy efficacy, pp. 363–379, *in* E.A. Heinrichs (ed.). Plant Stress–Insect Interactions. Wiley Interscience, New York.

SMIRNOFF, W.A., and HUTCHINSON, P.M. 1965. Bacteriostatic and bacteriocidal effects of extracts of foliage from various plant species on *Bacillus thuringiensis* var. *thuringiensis* Berliner. *J. Invert. Pathol.* 7:273–280.

STUBBLEBINE, W.H., and LANGENHEIM, J.H. 1977. Effects of *Hymenaea coubaril* leaf resin on the generalist herbiovore *Spodoptera exigua* (beet armyworm). *J. Chem. Ecol.* 3:633–647.

TYRELL, D.J. BULLA, L.A., JR., and DAVIDSON, L.I. 1981. Characterization of spore coat proteins of *Bacillus thuringiensis* and *Bacillus cereus*. *Comp. Biochem. Physiol.* 70B:535–539.

VAECK, M., HOEFTE, H., REYNAERTS, A., LEEMANS, J., VAN MONTAGU, M., and ZABEAU, M. 1987. Engineering of insect resistant plants using a *B. thuringiensis* gene, pp. 355–366, *in* C.J. Arntzen and C.A. Ryan (eds.). Molecular Strategies for Crop Protection. UCLA Symposia on Molecular and Cellular Biology, Vol. 48. Alan R. Liss, New York.

VAN SUMERE, C.F., ALBRECHT, J., DEDONDER, A., DE POOTER, H., and PE, I. 1975. Plant proteins and phenolics, pp. 211–264, *in* J.B. Harborne and C.F. van Sumere (eds.). The Chemistry and Biochemistry of Plant Proteins. Academic Press, London.

WINDHOLZ, M., BUDAVARI, S., BLUMETTI, R.F., and OTTERBEIN, E.S. (eds.). 1983. The Merck Index: An Enyclopedia of Chemicals, Drugs, and Biologicals, 10th ed. Merck & Co., Rahway, New Jersey.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

IDENTIFICATION OF HAIRPENCIL SECRETION FROM MALE *Mamestra brassicae* (L.) (LEPIDOPTERA: NOCTUIDAE) AND ELECTROANTENNOGRAM STUDIES

E. JACQUIN,* P. NAGNAN, and B. FREROT

Laboratoire des Médiateurs chimiques
Domaine de Brouessy
INRA
Magny les Hameaux. F-78114, France

(Received May 23, 1990, accepted September 10, 1990)

Abstract—Extracts of male *Mamestra brassicae* (L.) hairpencils were analyzed by capillary gas chromatography (GC) and by GC-mass spectrometry (GC-MS). The extracts were found to consist of six components. Benzaldehyde, 2-methylpropanoic acid, 2-methylbutanoic acid, and phenol were present in the extracts as well as the previously identified benzyl alcohol and phenyl ethanol. The two major components were 2-phenylethanol and 2-methylbutanoic acid. They represented, respectively, 74% and 12.5% of the total blend in 3-day-old male extracts. Electroantennograms were recorded on male and female antennae in response to stimulation by hairpencil compounds. Male and female antennae responded to each chemical but the female responses were significantly higher than those of the males.

Key Words—*Mamestra brassicae*, Lepidoptera, Noctuidae, hairpencils, male-produced compounds, identification, benzaldehyde, 2-methylpropanoic acid, 2-methylbutanoic acid, benzyl alcohol, 2-phenylethanol, phenol, electroantennography.

INTRODUCTION

Like many noctuid moths, male *Mamestra brassicae* (L.) possess evertable scent organs known as hairpencils, located in abdominal pockets situated on the first and second abdominal sternites. These structures are associated with two inter-

*To whom correspondence should be addressed.

0098-0331/91/0100-0239$06.50/0

nal glands, described by Stobbe (1912), and are everted during courtship behavior. In the vicinity of the female, they release an odor detectable by the human nose. In an attempt to elucidate the effect of male hairpencils and their associated odors on calling females, we compared in a previous work the courtship behavior of males with and without hairpencils (Jacquin, 1989). The number of attempted copulations, followed by either the rejection or the acceptation of the male by the female, was quantified. Our results, according to Birch et al. (1989), suggest that the presence of male hairpencils increases female acceptance. Observed behaviors suggested that hairpencil secretions could affect the recognition of the sexual partners and could be an important factor in sexual selection.

In order to provide further evidence for putative functions of the hairpencil secretions, biological tests using pheromonal blends could be used. A reinvestigation of the chemical composition of *M. brassicae* hairpencil extracts is therefore necessary since no recent study has been reported. Bestmann et al. (1977) identified two compounds in hairpencil extracts of males *M. brassicae*: 2-phenylethanol (96%) and benzyl alcohol (4%). This paper will discuss the identification of new components and their electrophysiological significance.

METHODS AND MATERIALS

Insects. The insects were reared on a cabbage semisynthetic diet (Poitout and Bues, 1974) and kept under a reversed light–dark 16:8 photoperiod at 20 $\pm$ 2°C at every life stage. The insects were sexed as pupae, and the adults were kept in hermetic plastic containers and fed with a sugar solution (10%). Only naive 3-day-old adults were used in this experiment.

Hairpencil Extracts. Four hours after the onset of the scotophase, hairpencils were carefully excised out of individual males that had previously been anesthetized with carbon dioxide. The third day of insect life corresponded to the optimum of male sexual activity under our experimental conditions. All dissections were carried out on males that presented a complete retraction of the hairpencils in their abdominal pockets. Each pair of individual male hairpencils was quickly immersed into a glass vial containing 200 μl of pentane (SDS, Vitry, France) and was left to extract at room temperature for one hour. This was repeated with 10 different males. Solutions were then condensed to 100 μl under a stream of nitrogen before analysis.

Chemical Analysis. Identifications were performed by capillary gas chromatography (GC) and by gas chromatography-mass spectrometry coupling (GC-MS). GC analyses were performed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) and a FFAP column 25 m $\times$ 0.32 mm (ID) (Chrompack) using helium as the carrier gas (P_{He} = 2.76

bar). Oven temperature was programmed to increase from 60°C to 140°C at 3°C/min. Samples were injected on a Ross injector after 10 sec of evaporation or on a split/splitless injector. Injector temperature was 220°C and detector temperature was 250°C.

GC-MS identification was conducted on a Nermag 10-10C quadrupolar mass spectrometer using a Sidar data system (EI, 70 eV, 30–400 amu). The GC was equipped with a nonpolar capillary column, CPSIL5 CB, 25 m × 0.32 mm (ID) (Chrompack) programmed to increase from 60°C to 140°C at 3°C/min.

Identifications of the extract components were determined by comparing their retention times on both polar and nonpolar columns and their spectra with those of reference compounds (Aldrich Chimie).

Electroantennography. Compounds identified in the crude extracts were tested on both male and female antennae. Electroantennograms (EAG) were recorded according to the technique described by Renou (1979). Chemicals were diluted in hexane and applied on filter papers at a dose of 10 μg. Stimulations with air alone and hexane alone (1 μl) were applied as check samples. The stimulation cartridges were only used twice and then renewed to prevent loss of charge due to the volatility of the chemicals. A 1-sec pulse of air, delivered at 60 liters/hr, was allowed to pass through the cartridge. Five male and five female antennae were tested twice each with the set of chemicals.

RESULTS

Identification of Hairpencil Components. GC analyses showed six peaks (Figure 1). The retention times of peaks 4 and 5 matched the retention times of, respectively, benzyl alcohol and 2-phenylethanol, which had previously been identified by Bestmann et al. (1977). GC-MS spectra confirmed these results. The first peak exhibited the same retention time as that of benzaldehyde. This identification was confirmed by the mass spectrum, which matched the reference benzaldehyde spectrum. Peak 6 had the same retention time and mass spectrum as phenol, whereas peaks 2 and 3 showed characteristic ions of 2-methylpropanoic acid and of 2-methylbutanoic acid. The retention times of both acids corresponded with those of the reference samples for the two types of columns.

Analyses of individual pairs of hairpencils from 10 individual males confirmed the occurrence of the six previously identified compounds. The percentage composition of the hairpencil extracts is: 1% benzaldehyde, 3.5% 2-methylpropanoic acid, 12.5% 2-methylbutanoic acid, 6.5% benzyl alcohol, 74% 2-phenylethanol, and 2.5% phenol (Table 1).

Studies of the correlations (Spearmann test) between the relative percent-

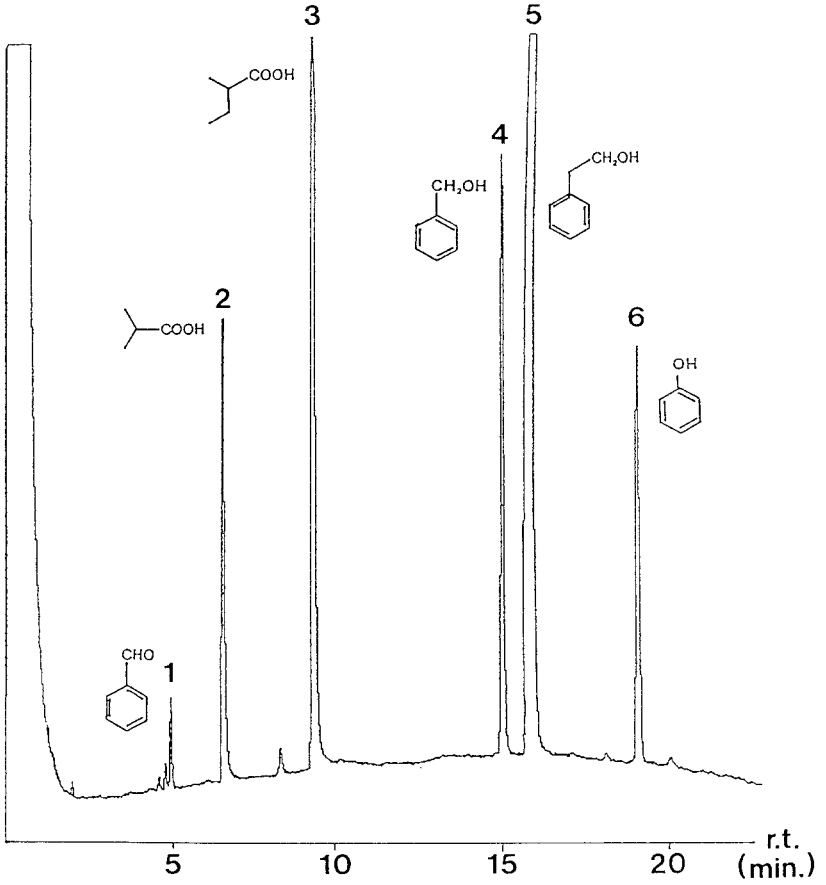


FIG. 1. Capillary column gas chromatogram of hairpencil extract of a virgin *Mamestra brassicae* male (2 μl out of 100 μl extract, other conditions in text). **1:** benzaldehyde; **2:** 2-methylpropanoic acid; **3:** 2-methylbutanoic acid; **4:** benzyl alcohol; **5:** 2-phenylethanol; **6:** phenol.

ages of these six compounds showed there to be negative correlations between phenylethanol and the other compound ratio ($P < 0.05$). However, phenol did not correlate with any of the five other compounds. The correlations were positive between each of the others ($P < 0.05$).

Electroantennographic Results. Electroantennogram recordings indicated that both male and female antennae responded to the six identified compounds (Figure 2). All six compounds were significantly better perceived by the female antennae than were the controls of hexane or air alone ($P < 0.01$). However, the responses of male antennae to the 2-methylpropanoic acid did not

TABLE 1. HAIRPENCIL EXTRACT COMPOSITION

Compounds	Percentage composition (means ± SEM, $N = 10$)
Benzaldehyde	1 ± 0.12
2-me.prop.ac.	3.5 ± 0.6
2-me.but.ac.	12.5 ± 1
Benzyl OH	6.5 ± 0.6
2-phenyl ethOH	74 ± 2.3
Phenol	2.5 ± 0.7

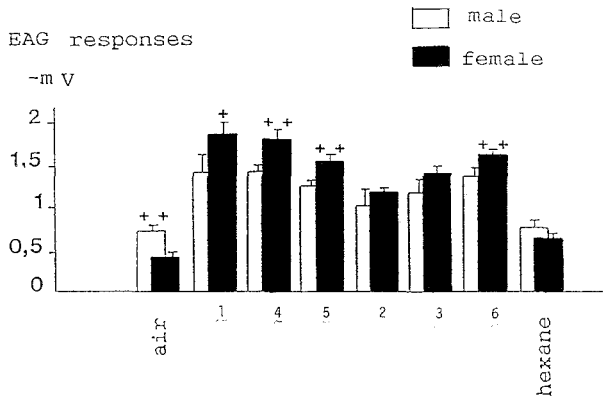


FIG. 2. Average values of EAG potentials evoked on five antennae of both male and female *Mamestra brassicae* by 10 μg of each identified compound. Vertical bars indicate the standard error. (+) or (++) above responses indicates that the responses are significantly different between male and female (Mann-Whitney: + $P < 0.05$, ++ $P < 0.01$). All the responses to the test compounds are significantly different from controls (air and hexane) ($P < 0.05$) except for the male response to 2-methylpropanoic acid (**2**).

significantly differ from the responses to the hexane control. The other compounds elicited significantly higher responses than hexane on male antennae ($P < 0.05$ for 2-methylbutanoic acid and $P < 0.01$ for all others).

Both males and females showed the same response pattern to the six components of hairpencil extracts. The highest responses were evoked by benzaldehyde, benzyl alcohol, 2-phenylethanol, and phenol for both sexes. The 2-methylpropanoic acid and the 2-methylbutanoic acid showed lower activities. The comparison of male and female EAG responses to the six compounds

showed that female antennae gave significantly higher responses, except for the two acids (Figure 2).

DISCUSSION

Low-molecular-weight compounds have been isolated previously from hairpencils of various noctuid males (reviewed by Zagatti, 1981; Boppré, 1984; Fitzpatrick and McNeil, 1988; Birch et al., 1990). All are simple terpenoids, aromatics, and carboxylic acids. The six compounds isolated in *Mamestra brassicae* hairpencil extracts are structurally similar and have been reported previously in other noctuids as hairpencil components (Aplin and Birch, 1970). However, phenol has never been identified in male hairpencils of any other lepidoptera. Nevertheless, phenols are described as defensive allomones of millipedes (Blum, 1987) and as a sex attractant for the grass grub beetle *Costelytra zealandica* (Henzell and Lowe, 1970). Phenols also are known to be produced by wounded trees and to act as a repellent or a toxic agent to bark beetles (Raffa and Berryman, 1983). Fitzpatrick et al. (1989) relate that many noctuids are attracted to exudates of tree wounds. It is curious to find phenol as a hairpencil constituent, and its biological function could differ from the other component functions. Further behavioral studies will be conducted to test the different functions of the hairpencil components.

Benzaldehyde is an extremely common hairpencil component and is found to be a major component of male scent brushes of many *Leucania* and *Mythimna* species, and a minor component in *Polia nebulosa* (Hufn.) and *Mamestra persicariae* (L.) (Aplin and Birch, 1970). Up to now, it has not been detected in *M. brassicae*. This was probably due to the low quantity present in hairpencil extracts (1%).

In our experiment, benzyl alcohol and 2-phenylethanol have been described as hairpencil components of *M. brassicae* with approximately the same relative ratios as those published by Bestmann et al. (1977). However, 2-methylpropanoic acid and 2-methylbutanoic acid have been newly identified in this study as components of *M. brassicae* hairpencil extracts. The 2-methylpropanoic acid has been found at a level of about 20% in hairpencil extracts of several Hadeninae [*Leucania impura* (Hueb.), *L. conigera* (Schiff.), *L. pallens* (L.)] (Aplin and Birch, 1970). 2-Methylbutanoic acid is also produced by some noctuid species (Amphipyrinae and Cucullinae) but has never been reported in Hadeninae. This compound appeared in our experiment to be one of the main compounds of *M. brassicae* male extracts (12.5%).

The 10 analyses of individual male hairpencil extracts reflected a low vari-

ability of the component percentages between individuals. However, the quantities detected seemed to be very variable from one male to another.

EAG recordings indicated that female antennae responded to the six compounds identified in male hairpencil extracts. It is of interest that male *M. brassicae* also responded to the majority of their own hairpencil components, except to 2-methylpropanoic acid. This suggests that the male scent could affect male behavior. Although Toth (1982) does not find any evidence for male-to-male inhibition, other effects should be considered.

Male and female antennae responded in the same way to the different hairpencil components. This is in accordance with the results on *Trichoplusia ni* (Hübn.) (Grant, 1970) and suggests that the antennae of both sexes possess similar receptors for detecting male scents. Nevertheless, nothing is known on the specificity of those receptors for the hairpencil components.

The female responses were higher than the male responses. As EAG is thought to be the summed potential of the activated receptor cells of the antennae (Boeckh, 1969), our results suggest that female antennae possess more acceptor sites for all of the compounds than do the male antennae.

The fact that the phenol ratio did not correlate with the ratios of the other identified compounds appears to be consistent with the hypothesis that there is a different metabolic origin between this chemical and the other aromatic compounds. Phenol is known to originate in tyrosine (Blum, 1987) while phenylethanol and benzaldehyde originate in L-phenylalanine (Clearwater, 1975). The two acid precursors are not known and could be valine and isoleucine for, respectively, 2-methylpropanoic acid and 2-methylbutanoic acid as described in other insects (Blum, 1987).

Identification of new components in *Mamestra brassicae* hairpencils may provide some clues for the understanding of the specificity of chemical communication between males and females. Many noctuid males within Hadeninae are considered to produce closely related scent blends, but it may be worthwhile to reinvestigate hairpencil secretions by GC on capillary columns and by GC-MS. Minor unidentified components could act to promote species isolation. To date, we do not know whether the female can discriminate variable blends of the same components or whether the quality of the blend can interact with sexual behavior and act on sexual selection. Males seem to produce variable quantities of the same blend of compounds. Does this fact interact with the female choice or acceptance? Further studies on this subject will be conducted on *Mamestra brassicae*.

Acknowledgments—The authors are pleased to thank Dr. P. Zagatti and Dr. M. Renou for their helpful comments, Dr. C. Descoins for reviewing the manuscript and C. Malosse for GC and GC-MS advice.

REFERENCES

APLIN, R.T., and BIRCH, M.C. 1970. Identification of odorous compounds from male Lepidoptera. *Experientia* 26:1193–1194.

BESTMANN, H.J., VOSTROWSKY, O., and PLATZ, H. 1977. Männchenduftstoffe von Noctuiden (Lepidoptera). *Experientia* 33:874–875.

BIRCH, M.C., LUCAS, D., and WHITE, P.R. 1989. The courtship behavior of the cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae), and the role of hairpencils. *J. Insect. Behav.* 2:227–239.

BIRCH, M.C., POPPY, G.M. and BACKER, T.L. 1990. Scents and eversible scent structures of male moths. *Annu. Rev. Entomol.* 35:25–58.

BLUM, M.S. 1987. Biosynthesis of arthropod exocrine compounds. *Annu. Rev. Entomol.* 32:381–413.

BOECKH, J. 1969. Electrical activity in olfactory receptor cells, pp. 34–51, *in* C. Pfaffman (ed.). Olfaction and Taste, Vol III. Rockefeller University Press, New York.

BOPPRÉ, M. 1984. Chemically mediated interactions between butterflies, pp. 261–275, *in* R.I. Vane-Wright and P.R. Ackery (eds.). The Biology of Butterflies. Academic Press, London.

CLEARWATER, J.R. 1975. Synthesis of a pheromone precursor in the male noctuid moth, *Mamestra configurata. J. Insect Biochem.* 5:737–746.

FITZPATRICK, S.M. and MCNEIL, J.N. 1988. Male scent in Lepidopteran communication: The role of male pheromone in mating behaviour of *Pseudaletia unipuncta* (Haw.) (Lepidoptera: Noctuidae). *Mem. Entomol. Soc. Can.* 146:131–151.

FITZPATRICK, S.M., MCNEIL, J.N., and MILLER, D. 1989. Age-specific titer and antennal perception of acetic acid, a component of male *Pseudaletia unipuncta* (Haw.) hairpencil secretion. *J. Chem. Ecol.* 15:641–648.

GRANT, G.G. 1970. Evidence for a male sex pheromone in the noctuid, *Trichoplusia ni. Nature* 227:1345–1346.

HENZELL, R.F., and LOWE, M.D. 1970. Sex attractant of the grass grub beetle. *Science* 168:1005–1006.

JACQUIN, E. 1989. Etude comportementale et électrophysiologique des phéromones mâles de la noctuelle du chou *Mamestra brassicae* (L.). Mémoire de DAA Protection des Cultures, INAPG Paris. 24 pp.

POITOUT, S., and BUES, R. 1974. Elevage de 28 espèces de lépidoptères Noctuidae et de 2 espèces d'Arctiidae sur milieu artificiel simplifié. Particularités selon les espèces. *Ann. Zool. Ecol. Anim.* 6:431–441.

RAFFA, K.F., and BERRYMAN, A.A. 1983. Physiological aspects of lodgepole pine wound responses to a fungal symbiont of the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytiidae). *Can. Entomol.* 115:723–734.

RENOU, M. 1979. Contribution à l'etude de la communication phéromonale chez trois Tinèides ravageurs des cultures: *Acrolepiopsis assectella* (Z.), *Scobipalpa ocellatella* (Boyd.), *Prays oleae* (Bern.), Thèse de 3ème cycle, Paris VI. 161 pp.

STOBBE, R. 1912. Die abdominalen Duftorgane der Männlichen Sphingiden und Noctuiden. *Zool. Jb.* 32:493–532.

TOTH, M. 1982. Male produced pheromone of *Mamestra brassicae* L. (Lepidoptera: Noctuidae): Its possible role in courtship behaviour and study of other effects. *Acta Phytopathol. Acad. Sci. Hung.* 17:123–132.

ZAGATTI, P. 1981. Micro-comportements induits par les phéromones sexuelles chez quelques lépidoptères ravageurs des cultures en milieu sahélien. Thèse de 3ème cycle, Paris VI. 161 pp.

Journal of Chemical Ecology, Vol. 17, No. 1, 1991

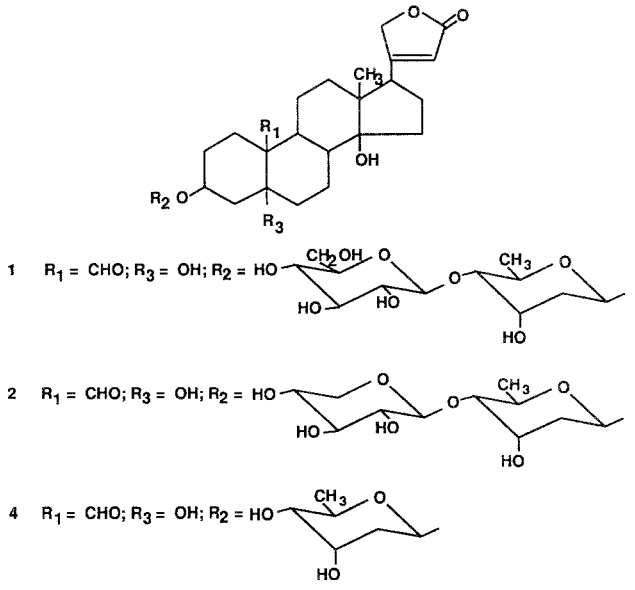
Erratum

ISOLATION AND IDENTIFICATION OF OVIPOSITION DETERRENTS TO CABBAGE BUTTERFLY, *Pieris rapae*, FROM *Erysimum cheiranthoides*

K. SACHDEV-GUPTA, J. A. A. RENWICK, and C. D. RADKE

Boyce Thompson Institute
Cornell University
Ithaca, New York 14853

Readers should note the following correction to Figure 1 (structures 1, 2, and 4).



REFERENCE

SACHDEV-GUPTA, K., RENWICK, J. A. A., and RADKE, C. D. 1990. *J. Chem. Ecol.* 16:1059–1067.

0098-0331/91/0100-0247$06.50/0

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

MINT ROOT BORER, *Fumibotys fumalis* (Guenee)[1]: IDENTIFICATION AND FIELD TESTS OF FEMALE SEX PHEROMONE GLAND COMPONENTS

H.G. DAVIS,[2] L.M. McDONOUGH,[2,*] C.L. SMITHHISLER,[2] D.F. BROWN,[2] B.A. LEONHARDT,[3] S. VOERMAN,[4] and P.S. CHAPMAN[2]

[2]*Agricultural Research Service, USDA*
Yakima, Washington 98902

[3]*Agicultural Research Service, USDA*
Beltsville, Maryland 20705

[4]*Research Institute for Plant Protection*
P.O. Box 9060
6700 GW Wageningen, The Netherlands

(Received July 12, 1990; accepted September 17, 1990)

Abstract—Compounds identified in sex pheromone gland extracts of female *Fumibotys fumalis* (Guenee) consisted of (*E*,*E*)-10,12-tetradecadienyl acetate, (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, and (*Z*)-9-tetradecenyl acetate in a ratio of 100:18:8:4, respectively. The compounds were identified by electroantennographic, gas chromatographic, mass spectrometric, and chemical derivatization procedures. In mint fields synthetic components in gray elastomeric septa at ratios found in the sex pheromone gland and at doses of 3 or 10 mg of the diene produced trap catch comparable to traps baited with three females.

Key Words—Lepidoptera, Pyralidae, Pyraustinae, Pyraustini, *Fumibotys fumalis*, sex pheromone, (*E*,*E*)-10,12-tetradecadienyl acetate, (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, (*Z*)-9-tetradecenyl acetate.

INTRODUCTION

The mint root borer, *Fumibotys fumalis* (Guenee) is widely distributed in North America (Monroe, 1976). It was first reported to be a pest of a cultivated crop

*To whom correspondence should be addressed.
[1]Lepidoptera: Pyralidae.

in 1971 when it was found damaging commercially grown peppermint, *Mentha piperita* L. in the Willamette Valley of Oregon (Berry, 1974). Subsequently, *F. fumalis* adapted to and damaged spearmint, *M. spicata* L., and became an established pest in the commercial mint fields of Washington, Idaho, and Oregon (Pike and Glazer, 1982). Damage occurs when the larvae bore into and feed on the mint rhizomes. Infested fields show declining yields and shortened stand life (Pike et al., 1988).

The taxonomy, morphology, and geographical distribution of *F. fumalis* were described by Monroe (1976). Studies of its biology and behavior (Forbes, 1923; Berry, 1974, 1977; Davis et al., 1984; Pike et al., 1988) and chemical and cultural control (Pike, 1979; Pike and Getzin, 1981; Pike and Glazer, 1982) have been reported.

Because control procedures would be enhanced by a lure to monitor adult flight periods, we undertook a chemical and biological study in order to develop an effective sex pheromone attractant. An earlier biological study (Davis et al., 1984) demonstrated a female sex pheromone in *F. fumalis*, that maximum mating occurred between 12 PM and 1 AM, that females were maximally attractive from age 0.5–7 days, and that females were not attractive the night following mating. Here we report the identification of four components of the female sex pheromone gland and the development of an effective field lure.

METHODS AND MATERIALS

Insects. F. fumalis hibernaculae containing prepupae were collected from soil samples taken from infested peppermint fields near Harrah, Washington, in February and early March in 1987, 1988, and 1989. The hibernaculae were placed in clear plastic shoe boxes (31 × 17 × 9 cm) on a 5- to 6-cm layer of moistened peat moss, covered with clear plastic lids, and held in a rearing room at a temperature of 12.8°C, relative humidity of 55–60%, and a 12 : 12 hr light–dark cycle. As adults were needed to conduct laboratory or field studies, boxes were removed and placed in another facility at a temperature of 21.1°C and a 15 : 9 hr light–dark cycle. The onset of the dark cycle was set at 0800 hr for the flight-tunnel studies and at 2100 hr for the field studies. Emergent adults were collected daily at least 1 hr before the dark period and placed in individual vials.

Collection of Pheromone. Female moths (3–4 days old) in their vials were collected 6 hr after the beginning of scotophase and placed in a refrigerator to inactivate them. They were removed individually and their abdominal tips containing the sex pheromone gland (SPG) were severed and steeped for 15–60 min in dichloromethane. Then the solution was removed with a syringe. To obtain pheromone that the females may have released while inside the vials, the vials were rinsed with dichloromethane and the rinses were combined.

Electroantennogram (EAG) Determinations. EAG determinations of model compounds were made with previously described apparatus (McDonough et al., 1980) except that 60-μg charges were used. Duplicate determinations were made of each compound. The model compounds used were synthesized mainly by the procedure of Voerman (1988). A few were obtained from commercial sources. All compounds were at least 98% pure by capillary gas chromatographic analysis and contained 1% or less of their geometric isomers. The compounds used were: all of the saturated and monoene 12- and 14-carbon alcohols and corresponding acetate; *Z* and *E* isomers of 7–, 9–, and 11–16 : Ac; *Z* and *E* isomers of 7–, 8–, and 9–12 : Al; *Z*7–14 : Al, *Z*9–14 : Al, *Z*11– and *E*11–14 : Al; *Z*5–16 : Al, *Z*7– and *E*7–16 : Al, *Z*9–16 : Al, *Z*11– and *E*11–16 : Al, *Z*13–16 : Al.

Gas Chromatography–Electroantennography (GC-EAG). Fractions of extracts from sex pheromone glands or vial washes containing female emissions were collected from a GC equipped with a 0.63-cm-OD × 180-cm-long glass column packed with 3% methyl silicone (SE-30) liquid phase on 80–100 mesh Gas Chrom Q and connected to an effluent splitter. The initial column temperature was 120°C for 8 min, then programmed at 4°/min to 210°C and held at that temperature. The fractions were collected from time zero to the time corresponding to the complete elution of 10 : Ac and then successively at times corresponding to the complete elution of each of the saturated acetates from 11 : Ac through 20 : Ac. Fractions were collected in 3-mm-OD, U-shaped, glass traps cooled by Dry Ice–acetone, and then dissolved in dichloromethane and deposited on the inside of glass tubes for EAG determinations. Fractions also were collected for EAG determination from a 0.63-cm-OD × 180-cm-long glass column packed with 5% Carbowax 20M liquid phase on 80–100 mesh Gas Chrom Q. Fraction 1 encompassed time zero to the beginning of the elution of 10 : Ac; succeeding fractions each encompassed two carbon increments to the beginning of the elution of 20 : Ac.

Gas Chromatography–Mass Spectrometry (GC-MS). A Hewlett-Packard (Avondale, Pennsylvania) gas chromatograph (model 5790) with a quadrupole mass spectrometer (model 5970) was equipped with either a DB-1 or DB-Wax capillary column, 60 m × 0.25 mm ID (J & W Scientific, Folsom, California). When the pheromone gland extracts were analyzed, the columns were held at 80°C for 2 min, programmed at 20°/min to 190°C for DB-1, and to 200°C for DB-Wax, and maintained isothermally thereafter. Total ion abundance was monitored. When the dimethyl disulfide derivatives of the monoenes were analyzed, the DB-1 column was used and was held at 80°C for 2 min, programmed at 20°/min to 255°C and held at that temperature for 30 min.

Determination of Positions and Configurations of Double Bonds of Monoenes. Monoenes from extract of sex pheromone glands were provisionally identified by comparing retention times on DB-1 (60 m × 0.25 mm; 2 min at 80°C then 20°/min to 190°C) and DB-Wax (45 m × 0.20 mm ID at 145°C)

GC capillary columns with synthetic monoenes. A Hewlett-Packard model 5880 GC was used.

Rigorous determination of double-bond positions was accomplished by mass spectral determinations of fragmentation patterns of the dimethyl disulfide (DMDS) derivatives (Buser et al., 1983). To prepare the derivatives, monoenes (15 ng) isolated as a GC fraction from extract of SPG in 200 μl of dichloromethane were transferred to a 1-ml Reacti-vial (Pierce Chemical Co., Rockford, Illinois). The solution was evaporated just to dryness in a stream of nitrogen, and the residue was dissolved in 50 μl heptane. Then 50 μl DMDS and 10 μl of a saturated solution of iodine in ether were added. The vial was sealed and heated overnight at 40°C in a convection oven. The solution was diluted with 200 μl of heptane and excess I_2 was removed by shaking with 100 μl of 5% of sodium bisulfite solution. The heptane solution was moved with a syringe and concentrated to 2 μl for injection into the GC-MS.

Determination of Positions and Configurations of Double Bonds of Diene. The GC purified diene (144 ng) was ozonized in predistilled dichloromethane at −70°C with an ultraviolet ozonizer (Orec Co. model 03V1, Phoenix, Arizona), and the ozonide was reduced with triply recrystallized triphenylphosphine. The ozonized sample was injected on a GC column (DB-1, 60 m × 0.25 mm), which was held at 80°C for 7 min, programmed at 6°/min to 200°C and held at that temperature. When the configuration of the diene was determined, the same column was held at 80°C for 2 min and programmed at 20°/min to 180°C and held at that temperature.

Flight Tunnel. A flight tunnel constructed as described by Miller and Roelofs (1978) was used. The flight tunnel room was maintained at 22°C. Males were released in groups of five. The number of males initiating and sustaining flight and contacting the lure were determined. All lures were formulated in gray elastomeric septa (formulation number 1888, size no. 1, West Co., Phoenixville, Pennsylvania) (Brown and McDonough, 1986) to prevent isomerization of the diene. Males were three to four days postemergence and were maintained on a reverse dark–light cycle (10 and 14 hr). Tests were conducted 3 hr after the onset of scotophase.

Field Tests. *Z*9–, *Z*11–, and *E*11–14:Ac used for the field tests were obtained from commercial sources and were pure by GC analysis and contained less than 1% of the geometric isomer. *E*10, *E*12–14:Ac was synthesized by a modification of the method for *Amorbia cuneana* (McDonough et al., 1982; McDonough and Smithhisler, 1989). The synthesis was carried through until a mixture of *E*10, *E*12– and *E*10, *Z*12–14:OH was obtained. Pure *E*10, *E*12–14:OH was obtained by low-temperature recrystallization from pentane and then was converted to the acetate. Isomeric purity was 98.5% *EE*, 1% *EZ* and 0.5% *ZE* for all of the experiments except the one in which the effect of purity was tested. Then the purity was 96.0% *EE*, 2.5% *EZ*, and 1.5 *ZE*. Candidate

lures in 100 μl of dichloromethane were impregnated into gray elastomeric septa; dichloromethane alone was added to control septa. Pherocon IC sticky traps (Trece, Corp., Salinas, California) were used. Traps were suspended from a movable metal arm (an 18 × 23-cm shelf bracket) attached by a metal hose clamp to a 120-cm-long wooden broom handle driven into the ground. This arrangement enabled the traps to be positioned just above the mint foliage, usually 45–60 cm above the ground, where most flight activity was believed to occur.

The position of each trap in the experimental plot was drawn randomly and traps were deployed at approximately 10-m intervals. Females used in the field tests were placed in aluminum wire screen cages (9 × 9 × 2 cm, three females/cage), which were suspended by a wire from the top of the trap equidistant between the top and bottom. There were four replicates of each lure tested and the trap data were transformed by $(x + 0.5)^{1/2}$ and compared by Duncan's (1955) multiple-range test ($P = 0.05$).

RESULTS AND DISCUSSION

Exploratory Study of Structure of Pheromone Components. Among the model compounds tested, the strongest EAG responses were obtained from the 14-carbon acetates (Figure 1). The 10 and 12 positions of unsaturation produced the strongest responses, and strong responses were also obtained from the 9 and 11 positions. After the 14-carbon acetates, the strongest responses were obtained from the 12-carbon acetates: *E*10- and *Z*10–12 : Ac produced responses of about 50% of the standard (*Z*10–14 : Ac) and the other positional isomers produced responses of about 25% of standard. Other tested compounds produced responses of 0–30% of the standard.

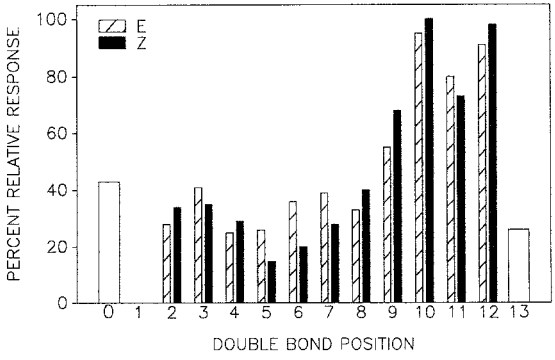


FIG. 1. Electroantennographic responses of male mint root borers (*Fumibotys fumalis*) to 14-carbon acetates. Numbers indicate double-bond position; zero indicates saturated.

The EAG response of GC fractions of SPG extract from the methyl silicone column was greatest for the fraction corresponding to the end of 14:Ac through the end of 15:Ac (1.9 mV vs. 0.1–0.8 mV for the other fractions), and from the Carbowax column for the fraction corresponding to the beginning of 16:Ac to the beginning of 18:Ac (0.7 mV vs. 0.2–0.4 mV for the other fractions). The EAG response to GC fractions (methyl silicone column) of the washes of the vials that held females was also greatest for the fraction corresponding to the end of 14:Ac through the end of 15:Ac (0.9 mV vs. 0.05–0.35 mV for the other fractions).

GC-MS analysis (DB-1 column) of SPG extract (50 female equivalents) at the retention time period that produced the strong EAG response indicated the presence of a tetradecadienyl acetate [M^+, 252; $(M\text{-}60)^+$, 192; $CH_3CO_2H_2^+$, 61] at a retention index (I_x) of 1838. Also, eluting prior to the tetradecadienyl acetate were three tetradecenyl acetates [$(M\text{-}60)^+$, 194; $CH_3CO_2H_2^+$, 61] at I_x values of 1776, 1781, and 1788. There was about 0.5 ng of the diene per female equivalent, and the ratio of the monoenes to the diene for each I_x value was 0.04:1 (1776), 0.08:1 (1781), and 0.18:1 (1788).

Positions and Configurations of Double Bonds of Pheromone Gland Components. Ozonolysis of the diene gave a single GC peak (methylsilicone column) with a retention time of 24.50 min near that of hexadecane (retention time = 24.77 min) and the same within experimental error ($\pm$0.04 min) as the ozonolysis product of 10,12–14:Ac (retention time = 24.52). The configuration of the diene was *EE* (retention time = 21.85 min) and the retention times of the four isomers of a synthetic standard (McDonough et al., 1982) were: *ZE*, 21.33; *EE*, 21.85; *EZ*, 22.10; *ZZ*, 22.29.

A comparison of retention times of the pheromone tetradecenyl acetates with those of the synthetic tetradecenyl acetates on polar and nonpolar capillary GC columns indicated the pheromone tetradecenyl acetates were *Z*9–, *Z*11–, and *E*11–. Mass spectral analysis of the DMDS derivatives confirmed these assignments. The monoene derivatives and their diagnostic peaks (Buser et al., 1983) were: *Z*9– [M^+, 348 (33%); A, 117 (53%); B, 231 (100%)]; *Z*11– [M^+, 348 (21%); A, 89 (34%); B, 259 (100%)]; *E*11– [M^+, 348 (19%); A, 89 (25%); B, 259 (100%)]. The retention times of the DMDS derivatives (in minutes) were: *Z*9–, 25.34; *Z*11–, 27.12; *E*11–, 27.54. The configurational assignments were confirmed by comparison of retention times with synthetic samples (*E*9– DMDS derivative at 25.66 min).

Male Response to Synthetic Pheromone Components. When lures containing 100 μg of *E*10,*E*12–14:Ac plus 18, 8, and 4 μg of *Z*11–, *E*11–, and *Z*9–tetradecenyl acetates were tested in a mint field (four replicated traps per test), total male captures (13) were not statistically different from blank traps but captures in female baited traps (98) were. In order to identify the reason for

the lack of significant capture, limited tests in a flight tunnel were conducted (Table 1).

When the pheromone components were tested individually (Table 1, tests 1–4), Z11–14:Ac was a weak attractant, but no response was obtained from the other components. When the four components were tested in the ratio found in the SPG and the dosage of *EE* was 100 μg (the same as the unsuccessful field test), 65% of males exhibited upwind flight and 50% touched the source (test 5). Lower doses of 33 and 10 μg of this ratio produced less response (tests 6 and 7), while a higher dose produced the highest percent responding (test 8). Subtraction of *E*11–14:Ac from the natural blend produced a response almost equivalent to the natural blend (test 9 vs. 5). Because of the limited numbers available for testing, more complete, systematic tests and statistical analysis were not undertaken. Nevertheless, collectively, these experiments established the attractiveness of the mixture of identified components and suggested that the initial field test may have produced low catches because the dose was too low.

In further field tests (test 1, Table 2) when the ratio of *EE*, Z11–, and Z9–

TABLE 1. RESPONSE IN FLIGHT TUNNEL OF ADULT MALE *F. fumalis* TO CANDIDATE LURES

Test	Lure (dose in μg)	Number of males tested	Number flying upwind	Number contacting source
1	*E*10,*E*12–14:Ac (100)	10	0	0
2	Z11–14:Ac (100)	10	3	1
3	Z9–14:Ac (100)	10	0	0
4	*E*11–14:Ac (100)	10	0	0
5	*E*10,*E*12–14:Ac (100) + Z11–14:Ac (18) + Z9–14:Ac (4) + *E*11–14:Ac (8)	20	13	10
6	*E*10,*E*12–14:Ac (33) + Z11–14:Ac (6) + Z9–14:Ac (1.3) + *E*11–14:Ac (2.7)	10	5	3
7	*E*10,*E*12–14:Ac (10) + Z11–14:Ac (1.8) + Z9–14:Ac (0.4) + *E*11–14:Ac (0.8)	10	2	2
8	*E*10,*E*12–14:Ac (300) + Z11–14:Ac (54) + Z9–14:Ac (12) + *E*11–14:Ac (24)	5	4	4
9	*E*10,*E*12–14:Ac (100) + Z11–14:Ac (18) + Z9–14:Ac (4)	10	6	4

TABLE 2. CAPTURES OF NATIVE ADULT MALE *F. fumalis* IN PHEROCON 1C TRAPS BAITED WITH VARIOUS CANDIDATE LURES IN COMMERCIAL MINT FIELDS, HARRAH, WASHINGTON, 1989[a]

Test	Lure	Dose (μg/septum)	Cumulative captures
1. July 27–28, 1989	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac	100:18:4	9 a
	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac	300:54:12	5 a
	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac	1000:180:40	22 a
	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac	3000:540:120	68 b
	3 females		61 b
	blank septa		4 a
2. August 4–6, 1989	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac	3000:540:120	105 a
	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac	3000:540:120[b]	128 a
	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac	10,000, 1800, 400	278 b
	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac, *E*11–14:Ac	3000, 540, 120, 240	119 a
	3 females		329 b
	blank septa		14 c
3. August 8, 1989	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac	3000:1800:120	125 ab
	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac	3000:5400:120	61 ac
	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac	10,000:1800:400	100 ab
	*E*10,*E*12–14:Ac, *Z*11–14:Ac, *Z*9–14:Ac	10,000:5400:400	164 b
	2 females		31 c
	blank septa		4 c

[a] Gray elastomeric septa were used as the controlled release substrate. There were four replicate traps per treatment. Letters after the numbers within a test number indicate significance according to Duncan's (1955) multiple range test ($P = 0.05$).

[b] The isomeric purity of *E*10,*E*12–14:Ac in this test was 96%; in all other tests the isomeric purity was 98.5%.

found in the SPG was used and the dose of *EE* was varied from 100 to 3000 μg, the 3000-μg dose produced significant trap catch compared to controls and was equivalent to that produced by females. Captures produced by the lower doses were not statistically significant. In test 2, Table 2, lower isomeric purity of *EE* did not decrease catch; the absence of *E*11–14:Ac did not decrease catch; and the 10-mg dose produced higher catch than the 3-mg dose. Catches from both doses were statistically significant but only the 10 mg dose was equivalent to females. Test 3 was conducted to determine if a higher proportion of *Z*11–14:Ac would increase catch. At neither the 3- nor the 10-mg dose level did the increase in the proportion of *Z*11–14:Ac produce statistically significant increases in trap catch.

Collectively, these experiments demonstrated that the three- or four-component lure in the ratio found in the sex pheromone gland and at a dose of 3–10 mg of *E*10,*E*12–14:Ac in gray elastomeric septa produced a viable attrac-

tant, competitive with female baited traps in field tests. Flight-tunnel studies indicate at least two components are necessary to produce an effective attractant. *E*11–14 : Ac may not be a component of the pheromone emitted by females, since it is the expected biosynthetic precursor to *E*10,*E*12–14 : Ac (Löfstedt and Bengtsson, 1988). To fully define the importance of each of the four components, field or flight-tunnel tests with subtraction experiments over a range of concentrations are needed.

The relatively high septa doses required, coupled with the small amount of pheromone found in the glands, were unexpected. Nevertheless, these facts do not necessarily indicate there are undetected pheromone components. The low amount of pheromone in the glands could indicate close synchronization of biosynthesis with emission rather than a low emission rate. Moreover, the emission rate of septa at the 3- to 10-mg doses is not excessive compared with that required for some other Lepidoptera. Gray septa emit at rates about the same as red rubber septa (McDonough and Aller, unpublished). Also 16-carbon aldehydes and 14-carbon acetates emit at similar rates (McDonough, 1990). The most effective doses of the sex pheromone of *Heliothis zea* (16-carbon aldehydes) in red rubber septa are 3–10 mg (Halfhill and McDonough, 1985), and the most effective doses of *Heliothis virescens* sex pheromone (mainly a 16-carbon aldehyde) are even higher (Flint et al., 1979).

Although this is the first report of the chemical identification of *E*10,*E*12–14 : Ac in the female sex pheromone gland of a pyralid, in field screening tests Reed and Chisholm (1985) reported that *Pyrausta fodinalis* (L.) was captured in traps baited with *E*10,*E*12–14 : Ac. Taxonomically, *P. fodinalis* and *F. fumalis* are both grouped as *Pyraustini*.

Acknowledgments—We thank Dr. Keith Pike, Washington State University Experiment Station, Prosser, for his suggestions; and L. Bert Dekker, Matthew Lommers, and on Rice for use of their mint fields.

REFERENCES

BERRY, R.E. 1974. Biology of *Pyrausta fumalis* on peppermint in Oregon. *Ann. Entomol. Soc. Am.* 67:580–582.

BERRY, R.E. 1977. Insects on mint. Pacific Northwest Coop. Ext. Pub. 182. 15 pp. Joint issue of the State Universities of Washington, Oregon, and Idaho.

BROWN, D.F., and MCDONOUGH, L.M. 1986. Insect sex pheromones: Formulations to increase the stability of conjugated dienes. *J. Econ. Entomol.* 79:922–927.

BUSER, H.R., ARN, H., GUERIN, P., and RAUSCHER, S. 1983. Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts. *Anal. Chem.* 55:818–822.

DAVIS, H.G., MCDONOUGH, L.M., and PIKE, K.S. 1984. Attraction of male *Fumibotys fumalis* to females of the species. *J. Entomol. Soc. B.C.* 81:25–28.

DUNCAN, O.B. 1955. Multiple range and multiple F tests. *Biometrics* 11:1–41.

FLINT, H.M., MCDONOUGH, L.M., SALTER, S.S., and WALTERS, S. 1979. Rubber septa: A long lasting substrate for (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenal, the primary components of the sex pheromone of the tobacco budworm. *J. Econ. Entomol.* 72:798–800.

FORBES, W.T.M. 1923. The Lepidoptera of New York and neighboring states. Memoir 68. Cornell Univ. Agric. Exp. Sta. Ithica, New York. 729 pp.

HALFHILL, J.E., and MCDONOUGH, L.M. 1985. *Heliothis zea* (Boddie): Formulation parameters for its sex pheromone in rubber septa. *Southwest. Entomol.* 10:176–180.

LÖFSTEDT, C., and BENGTSSON, M. 1988. Sex pheromone biosynthesis of (*E,E*)-8,10-dodecadienol in codling moth *Cydia pomonella* involves E9 desaturation. *J. Chem. Ecol.* 14:903–915.

MCDONOUGH, L.M. 1990. Controlled release of insect sex pheromones from a natural rubber substrate, *in* P. Hedin (ed.). Naturally Occurring Pest Bioregulators. ACS Symposium Series. American Chemical Society, Washington, D.C. In press.

MCDONOUGH, L.M., and SMITHHISLER, C.L. 1989. Improved synthesis of the sex pheromone of the avocado leafroller, *Amorbia cuneana* (Walsingham). *Southwest. Entomol.* 14:153–157.

MCDONOUGH, L.M., KAMM, J.A., and BIERL-LEONHARDT, B.A. 1980. Sex pheromone of the armyworm (*Pseudoletia unipuncta* (Haworth) (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 6:565–572.

MCDONOUGH, L.M., HOFFMANN, M.P., BIERL-LEONHARDT, B.A., SMITHHISLER, C.L., BAILEY, J.B., and DAVIS, H.G. 1982. Sex pheromone of the avocado pest, *Amorbia cuneana* (Walsingham) (Lepidoptera: Tortricidae): Structure and synthesis. *J. Chem. Ecol.* 8:255–265.

MILLER, J.R., and ROELOFS, W.L. 1978. Sustained flight tunnel for measuring insect responses to wind-borne sex pheromones. *J. Chem. Ecol.* 4:187–198.

MONROE, E. 1976. Pyraloidea, pp. 26–28, *in* The Moths of America North of Mexico. Pyralidae (part) Fascicle 13.2A: E.W. Classey Ltd. and The Wedge Entomological Research Foundation, London. 304 + xx pp.

PIKE, K.S. 1979. Peppermint, *Fumibotys fumalis* control, Washington. *Insecticide Acaracide Tests* 4:88.

PIKE, K.S., and GETZIN, L.W. 1981. Persistence and movement of chlorpyrifos in sprinkler-irrigated soil. *J. Econ. Entomol.* 74:385–388.

PIKE, K.S., and GLAZER, M. 1982. Strip rotary tillage: A management method for reducing *Fumibotys fumalis* (Lepidoptera: Pyralidae) in peppermint. *J. Econ. Entomol.* 75:1136–1139.

PIKE, K.S., BAIRD, C.R., and BERRY, R.E. 1988. Mint root borer in the Pacific Northwest. Pacific Northwest Coop. Ext. Pub. 322. 8 pp. Joint issue of the State Universities of Washington, Oregon, and Idaho.

REED, D.W., and CHISHOLM, M.D. 1985. Attraction of moth species of Tortricidae, Gelechiidae, Geometridae, Drepanidae, Pyralidae, and Gracillariidae families to field traps baited with conjugated dienes. *J. Chem. Ecol.* 11:1645–1657.

VOERMAN, S. 1988. The pheromone bank: A collection of unsaturated compounds indispensable for discovery of sex attractants for Lepidoptera. *Agric. Ecosyst. Environ.* 21:31–41.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

FLIGHT ACTIVITY OF *Heliothis virescens* (F.) FEMALES (LEPIDOPTERA: NOCTUIDAE) WITH REFERENCE TO HOST-PLANT VOLATILES[1]

E.R. MITCHELL,* F.C. TINGLE, and R.R. HEATH

Insect Attractants, Behavior, and Basic Biology Research Laboratory
Agricultural Research Service, U.S. Department of Agriculture
Gainesville, Florida 32604

(Received June 11, 1990; accepted September 18, 1990)

Abstract—*Heliothis virescens* (F.) females responded positively via upwind flight in laboratory assays to volatiles emitted from methylene chloride washes of fresh whole leaves of host plants including cotton, tobacco, and a weed species, *Desmodium tortuosum* (Swartz) de Candolle. Except for *D. tortuosum*, the response increased positively with dose; the steepest slope occurred with an extract of cotton squares (flower buds). Almost all of the moths that landed on the extract dispenser also oviposited. Moths simulated by extracts from cotton squares exhibited a full array of behaviors (upwind flight, contact with the dispenser, examination of the cloth substrate with antennae, and oviposition) expected of gravid individuals seeking sites to propagate the species.

Key Words—Plant attractant, host phenology, oviposition stimulant, *Heliothis subflexa*, *Heliothis armigera*, Lepidoptera, Noctuidae.

INTRODUCTION

Many phytophagous insects use airborne volatiles emitted from plants to locate their hosts. The recent development of bioassay systems for studying host-plant finding and ovipositional behavior under controlled environmental conditions in the laboratory has intensified interest in characterization of the specific behaviors regulated by volatile emissions from plants and identification of the

*To whom correspondence should be addressed.

[1]This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or the recommendation of its use by USDA.

active compounds. Information on volatile chemicals that attract phytophagous insects to their host to stimulate oviposition upon arrival could be of benefit to plant-breeding programs. For example, selective elimination or reduction in the level of these chemicals possibly could impart a degree of "resistance" to otherwise acceptable cultivars.

Heliothis and *Helicoverpa* spp. rank among the most destructive pests of crops in the United States and around the world. The evidence is growing that many of these species use plant constituents for host location and accep- tance. Allelochemicals that stimulate females to oviposit have been isolated for *Helicoverpa zea* (Boddie) (corn) (Wiseman et al., 1988), *Heliothis subflexa* (Guenée) (*Physalis angulata* L.) (Mitchell and Heath, 1987), and *Heliothis virescens* (F.) (tobacco) (Jackson et al., 1984; Mitchell et al., 1990). Mitchell et al. (1990) also isolated oviposition stimulant compounds for *H. virescens* from a late-season wild host plant, *Desmodium tortuosum* (Swartz) de Candolle, and leaves and squares (i.e., flower buds) of cotton.

In each of the cases cited above, the techniques used to demonstrate oviposition activity were unable to differentiate between tactile and olfactory stimuli. Other workers, however, have demonstrated a relationship between plant volatiles and attraction/oviposition stimulation in *Heliothis*. Rembold and Tober (1985) showed that *Heliothis armigera* (Hübner) females (an oligophagous species) responded differentially in oviposition trials to odors obtained by pulling air over seedlings of two cultivars of pigeonpea, *Cajanus cajan* L. Millsp. Tingle et al. (1989) showed that mated *Heliothis subflexa* females (a monophageous species) flew upwind in wind-tunnel bioassays to a methanol wash of fresh whole leaves from its host plant, groundcherry (*P. angulata*), culminating in ca. 50% of the responding moths depositing one or more eggs. The present study reports on the flight and ovipositional responses of females of a sibling species of *H. subflexa*, *H. virescens*, in a wind-tunnel system to extracts of selected cultivated host plants (cotton and tobacco) and *D. tortuosum*, a late-season weed host.

METHODS AND MATERIALS

A Plexiglas flight tunnel (60 cm wide × 60 cm high × 195 cm long) was used to observe flight and ovipositional responses of females to volatiles from crude extracts of three different plant hosts. The extracts were obtained by dipping ca. 400 g fresh plant material for 30 sec in 1 liter of solvent (methylene chloride), which was filtered and stored in 1-liter glass containers at 0°C until needed (Mitchell and Heath, 1987). Extracts were prepared from cotton (variety McNair 220) leaves and squares (flower buds) and from leaves of *D. tor-*

tuosum and tobacco [susceptible: NC 2326, and resistant: TI 1112 (Jackson et al., 1983)]. All plants were grown in small field plots using conventional cultural practices. The extracts were concentrated in a rotary evaporator before testing. All plants were in the flowering stage except tobacco, which was in the prebloom stage.

All test insects were reared in our laboratory on the modified pinto bean diet using methods described by Guy et al. (1985) and Mitchell et al. (1988). The insects were sexed in the pupal stage and held separately in 3.8-liter paper cans with screened tops until emergence. Upon eclosion, the sexes were confined together for two days for mating (21 females and 14 males/cage) in 5.5-liter plastic cages with screened tops. The moths were fed a 10% honey–water solution and held under a reversed 14 : 10-hr light–dark cycle in a holding room that was maintained at 25–26°C and 60–70% relative humidity. Preliminary studies indicated that >95% of the females mated under these conditions, as evidenced by the presence of a spermatophore in the bursa copulatrix.

On the day of testing, the moths were sexed by gently squeezing the abdomen to extrude the genitalia, and the females were confined in a 25 × 25 × 25-cm Plexiglas holding cage and placed in the flight tunnel room (3 × 2.6 × 2.1 m) ca. 1 hr before scotophase. An intake vent in the wall allowed a continuous flow of fresh unfiltered air from the outside into the room, which was maintained at the same environmental conditions as the holding room. An electric timer was used to control overhead fluorescent lights (two banks of two 40-W bulbs). Three incandescent 25-W red light bulbs that were equally spaced above the tunnel remained on continuously. The light level during scotophase was 1.4 lux. Air was pulled through the tunnel at ca. 0.4 m/sec. when measured at the center of the tunnel, and exhausted opposite the intake duct via a 30-cm-diam. flexible pipe equipped with a fan. The flight tunnel was the same as described by Tingle et al. (1989).

Crude extract (gram equivalent dosages) was deposited on white muslin cloth dispensers secured over the end of a glass cylinder (3.5-cm opening) with a rubber band. After a 5-min evaporation period, the dispenser was placed at the upwind end of the tunnel. Air was blown through the cylinder with an aquarium pump at the rate of 1 liter/min to provide a continuous flow (plume) of the extract odor through the tunnel. The location and form of the plume was verified by introducing smoke into the dispenser system and observing the smoke trail.

A moth was removed from the holding cage and placed into a cylindrical 4 × 6.5-cm plastic release cage with screened ends. After placement of the release cage into the downwind end of the flight tunnel, the moth was released immediately and observed for 2 min. Behavioral responses, including random and oriented flight, and contacts, landings, and oviposition on extract-treated substrates were recorded. Ten to 16 *H. virescens* females (mean = 14) were

tested individually in each replicate; each treatment was replicated 3–13 times. The data were converted to arcsin $\sqrt{\text{percentage}}$ and analyzed using ANOVA or regression analysis (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Heliothis virescens (Hv) females demonstrated upwind flight to volatiles emitted from cloth dispensers treated with each of the host-plant extracts (Figure 1). The flight sequence was similar to that previously described for a sibling species, *H. sublfexa*, to extracts of whole leaves from its host, groundcherry (Tingle et al., 1989). Typically, most of the females began nondirectional (random) flight after emerging from the release cage at the downwind end of the tunnel. Random flight usually began in an upwind direction. Shortly after exiting the release cage, moths detecting the odor plume flew upwind in a zigzag pattern towards the extract dispenser. A few moths flew directly to the dispenser and landed, probed the cloth substrate with their ovipositor, and deposited an egg. Others flew to within 5 cm of the dispenser and hovered for a short time

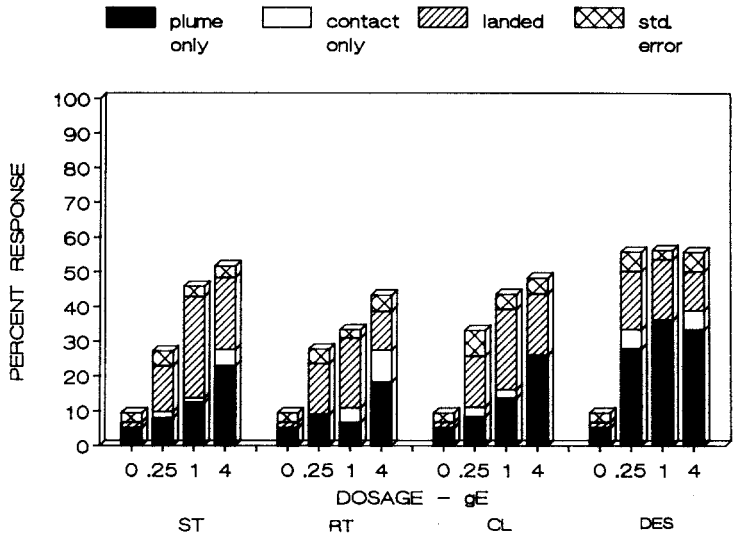


FIG. 1. Behavioral responses of *Heliothis virescens* females to volatiles from different dosages (gram equivalents) to methylene chloride washes of whole leaves of susceptible (ST) and resistance (RT) tobacco, cotton (CL), and *Desmodium tortuosum* (DES) in wind-tunnel assays. Differences between means within each response category were not significant ($P < 0.05$; ANOVA) (Steel and Torrie, 1960). The response totals do show a progressive increase with dosage for whole-leaf washes of tobacco and cotton.

before veering away or landing on the cloth substrate. The mean oriented flight response to control dispensers treated with methylene chloride was only 6.7 ± 2.6% (eight replicates). The few females orienting to the control dispenser flew upwind < 100 cm before veering away and continued flying about in random fashion or landed on the tunnel wall. None of these females contacted the control dispenser.

With the exception of *D. tortuosum*, the total response (oriented upwind flight, contact, and landing) recorded for Hv to each leaf extract showed a progressive increase with dose (Figure 1). The steepest slope occurred with an extract from cotton squares (Figure 2). It is unknown whether the apparent difference in response to extracts of cotton squares and cotton leaves was quantitative or qualitative. A comparable situation also might exist with tobacco and *D. tortuosum*; however, floral parts of these plants were not examined.

The flowering stage of corn, cotton, soybean, and tobacco is the most preferred phenological state for oviposition by *H. zea* (Johnson et al., 1975), although females will oviposit on certain host plants in the absence of flowering parts. Roome (1975) reached similar conclusions concerning the oviposition behavior of *H. armigera* in corn and sorghum. He also suggested that "flowering" corn and sorghum crops attract adults that, once in the crop, are "trapped" by suitable physiological cues from the plants. Thus, the increased response of females to extracts of cotton squares versus cotton leaves suggests

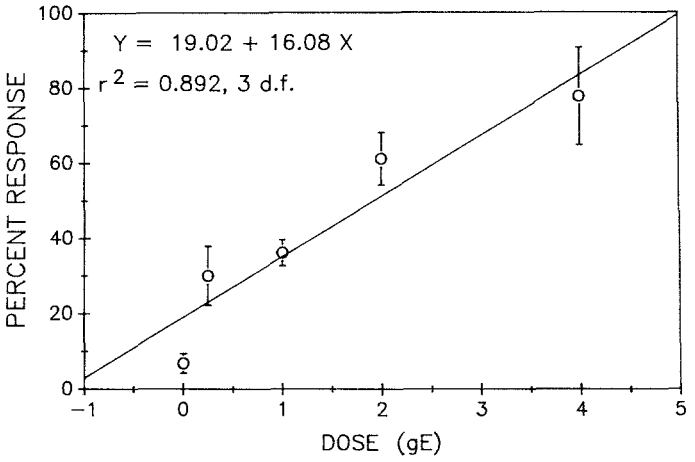


FIG. 2. Effect of dosage (gram equivalents) of a methylene chloride wash of cotton squares (flower buds) on the total response (upwind flight, contact, and landing) of *Heliothis virescens* females in wind-tunnel assays (regression analysis) (Steel and Torrie, 1960).

that, like the aforementioned species, Hv also are more strongly attracted to plants in the reproductive phase of their life cycle.

A test was conducted to establish whether attraction of Hv to cotton and tobacco was due to specific chemicals emitted by these plants or if the attraction response was due to the ubiquitous "green odor" factor. Using the flight-tunnel assay described earlier, female Hv were exposed to a 1-g equivalent dose of a methylene chloride wash of fresh whole leaves of cotton, susceptible tobacco, or elderberry (*Sambucus simpsonii* Rehd.), a nonhost weed species. Tingle and Mitchell (1986) showed that a methylene chloride wash (among others) of whole elderberry leaves was effective in deterring oviposition by Hv in laboratory assays. The mean percent flight responses for the three extracts were: cotton, 55.6 ± 3.5a; tobacco, 50.0 ± 5.4a; and elderberry, 23.6 ± 7.4 b [means ±SE, with different letters indicating significant differences, $P < 0.05$, Duncan's multiple-range test (Duncan, 1955)]. These results suggest that the flight responses recorded for Hv to cotton and tobacco extract were due to chemical qualities different from what is typically referred to as the green factor. It is unknown, however, whether the attractants from cotton and tobacco are the same compounds.

The total flight response (upwind flight, contact, and landing) by Hv to *D. tortuosum* was ca. 50% for each of the three doses tested. However, ca. two-thirds of the moths flying upwind and contacting the dispensers treated with *D. tortuosum* extract did not land. Mitchell et al. (1990) reported that *D. tortuosum* possessed compounds that stimulated oviposition by Hv females in laboratory assays. In Florida, *D. tortuosum* typically becomes abundant in late August and September in tobacco fields following harvest. Jackson and Mitchell (1984) showed that although Hv can survive to pupation on *D. tortuosum*, this species is less suitable than tobacco as a host. Thus, due to the proximity of *D. tortuosum* to moth emergence sites in abandoned tobacco fields, a strong long-range attractant is not needed for Hv females to locate and oviposit on *D. tortuosum*. The propensity for Hv to oviposit on *D. tortuosum* located outside of tobacco fields relative to *D. tortuosum* in tobacco fields is unknown.

There was no significant difference among treatments in the mean percentage of Hv moths (13.9 ± 1.5) depositing an egg upon contact with the cloth substrates. In a recent study using a system of individual chambers specifically designed to evaluate oviposition responses, Mitchell et al. (1990) showed that Hv females laid significantly more eggs on cloth substrates treated with whole-leaf wash extracts of leaves from susceptible or resistant tobacco, *D. tortuosum*, and cotton leaves or squares compared to the number of eggs laid on control cloths. Moreover, in competitive trials in which Hv females were given a choice between susceptible tobacco-leaf extract and extract from leaves of resistant tobacco, cotton, or *D. tortuosum*, significantly more eggs

were deposited on cloths treated with susceptible tobacco extract (Mitchell et al., 1990).

In this study, the response period was limited to 2 min/moth. This necessarily restricted the total number of eggs that a female could deposit within the observation period. Nevertheless, females were stimulated to exhibit an array of behaviors (upwind flight, contact, surface examination with antennae, and oviposition) characteristic of gravid individuals seeking sites to propagate the species. Work by Jackson et al. (1984) suggests that the chemicals eliciting egg laying in Hv most likely are contact stimuli. Thus, the chemicals that elicit long-range orientation (>1 m) and landing probably are different from those eliciting the oviposition response.

The role of plant volatiles in the selection and colonization process of oligophagous species such as Hv, and indeed most insect species regardless of their degree of host specificity, is poorly understood. Elucidation of the phytochemicals governing these processes will enhance greatly an understanding of the factors driving the establishment and development of insect pest populations on crops. Knowledge of the behavioral effects of such phytochemicals offers opportunities for creative management of crop insect pests via genetic modification of the host's chemical profile. Alternately, chemically defined plant attractants may be used alone or combined with insect sex attractant pheromones to monitor pest populations or for direct control as toxic baits by combining plant attractants, pheromones, and insecticides.

Acknowledgments—We gratefully acknowledge the technical assistance of P. Hall, W. Copeland, F. Adams, and B. Dueben of this laboratory; and D.M. Jackson, ARS USDA, Oxford, North Carolina, and G. Herzog, Tifton, Georgia, for providing the tobacco and cotton seed, respectively.

REFERENCES

DUNCAN, D.B. 1955. Multiple range and multiple F tests. *Biometrics* 11:1–2.

GUY, R.H., LEPPLA, N.C., RYE, J.R., GREEN, C.W., BARRETTE, S.L., and HOLLIEN, K.A. 1985. *Trichoplusia ni*, pp. 487–494, *in* P. Singh and R.F. Moore, (eds.). Handbook of Insect Rearing, Vol. 2. Elsevier Science Publishers B.V., Amsterdam.

JACKSON, D.M., and MITCHELL, E.R. 1984. Growth and survival of tobacco budworm (Lepidoptera: Noctuidae) larvae fed Florida beggarweed (Fabaceae) and tobacco (Solanaceae). *J. Econ. Entomol.* 77:960–965.

JACKSON, D.M., CHEATHAM, J.S., PITTS, J.M., and BAUMHOVER, A.H. 1983. Ovipositional response of tobacco budworm moths (Lepidoptera: Noctuidae) to tobacco Introduction 1112 and NC 2326 in cage tests. *J. Econ. Entomol.* 76:1303–1308.

JACKSON, D.M., SEVERSON, R.F., JOHNSON, A.W., CHAPLIN, J.F., and STEPHENSON, M.G. 1984. Ovipositional response of tobacco budworm moths (Lepidoptera: Noctuidae) to cuticular chemical isolates from green tobacco leaves. *Environ. Entomol.* 13:1023–1030.

JOHNSON, M.W., STINNER, R.E., and RABB, R.L. 1975. Ovipositional response of *Heliothis zea* (Boddie) to its major hosts in North Carolina. *Environ. Entomol.* 4:291–297.

MITCHELL, E.R., and HEATH, R.R. 1987. *Heliothis subflexa* (Gn.) (Lepidoptera: Noctuidae): Demonstration of oviposition stimulant from groundcherry using novel bioassay. *J. Chem. Ecol.* 13:1849–1858.

MITCHELL, E.R., HINES, R.W., and COPELAND, W.W. 1988. *Heliothis subflexa* (Lepidoptera: Noctuidae): Establishment and maintenance of a laboratory colony. *Fla. Entomol.* 71:212–214.

MITCHELL, E.R., TINGLE, F.C., and HEATH, R.R. 1990. Oviposition response of three *Heliothis* species (Lepidoptera: Noctuidae) to allelochemicals from cultivated and wild host plants. *J. Chem. Ecol.* 16:1817–1827.

REMBOLD, H., and TOBER, H. 1985. Kairomones as pigeonpea resistant factors against *Heliothis armigera*. *Insect Sci Appl.* 6:249–252.

ROOME, R.E. 1975. Activity of adult *Heliothis armigera* (Hb.) (Lepidoptera: Noctuidae) with reference to the flowering of sorghum and maize in Botswanna. *Bull. Entomol. Res.* 65:523–530.

STEEL, R.G.D., and TORRIE, J.H. 1960. Principles and Procedures of Statistics. McGraw-Hill, New York.

TINGLE, F.C., and MITCHELL, E.R. 1986. Behavior of *Heliothis virescens* (F.) in presence of oviposition deterrents from elderberry. *J. Chem. Ecol.* 12:1523–1531.

TINGLE, F.C., HEATH, R.R., and MITCHELL, E.R. 1989. Flight response of *Heliothis subflexa* (Gn.) females to an attractant from groundcherry, *Physalis angulata* L. *J. Chem. Ecol.* 15:221–231.

WISEMAN, B.R., GROSS, H.R., WIDSTROM, N.M., WAISS, A.C., and JONES, R.L. 1988. Resistance of corn to *Heliothis zea*. *South. Coop. Ser. Bull.* 337:21–30.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

UNUSUAL POLYMETHYL ALKENES IN TSETSE FLIES ACTING AS ABSTINON IN *Glossina morsitans*

D.A. CARLSON[1] and Y. SCHLEIN[2,*]

[1]*USDA, ARS, MAVERL*
Gainesville, Florida 32604
[2]*Department of Parasitology, Hadassah Medical School*
The Hebrew University of Jerusalem
Jerusalem, Israel

(Received July 30, 1990; accepted September 18, 1990)

Abstract—The major alkene of the male tsetse fly, *Glossina morsitans morsitans*, was isolated for characterization by thin-layer and gas chromatography (GC). The mass spectra of the alkene and the alkene DMDS derivative indicated one isomer, 19,23-dimethyltritriacont-1-ene. The material is present at 1–2 μg/male fly and is partially transferred to the female preparatory to or during mating. A dose-dependent antiaphrodisiac effect was seen with exposed male flies using the isolated natural product, with 2 and 4 μg causing 80% loss of copulatory attempts, and 10 μg extinguishing the attempts. This effect was increased by addition of male-produced alkane. This compound and a 31-carbon homolog also appear in *G. m. submorsitans*. Similar quantities of alkenes that are species-specific appear in all tsetse males. Structures of male-produced trimethylalkenes that appear in two other species, *G. palpalis palpalis* and *G. fuscipes fuscipes*, were investigated.

Key Words—Mating inhibitor, abstinon, stimulatory, cuticle waves, diptera, extract, hydrocarbon, dimethylalkene, trimethylalkene, aphrodisiac, Diptera, Muscidae, *Glossina morsitans morsitans*, tsetse fly, 19,23-dimethyltritriacont-1-ene.

INTRODUCTION

Glossina morsitans morsitans (Westwood) males were reported to produce a chemical deterrent to mating (termed "abstinon"), that was hexane soluble and could be transferred to otherwise stimulatory decoys. Application of as little as

*To whom correspondence should be addressed.

0.5 and 0.25 male equivalent of crude extract caused 100% and 96% abolishment, respectively, of male *G. m. morsitans* response to freshly killed females. Application of up to 0.4 ml of solvent had no effect on the response of males. No chemical isolation accompanied this work, and no candidate material was identified (Schlein et al., 1981a).

Reports of the existence of antiaphrodisiac pheromones (abstinons) in tsetse flies were challenged by other workers who described solvent effects that moved cuticular hydrocarbon components about the cuticle of otherwise stimulatory female test flies (Coates and Langley, 1982). Recently, the physical masking of active sex pheromone by inactive compounds was described as more likely than the decline or loss of activity due to the addition of antiaphrodisiacs (Langley et al., 1987).

Sex-stimulant hydrocarbons are produced by female tsetse flies that stimulate the male to attempt copulation. Pheromones have been isolated, identified, and synthesized for *Glossina morsitans morsitans* (Westwood) including 15,19,23-trimethylheptatriacontane (morsilure, Carlson et al., 1978), and 13,23-dimethylpentatriacontane in *G. pallidipes* (Austen) (Carlson et al., 1981, 1984). The presence of a sex stimulant pheromone has been reported in *G. palpalis palpalis* (Rob-Des) (Offor et al., 1981). Synthetic 15,19-dimethyltritriacontane was stimulatory to male *G. austeni* (Newstead) (Huyton et al., 1980). Alkanes that are predominant in males are usually also present in females of the same species, (Huyton et al., 1980; Nelson and Carlson, 1986; Nelson et al., 1988), but sexual dimorphism is observed in higher chain-length alkanes that are present only in females.

The gas chromatographic (GC) analysis of cuticular hydrocarbons of several species of male *Glossina*, including *G. m. morsitans*, *G. austeni*, *G. fuscipes fuscipes* (Newstead), and *G. p. palpalis*, showed that sex-specific alkenes were present in each species of fly (Carlson and Langley, 1986). Laboratory-reared *G. m. morsitans* contained a major 33-carbon backbone alkene, whereas virgin females had only traces. However, mated females contained appreciable quantities of this compound for several days after copulation. Identical alkenes were found in males of *G. m. centralis* (Machado) and *G. m. submorsitans* (Newstead). *G. austeni* males produced a 31-carbon backbone alkene, whereas *G. p. gambiensis* (Vanderplank) and *G. p. palpalis* males produced a major 26-carbon backbone alkene. The compounds were also found in wild females. Gas chromatography showed that relatively large amounts of these alkenes are transferred from males to females, apparently upon contact (Carlson and Langley, 1986).

The concept that male-produced compounds affect conspecific male behavior has been extended to other insects that have female-produced sex pheromones. *Drosophila melanogaster* (L.) males produce *cis*-vaccenyl acetate (CVA) that is transferred to the female and functions to repel males in subse-

quent mating attempts (Jallon et al., 1981). Recently Scott et al., (1988) found that mutual pheromone exchange in *D. melanogaster* decreases the sexual attractiveness of these flies, due to (*Z*)-7-tricosene, a 23-carbon alkene, and not CVA. The male stable fly *Stomoxys calcitrans* (L.) possesses a large quantity of a triply unsaturated alkene, (*Z,Z*)-1,7,13-pentacosatriene (Muhammed et al., 1975, Sonnet et al., 1977), which has been synthesized (Sonnet, 1979, Carlson and Mackley, 1985). It is transferred in significant quantities to females upon mating (Carlson and Mackley, 1985). The reason for such a transfer is not known, but the alkene is recovered by extraction of old mated females (Muhammed et al., 1975). In insects other than diptera, studies of the cockroach *Nauphoeta cinerea* showed that an unsaturated ester, octadecyl (*Z*)-9-tetracosenoate, inhibits the wing-raising behavior released by contact with the stimulatory, wing-raising hydrocarbon fraction from conspecific females (Fukui and Takahashi, 1983). A similar phenomenon was reported in the tobacco budworm moth (Jacobson et al., 1984).

The only reported methyl alkenes are three 2-methyl alkenes from male house crickets, including 2-methyl-24-hexatriacontene and minor amounts of the 22- and 26- isomers (Warthen and Uebel, 1980). These structures were determined by ozonolysis and GC retention indices to establish the presence of branched 23-, 25-, and 27-carbon aldehydes. GC-MS was not helpful in determining which aldehydes were isomethyl-branched.

We studied the effect of male-produced alkene upon the sexual responses of male tsetse flies to show that it is primarily responsible for the antiaphrodisiac effect seen in *G. m. morsitans* males. Natural alkenes and synthetic alkanes and alkenes were assayed against male *G. m. morsitans*, establishing dose–response relationships to show the abstinon effect. We also describe the chemical structures of these unusual alkenes transferred between sexes in four species of tsetse flies.

METHODS AND MATERIALS

Biological Materials. Tsetse flies were obtained as pupae from the Tsetse Research Laboratory, University of Bristol. Adults were sexed on the first day, maintained at 26°C, and fed on guinea pigs. Flies used for the experiments were 5–10 days old. Treatments of extracts and samples onto subject decoys were as previously described (Schlein et al., 1981a). Freshly killed females with amputated legs and wings were pin-mounted on corks and used as decoys with or without test materials, which were applied to each in 50-μl *n*-hexane aliquots using a microsyringe. Other females were washed with *n*-hexane three times following amputation to remove the cuticular lipids. The bodies then were dried in an air stream and each was dosed with 20 μl hexane solution containing

4 μg synthetic morsilure (15,19,23-trimethylheptatriacontane) and different quantities of test materials. Test materials were: *G. m. morsitans* alkenes from males, *G. m. morsitans* and *G. pallidipes* alkanes, and vaseline, which consists of purified hydrocarbons (Merck Index, 1976). Treated decoy females were dried in an air stream for 30 min. Males that had been fed 24 hr earlier were each placed in a corked glass tube 7 × 2.5 cm diameter and allowed 1 hr acclimatization. For testing, the cork of each tube was replaced by one bearing a mounted female, and males were brought into contact with the decoy three times. The behavior of the male was scored "nil" for no response, stage I for a short arrest in the male's movements on the decoy female, stage II for correct positioning of the male, either with or without flexing of the genitalia, and stage III for an attempt at copulation.

Chemical Separations and Identifications. Extracts were made of frozen laboratory flies or wild flies by washing with hexane or diethyl ether solvent for 1 hr at the rate of 1 ml/fly (Carlson et al., 1978, 1984). The hydrocarbon fraction was obtained by chromatography on silica gel and was further fractionated by chromatography on silver nitrate-impregnated silica gel. Samples of alkenes were collected by preparative argentation TLC to ensure purity (Carlson et al., 1978).

Analyses by fused silica gas chromatography (FSGC) were conducted using a Varian 3700 GC with a 15-m × 0.32-mm-ID FS column of DB-1 via a split/splitless injector, temperature programmed at 150°C to 300°C at 12°/min and with H_2 carrier gas. Alkane standards were used to determine retention indices.

GC–mass spectrometry (GC-MS) utilized a Finnigan model 4000 fitted with a 15-m × 0.32-mm-ID FSGC column (DB-1, J & W Co., Rancho Cordoba, California) that was temperature programmed as above for GC, and an INCOS data system. Alternatively, a Hewlett-Packard model 5988A GC-MS was used, fitted with a 30-m × 0.32-mm-id FSGC column of DB-1, temperature programmed as above. Alkenes were derivatized using dimethyl disulfide (DMDS) and analyzed by GC-MS (Dunkelblum et al., 1985, Carlson et al., 1989).

Peak ratios for a small but consistently appearing alkane (KI 3265) were obtained by dividing the quantity found in each fly by that of another alkane (KI 3355). Similarly treated were the KI 3755 and 3775 alkanes, using the same samples used in Carlson and Langley (1986) in order to evaluate transfer of cuticular alkanes between flies.

RESULTS

Isolation and Characterization of Alkenes. Alkanes from male *G. m. morsitans* included about equal quantities of 2-methyltriacontane (KI 3065) and 11,15-dimethyltritriacontane (KI 3355). The latter was present at 3.3 μg/male

(range 1–4 μg), which comprised an average of 22% of the extracted hydrocarbon (range 13–28%). This quantitation is consistent with Nelson and Carlson (1986), in which alkanes were identified by GC-MS. A minor KI 3155 compound was homologous, as was one of the two homologs at KI 3555. Hydrogenation of the corresponding KI 3355 alkene gave an alkane, 11,15-dimethyltritriacontane, that produced major fragment ions at m/z 168, 169, 239, 280, and 351 (Carlson and Langley, 1986), but did not locate the double-bond position.

Here, the alkene fraction (MA) containing the major male alkene (MMA KI 3355) was isolated as previously described (Carlson and Langley, 1986). MMA was present at 1–2 μg/male (Figure 1), with much smaller quantities of homologs. Milligram quantities were obtained from extracts of large collections of each sex of *G. m. morsitans* and checked for the absence of appreciable quantities of female-produced alkenes (Figure 1) before bioassay. After development with hexane–20% benzene, the alkenes were collected from the region of a silver nitrate silica gel TLC plate consistent with (*Z*)-alkene standards (*Rf* = 0.5). No evidence was seen for (*E*)-alkenes (*Rf* = 0.65) by TLC or GC. Alkenes were collected from pooled samples of males of the other species and purified by the same procedures, with the resulting composition essentially the same as in the previous study (Carlson and Langley, 1986).

The EI mass spectra of the minor KI 3155 alkene homolog from *G. m. submorsitans* showed fragment ions at m/z 167, 250–251, and 462 (M) that were consistent with the structure 19,23-dimethylhentriacont-1-ene. The major KI 3355 alkene from both *G. m. submorsitans* and *G. m. morsitans* showed fragment ions at m/z 167 (C_{12}), 278–279 ($C_{20:1}$), 349 ($C_{25:1}$), and 490 (M), indicating a structure consistent with 19,23-dimethyltritriacont-1-ene (Figure 2). The minor ions at m/z 181 and 295 are consistent with "apparent β-cleavage fragmentation" internal to methyl branch points that was described for EI-MS of dimethyl-branched long-chain alcohols and their acetate esters (Nelson et al., submitted). This β-cleavage is apparently suppressed in saturated methyl-branched alkanes that normally undergo α-cleavage but is observed in unsaturated compounds, here causing the appearance of additional fragment ions that are otherwise difficult to explain, and also causing loss of 1–3 amu from diagnostic fragment ions. However, the homologous alkenes at KI 3155 and KI 3555 were consistent with the presence of the double bond on the longer end of this structure and with alkanes produced by a previous hydrogenation of similar alkene fractions.

The EI mass spectra of the major KI 2720 alkene from *G. p. palpalis* showed fragments at m/z 139 (C_{10}), 209 (C_{15}), 222/223 ($C_{16:1}$), and 406 (M), indicating a structure consistent with 4,8,12-trimethylhexacos-25-ene (Figure 3). Additional ions at m/z 154, 224, 238, and 308 could be ascribed to β-cleavage as described above. The minor alkene at KI 2640 showed fragments

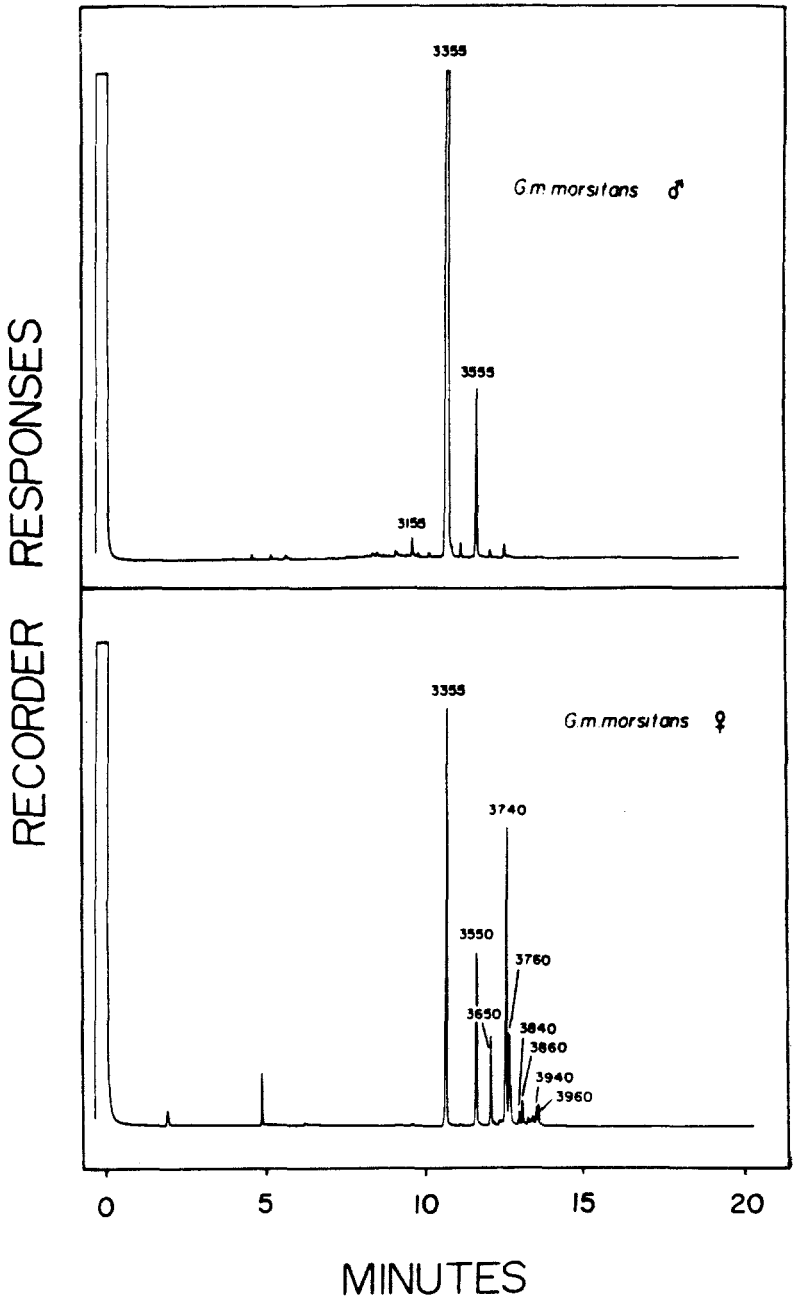


FIG. 1. Capillary gas chromatogram of alkenes from male and female *G. m. morsitans*.

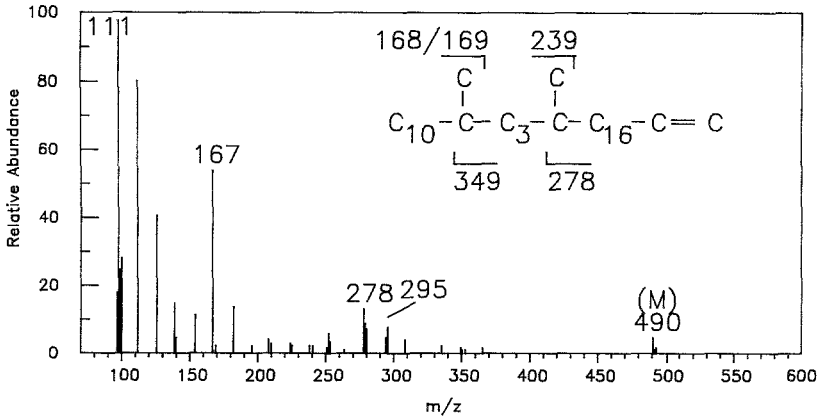


FIG. 2. Electron-impact GC-MS of major alkene from *G. m. morsitans* males.

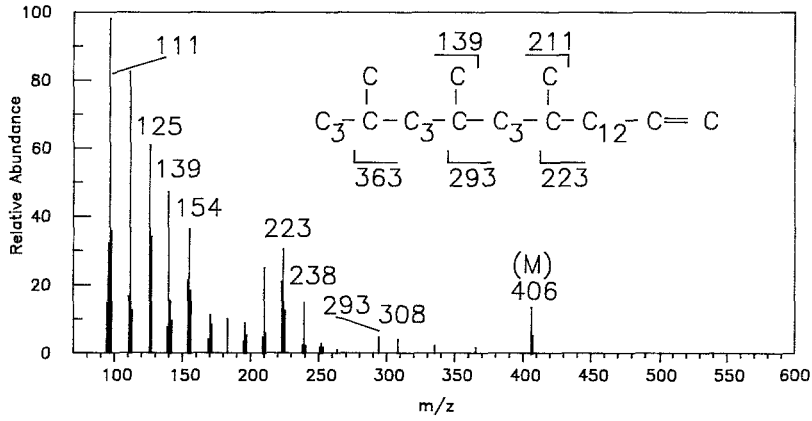


FIG. 3. Electron-impact GC-MS of major alkene from *G. p. palpalis* males.

at m/z 125 (C_9), 223 ($C_{16:1}$), and 392 (M), indicating a structure consistent with 3,7,11-trimethylpentacos-24-ene. Thus, the methyl branchings are analogous with the corresponding trimethylalkanes from these species (Nelson et al., 1988).

DMDS Location of Double-Bond Position in Alkenes from Males. The alkene fraction from *G. m. morsitans* males was derivatized with DMDS. The resulting major adduct eluted at KI 4000, indicating that an equimolar amount of DMDS had been added to the major male alkene KI 3355. Electron-impact GC-MS did not show a molecular ion for the monoadduct at m/z 582 or M-47 (m/z 537) (Figure 4). However, the adduct showed one major fragment ion at

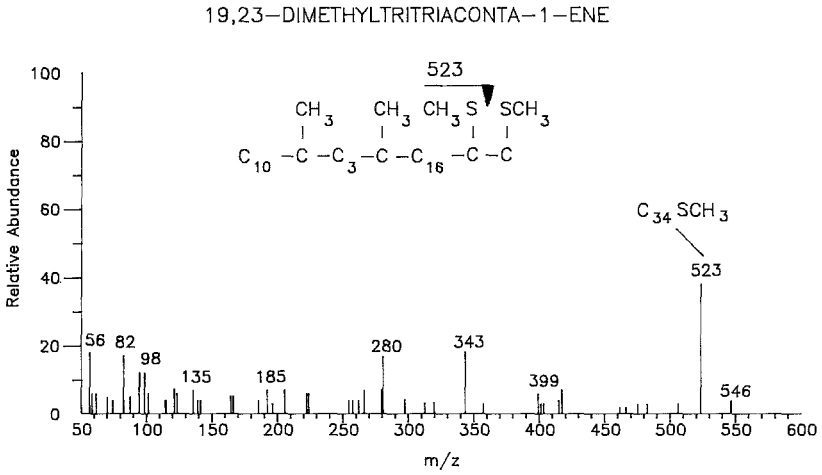


FIG. 4. Electron-impact GC-MS of major male alkene dimethyl disulfide adduct from *G. m. morsitans* males.

m/z 523 that was consistent with a structure of $C_{34}SCH_3$ in both species. This was consistent with addition of DMDS to a terminal position of unsaturation. The mass spectra presented fragment ions for $C_{18}SCH_3$ (*m/z* 297) and $C_{18}(SCH_3)_2$ (*m/z* 343), indicating fragmentation at a methyl branch located 19 carbons from the unsaturated end of the molecule. This is consistent with an alkene of structure 19,23-dimethyltritriacont-1-ene, in which the double bond is on the long alkyl chain. The fragments are inconsistent with the isomer 11,15-dimethyltritriacont-1-ene, for which cleavage at a methyl branch cannot be drawn to give an 18-carbon fragment, as the assignments do not fit.

The alkene fractions from *G. p. palpalis* and *G. f. fuscipes* males contained a major alkene at KI 2720, with minor quantities of an alkene, apparently homologous, at KI 2640 as reported by Carlson and Langley (1986). Derivatization with DMDS yielded one major adduct that eluted at KI 3300, indicating that an equimolar amount of DMDS had been added. Electron-impact GC-MS showed a molecular ion for the monoadduct (M+, *m/z* 500) in *G. p. palpalis*, and M-47 (*m/z* 453) was seen in both species (Figure 5). Both alkene adducts showed one major fragment ion at *m/z* 439 that was consistent with a structure of $C_{28}SCH_3$ in both species. This was consistent with addition of DMDS to a terminal position of unsaturation on the long alkyl chain. These mass spectra did not present any fragment ions suggesting fragmentation at methyl branches.

High-resolution proton NMR spectra were recorded on original alkene fractions from males of these species. The samples contained microgram quantities of the major alkene and minor amounts of other compounds as shown in Carlson and Langley, 1986. Chemical shifts of two allylic hydrogens (α-meth-

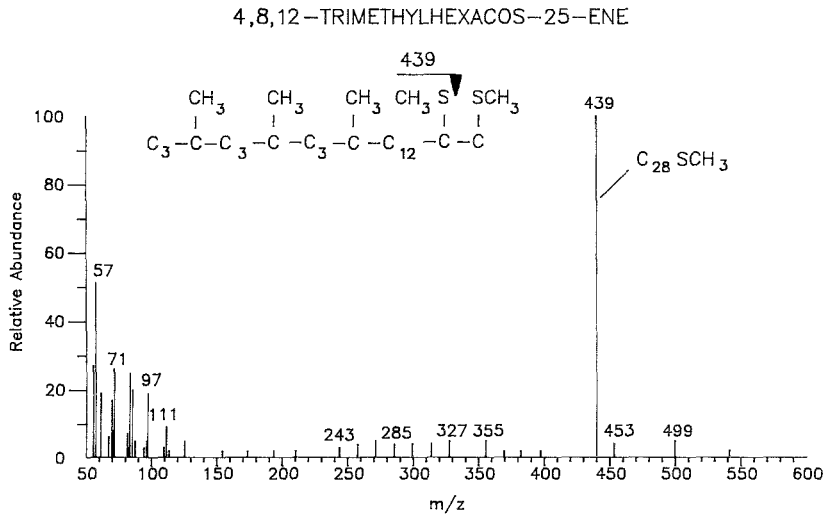


FIG. 5. Electron-impact GC-MS of major male alkene dimethyl disulfide adduct from *G. p. palpalis* males.

ylenes) were seen in all three samples, as were two homoallylic hydrogens (β-methylenes). Thus, the position of unsaturation could not be located on the short, two-carbon alkyl chain of the *palpalis* or *fuscipes* alkenes (A. Allerhand, unpublished data).

Bioassays. A clear dose–response relationship was obtained to natural MA treated onto unwashed but legless and wingless female decoys. Increasing dosages resulted in a clear antiaphrodisiac effect, with loss of all three progressive stages of sexual response, and concomitant increase of nil responses (Table 1). Stage III responses declined steadily to 24% with 1 μg MA, declined still more to 19% with 4 μg of MA, and were eliminated with 10 μg of MA, with nil responses increasing to 91%. In contrast, nearly obligatory stage III responses (93, 91, and 96%) were obtained to wingless, legless, untreated female controls; solvent-treated controls; and those treated with 5 μg vaseline, respectively. Even 25 μg of vaseline did not abolish male response, although 15% of males ignored decoys.

Similar dose–response relationships were obtained using hexane-washed female decoys that were treated with a uniform quantity (4 μg) of synthetic female pheromone (morsilure) mixed with increasing quantities of MA. A linear decline in response was observed with increasing quantities of MA, until a mixture of 1 : 1 proportion abolished all but 3% of stage III responses, or 85% of total male responses (Table 2). Similarly, *G. morsitans* alkanes at 4 μg and hydrocarbons of *G. pallidipes* at 8 μg [2 male equivalents (ME)] also had an

TABLE 1. RESPONSE OF *G. morsitans* MALES TO FRESHLY KILLED FEMALES FOLLOWING APPLICATION OF MALE ALKENES (MA) OR VASELINE DISSOLVED IN 50 μl HEXANE

Substance applied	No. of males	Degree of response (%)[a]			
		III	II	I	0
None	60	93.3	0	0	6.6
Hexane only	60	91.7	0	1.7	6.7
MA					
0.25 μg	69	59.4	8.7	7.2	24.6
0.5 μg	71	38.0	9.9	24.0	28.2
1 μg	82	24.4	7.3	19.5	48.8
2 μg	74	21.6	6.8	17.6	54.1
4 μg	73	19.2	5.8	11.0	64.4
10 μg	66	0	0	9.1	90.9
Vaseline					
5 μg	60	96.7	0	0	3.3
25 μg	60	76.7	3.3	5.0	15.1

[a] 0 = no response; I = short arrestment in male movement on decoy; II = correct positioning of male, with or without flexing of genitalia; III = copulatory attempt.

inhibitory effect on male courting behavior and reduced stage III responses to 4.7% and 12.1%, respectively (2 ME) (Table 2). There was, however, a difference in the stage in which the inhibition was elicited by MA or alkanes. The *G. morsitans* MA and alkanes differed in their effects on stages II and III of the courting behavior. This is evident from the frequency of responses at these stages in pairs of series where the proportion of nonresponses and stage I responses did not differ statistically (Fisher exact text, Table 2, group 3 vs. 6, group 4 vs. 7, group 5 vs. 8). Differences in the proportions of stage II responses were highly significant between series 3 (10.0%) and series 6 (25.6%), also between series 4 (8.3%) and series 7 (22.5%), and differences were significant between series 5 (3.2%) and series 8 (11.6%). Similarly there was a highly significant difference in courting cessation at stage III between series 3 (52.9%) and 6 (20.9%) and between series 4 (24.0%) and 7 (9.2%). The number of these responses in series 5 (2 flies) and 8 (4 flies) was too low for statistical analysis. Series of tests were compared where the overall effect of the inhibition was similar, regardless of the various stages of response.

Thus, with the alkenes, very few reactions stopped at stage II and most males completed the course of mating. In contrast, the largest group among the flies exposed to alkanes ceased courting at stage II. The effect of male hydrocarbons was enhanced when aliquots of both chemical classes were applied together. Abolishment of courting at stages II and III by 0.5 μg MA plus 1 μg

TABLE 2. RESPONSE OF *G. morsitans* MALES TO FRESHLY KILLED, HEXANE-WASHED FEMALES TREATED WITH MORSILURE ALONE OR MIXED WITH HYDROCARBON FROM MALES: *G. morsitans* ALKENES (MA) AND ALKANES OR *G. pallidipes* HYDROCARBONS

Test series	Male substance plus 4 μg morsilure	No. of males	Degree of response (%)[a]			
			III	II	I	0
1	none	60	78.7	6.6	8.3	6.6
2	MA					
	0.25 μg	70	62.9	8.6	10.0	18.6
3	0.5 μg	70	52.9	10.0	8.6	28.6
4	1 μg	60	24.0	8.3	8.3	58.3
5	4 μg	62	3.2	3.2	8.1	85.5
6	*G. morsitans* 2 μg alkanes	32	20.9	25.6	16.3	37.2
7	*G. morsitans* 4 μg alkanes	56	9.2	22.5	11.2	57.2
8	*G. morsitans* 8 μg alkanes	64	4.7	11.6	9.3	74.4
9	MA					
	0.5 μg + 1 μg alkanes	44	1.6	8.1	19.4	71.0
10	1 μg + 2 μg alkanes	41	0	0	18.0	82.0
11	*G. pallidipes* 8 μg hydrocbn.	39	11.8	8.8	22.1	57.4
12	Dotriacosane 4 μg	40	20	25	25	20
13	Tetracontane 4 μg	40	13	25	25	40

[a] Same legend as Table 1. Results of Fisher exact test:

Group MA vs. *G. morsitans* sum of all doses:

deg. response	0 — $P < 0.5$	NS
	I — $P < 0.09$	NS
	II — $P < 7.2 \times 10^{-5}$	highly sig.
	III — $P < 2.7 \times 10^{-6}$	highly sig.

Group 3 vs 6

deg. response	0 — $P < 0.13$	NS
	I — $P < 0.08$	NS
	II — $P < 6.4 \times 10^{-3}$	highly sig.
	III — $P < 1.7 \times 10^{-3}$	highly sig.

Group 4 vs 7

deg. response	0 — $P < 0.44$	NS
	I — $P < 0.28$	NS
	II — $P < 0.01$	sig.
	III — $P < 3.59 \times 10^{-3}$	sig.

Group 5 vs 8

deg. response	0 — $P < 0.05$	NS
	I — $P < 0.30$	NS
	II — $P < 0.03$	sig.
	III — $P < 0.33$	NS

alkanes was similar to that of 4 μg alkenes and greater than that of 4 μg alkanes (Table 2).

Application to decoys of *n*-hydrocarbons at 4 μg with 4 μg morsilure also reduced male response. Of 40 repetitions with dotriacontane, the results for stages 0, I, II, and III were 12, 10, 10, and 8, respectively, and with tetracosane were 16, 10, 9, and 5, respectively (Table 2).

Transfer of Alkanes. Extracts of mated and unmated flies (5–10 days old) were analyzed by GC after Carlson and Langley (1986). Chemical analyses showed that quantities of alkanes were also transferred from males to females (Figure 6). Both KI 2965 and KI 3355 were more prominent in males than females, and both increased in females after mating, while KI 3065 and 3265 remained constant in all flies. In a pooled sample of female flies, the peak ratios R1 for KI 3355/KI 3265 were nearly double in mated females (Table 3). Similarly, small amounts of female-produced sex pheromone, KI 3755 and 3775, were found on males that were held for a short time (4 hr) with conspecific females, as the ratios R2 and R3 also increased (Figure 7, Table 3).

DISCUSSION

Dipteran sex pheromone receptors were described as pairs of shallow convex structures on both the inner and outer surfaces of the upper tibiae (Schlein et al., 1981b). However, Langley et al. (1987) describe active contact chemoreceptors as setae located on the tibiae and tarsomeres that possess straight longitudinal ribbing with pores and are confined primarily to the first two pairs of legs. All males have these setae (770), with nearly equal numbers on each leg, although females have more than half as many.

By isolating, identifying, and quantifying a compound responsible for "abstinon" behavior in tsetse males, we have made significant advances in understanding the nature of this phenomenon. Regardless of the current disagreement over the types and location of sex pheromone receptors and/or abstinon receptors, this information should allow further attack upon the type and location of receptors. Perhaps more importantly, we can then study the still obscure mechanism of reception for hydrocarbon pheromones.

It appears that in the higher diptera, production of sex stimulant pheromone is under gonadotrophic control, that is, its production is coincident with ovarian maturation and unavoidably continues with age. Thus, in several species there are indications of an antiaphrodisiac that would serve to lessen mating attempts by males upon mated females, as well as protecting males from excessive homosexual assault.

While somewhat variable, the average quantity of natural sex stimulant pheromone on a female is 9.4 μg (Carlson et al., 1978). When applied alone,

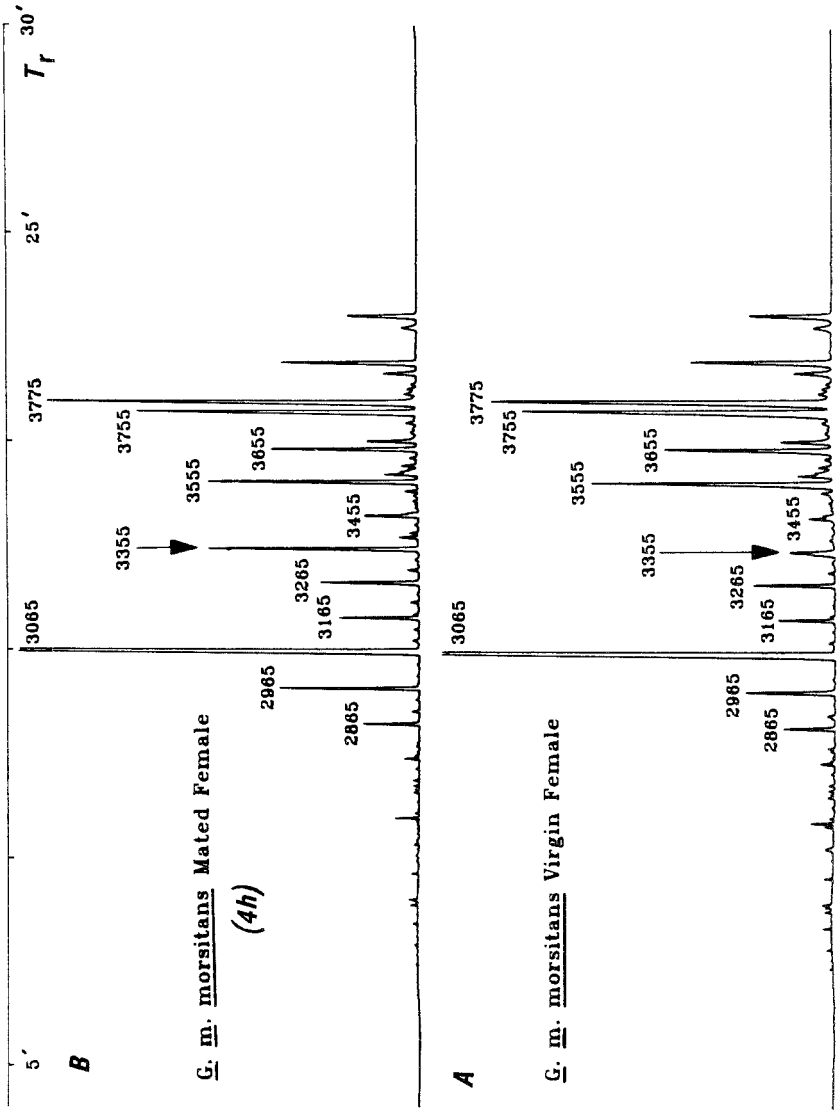


FIG. 6. Capillary GC of alkanes from *G. m. morsitans* females: (A) virgin; (B) mated.

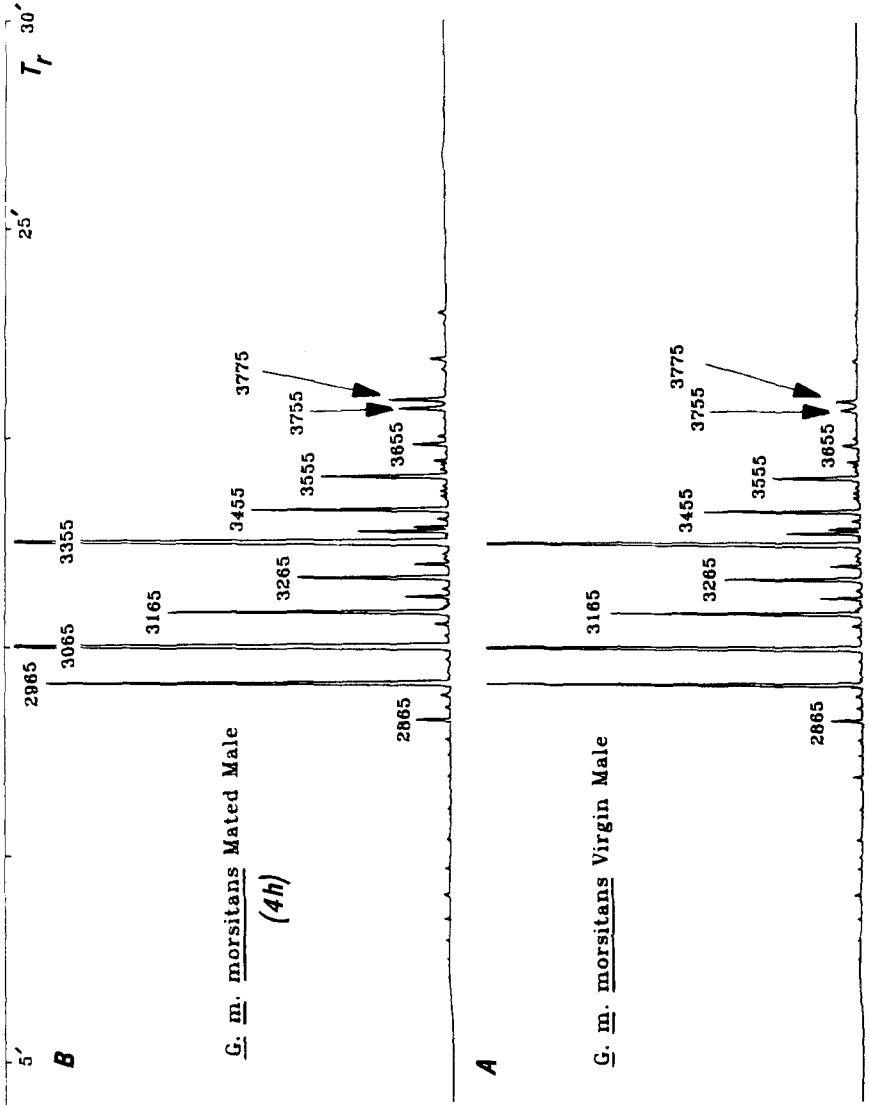


FIG. 7. Capillary GC of alkanes from *G. m. morsitans* males: (A) virgin; (B) mated.

TABLE 3. PEAK RATIOS OF ALKANES PRESENT ON CUTICLE OF *G.m. morsitans* MALES OR FEMALES HELD WITH OPPOSITE SEX

Ratios of alkanes	No. of insects	Females		Males	
		Virgin	Mated	Virgin	Mated
R1 = 3355/3265	10	0.9	2.1	4.6	5.2
R2 = 3755/3265	10	7.1	3.9	0.2	0.3
R3 = 3775/3265	10	7.8	5.2	0.2	0.4

4 μg of synthetic morsilure elicited copulatory responses in more than 90% of the males (Table 2), which is consistent with many previous tests.

Synergism is indicated between male-produced alkanes and alkenes: While it is obvious here that MMA are transferred, transfer of alkanes is less obvious. Nelson and Carlson (1986) show that the saturated analog of MMA (11,15-dimethyltritriacontane, KI 3355) is male-produced and not present in virgin females. Therefore, we rechecked samples of mated females for the male-produced KI 3355 alkane. It appears that small amounts of male alkanes are, in fact, transferred between the sexes with alkenes, as female-to-male transfer can be observed. It is known that male *G. m. morsitans* can be held in cages of conspecific females, then returned to cages of male flies, only to undergo prolonged attacks by sexually stimulated male flies (P.A. Langley, unpublished observations). Chemical analysis shows a small amount of female-produced sex pheromone in extracts of such males. Apparently, not much material is needed to evoke sexual stimulation in males, especially if it has been applied by physical contact rather than by a rather crude application in solvent. If surface contact is enough to transfer biologically active female material onto seti or other outer body parts of males, by analogy, not much material should be necessary to evoke an abstinon-type response in attacking males. A very small amount of male alkene may be sufficient to release this response. We believe that we have observed this effect. As mentioned in Carlson and Langley (1986), MMA or other alkanes are not present in seminal fluid but seem to be cuticular in nature. It appears that both male alkenes and alkanes are deposited on the cuticle of females.

The present tests demonstrated that male *G. morsitans* hydrocarbons include abstinons, which can inhibit male stimulation by female pheromones. Males have 1.3–4 μg of MA (Carlson and Langley, 1986), which was enough to demonstrate dose–response activity when applied to females, and inhibited all but 9% of stage I behavior at a quantity of 10 μg (Table 1). A similar effect was obtained when MA was applied to washed females in a mixture of 4 μg

together with 4 μg of morsilure. Tests with male alkanes similarly showed dose–response activity and inhibited most male response when used at 8 μg together with 4 μg of morsilure on hexane-washed females (Table 2). The alkenes and alkanes functioned synergistically and a combination of 1.5 or 3 μg of both was as effective as higher quantities of each of these compounds alone. The observed degree of synergistic effect is similar to the 96% inhibition of male response by the application of unfractionated extract containing 0.25 male equivalent, reported by Schlein et al. (1981a). The effect of male abstinons in the natural situation must be much stronger since they function without antagonists, and their unequivocal message is the one that inhibits courting. The perception of the female cue by the male must also be very specific to prevent courting of different species in the biotope. Thus, it is not surprising that (synthetic) linear hydrocarbons, which do not occur except as very minor components in tsetse flies of any species, were somewhat effective in reducing male sexual responses.

The classification of the response to alkenes and alkanes indicated differences in their effect. The quantities of MA and alkenes tested in the experiments were not the same, although the quantities of test materials can be compared most readily when their quantities are the same. In this situation, dissimilar quantities of test materials were used because the activity was similar, since the final stages of courting behavior were usually eliminated when responses were minimal. Conversely, initial and intermediate stages of behavior would not be represented adequately in tests with high doses of inhibitor. The alkanes of male *G. morsitans* produced alternating degrees of courting inhibition at stages II and III in series where their total effect was similar. The proportion of no responses in series 3, 4, 5 (192 flies), and also in series 6, 7, 8 (270 flies) was 56.3% The response to MA in the first group of these series amounted to 7.3% at stage II and 28.1% at stage III. The relative proportion of responses at these stages was reversed when alkenes were used and in the second group of experiments. The percentage of stage II responses was 20.0% and stage III was 11.5%. The increase in cessation at stage II in the presence of alkanes involved, in many cases, an unusual behavior expressed by prolonged grasping of the females without moving the genitalia. This behavior was not observed in other tests, including those carried out with *G. pallidipes* male alkanes. In view of these differences, it is suggested that the species-specific male alkenes and alkanes exert a different type of inhibition. The alkenes seem to block mostly the initial cue for courting, and copulation is completed once their effect is overcome, while the alkanes exert their effect in the initial stage, and in addition they mainly inhibit the engagement of the genitalia.

In this context, it is interesting to note that each of the three synthetic candidate pheromones treated against *G. morsitans* males in the first report (Carlson et al., 1978) appeared to elicit male reaction mostly to one specific

stage. For example, following the application of 10 μg of each to shoelace decoys, the respective responses at stages I, II, and III were, to 15,19-dimethylheptatriacontane (compound 1), 4%, 44%, and 8%; to the least active 17,21-dimethylheptatriacontane (compound 2), 16%, 0%, and 0%; to morsilure (compound 3), 4%, 36%, and 40%. Responses to application of a larger quantity of 40 μg of morsilure for stages I, II, and III were 0%, 16%, and 80%. Note that there were errors in numbering compounds 1 and 2 at two locations in this reference; in the center of p. 471 in the text and in Figure 1, compound 2 should refer to the 15,19-dimethyl isomer, and compound 1 to the least-active symmetrical 17,21-dimethyl isomer, as above, and elsewhere (Huyton et al., 1980).

It is tempting to suggest that each of the natural pheromones can initiate at least some courting stimulus, and afterwards each is mostly responsible for one step of the male's sexual behavior. Correspondingly, males possess a set of inhibitory compounds that are clearly transferred to females during courtship. Further studies with fractionated hydrocarbons are needed to clarify the actual role of the different compounds in *G. morsitans* courting.

It is interesting that MMA is structurally related to 15,19-dimethyltritriacontane, which was found to be a candidate pheromone in *G. austeni* and also had excellent sex stimulant activity against *G. morsitans*. Thus we present the first evidence that the unsaturated analog of a sex stimulant is a potent antipheromone.

The decline in responses to decoys treated with *n*-dotriacontane and *n*-tetracosane showed that a physical masking effect is partially responsible for the reduced activity. This effect is difficult to quantitate, but cannot be disregarded in evaluating behavioral effects of natural alkanes.

Acknowledgments—We thank S.K. Milstrey for technical assistance. We thank A.M. Jordan and P.A. Langley of the Tsetse Research Laboratory, University of Bristol, Bristol, England for biological material; and A. Allerhand, University of Indiana, for high-resolution MMR spectra.

REFERENCES

CARLSON, D.A., and LANGLEY, P.A. 1986. Tsetse alkenes: Appearance of novel sex-specific compounds as a result of mating. *J. Insect Physiol.* 32:781–790.

CARLSON, D.A., and MACKLEY, J.W. 1985. Polyunsaturated hydrocarbons in the stable fly. *J. Chem. Ecol.* 11:1485–1496.

CARLSON, D.A., LANGLEY, P.A., and HUYTON, P. 1978. Sex pheromone of the tsetse fly: Isolation, identification and synthesis of contact aphrodisiacs. *Science* 201:750–753.

CARLSON, D.A., LANGLEY, P.A., and COATES, T.W. 1981. Sex pheromones in *Glossina pallidipes*. Proc. 17th ISCTRC Meeting, Arusha, Tanzania, 1981. Organization of African Unity, Nairobi, Kenya. pp. 431–440, 1983.

CARLSON, D.A., NELSON, D.R., LANGLEY, P.A., COATES, T.W., DAVIS, T.L., and

LEEGWATER-VANDERLINDEN, M. 1984. Contact sex pheromone in the Tsetse Fly *Glossina pallidipes* (Austen: Identification and synthesis. *J. Chem. Ecol.* 10:429–450.

CARLSON, D.A., ROAN, C.S., YOST, R.A., and HECTOR, J. 1989. Dimethyl Disulfide Derivatives of Long Chain Alkenes, Alkadienes and Alkatrienes for Gas Chromatography-Mass Spectrometry. *Anal. Chem.* 61:1564–1571.

COATES, T.W., and LANGLEY, P.A. 1982. The causes of mating abstention in male tsetse flies *Glossina morsitans. Physiol. Entomol.* 7:235–242.

DUNKELBLUM, E., TAN, S.H., and SILK, P.J. 1985. Double-bond location in monounsaturated fatty acids by dimethyl disulfide derivatization and mass spectrometry: Application to analysis of fatty acids in pheromone glands of four Lepidoptera. *J. Chem. Ecol.* 11:265–278.

FUKUI, M., and TAKAHASHI, S. 1983. Studies on the mating behavior of the cockroach *Naophoeta cinerea* Oliver (Dictyoptera: Blaberidae) *Mem. Coll. Agric. Kyoto Univ.* 122:25.

HUYTON, P.M., LANGLEY, P.A., CARLSON, D.A., and SCHWARZ, M. 1980. Specificity of contact sex pheromones in tsetse flies, *Glossina* spp. *Physiol. Entomol.* 5:253–264.

JACOBSON, M., ADLER, V.E., and BAUMHOVER, A.H. 1984. A male tobacco budworm pheromone inhibitory to courtship. *J. Environ. Sci. Health.* A19:469–476.

JALLON, J.M., ANTHONY, C., and BENAMAR, O. 1981. Un antiaphrodisiaque produit par les mâles de *Drosophila melanogaster* et transféré aux femelles lors de la copulation. *C.R. Acad. Sci. Paris* 292:1147–1149.

LANGLEY, P.A., HUYTON, P.M., and CARLSON, D.A. 1987. Sex pheromone perception by males of the tsetse fly, *Glossina morsitans morsitans. J. Insect. Physiol.* 33:981–986.

Merck Index. 1976. M. Windholz, ed. 9th edition. Merck and Co., Rahway, New Jersey.

MUHAMMED, S., BUTLER, J.F., and CARLSON, D.A. 1975. Stable fly sex attractant found in female body hydrocarbons. *J. Chem. Ecol.* 1:387–398.

NELSON, D.R., and CARLSON, D.A. 1986. Cuticular hydrocarbons of the tsetse flies *Glossina morsitans, G. austeni* and *G. pallidipes. Insect Biochem.* 16:403–416.

NELSON, D.R., CARLSON, D.A., and FATLAND, C.L. 1988. Cuticular hydrocarbons of the tsetse flies, II: *G. p. palpalis, G. p. gambiensis, G. fuscipes, G. tachinoides* and *G. brevipalpis. J. Chem. Ecol.* 14:963–987.

OFFOR, I.I., CARLSON, D.A., GADZAMA, N.M., and BOZIMO, H.T. 1981. Sex recognition pheromone in the West African tsetse fly, *Glossina palpalis palpalis* (Robineau-Desvoidy). *Insect Sci. Appl.* 1:417–20.

SCHLEIN, Y., GALUN, R., and BEN-ELIAHU, M.N. 1981a. Abstinons-male produced deterrents of mating in flies. *J. Chem. Ecol.* 7:285–290.

SCHLEIN, Y., GALUN, R., and BEN-ELIAHU, M.N. 1981b. Receptors of sex pheromones and abstinons in *Musca domestica* and *Glossina morsitans. J. Chem. Ecol.* 7:291–303.

SCOTT, D., RICHMOND, R.C., and CARLSON, D.A. 1988. Mutual pheromone exchange during mating decreases the sexual attractiveness of *Drosophila* males and females. *Anim. Behav.* 36:1164–1173.

SONNET, P.E. 1979. Synthesis of the male stable fly polyene (*Z, Z*)-1,7,13-pentacosatriene and its geometrical isomer. *J. Chem. Ecol.* 5:415–422.

SONNET, P.E., UEBEL, E.C., and MILLER, R.W. 1977. An unusual polyene from male stable flies. *J. Chem. Ecol.* 3:251–255.

WARTHEN, J.D., JR., and UEBEL, E.C. 1980. Comparison of the unsaturated cuticular hydrocarbons of male and female house crickets, *Acheta domesticus* (L.) (Orthoptera: Gryllidae). *Insect Biochem.* 10:435–439.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

HOST-DERIVED VOLATILES AS ATTRACTANTS AND PHEROMONE SYNERGISTS FOR DRIEDFRUIT BEETLE, *Carpophilus hemipterus*[1]

PATRICK F. DOWD[2,*] and ROBERT J. BARTELT[3]

[2]*Mycotoxin and* [3]*Bioactive Constituents Research Units*
National Center for Agricultural Utilization Research
USDA, Agricultural Research Service
Peoria, Illinois 61604

(Received July 10, 1990; accepted September 21, 1990)

Abstract—The attractiveness of representative host materials, host extracts, and individual host volatiles (primarily carboxylic acids, alcohols, and esters) to *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae) adults in wind-tunnel bioassays was examined. Attractiveness of the materials was examined alone and in combination with the aggregation pheromone. Host materials and extracts were often attractive on their own, and the attractancy was synergized when they were combined with the pheromone. Propanoic and butanoic acids, methanol, 2-propanol, 1-heptanol, methyl butanoate, and propanal were among the most effective attractants relative to the pheromone, but many other compounds significantly synergized the pheromone (typically three- to four fold). Attractiveness and synergism were influenced by the carbon chain length and branching of the substitutents. Straight-chain compounds that had at least three carbon atoms were generally effective as synergists. Many branched-chain compounds were also effective synergists. In general, the degree of attractiveness and synergism could be predicted fairly well with the physicochemical steric (Es) parameter, although the lipophilicity (Pi) parameter also appeared to be useful in explaining the lower activity of short-chain substituents. Thus, many compounds that had only limited attractiveness on their own may nevertheless play and important role in synergizing the pheromone. Structure–activity studies appear to be appropriate not only for determining optimal attractants for these insects, but also for determining effective synergists for the pheromone.

*To whom correspondence should be addressed.

[1]The mention of firms or trade names does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Key Words—Pheromone, aggregation, synergism, ester, carboxylic acid, alcohol, carboxylic ester, driedfruit beetle, *Carpophilus hemipterus*, Coleoptera, Nitidulidae.

INTRODUCTION

The use of host-derived volatiles to locate suitable hosts is important in many insects, including the Coleoptera (Metcalf, 1987). Aggregation pheromones also are produced by many Coleoptera (Jones, 1985). Host-derived volatiles and aggregation pheromones also can interact to produce synergized attraction of bark beetles (Jones, 1985). An increasing number of studies (e.g., Walgenbach et al., 1987; Burkholder, 1988; Oehlschlager et al., 1988) also have demonstrated that stored-product beetle attraction is synergistically enhanced by combining aggregation pheromones and host volatiles. However, synergized attraction of insects by combinations of host volatiles and pheromones is just beginning to be appreciated as a widespread phenomenon.

The driedfruit beetle, *Carpophilus hemipterus*, is a cosmopolitan pest of fresh and dried fruit, as well as many fresh and stored grains, spices, drugs, and seeds (Hinton, 1945). Past information indicates these insects also are attracted by host odors (especially fermenting ones) or isolated volatiles (Smilanick et al., 1978; Blackmer and Phelan, 1988). For example, a 1 : 1 : 1 combination of ethanol–acetaldehyde–ethyl acetate was a highly effective combination for attracting *C. hemipterus* (Smilanick et al., 1978). Fermented baits have been used successfully to reduce populations of this insect when the insects collected by the traps were not immediately killed (Warner, 1960, 1961). However, poisoned attractive baits were not able to outcompete naturally ripe (and presumably fermenting) figs in orchards (Smilanick, 1979). This information suggests that a combination of aggregation pheromone and host volatiles is necessary to equal the attraction of natural host materials with insects present and could potentially involve synergism as well. Bartelt et al. (1990a) recently reported the first example of an aggregation pheromone from nitidulids, which interacts synergistically with some host volatiles. To further examine this important interaction, host materials, host extracts, and individual host-derived volatiles were combined with the pheromone of *C. hemipterus* and tested for relative attractiveness versus individual components in wind-tunnel bioassays. Structure–activity studies of individual host components combined with the pheromone extract also were run to determine if optimal host-derived individual attractancy, synergism, and overall attractancy could be predicted by quantitative analysis of physiocochemical parameters or use of other structural relationships.

METHODS AND MATERIALS

Insects. The *C. hemipterus* were reared according to previously described methods (Dowd, 1987). Adults used in assays were 7–10 days old. The insects were conditioned for flight by 16 hr starvation (see Bartelt et al., 1990a).

Hosts and Chemicals. Whole oranges, bananas, and apple juice were obtained from a local grocery store. Oranges were squeezed for juice immediately before the assays. Milk-stage dent corn was obtained from greenhouse-grown plants. The *Saccharomyces cerevisae* used was a baker's yeast strain (Fleishman's dried activated). The culture of the wild yeast, *Zygosaccharomyces bailii*, a common contaminant of fermenting fruit (NRRL Y-2227), was obtained from the Northern Regional Research Center culture collection. Bananas were fermented with the two yeast strains by sprinkling or loop inoculating the freshly cut surface and capping in 35 ml cups. The liquid produced by the fermentation (after two months) was used in assays. Fresh *S. cerevisae* were obtained by sprinkling the dry yeast onto potato dextrose agar and scraping the colonies off the surface after two weeks. These yeast were made up as a 10% suspension in distilled water. Individual host volatiles and sources are reported in Table 1. A series of acids, alcohols, methyl esters, and acetate esters were used to determine structure–activity relationships.

Wind-Tunnel Bioassays. Assays were performed according to previously reported methods (Bartelt et al., 1990a). Briefly, ca. 300–600 starved insects were assayed at one time. Disks of filter paper (Whatman 541 or related, 7 cm diameter) were folded into quarters, treated with the liquid attractant(s), and hung with binder clips ca. 30 cm apart and 40 cm above the floor in the upwind end of the wind tunnel. The pheromone and host volatile were applied to different areas of the filter paper when tested in combination. For the liquid attractants, 20-μl quantities were applied to the folded filter paper. Esters, alcohols, aldehydes, ketones, and acids were applied as 10% solutions/suspensions in mineral oil, since these conditions provided a concentration and release rate previously proven effective in the field (Smilanick et al., 1978) and found to be effective in our wind-tunnel assays (see Results). When chemicals were combined in solution, the total contribution of an individual chemical was 10%. The pheromone source was the hydrocarbon fraction from extracts of cultures that contained male beetles (Bartelt et al., 1990). The concentration of the most abundant pheromone component, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene, was ca. 50 pg/μl when quantitated by gas chromatography; 20 μl of this extract was used in each test (ca. 1 ng of the major component and proportional amounts of the cooccurring components). Comparisons of this initial material and an isolated Tenax-trapped volatile extract developed after the start of these assays (Bartelt et al., 1990a) did not yield significantly different results for representative combinations, so the diet extract was used throughout

TABLE 1. CHEMICALS, SOURCES, AND PURITIES[a]

Compound	Source	Purity (%)
Alcohols		
Methanol	EM Labs	99.9
Ethanol	USICC	100
1-Propanol	Aldrich	99.7
2-Propanol (isopropyl alcohol)	Baker	99.8
1-Butanol	Fisher	99.95
2-Methyl-1-propanol (isobutyl alcohol)	Aldrich	99.9
2-Butanol (*s*-butyl alcohol)	Aldrich	99+
2-Methyl-2-propanol (*t*-butyl alcohol)	Aldrich	99+
2-Methyl-1-butanol	Aldrich	99+
1-Pentanol	Aldrich	99+
1-Heptanol	Sigma	99
Acids		
Formic	Aldrich	95
Acetic	EM Labs	99.7
Propanoic	Sigma	99
Butanoic	Aldrich	99+
2-Methylpropanoic (isobutyric)	Aldrich	99+
Pentanoic	Kodak	98
3-Methylbutanoic (isovaleric)	Aldrich	99
2-Methylbutanoic	Kodak	99
2,2-Dimethylpropanoic	Aldrich	99
3-Methylpentanoic	Aldrich	97
4-Methylpentanoic	Kodak	99
2,2-Dimethylbutanoic	Aldrich	96
Acetate esters		
Methyl acetate	PolySciences	98+
Ethyl acetate	MCB	99.5
Propyl acetate	PolySciences	98+
1-Methylethyl (isopropyl) acetate	PolySciences	98+
Butyl acetate	Aldrich	99+
2-Methylpropyl (isobutyl) acetate	PolySciences	98+
1-Methylpropyl (*s*-butyl) acetate	PolySciences	98+
1,1-Dimethylethyl (*t*-butyl) acetate	PolySciences	98+
Pentyl acetate	MCB	98
3-Methylbutyl (isopentyl) acetate	PolySciences	98+
1-Methylbutyl (*s*-pentyl) acetate	PolySciences	98+
2-Methylbutyl acetate	PolySciences	98+
1-Ethylpropyl acetate	PolySciences	98+
Octyl acetate	Aldrich	99+
Methyl esters		
Methyl formate	PolySciences	98+
Methyl acetate	PolySciences	98+
Methyl propanoate	PolySciences	98+

TABLE 1. Continued

Compound	Source	Purity (%)
Methyl butanoate	PolySciences	98+
Methyl 2-methylpropanoate (isobutyrate)	PolySciences	98+
Methyl pentanoate	PolySciences	98+
Methyl 3-methylbutanoate (isovalerate)	PolySciences	98+
Methyl 2-methylbutanoate	PolySciences	98+
Methyl 2,2-dimethylpropanoate (*t*-valerate)	PolySciences	98+
Methyl 4-methylpentanoate	PolySciences	98+
Other esters		
Ethyl propanoate	Sigma	99+
Ethyl butanoate	Aldrich	99+
Ethyl 2-methylpropanoate (isobutyrate)	Aldrich	99+
Ethyl 3-methylbutanoate (isovalerate)	Sigma	NS
Propyl propanoate	Aldrich	99+
Heptyl hexanoate (caproate)	Pfaltz & Bauer	NS
Other compounds		
Water	In-house	99+
Acetaldehyde	Baker	NS
Propanal	Aldrich	99+
Pentanal	Aldrich	99
2-Pentanone	Aldrich	97
2-Hydroxypropanoic acid (lactic acid)	Fisher	85

[a] Branched compounds listed as methyl or ethyl derivitives for ease of considering location of branching to synergistic effects. NS = not specified.

this study for the sake of consistency. Two treatments were always compared in the wind tunnel at one time. The test duration was always 3 min. The number of beetles landing on each treatment was recorded.

Experimental Design. To evaluate each host volatile for attractiveness and synergistic activity, an experiment was conducted involving three treatments: the host volatile (A), the pheromone (P), and the host volatile plus the pheromone (A + P). These treatments were compared two at a time, and all possible combinations were tested an equal number of times (a balanced, incomplete block design). In general, each treatment was used a total of eight times. Treatment positions in the wind tunnel were reversed in successive replications to eliminate potential bias due to position effects. The pheromone served as a relative control in these experiments.

Our experience has been that counts for a particular treatment over the 3-min bioassay period varied widely over different days, depending on the number of beetles in the wind tunnel. The paired experimental design was used to

compensate for this variability, and the ratio of counts for two paired treatments tended to remain very constant despite changes in overall beetle numbers or activity.

Three ratios were of particular interest in this study. The first ratio, the bioassay count for the test volatile alone plus the bioassay count for the pheromone alone, divided by the counts for the pheromone treatments alone (*relative attractancy*, RA), expressed the activity of the volatiles relative to a "standard" attractant (the pheromone). An RA of 1.0 indicated no attractancy of the attractant by itself. The second value, the *synergist ratio* (SR), was the bioassay count for the pheromone combined with the host volatile, divided by the sum of the counts for the separated pheromone and volatile treatments. This ratio expressed the degree that the activity of the combined attractants exceeded the total activity of the separate attractants. An SR of 1.0 indicated the absence of synergism, in this case the resulting attractancy was merely additive. The third value, the *total attractancy* (TA), is the same as the relative attractancy times the synergist ratio. It is defined as the counts for the pheromone combined with the host volatile divided by the counts for the pheromone alone. Once again, a TA value of 1.0 indicated no attractancy relative to the pheromone. These values are more fully defined in the Results section, where actual data are examined.

One initial test involved a filter paper blank to see if a blank yielded useful information. Two additional experiments were run to evaluate the stability of beetle activity and the relative attractiveness of a treatment over time. Stability of these values is necessary to make relevant comparisons of synergistic activity.

The first of these two experiments involved examining the attractancy of apple juice and the pheromone over time after the insects were released directly into the cage. The activity was examined at constant intervals until a few hours after the number of responses peaked (22 total hr). Three 3-min tests were run every half hour, with 15 min between each group of tests.

The second experiment involved pheromone, octyl acetate, and octyl acetate plus pheromone in the balanced, incomplete block design and was run repeatedly for eight consecutive hours after starving the beetles overnight in the same manner as described for the apple juice assays. RA, SR, and TA ratios were calculated for each hourly segment (see Results).

The bulk of the study involved testing host materials, extracts, and volatiles in a balanced, incomplete block design such that there were eight replications of each treatment per hour (twelve 3-min tests per hour, with 2 min between tests). In cases where responses to the compounds were weak, the response of the insects was retested with a complex known to be effective (e.g., pheromone plus propyl acetate or octyl acetate) in order to ensure the insects were in a responsive mode.

In the final series of experiments, octyle acetate was used as a standard attractant for evaluating the relative synergistic activity of other host volatiles. The three treatments were: the pheromone, octyl acetate plus the pheromone, and the host volatile plus the pheromone. Once again, the balanced, incomplete block design was used.

Statistical Analyses. All bioassay experiments were analyzed by categorical methods (chi-square tests) applying a model of quasiindependence to the incomplete block designs (Fienberg, 1977; Bartelt et al., 1990b). Statistical tests were performed first to determine whether departures from this model were within allowable limits. A small chi-square statistic (large P value) for the model of quasiindependence indicated the model described the data set adequately. In our large series of experiments, we expected 95% of the chi-square statistics to have P values >0.05. This expectation was largely met ($P > 0.05$ for 109 of 116 experiments), validating subsequent tests on treatment effects. Thus, categorical analysis appeared to be appropriate for our data. The use of ratios of fitted values to summarize treatment effects followed naturally from the quasiindependence model. Conditional G^2 tests (analogous to t tests in analysis of variance; see Feinberg, 1977, p. 47) were used to test for the equivalence of treatments or the importance of other effects of interest, such as blocking effects. The treatments were concluded to differ if $P < 0.05$ when this statistic was compared with the chi-square distribution. Further details and examples of the statistical tests are provided in the Results.

Since prior work (Bartelt et al., 1990a) indicated synergism between the pheromone and host-derived volatiles could occur, we wished to determine when synergism was statistically significant. The presence of synergism could be tested for if the number of beetles responding to a treatment remained effectively constant within each set of three consecutive tests [P vs. A, P vs. (P + A), and A vs. (P + A)], regardless of what the treatment was paired with (in other words, if the six data values could be viewed as coming from one large block rather than three incomplete blocks—without inflating residual variance). Conditional tests indicated this interpretation was usually justified. A conditional G^2 statistic was constructed to determine whether the total responses to P plus the total responses to A differed from the total for (P + A) (i.e., whether SR > 1).

Quantitative Structure–Activity Analysis. The use of physiocochemical parameters to allow mathematical correlation between chemical structure and biological activity permits a better understanding of biological phenomena as influenced by chemical perturbation. Due to the obvious influence of branching and chain length on different types of attractancy (RA, SR, and TA), we concentrated on use of the sigma * electronic parameter, the Taft steric parameter (Es), and the Pi constant, respectively, in an attempt to evaluate the quantitative contribution of these types of modifications to the relative activity of alcohols,

acids, and esters tested. Values from the monograph of Hansch and Leo (1979) were used to attempt to evaluate activity based on electronic, steric, and lipophilic influences. Values were not available for all compounds that were tested. Initially, values were plotted to determine appropriate models for correlation analyses (e.g., linear, logarithmic) and to determine appropriate portions to consider in substituent analysis (e.g., include or exclude the carbon atom with the functional group in the carbon chain length). The MAXR STEPWISE procedure (SAS Institute, 1985) was used to obtain substituent constants for one- and two-parameter models and included reports of correlation coefficients and probability values. Plots were examined in light of correlation values to visually determine outliers, which might obscure underlying relationships. The procedure then was rerun without these values to see if better correlation resulted—up to an r^2 of 0.7 or 0.8. Typically, this only involved the removal of one or two points.

RESULTS

Properties of Bioassays

One property of the bioassay is that beetles land extremely rarely on filter paper blanks. The mean count for such a control was near zero in all cases we observed. For example, during 54 min or bioassay time in one experiment, 579 beetles landed at a combination of pheromone plus coattractant, but only one landed on the filter paper blank (Table 2). The filter paper blank did not provide any information on the activity of the beetles during the bioassays or any information on the relative attractiveness of other treatments. Thus, it was generally excluded in the experiments, and the response to the pheromone treatment was used as a standard (as described previously).

As our previous observations indicated (Bartelt et al., 1990a), a starvation period was necessary for the beetles to respond. The beetles did not respond to the apple juice, or pheromone, or even take flight, for several hours after being

TABLE 2. RESPONSIVENESS OF *C. hemipterus* TO FILTER PAPER BLANK

Treatment	Total count	Mean count (N = 18)
Filter paper blank	1	0.06
Pheromone	92	5.11
Ethanol + ethyl acetate + acetaldehyde	172	9.56
Ethanol + ethyl acetate + acetaldehyde + pheromone	579	32.17

transferred from rearing cups into the wind tunnel (Figure 1). After this time, the response level increased steadily until at 9–13 hr after release, an average of over 25 beetles responded to the apple juice–pheromone combination per 3-min test. During this period, the dramatic enhancement of the attraction of the apple juice by the pheromone was clearly evident, as we observed previously (Bartelt et al., 1990a). After 13 hr, however, the response level decreased again and became more erratic. This study demonstrated that ratios of numbers of beetles responding to the different attractants was quite consistent over a 6-hr period (between 7 and 13 hr after release). The quasiindependence model described the experiment adequately when just one parameter for each treatment was fitted over the entire 6-hr period ($G^2 = 46.82$, 34 *df*, $P > 0.05$), indicating essentially the same results could be expected at any interval during the 6-hr period. In addition, there was no significant interaction between the treatments and the time period ($G^2 = 7.38$, 4 *df*, $P > 0.10$), again indicating that the data obtained at any instant over the 6-hr time period would be reasonable. For this experiment, the fitted ratios were: apple juice/pheromone = 0.35, pheromone + apple juice/apple juice = 12.42, and pheromone + apple juice/pheromone = 4.37 (note that the product of the first two ratios essentially equals the third ratio; this is implicit in the quasiindependence model). The activity profile illustrated in Figure 1 is throught not to represent a circadian activity rhythm because the time of greatest activity depended more on when the beetles were separated from their food than on the time of day (see below).

The peak activity period could be adjusted so that it occurred at a more convenient time if the beetles were placed in the wind tunnel in the evening before the day the tests were to be run, with lights and fan off (see Methods and Materials). When the lights and fan were turned on the following morning, the beetles usually began to fly actively within 1 hr and responded consistently

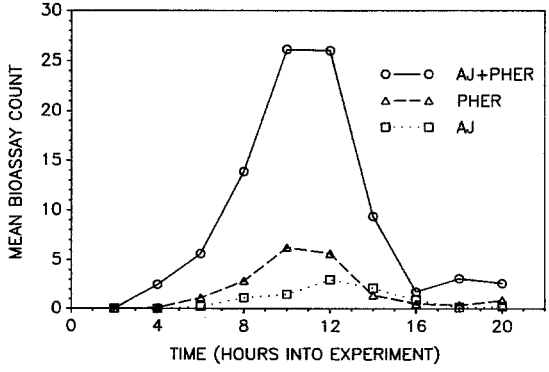


FIG. 1. Response of *C. hemipterus* adults to attractants over time in the wind-tunnel assays. AJ = apple juice, PHER = pheromone.

for about 6 hr once they became active (Table 3). Although there were obvious overall differences in the response to the different treatments ($G^2 = 512.7$, 2 *df*, $P \ll 0.001$), the treatment by hour interaction was not significant ($G^2 = 16.47$, 10 *df*, $P \geq 0.05$). Thus, the bioassay could give reliable data for as long as 6 hr in one day. The column for the TA in Table 3 represents an "acceptable" degree of variability, one that is not statistically significant.

Finally, the appropriateness of the 10% formulation of the coattractants in mineral oil was investigated. Methyl butyrate was used because it was one of the most active single compounds tested. This ester appeared to be inactive at a 0.1% concentration and exhibited only slight activity at 1.0% (Table 3). Thus, use of a 10% formulation appeared appropriate.

Representative compounds also were tested on different days, and variability remained within a reasonable range. For example, trials on completely different days yielded synergist ratios of 0.69 and 1.34 for 2-methylpropionic acid, 2.93 and 4.32 for 2-methylbutyric acid, 3.63 and 2.69 for ethanol, 1.75 and 1.68 for 3-methylpropionic acid. Thus, the values appear to be generally reliable $\pm 20\%$ of the synergist ratio.

Representative Hosts and Host Derivatives

Whole host materials, fermented host materials, and host extracts generally exhibited some attractancy on their own; fermented banana was among the most attractive based on the RA value (Table 4). They all acted as effective synergists for the pheromone in spite of the fact that relative attractancy was low in some caes. Significant synergism (SRs) occurred in all cases, and effective compounds were more widely distributed compared to those producing high RAs. Again, one of the fermented materials was most effective. Thus, it is not surprising that the total attractancy (TA) for the fermented banana material was among the greatest since it incorporates both high RA and SR values, although the TA of the orange juice was comparable.

Effectiveness of the individual volatiles was even more variable. Other than 2-methylpropanoic acid, many of the mono-branched acids were relatively effective attractants, having RAs above 1.5 (Table 4). The C_2 acids and those with greater chain length, except for those that had two methyl groups in the 2-position, were effective synergists. Propanoic and butanoic acid had the greatest TAs.

The pattern for alcohols was somewhat different. Methanol and 2-propanol were particularly effective as individual attractants. Significant synergism was seen in many cases and was of approximately the same level (2–3×). Most of the C_1–C_4 straight-chain and branched alcohols, and C_5 and longer straight-chain alcohols were the effective synergists, Methanol, 2-propanol, 1-heptanol, and 1-methyl-2-propanol had greatest TAs.

TABLE 3. TIME AND DOSE-RELATED RESPONSES OF *C. hemipterus*[a]

Treatment	Mean beetles attracted			Fitted ratios		
	P	A	P + A	RA	SR	TA
Time interval test with octyl acetate						
Hour 1	2.9	1.0	16.0	1.37	4.13	4.44
Hour 2	4.9	2.0	20.4	1.43	2.96	3.10
Hour 3	3.9	1.0	20.1	1.25	4.13	5.44
Hour 4	3.1	1.4	12.0	1.33	2.67	2.59
Hour 5	2.8	1.9	13.4	1.49	2.89	3.04
Hour 6	3.4	0.6	11.5	1.18	2.88	4.13
Overall	3.5	1.3	15.6	1.35	3.25	3.54
Concentration test with methyl butyrate (in mineral oil)						
0.1%	11.1a	0.6b	10.8a	1.06	0.91	0.86
1.0%	3.5a	2.4a	9.5b	1.59	1.62	1.90
10.0%	1.8a	2.6a	10.9b	2.52	2.49	5.46

[a] Treatments whose means are followed by the same letter are not significantly different ($P > 0.05$, conditional G^2 tests). The means were not used in calculation of statistical tests but are presented to show the magnitude of the biological responses.

Of the methyl esters, methyl butanoate had the greatest individual attractancy, followed distantly by methyl propanoate. Once again, significant synergism occurred in many cases, and generally was 2–3×. Methyl esters with straight-chain acids other than formate and branched-acid moieties having no more than one methyl group tended to be effective synergists. As would be expected by the high RAs, methyl butanoate and methyl propanoate had very high TAs.

For the acetate esters, pentyl acetate and propyl acetate had the highest RAs. Those acetate esters with straight-chain substitutions longer than methyl, as well as 1-methylethyl and 2-methylpropyl-substituted compounds were effective synergists, while those with other branched or secondary alcohols were not effective. Synergist ratios were fairly consistent for all of the linear esters, but some branched esters appeared antagonistic. Pentyl acetate and ethyl acetate had the highest TAs. Other esters with notable TAs were ethyl butanoate, ethyl propanoate, and heptyl hexanoate; all three had high and significant SRs.

Of the additional compounds, propanal had the highest RA of any individual compound tested. The aldehydes, ketones, hydroxy acid, and even water showed significant synergism. Propanal, 2-pentanone, and acetaldehyde had highest TAs. The mixtures of components all had very high individual attractancy. Synergist ratios were significant and comparable except for that of the ethanol–ethyl butanoate–2-hydroxypropanoic acid combination, which was at

TABLE 4. HOST EXTRACTS, AND REPRESENTATIVE COMPOUNDS AS *C. hemipterus* ATTRACTANTS AND PHEROMONE SYNERGISTS[a]

Attractant	Mean beetles attracted			Fitted ratios		
	P	A	P + A	RA	SR	TA
Natural materials						
Apple juice	4.9a	1.9b	22.0c	1.34	3.24***	4.37
Orange juice	1.1a	0.6a	11.5b	1.50	6.57***	6.73
Corn juice	2.0a	0.0b	6.3c	1.00	2.56***	2.89
Baker's yeast	2.6a	0.6b	6.6c	1.26	2.04**	4.53
S.c.[b] banana	3.5a	2.3a	30.4b	1.50	5.24*	7.64
Z.b.[b] banana	3.0a	3.3a	24.8b	1.92	3.94*	7.59
Acids						
Formic	1.6a	0.1b	2.0a	1.09	1.14	1.80
Acetic	3.4a	0.4b	9.4c	1.09	2.50***	1.94
Propanoic	9.9a	6.8b	76.5c	1.68	4.60***	9.91
Butanoic	3.3a	0.5b	20.3c	1.17	5.40***	6.68
2-Methylpropanoic	17.7a	0.4b	22.4c	1.02	1.24*	1.29
Pentanoic	6.4a	1.9b	27.9c	1.26	3.38***	3.84
3-Methylbutanoic	7.4a	1.0b	22.0c	1.13	2.63***	2.83
2-Methylbutanoic	4.6a	4.6b	27.5c	1.53	3.73***	5.03
2,2-Dimethylpropanoic	6.9a	0.1b	6.8a	1.02	0.96	0.79
3-Methylpentanoic	6.0a	3.3a	37.4b	1.62	4.04***	4.83
4-Methylpentanoic	4.1a	3.4a	25.5b	1.91	3.40***	6.52
2,2-Dimethylbutanoic	11.9a	0.1b	16.9a	1.01	1.41*	1.23
Alcohols						
Methanol	2.6a	3.9a	15.0b	2.51	2.33***	7.93
Ethanol	6.0a	1.3b	26.0c	1.22	3.61***	4.25
1-Propanol	16.0a	5.4b	62.5c	1.37	2.92***	5.01
2-Propanol	9.4a	7.5a	42.0b	1.86	2.49***	5.63
Butanol	3.3a	0.1b	8.3c	1.04	2.44***	2.26
2-Methyl-1-propanol	35.5a	0.3b	25.5a	1.01	0.71(*)	0.79
2-Butanol	8.1a	0.5b	18.6c	1.07	2.16***	3.21
2-Methyl-2-propanol	8.5a	1.6b	37.8c	1.22	3.73***	5.28
2-Methyl-1-butanol	5.0a	0.3b	3.6a	1.05	0.69	0.78
3-Methyl-1-butanol	17.0a	1.6b	12.0c	1.12	0.64(*)	0.78
1-Heptanol	1.8a	0.3b	6.0c	1.17	3.0***	5.45
Methyl esters						
Formate	17.1a	2.1b	20.0a	1.15	1.04	1.10
Acetate	6.5a	1.8b	24.1c	1.23	2.92***	2.46
Propanoate	6.9a	6.9a	45.1b	2.05	3.28***	8.20
Butanoate	3.6a	10.0a	37.3b	3.75	2.73***	14.01
2-Methylpropanoate	3.4a	1.3b	16.1c	1.39	3.49***	5.79
Pentanoate	6.5a	1.5b	26.6c	1.26	3.33***	5.13
3-Methylbutanoate	6.1a	0.1b	10.1c	1.02	1.62***	1.75
2-Methylbutanoate	4.5a	0.4b	17.1c	1.09	3.51***	3.63
2,2-Dimethylpropanoate	3.1a	0.4b	4.9a	1.12	1.39	1.56
4-Methylpentanoate	6.9a	0.9b	22.8c	1.12	2.94***	3.60

TABLE 4. Continued

Attractant	Mean beetles attracted			Fitted ratios		
	P	A	P + A	RA	SR	TA
Acetate esters						
Methyl	6.5a	1.8b	24.1c	1.23	2.92***	2.46
Ethyl	3.3a	0.4b	15.1c	1.12	4.17***	6.01
Propyl	3.3a	1.4b	18.3c	1.47	3.91***	5.41
1-Methylethyl	7.1a	1.6b	26.3c	1.22	3.00***	3.32
Butyl	3.0a	0.1b	10.8c	1.04	3.44***	3.58
2-Methylpropyl	2.9a	0.0b	9.3c	1.00	3.21***	3.78
1-Methylpropyl	2.4a	0.3b	3.9a	1.11	1.48	1.75
1,1-Dimethylethyl	2.8a	0.0b	1.5a	1.00	0.55	0.55
Pentyl	3.1a	2.0a	14.6c	1.71	2.85***	7.13
3-Methylbutyl	5.1a	0.3b	5.4a	1.06	1.01	1.45
2-Methylbutyl	5.9a	0.0b	3.4a	1.00	0.57(*)	0.90
1-Methylbutyl	6.4a	1.0b	10.3c	1.18	1.39	2.16
1-Ethylpropyl	6.3a	0.4b	5.0a	1.10	0.75	0.97
Octyl	3.3a	0.5b	14.4c	1.16	3.83***	5.78
Benzyl	1.0a	0.1b	2.0a	1.11	1.78	2.13
Other esters						
Ethyl 3-methylbutanoate	5.1a	0.4b	4.9a	1.08	0.89	0.89
Ethyl 2-methylpropanoate	1.8a	0.1b	3.8a	1.05	2.00*	1.18
Ethyl propanoate	5.9a	1.8b	37.9c	1.31	4.97***	7.57
Ethyl butanoate	2.9a	0.5b	19.0c	1.20	5.63***	8.34
Heptyl hexanoate	1.4a	0.4a	9.4b	1.32	5.36***	6.72
Other compounds						
Acetaldehyde	2.6a	0.0b	9.0c	1.00	3.43***	5.14
Propanal	1.0a	2.9b	7.3c	4.54	1.87**	6.62
2-Pentanone	0.3a	1.0a	8.1b	1.31	6.50***	6.53
2-OH-propanoic acid	4.3a	0.3b	9.4a	1.06	2.08***	1.84
Water	6.3a	0.3b	10.9c	1.04	1.67**	1.64
Mixtures						
Ethanol–ethyl acetate–acetaldehyde	5.1a	9.6b	32.2c	3.73	2.19***	8.29
Ethanol–ethyl propanoate–acetaldehyde	3.9a	7.6b	32.6c	2.88	2.84***	7.03
Ethanol–ethyl propanoate–propanoic acid	5.8a	9.9b	43.3c	2.70	2.77***	9.37
Ethanol–ethyl butanoate–hydroxypropanoic acid	2.5a	2.4a	33.3b	2.03	6.82***	19.30

[a] Treatments whose means are followed by the same letter are not significantly different ($P \geq 0.05$, conditional G^2 tests). The means were not used in the calculation of statistical tests but are presented to show the magnitude of the biological responses. The symbols *, **, *** indicate that synergism was significant at the 0.05, 0.01, or 0.001 level, respectively (conditional G^2 tests). (*) indicates that the response to the mixture was significant less than an "additive" response.

[b] *Saccharomyces cerevisae* and *Zygosaccharomyces bailii*.

least 2× the others. This mixture also had the highest TA, but TAs for other mixtures were generally higher than those of any individual components.

Many of these combinations were included in octyl acetate comparisons and yielded relative attractancy values that might be expected based on the previous results. The TA for octyl acetate did fluctuate throughout the experiment, but generally averaged around 6.0 (Table 5). As would be expected, the most potent individual compound tested, methyl butanoate, gave the highest attractancy relative to the octyl acetate, and one of the highest TAs. Poor attractants/synergists as revealed in prior assays, such as methyl formate, were again proven poor relative to octyl acetate. Thus, using the pheromone alone for a benchmark appeared generally reliable, although a simple combined comparison vs. pheromone plus octyl acetate may also prove useful for rapid determinations of relative effectiveness.

Quantitative Structure–Activity Relationships

Initial examinations of the electronic parameter, sigma *, indicated it was of little use in explaining differences of activity in compounds with different substituents, so it was excluded from further consideration. Transformations of the Es parameter were necessary to linearize equations for RA [(natural log of the absolute value of Es)/Es] and TA (natural log of the absolute value of (Es); the relationship between Es and SR appeared linear. In addition, consideration of whether or not to include the functional groups in the carbon count was important. They were excluded for free alcohols, free acids, and methyl esters (in other words, propyl alcohol was considered to have an ethyl substituent, and so on). Steric parameters were available for all linear chains except for heptyl, and all 5-carbon branched chains except for 1-methylbutyl and 2-methylbutyl. Lipophilic parameters were only available for 1- to 4-carbon compounds, but included all branched substitutents. In some cases, visual inspection of plotted curves suggested outliers. Exclusion of these outliers will be detailed for each set of equations.

Free Acids. The relative attractancy of acids was poorly described when all available compounds were included, but elimination of methyl, 1-methylpropyl, and 3-methylbutyl groups increased the r^2 to 0.81 (Table 6), indicating a high correlation between (log Es)/Es and RA. Inclusion of the Pi parameter helped somewhat (probably to compensate for the methyl derivitive), but the relationship was still poorly described when the 1-methylethyl value was included. Correlation for all included values was much better for the SR, but elimination of 1-methylpropyl and 1-methylethyl was necessary to get a significantly high correlation. Inclusion of the PI value did not appear to compensate for the low activity of the methyl derivative. The log (Es) factor was also a fairly good predictor of TA, but again correlation became highly significant

TABLE 5. ATTRACTANCY OF PHEROMONE–ATTRACTANT COMBINATIONS TO *C. hemipterus* RELATIVE TO OCTYL ACETATE STANDARD[a]

Compound	Mean beetles attracted			Fitted ratios		
	P	C + P	O + P	TAA	TAO	RAO
Propyl acetate	3.4a	13.3b	11.8b	4.48	4.10	1.09
1-Methylethyl acetate	2.6a	9.0b	14.5c	3.76	6.19	0.61*
2-Methylpropyl acetate	2.9a	9.3b	15.1c	3.78	6.92	0.55*
1-Methylpropyl acetate	2.4a	3.9a	10.8b	1.75	4.65	0.38**
1,1-Dimethylethyl acetate	2.8a	1.5a	18.4b	0.54	6.65	0.08***
Methyl formate	2.3a	2.3a	8.0b	0.82	3.18	0.26***
Methyl acetate	3.6a	11.5b	28.3c	3.65	10.94	0.33***
Methyl propanoate	3.0a	34.8b	19.0c	19.26	7.29	2.64***
Methyl butanoate	2.8a	36.3b	12.9c	25.00	4.10	4.70***
Methyl pentanoate	4.0a	26.1b	18.4c	7.47	5.19	1.44*
Propyl propanoate	6.1a	60.6b	27.3c	16.66	4.92	3.39***
Butyl propanoate	8.5a	31.1b	28.3b	4.39	3.81	1.16
Ethanol–ethyl acetate–acetaldehyde 1 : 1 : 1	3.0a	58.9b	23.4c	23.15	8.57	2.70***
Methanol–propanoic acid–methyl butanoate 1 : 1 : 1	3.8a	39.4b	16.1c	34.92	8.15	4.28***

[a]TAA = total attractancy of attractant, TAO = total attractancy of octyl acetate, RAO = ratio of attractancy of attractant to octyl acetate. Treatments whose means are followed by the same letter are not significantly different ($P \geq 0.05$, conditional G^2 tests). The means were not used in the calculation of statistical tests but are presented to show the magnitude of the biological responses. The symbols *, **, *** indicate that the ratios were significantly different from 1.0 at the 0.05, 0.01, or 0.001 level, respectively (conditional G^2 tests).

when the methyl and 1-methylethyl values were removed. Inclusion of the Pi parameter in the model again did not help to explain the variation.

Free Alcohols. The RA of alcohols was reasonable, but not significant, when all available compounds were included. Elimination of the methyl group increased the r^2 somewhat, indicating some correlation between (log Es)/Es and RA. However, inclusion of the Pi parameter yielded a significant r^2 of 0.88, indicating both steric and lipophilic functions are important in predicting activity. A similar relationship was noted for the SR, although in this case the Es parameter alone provided a significant correlation. Exclusion of the 1-methylethyl group did provide some improvement for the Es model. Once again, in the case of the TA, the Es parameter appeared to most effectively describe the relationship. Exclusion of the 1-methylethyl group provided some increase in the r^2, but inclusion of the Pi parameter only slightly increased the r^2.

Methyl Esters. The relative attractancy of methyl esters was poorly described when all available compounds were included, but elimination of

TABLE 6. REGRESSION EQUATIONS DERIVED FROM PHYSICOCHEMICAL COEFFICIENTS[a]

Group		N	Coefficients			r^2	Prob > F
			Es	Pi	INT		
Acids							
RA		10	1.20		1.65	0.07	0.471
RA	1B,2A,3A	7	3.84		2.39	0.81	0.014
RA		8	3.26	0.35	1.65	0.15	0.665
SR		10	1.35		5.55	0.32	0.092
SR	2A,4A	8	2.03		7.36	0.70	0.010
SR		8	2.22	1.18	5.26	0.29	0.422
TA		10	−5.22		7.24	0.28	0.116
TA	1B,4B	8	−9.06		10.77	0.78	0.004
TA		8	−8.85	3.35	7.83	0.28	0.434
Alcohols							
RA		6	1.30		1.50	0.55	0.090
RA	4B	5	1.79		1.67	0.60	0.123
RA		6	6.74	0.74	1.97	0.88	0.044
SR		6	2.55		6.25	0.77	0.021
SR	4B	5	2.58		6.54	0.96	0.004
SR		6	0.82	−1.41	5.34	0.82	0.075
TA		6	−6.47		5.68	0.79	0.018
TA	4B	5	−6.39		5.93	0.91	0.012
TA		6	−5.26	−0.70	5.26	0.80	0.092
Methyl esters							
RA		9	2.90		2.42	0.05	0.561
RA	1B,5A	7	5.54		3.03	0.79	0.007
RA		8	1.97	−0.14	2.42	0.06	0.866
SR		9	0.91		4.47	0.34	0.127
SR	2B,4A	7	1.30		4.89	0.81	0.006
SR		8	1.27	0.46	4.39	0.39	0.292
TA		9	−6.02		8.55	0.17	0.277
TA	1B,5A	7	−7.57		9.02	0.72	0.016
TA		8	−14.29	6.18	11.13	0.39	0.295
Acetate esters							
RA		13	0.81		1.43	0.06	0.420
RA	*	5	5.43		3.00	0.86	0.023
RA		8	−0.84	−0.23	1.27	0.24	0.050
SR		13	1.58		5.60	0.56	0.003
SR	3B	12	1.77		6.14	0.79	0.0001
SR		8	2.81	1.13	6.21	0.84	0.0103
TA		13	−4.47		6.18	0.38	0.025
TA	1B,3B	11	−6.76		8.21	0.75	0.0005
TA		8	−7.81	2.90	6.70	0.65	0.071

[a]Es = steric parameter, Pi = pi parameter, INT = intercept. RA = relative attractancy = P + A/P; SR = synergist ratio = (P + A)/P + A; TA = total attractancy = (P + A)/P or (RA × SR). Values following group designations indicated those removed from consideration: 1 = methyl, 2 = 1-methylpropyl, 3 = 3-methylbutyl, 4 = 1-methylethyl, 5 = propyl, * = only contains propyl, 1-methylpropyl, 2-methylpropyl, 1-methylethyl, and 2,2-dimethylethyl; A = above, B = below original regression line.

methyl and propyl groups increased the r^2 to 0.79 (Table 6), indicating a high correlation between (log Es)/Es and RA. Inclusion of the Pi parameter helped slightly, but the relationship was still poorly described when the propyl value was included. Correlation for all included values was much better for the SR, but elimination of 1-methyethyl and 1-methylpropyl was necessary to get a significantly high correlation. Inclusion of the Pi value again appeared to help compensate for the low activity of the methyl derivative. The log (Es) factor also was a poor predictor of TA, but again correlation became highly significant when the methyl and propyl values were removed. Inclusion of the Pi parameter in the model helped to explain the variation.

Acetate Esters. The relative attractancy of the acetate esters was poorly described when all available compounds were included; elimination of over half the compounds (leaving only 2,2-dimethyl ethyl, 1-methyethyl, propyl, 1-methyl propyl and 2-methylpropyl) was necessary to obtain a significant r^2 of 0.86. Inclusion of the Pi parameter helped a great deal, but again this involved the elimination of a large part of the values. In contrast, correlation for all included values was much better for the SR and significant, but elimination of the 3-methylbutyl substituent was necessary, to get an r^2 of 0.79. Inclusion of the Pi value again appeared to help a great deal, but at the same time the 3-methylbutyl group was excluded due to the unavailability of the Pi parameter. The log (Es) factor also was a significant predictor of TA, but again correlation became highly significant when the methyl and 3-methylbutyl values were removed. Inclusion of the Pi parameter in the model helped to explain the variation.

DISCUSSION

Nitidulid Attractants

Attractancy. Prior work with *C. hemipterus* has involved testing a series of "plant" volatiles for field attractancy in fig orchards (Smilanick et al., 1978). Acetaldehyde was at least 10× more effective by itself than any other chemical tested, which included ethyl acetate, propyl acetate, methyl butyrate, ethyl butyrate, ethyl isobutyrate, and ethyl propionate. The combination of acetaldehyde–ethanol–ethyl acetate (1 : 1 : 1) was more than 10× better than acetaldehyde alone. The paper concludes that these insects appear to use a restricted number of "plant" volatiles, which are common to hosts such as fig (Jennings, 1977), and the more tropical pineapple (Dupaigne 1970) and guava (MacLeod and DeTroconis, 1982). However, the volatiles of corn tassels, which are highly attractive to these and other nitidulids, contain no acetaldehyde, only a trace of ethyl acetate, and some ethanol (Flath et al., 1978), which suggests other components also may be involved. Although acetaldehyde, ethanol, and ethyl ace-

tate are reported from these fruits, Jennings (1977) indicated that they are probably fermentation products from figs, which suggests the same could be the case for the other fruits. Fogleman (1982) also indicates in a review that these three compounds are common products of fermenting yeast. This information may explain why fermenting materials, which would produce these attractive compounds (especially ethanol) in greater abundance, are more attractive than unfermented materials to these insects. The greater attractancy of fermenting material would also be logical, considering *C. hemipterus* may actually prefer to feed on the yeast as opposed to the plant material (Miller, 1952; Miller and Mrak, 1954). In other studies on individual plant attractants for nitidulids, Alm et al. (1985, 1986), demonstrated the relatively high attractive specificity of butyl acetate in the field for *Glischrochilus guadrisignatus* (ca. 14× ethyl butyrate, the next best). Propyl acetate was similar in effectiveness to butyl acetate. Bouchier and Stewart (1986) reported that *G. quadrisignatus* could distinguish between isobutyl acetate and *n*-butyl acetate (the branched ester was more attractive). Alm et al. (1986) suggested the receptors are very specific.

In the present work, we found that some volatiles were obviously superior attracts when used alone. The most effective (having RAs above 2.0) were methanol, methyl butyrate, methyl propionate, and propanal. However, many other compounds were also obviously attractive on their own. These compounds are different from those previously reported to be effective attractants for *C. hemipterus* (Smilanick et al., 1978). Acetaldehyde had no attractancy in its own in our study, while methyl butyrate was more potent than any of the other compounds previously reported (Smilanick et al., 1978) that we also examined. In addition, propanal was found to be extremely attractive on its own. These insects are known to respond to humidity gradients (Amos and Waterhouse, 1967; Amos, 1969), so the attractiveness of water is not unexpected. However, although these particular compounds were very attractive on their own (possibly representing specific interactions), when combined with the pheromone, many other compounds produced a TA that was comparable to that for those attractants that were very effective on their own. The synergistic interaction with the pheromone was a potent phenomenon that needs to be considered in predicting the overall effect of any particular host volatile.

Synergism. Due to differences in relative attractancy of butyl acetate vs. banana baits to the nitidulid *G. quadrisignatus* when adults were excluded vs. allowed to feed on the baits, Alm et al. (1986) suggested an aggregation pheromone may be present but did not address potential synergism. Synergism between host volatiles and pheromones is reported for some insects [e.g., Coleoptera (Birch, 1984; Oehlschlager et al., 1988) and Diptera (Bartelt et al., 1988)]. Our prior results indicated that some representative host volatiles can synergize the attractancy of the pheromone from *C. hemipterus* (Bartelt et al., 1990a). The present study indicates that for *C. hemipterus* a wide variety of

hosts and host volatiles is capable of synergizing the pheromone. This information suggests that, in contrast to the limited number of individual compounds that act as attractants on their own, synergism in *C. hemipterus* is a more generally widespread phenomenon. The broad spectrum of compounds capable of synergism is consistent with the known wide host range of *C. hemipterus*, which also would be expected to involve a diverse range of volatiles. Nitidulids with a more restricted host range may not be synergistically attracted by such a wide range of potential host volatiles (theoretically being restricted to volatiles in common from suitable hosts). While a few specific volatiles may be effective in initially bringing *C. hemipterus* to a potential host, once the insects arrive and start producing the aggregation pheromone, the overall attractancy of the host will be dramatically increased due to the interaction of the pheromone with the many host volatiles that are likely to be present. Our discovery that heptanol, which is produced in relatively large amounts by corn (Buttery et al., 1978; Flath et al., 1978), is a potent overall attractant for *C. hemipterus* helps explain how these insects are able to infest corn in the absence of more commonly recognized nitidulid attractants such as esters.

Attractant Combinations. Substituting components into the complex reported by Smilanick et al. (1978) resulted in little change in synergistic potency in the present study, except when better or worse individual synergistic components (esters) were added (see Discussion on structure–activity relationships). Notably, acetaldehyde was found not to be necessary. This is important to consider in formulating a field-oriented synergistic combination, since acetaldehyde is inherently more volatile and unstable than potential ester, alcohol, or acid components. The importance of acetaldehyde as an attractant reported by Smilanick et al. (1978) was not noted in the present study. It is possible that insect strain differences may explain differences in acetaldehyde effects, although the strain used in the present study also was obtained originally from California. However, it is also possible that acetaldehyde oxidized in the field to form acetic acid, which was effective as a synergist in our study.

Structure–Activity Relationships

Overall the Es (bulkiness) parameter was a good predictor for many of the relationships, although an apparent outlier or two had to be removed to give a significant correlation. The outlier was frequently methyl, which appeared to be compensated for when lipophilicity (Pi) was entered into the model. Another frequent outlier was the 1-methylethyl moiety, since it became important in some cases for acids, alcohols, and methyl esters. It is possible that specific receptors may exist for these types of compounds. The equations generated were generally similar to each other, although RA equations typically had lower intercepts (probably a function of the linearization), and TA equations had neg-

ative coefficients (again caused by the transformations). The equations for the RAs tended to be the most variable for the different classes of substituents. The coefficients for the SR equations appeared most similar to one another, although the intercepts were more variable. This information suggests that the receptors, or entire transport–receptor–processing phenomenon is similar for all of the compounds. However, individual differences between attractant class and type of attractancy did exist and are worth considering in more detail.

Relative Attractancy. The optimized equations predicting the RA for the methyl esters and acetate esters were very similar, suggesting that the receptor involved in the generalized attractancy is similar and symmetrical (although specific receptors for more attractive "outliers" such as methyl butyrate are likely). The derived optimized equation for the RA of the acids was somewhat similar to those derived for the esters, but different enough to suggest that two different receptors may be involved for acid and ester attractancy. This was even more obvious for the alcohols, where both coefficients for Es and the intercept were very different from those for the esters and acids. The Es parameter appeared to have the greatest influence on the attractancy of the compounds, but lipophilicity (Pi) also appears to be involved. However, of the three types of attractancy examined, the relative attractancy was most difficult to fit with regression equations. This may have been due to the relatively lesser sensitivity of the assays involving the attractants alone, due to the smaller numbers of beetles that were usually attracted compared to when the attractants were combined with the pheromone.

Synergism. The synergism between the pheromone and the attractants appeared to be a more generalized phenomenon and, due to the higher counts involved, appears to be more suitable for considering relationships of the different classes of substituted compounds. If a single site is assumed for all esters, the binding site would appear to be relatively symmetrical, based on studies with the methyl- and acetate-substituted esters. Long-chain substituents, including compounds such as heptyl caproate, were generally effective, as were some branched compounds. However, the position of the branch affected activity differently depending on which "side" of the carbonyl it was positioned. Branching at the 2-position was acceptable for the methyl esters, but not always acceptable for the acetate esters.

It is possible that acids, alcohols, and esters were binding to the same site or that esters were hydrolyzed to acids and alcohols that then bound to the same or two different respective sites. However, in spite of the general similarity in effectiveness of the many analogous alcohols, acids, and esters, individual effectiveness of analogous compounds did significantly differ in some cases, suggesting different receptors are involved for all three classes of compounds. For example, 3-methyl butanoate was more active synergist than 2-methyl pro-

panoate was; the opposite was true for the corresponding methyl esters. Different effectiveness as synergists of corresponding alcohols and acetate esters also occurred for 1-methyl propyl, 2-methylpropyl, and 1,1-dimethylethyl analogs. However, highly effective "outliers" such as methyl butyrate suggest specific receptors are also likely to be present.

Total Attractancy. Based on structure–activity relationships of acid, alcohol, and ester synergists, it is clear that the best synergists are linear compounds and that there is a series of each of these components that is similar in effectiveness. However, since the total attractancy is a function of the relative attractancy of the individual compound alone coupled with the synergist ratio, the compound with the highest attractancy (RA) and the highest synergist ratio (SR) would be the most effective compound to use in combination with the pheromone. Theoretically, for the esters this would involve the most attractive alcohol and acid portion. For the methyl esters, clearly this would involve either a propanoate or *n*-butanoate moiety, since these functional groups had the greatest single attractancy as well as a high synergist ratio and, indeed, the highest TA. For the alcohol portion, this would involve most of the straight-chain moieties. Ethyl acetate was among the most effective esters in terms of total attractancy and was found to be very effective in the field as well (Smilanick et al., 1978). Predictably, propyl propanoate would be one of the most effective compounds to use as a synergist, and it yielded a octyl acetate–ester ratio of 3.26. Only methyl butanoate yielded a comparable ratio, and this is likely to involve a specific attractancy receptor for this compound. For the alcohols, since a different receptor appears to be involved, methanol, 2-propanol, or heptanol would appear to be the most effective alcohols to use, although the fermentatively obvious ethanol is also very effective. Propanoic acid is clearly the superior acid attractant, but butanoic and 4-methylpentanoic acids are also very effective. Prior success with combinations including an alcohol and ester (Smilanick et al., 1978) may reflect multiple receptor stimulation, which would logically be further enhanced if the acetaldehyde were converted to acetic acid, producing a stimulant for an additional receptor. However, the results of the present study suggest attractancy could be significantly improved by use of the appropriate ester, alcohol, and acid (propanoate). This combination was approximated with ethyl propanoate, ethanol, and propanoic acid, which were readily available. Additional attractancy was achieved by adding water to this mix, which may represent and additional receptor. Propanal also would appear to be an appropriate compound to incorporate in the mixture. The determination of appropriate ratios would be of value also. Of course, formulation and cost may limit the selection of suitable components, but substituent optimization as it influences the synergist ratio should be considered in developing an appropriate blend.

CONCLUSIONS

We believe the use of the pheromone of *C. hemipterus* in combination with the appropriate esters, alcohols, acids, and potentially other compounds such as water and propanal, is likely to yield a potent attractant for this insect. Appropriate selection of the component from a particular class is necessary to obtain optimum attractancy, as indicated by structure–activity relationships reported here. These combinations will be valuable tools for monitoring the population of *C. hemipterus* as well as controlling them by trapping, a method that provided economically acceptible control of insects infesting figs. Bait stations with simple fermentation products have been used successfully in the past to increase quality and quantity of figs produced (Warner, 1960, 1961). A synergistic combination of pheromone and host volatiles should overcome problems of poor competition of attractants with natural hosts cited in past reports. However, *C. hemipterus* is also a pest of other ripe and dried fruit, as well as stored maize, corn meal, wheat, oats, rice, beans, nuts, peanuts, cotton seed, copra, spices, drugs, bread, sugar, honey, and other items (Hinton, 1945). It is also responsible for vectoring organisms responsible for souring of figs (Hinton, 1945) and fungi that contaminate corn and produce mycotoxins (Lussenhop and Wicklow, 1990). Thus, the utility of the components we have discovered is likely to be widespread and may be applicable to the control of other nitidulids as well. Although specific receptors may sometimes be involved, structure–activity relationships appear to be useful in predicting optimal synergists for pheromones, a methodology that should prove useful in developing optimal synergistic blend for many other insects.

Acknowledgments—We thank C.M. Weber for technical assistance and T.C. Nelsen for suggestions on appropriate statistical analyses.

REFERENCES

ALM, S.R., HALL, F.R., and LADD, T.L., JR., and WILLIAMS, R.N. 1985. A chemical attractant for *Glischrochilus quadrisignatus* (Coleoptera: Nitidulidae). *J. Econ. Entomol.* 78:839–843.

ALM, S.R., HALL, F.R., MCGOVERN, T.P., and WILLIAMS, R.N. 1986. Attraction of *Glischrochilus quadrisignatus* (Coleoptera: Nitidulidae) to semiochemical: Butyl acetate and propyl propionate. *J. Econ. Entomol.* 79:654–658.

AMOS, T.G. 1969. Reactions of *Carpophilus* spp. (Coleoptera, Nitidulidae) to humidity with reference to gradient steepness. *Anim. Behav.* 17:9–13.

AMOS, T.G., and WATERHOUSE, F.L. 1967. Plastic behavior shown by two *Carpophilus* spp. (Coleoptera, Nitidulidae) in various humidity gradients and its ecological significance. *Oikos* 18:345–350.

BARTELT, R.J., SCHANER, A.M., and JACKSON, L.L. 1988. Aggregation pheronomes in *Drosophila borealis* and *Drosophila littoralis*. *J. Chem. Ecol.* 14:1319–1327.

BARTELT, R.J., DOWD, P.F., PLATTNER, R., and WEISLEDER, D., 1990a. Aggregation pheromone of the driedfruit beetle, *Carpophilus hemipterus:* Wind-tunnel bioassay and identification of novel tetraene hydrocarbon components. *J. Chem. Ecol.* 16:1015–1039.

BARTELT R.J., MCGUIRE, M.R., and BLACK, D.A. 1990b. Feeding stimulants for the European corn borer (Lepidoptera: Pyralidae): Additives to a starch-based formulation for *Bacillus thuringiensis. Environ. Entomol.* 19:182–189.

BIRCH, M.C. 1984. Aggregation in bark beetles, pp. 331–353 *in* W.J. Bell and R. J. Cardé (eds.), Chemical Ecology of Insects. Sinauer Assoc. Sunderland, Massachusetts.

BLACKMER, J.L., and PHELAN, P.L. 1988. Flight behavior of *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae): Transition from dispersive to vegetative flight. Proceedings XVIII International Congress on Entomology, p. 217.

BOUCHIER, R.S., and STEWART, R.K. 1986. Attraction of *Glischrochilus quadrisignatus* (Coleoptera: Nitidulidae) adults to food plant volatiles. *Proc. Entomol. Soc. Manitoba* 42:37.

BURKHOLDER, W.E. 1988. Some new lures, traps, and sampling techniques for monitoring stored-product insects. Proceedings XVIII International Congress on Entomology, p. 444.

BUTTERY, R.G., LING, L.C., and CHAN, B.G. 1978. Volatiles of corn kernels and husks: possible corn earworm attractants. *J. Agric. Food Chem.* 26:866–869.

BUTTERY, R.G., LING, L.C., and R. TERANISHI, 1980. Volatiles of corn tassels: Possible corn ear worm attractants. *J. Agric. Food Chem.* 28:771–774.

DOWD, P.F. 1987. A labor-saving method for rearing the driedfruit beetle (Coleoptera: Nitidulidae) on pinto bean-based diet. *J. Econ. Entomol.* 80:1351–1353.

DUPAIGNE, P. 1970. The aroma of pineapples. *Fruits* 25:793–805.

FIENBERG, S.E. 1977. The Analysis of Cross-Classified Categorical Data. MIT Press, Cambridge, Massachusetts. 151 pp.

FLATH, R.A., FOREY, R.R., JOHN, J.O., and CHAN, B.C. 1978. Volatile components of corn silk (*Zea mays* L.): Possible *Heliothis zea* (Boddie) attractants. *J. Agric. Food Chem.* 26:1290–1293.

FOGLEMAN, J.C. 1982. The role of volatiles in the ecology of cactophilic *Drosophila*, pp. 191–206, *in* J.S.F. Barker and W.T. Starmer (eds.). Ecological Genetics and Evolution: The Cactus–Yeast–*Drosphila* Model System. Academic Press, New York.

HANSCH, C., and LEO, A. 1979. Substituent Constants for Correlation Analysis in Chemistry and Biology. Wiley, New York. 339 pp.

HINTON, H.E. 1945. A Monograph of the Beetles Associated with Stored Products. Jarrold and Sons, Norwich, England. 443 pp.

JENNINGS, W.G. 1977. Volatile components of figs. *Food Chem.* 2:185–191.

JONES, O.T. 1985. Chemical mediation of insect behavior, pp. 311–373, *in* D.H. Hutson and T.R. Roberts (eds.). Progress in Pesticide Biochemistry and Toxicology, Vol. 5, Insecticides. Wiley, New York.

LUSSENHOP, J.L., and WICKLOW, D.T. 1990. Nitidulid beetles as a source of *Aspergillus flavus* infective inoculum. *Trans. Jpn. Mycol. Soc.* 31:63–74.

MACLEOD, A.J., and DE TROCONIS, N.G. 1982. Volatile flavour components of guava. *Phytochemistry* 21:1339–1342.

METCALF, R.L. 1987. Plant volatiles as insect attractants. *C.R.C. Critical Rev. Plant Sci.* 5:251–300.

MILLER, M.W. 1952. Yeast associated with the dried-fruit beetle in figs. *Proc. Cal. Fig. Inst.* 6:8–9.

MILLER, M.W., and MRAK, E.M. 1954. Yeast associated with dried-fruit beetles in figs. *Appl. Environ. Microbiol.* 1:174–178.

OEHLSCHLAGER, A.C., PIERCE, A.M., PIERCE, H.D., JR. and BORDEN, J.H. 1988. Chemical communication in cucujid grain beetles. *J. Chem. Ecol.* 14:2071–2098.

SAS INSTITUTE. 1985. SAT/STAT Guide for Personal Computers, Version 6. SAS Institute, Cary, North Carolina.

SMILANICK, J. 1979. Colonization of ripening figs by *Carpophilus* spp. *J. Econ. Entomol.* 72:557–559.

SMILANICK, J.M., EHLER, L.E., and BIRCH, M.C. 1978. Attraction of *Carpophilus* sp. to volatile compounds of figs. *J. Chem. Ecol.* 4:700–701.

WALGENBACH, C.A., BURKHOLDER, W.E., CURTIS, M.J., and KHAN, Z.A. 1987. Laboratory trapping studies with *Sitophilus zeamais* (Coleoptera: Curculionidae). *J. Econ. Entomol.* 80:763–767.

WARNER, R.M. 1960. Area baiting to control *Drosophila* and nitidulid beetles. *Proc. Cal. Fig Inst.* 14:35–38.

WARNER, R.M. 1961. Area baiting program 1960 results. *Proc. Cal. Fig Inst.* 15:36–40.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

SELECTION OF PHEROMONE TRAP AND ATTRACTANT DISPENSER LOAD TO MONITOR BLACK ARMY CUTWORM, *Actebia fennica*

T.G. GRAY,[1,*] R.F. SHEPHERD,[1] D.L. STRUBLE,[2,4] J.B. BYERS,[2] and T.F. MAHER[3]

[1]*Forestry Canada, Pacific Forestry Centre*
506 West Burnside Road
Victoria, British Columbia, Canada V8Z 1M5

[2]*Agriculture Canada, Research Station*
Lethbridge, Alberta, Canada T1J 4B1

[3]*TFM Forestry Limited*
P.O. Box 364
Kamloops, British Columbia, Canada V2C 5K9

(Received November 1, 1989; accepted September 24, 1990)

Abstract—Catch rates of black army cutworm moths, *Actebia fennica* (Lepidoptera: Noctuidae), were determined for different types of traps and different dispenser loads of sex attractant. Of the five traps tested, highest catch (~35 males/per night) was obtained with Uni-Traps, whereas Pherocon 1CP, Delta and Hara traps rapidly became saturated at ~9, 6, and 11 males/per night, respectively. Multi-Pher traps, like the Uni-Traps, have a covered vertical cone with a large collecting bucket but only catch about 1/3 of the number of moths (~9 males/per night) as the Uni-Traps, thus reducing potential saturation. Red rubber septa loaded with 500 μg of attractant blend gave a consistent catch for at least 60 days; 1000 and 2000 μg loadings lasted for a minimum of 90 days. Polyvinylchloride and red rubber septa dispensers containing 100 μg of attractant had similar catch rates, which decreased rapidly with age. Based on these trap and lure-loading experiments, Multi-Pher traps baited with 1000 μg of attractant in a red rubber septum were selected for future calibration studies between catch density and population fluctuations on susceptible sites. Two years of monitoring results also are reported.

*To whom correspondence should be addressed.
[4]Present address: Agriculture Canada, Research Station, Vancouver, British Columbia V6T 1X2, Canada.

0098-0331/91/0200-0309$06.50/0

Key Words—*Actebia fennica*, Lepidoptera, Noctuidae, cutworm, pheromone traps, pheromone, monitoring.

INTRODUCTION

Black army cutworm, *Actebia fennica* (Tauscher), recently has been recognized as an economic pest of forest regeneration (Ross and Ilnytzky, 1977). It has been a minor problem in agriculture for many years, causing damage to alfalfa, clover, barley, creeping fescue, and blueberries (Wood and Neilson, 1956). The first outbreak of significance to forestry occurred in eastern British Columbia in 1973 (Ross and Ilnytzky, 1977). Prior to that date there were only three recorded minor outbreaks in British Columbia, dating back to 1943; since 1983 outbreaks have occurred annually. Occasional outbreaks also have occurred on regenerated sites in Ontario and Newfoundland.

Females lay their eggs in the soil from mid-July to early September. The eggs hatch in autumn and first- or second-instar larvae overwinter in the soil. Spring feeding commences as soon as the snow disappears, resulting in patches of denuded herbaceous growth by June. Pupation occurs in June or early July followed by a flight period that can occur between July 1 and September 30, depending on the developmental season.

High densities of black army cutworm often appear one or two years after forest sites are burned by wild or prescribed fires. Planted seedlings are consumed, along with other herbaceous plants. Damage is usually evident for two successive years and then the population returns to endemic levels.

An early-warning system to predict impending high populations before feeding commences would allow land managers to take appropriate action to avoid damage. A pheromone-trap monitoring system is being developed to meet this need (Struble et al., 1989). This paper describes tests conducted to select an appropriate trap and lure for such a system. For this, a lure is required that retains a constant rate of capture throughout a flight period of at least 60 days (Humble et al., 1989) and a trap that catches sufficient moths to indicate an impending outbreak, without becoming saturated.

METHODS AND MATERIALS

Trapping studies were carried out 16 km southeast of Smithers, British Columbia, in the Telkwa River Valley in 1985 and 50 km northeast of Smithers near Torkelsen Creek in 1986. Each area consisted of several square km of a regenerated site, which had been cut and burned two years previous to our studies. Larval populations in 1985 and 1986 were high and caused patches of severe defoliation of vegetation and destruction of conifer seedlings. Thus,

numbers of moths caught in this study are representative of catches made under outbreak conditions.

Traps were hung in rows from stakes at a height of 1 m with 40 m between traps and at least 100 m between rows. The order of treatments was randomized along each row and a "guard" trap, baited with 105 μg of attractant, was placed at the ends of each row to reduce concentrating immigrating moths. Catches in guard traps were not analyzed. When traps and the first dispenser load tests were run in 1985, trap positions were randomized daily. When dispenser loads were being tested in 1986, the tests were so large that a modified design had to be used in order to complete changes on a daily basis. Therefore, dispensers were initially randomized and then advanced one position daily.

Data for trap-nights were deleted from the analysis whenever there was interference with the trap because of vole or wasp predation, trap breakage, etc. This resulted in a variable number of replications in any one test. Uni-Traps were used exclusively during dispenser load tests. Counts were made daily, and the captured dead moths were discarded to prevent both possible reduction of attraction (Sanders, 1986) and having to count the same moths repeatedly.

Type of Trap. Three types of nonsticky and two types of sticky traps were evaluated. The nonsticky traps were: Uni-Trap (International Pheromone Systems Limited, Wirral, Merseyside, United Kingdom), a white, yellow, and green trap; Multi-Pher (Bio-Contrôle Services, Ste. Foy, Quebec, Canada), a green and white trap but also available as all green; and Hara (Hara Products, Swift Current, Saskatchewan, Canada), a double cone, totally white trap. Squares of insecticide strip, approximately 2.5 cm^2, containing 18.6% dichlorvos (Zoecon Industries Limited, Port Perry, Ontario, Canada), were placed inside each of these traps. The sticky traps were Pherocon 1CP (Trécé, Salinas, California) and Delta (made by T.G.G. from 2-liter cardboard milk cartons and coated inside with Tangle-trap from Tanglefoot Co., Grand Rapids, Michigan). In initial tests the Hara, Pherocon, and Delta traps reached saturation after only a few nights of exposure. These were therefore eliminated from future consideration and performance of the Uni-Trap and Multi-Pher traps was further tested using a matched-pair design baited with a septum load of 105 μg. The test was run for two nights with the position of each pair exchanged on the second night. There were 15 pairs, resulting in 30 trap-nights for each type of trap.

Quantity of Attractant per Dispenser. Two types of pheromone dispensers were used. The first consisted of a red rubber septum (A.H. Thomas Co., Catalog No. 1780-J07, Philadelphia, Pennsylvania) impregnated with a hexane dilution of 105 μg of (*Z*)-7-dodecenyl acetate (*Z*7–12 : Ac) and (*Z*)-11-tetradecenyl acetate (*Z*11–14 : Ac) each, at a ratio of 1 : 20. In 1985 0.15% of (*Z*)-5-tetradecenyl acetate (*Z*5–14 : Ac) also was added, but subsequent tests indicated this ingredient had little or no effect on catches (Struble et al., 1989); therefore, it was deleted from the mixture in 1986. The hexane was allowed to evaporate

before the septa were stored at 0°C until required. The second type of dispenser was polyvinylchloride (PVC) rods (5 × 3 mm diam.) (Daterman, 1974) containing 100 μg (0.4% w/w) of pheromone. Both types of dispensers were pinned under the lid or inside of each trap.

Two tests were run to compare dispenser load. In the first test, carried out in 1985, dispenser loads of 50, 100, 500, and 1000 μg were placed in rubber septa and used without aging. Six traps per treatment were run for five nights. In the second test, run in 1986, pheromone dispensers were aged for 0, 30, 60, and 100 days before use. Red rubber septa containing 100, 500, 1000, and 2000 μg of the two acetates at a ratio of 1:20 were placed inside cone-orifice traps, with the cone openings blocked to prevent insect entry, and aged outdoors near Lethbridge, Alberta, starting on April 9, 1986. The PVC dispensers, containing 100 μg of pheromone, were aged inside a Stevenson screen within an unheated shadehouse in Victoria, British Columbia, starting on May 9, 1986. Temperatures were recorded at each site and the degree-days above 0°C were calculated for each aging period. When the dispensers had aged for a specific period, they were sealed in separate containers and stored at −15°C until used in field tests. Two additional traps baited with unmated females reared from field-collected larvae were added to this test. Each treatment was exposed for 26 trap-nights.

Data were transformed, after the method of Iwao and Kuno (1968), using the formula $y = \log(\sqrt{0.163x} + \sqrt{0.163x + 1})$. Bartlett's test of homogeneity of variance indicated no significant differences after transformation. An analysis of variance and Tukey's comparison of means was carried out on the transformed data.

Assessment of Traps under Operational Conditions. In 1987 and 1988, all-green Multi-Pher traps baited with 1000 μg of the two-component pheromone were used to monitor adult populations in eastern and northeastern British Columbia. Each trap was hung 1 m high within burned areas, which had been restocked with conifer seedlings, with the intent of evaluating the traps as tools to detect populations in the autumn before damaging populations occurred. Traps were left out for the entire flight period and captured moths were sorted and counted volumetrically (Shepherd and Gray, unpublished data).

RESULTS AND DISCUSSION

Type of Trap. The mean catches per trap night for the Uni-Trap and Multi-Pher traps were significantly different: 17.9 (SD = 13.7) and 5.8 (SD = 5.4) moths, respectively ($P < 0.005$, Wilcoxon matched-pairs signed-ranks test). This indicated that the Multi-Pher trap could adequately detect damaging populations at lower trap densities than the Uni-Traps and, therefore, would be less likely to become saturated before reaching action threshold densities. Incidental

to these trap tests, it was found that large numbers of bumblebees were attracted to yellow or white traps. Use of all-green traps reduced this problem.

Quantity of Attractant per Dispenser. The first test on the quantity of attractant in septa resulted in the highest mean catch occurring with 100 μg; slightly fewer were caught with 50 μg and progressively fewer were caught with higher quantities (Figure 1). The catch at 1000 μg was less variable than the 500-μg and 2000-μg lures and was significantly different ($P < 0.05$, Tukey's comparison of means) than that for 50 and 100 μg.

In the second test the average catch per night for female-baited traps was 17.2 (SD = 25.4) males, while that for fresh 100-, 500-, 1000-, and 2000-μg septa were 35.2 (SD = 30.5), 24.1 (SD = 16.1), 24.6 (SD = 18.1), and 21.5 (SD = 16.6) males, respectively. The relative variability of the catch in traps with females (CV = 148%) was greater than that for any of the four dispenser loads (CV = 87, 67, 74, and 77%, respectively). Thus the fresh synthetic dispensers attracted higher numbers per night and were more consistent in their catch than females due, in part, to their age and attractiveness.

The results of using PVC dispensers containing 100 μg of attractant and aged 0, 30, 60, and 90 days are included in Figure 2. This test is not strictly comparable to the test using septa as it was run at a different time and thus

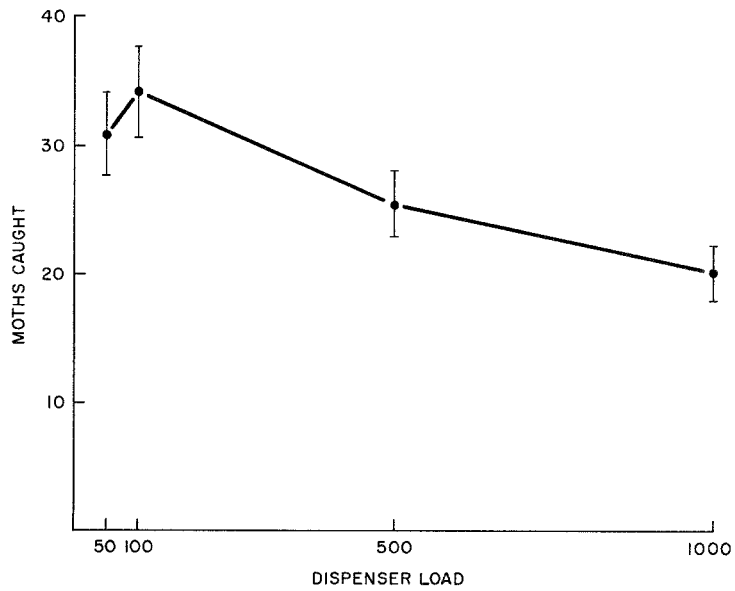


FIG. 1. Effect of dispenser load (μg) on average moth catch per night (N = 30 trap-nights per treatment).

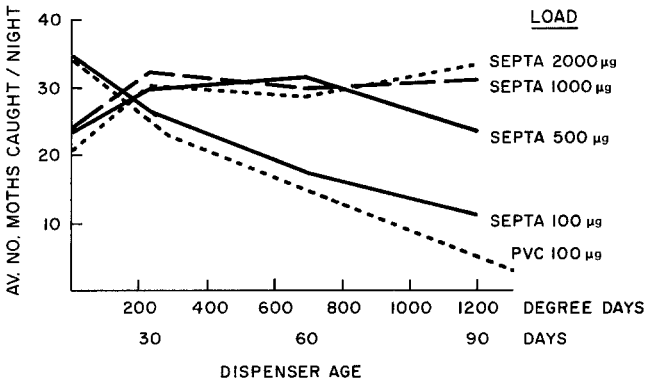


FIG. 2. Effect of dispenser age and load (μg) on average moth catch per night (N = 17–22 trap-nights per treatment).

exposed to different conditions, but the relative decrease with age appears to be similar with PVC as with rubber septa.

When exposing the pre-aged dispensers containing different quantities of attractant, the septum with 100 μg of attractant again gave a slightly higher initial catch than the other dosages. However, after 60 and 90 days of aging, 100 μg produced significantly lower catches than higher dosages aged for the same period of time ($P = < 0.05$, Tukey's comparison of means) (Figure 2). Catches in traps with 500, 1000, and 2000 μg/septum remained relatively consistent after 60 days and, even after 90 days of aging, catches were not reduced for traps with 1000- and 2000-μg dispensers. The reason for the consistency over time at higher dosages is not clear, but two other species of Noctuidae, responding to different attractants and traps, responded in a similar way (Struble, 1981; Struble and Byers, 1987). Thus, baits of 1000 or 2000 μg gave constant catch rates, which is ideal for a trap monitoring system. Based on the results of these experiments, a trap/lure combination of a totally green Multi-Pher trap and a septum lure with 1000 μg of pheromone was recommended for further field evaluation.

Assessment of Traps under Operational Conditions. By 1987 the populations at Smithers and Telkwa had collapsed but new populations were located near McBride, Golden, Clearwater, and Invermere, British Columbia where monitoring was carried out in 1987 and 1988 (Tables 1 and 2). The trap catches ranged from 0 to 1667 with a mean of 692 in 1987, and ranged from 89 to 1161 with a mean of 227 in 1988. Flight commenced in early July and lasted until late September; over 50% occurred in August during both years. Preliminary results indicate that when low catches (< 350 moths) were obtained, there was no risk of seedling damage and restocking could proceed the next spring. When

TABLE 1. SELECTED PHEROMONE TRAP CATCHES AND SUBSEQUENT POPULATION LEVELS OF LARVAE AT 10 LOCATIONS IN INVERMERE AND GOLDEN FOREST DISTRICTS, BRITISH COLUMBIA

Trap location	Male moths caught 1987	Larval populations 1988
1	0	None
2	56	None
3	144	None
4	198	None
5	449	Trace
6	636	Trace
7	776	Trace
8	939	Light
9	1181	Moderate
10	1667	Large

TABLE 2. SELECTED PHEROMONE TRAP CATCHES AND SUBSEQUENT POPULATION LEVELS OF LARVAE AT 8 LOCATIONS IN INVERMERE AND MCBRIDE FOREST DISTRICTS, BRITISH COLUMBIA

Trap location	Male moths caught 1988	Larval population 1989
1	89	None
2	156	None
3	373	None
4	397	Light
5	434	Light
6	486	Light
7	986	Light
8	1123	Light

high catches (>600 moths) were obtained, there was a potential for seedling damage and follow-up surveys should be conducted the following spring prior to planting. Evaluation of the monitoring system is continuing to further define these action thresholds.

REFERENCES

DATERMAN, G.E. 1974. Synthetic sex pheromones for detection survey of European pine shoot moth. U.S.D.A. Forest Service Res. Pap. PNW-180. 12 pp.

HUMBLE, L.M., SHEPHERD, R.F., and MAHER, T.F. 1989. Biology, outbreak characteristics and

damage caused by the black army cutworm (Lepidoptera: Noctuidae), pp. 82–88, *in* R.R. Alfaro and S. Glover (eds.). Insects Affecting Reforestation: Biology and Damage, Proceedings, Symposium IUFRO and International Congress of Entomology, July 3–9, 1989, Vancouver, British Columbia. Forestry Canada, Victoria, British Columbia.

IWAO, S., and KUNO, E. 1968. Use of the regression of mean crowding on mean density for estimating sample size and the transformation of data for the analysis of variance. *Res. Popul. Ecol.* 10:210–214.

ROSS, D.A., and ILNYTZKY, S. 1977. Black army cutworm, *Actebia fennica* (Tauscher), in British Columbia. Can. For. Serv., Pac. For. Res. Cent., Inf. Rep. BC-X-154. 23 pp.

SANDERS, C.J. 1986. Accumulated dead insects and killing agents reduce catches of spruce budworm (Lepidoptera: Tortricidae) male moths in sex pheromone traps. *J. Econ. Entomol.* 79:1351–1353

STRUBLE, D.L. 1981. A four-component pheromone blend for optimum attraction of redbacked cutworm males, *Euxoa ochrogaster* (Guenee). *J. Chem. Ecol.* 7:615–625.

STRUBLE, D.L., and BYERS, J.R. 1987. Identification of sex pheromone components of darksided cutworm, *Euxoa messoria*, and modification of sex attractant blend for adult males. *J. Chem. Ecol.* 13:1187–1199.

STRUBLE, D.L., BYERS, R.J., SHEPHERD, R.F. and GRAY, T.G. 1989. Identification of sex pheromone components of the black army cutworm, *Actebia fennica* (Tauscher) (Lepidoptera: Noctuidae) and a sex attractant blend for adult males. *Can. Entomol.* 121:557–563.

WOOD, G.W., and NEILSON, W.T.A. 1956. Notes on the black army cutworm, *Actebia fennica* (Tausch.) (Lepidoptera: Phalaenidae) a pest of low-bush blueberry in New Brunswick. *Can. Entomol.* 88:93–96.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

EUROPEAN CORN BORER: PHEROMONAL CATABOLISM AND BEHAVIORAL RESPONSE TO SEX PHEROMONE

J.A. KLUN,* M. SCHWARZ, and E.C. UEBEL

Insect Chemical Ecology Laboratory
U.S. Department of Agriculture, Agricultural Research Service
Beltsville Agicultural Research Center
Beltsville, Maryland 20705

(Received July 10, 1990; accepted September 24, 1990)

Abstract—When physiologically excessive amounts of the female sex pheromone of the European corn borer (ECB) or esters analogous to the pheromone were applied to the antennae of males, their behavioral responsiveness to pheromone in a flight tunnel was significantly impaired for 2 hr. Concurrent quantitative analyses of heptane extracts of the male antennae by gas–liquid chromatography showed that the compounds applied to antennae were hydrolyzed and, at 2 and 4 hr posttreatment, little or none of the compound applied or hydrolysis product was detectable in the antennal extracts. After 4 hr of in vivo incubation, male responsiveness to pheromone was restored among moths treated with the analogs but not among moths treated with pheromone. Esterase activity on the antennae was moderately inhibited in vivo by a pheromone analog that is a so-called transition-state esterase inhibitor, 1,1,1-trifluoro-14-heptadecen-2-one. However, the analog did not inhibit male behavior when it was coevaporated with pheromone in a flight-tunnel assay. Therefore, in the presence of pheromone, the analog did not compete well for esterase or the pheromone receptor. Treating the antennae of intact males with tetrahydrofuran obliterated sex pheromone response capability in males, but the treatment did not significantly attenuate esterase and other catabolic activity of the antennae. Indications are that degradation of esters on the ECB antennae involves substrate-nonspecific esterase activity and other metabolic processes that in turn remove hydrolysis products from the antennae. Maintenance of a male's ability to respond to pheromone is linked to these processes.

Key Words—Antennal catabolism, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, pheromone analogs, 11-tetradecenyl acetate.

*To whom correspondence should be addressed.

INTRODUCTION

Degradation of female sex pheromones on the antennae and other body parts of male moths has been studied in a number of species. Earliest works involved studies of pheromone degradation in *Bombyx mori* (Kasang, 1971), *Lymantria dispar* (Kasang et al., 1974), and *Trichoplusia ni* (Ferkovich et al., 1972; Ferkovich, 1982) males. These studies generally showed that degradative activity against pheromone exists on various body parts of the moths and that the activity was usually greatest on the antennae. In *B. mori*, its major pheromone component, (*E*,*Z*)-10,12-hexadecadien-1-ol, was found to be converted to fatty acid and fatty acid ester. Kasang et al. (1989) confirmed this finding by using a radiotracer and presented evidence of formation of water and other polar metabolites on the antennae. Prestwich et al. (1989, 1990) showed in vitro that epoxide hydrolase from *L. dispar* males degrades its pheromone substrate, (7*R*,8*S*)-7,8-epoxy-2-methyloctadecane, to the corresponding *threo*-7,8-diol. In *T. ni*, esterase is known to convert (*Z*)-7-dodecen-1-ol acetate (*Z*7-12:OAc) to the corresponding alcohol.

It is believed that antennal catabolic activity is somehow tied to the transductory processes of olfaction and behavioral response; however, belief in this connectivity is based mostly on conjecture. As examples, Mayer (1975) found that *T. ni* male antennae hydrolyzed pheromone at a rate that was twice as fast as that of female antennae and that the esterase activity on antennae and legs of *T. ni* males was greater against *Z*7-12:OAc than its analogs in brief (4 sec) in vivo incubations. Based upon these findings, Mayer speculated that this enzyme activity might be related to the transductory process. Later, Taylor et al. (1981) observed that pheromone esterase activity on the antennae of *T. ni* against its pheromone was low before adult eclosion, but the activity rose sharply 24–72 hr after eclosion, a time when males become maximally responsive to female sex pheromone. The concurrent rise in esterase activity and behavioral responsiveness to pheromone was considered circumstantial evidence that ester hydrolysis and the olfaction process were associated.

More recently, in vitro studies of esterase activity associated with integument and body scales (Vogt and Riddiford, 1986) and sensilla (Vogt et al., 1985; Prestwich et al., 1986) of *Antheraea polyphemus* have been reported. These studies showed that the esterase associated with scales was not extractable, but esterase residing with the integument was soluble. Vogt and Riddiford surmised that the role of esterase on the scales of males and females was to prevent the adsorptive body surfaces from becoming uncontrolled pheromone sources. Vogt et al. and Prestwich et al. also speculated that sensillar esterase in *A. polyphemus* acts as a rapid acetate inactivator and maintains a low stimulus noise level in sensory hairs. The female sex pheromone of *A. polyphemus* is a 9:1 mixture of (*E*,*Z*)-6,11-hexadecadienyl acetate and (*Z*)-11-hexadecenal

(Kochansky et al. 1975), but it is not yet known what metabolic process limits stimulus noise level related to the aldehyde component of the species' pheromone. Furthermore, all studies of pheromone catabolism in *A. polyphemus* have been restricted to acetate hydrolysis, and the metabolic fate of the resulting alcohol has not yet been probed in detail, although radiotracer studies by Kasang et al. (1988) have indicated that the alcohol is degraded to water, aldehydes, acids, and long-chained fatty acid esters.

In the tobacco budworm (*Heliothis virescens*), a species also known to utilize aldehydes in its pheromone complement, in vitro studies have shown that the pheromonal aldehydes are converted to carboxylic acids by aldehyde dehydrogenase and oxidase enzymes (Tasayco and Prestwich, 1990). It is conceivable that similar enzymes occur in *A. polyphemus*.

Prestwich and Streinz (1988) have evaluated the in vitro esterase inhibition and electroantennogram (EAG) activities of various haloacetate analogs of a trace pheromone component, (*Z*)-11-hexadecenyl acetate (Z11–16:OAc), of *Plutella xylostella;* the major pheromone component for the species is (*Z*)-11-hexadecenal. The purpose of their research was to "correlate analogs active in signal transduction with the recognition of these analogs by catabolic proteins." Among the compounds they studied was 1,1,1-trifluoro-(*Z*)-14-nonadecen-2-one, a potential esterase inhibitor and putative mimic of Z11–16:OAc. An analogous trifluoroketone was known to be a transition state inhibitor of juvenile hormone esterase (Hammock et al., 1982). Results of their research indicated that fluoroacetate analogs elicited greater EAG responses than other haloacetate analogs and that the fluoroacetates were also better esterase inhibitors than the haloanalogs. They concluded that the steric bulk of the chloro and bromo analogs accounted for lesser activities in the esterase inhibition and EAG assays. They also reported that 1,1,1-trifluoro-(*Z*)-14-nonadecen-2-one had weak EAG activity and was a poor inhibitor of esterase. This result differs from the findings of Vogt et al. (1985), who found that 1,1,1-trifluorotetradecan-2-one was a potent inhibitor of sensillar esterase in *A. polyphemus*. Ostensibly, substitution of a CH_2 for oxygen in the acetate moiety is allowable for interaction with esterase in one species but not in the other. This indicates that esterases may differ from one species to another.

Most of the foregoing studies involved in vitro approaches using excised antennae, antennal homogenates, or extracted enzymes. It therefore became of interest to us to probe the in vivo effects of pheromone and analogs applied topically to the antennae of intact European corn borer (ECB), *Ostrinia nubilalis*, males, to determine what influence the compounds might have upon male behavioral response to pheromone and to follow, at the same time, loss of the compounds from the antennae in a time-course fashion. Although the whole-organism approach to study the phenomenon of pheromone perception has been discredited by some as being "black box" experimentation (Prestwich, 1987),

we were of the opinion that such a study might yield biologically pertinent insight into the overall characteristics of the system, which then eventually could be correlated with its mechanistic components.

Our research involved the pheromone of the *E* and *Z* types of ECB (Klun and Huettel, 1988) and ECB pheromone analogs. Compounds were applied to moth antennae at physiologically excessive doses, degradation and removal of the compounds from the antennae were monitored chromatographically, and the influence of these doses on male responsiveness to pheromone was evaluated behaviorally. We report evidence of intense esterase and other catabolic activity on the antennae of ECB adults and behavioral data, which indicates that catabolic activity is somehow linked to the behavioral sensitivity of ECB males to pheromone.

METHODS AND MATERIALS

ECB Adults. All ECB adults used in this study were from colonies that had been established in the laboratory using females collected from fields in the vicinity of Beltsville, Maryland. They were reared continuously since 1985 (Klun and Huettel, 1988). One colony had been selected for homozygosity of *ZZ* female sex pheromone production genes, and the other was selected for homozygosity in *EE* pheromone production genes. Females in these homozygous cultures produced 97 : 3 (*Z*,*E*)- and 3 : 97 (*Z*,*E*)-11-tetradecen-1-ol acetate, respectively, as their pheromone, based upon gas chromatographic analyses of the females. The colonies were maintained in incubators (80% relative humidity; 16 hr light–8 hr dark, 26°C : 20°C). Although the colonies were selected for homozygosity of females sex pheromone production genes, males in the respective colonies also were predisposed to respond to sibling female sex pheromone; flight tunnel assays showed that males from the *ZZ* culture responded only to 97 : 3 (*Z*,*E*)- and *EE* culture males responded only to 97 : 3 (*E*,*Z*)-11-tetradecen-1-ol acetate. Correspondingly, pheromone processing by the antennae was expected to be specific to either *trans* or *cis* pheromone, depending on the colony from which the males were derived.

ECB Pheromonal Analogs. The compounds selected for study were among those that we used in previous studies of chemical structure–activity relationships in the ECB (Schwarz et al., 1989, 1990). Details of the synthesis, purification, and characterization of the compounds are described in these publications.

Gas Chromatographic Analyses. In all cases, antennae treated with compound were extracted with ca. 50 μl heptane at room temperature for 30 min and then removed from the solvent. Extracts were analyzed quantitatively for starting material (compound applied to the tissue) and a presumed first product

(alcohol or acid) by capillary gas–liquid chromatography using the internal standard technique. Pentadecane, 100 ng, served as the internal standard. Analytical sensitivity was ca. 0.5 ng/compound. We used four 60 m × 0.25 mm (ID) fused silica capillary columns for the analyses; two SPB-1 columns (Supelco Inc., Bellefonte, Pennsylvania 16823-0048) and two DBWAX-60N columns (J & W Scientific, Folsom, California 95630-4714). The columns were installed in Hewlett-Packard models 5830, 5840, 5880A, and 5890 gas chromatographs that were equipped with flame ionization detectors (nitrogen carrier makeup gas flow) and splitless sample injectors. Injector and detector temperatures were 225°C and 300°C, respectively. Hydrogen was used as carrier gas at 30 psi. Oven temperatures and programmed heating rates were optimized for each compound. SPB-1 columns were used to analyze for free fatty acids because this liquid phase permitted direct analysis of the acids without derivatization.

Brief In Vivo Incubation of Pheromone and Analogs on Male and Female Antennae. Antennae of each sex and moths of both ECB pheromonal types were immersed in 10 μg/μl solutions of compounds, and, after 5–10 sec of incubation, antennae were excised and extracted for 30 min in heptane containing internal standard. The proportional amount of hydrolysis product formed was determined to evaluate comparative rates of hydrolysis for each compound. The purpose of the test was to document existence of esterase on the antennae and to determine if esterase activity on the antennae would exhibit substrate specificity during the first few seconds of reaction, a time when zero-order kinetics should predominate. Adults used in the studies were 48–72 hr old. Reciprocal tests were conducted in which *cis* and *trans* pheromones each were tested against both sexes of *E*- and *Z*-type moths. The brief incubation tests were replicated 12–18 times.

Application of Compounds to ECB Antennae, Extraction of Antennae, and Bioassay of Upwind Flight Response of Treated Males to Pheromone in a Flight Tunnel. Typically, study of the effects of a given compound applied to the insect's antennae involved a set of 36 males. Each male represented a replicate, and each treatment was replicated at least 12 times. Approximately 1 hr before the onset of scotophase, males were inactivated by placing them individually into a 2.5 × 8.5-cm plastic tube containing a bed of crushed Dry Ice covered with a 3-cm plug of tissue paper. They became anesthetized within a few seconds and were treated immediately. The antennae of 24 individuals were immersed in heptane solution held in a microconical vial and containing test compound. The concentration of compound in the heptane solution was always 10 μg/μl, with the exception of the treatment involving 11-dodecenyl fluoroacetate where a 1 μg/μl solution was used. A low dose of this compound was required because preliminary tests showed that immersion of antennae in a 10 μg/μl heptane solution was lethal to the moths; all moths treated with the solution were dead within 30 min. This toxicity is analogous to the toxic action of

a fluorinated pheromone analog of the western spruce budworm (McLean et al., 1989). In our case, the analog undoubtedly serves as a suicidal substrate; hydrolysis of the fluoroacetate produces highly toxic fluoroacetic acid. The *cis* and *trans* compounds were applied to *Z*-type and *E*-type males, respectively. As a control, six males were anesthetized and treated with heptane (control; C_7), and an additional six were anesthetized only (control; CO_2). A dissecting microscope was used in the treatment procedures to observe the rise of heptane to the base of the antennae by capillary action and to confirm complete coverage of the antennae. Care was exercised in this procedure because the insect was killed instantly if its head and mouth parts contacted solvent.

Once they were treated, all males were held individually in jelly cups. The antennae of six compound-treated males were excised, using fine dissecting scissors, at their bases after 3–9 min incubation (T_0). Each pair of antennae was transferred to 50 μl heptane containing 100 ng pentadecane as internal standard. The 30 remaining males then were placed into the scotophase (2.5 lux red light) of a flight tunnel room at 20°C.

Two hours later (T_2), the antennae of six males that had been treated with compound were excised and extracted with heptane containing the internal standard. At the same time, the remaining treated and control males were assayed for behavioral responsiveness to pheromone in the flight tunnel. The operating conditions for the tunnel bioassay were the same as described by Schwarz et al. (1989). Six males each from the compound-treated and the control groups were tested against pheromone alone; each male was positioned at the downwind end of tunnel in a stimulus plume generated by a 0.5 × 3-cm filter paper strip 3 m upwind that had been treated with 100 ng ECB pheromone in 5 μl heptane. The remaining six males from the compound-treated group were exposed to 3 μg of the same analog that had been applied topically to antennae plus 100 ng pheromone on a filter paper strip. The analog plus pheromone stimulus (A+P) was used in the assay because other experiments (Schwarz et al., 1990) had shown that when these analogs were presented at a 3-μg dose along with pheromone, they either inhibited or had no influence on male upwind flight response to 100 ng pheromone, depending on the structure of the analog. We wished to determine what effect dipping antennae in a solution of analog might have on subsequently male behavior as compared to the effects that were observed previously by Schwarz et al. (1990). In all cases, each male was allowed 3 min to respond after being exposed to stimulus. If a male responded by flying upwind in the stimulus plume and landing on the paper strip or by flying up to within 2 cm of the strip, a positive upwind response was recorded for that individual. A negative response was recorded for males that failed to respond in this manner within 3 min after exposure to pheromone. All males tested at T_2, other than those of control groups, were retrieved from the flight tunnel and assayed as a combined group for behavioral sensitivity to

pheromone alone at 4 hr after treatment (T_4), and then their antennae were excised and extracted.

In this time-course study, the antennal extracts of males that had been treated with tetradecyl acetate or 11-tetradecenyl acetate were analyzed for tetradecanoic acid and 11-tetradecenoic acid, respectively, as well as for the corresponding acetates and alcohols. We surmised that the acids might be logical metabolic products if the alcohols formed on the antennae served as substrate for an oxidative attack on C-1.

1,1,1-Trifluoro-14-heptadecen-2-one (TF). The *cis* and *trans* isomers of TF were synthesized as described by Shaw and Tuominen (1985). In our case, TFs were designed to be steric mimics of the geometric isomers of ECB pheromone and transition-state inhibitors of esterase. In a test of the in vivo effects of TF on antennal esterase, we dipped the antennae of *Z*- and *E*-type males in 10 μg/μl heptane solutions of *cis*- and *trans*-TF, respectively. Three minutes afterward, antennae of these males were treated with pheromone solution, and then the antennae were extracted at T_0, T_2, and T_4 posttreatment. The extracts were analyzed for starting material, alcohol, and residual TF. As control, the same number of males were treated with pheromone and their antennae extracted at the three time periods. The test was replicated 12 times with each ECB pheromonal type. The effect of *cis*- and *trans*-TF isomers upon *Z*- and *E*-type males' upwind flight response to pheromone in the flight tunnel was tested, respectively, by exposing 20 individual males to 3 μg TF plus 100 ng pheromone on a filter paper and by using the same assay methods described earlier. As a control, 10 untreated male cohorts of each ECB type were tested against their pheromone alone.

Tetrahydrofuran and Antennal Esterase Activity. In earlier tests of the effect of various solvents applied to male antennae upon male reactivity to pheromone in the flight tunnel, we found that immersing male ECB antennae in tetrahydrofuran destroyed the ability of males to respond to pheromone. Therefore, it became of interest for us to determine if the solvent also destroyed catabolic activity on the antennae. The antennae of 10 *Z*-type males were each dipped in tetrahydrofuran and then immediately into 10 μg 11–12:OAc/μl heptane. As control, a second set of 10 males was treated with 11–12:OAc solution alone. Individual pairs of antennae were excised and extracted from each of the two groups at T_0, T_2, and T_4. The extracts then were analyzed for starting ester and alcohol.

Statistical Methods. The ester and alcohol quantitative data exhibited large variance of heterogeneity, nonnormality, and there was a large number of zero-value observations. Consequently, the nonparametric median test was used to make comparisons among treatment groups. Pairwise median tests were protected by overall median tests, and the significance level was adjusted in order to hold the experimentwise error below 5%. The frequencies of ECB males

flying upwind were compared using Fisher's exact test protected by stratified Cochran-Mantel-Haenszel analyses, and the significance level also was adjusted to hold the experimentwise error rate below 5%. (SAS Institute, 1988).

RESULTS AND DISCUSSION

Brief In Vivo Incubation of Pheromone and Analogs. Analyses of antennal extracts for acetate to alcohol proportions showed (Table 1) that hydrolysis of most esters took place within seconds after they were applied, with the exception of the methyl-11-tetradecenoate; no free acid was formed in the 5 to 10-sec incubation period. This compound clearly was not processed well by males of either ECB type. In *Z*-type males, 11-dodecenyl fluoroacetate was hydrolyzed more rapidly than all other esters with exception of *Z*-11,13-tetradecadienyl acetate. The fluoroacetate was also hydrolyzed more rapidly than any of the esters tested against the *E*-type males. The rapid hydrolysis of the fluoroacetate is most likely due to the inductive effect of the fluorine atom, which makes it

TABLE 1. HYDROLYSIS OF PHEROMONE AND PHEROMONAL ANALOGS IN 5–10 SEC AFTER APPLICATION TO ECB ANTENNAE[a]

Compound		Proportion[b] median acetate : OH	
		Z-type Male	*E*-type Male
11-Dodecenyl acetate		97:3 b	93:7 b
11-Dodecenyl fluoroacetate		88:12 a	85:15 a
Tetradecyl acetate		96:4 b	96:4 b
Methyl-11-tetradecenoate		100:0 c	100:0 c
12-Cyclopropyl-11-dodecenyl acetate		95:5 b	95:5 b
11,13-Tetradecadienyl acetate		88:12 a	95:5 b
11-Tetradecenyl acetate		97:3 b	91:9 ab
	Reciprocal test Sex		
(*Z*)-11-Tetradecenyl acetate	M	97:3 a	92:8 a
(*E*)-11-Tetradecenyl acetate	M	95:5 a	91:9 ab
(*Z*)-11-Tetradecenyl acetate	F	95:5 a	92:8 ab
(*E*)-11-Tetradecenyl acetate	F	95:5 a	95:5 b

[a] In cases where a compound has two geometric forms, the *cis* isomer was applied to the *Z*-type male and *trans* isomer was applied to the *E*-type male. In the reciprocal test, both isomers were applied to each type and sex. Medians withn the same column followed by the same letter are not significantly different at the 5% level.

[b] The ester : free acid was determined in case of methyl-11-tetradecenoate.

more susceptible to hydrolysis. Pheromone, 11-tetradecenyl acetate, was not hydrolyzed more rapidly than any of the analogs in both types of insects. Therefore, the data indicate that esterase activity of the antennae is substrate-nonspecific. This finding has relevance to the observation of Schwarz et al. (1990) that when pheromone analogs that mimicked the biological activity of natural pheromone at a low dose were coevaporated at a high dose along with pheromone, male response to the pheromone was not inhibited. However, when analogs that lacked pheromone mimicking quality, such as 11-dodecenyl acetate and 11,13-tetradecadienyl acetate, were evaporated at high dose with pheromone, male response to pheromone was inhibited. They speculated that the inhibitory analogs bind to the pheromone receptors without provoking behavioral response but are not cleared from the receptor as efficiently as the pheromone or pheromonally active analogs, which are more effectively recognized and removed by substrate-specific catabolic enzymes. Data in Table 1 show that the behavioral inhibitors identified by Schwarz et al. (1990), 11-dodecenyl acetate and 11,13-tetradecadienyl acetate, were hydrolyzed as effectively as pheromone. Therefore, it would seem that if the speculation by Schwarz et al. (1990) was correct, then the catabolic specificity they reported must reside with catabolic enzymes other than esterase.

As has proven to be the case for other species of moths in which esterase activity has been assayed, results of the reciprocal test (Table 1) showed that esterase activity in the ECB is associated with female antennae as well as with male antennae. The proportions of alcohol formed indicate that the rate of hydrolysis of *cis* and *trans* pheromone by Z-type ECB did not differ from one geometric isomer to another or from one sex to another. This was also generally true with the *E*-type; however, *E*-type males hydrolyzed *cis* isomer more rapidly than *E*-type females hydrolyzed *trans*. Moreover, esterase activity toward pheromonal isomers did not differ significantly between types of insects. This is, perhaps, not surprising because the pheromones in the two types are comprised of both isomers, albeit in different proportions, and it is reasonable that they should be capable of processing each isomer.

Time-Course Assay of Upwind Flight Response of Males Treated with Compound and Concomitant Analysis of Male Antennal Extracts. Chromatographic analyses (Table 2) showed that at T_0 (3 min after the antennae were treated) predominantly starting ester and somewhat lesser amounts of hydrolysis product were detected in the antennal extracts of all males independent of the ester with which they were treated. In the case of methyl decanoate at T_0, only small amounts of ester and acid were detectable in the extracts, and there was proportionately more acid present than ester at that time, an indication that the latter was removed from the antennae more rapidly than any other compound. The amount of 11-dodecenyl fluoroacetate detected at T_0 was about one order of magnitude less than that observed in other treatments at T_0. This was expected

TABLE 2. THE ESTER AND ALCOHOL (OH) COMPOSITION OF ANTENNAL EXTRACTS 3 MIN (T_0), 2 HR, OR 4 HR AFTER MALE ECB ANTENNAE WERE IMMERSED IN 10 $\mu g/\mu l$ HEPTANE SOLUTIONS OF INDICATED COMPOUNDS[a]

Compound	Posttreatment time (hr)	Median (ng)			
		Z-type male		E-type male	
		ester	OH	ester	OH
11-Dodecenyl acetate	0	543 a	325 a	492 a	396 a
	2	0 b*	0 b*	0 b*	9 b*
	4	0 b	0 b	0 b	0 c
11-Dodecenyl fluoroacetate[b]	0	30 a	43 a	18 a	43 a
	2	0 b*	0 b*	0 b*	0 b*
	4	0 b	0 b	0 b	0 b
11-Tetradecenyl acetate	0	1415 a	355 a	846 a	285 a
	2	1 b*	39 b*	0 b*	30 b*
	4	0 b	0 c	0 b	0 b
Tetradecyl acetate	0	680 a	188 a	1495 a	378 a
	2	0 b*	204 a*	0 b*	461 a*
	4	0 b	29 b	5 b	16 b
Methyl-11-tetradecenoate[c]	0	2451 a	166 a	1752 a	213 a
	2	0 b*	0 b*	0 b*	0 b*
	4	0 b	0 b	0 b	0 b
12-Cyclopropyl-(*E*)-11-dodecenyl acetate	0	nt	nt	947 a	498 a
	2	nt	nt	23 b*	386 a*
	4	nt	nt	7 b	44 b
11,13-Tetradecadienyl acetate	0	1579 a	419 a	1693 a	538 a
	2	0 b*	152 b*	0 b*	197 b*
	4	0 b	0 c	0 b	0 c
Hexadecane	0	nt	nt	na	2225 a
	2	nt	nt	na	8 b
	4	nt	nt	na	nt
Methyldecanoate[c]	0	nt	nt	71 a	120 a
	2	nt	nt	0 b	0 b
	4	nt	nt	nt	nt

[a] Where geometrical isomers exist, *cis* isomer was applied to Z-type males and *trans* isomer was applied to *E*-type. Medians within the same column and compound followed by a different letter are significantly different at the 5% level. An asterisk after a letter indicates that upwind flight response of compound-treated cohort males to pheromone in a flight response of compound-treated cohort males to pheromone in a flight tunnel was significantly inhibited ($P = 0.05$) at 2 hr post-treatment compared to control moths. nt = not tested. na = not applicable.

[b] Antennae were dipped in a 1 $\mu g/\mu l$ heptane solution; treatment of males with a 10 $\mu g/\mu l$ solution was lethal.

[c] In cases of methyl esters, the hydrolysis product quantified was the corresponding free fatty acid.

because, as was mentioned earlier, the compound was applied at one-tenth the dose of other compounds.

In subsequent time-course samples, none of the esters applied to the antennae were detectable in the antennal extracts at T_2, but hydrolysis product was often found in the extracts. When hydrolysis product was detected at T_2, it was most often at a level that was significantly lower than the amount detected at T_0, an indication that alcohols were removed from the antenna as they formed. However, inspection of all chromatograms from the polar and nonpolar columns showed no other apparent chromatographic components that might have represented other metabolites, and the specific chromatographic analyses of the extracts of antennae treated with tetradecyl acetate or 11-tetradecenyl acetate showed no evidence of tetradecanoic or 11-tetradecenoic acid, respectively. Therefore, indications are that degradation of alcohol on the ECB antenna does not involve metabolism resulting in formation of fatty acid as has been observed in studies of *B. mori* (Kasang et al., 1989) and *A. polyphemus* (Kasang et al. 1988).

Analyses of insects treated with tetradecyl acetate and 12-cyclopropyl-(*E*)-11-dodecenyl acetate indicated that these compounds represent a special case. Although no tetradecyl acetate was detected in T_2 extracts of *Z*- and *E*-type males, the amounts of tetradecanol present were not significantly different from the amounts detected at T_0. This result indicates that both moth types processed tetradecanol less effectively than the pheromone-derived alcohol 11-tetradecenol. In the case of 12-cyclopropyl-(*E*)-11-dodecenyl acetate, antennal extracts of *E*-type males at T_2 also showed that the amount of the corresponding alcohol present was not significantly different from the amount present at T_0 and that residual acetate was present at T_2. Thus, among all the compounds tested, tetradecyl acetate and the cyclopropyl analog were cleared least effectively from the antennae. This conclusion is reinforced by the fact that trace amounts of only these compounds persisted in the antennal extracts at T_4. Assuming that enzymatic degradation of the alcohols involves attack of the olefinic site, we surmise that the slowed catabolism of these two compounds may be related to the absence of a double bond in tetradecyl acetate and steric hinderance caused by the cyclopropyl group in the vicinity of the double bond in the other compound.

Flight-Tunnel Assay. Statistical analyses revealed that responses of control groups, C_7 and CO_2, to pheromone in the tunnel were not different from each other. Similarly, upwind flight responses of compound-treated males exposed to pheromone alone (P stimulus) or a mixture of pheromone and 3 μg analog (A+P stimulus) at 2 hr after treatment were not different from one another for all compounds tested. Therefore, upwind flight response values of the P and A+P groups and the control groups were pooled, respectively, to test for statistically different responses between compound-treated moths and control

moths. The mean percentage upwind flight response to pheromone among Z-type control males was 67% (N = 154), and in the E-type it was 65% (N = 216).

Analyses of the flight-tunnel data showed that the upwind flight response to pheromone of all males that had been treated with pheromone or analogs (including the 11-dodecenyl fluoroacetate, which was applied at a 1/10 dose) was significantly impaired at T_2 relative to the control males. Treatments causing significant impairment of response are indicated by asterisks in Table 2, and the mean percentage upwind responses of the males at T_2 are presented in Table

TABLE 3. COMPARISON OF PERCENTAGE ECB MALE UPWIND FLIGHT RESPONSE TO PHEROMONE IN TUNNEL AT 2 AND AT 4 HR AFTER THEIR ANTENNAE WERE TREATED WITH INDICATED COMPOUND[a]

Compound	Posttreatment time (hr)	$\bar{X}$ percentage upwind flight	
		Z-type male	E-type male
11-Dodecenyl acetate	2	3 b (36)	13 b (24)
	4	38 a (34)	59 a (22)
11-Dodecenyl fluoroacetate	2	0 b (12)	27 b (11)
	4	33 a (12)	40 a (10)
11-Tetradecenyl acetate	2	0 a (24)	0 a (24)
	4	4 a (23)	0 a (24)
Tetradecyl acetate	2	0 b (22)	0 b (24)
	4	39 a (18)	25 a (24)
Methyl-11-tetradecenoate	2	17 b (36)	19 b (48)
	4	59 a (34)	50 a (46)
12-Cyclopropyl-(E)-11-dodecenyl acetate	2	nt	0 b (12)
	4	nt	64 a (11)
11,13-Tetradecadienyl acetate	2	0 b (24)	0 a (24)
	4	50 a (22)	17 a (24)
Hexadecane	2	nt	50 a (12)
	4	nt	40 a (12)
Methyl decanoate	2	nt	67 a (12)
	4	nt	75 a (12)

[a] Where geometrical isomers exist, *cis* isomer was applied to Z-type males and *trans* isomer was applied to E-type. Percentages within the same column and same compound followed by the same letter are not significantly different at the 5% level. The number of individual males tested in each time period is in parentheses. In some cases, the number of males tested at 4 hr within compounds was fewer than the number tested at 2 hr because some males escaped recapture after testing at 2 hr. nt = not tested.

3. The impairment was often independent of whether or not GC-detectable amounts of applied compound or hydrolysis product were present on the antennae at the time of flight-tunnel assay. As examples, among all males treated with 11-dodecenyl fluoroacetate or methyl-11-tetradecenoate, or *Z*-type males treated with 11-dodecenyl acetate, no esters or hydrolysis products were detected in the antennal extracts; yet male response in these groups was significantly impaired. This indicates that impairment of response may be related to a build-up of pools of catabolic products beyond the hydrolysis product, which tie up olfactory-behavioral response capability. However, we cannot rule out the possibility that physiologically relevant levels of ester or hydrolysis product might have been present on the antennae at levels below GC detection limits. In contrast, hexadecane and methyl decanoate, compounds that have no structural analogy to pheromone, did not impair response capability. It would seem that, owing to their dissimilarity to pheromone structure, they were processed differently from pheromone or pheromone analogs and therefore did not tie up the olfactory response system.

Data in Table 3 compare the mean upwind responses of males to pheromone at T_2 and T_4 posttreatment. All males treated with pheromone analogs, with the exception of the *E*-type males treated with (*E*)-11,13-tetradecadienyl acetate, showed a significant recovery of response capability 4 hr after they were treated with compound. In the case of the *E*-type males against 11,13-tetradecadienyl acetate, the flight assay data indicated that the males also were showing signs of response recovery because a 17% upwind flight response was recorded at T_4, whereas it had been zero at T_2. Insects that were treated with tetradecyl acetate or 12-cyclopropyl-(*E*)-11-dodecenyl acetate were among those showing a significant recovery of response despite the fact that extracts of their antennae at T_4 (Table 2) showed that trace amounts of acetate and/or alcohol were still present on their antennae. In this case, the amount of compound remaining on the antennae presumably fell to a level that no longer disrupted response capability.

Quite unexpectedly, the only instance where males did not show recovery of behavioral response at T_4 was among moths treated with pheromone, 11-tetradecenyl acetate. Application of physiologically excessive doses of pheromone to the moth clearly had a traumatizing effect that was measurably greater than similar does of any of the pheromone analogs. This was true despite the fact that GC analyses of the antennal extracts of the pheromone-treated males indicated that their antennae had been freed of compound at T_4. This fact is reminiscent of observations made at T_2 where neither ester nor alcohol of several analogs was detected in the antennal extracts, yet male response to pheromone was inhibited. Therefore, it seems that esterase activity alone or removal of hydrolysis product from the antennae is not a limiting factor involved in recovery of response capability; if they were, one might have expected response

capability to be restored as soon as ester or hydrolysis product were depleted from the moth antennae at T_2 or T_4. This was not the case whether pheromone or its analogs were involved. However, as discussed earlier, it is also likely that the compounds merely fell below GC detection limits and that the undetected, but physiologically significant, amounts of compound persisted on the antennae. Because all males treated with analogs recovered response capability before they were treated with pheromone, it appears that the males processed the physiologically excessive analog doses more effectively than they processed an equivalent dose of pheromone.

Influence of 1,1,1-Trifluoro-14-heptadecen-2-one (TF) on 11-Tetradecenyl Acetate In Vivo Degradation and Male Flight Response to Pheromone. Results reported in Table 4 show that TF was a weak inhibitor of esterase activity in both *Z*- and *E*-type ECB. The inhibitory effect of the compound is evidenced by the facts that at T_0 (3 min incubation) significantly smaller amounts of alcohol were present in TF-treated males than in the controls and significantly larger amounts of acetate were present in the treated males than in the control insects at 2 hr. After 4 hr of incubation, trace amounts of acetate were detectable in the TF-treated group, whereas none was detected in the controls. It is noteworthy that the data show that TF also was removed from the antennae over time, an indication that degradative activity related to the antennae may not be restricted to attack on esters.

Although TF had a modicum of inhibitory activity against 11-tetradecenyl acetate hydrolysis, the tunnel assay (Table 4) showed that the compound had no effect upon male upwind flight response to pheromone by the two ECB types. Moreover, the esterase inhibition and the behavioral data indicate that TF was not a good competitive inhibitor of ECB esterase and did not effectively compete for putative behavior-inducing pheromone receptor sites on the antennae because it did not interfere with male behavioral response to pheromone. These results are similar to those obtained by Prestwich and Streinz (1988) inasmuch as they found that their trifluoromethylketone stereochemical mimic of the *P. xylostella* pheromone component, (*Z*)-11-hexadecenyl acetate, was also only a weak esterase inhibitor and elicited a negligible electrophysiological response in EAG assays. Thus, in the ECB and *P. xylostella* the trifluoromethylketones have proven to be ineffectual as chemical probes in efforts to further our understanding of the role of antennal esterase activity in pheromone perception.

Influence of Tetrahydrofuran on Antennal Esterase and Catabolic Activity. Inasmuch as THF was found to destroy the ability of males to respond to pheromone, one might have expected the treatment to obliterate esterase activity and other degradative processes on the antennae. However, data in Table 5 show that immersion of male antennae in tetrahydrofuran (THF) did not have such an effect. The data show that the amounts of acetate and alcohol on THF-treated and control insects did not differ significantly 3 min (T_0) after ester was

TABLE 4. INFLUENCE ON IN VIVO ANTENNAL DEGRADATIVE ACTIVITY AGAINST 11-TETRADECENYL ACETATE IN *Z*- AND *E*-TYPE ECB MALES AND EFFECT OF TF ON MALE RESPONSE TO PHEROMONE[a]

Treatment	Post-treatment time (hr)	Median (ng)						Flight-tunnel assay (mean percentage upwind flight)	
		Acetate		Alcohol		TF			
		Z-type	*E*-type	*Z*-type	*E*-type	*Z*-type	*E*-type	*Z*-type	*E*-type
TF	0	2417 a	3120 a	34 b	53 cd	944 a	699 a		
	2	412 b	305 b	70 ab	99 bc	249 b	183 b		
	4	6 c	20 c	3 b	12 d	20 b	18 b		
None	0	1478 b	1660 a	161 a	259 ab				
	2	2 c	7 c	61 ab	509 a				
	4	0 c	0 c	2 b	0 e				
3 μg TF + 100 ng pheromone								85 a	70 a
100 ng pheromone								90 a	80 a

[a]The *cis* isomer of TF was applied to *Z*-type males and opposite isomer was applied to the *E*-type. Medians or means within the same column followed by the same letter are not significantly different at the 5% level.

TABLE 5. CATABOLIC ACTIVITY IN MALE ANTENNAE TREATED WITH TETRAHYDROFURAN (THF)[a]

Treatment	Posttreatment time (hr)	Median (ng)	
		Acetate	Alcohol
THF	0	1428 a	155 a
	2	40 b	84 b
	4	9 b	24 c
Control	0	1000 a	198 a
	2	3 c	2 d
	4	2 c	0 e

[a] Medians with columns followed by the same letter are not significantly different at the 5 % level.

applied to the antennae. However, at T_2 and T_4 after treatment, there were significantly more residual acetate and alcohol detected in extracts of THF-treated males than in control insects. Thus, the THF treatment had only a slight attenuating effect on antennal catabolic activity. This indicates that male behavioral response capability to pheromone and catabolism are separate but presumably coupled elements of the antennal chemosensory system. THF uncoupled the elements but did not have a significant adverse impact upon the catabolic processes. It also is interesting to note that the solvent, heptane, used in the application of compounds to moths' antennae did not exert this denaturing effect, and the disassociating effect of THF must be attributed to its comparatively polar solubilization characteristic.

SUMMARY

Overall, our studies indicate that catabolism of esters on the ECB antennae involves a combination of substrate-nonspecific esterase activity as well as other undiscovered processes that in turn remove hydrolysis products from the antennae. The catabolism takes place on the antennae of both sexes of the moth. The processes responsible for degradation of the hydrolysis products need to be elucidated because esterase activity does not appear to be the only factor involved in the maintenance of behavioral response capability in the male. Chromatographic evidence showed that degradation of hydrolysis products in the ECB does not involve conversion to carboxylic acid, although such conversion is known to take place in other lepidopteran species. Behavioral data show that catabolism is linked to the ability of males to respond to pheromone inasmuch

as results showed that males exposed to the physiologically excessive doses of pheromonal analogs were incapable of responding to pheromone, but once the compounds were cleared from the antennae, response capability to pheromone was restored. In the time-course study, we did not observe a similar restoration of response among moths treated with pheromone, and there is no ready explanation for this result. Treatment of antennae with THF showed that catabolic activity persisted on the antennae even though the treatment destroyed the ability of males to respond to pheromone. Thus, response and catabolism must be considered as separate but coupled components in the insect's chemosensory system. No doubt, intense catabolic activity is operative when physiologically relevant doses of pheromone impinge on the insect antennae, but the exact molecular linkage between the catabolism, responsiveness to pheromone, and the transductory process remain to be discovered.

Acknowledgments—We thank William Potts for conducting statistical analyses of the data and Ingrid Orentas for technical assistance in the flight-tunnel studies.

REFERENCES

FERKOVICH, S.M. 1982. Enzymatic alternation of insect pheromones, pp. 165–186, *in* D.M. Norris (ed.), Perception of Behavioral Chemicals. Elsevier/North Holland, Amsterdam.

FERKOVICH, S.M. MAYER, M.S., and RUTTER, R.R. 1972. Conversion of the sex pheromone of the cabbage looper. *Nature* 242:53–55.

HAMMOCK, B.D., WING, K.D., MCLAUGHLIN, J., LOVELL, V., and SPARKS, T.C. 1982. Trifluoromethyl ketones as possible transition state analog inhibitors of juvenile hormone esterase. *Pestic. Biochem. Physiol.* 17:76–88.

KASANG, G. 1971. Bombykol reception and metabolism on the antennae of the silkworm *Bombyx mori*, pp. 245–250, *in* G. Ohloff and A.F. Thomas (eds.). Gustation and Olfaction. Academic Press, New York.

KASANG, G., KNAUER, B., and BEROZA, M. 1974. Uptake of the sex attractant ^{3}H-disparlure by male gypsy moth antennae (Lymantria dispar) (Porthetria dispar). *Experientia* 30:147–148.

KASANG, G., VON PROFF, L., and NICHOLLS, M. 1988. Enzymatic conversion and degradation of sex pheromones in antennae of the male silkworm moth *Antheraea polyphemus*. *Z. Naturforsch.* 43:275–284.

KASANG, G., NICHOLLS, M. and VON PROFF, L. 1989. Sex pheromone conversion and degradation in antennae of the silkworm moth *Bombyx mori* L. *Experientia* 45:81–87.

KLUN, J.A., and HUETTEL, M.D. 1988. Genetic regulation of sex pheromone production and response: Interaction of sympatric pheromonal types of European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *J. Chem. Ecol.* 14:2047–2061.

KOCHANSKY, J., TETTE, J., TASHENBERG, E.F., CARDÉ, R.T., KAISSLING, K.-E., and ROELOFS, W.L. 1975. Sex pheromone of the moth, *Antheraea polyphemus*. *J. Insect Physiol.* 21:1977–1983.

MAYER, M.S. 1975. Hydrolysis of sex pheromone by the antennae of *Trichoplusia ni*. *Experientia* 31:452–454.

MCLEAN, J. MORGAN, B., SWEENEY, J.D., and WEILER, L. 1989. Behavior and survival of western spruce budworm, *Choristoneura occidentalis* Freeman, exposed to an ω-fluorinated pheromone analog. *J. Chem. Ecol.* 15:91–103.

PRESTWICH, G.D. 1987. Chemical studies of pheromone reception and catabolism, pp. 473–527, *in* G.D. Prestwich and G.J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, Orlando, Florida.

PRESTWICH, G.D., and STREINZ, L. 1988. Haloacetate analogs of pheromones: Effects on catabolism and electrophysiology in *Plutella xylostella*. *J. Chem. Ecol.* 14:1003–1021.

PRESTWICH, G.D., VOGT, R.G., and RIDDIFORD, L.M. 1986. Binding and hydrolysis of radiolabeled pheromone and several analogs by male-specific antennal proteins of the moth pheromone *Antheraea polyphemus*. *J. Chem. Ecol.* 12:323–333.

PRESTWICH, G.D., GRAHAM, S.M., and KOENIG, W.A. 1989. Enantioselective opening of the (+)- and (−)-disparlure by epoxide hydrolase in gypsy moth antennae. *J. Chem. Soc., Chem. Commun.* 1989:575–577.

PRESTWICH, G.D., GRAHAM, S.M., KUO, J.-W., and VOGT, R.G. 1990. Tritium-labeled enantiomers of disparlure. Synthesis and in vitro metabolism. *J. Am. Chem. Soc.* 111:636–642.

SAS Institute, 1988. SAS Users Guide: Release 6.03. SAS Institute Inc., Cary, North Carolina.

SCHWARZ, M., KLUN, J.A., FRITZ, G.L., UEBEL, E.C., and RAINA, A.K. 1989. European corn borer sex pheromone: Structure–activity relationships. *J. Chem. Ecol.* 15:601–617.

SCHWARZ, M., KLUN, J.A., and UEBEL, E.C. 1990. European corn borer sex pheromone inhibition and elicitation of behavioral response by analogs. *J. Chem. Ecol.* 16:1591–1604.

SHAW, D.A., and TUOMINEN, T.C. 1985. An efficient synthesis of 3-hydroxy-3-trifluoromethylphthalide. *Synth. Commun.* 15:1291–1297.

TASAYCO, M.L., and PRESTWICH, G.D. 1990. Aldehyde-oxidizing enzymes in an adult moth: In vitro study of aldehyde metabolism in *Heliothis virescens*. *Arch. Biochem. Biophys.* 278:444–451.

TAYLOR, T.R., FERKOVICH, S.M., and VAN ESSEN, F. 1981. Increased pheromone catabolism by antennal esterases after adult eclosion of the cabbage looper moth. *Experientia* 37:729–731.

VOGT, R.G., and RIDDIFORD, L.M. 1986. Scale esterase: A pheromone-degrading enzyme from scales of silk moth *Antheraea polyphemus*. *J. Chem. Ecol.* 12:469–482.

VOGT, R.G., RIDDIFORD, L.M., and PRESTWICH, G.D. 1985. Kinetic properties of a sex pheromone-degrading enzyme: The sensillar esterase of *Antheraea polyphemus*. *Proc. Natl. Acad. Sci.*, U.S.A. 82:8827–8831.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

CHEMOTAXONOMIC STUDY OF UNDESCRIBED SPECIES[1] OF *Myrmica* ANT FROM IDAHO

BRIAN D. JACKSON,[2] SARAH J. KEEGANS,[2] E. DAVID MORGAN,[2,*] WILLIAM H. CLARK,[3] and PAUL E. BLOM[4]

[2]*Department of Chemistry*
University of Keele
Staffordshire, ST5 5BG, England

[3]*College of Idaho*
Museum of Natural History
Caldwell, Idaho 83605

[4]*Department of Plant, Soil and Entomological Sciences*
University of Idaho
Moscow, Idaho 83843

(Received July 11, 1990; accepted September 24, 1990)

Abstract—An undescribed species of *Myrmica* collected in Idaho has been shown to have the same substances in its mandibular glands (3-octanol and 3-octanone and related 3-alkanols and 3-alkanones) and in its Dufour gland (linear alkanes, alkenes, and farnesene isomers and homologs) as previously examined European species of *Myrmica*. The poison gland contains the trail pheromone 3-ethyl-2,5-dimethylpyrazine, common to all *Myrmica* species studied so far. The Dufour gland contains large amounts of bishomofarnesene, which easily distinguishes it from some 13 other *Myrmica* already known.

Key Words—Ant, *Myrmica*, Hymenoptera, Formicidae, exocrine secretions, mandibular gland, Dufour gland, trail pheromone, 3-ethyl-2,5-dimethylpyrazine, bishomofarnesene.

INTRODUCTION

Yensen et al. (1977), in their checklist of Idaho ants, list 12 species of *Myrmica*. Recently, an undescribed species was discovered in the Idaho National Envi-

*To whom correspondence should be addressed.
[1] Species description to appear elsewhere.

0098-0331/91/0200-0335$06.50/0

ronmental Research Park, which is located in the desert area of southeastern Idaho. André Francoeur has recognized this as a new species in the course of revision of the Nearctic species and will describe it in the near future. The species is not confined to Idaho; it is a western species found infrequently in warm habitats (Francoeur, personal communication). In order to define this new species more fully, a chemotaxonomic study has been made of its exocrine secretions. There is an increasing awareness that such characteristics can be a valuable addition to the use of morphological characters in the diagnosis of a species. Numerous studies of ants have shown that in all cases examined, the Dufour gland in particular contains a species-specific mixture of substances, dominated by oily hydrocarbons.

Crewe and Blum (1970a,b) carried out the earliest studies on the mandibular gland secretions of *Myrmica* species and showed that 3-octanol and 3-octanone are the principal constituents in each one, although the proportions varied. Subsequently a systematic study was carried out on the eight species of *Myrmica* commonly found in Great Britain (Cammaerts et al., 1982; Attygalle et al., 1983a), and it was further extended to five other European species (Jackson et al., 1989, and unpublished). In each case we have found a species-specific mixture of alcohols and ketones, usually dominated by 3-octanol and 3-octanone. Similarly, we have studied the Dufour gland contents of the eight species of British *Myrmica* (Cammaerts et al., 1983; Attygalle et al., 1983b) and the five European species (Jackson et al., 1989, and unpublished) and have found a mixture of alkanes, alkenes, and (*Z*,*E*)-α-farnesene and it homologs with one, two, or three extra carbon atoms (Attygalle and Morgan, 1982). Whether the linear or terpenoid hydrocarbons are dominant in the gland varies widely with species (Attygalle et al., 1983b).

Ant trail pheromones commonly are shared by more than one species (Attygalle and Morgan, 1985). Our investigation of the trail pheromone of *Myrmica* (Evershed et al., 1981, 1982; Jackson et al., 1989, and unpublished), has shown that the same single substance 3-ethyl-2,5-dimethylpyrazine (EDMP), present in the poison gland as a trace component, provides the trail pheromone in each of 12 species examined.

We show here that this undescribed species from Idaho, while fitting perfectly within the pattern found for its European cousins, has a specific composition of secretion in its mandibular and Dufour glands that should distinguish it from other species of *Myrmica*. It shares the same trail pheromone as all the other *Myrmica* species.

METHODS AND MATERIALS

A colony of this *Myrmica* sp. collected at the Idaho National Environmental Research Park (WHC #8919, 30-IX-1989) was sent live to Keele, where it was maintained in an artificial nest until analysis could be carried out. A colony

of *Myrmica rubra*, collected near Keele was maintained in a similar artificial nest and used in the trail pheromone tests.

For analysis of the mandibular gland substances, whole worker heads were sealed in soft glass capillaries (2 × 20 mm). For the analysis of the Dufour glands, whole poison apparatuses (sting lance, Dufour gland, poison filaments, and reservoir) were similarly sealed in soft glass capillaries. These capillaries were introduced into the gas chromatograph as described by Morgan and Wadhams (1972) and there heated and crushed to produce the chromatogram.

The samples were analyzed by GC-MS, on a Hewlett Packard 5890 gas chromatograph and 5970B mass selective detector with HP59970C chemstation software. A fused silica capillary column (12 m × 0.2 mm) coated with immobilized dimethylsiloxane of 0.33 μm film thickness was used. The injection port temperature was 140°C, and the capillary tubes were equilibrated in the solid injector for 2 min. The oven temperature was initially set at 30°C for two minutes and then increased at 8°C/min. The flow rate of the helium carrier gas was 1 ml/min. The mass selective detector was set to detect ions with *m/z* 35–350. Calibration was achieved by injecting external standards of C_{15} to C_{18} hydrocarbons for Dufour gland substances 3-octanone, 3-octanol, 3-nonanone, and 3-nonanol for mandibular gland substances, and EDMP for the trail pheromone.

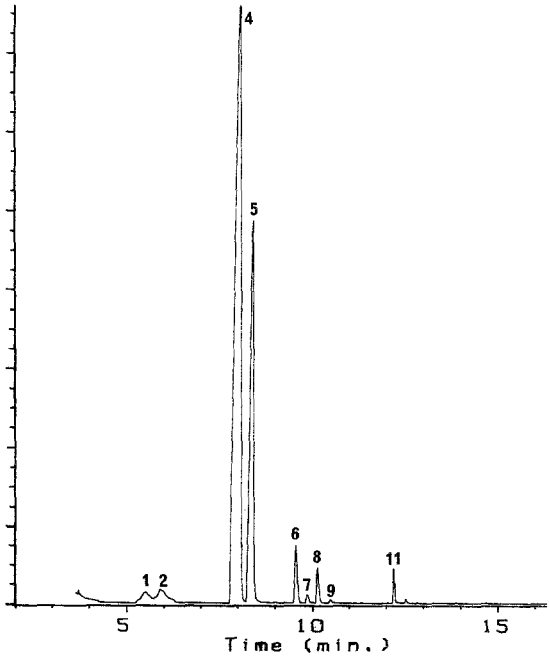


FIG. 1. Gas chromatogram of the volatiles from the mandibular glands of a single worker of *Myrmica* sp. (undescribed). Peak numbers refer to compounds listed in Table 1.

These substances also were used for comparison of retention times and mass spectra in identification of the various substances. Farnesenes were identified by comparison with retention times and mass spectra of those identified in other *Myrmica* species (cf., Attygalle and Morgan, 1982).

The method of Pasteels and Verhaege (1974), using circular trails, was used for determination of the activity of the trail pheromone and for cross-activity testing between *Myrmica rubra* and *Myrmica* sp. Solutions of extracts were prepared in hexane (100 μl) applied to the circumference of a circle (r = 5 cm) drawn on paper, with 1-cm arcs marked off on the circumference, and after allowing 2 min for the hexane to evaporate, the paper was presented to foraging workers. The number of 1-cm arcs for which each worker followed the circular trail before deviating from it were recorded. The median value of 30 such observations was calculated for each test. Hexane solvent alone was used for control tests. Synthetic 3-ethyl-2,5-dimethylpyrazine was tested similarly.

Voucher specimens are deposited in the collection of College of Idaho

TABLE 1. AMOUNTS AND PERCENTAGES OF VARIOUS SUBSTANCES FOUND IN MANDIBULAR GLANDS OF WORKERS OF UNDESCRIBED SPECIES OF *Myrmica* FROM IDAHO[a]

	Compound	Amount ± SD (ng/ant)	Percentage (% ± SD)
1	3-Heptanone	35 ± 23	1.9 ± 0.4
2	3-Heptanol	39 ± 43	1.8 ± 0.7
3	Unknown	1 ± 2	t[b]
4	3-Octanone	1186 ± 788	65.0 ± 11.0
5	3-Octanol	456 ± 322	25.6 ± 10.4
6	6-Methyl-3-octanone	33 ± 19	1.8 ± 0.7
7	6-Methyl-3-octanol	8 ± 5	0.5 ± 0.2
8	3-Nonanone	32 ± 24	1.7 ± 0.4
9	3-Nonanol	5 ± 3	0.3 ± 0.2
10	Unknown	1 ± 1	t
11	3-Decanone	20 ± 12	1.2 ± 0.4
12	3-Decanol	3 ± 2	0.2 ±
13	Unknown	1 ± 1	0.1 ±
14	3-Undecanone	1	0.1 ±
15	3-Undecanol	1 ± 1	t
	Total	1820	

[a] Results are the means (with sample standard deviations) of analysis of 10 individuals.
[b] t = less than 0.1%.

Museum of National History (CIDA), André Francoeur, and the William F. Barr Entomological Museum, University of Idaho (UICM).

RESULTS

The mandibular glands of this undescribed species contain chiefly 3-octanone, with less of 3-octanol and traces of other related alcohols and ketones (Figure 1). The mean amount of each substance and the mean percent that each substance represents of the total, plus their sample standard deviations are given in Table 1. As found elsewhere, the amounts in the gland vary widely, but the composition remains within a fairly narrow band of values.

The Dufour gland contained very little linear hydrocarbons but was dominated by a large amount of (*Z*,*E*)-α-bishomofarnesene (Figure 2). The amounts and percentages of the major components are given in Table 2.

The presence of EDMP was evident in the chromatogram obtained from the poison apparatus (Figure 2). The amount was similar to that found in *M.*

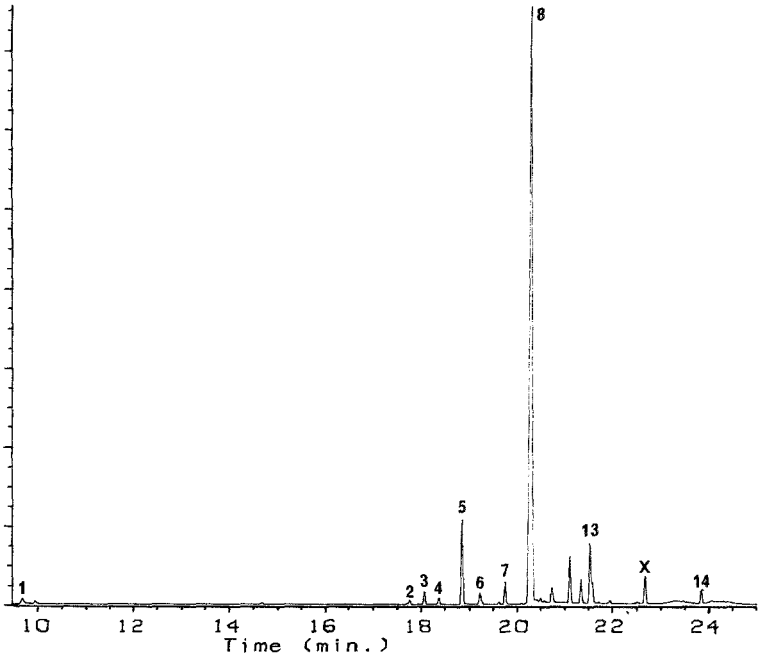


FIG. 2. Gas chromatogram of the poison apparatus of a single worker of *Myrmica* sp. (undescribed). Peak numbers refer to compounds listed in Table 2; X is isopropyl myristate, a contaminant.

TABLE 2. AMOUNTS OF 3-ETHYL-2,5-DIMETHYLPYRAZINE (EDMP) IN POISON RESERVOIR AND AMOUNTS AND PERCENTAGES OF VARIOUS SUBSTANCES FOUND IN DUFOUR GLAND OF WORKERS OF UNDESCRIBED SPECIES OF *Myrmica* FROM IDAHO[a]

	Compound	Amount (ng/ant ± SD)	Percentage (% ± SD)
1	EDMP (from poison gland)[b]	4.9 ± 5.4	
2	*(Z,E)*-α-Farnesene	5.5 ± 6.2	3.1 ± 5.0
3	Pentadecane	10.4 ± 14.8	1.9 ± 2.8
4	Homofarnesene isomer 1	6.0 ± 11.8	0.5 ± 0.3
5	*(Z,E)*-α-Homofarnesene	60.6 ± 100	7.4 ± 3.6
6	Homofarnesene isomer 2	13.3 ± 24.5	1.2 ± 0.4
7	Bishomofarnesene isomer 1	23.4 ± 47.8	2.0 ± 1.0
8	*(Z,E)*-α-Bishomofarnesene	514 ± 633	67.4 ± 7.6
9	Bishomofarnesene isomer 2	1.4 ± 2.3	0.2 ± 0.3
10	Heptadecene	6.3 ± 6.8	1.2 ± 1.0
11	Heptadecane	29.7 ± 66.7	5.5 ± 1.4
12	Trishomofarnesene isomer 1	30.4 ± 61.5	2.3 ± 1.3
13	Trishomofarnesene 2	67.8 ± 121	6.0 ± 2.9
14	Nonadecane	11.6 ± 14.5	1.4 ± 0.7
	Total	780	

[a] Results are the means (with sample standard deviations) of 10 determinations on individual workers.

[b] The whole poison apparatus was used, consisting of poison glands, poison reservoir, and Dufour gland. The protein venom of the poison reservoir is not seen in the chromatogram, and the EDMP is the only substance not coming from the Dufour gland.

rubra (Evershed et al., 1981). All control experiments gave zero median values, and all test results were significantly different ($P < 0.001$) from controls using the Mann-Whitney-Wilcoxon test (Siegel and Castellan, 1988). The results of the activity tests are shown in Table 3.

DISCUSSION

The results obtained here are conveniently compared with those recently obtained for *M. gallieni* and *M. specioides*, collected in Switzerland (Jackson et al., 1989) and examined under almost identical conditions. The mandibular gland contents of *Myrmica* sp. are superficially very similar to that of *M. gallieni*, with octanone and octanol comprising over 80% of the total contents, but there are minor differences, such as the absence of undecanone and undecanol in *M. gallieni*. The size of the gland is somewhat larger here (1.8 μg secretion)

TABLE 3. RESULTS OF TRAIL-FOLLOWING TESTS ACCORDING TO METHOD OF PASTEELS AND VERHAEGHE (1974) ON CIRCULAR TRAIL (RADIUS 5 cm)[a]

Source of material for tests	Species	
	M. rubra	*Myrmica* sp. (undescribed)
Myrmica sp. (undescribed), 1 gaster extracted in 100 μl hexane	7	4
Myrmica rubra, 1 gaster extracted in 100 μl hexane	7	4
EDMP		
1 ng in 100 μl hexane	2	3
7 ng in 100 μl hexane	[b]	6

[a]Results are expressed as the median number of 1-cm arcs on the circumference followed on the trail by 30 worker ants. Hexane controls in the same test gave 0 cm.
[b]Not tested.

than in either *M. gallieni* (1.3 μg) or *M. specioides* (1.1 μg). The unknown compounds are trace substances from the head. They have been recognized in other chromatograms where large amounts of insect tissue have been injected. The sample standard deviations of amount are very large because the quantity of substance in an individual gland varies widely; for example, there was 3.0 μg of 3-octanone in the largest gland, and 0.2 μg in the smallest.

The Dufour gland is rather distinct from the European species in having bishomofarnesene representing 70% of the total contents. It also is unusual in having small amounts of other isomers of homofarnesene, bishomofarnesene, and trishomofarnesene. If these have been present in other species, the amounts have been too small to detect. Comparatively, the Dufour gland is smaller in this species (contains 0.8 μg) compared to *M. rubra*, *M. gallieni* (1.8 μg), or *M. specioides* (0.9 μg). Large variations in the amount per gland were again observed.

The quantity of EDMP found in the poison glands (4.9 ng) is comparable with what has been found in other *Myrmica* species, and the trail tests (Table 3) show that the trail-following ability of this species is similar to, or slightly less than, that of *M. rubra*. Moreover, *M. rubra* readily followed artificial trails made with extracts of the gaster of this species and vice versa. It can be seen from Table 3 that the activity of a single gland and the equivalent amount of pure EDMP are comparable.

Myrmica sp. clearly fits into the pattern of trail pheromone and exocrine secretions of its geographically distant cousins from Europe.

Acknowledgments—B.D.J. and S.J.K. thank the SERC for the award of studentships, and E.D.M. thanks SERC for a grant for the purchase of the GC-MS equipment. We thank O.D. Markham, T.D. Reynolds, and J.B. Johnson for their assistance. Partial support was received from the Idaho National Engineering Laboratory, Radioecology and Ecology Programs sponsored by the Office of Health and Environmental Research, U.S. Department of Energy.

REFERENCES

ATTYGALLE, A.B., and MORGAN, E.D. 1982. Structures of homofarnesene and bishomofarnesene isomers from *Myrmica* ants. *J. Chem. Soc. Perkin I* 949–951.

ATTYGALLE, A.B., and MORGAN, E.D. 1985. Ant trail pheromones. *Adv. Insect Physiol.* 18:1–30.

ATTYGALLE, A.B., CAMMAERTS, M.C., and MORGAN, E.D. 1983a. Dufour gland secretion of *Myrmica nigulosa* and *Myrmica schencki* workers. *J. Insect Physiol.* 29:27–32.

ATTYGALLE, A.B., EVERSHED, R.P., MORGAN, E.D., and CAMMAERTS, M.C. 1983b. Dufour gland secretions of workers of the ants *Myrmica sulcinodis* and *Myrmica lobicornis*, and comparison with six other species of *Myrmica*. *Insect Biochem.* 13:507–512.

CAMMAERTS, M.C., EVERSHED, R.P., and MORGAN, E.D. 1982. Mandibular gland secretions of workers of *Myrmica rugulosa* and *M. schencki*: Comparison with four other *Myrmica* species. *Physiol. Entomol.* 7:119–125.

CAMMAERTS, M.C., EVERSHED, R.P., and MORGAN, E.D. 1983. The volatile components of the mandibular gland secretion of workers of the ant *Myrmica lobicornis* and *Myrmica sulcinodis*. *J. Insect Physiol.* 29:659–664.

CREWE, R.M., and BLUM, M.S. 1970a. Identification of the alarm pheromones of the ant *Myrmica brevinodis*. *J. Insect Physiol.* 16:141–146.

CREWE, R.M., and BLUM, M.S. 1970b. Alarm pheromones in the genus *Myrmica* (Hymenoptera: Formicidae), Their composition and species specificity. *Z. Vergl. Physiol.* 70:363–373.

EVERSHED, R.P., MORGAN, E.D., and CAMMAERTS, M.-C. 1981. Identification of trail pheromone of the ant *Myrmica rubra* L., and related species. *Naturwissenschaften* 67:374–375.

EVERSHED, R.P., MORGAN, E.D., and CAMMAERTS, M.-C. 1982. 3-Ethyl-2,5-dimethylpyrazine, the trail pheromone from the venom gland of eight species of *Myrmica* ants. *Insect Biochem.* 12:383–391.

JACKSON, B.D., MORGAN, E.D., and COLLINGWOOD, C.A. 1989. The chemical secretions of *Myrmica specioides* Bondroit and *Myrmica gallieni* Bondroit (Myrmicinae). *Actes Coll. Insectes Soc.* 5:315–321.

MORGAN, E.D., and WADHAMS, L.J. 1972. Gas chromatography of volatile materials. *J. Chromatogr. Sci.* 10:528–529.

PASTEELS, J.M., and VERHAEGHE, J.C. 1974. Dosage biologique de la phéromone de piste chez les fourrageures et les reines de *Myrmica rubra*. *Insectes Soc.* 21:167–180.

SIEGEL, S., and CASTELLAN, N.J. 1988. Nonparametric Statistics For The Behavioural Sciences. McGraw-Hill, Singapore. p. 128.

YENSEN, N.P., CLARK, W.H., and FRANCOEUR, A. 1977. A checklist of Idaho ants. *Pan-Pac. Entomol.* 53:181–187.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

INVESTIGATIONS ON SOME ASPECTS OF CHEMICAL ECOLOGY OF COGONGRASS, *Imperata cylindrica* (L.) BEAUV.

INDERJIT and K.M.M. DAKSHINI*

Department of Botany
University of Delhi
Delhi 110007, India

(Received August 20, 1990; accepted September 27, 1990)

Abstract—To understand the interference mechanism of the weed, cogongrass, *Imperata cylindrica* (L.) Beauv., its effect on nutrient availability and mycoflora of its soil rhizosphere as well as nodule characteristics, root length, and root/shoot ratio of *Melilotus parviflora* Desf. were investigated. Additionally, the effect of the leachates of leaves and root/rhizome of cogongrass on seed germination and seedling characteristics of radish, mustard, fenugreek, and tomato were examined. Furthermore, to assess the qualitative and quantitative differences in phytochemical components, the leachates and the soils from three sampling sites (with cogongrass and 1.5 m and 3 m away from cogongrass) were analyzed with high-performance liquid chromatography (HPLC) on a C18 column. No significant difference in nutrient availability was found, but qualitative and quantitative differences in phenolic fractions were recorded in the three sampling sites. Furthermore, of the 19 fungi recorded in the soils, decreases in the number of colonies (per gram of soil) of *Aspergillus fumigatus, A. niger, A. candidus*, and an increase of *A. flavus* was recorded in the soils with cogongrass. The inhibition in nodule number, weight, nitrogen fixation (acetylene reduction activity), root length, and root/shoot ratio of *Melilotus parviflora* were noted. Percent seed germination, root and shoot length, fresh and dry weight of seedlings of different seeds were affected by the leachates of leaves and root/rhizome. It was found that root/rhizome leachate was more inhibitory than leaf leachate. However, the inhibition was higher in soil + leaves leachate than soil + root/rhizome leachate. HPLC analysis established that four compounds were contributed by the weed to the soil system even though their relative concentration varies in various leachates. It is surmised that these compounds cause allelopathic inhibition of growth characteristics of seeds tested. Significance of the data vis-à-vis the interference potential of the cogongrass is discussed.

*To whom correspondence should be addressed.

0098-0331/91/0200-0343$06.50/0

Key Words—Allelopathy, cogongrass, competition, *Imperata cylindrica*, HPLC, interference, weed.

INTRODUCTION

Cogongrass, *Imperata cylindrica* (L.) Beauv. is among the 10 worst weeds of the warmer regions of the world (Holm, 1969). However, how this weed achieves success has not been thoroughly explained. Abdul-Wahab and Al-Naib (1972) identified scopoletin, scopolin, chlorogenic acid, and isochlorogenic acid in water extract of leaves and culm of this weed. These phenolic acids have been reported to effect growth and physiological characteristics of plant species (Rice, 1984; Einhellig and Kuan, 1971; Lodhi and Rice, 1971), but whether these compounds were present in the rhizosphere of this weed, thus giving allelopathic success to the weed, has not been established. In view of such a gap in our understanding, the present investigations on this weed have been undertaken.

METHODS AND MATERIALS

These investigations were conducted at the Botanical Garden, University of Delhi, where cogongrass is distributed in various densities. Data to study the effect of the weed on nutrient availability, soil mycoflora, nodule forming characteristics of *Melilotus parviflora* Desf., seed germination, seedling characteristics, and phenolic fractions of soils and test solutions were collected. The methods for each of these investigations are described below.

Analysis of Phenolic Fractions. Different test solutions [leaf leachate (TS1), soil + leaves leachate (TS2), root/rhizome leachate (TS3), soil + root/rhizome leachate (TS4), garden soil leachate, as well as soils from the three different sampling sites (with cogongrass and 1.5 and 3 m away from cogongrass)] were analyzed for phenolic fractions by high-performance liquid chromatography (HPLC). Soils (5 g) and plant materials (0.1 g) were shaken with 10 ml of methanol for 1 hr at room temperature. The ratio of extractant to soil was 2 : 1 (v/w) and that for plant material was 100 : 1 (v/w). The extracts were carefully filtered and subjected to HPLC (Shimadzu LC-4A) using a variable wavelength UV detector set at 275 nm. Reversed-phase chromatography was carried out using a steel column (15 cm × 4.6 mm ID) containing Zorbax C18 with the flow rate of 1 ml/min. The volume injected each time was 10 μl and 30 μl for leaf and soil leachates, respectively. The phenolic fractions, differentiated on the basis of their respective retention times and relative concentrations, were recorded (Table 1 below).

Effect of Weed on Root Length, Root/Shoot Ratio and Nodule-Forming

Characteristics of Melilotus parviflora. *Melilotus parviflora* Desf. growing wild with (considered as treated in the investigation) and without (considered as control) cogongrass was selected to study the effect of this weed on its root length, root/shoot ratio, and nodule characteristics. After careful uprooting of the plants, number and weight of nodules, root length, and root/shoot ratio were recorded (Table 2 below). Nodules of each of the sites were carefully washed with double distilled water (DDW), and then the N_2 fixation rate following the acetylene reduction activity was determined. Acetylene was injected into vials containing weighed nodules so as to give a partial pressure of 0.1 atmosphere. Samples of gas phase were drawn after 45 min and their acetylene and ethylene concentrations were assessed by gas chromatography (GC). Acetylene reduction was calculated as per gram fresh weight of the nodules. All trials were made in triplicate.

Effect of Weed on Soil Mycoflora. For analysis of the effect of the weed on mycofloral components, 12 localities, with (considered as treated) and without (considered as control) cogongrass were selected. Soils for fungal analyses were collected by carefully inserting vertically a sterilized glass tube. These tubes were immediately plugged to avoid any contamination and then were stored at 0–4°C. The soil plating technique was employed to isolate fungal colonies. For preparation of the soil suspension, 1 g of soil was shaken with 100 ml of DDW. Three replicates were made by pouring 1 ml of the aliquot from each of the soil suspensions into Petri plates containing 20 ml of cooled and melted Czapek's Dox yeast extract agar medium (Dickinson, 1971) supplemented with streptomycin. Culture plates were thus made and incubated in the culture room at 25 ± 3°C. Total population of fungal colonies was calculated using the following formula:

$$\text{Total number of fungal colonies} = \frac{\text{Mean No. of fungal colonies} \times \text{dilution factor}}{\text{weight of dry soil}}$$

Effect of Weed on Nutrient Availability. To study the effect of this weed on nutrient availability, three contiguous regions, one with the weed present, and the others 1.5 m and 3 m away from it were selected. Soil samples from the rhizosphere (to a depth of 15 cm) of these three sampling sites were collected, air dried, sieved, stored in paper bags and analyzed following standard procedures (Allen, 1989) for pH, electrical conductivity, organic matter, Cu^{2+}, Zn^{2+}, K^{+}, Na^{+}, Mg^{2+}, Ca^{2+}, Cl^{-}, and PO_4^{-3}.

Effect of Weed on Seed Germination and Growth. For this, the effect of different test solutions (TS1, TS2, TS3, and TS4) on seed germination and seedling characteristics of radish (*Raphanus sativus* var. pusa desi), mustard (*Brassica juncea* CV PR 45), fenugreek (*Trigonella foenum-graecum* var. pusa early bunch), and tomato (*Lycopersicon esculentum* var. pusa ruby), the commonly cultivated crops of the region, were investigated. These leachates were

prepared by taking 15 g of each of the plants' parts; leaf and root/rhizome, either alone (for TS1 and TS3) or mixed with garden soil (for TS2 and TS4), were soaked, the former in 100 ml of DDW and the latter in 1:5 soil–DDW for 72 hr. Each of these was filtered and made to 100 ml with DDW. These leachates were used in the experiments without any further dilution. For germination studies, 50 seeds of the respective crop plants were sown on filter paper moistened with DDW (control for plant leachate), garden soil leachate (control for soil leachate), or test solutions, each in equal volume and placed in 15-cm-diameter Petri plates. To maintain uniform moisture status in Petri plates, a cotton pad soaked in DDW or any of the test solutions was placed below the filter paper. Observations on root and shoot length were made every 24 hr up to seven days. Each treatment was replicated thrice. During the period of experimentation, the temperature regime of 22 $\pm$ 5°C and the diurnal regime of light conditions were maintained. After seven days, fresh and dry weights (after keeping at 60°C for 24 hr) of seedlings were recorded (see Table 5 below).

RESULTS

Phenolic Fraction Analysis through HPLC. The presence of this weed brought about distinct qualitative and quantitative changes in the phenolic fractions of the soil samples of different sampling sites (1–3) and the test solutions (TS1, TS2, TS3, and TS4) analyzed (Table 1). In all, 18 phenolic fractions were detected from eight extracts, of which fractions 6 and 14 were common to all. Fraction 8 was present in all the extracts except TS1, and fraction 3 was present in all except sampling site 3 and TS4. However, since these fractions also were detected in control soil, it could be surmised that these must be present in traces but did not show up on the HPLC. On the contrary, fraction 9, although absent in control soil and TS4, was present in rest of the extracts. Furthermore, the relative concentrations of fractions 3, 8, and 9 decreased with the increase in distance from the immediate vicinity (rhizosphere zone) of the weed. The relative concentrations of fractions 3, 5, and 7 were higher in plant extracts (TS1 and TS3) than in soil extracts (TS2 and TS4), while the reverse was true of the quantitative distribution of fraction 8. However, the phenolic fractions 4, 12, and 17 present in the soil with the weed were found to be absent in the test solutions (except TS1 with fraction 4). Phenolic fractions 1, 2, 11, and 13 could be detected only in the HPLC profiles of the soil extracts. It should be noted that even the concentrations of fractions that were common to all the extracts (fractions 6 and 14) exhibited a relative increase wherever the influence of the weed plant part was present.

Nodule Experiments. In the present investigation nodule characteristics, root length, and root/shoot ratio of *Melilotus parviflora* were significantly

TABLE 1. HPLC ANALYSIS OF PHENOLIC FRACTIONS OF SOILS AND TEST SOLUTIONS[a]

Phenolic fraction		Relative concentration (percent)							
		Soil				Test Solution			
No.	Retention time (min)	CS	SS1	SS2	SS3	TS1	TS2	TS3	TS4
1.	0.308	0.208							
2.	1.408	9.35							
3.	1.558	15.12	18.00	7.04		11.51	3.62	19.02	
4.	1.60			6.84	8.90	3.62			
5.	1.79					34.40	4.78	38.40	7.94
6.	2.10	25.57	26.80	38.60	29.20	21.30	19.07	16.75	63.32
7.	2.30					6.92	1.49	4.64	
8.	2.46	36.85	27.20	20.21	18.37		2.73	2.12	11.70
9.	2.80		17.00	15.40	14.13	5.27	7.20	3.23	
10.	3.07					4.74	3.41		4.04
11.	3.27	4.05							
12.	3.60		3.90	2.42	3.68				
13.	3.73	2.62							
14.	4.05	1.95	6.13	7.34	11.07	1.04	2.38	10.09	1.14
15.	4.25					10.70			
16.	4.35	2.63					49.80		2.84
17.	5.10			1.49	3.40				
18.	5.70				2.90				

[a] CS, control soil; SS1, sampling site 1; SS2, Sampling site 2; SS3, Sampling site 3; TS1, Leaf leachate; TS2, Soil + leaves leachate; TS3, Root/rhizome leachate; TS4, Soil root/rhizome leachate.

TABLE 2. ROOT LENGTH, ROOT/SHOOT RATIO, AND NODULE CHARACTERISTICS OF *Melilotus parviflora* GROWING WITH (TREATED) AND WITHOUT (CONTROL) COGONGRASS

Characteristics	Control	Treated
Nodule number	58.50 ± 19.01	26.00 ± 7.88**[a]
Nodule weight (g)	0.0073 ± 0.0026	0.0041 ± 0.0017*
N_2 fixation (μmol C_2H_4/g fresh wt)	10.9588 ± 3.4078	2.1814 ± 0.8494***
Root length (cm)	10.540 ± 1.390	7.460 ± 2.48*
Root/Shoot ratio	0.367 ± 0.101	0.228 ± 0.055**

[a] *$0.05 \leq P < 0.1$, **$0.01 \leq P < 0.05$, ***$0.001 < P < 0.01$.

affected by the weed (Table 2). Nodule weight, root length ($0.05 \leq P < 0.1$), nodule number, root/shoot ratio ($0.01 \leq P < 0.05$) and nitrogen fixation ($P < 0.1$) were reduced in plants occurring with the cogongrass.

Soil Mycoflora Studies. The fungal components of soil were found to be affected by this weed. Significant differences in the number of colonies per gram of soil of four species of *Aspergillus* were recorded in soils associated with the weed (Table 3). Thus, in comparison to control, reductions in the number of colonies were as follows: 50.13% in *Aspergillus fumigatus* ($P < 0.25$), 36% in *A. niger* ($P < 0.25$), 49.35% in *A. candidus* ($0.05 < P < 0.07$); whereas augmentation (65.27%) was recorded in *A. flavus* ($0.05 < P < 0.07$).

Soil Nutrient Studies. Data from Table 4 suggest that no significant changes were brought about by the weed in the concentration of nutrients in the soil samples analyzed.

Seed Germination and Growth Experiments. All the four leachates affected variously the seed germination and seedling characteristics of the different seeds tested (Table 5). Inhibition in percentage seed germination and shoot length in

TABLE 3. FUNGAL COMPOSITION OF SOILS WITH (TREATED) AND WITHOUT (CONTROL) COGONGRASS

Name of fungus	Number of Colonies of[a]	
	Control soil	Treated soil
Aspergillus flavus Link	108	288**
A. fumigatus Fresenius	375	187*
A. niger Van Teighem	750	480*
A. candidus Link	616	312**
A. terreus Thom	375	187
A. nidulans (Eidam) Winter		200[b]
A. humicola Chaudhuri	200	200
A. luchuensis Inui	300[b]	
Curvularia pallescens Boedijn	100[c]	100[b]
Penicillium javanicum Van Beyma	200[c]	
P. chrysogenum Thom	658	562
Trichoderma viride Pers ex Fries	100[b]	266
Cladosporium cladosporides (Fries.) De Vries		200[b]
Rhizopus nigricans Ehrenberb	300[c]	
Fusarium oxysporum (Schl ex Fr.) Snyder et Hansen	200[c]	
Epicoccum purpurascens Ehrenb ex Schlecht		200[b]
Aurobasidium pullulans (de Bary) Arnaud	600[b]	
Alternaria alternata (Fr.) Keissler		200[b]
Yeast-like fungi	300[b]	

[a] *$P < 0.25$, **$0.05 < P < 0.07$.
[b] Colonies detected only in one replicate.
[c] Colonies detected only in two replicates.

TABLE 4. CHEMICAL CHARACTERISTICS OF SOILS FROM THREE DIFFERENT SAMPLING SITES[a]

Chemical characteristics	Soil samples collected from		
	Rhizosphere zone of cogongrass	1.5 m away from cogongrass	3 m away from cogongrass
pH	8.227 ± 0.0808	8.070 ± 0.098**[d]	8.353 ± 0.341
EC[b] (μmho/cm)	427.293 ± 82.75	795.120 ± 335.90*	710.830 ± 226.250*
OM (%)[c]	0.879 ± 0.039	1.016 ± 0.204	0.963 ± 0.100
Cl^- (%)	0.013 ± 0.0028	0.021 ± 0.008*	0.041 ± 0.030*
PO_4^{-3} (mg/100 g)	0.958 ± 0.141	1.017 ± 0.117	1.189 ± 0.091**
Cu^{2+} (mg/100 g)	0.189 ± 0.083	0.107 ± 0.031*	0.111 ± 0.008*
Zn^{2+} (mg/100 g)	1.356 ± 0.505	1.147 ± 0.087	0.549 ± 0.177
Na^+ (mg/100 g)	40.417 ± 15.97	58.00 ± 40.420	110.670 ± 68.030*
K^+ (mg/100 g)	34.833 ± 3.02	56.16 ± 17.710*	33.750 ± 19.540
Mg^{2+} (mg/100 g)	33.660 ± 6.80	32.50 ± 10.64	33.730 ± 3.400
Ca^{2+} (mg/100 g)	313.133 ± 49.47	313.56 ± 126.57	304.230 ± 38.100

[a] See text for details.
[b] Electric conductivity.
[c] Organic matter.
[d] *$0.05 < P < 0.1$, **$P < 0.05$.

tomato was brought about in all test solutions. In general, tomato was found to be the most affected by the test solutions. In fact, except for dry weight (TS1, TS2, and TS3) and root length (TS2, TS3, and TS4), all other parameters studied showed maximum inhibition in tomato. TS1 inhibited most of the seedling characteristics of all the seeds studied except a marginal increase in dry weight in fenugreek and tomato. Inhibition by TS2 was least in radish as compared to other systems investigated. Similarly, TS3 suppressed all the seedling characteristics except dry weight in radish, mustard, and tomato, where it showed an increase. TS4 did not decrease the fresh weight of the seedlings of any of the seeds tested. Even the dry weight was not reduced except for the significant reduction (23.21%) noted in tomato. The germination response of different seeds varied: increases in radish and fenugreek and reductions in mustard and tomato were observed. It may be noted that the reduction in values of all the characters by TS1 and TS3 was markedly significant as compared to TS2 and TS4, respectively. Moreover, inhibition in TS2 and TS3 was more than in TS1 and TS4.

DISCUSSION

These data clearly establish that cogongrass produces phenolic compounds, especially those represented by phenolic fractions 5, 7, 9, 10, and 12, which get incorporated in the soil and cause an allelopathic effect on other plant spe-

TABLE 5. EFFECT OF DIFFERENT COGONGRASS LEACHATES[a] ON SEED GERMINATION AND SEEDLING CHARACTERISTICS OF DIFFERENT CROP SEEDS

Crop seeds	Source of test solution	Seed germination	Root length[b]	Shoot length[b]	Fresh weight[c]	Dry weight[c]
Radish	L[a]	0.00	−22.37	−4.09	−11.11	−15.59
	S(L)	+10.00	−1.37	+2.39	+6.58	+12.67
	R	−14.90	−81.12	−64.55	−46.43	+12.47
	S(R)	+2.22	−8.13	−5.30	+2.52	+16.48
Mustard	L	−2.08	−36.30	−6.59	−16.10	−15.47
	S(L)	+2.12	−33.24	−18.85	−11.43	−6.77
	R	0.00	−94.25	−62.25	−44.52	+51.69
	S(R)	−2.04	−22.40	−1.40	+2.63	−0.99
Fenugreek	L	−8.88	−21.54	−34.70	−12.00	+7.11
	S(L)	−2.27	−10.55	−18.70	+3.43	−9.45
	R	−14.58	−49.36	−10.56	−30.29	−9.15
	S(R)	+4.34	−3.48	−8.60	+9.54	+1.02
Tomato	L	−43.24	−67.21	−77.87	−51.24	+4.42
	S(L)	−20.00	−17.91	−48.20	−17.14	−3.60
	R	−39.47	−90.64	−72.51	−53.25	+16.50
	S(R)	−15.15	+7.40	−97.78	+5.15	−23.21

[a] See text for details.
[b] Average percent decrease (−) or increase (+) in length as compared to control.
[c] Average percent decrease (−) or increase (+) in weight as compared to control.
[d] L, Leaf leachate; S(L), soil + leaf leachate; R, root/rhizome leachate; S(R), Soil + root/rhizome leachate.

cies. This also is supported by the fact that even the concentrations of those compounds that are common to control soils and test solutions were higher in the soils collected either from or near the rhizosphere of the weed. Furthermore, that these compounds move from the weed plant to the soil through the medium of water (as leachates were prepared in the aqueous medium) explains the success of the weed in lawns, orchards, and cultivated fields where irrigation is frequent. Furthermore, since the contribution of allelochemics is more by the root/rhizome than by the leaves, etc. (Table 1), and the percentage germination and seedlings is concentration-dependent, it is not difficult to conclude that the weed succeeds through allelochemics in the rhizosphere zone. As a matter of fact, this may be the most important means to totally eliminate any other plant species from its vicinity, as no buried or sown propagules would be able to establish themselves beyond the germination stage. This is borne out also by the observation that both root and shoot lengths of the germinated seeds tested were inhibited by the test solutions. Furthermore, the relative decrease in the

concentrations of phenolic fractions in the different sampling sites suggests that the zone of influence of the weed is much beyond the immediate vicinity of the weed. That the allelopathic effect is very efficient is suggested also by the reduction in the nodule characteristics and thus the N_2 fixation capacity of the legume *Melilotus parviflora*, as well as by an overall reduction in the soil mycoflora. The inhibitory effect of the weed on rhizosphere mycoflora may account for the insignificant change in the nutrient status of the rhizosphere soil of the weed, as the release of the nutrients would be affected under such conditions. Although this is in contrast to the observations of Glass (1973) and Mersie and Singh (1988), it proves that cogongrass is a very effective competitor because, in spite of its aggressiveness toward other plant species, the weed does not create stress for itself by changing the substratum. Interestingly, despite such allelopathic potential, the weed seems to be selective, as exhibited by the data on seed germination and seedling characteristics (Table 5). However, as opined by Williams and Hoagland (1982), since such a selection can also be because of seed characteristics such as size or seed coat permeability or other physiological characteristics, any inference in this regard requires further investigation.

The data in Table 1 show that the concentrations of the common phenolic fractions were higher in field soil leachates than in any of the test solutions. This could be due to the constancy of availability of the phenolic compounds for longer periods as compared to the test solutions, where the time allowed for leaching was shorter and the quantity of plant parts per gram of soil was less than the plant biomass available in the sampling sites. Furthermore, a decrease in concentration of phenolic fractions (Table 1) and percent germination and seedling characteristics in TS2 and TS4 as compared to TS1 and TS3, is now shown. This suggests selective release of the compounds in the medium. Similarly, this may be the explanation for the detection of phenolic fractions 5, 7, and 10 in the test solutions and 9 and 12 in the soils from the sampling sites. This observation is of significance since the quantity leached and made available to bring allelopathic effects should be crucial for understanding the mode of interference. The presence of fractions 4, 12, and 17 only in the soil with the weed (except TS1 with fraction 4) but their absence in the test solutions where fractions 5, 7, and 10 are present, could be due to phased release (quantitative as well as qualitative) of both these groups of compounds, as well as their stability outside the tissue of the plant. As suggested by Rice (1984), the role of mycoflora may also be important in the breakdown of the compounds in the soil system and may explain the differences in the phenolic fractions as noted above. Furthermore, it is very likely that the compounds released later are resistant to such processes as compared to those released earlier. Detailed studies on these aspects are necessary before any final comment is made on this aspect.

These investigations also brought out that tomato seeds can be used as an

assay system for such studies. A major outcome of the present investigation has been importance of HPLC analysis of soils and leachates as a tool for rapid and routine delimitation of the causes or the mode of achieving interference success by a weed. However, there remains the need to characterize the allelochemics to understand the interference mechanism.

Acknowledgments—We are grateful to Professor K.G. Mukerji for his help in identifying fungal members. We would like to thank Ms. Neena of University Science Instrumentation Centre, Delhi University, for help in HPLC analysis. The financial grant of University Grants Commission, India, is gratefully acknowledged. We are extremely grateful to Professor Elroy L. Rice, Oklahoma, U.S.A., for his critical evaluation and valuable suggestions.

REFERENCES

ABDUL-WAHAB, A.S., and AL-NAIB, F.A.G. 1972. Inhibitional effects of *Imperata cylindrica* (L.) *P.B. Bull. Iraq Nat. Hist. Mus.* 5:17–24.

ALLEN, S.E. (ed.). 1989. Chemical Analysis of Ecological Materials. 2nd ed. Blackwell Scientific, Oxford.

DICKINSON, C.H. 1971. Cultural studies of leaf saprophytes, pp. 293–324, *In* C.H. Dickinson and T.F. Preece (eds.). Ecology of Leaf Surface Micro-organisms. Academic Press, London.

EINHELLIG, F.A., and KUAN, L.A. 1971. Effect of scopoletin and chlorogenic acid on stomatal aperture in tobacco and sunflower. *Bull. Torrey Bot. Club* 98(3): 155–162.

GLASS, A.D.M. 1973. Influence of phenolic acids on ion-uptake: Inhibition of phosphate uptake. *Plant Physiol.* 51:1037–1041.

HARTLEY, R.D., and BUCHEN, H. 1979. High performance liquid chromatography of phenolic acids and aldehydes derived from the decomposition of organic matter in the soil. *J. Chromatogr.* 180:139–143.

HOLM, L. 1969. Weed problems in developing countries. *Weed Sci.* 17:113–118.

LODHI, M.A.K., and RICE, E.L. 1971. Allelopathic effects of *Celtis laevigata. Bull. Torrey Bot Club* 98:83–89.

MERSIE, W., and SINGH, M. 1988. Effects of phenolic acids and ragweed Parthenium (*Parthenium hysterophorus*) extract on tomato (*Lycopersicon esculentum*) growth and nutrient and chlorophyll content. *Weed Sci.* 25:278–281.

OLSEN, R.A., ODHAM, G., and LINDEBERG, G. 1971. Aromatic substances in leaves of *Populus tremula* as inhibitors of mycorrhizal fungi. *Physiol. Plant.* 25:122-129.

RICE, E.L. 1984. Allelopathy, 2nd ed. Academic Press, Orlando, Florida.

ROSE, S.L., PERRY, D., PILZ, D. and SCHOENEBERGER, M.M. 1983. Allelopathic effect of litter on growth and colonization of mycorrhizal fungi. *J. Chem. Ecol.* 9(8):1153–1162.

WILLIAMS, R.D., and HOAGLAND, R.E. 1982. The effects of naturally occurring phenolic compounds on seed germination. *Weed Sci.* 30:206–212.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

THE *t* COMPLEX OF THE MOUSE: CHEMICAL CHARACTERIZATION BY URINARY VOLATILE PROFILES[1]

B. JEMIOLO,* T-M. XIE,[2] F. ANDREOLINI,[3] A.E.M. BAKER,[4] and M. NOVOTNY

Department of Chemistry
Indiana University
Bloomington, Indiana 47405

(Received July 3, 1990; accepted September 28, 1990)

Abstract—Urine samples from C3H congenic house mice (*Mus domesticus*) differing only at the *t* complex were examined by capillary gas chromatography to assess variations in the volatile components that may cause olfactory discrimination between animals bearing *t* lethal and + (wild-type) haplotypes. Urine was collected from 192 males and females varying in age from 1 to 9 months. C3H congenic mice that have the same genetic background at all loci but differed in their *t* complex genotypes: +/+, $+/t_{w1}$, T/t_{w1}, *T*/+ were used. No urinary volatiles were unique to the *t* complex. However, significant differences among *t* complex genotypes and among ages occurred for concentrations of 12 male volatiles and four female volatiles. Usually young males (1–2 months of age) had significantly higher concentrations of cyclic enol ethers and ketones than older males (4–9 months of age). Moreover, some urinary volatiles (cyclic enol ethers, one ketone, dehydrobrevicomin, and thiazoline) were excreted in the urine of *T*/+ and/or *T*/*t* males in significantly higher concentration than in the urine of +/+ males. Age and *t* complex genotype influences on the urinary volatiles in females were observed for four ketones. Gas chromatography of urinary components has the potential to be used in field studies of the *t* complex because the two *t* complex genotypes found in wild populations, +/+ and +/*t*, had significant differences in concentration for two males volatiles and three female volatiles.

Key Words—Urinary volatiles, house mouse, chemosignals, *T* locus, *t* complex, *Mus domesticus*, capillary gas chromatography.

*To whom correspondence should be addressed.

[1] We dedicate this paper to the memory of Dorothea Bennett.

[2] On leave from the Environmental Protection Institute, Ministry of Light Industry, Beijing, China.

[3] Present address: Carlo Erba Strumentazione, Strada Rivoltana, 20090 Rodano-Milano, Italy.

[4] Present address: Department of Biology, Colorado State University, Fort Collins, Colorado 80523.

A.E.M.B. supported in part by NSF BSR 8909172.

0098-0331/91/0200-0353$06.50/0

INTRODUCTION

The house mouse (*Mus domesticus*) *t*-complex covers the proximal one-third of chromosome 17 of the mouse, and includes the *T* locus (defined by mutations that express both a recessive lethal phenotype and a dominant tail-length phenotype) as well as the major histocompatibility complex (Bennett, 1975; Klein, 1975; Silver, 1985). The *t* complex in the mouse is a set of dominant and recessive mutations, some of which have profound effects on embryonic development, sperm production and function, genetic recombination, and behavior. Approximately 20–25% of wild mice are heterozygous (+/*t*) for various recessive *t* haplotypes and 75–80% are homozygous (+/+) for the wild-type haplotype (Bennett, 1978; Lenington et al., 1988b). Despite strong selection against *t* haplotypes (prenatal deaths of *t* lethal homozygotes), they are maintained with high frequency because of their association with strong segregation distortion in males (Silver, 1985). When a heterozygous (+/*t*) male reproduces, about 90–95% of his offspring will carry his *t* haplotype. The frequency of *t* haplotypes in wild population of house mouse is considerably lower than predicted by a deterministic model incorporating selection against homozygotes and segregation distortion (Bruck, 1957).

Factors controlling the frequency of *t* haplotypes in wild populations are not known. One possibility explored by Lenington et al. (1988a), is that social behavior associated with this polymorphism may select against mice bearing *t* haplotypes. They found that both male and female mice can discriminate +/+ from +/*t* individuals of the opposite sex on the basis of their odor alone and prefer +/+ odors. This odor preference for +/+ over +/*t* individuals could provide a cue for selective mating that might reduce the frequency of *t* haplotypes in wild populations (Lenington, 1983; Lenington et al., 1988a,b; Drickamer and Lenington, 1987).

Mouse urine is a rich source of specific olfactory messengers (Novotny et al., 1991), which include another example of "genetic signaling," also on chromosome 17, the major histocompatibility complex mating preference (Yamazaki et al., 1978, 1979). The purpose of our study was to examine the urinary volatile profiles of male and female mice that differed only in genotype at the *t* complex.

METHODS AND MATERIALS

The mice used in this study were F_1, F_2, and F_3 progeny derived from C3H congenic laboratory animals, heterozygous for a dominant marker allele (*T*) at the *T* locus. Congenic mice have the same genetic background at all loci and differ only at the *t* complex. This allows us to distinguish if differences in urinary volatiles are the result of differences among *t* complex genotypes. The

founders of our colony were a gift from Dr. Dorothea Bennett of the Department of Zoology, University of Texas, Austin.

Urine was collected from four *t* complex genotypes: $T/+$; T/t_{w1} (hereafter T/t); $+/+$ and $+/t_{w1}$ (hereafter $+/t$). The marker allele (T), in conjunction with the + haplotype ($T/+$), produces mice with a short tail and, in conjunction with a *t* haplotype (T/t), produces tailless offspring. The *t* complex genotype $+/+$ (normal tail phenotype) was verified by breeding our mice until they produced at least six $+/+$ and no $+/t$ progeny. Because $+/t$ mice (normal tail phenotype) had to be bred to $T/+$ mice to determine their *t* complex genotype, all our $+/t$ mice were reproductively experienced and more than 6 months old.

All mice were weaned when 21 days old and kept as two of the same sex per cage until they reached an established age (1–2, 2–4; 4–6, or 6–9 months) for urine collection or mating.

All mice used as urine donors were housed in plastic cages (12 × 28 × 27 cm), with metal tops. The animals were kept in an air-conditioned room at 21 ± 1°C and 50–70% humidity with a 12-hr light–12-hr dark daily regime. Unlimited amounts of Purina Mouse Chow (Ralston Purina Corp., St. Louis, Missouri) and water were supplied throughout the entire experimental period. Bedding was changed weekly.

Fresh urine was collected by holding a mouse over a glass vial and gently squeezing the abdomen and flanks. Each 1-ml of sample of female urine was collected for six days and pooled from six to eight animals to reduce the individual variation and estrous cycle effect on the urinary volatiles concentration. For each 1-ml sample of male urine, the specimens were collected for two days and pooled from six to eight animals. Thus, each 1-ml sample was a good representative mixture of urine from different animals (six to eight) collected for same period of time (two to six days). Immediately after collection, samples were stored at −20°C and analyzed within 10 days. All urine was analyzed three times by using a new 1-ml sample each time, obtained from three different collections, from the same six to eight animals for a total of 3 ml of urine per *t* haplotype/age/sex. Different mice were used for each age period: 1–2, 2–4, 4–6, and 6–9 months. We used a total of 192 mice as follows: (3 ages) × (3 haplotypes) × (2 sexes) × (8 animals per group) = 144 mice, and (1 age) × (4 haplotypes) × (2 sexes) × (6 animals per group) = 48 mice.

The urinary volatiles were analyzed using the headspace technique, employing Tenax GC (a porous polymer) as the adsorption medium (Novotny et al., 1974). The volatiles were sparged from 1-ml urine samples at room temperature with purified helium gas at a flow rate of 100 ml/min and adsorbed onto a precolumn packed with 4 mg of Tenax GC. The sample was subsequently desorbed in the heated injection port (220–240°C) of a Varian 1400 gas chromatograph equipped with a flame ionization detector, and retrapped into a cooled section of a glass capillary column [60 m × 0.25 mm ID, soda-lime glass

capillary, coated statically with UCON 50-HB-2000 (Schwende et al., 1984a,b)].

While quantitative comparisons were obtained through peak integration routines (Sigma 10 Data System, Perkin-Elmer, Norwalk, Connecticut), identification of the individual profile constituents was established through a combined gas chromatograph–mass spectrometer (Hewlett-Packard 5980 A dodecapole instrument, Palo Alto, California) using electron impact ionization at 70 eV. Identification made through mass spectrometry was further verified through chromatographic retention time of authentic compounds, either purchased or synthesized in the laboratory.

Statistical comparisons of the levels of excreted volatiles were made using the single-factor analysis of variance with the Duncan new multiple-range test and two-factor analysis of variance with the Tukey test for multiple comparison (Zar, 1984).

RESULTS

Preliminary Assessment of Urinary Profiles

Volatiles we identified (Table 1) are characteristic of other laboratory house mouse strains (Andreolini et al., 1987; Jemiolo et al., 1987; Harvey et al., 1989). Certain urinary volatiles of *t* complex mice exhibited strong dependency on age and *t* complex genotype. These compounds are marked in the chromatographic profiles shown on Figures 1 and 2.

The gas chromatograms shown in Figures 1 and 2 are representative of the substance profiles obtained from externally voided urines of the *t*-bearing mice. Figure 1A and B represent the urinary profiles of males, 1–2 months of age, whose genotypes at the *t* complex were *T*/*t* and +/+, respectively, while Figure 1C and D represent the urinary profiles of males, 6–9 months old, whose genotypes were +/+ and +/*t*, respectively. A visual inspection of these chromatograms easily reveals that heights of the individual peaks greatly decrease in the older males, (A, B vs. C, D), and that the urinary profile of *T*/*t* males at the age of 1–2 months contains volatile compounds in higher concentration than +/+ mice at this same age (A vs. B). Urinary profiles presented on Figure 2A and B are representative for +/+ and *T*/+ females, 1–2 months old. Few female urinary profiles varied with age and *t* complex genotype, whereas more male urinary profiles varied with age and *t* complex genotype.

The urinary constituents have been identified by mass spectrometry as cyclic enol esters, acetate esters, various ketones, *o*-toluidine, dehydro-*exo*-brevicomin and 2-(*sec*-butyl)-4,5-dihydrothiazole (Table 1). These constituents are the same as in other *Mus domesticus* strains (Andreolini et al., 1987; Jemiolo et al., 1987; Harvey et al., 1989). Seventeen urinary volatiles occurred in con-

TABLE 1. MAJOR URINARY VOLATILES FROM MALE AND FEMALE MICE BEARING *t* HAPLOTYPES IDENTIFIED BY CAPILLARY GC-MS

Structural type and compound name	Peak number
Cyclic enol ethers	
5,5-Dimethyl-2-ethyl-4,5-dihydrofuran	1*
(*E*)-5,5-Dimethyl-2-ethylenetetrahydrofuran	2*
(*Z*)-5,5-Dimethyl-2-ethylenetetrahydrofuran	4*
para-Xylene (a contaminant)	3***
Acetate esters	
n-Pentyl acetate	6
2-Penten-1-yl acetate	7
Ketones	
2-Hexanone	A**
2-Methyl-3-hexanone	B
2-Heptanone	5* **
5-Hepten-2-one	8
4-Hepten-2-one	9* **
3-Hepten-2-one	10* **
6-Methyl-6-hepten-3-one	11*
6-Methyl-5-hepten-3-one	12*
Acetophenone	15*a
Dehydro-*exo*-brevicomin (dehydrobrevicomin)	13*
2-(*sec*-butyl)-4,5-Dihydrothiazole (thiazoline)	14*a
o-Toluidine	16*a

*Compounds exhibiting genetic and age dependency in male urine;
**compounds exhibiting genetic and age dependency in female urine; a, compounds absent in female urine; A and B, compounds present reliably in female urine only;
***contaminant omitted from analysis.

sistently measurable quantities. Significant differences in concentations were caused by age for 12 male volatiles, by *t* complex genotype for seven male volatiles, by age for three female volatiles, and by *t* complex genotype for three female volatiles.

Male Volatile Constituents

Age. Quantitative comparisons of the selected urinary compounds from males of different ages revealed that the youngest males (1–2 months old) had significantly higher concentrations of urinary volatiles than older males (2–9 months old). This trend occurred in +/+, *T*/+, and *T*/*t* males for the ketones and cyclic enol ethers. After two to four months, there is little change in excretion of these two type of compounds (Table 2). For +/+, *T*/+, and *T*/*t* males,

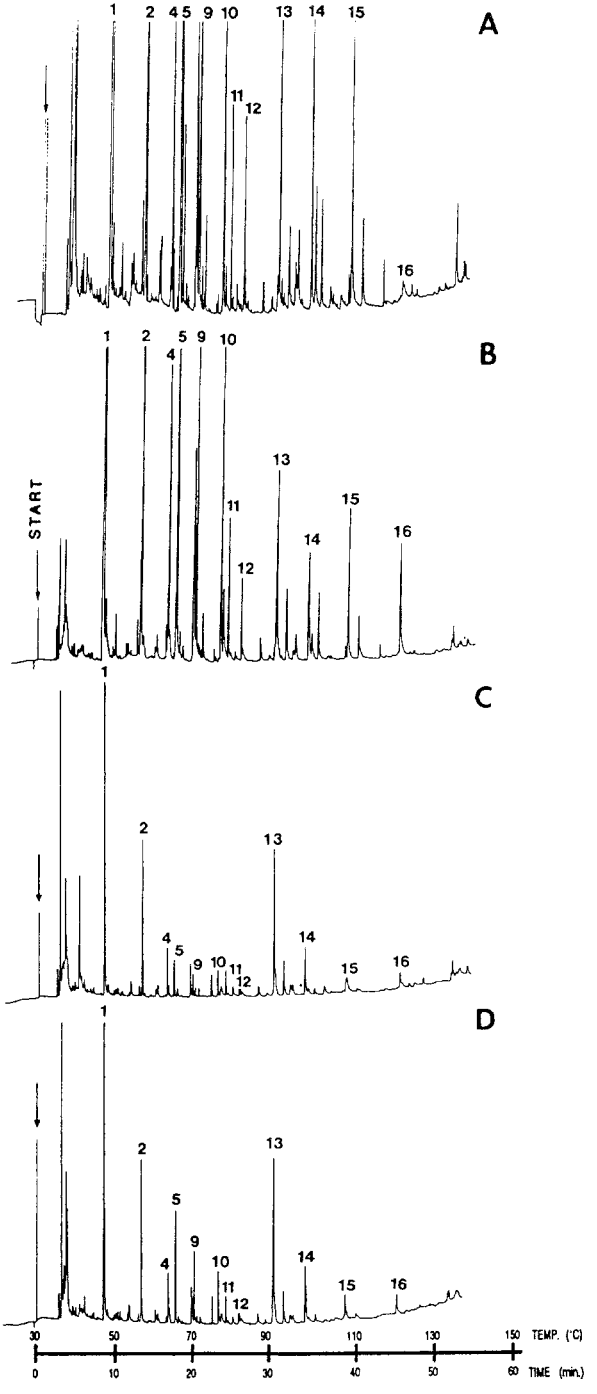


FIG. 1. Gas-chromatographic urinary volatile profiles from (A) 1 to 2-month-old *T/t* males; (B) 1 to 2-month-old +/+ males; (C) +/+ males over 6 months of age; (D) +/*t* males over 6 months of age.

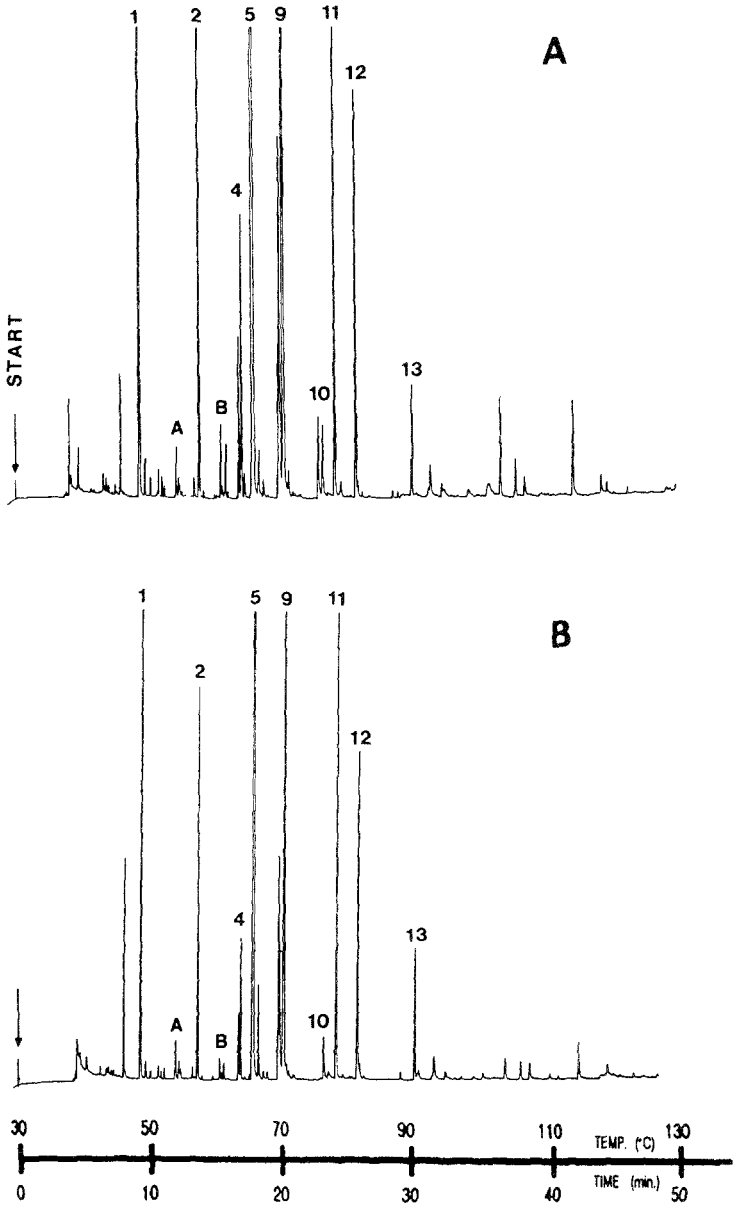


FIG. 2. Gas-chromatographic urinary volatile profiles from (A) 1 to 2-month-old +/+ females and (B) 1 to 2-month-old *T*/+ females.

TABLE 2. MEAN PEAK AREAS (SEM) IN ARBITRARY UNITS FOR URINARY VOLATILES FROM MALE MICE OF DIFFERENT AGES AND *t* COMPLEX GENOTYPES[a]

	Cyclic enol ethers									Ketones								
	Peak 1			Peak 2			Peak 4			Peak 5			Peak 9			Peak 10		
Age of Male [mo.]	+/+	T/+	T/t	+/+	T/+	T/t	+/+	T/+	T/t	+/+	T/+	T/t	+/+	T/+	T/t	+/+	T/+	T/t
1–2	25.73^{a}	28.42^{a}	37.81^{aA}	7.19^{a}	7.44^{a}	9.82^{aA}	2.81^{a}	2.90^{a}	3.35^{aA}	7.10^{a}	5.01^{a}	7.97^{a}	3.11^{a}	3.84^{a}	4.75^{a}	3.62^{a}	3.76^{a}	3.69^{a}
	(0.96)	(1.52)	(1.62)	(0.21)	(0.70)	(0.06)	(0.12)	(0.28)	(0.35)	(0.06)	(0.19)	(0.92)	(0.43)	(0.54)	(1.11)	(0.29)	(0.83)	(0.57)
2–4	5.41	4.92	8.70	1.46	1.21^{b}	2.31^{A}	0.67^{AB}	0.55^{bB}	0.82^{A}	2.35^{b}	1.62	2.19	1.87	0.64	1.42	1.14	0.72	0.88
	(0.72)	(0.55)	(0.42)	(0.21)	(0.07)	(0.12)	(0.13)	(0.05)	(0.09)	(0.29)	(0.08)	(0.22)	(0.42)	(0.04)	(0.22)	(0.12)	(0.08)	(0.07)
4–6	4.32	8.09	7.49	1.23	2.21^{A}	2.12^{A}	0.46	0.84^{A}	0.80^{A}	0.43	1.11	0.81	1.51	1.51	0.82	0.96	1.15	0.95
	(0.22)	(1.14)	(1.21)	(0.05)	(0.32)	(0.36)	(0.01)	(0.07)	(0.09)	(0.02)	(0.09)	(0.09)	(0.31)	(0.22)	(0.08)	(0.09)	(0.07)	(0.15)
6–9	4.13	8.51^{A}	8.98^{A}	1.16	2.33^{A}	2.29^{A}	0.49	0.86^{A}	0.84^{A}	0.41	1.30	1.65	0.43^{b}	1.16	1.27	1.02	0.97	0.96
	(0.03)	(1.14)	(0.63)	(0.01)	(0.21)	(0.29)	(0.02)	(0.06)	(0.17)	(0.01)	(0.18)	(0.57)	(0.02)	(0.22)	(0.06)	(0.06)	(0.22)	(0.18)
Age factor		44.4			107.6			149.6			18.9			11.8			16.8	
$F(df = 3, 24)$		$P < 0.0005$			$P < 0.0005$			$P < 0.0005$			$P < 0.0005$			$P < 0.0005$			$P < 0.0005$	
Genotype factor		10.1			7.4			5.9										
$F(df = 2, 24)$		$P < 0.001$			$P < 0.005$			$P < 0.01$			NS			NS			NS	
Interaction age × genotype					5.5													
$F(df = 6, 24)$		NS			$P < 0.001$			NS			NS			NS			NS	

[a] Effect of age and *t* complex genotype on mean areas of each peak was calculated by two-factor of analysis of variance; multiple comparison was done using the Tukey test at the 0.05 level. Those means in the same vertical column (within each peak) not marked with the same superscript letter (a, b, c, d) are significantly different. Those means in the same horizontal row (within each peak) not marked with the same superscript letter (A, B, C) are significantly different. Where there are no superscript letters in a row or column, there are no significant differences among the means in that row or column.

TABLE 2. Continued

Age of Male [mo.]	Ketones									Dehydrobrevicomin			Thiazoline			Toluidine		
	Peak 11			Peak 12			Peak 15			Peak 13			Peak 14			Peak 16		
	+/+	T/+	T/t	+/+	T/+	T/t	+/+	T/+	T/t	+/+	T/+	T/t	+/+	T/+	T/t	+/+	T/+	T/t
1–2	1.41^{a}	1.10^{a}	1.66^{a}	0.81^{a}	0.98^{a}	1.17^{a}	1.72^{aA}	2.06^{aB}	3.69^{aC}	2.83	3.24^{a}	2.88^{a}	1.46	2.71^{A}	2.78^{aA}	1.31^{aA}	2.78^{aB}	0.80^{abC}
	(0.01)	(0.07)	(0.11)	(0.11)	(0.02)	(0.19)	(0.01)	(0.05)	(0.04)	(0.17)	(0.18)	(0.35)	(0.19)	(0.26)	(0.26)	(0.14)	(0.14)	(0.12)
2–4	0.43	0.51	0.50	0.24	0.24	0.33	0.63	0.94	0.99^{b}	2.60	2.07^{b}	3.14^{aA}	1.31	2.89^{A}	1.77	1.92^{b}	1.51^{b}	1.27^{bc}
	(0.02)	(0.05)	(0.04)	(0.01)	(0.01)	(0.05)	(0.04)	(0.01)	(0.04)	(0.36)	(0.31)	(0.09)	(0.06)	(0.05)	(0.03)	(0.51)	(0.11)	(0.19)
4–6	0.48	0.42	0.35	0.32	0.30	0.23	0.52	0.51^{b}	0.41^{c}	3.51^{a}	4.54^{cA}	3.62^{b}	1.61	2.87^{A}	1.77	1.63^{ab}	2.47^{aA}	1.71^{c}
	(0.16)	(0.03)	(0.01)	(0.05)	(0.03)	(0.02)	(0.04)	(0.13)	(0.02)	(0.17)	(0.33)	(0.35)	(0.13)	(0.25)	(0.19)	(0.24)	(0.18)	(0.25)
6–9	0.40	0.42	0.57	0.41	0.36	0.36	0.02^{bA}	0.93^{B}	1.65^{dC}	2.53	3.95^{cA}	4.31^{bA}	1.07^{A}	2.35^{B}	1.68^{C}	0.66^{c}	0.91^{c}	0.49^{d}
	(0.04)	(0.04)	(0.01)	(0.05)	(0.01)	(0.06)	(0.01)	(0.11)	(0.04)	(0.01)	(0.44)	(0.53)	(0.02)	(0.21)	(0.12)	(0.09)	(0.02)	(0.02)
Age factor	9.3			12.7			57.7			4.6			4.1			9.7		
$F(df = 3, 24)$	$P < 0.0005$			$P < 0.0005$			$P < 0.0005$			$P < 0.02$			$P < 0.02$			$P < 0.0005$		
Genotype factor							16.8			4.6			33.2			7.8		
$F(df = 2, 24)$	NS			NS			$P < 0.0005$			$P < 0.02$			$P < 0.0005$			$P < 0.002$		
Interaction age × genotype							6.9			2.9								
$F(df = 6, 24)$	NS			NS			$P < 0.0005$			$P < 0.05$			NS			NS		

concentrations of cyclic enol ethers (Table 2, peaks 1, 2, 4) decreased by 71–84% and remained at low values throughout the remaining age intervals.

The average concentration of the urinary ketones for +/+, *T*/+, and *T*/*t* older males (Table 2, peaks 5, 9, 10–12, 15), is 61–84%, lower than in 1 to 2-month-old males of the same *t* complex genotypes. Among ketones, only acetophenone (peak 15) from *T*/*t* male urine exhibited a significant increase in concentration for old (6–9 months) animals when compared to 2 to 4- and 4 to 6-month age groups. However, this concentation increase was not significantly greater than the level originally found in the urine of animals 1–2 months old.

As seen in Table 2, peak 13, dehydro-*exo*-brevicomin exhibited a very different trend from the ketones and cyclic enol ethers, as its urinary concentration varies with the age of *t*-bearing animals (significant interation age × *t* complex genotype). The 4 to 6-month-old +/+, *T*/+, and *T*/*t* males showed a significantly higher concentration of dehydro-*exo*-brevicomin (peak 13), than the younger males. Higher levels of dehydro-*exo*-brevicomin were still observed in *T*/+ and *T*/*t* males 6–9 months of age, but dropped significantly in +/+ males. 2-(*sec*-butyl)-4,5-Dihydrothiazole (peak 14) was produced at comparable levels by +/+ and *T*/+ males of all ages. Only the *T*/*t* males exhibited age dependency in peak 14. The highest level of this compound was observed in the urine of animals 1–2 months old. *o*-Toluidine (peak 16) exhibited fluctuations with age for all investigated *t* complex genotypes. The lowest concentration of *o*-toluidine, however, was commonly observed in urine of all *t* complex genotypes at ages 6–9 months (Table 2, peaks 16).

t Complex Genotype. A quantitative comparison of the selected urinary compounds from males of different *t* complex genotypes revealed that certain constituents were excreted in the urine of *T*/+ and *T*/*t* males in significantly higher concentrations than in the urine of +/+ males. As shown in Table 2, peaks 1, 2, 4, the urine of *T*/*t* males at age 1–4 months contained significantly more cyclic enol ethers than the urine of +/+ and *T*/+ males (exception for peak 1 in 2 to 4-month-old *T*/*t* animals, where their concentration was found elevated but not significantly). Furthermore, these same cyclic enol ethers were found at significantly higher concentrations in *T*/*t* and *T*/+ mice of older age (4–9 months) when compared to +/+ males (exception for peak 1 in 4 to 6-month-old *T*/*t* and *T*/+ animals, where their concentration was elevated non-significantly.

A *t* complex genotype influence on concentration of a ketone (peak 15) was evident for the youngest (1–2 months) and oldest (6–9 months) *T*/+ and *T*/*t* males in comparison to +/+ mice of the same age (Table 2, peak 15). In the urine of *T*/+ males of all ages, 2-(*sec*-butyl)-4,5-dihydrothiazole (Table 2, peak 14) exhibited significant increase of concentrations compared to urine of +/+ and *T*/*t* males (exception for 1 to 2-month-old *T*/*t* mice, which exhibited a similar urinary concentration of peak 14 as *T*/+ males). Urinary concentration

of dehydro-*exo*-brevicomin (peak 13) was significantly elevated for *T/t* males at ages 2–4 and 6–9 months and for *T/+* males at age 4–9 months. As shown in Table 2, peak 16, *o*-toluidine exhibited significant increase of concentration in *T/+* males in comparison to *+/+* and *T/t* males, but only in 1 to 2- and 4 to 6-month-old animals.

The changes in concentration of urinary volatiles from *+/+*, *+/t*, *T/+*, and *T/t* males, at the age of 6–9 months, are presented in Table 3. The *t* complex genotypes *T/t* and *T/+* at this age clearly exhibited a greater concentration of most investigated compounds. Statistically significant differences, however, were found for the cyclic enol ethers (peaks 1, 2 and 4), two ketones (peaks 9 and 15), dehydro-*exo*-brevicomin (peak 13), and 2-(*sec*-butyl)-4,5-dihydrothiazole (peak 14) when compared to the genotypes *+/+*, and *+/t*. At 6–9 months, *+/+* males had significantly higher concentration than *+/t* males for a cyclic enol ether (peak 4) and a ketone (peak 10). These significant differences have a potential use in field studies because only *+/+* and *+/t* mice occur in field populations.

Female Volatile Constituents

Age. Age had no influence on most female urinary volatiles. However, three ketones were exceptions (Table 4). *T/+*, *T/t*, and *+/+* females at 2–4 months of age had greater concentrations of 2-hexanone (peak A), 2-heptanone (peak 5) and 4-hepten-2-one (peak 9) than females of older ages.

t Complex Genotype. Quantitative comparisons among different *t* complex genotypes of the same age revealed that 2-heptanone (peak 5) and 4-hepten-2-one (peak 9) were significantly elevated in *T/+* and/or *T/t* females when compared to 1 to 4-month-old *+/+* females. Due to high excretion of volatile compounds by 2 to 4-month-old females, the *T/t* haplotype can easily be distinguished from *T/+* and *+/+* on the basis of the levels of 2-heptanone (peak 5) and 4-hepten-2-one (peak 9), while *+/+* mice appear to differ significantly from *T/+* and *T/t* mice on the basis of 2-hexanone (Table 4, peak A).

At the age of 6–9 months *+/t* females had significantly more 2-hexanone (peak A) and 3-hepten-2-one (peak 10) than *T/+*, *T/t*, and *+/+* females (Table 5). A significantly higher concentration of 4-hepten-2-one (peak 9) was found in the urine of *T/+* and *+/t* females when compared to *+/+* and *T/t* females. No statistically significant haplotype differences were found in the excretion of remaining volatiles by the females at the age of 6–9 months.

DISCUSSION

The urinary volatile constituents obtained from C3H congenic mice with the *t* complex had been found in other strains [C57BL/6J; BALB/cWt; ICR/Alb; C57BL/10; B10(H-2^b); B10.A(H-2^a); B10.BR(H-2^k); B10.RIII(H-2)^r]

TABLE 3. MEAN PEAK AREAS (SEM) IN ARBITRARY UNITS FOR URINARY VOLATILES FROM +/+, *t*/+, *T*/+, AND *T*/*t* MALES AT 6–9 MONTHS OLD[a]

	t Complex genotype			
Peak	+/+	*T*/+	+/*t*	*T*/*t*
1	4.13 (0.03)	8.51 (0.14)^{A}	3.25 (0.60)	8.98 (0.63)^{A}
2	1.16 (0.01)	2.33 (0.29)^{A}	0.89 (0.17)	2.29 (0.29)^{A}
4	0.49 (0.02)^{B}	0.86 (0.06)^{A}	0.13 (0.03)^{C}	0.84 (0.17)^{A}
5	0.41 (0.01)	1.30 (0.18)	0.45 (0.04)	1.65 (0.57)
9	0.43 (0.02)	1.16 (0.22)^{A}	0.61 (0.15)	1.27 (0.06)^{A}
10	1.02 (0.06)	0.97 (0.22)	0.38 (0.08)^{A}	0.96 (0.18)
11	0.40 (0.04)	0.42 (0.04)	0.40 (0.05)	0.57 (0.01)
12	0.41 (0.05)	0.36 (0.01)	0.41 (0.05)	0.36 (0.06)
15	0.02 (0.01)	0.93 (0.11)^{A}	0.03 (0.01)	1.65 (0.44)^{B}
13	2.53 (0.01)	3.95 (0.44)^{A}	2.39 (0.09)	4.31 (0.53)^{A}
14	1.07 (0.02)	2.35 (0.21)^{A}	1.03 (0.14)	1.68 (0.12)^{B}
16	0.66 (0.09)	0.91 (0.02)	0.81 (0.01)	0.49 (0.02)

[a]Effect of *t* complex genotype on mean areas for each peak was calculated by single-factor of analysis of variance; $F(df = 3, 8)$, $P < 0.05$, using Duncan's new multiple-range test for genotype comparison at the 0.02 level. Those means in the same row not marked with the same superscript letter (A, B, C) are significantly different. Where there is no superscript letter in a row there are no significant differences among the means.

(Harvey, 1988; Jemiolo et al., 1987; Andreolini et al., 1987; Schwende et al., 1984a). No C3H congenic mice differing only at the *t* complex have urinary volatiles unique to *t* complex genotypes.

Comparison of the urinary profiles from all *t* complex genotypes leads to the following conclusions: (1) younger males (1–2 months) have higher concentrations of urinary cyclic enol ethers and ketones than older (2–9 months) ones, (2) the known male mouse pheromone (Novotny et al., 1985; Jemiolo et al., 1985, 1986) dehydro-*exo*-brevicomin (peak 13) is in higher concentration in the urine of older (4–9 months) males; (3) males with a *T* allele produce considerably more urinary volatiles than males carrying a *t* haplotype or wild-type + haplotype; (4) 6 to 9-month-old +/+ males can be distinguished from +/*t* males on the basis of differences in cyclic enol ether (peak 4) and 3-hepten-2-one (peak 10) concentration; (5) younger (1–4 months old) +/+, *T*/+, and *T*/*t* females have greater concentrations of three ketones than older females; (6) +/*t* females 6–9 months old have greater concentrations of ketones 2-hexanone (peak A), 4-hepten-2-one (peak 9), and 3-hepten-2-one (peak 10) than +/+ mice.

The preference tests conducted by Egid and Lenington (1985) demonstrated that *t* complex genotype recognition signals were airborne. Males showed

TABLE 4. MEAN PEAK AREAS (SEM) IN ARBITRARY UNITS FOR URINARY VOLATILES FROM FEMALE MICE OF DIFFERENT AGE AND *t* COMPLEX GENOTYPES[a]

Age of male (mo.)	Ketones								
	Peak 5			Peak 9			Peak A		
	+/+	T/+	T/t	+/+	T/+	T/t	+/+	T/+	T/t
1-2	1.90^{A}	4.10^{aB}	3.10^{aC}	1.20	3.60^{aA}	1.00	0.14^{a}	0.09	0.09
	(0.15)	(0.72)	(0.35)	(0.11)	(0.52)	(0.25)	(0.01)	(0.01)	(0.01)
2-4	3.51^{a}	3.70^{a}	6.50^{bA}	2.34^{a}	2.43^{b}	3.66^{aA}	0.52^{bA}	0.43^{aB}	0.22^{aC}
	(0.40)	(0.12)	(0.10)	(0.06)	(0.18)	(0.54)	(0.03)	(0.03)	(0.07)
4-6	1.91	1.82	2.00^{c}	1.01	1.20	1.59	0.24^{cA}	0.14	0.10
	(0.34)	(0.32)	(0.42)	(0.21)	(0.16)	(0.47)	(0.01)	(0.02)	(0.03)
6-9	1.43	1.55	0.90^{d}	0.82	1.83^{A}	0.77	0.06^{d}	0.15^{A}	0.08
	(0.66)	(0.09)	(0.14)	(0.48)	(0.28)	(0.27)	(0.03)	(0.06)	(0.05)
Age factor	27.1			5.8			24.1		
$F(df = 3, 24)$	$P < 0.0005$			$P < 0.005$			$P < 0.0005$		
Genotype factor	3.9			3.0			6.0		
$F(df = 2, 24)$	$P < 0.05$			$P < 0.05$			$P < 0.01$		
Interaction age × genotype $F(df = 6, 24)$	4.7 $P < 0.0025$			NS			NS		

[a] Effect of age and *t* complex genotype on mean areas of each peak was calculated by two-factor of analysis of variance; multiple comparison was done using the Tukey test at the 0.05 level. Those means in the same vertical column (within each peak) not marked with the same superscript letter (a, b, c, d) are significantly different. Those means in the same horizontal row (within each peak) not marked with the same superscript letter (A, B, C) are significantly different. Where there is no superscript letter in a row or column, there are no significant differences among the means in that row or column.

strong preference for the odors of +/+ females over +/*t*. However, when males were tested with the odor or recombinant females (differing at the *t* complex but carrying the same *H-2* haplotype), males had no preference (Egid and Lenington, 1985). In contrast, females were able to discriminate +/+ from +/*t* recombinant males on the basis of *t* complex genotype alone.

A lack of unique *t* complex compounds responsible for the haplotype discrimination supports the concept of a nonspecific influence of *t* complex on olfactory cues. Significant olfactory differences between +/+ and +/*t* mice may well be due to a variation in the overall pattern of general secondary metabolites rather than differences in metabolic production of specific components. Results presented in this paper have shown that the T/+ and T/*t* males differed in volatile profiles from +/+ and +/*t* males. As well +/+ and +/*t* males had significantly different concentration for two compounds, and females for three compounds.

TABLE 5. MEAN PEAK AREAS (SEM) IN ARBITRARY UNITS FOR URINARY VOLATILES FROM +/+, +/*t*, *T*/+, AND *T*/*t* FEMALES AT 6–9 MONTHS OLD[a]

	t Complex genotype			
Peak	+/+	*T*/+	+/*t*	*T*/*t*
1	2.77 (0.62)	3.50 (0.78)	2.50 (1.45)	3.68 (0.07)
2	0.82 (0.18)	1.10 (0.22)	0.96 (0.01)	1.04 (0.05)
4	0.30 (0.06)	0.40 (0.08)	0.30 (0.09)	0.40 (0.04)
A	0.06 (0.02)	0.15 (0.06)^{A}	0.29 (0.06)^{B}	0.08 (0.05)
B	0.30 (0.16)	0.10 (0.02)	0.30 (0.02)	0.10 (0.01)
5	1.43 (0.66)	1.55 (0.09)	1.65 (0.29)	0.90 (0.14)
9	0.82 (0.48)	1.83 (0.28)^{A}	1.64 (0.18)^{A}	0.77 (0.27)
10	0.22 (0.04)	0.10 (0.03)	0.64 (0.15)^{A}	0.24 (0.03)
11	1.01 (0.24)	0.73 (0.36)	0.70 (0.22)	1.08 (0.19)
12	0.63 (0.21)	0.39 (0.13)	0.50 (0.21)	1.01 (0.37)
13	0.49 (0.05)	0.46 (0.39)	0.63 (0.09)	0.73 (0.19)

[a]Effect of *t* complex genotype on mean areas for each peak was calculated by single-factor of analysis of variance; $F(df = 3, 8)$, $P < 0.05$, using Duncan's new multiple-range test for genotype comparison at the 0.02 level. Those means in the same row not marked with the same superscript letter (A, B) are significantly different. Where there are no superscript letters in a row, there are no significant differences among the means.

Furthermore, most significant differences occurred among younger mice. Future studies might find more significant differences in concentations of urinary volatiles of +/+ and +/*t* mice when mice younger than 6 months old are tested. The lack of a significant interaction between haplotype and age for most volatiles means that mice of unknown ages, such as trapped in the field, might be accurately assessed for *t*-complex genotype (+/+ or +/*t*) using gas chromatography of urine.

Acknowledgments—We would like to express our thanks to Phillip Chapman and James zum Brunnen for helpful comments and statistical advice during preparation of our manuscript.

REFERENCES

ANDREOLINI, F., JEMIOLO, B., and NOVOTNY, M. 1987. Dynamics of excretion of urinary chemosignals in the house mouse (*Mus musculus*) during the natural estrous cycle. *Experientia* 43:998–1002.

BENNETT, D. 1975. The *T*-locus of the mouse. *Cell* 6:444–454.

BENNETT, D. 1978. Population genetics of *T*/*t* complex mutation, pp. 615–632, *in* Origins of Inbred Mice, H. Morse (ed.). Academic Press, New York.

BRUCK, D. 1957. Male segregation ratio advantage as a factor in maintaining lethal alleles in wild populations of house mice. *Proc. Natl. Acad. Sci. U.S.A.* 43:152–158.

DRICKAMER, L.C., and LENINGTON, S. 1987. *T*-locus effect on the male urinary chemosignal that accelerates puberty in female mice. *Anim. Behav.* 35:1581–1582.

EGID, K., and LENINGTON, S., 1985. Responses of male mice to odor of females: Effects of *T*- and *H-2*-locus genotype. *Behav. Gen.* 15:287–295.

HARVEY, S. 1988. Chemical investigations of the pheromone system of the house mouse. PhD thesis. Indiana University, Bloomington.

HARVEY, S., JEMIOLO, B., and NOVOTNY, M. 1989. Pattern of volatile compounds in dominant and subordinate male mouse urine. *J. Chem. Ecol.* 15:2061–2072.

JEMIOLO, B., ALBERTS, J., SOCHINSKI-WIGGINS, S., HARVEY, S., and NOVOTNY, M. 1985. Behavioral and endocrine responses of female mice to synthetic analogues of volatile compounds in male urine. *Anim. Behav.* 33:1114–1118.

JEMIOLO, B., HARVEY, S., and NOVOTNY, M. 1986. Promotion of the Whitten effect in female mice by synthetic analogs of male urinary constituents. *Proc. Natl. Acad. Sci. U.S.A.* 83:4576–4579.

JEMIOLO, B., ANDREOLINI, F., WIESLER, D., and NOVOTNY, M. 1987. Variation in mouse (*Mus musculus*) urinary volatiles during different periods of pregnancy and lactation. *J. Chem. Ecol.* 13:1941–1956.

KLEIN, J. 1975. Biology of the Mouse Histocompatibility-2 Complex. Springer Verlag, Berlin. pp. 251–274.

LENINGTON, S. 1983. Social preference for partners carrying "good genes" in wild house mice. *Anim. Behav.* 31:325–333.

LENINGTON, S., EGID, K., and WILLIAMS, J. 1988a. Analysis of a genetic recognition system in house mice. *Behav. Genet.* 18:549–564.

LENINGTON, S., FRANKS, P., and WILLIAMS, J. 1988b. Distribution of *t*-haplotypes in nautral populations of wild house mice. J. Mammal. 69:489–499.

NOVOTNY, M., LEE, M.L., and BARTLE, K.D. 1974. Some analytical aspects of the chromatographic headspace concentration method using a porous polymer. *Chromatographia* 7:333–338.

NOVOTNY, M., HARVEY, S., JEMIOLO, B., and ALBERTS, J. 1985. Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl. Acad. Sci. U.S.A.* 82:2059–2061.

NOVOTNY, M., JEMIOLO, B., and HARVEY, S. 1991. Chemistry of rodent pheromones: Molecular insights into chemical signaling in mammals, *in* D.M. Macdonald (ed.). Chemical Signals in Vertebrates, V. Oxford University Press, New York. In press.

SCHWENDE, F.J., JORGENSON, J.W., and NOVOTNY, M. 1984a. A possible chemical basis for histocompatibility-related mating preference in mice. *J. Chem. Ecol.* 11:1603–1615.

SCHWENDE, F.J., WIESLER, D., and NOVOTNY, M. 1984b. Volatile compounds associated with estrus in the mouse: Potential pheromones. *Experientia* 40:213–215.

SILVER, L.M. 1985. Mouse *t* haplotypes. *Annu. Rev. Genet.* 19:179–208.

YAMAZAKI, K., YAMAGUCHI, M., ANDREWS, P.W., PEAKE, B., and BOYSE, E.A. 1978. Mating preference of F_2 segregants of crosses between MHC-congenic mouse strains. *Immunogenetics* 6:253–259.

YAMAZAKI, K., YAMAGUCHI, M., BARANOSKI, L., BARD, J., BOYSE, E.A., and THOMAS, L. 1979. Recognition among mice: evidence from the use of a Y-maze differentially scented by congenic mice of different major histocompatibility types. *J. Exp. Med.* 150:755–760.

ZAR, J.H. 1984. Biostatistical Analysis. Prentice-Hall, New York.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

INFLUENCE OF PHENOLIC ACIDS ON MICROBIAL POPULATIONS IN THE RHIZOSPHERE OF CUCUMBER[1]

STEVEN R. SHAFER[2,*] and UDO BLUM[3]

[2]*United States Department of Agriculture, Agricultural Research Service and Departments of Plant Pathology and Soil Science*

[3]*Department of Botany*
North Carolina State University
Raleigh, North Carolina 27695-7616

(Received June 27, 1990; accepted October 1, 1990)

Abstract—Experiments were conducted to determine whether changes in soil microbial populations that occur in response to additions of certain allelopathic phenolic acids to bulk soil also occur in the rhizosphere. Cucumber seedlings were transplanted into cups containing a nutrient-enriched mixture of Portsmouth B_1 soil and sand and were watered five times (once every 48 hr) with aqueous solutions of ferulic, *p*-coumaric, or vanillic acid (each at 0, 0.25, or 0.50 μmol/g soil material). Nutrient solution was applied on alternate days. Leaf growth was suppressed by up to 42% by phenolic acids, but changes in root growth varied with the compound and concentration in solution. Significant increases (over 600% relative to controls) in populations of fast-growing bacteria in the rhizosphere were detected after two but not after five treatments, and increases (400% relative to controls) in numbers of fungal propagules were detected after five treatments. Such increases suggested that chronic exposure to a phenolic acid might result in high populations of rhizosphere microorganisms that could metabolize the compounds and thus alter observable responses by the plant. To test this, plants were watered repeatedly with a low-concentration solution of ferulic acid (chronic treatments; 0.0 or 0.1 μmol/g soil material in one experiment, 0.000 or 0.025 μmol/g soil material in a second) and then once with a high-concentration solution (acute treatment; 0.0, 0.5, or 1.0 μmol/g soil material in the first experiment; 0.000, 0.125, or 0.250 μmol/g soil material in the second).

*To whom correspondence should be addressed.

[1]Paper No. 12385 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, North Carolina. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service or the United States Department of Agriculture of the products named, nor criticism of similar ones not mentioned.

Acute treatments and some chronic treatments suppressed leaf growth, but results were inconsistent for root growth. Acute treatments increased numbers of several types of bacteria in the rhizosphere but had inconsistent effects on fungi. Chronic treatments had no effect on numbers of bacteria or fungal propagules in the rhizosphere. Furthermore, chronic treatments did not alter responses of plants or microbial populations to the subsequent acute treatment. Results demonstrated that phenolic acids in soil, which must pass through the rhizosphere before interaction with plant roots can occur, alter the microbial ecology of the rhizosphere. However, microbially mediated acclimation of plants to relatively high concentrations of ferulic acid was not observed.

Key Words—Allelopathy, ferulic acid, *p*-courmaric acid, vanillic acid, *Cucumis sativus*, bacteria, fungi.

INTRODUCTION

Plant growth and function in nature can be influenced by allelopathic interactions (Rice, 1984; Thompson, 1985; Putnam and Tang, 1986). The intensity of allelopathic activity by different chemicals varies with the compounds involved, concentrations available for uptake, and plant sensitivity to the compounds.

Derivatives of benzoic and cinnamic acids with allelopathic properties are released during the decomposition of plant debris (Flaig, 1964; Turner and Rice, 1975; Martin and Haider, 1976). Ferulic acid is a cinnamic acid derivative with allelopathic properties (Rice, 1984) that suppresses photosynthetic rate (Patterson, 1981), changes osmotic pressure and closes stomata (Einhellig et al., 1985), modifies root function such that water utilization and ion uptake are suppressed (Balke, 1985; Blum and Dalton, 1985; Blum et al., 1985), and generally inhibits leaf expansion, root elongation, and biomass accumulation (Blum and Rebbeck, 1989; Einhellig and Eckrich, 1984; Rice, 1984). Other phenolic acids identified in soil, such as *p*-coumaric acid and vanillic acid, also may exhibit allelopathic activities similar to those described for ferulic acid (Blum et al., 1985; Rice, 1984).

Soilborne microorganisms may modify allelopathic interactions among plants. Some microorganisms produce herbicidal compounds that can affect plant growth and function (Cutler, 1986). Others utilize phenolic acids as carbon sources and deplete the chemicals from the soil solution (Blum et al., 1987; Vaughan et al., 1983). Some microorganisms catabolize ferulic acid and produce compounds such as caffeic, protocatechuic, and vanillic acids (Evans, 1963; Flaig, 1964; Dagley, 1971; Turner and Rice, 1975; Martin and Haider, 1976) that also exhibit allelopathic characteristics under appropriate conditions (Wang et al., 1967; del Moral and Muller, 1970; Blum et al., 1985). Conversely, phenolic acids in soil can alter the indigenous microbial community.

Ferulic, *p*-hydroxybenzoic, vanillic, or caffeic acid added to soil from cropped fields stimulated soil respiration, microbial biomass, and amylase activity (Sparling et al., 1981). Applications of ferulic, *p*-coumaric, *p*-hydroxybenzoic, or vanillic acid to nonsterile soil induced numerous changes among population densities of bacteria, fungi, and actinomycetes, and applications of ferulic acid stimulated specific populations of bacteria (Blum and Shafer, 1988). Clearly, nonbiological properties of soil are important in mediating the activity of allelopathic compounds (Blum et al., 1987; Dalton et al., 1983). However, certain aspects of plant response to allelopathic phenolic acids depend upon the microbiological characteristics of the soil and must be investigated with nonsterile soil (Schmidt, 1988).

Microbiological characteristics of the rhizosphere are usually quite different from the bulk soil and are very important to plant development (Curl and Truelove, 1986). Allelopathic compounds exude from roots into the rhizosphere, leach from leaves, or leach from plant debris (Hoagland and Williams, 1985). Such compounds in the soil solution must pass through the rhizosphere before uptake by plants can occur. Interactions that involve allelopathic chemicals in soil are probably mediated by rhizosphere microorganisms (Hoagland and Williams, 1985). Indeed, a *Pseudomonas* sp. and *Syncephalastrum racemosum* Cohen ex J. Schrot., a bacterium and fungus, respectively, isolated from field soil reduced the toxic effects of ferulic, *p*-coumaric, and *p*-hydroxybenzoic acids on wheat (*Triticum aestivum* L.) in the laboratory (Vaughan et al., 1983). Another pseudomonad, capable of degrading the allelotoxin juglone, was isolated from soil under *Juglans nigra* L. (Schmidt, 1988). Conversely, a bacterium found in the seed carpels of *Sida spinosa* L. modified ferulic acid into 2-methoxy-4-ethylphenol, a styrene derivative that was more phytotoxic than ferulic acid (Liebl and Worsham, 1983). Studies on allelotoxins in nonsterile soils are required to evaluate further the importance of allelopathic compounds in plant–plant interactions (Schmidt, 1988; Williamson and Weidenhamer, 1990).

The experiments described here were designed to examine the influence of phenolic acids on certain aspects of the ecology of the rhizosphere and were based on results of prior studies. Previous experiments demonstrated that population densities of phenolic-acid-metabolizing microorganisms could be stimulated by repeated applications of the compounds (Blum and Shafer, 1988). Therefore, the first experiment described here was conducted to determine whether several phenolic acids cause such effects in the rhizosphere, in which microbial populations are already strongly influenced by physical and chemical conditions caused by the root.

In the second experiment, the mediating effect of rhizosphere microorganisms on plant response to ferulic acid was examined. Responses of cucumber plants (*Cucumis sativus* L.) to ferulic acid have been quantified in several other

investigations (e.g., Blum and Dalton, 1985; Blum and Rebbeck, 1989; Blum et al., 1984, 1985, 1987). In solution culture, chronic concentrations of ferulic acid did not affect plant growth responses to the subsequent occurrence of a high concentration (Blum and Dalton, 1985). However, a typical rhizosphere cannot develop in a frequently changed solution. If rhizosphere populations of ferulic-acid-metabolizing microorganisms could be stimulated by chronic exposure to ferulic acid, plant response to a high concentration of the compound might be weaker than expected because microorganisms near the root could decrease the availability of ferulic acid. This possibility was examined in experiments described here, in which plant measurements were collected to confirm that effects of the phenolic acids on plant growth were consistent with earlier studies and, therefore, that effects on the rhizosphere occurred in relation to typical plant responses.

METHODS AND MATERIALS

General Procedure. All experiments were conducted with nonsterile Portsmouth B_1-horizon soil mixed with quartz sand (1 soil:2 sand), adjusted to pH 5.2 with $Ca(OH)_2$ (to maximize availability of the phenolic acids yet maintain the plants), and enriched with $Ca(H_2PO_4)_2 \cdot H_2O$, $KHCO_3$, $ZnSO_4 \cdot 7H_2O$, and $MnCl_2 \cdot 4H_2O$ to support plant growth (Blum et al., 1987). Microbiological properties of this modified soil, hereafter designed "soil material," were quantified (Blum and Shafer, 1988). Polyethylene cups (150 ml capacity) each were filled with 150 g of soil material. Germination of cucumber seeds (cv Early Green Cluster), transplanting of the seedlings, and culture of the plants under light banks in a laboratory were conducted according to procedures developed in earlier experiments (Blum et al., 1987). Periodic nondestructive measurements of total leaf area per plant (Blum and Dalton, 1985) were collected to assess effects of treatments on plant growth.

All phenolic acids were applied in filter-sterilized, aqueous solutions adjusted to pH 5.2 with NaOH. All control treatments were sterile distilled water adjusted to pH 5.2. On all days on which no phenolic acid or control treatment was applied, plants were watered with the nutrient solution described above.

Microbial populations in the rhizosphere, cumulative length of primary plus secondary roots, and dry weight of the entire root system were quantified for each plant. The root system of each plant was dipped briefly into 250 ml of sterile deionized water to dislodge large aggregates of soil material. The entire root system was adhering soil material was transferred to a 99-ml blank of sterile diluent (0.1% aqueous agar) and shaken for 30 min on a wrist-action shaker. The resulting suspension of rhizosphere soil was diluted and assayed

by the plate-dilution frequency technique on agar media in Petri plates (Harris and Sommers, 1968). The roots were recovered from the diluent, and the cumulative length of primary + secondary roots was measured (Blum and Rebbeck, 1989). Fragments of fine roots in the diluent bottle were recovered on a sieve, and all roots were dried for 48 hr at 70°C and weighed. Microbial populations were quantified after plates had been incubated at 30°C in the dark for six days. Media and procedures used for quantification of microbial populations in these studies have been described (Blum and Shafer, 1988; Shafer, 1988). Media included a bacteriological mineral salts–glucose agar (BMS) for bacteria; a modified bacteriological nutrient agar (BNA) for "total" and phosphatase-positive bacteria; a modified nutrient agar for gram-negative bacteria (GNB); and a medium (FGI) for fungi modified from BMS. A ferulic acid medium (FAM) was modified from BMS by adjustment to pH 5.5 and substitution of glucose with ferulic acid (0.5 mmol/liter) to select for bacteria that could use ferulic acid as a carbon source. Fast-growing bacteria, which were defined as those that produced colonies at least 1 mm in diameter after six days, were quantified on FAM. Fast-growing bacteria also were quantified in addition to the total recovered on BMS and BNA. Microbial populations were expressed as the logarithm of the number of colony-forming units per gram of root ($\log_{10}$ CFU/g), and conclusions concerning statistical significance of treatment effects were based on analyses conducted with the log-transformed data. However, for ease in interpretation, all data sets that include statistically significant differences are presented as graphs of arithmetic values (i.e., CFU/g).

Effects of Different Phenolic Acids on Rhizosphere Populations and Plant Growth (Experiment 1). Cucumber seeds were sown on day 0, and seedlings were transplanted into cups of soil material on day 4. Treatments were initiated on day 7. Each cup received up to 0.5 ml of a solution of ferulic, *p*-coumaric, or vanillic acid (Sigma Chemical Company, St. Louis, Missouri) so that the total applied was equivalent to 0, 0.25, or 0.50 μmol/g soil material. Water then was added to bring the total application to 25 ml. Each solution was applied to eight seedlings, and seedlings were arranged in a randomized complete block design. Treatment applications were repeated on day 9, and four blocks per treatment (28 seedlings total) were harvested and assayed on day 10 with media BMS, GNB, and FGI. The remaining seedlings (28 total) received additional treatments on days 11, 13, and 15, and leaf areas were measured before each treatment. These plants were harvested and assayed on day 16 with BNA and the three media used for the day-10 assays. Final leaf area, primary + secondary root length, root dry weight, and microbiological data were analyzed by analysis of variance (ANOVA) for each date as a randomized complete block design with four blocks. An ANOVA also was conducted with day-16 leaf area data. For each phenolic acid, orthogonal contrasts (SAS Institute, 1988) compared populations associated with the 0.25 μmol/g treatment versus the 0.50

μmol/g treatment. Significant differences between the control treatment versus each ferulic, *p*-coumaric, or vanillic acid treatment were detected with Dunnett's procedure (Steel and Torrie, 1980). Significance was accepted at $P < 0.05$ in all statistical analyses.

Effect of Repeated Low-Concentration Applications of Ferulic Acid on Rhizosphere Populations and Plant Growth Responses to Subsequent High-Concentration Application (Experiments 2A and 2B). Cucumber seeds were sown on day 0, and seedlings were transplanted into cups of soil material on day 4. Repeated low-concentration applications (hereafter designated "chronic treatments") were initiated on day 7. On days 7, 9, 11, and 13, each cup received 0.1 ml of distilled water or ferulic acid solution to provide 0 or 0.1 μmol, respectively, of ferulic acid per gram of soil material. Water then was added to bring the total application to 25 ml. On day 15, each cup received a high-concentration application (hereafter designated "acute treatment") of up to 1 ml of solution so that the total applied was equivalent to 0, 0.5, or 1.0 μmol ferulic acid per gram of soil material (experiment 2A). Water again was added to bring the total application to 25 ml. This factorial treatment design (2 chronic levels × 3 acute levels) was replicated with six cups per combination arranged in a completely randomized design. Leaf area per plant was measured on days 15, 16, and 17. Plants were harvested on day 17 for root length and dry weight measurements and quantification of microbial populations on all media. The experiment was designed to test (by ANOVA) the significance of the chronic × acute interaction, which, if significant, would indicate that chronic treatments modified responses of the plant–rhizosphere system to an acute treatment. In the absence of a significant interaction, independent effects of acute and chronic treatments could be identified. Analyses of leaf area data were conducted for each date.

A similar experiment was conducted at a later date (experiment 2B) with chronic treatments at 0 or 0.025 μmol/g of soil material and acute treatment at 0, 0.125, or 0.250 μmol/g of soil material. Because the experiments involved different application concentrations, ANOVAs were conducted separately for each experiment.

RESULTS

Effects of Different Phenolic Acids on Rhizosphere Populations. Final leaf area per plant was suppressed significantly by each of the three phenolic acids (Figure 1). By day 16, leaf area per plant was suppressed by 37, 42, or 37% after five applications of ferulic, vanillic, or *p*-coumaric acid, respectively (0.50 μmol/g treatments). Differences between 0.25 and 0.50 μmol/g treatments for each phenolic acid were not significant. These results confirmed that the com-

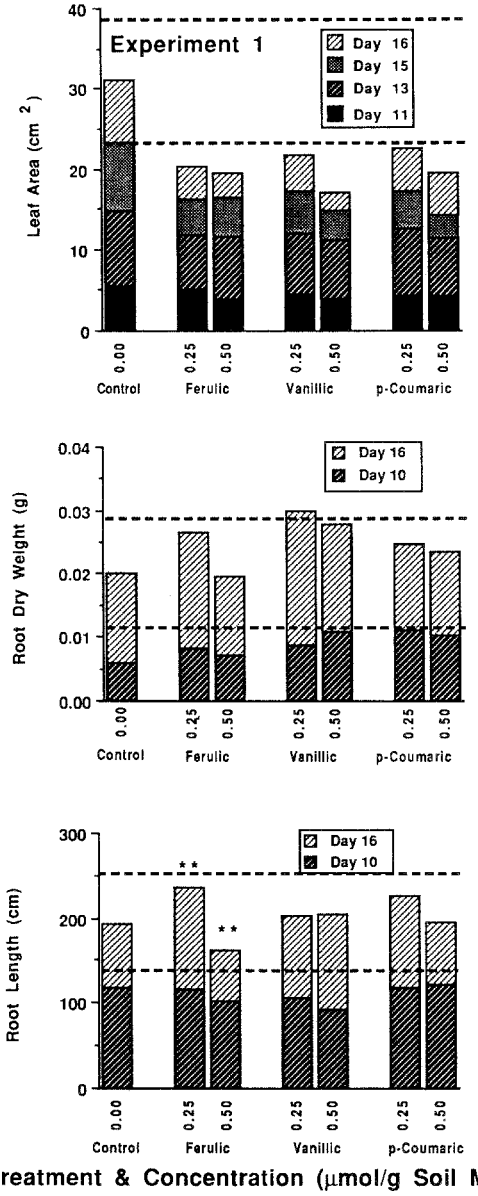


FIG. 1. Effect of multiple additions of phenolic acids to Portsmouth B_1 soil–sand mix on cucumber seedlings. Seeds were germinated on day 0, seedlings were transplanted into the soil-sand mix on day 4, and treatments were applied on days 7, 9, 11, 13, and 15. The horizontal dashed lines indicate the range of values that are not significantly different from the control on day 16 (according to Dunnett's procedure, $P < 0.05$). Means represented by columns marked with ** are significantly different from each other on day 16 (according to a single-degree-of-freedom contrast, $P < 0.01$).

pounds elicited the previously described suppression of foliar growth (Blum et al., 1985). Effects on root growth were less clear (Figure 1). By day 10, cumulative length of primary and secondary roots and dry weight of the entire root systems were not significantly affected by two applications of ferulic, *p*-coumaric, or vanillic acid. By day 16, total root length of plants treated with five applications of 0.50 μmol/g of ferulic acid was significantly less (32%) than that of plants treated with 0.25 μmol/g of ferulic acid. Final root dry weight of plants treated with 0.25 μmol/g vanillic acid was significantly greater (50%) than that of controls, but the 0.50 μmol/g treatment did not have this effect. Applications of *p*-coumaric acid did not affect root growth by day 16.

Only ferulic acid affected any microbial population in the rhizosphere by day 10 (after two applications). Numbers of fast-growing bacteria recovered on BMS from the rhizospheres of plants treated twice with 0.50 μmol ferulic acid per gram of soil material averaged approximately seven times that associated with controls (Figure 2). Fungal populations were not changed by any treatment relative to controls.

By day 16, bacterial populations after five treatments with phenolic acids were not different from populations associated with controls. Although numbers of fungal propagules after treatments with either concentration of *p*-coumaric acid were not significantly different from that of controls, the 0.50-μmol treatments resulted in numbers of fungal propagules that were significantly greater (nearly 10×) than those in the rhizosphere of plants that received the 0.25-μmol treatments (Figure 2).

Effect of Repeated Low-Concentration Applications of Ferulic Acid on Rhizosphere Populations and Plant Growth Responses to Subsequent High-Concentration Application. In experiment 2A, the chronic × acute interaction for leaf area was not significant for any day. However, repeated chronic treatments of 0.1 μmol ferulic acid per gram of soil material were sufficient to suppress leaf area significantly relative to controls by day 15, and this effect persisted through day 17 (Table 1). The suppressive effect of an acute treatment of 0.5 or 1.0 μmol/g on day 15 was not significant until day 17 (Table 1). Leaf expansion halted after day 16 for plants that received the 1.0 μmol/g acute treatment regardless of chronic treatment (Figure 3). In experiment 2B, the chronic × acute interaction again was not significant for leaf area (Table 1). Chronic treatments had no effect on leaf area on any day, but acute treatments applied on day 15 significantly suppressed leaf area on days 16 and 17 (Figure 3, Table 1).

The chronic × acute interaction was not significant for root length in either experiment. In experiment 2A, length of primary + secondary roots was stimulated by chronic treatments but was not significantly affected by acute treatments (Figure 4, Table 1). In experiment 2B, acute treatments (mainly the

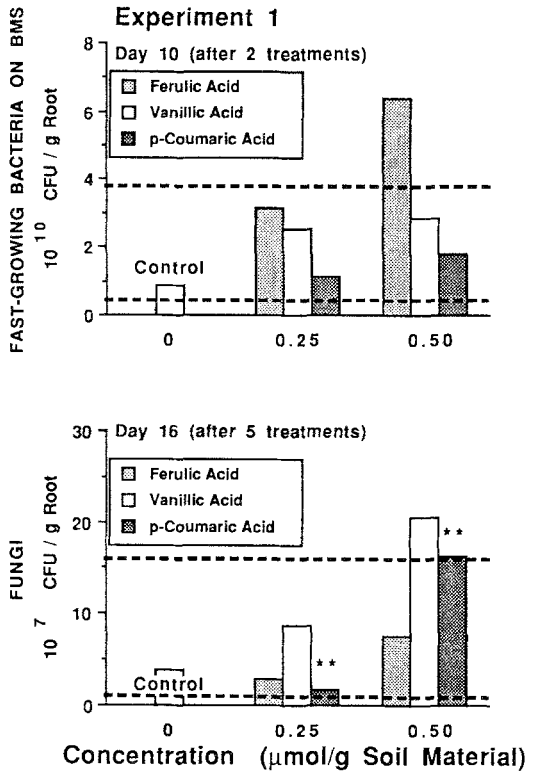


FIG. 2. Effect of multiple additions of phenolic acids to Portsmouth B_1 soil–sand mix on population densities of microorganisms in the rhizosphere of cucumber seedlings. Legend for Figure 1 details planting and treatment schedules. Data are shown only for populations that exhibited statistically significant differences. The horizontal dashed lines indicate the range of values that are not significantly different from the control on day 16 (according to Dunnett's procedure, $P < 0.05$). These ranges do not appear to be symmetrical about the control means because statistical calculations were performed with $\log_{10}$-transformed data, and arithmetic values are graphed. Means represented by columns marked with ** are significantly different from each other (according to a single-degree-of-freedom contrast, $P < 0.01$).

0.250 μmol/g application) suppressed root elongation, but effects of chronic treatments were not significant (Figure 4, Table 1).

Root dry weights provided the clearest indication of treatment effects on roots. The chronic × acute interaction was not significant for root dry weight in either experiment (Table 1). Chronic treatments significantly stimulated root dry weight in experiment 2A, and a similar (but not significant) trend occurred

TABLE 1. SUMS OF SQUARES VALUES FROM ANALYSES OF VARIANCE FOR EFFECTS OF CHRONIC AND ACUTE APPLICATIONS OF FERULIC ACID ON CUCUMBER PLANTS AND RHIZOSPHERE POPULATIONS[a]

			Plants						Rhizosphere microorganisms[e]							
									Media							
			Leaf area[b]													
			Days after germination				Roots		BNA			BMS		GNB	FAM	FGI
Experiment	Source of variation	Degrees of freedom	13	15	16	17	Length (primary and secondary)[c]	Total dry weight[d]	Total	Fast-growing	Phosphatase-positive	Total	Fast-growing	Gram-negative	Fast-growing	Total
2A	Total	35	ND[g]	2538.72	3387.67	5306.67	70556	12510	3.7630	5.0364	4.9214	3.4569	4.5618	6.0767	5.4564	7.1641
	Chronic	1	ND	1396.68**	2181.50**	1369.70**	17733**	2834**	0.0033	0.0587	0.0087	0.0043	0.0009	0.1267	0.0116	0.0335
	Acute	2	ND	12.68	99.70	2413.38**	8547	3282**	1.3012**	2.9031**	2.2654**	0.4698	1.7564**	3.4102**	2.9960**	0.7776
	C × A[f]	2	ND	12.68	38.61	195.52	1879	142	0.0396	0.0254	0.0712	0.1890	0.1065	0.1352	0.0084	0.6390
	Error	30	ND	1116.68	1067.86	1328.08	42397	6252	2.4188	2.0491	2.5761	2.9737	2.6981	2.4046	2.4403	5.7143
2B	Total	35	759.64	2032.15	3486.00	7469.31	39658	18508	2.8122	3.7510	5.5117	2.8402	4.5866	4.9495	4.7385	2.8062
	Chronic	1	0.48	29.23	107.11	34.13	2264	147	0.0012	0.0122	0.0001	0.0163	0.0009	0.2934	0.1389	0.0045
	Acute	2	13.75	34.07	833.64*	4263.30**	12140**	8247**	0.4601	1.0138**	2.3964**	0.3639	2.6368**	2.1729**	1.8880**	0.5930*
	C × A[f]	2	52.58	58.21	93.91	174.89	3436	2	0.1108	0.1021	0.1298	0.2198	0.0077	0.0948	0.0550	0.0167
	Error	30	692.83	1910.64	2451.34	2996.99	21818	10112	2.2400	2.6229	2.9855	2.2403	1.9411	2.3883	2.6566	2.1920

[a] Symbol * or ** indicates that the probability of obtaining a greater F value is less than 0.05 or 0.01, respectively.
[b] Measured in cm^2/plant.
[c] Measured in cm/plant.
[d] Measured in g/plant. Tabulated values have been multiplied by 10^7.
[e] Analyzed as $\log_{10}$ colony-forming units per g of root (dry weight). See Methods and Materials for specification of populations quantified on each medium.
[f] Chronic × acute interaction term.
[g] Data not determined.

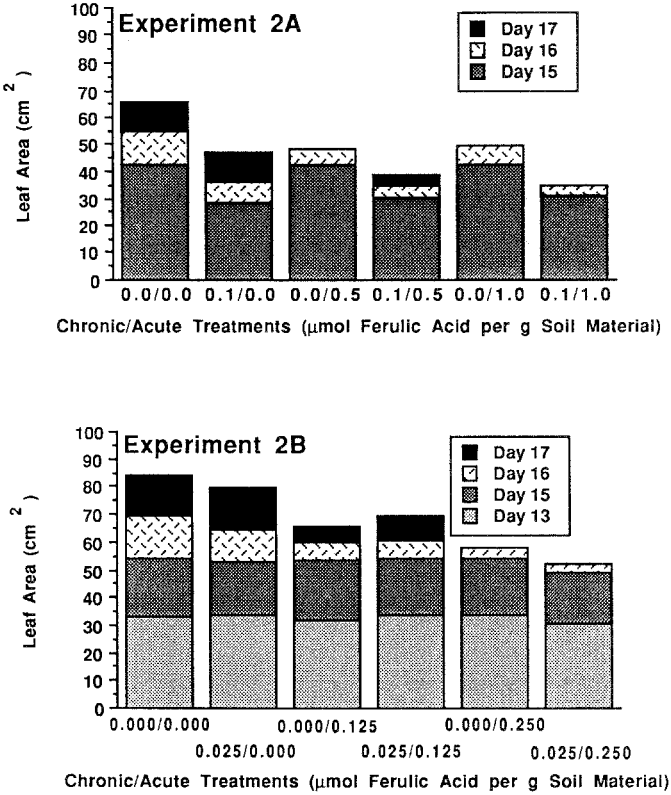


FIG. 3. Effect of chronic and acute applications of ferulic acid to Portsmouth B_1 soil–sand mix on leaf area of cucumber seedlings. Seeds were germinated on day 0, seedlings were transplanted into the soil–sand mix on day 4, and chronic treatments were applied on days 7, 9, 11, and 13. An acute treatment was applied on day 15. Experiments 2A and 2B were conducted at different times.

with the lower-concentration chronic treatments in experiment 2B (Figure 5, Table 1). In both experiments, acute treatments suppressed root dry weight (Figure 5, Table 1).

Populations of microorganisms in the rhizosphere were significantly altered by acute treatments of ferulic acid. However, neither the chronic treatments nor the chronic × acute interaction had significant effects on populations in either experiment 2A or 2B (Table 1). Therefore, effects of the acute treatments were averaged over all chronic treatments and indicated that numbers of most types of bacteria were increased by the acute treatments (Figure 6). For example, populations of fast-growing bacteria recovered on FAM from the rhizosphere of plants that had received the highest acute treatment in either experiment were

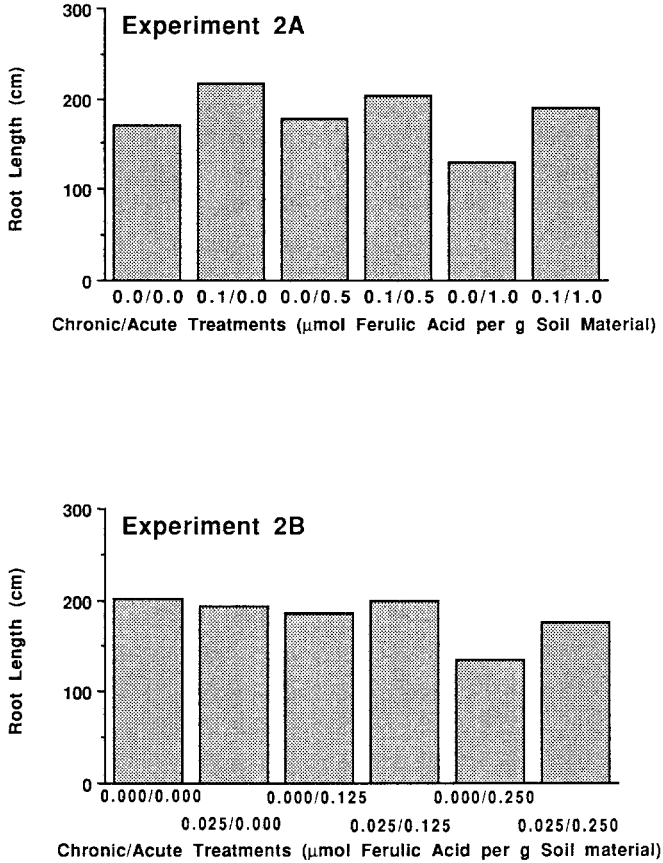


FIG. 4. Effect of chronic and acute applications of ferulic acid to Portsmouth B_1 soil–sand mix on final length of primary + secondary roots of cucumber seedlings. Legend for Figure 3 details planting and treatment schedules.

four to five times the populations in the rhizosphere of control plants. However, increases caused by acute treatments for "total" populations of bacteria (quantified on BNA and BMS) in the rhizosphere were significant only with BNA in experiment 2A.

Changes in different populations relative to each other were induced by the acute treatments and are illustrated by proportional changes averaged over chronic treatments (Table 2). In both experiments, ferulic acid treatments had no significant effect on the "total" bacterial that were isolated from the rhizosphere on BMS medium, yet the fast-growing isolates on this medium significantly increased with acute treatments. Among control plants, fast-growing isolates represented approximately 20% of the population recovered on BMS from

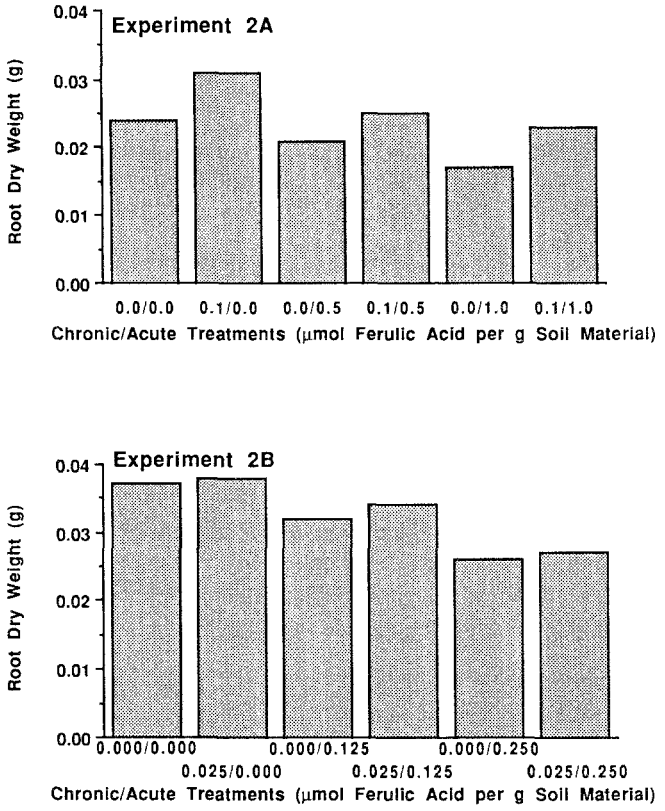


FIG. 5. Effect of chronic and acute applications of ferulic acid to Portsmouth B_1 soil–sand mix on root biomass of cucumber seedlings. Legend for Figure 3 details planting and treatment schedules.

control plants (averaged across experiments and chronic treatments). However, fast-growing isolates increased to approximately 45% of the population recovered on this same medium after plants had received the highest acute treatments. The highest acute treatments in both experiments more than doubled the proportion represented by fast-growing bacteria on FAM relative to the total numbers recovered on BMS (in which glucose was the carbon source). Similarly, the highest acute treatments increased the average proportion represented by gram-negative bacteria from 22% (from control plants) to 44% of the total numbers recovered on BNA. Acute treatments increased the total recovered on BNA only in experiment 2A. However, numbers of fast-growing bacteria and phosphatase-positive bacteria recovered on BNA were increased two to five times by acute treatments compared to controls in both experiments. Although

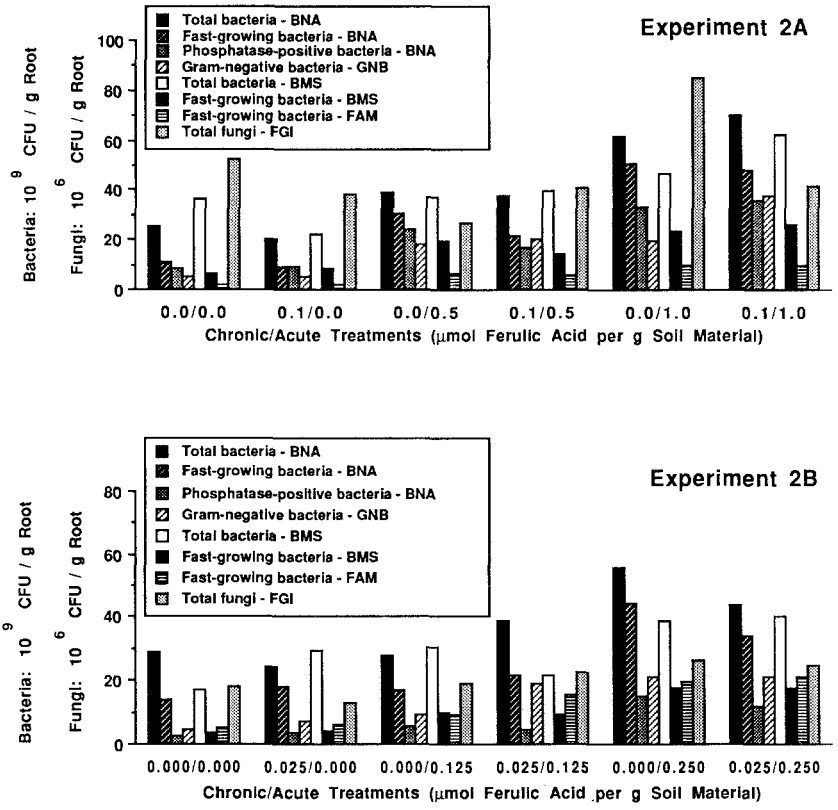


FIG. 6. Effect of chronic and acute applications of ferulic acid to Portsmouth B_1 soil–sand mix on population densities of bacteria and fungi in the rhizosphere of cucumber seedlings. Legend for Figure 3 details planting and treatment schedules. Populations recovered on different media (see Methods and Materials for media abbreviations) are shown.

ferulic acid treatments tended to increase numbers of fungal propagules, increases were significant only in experiment 2B.

DISCUSSION

Phenolic acids occur in all higher plants and often have allelopathic properties when released into the environment. Once in the soil, the compounds modify the microbial ecology of the soil as demonstrated with experiments reported here for the rhizosphere and earlier (Blum and Shafer, 1988) for the bulk soil. Thus, responses of plants to phenolic acids in soil may be determined

TABLE 2. INFLUENCE OF ACUTE APPLICATION OF FERULIC ACID ON POPULATION RATIOS OF MICROORGANISMS IN THE RHIZOSPHERE OF CUCUMBER[a]

Population ratio[b]	Acute treatment (μmol/g)					
	Experiment 2A			Experiment 2B		
	0.0	0.5	1.0	0.000	0.125	0.250
FAM – Fast / BMS – Total	0.07	0.16	0.17	0.24	0.47	0.51
BMS – Fast / BMS – Total	0.25	0.44	0.45	0.17	0.36	0.44
GNB – Total / BNA – Total	0.23	0.50	0.44	0.22	0.43	0.43
BNA – Fast / BNA – Total	0.44	0.68	0.75	0.61	0.58	0.79
BNA – Ph / BNA – Total	0.38	0.53	0.53	0.12	0.16	0.27
FGI – Total / BNA – Total	0.0020	0.0009	0.0010	0.00058	0.00063	0.00051

[a] All values averaged over chronic treatments, which were not significant.

[b] Based on recovery on different media (see Methods and Materials): FAM = bacteriological ferulic acid medium; BMS = bacteriological mineral salts medium; GNB = nutrient agar selective for gram-negative bacteria; BNA = bacteriological nutrient agar modified for identification of phosphatase-positive bacteria among the total recovered; FGI = medium for fungi. Fast = bacteria that produced colonies >1 mm diam.; Ph = phosphatase-positive bacteria.

in part by prior responses of microorganisms and changes in the rhizosphere environment.

Controlled experiments with allelochemicals require selection of concentrations for study, and selecting such concentrations is difficult. Actual uptake of allelotoxins from the soil solution determines plant response. Data on concentrations of the compounds in the soil are not necessarily representative of plant uptake. Unfortunately, no data on plant uptake of allelotoxins from field soil are available, so treatments presently used in studies of allelopathy can only reflect information from the soil. Treatments may be based on concentrations of a particular compound (e.g., ferulic acid) or an entire class of compounds (e.g., phenolic acids) extracted from field soil. In the latter case, a single compound at times may be used to represent the entire class. Such approaches clearly present problems. Hypothetically, a large quantity of an allelotoxin may be available in soil over time, but the concentration measured in the soil solution may be low if high rates of input are balanced by equivalent losses. For exam-

ple, concentrations of ferulic, vanillic, and *p*-coumaric acid in soil in a no-till field [rotation of wheat and soybean, *Glycine max* (L.) Merr.] exhibited only minor fluctuations during the first $3\frac{1}{2}$ months of the growing season, suggesting similar rates of input and loss (Blum, unpublished data). Treatments also can be based on inputs to soil, but input estimates are based on litter pools and are crude at best. For example, as much as 600 g wheat straw/m^2 can accumulate in agricultural fields (Greb et al., 1967, 1974). Wheat, barley (*Hordeum vulgare* L.), and oat (*Avena sativa* L.) straw contain 0.3% and 0.21% *p*-coumaryl and ferulyl, respectively (Thelander, 1985), most of which is bound to hemicellulose and cellulose in cell walls. Therefore, the potential inputs of *p*-coumaric and ferulic acids in an agricultural field can be estimated at 1800 and 1260 mg/m^2, respectively, although these estimates are conservative because plant residues involve leaf and root tissue in addition to stems. Based on these input estimates, equivalent inputs to the soil cups used in the present experiments would be 34 μg *p*-coumaric acid/g soil and 24 μg ferulic acid/g soil, respectively. These values exceed the chronic treatment applications of ferulic acid used in the first chronic/acute experiment (0.1 μmol/g soil material = 19.4 μg/g soil material) and in the second (0.025 μmol/g soil material = 4.9 μg/g soil material).

Phenolic acids are depleted rapidly from soil due to microbial utilization of the compounds under favorable environmental conditions in laboratory studies (Blum et al., 1987; Blum and Shafer, 1988), so the compounds were added to the system periodically to maintain desired levels. The sequence of experiments described here allowed examination of responses of the plant–soil system to successively smaller concentrations of ferulic acid, based on the supposition that concentrations in soil are low and relatively stable.

Although plant responses to phenolic acids have been studied earlier, foliar area data were collected in the present experiments to confirm that the compounds had typical effects on the plants (Blum et al., 1987), and rhizosphere responses are interpreted in that context. Experiment 1 was a test of several phenolic acids that had induced substantial changes in microbial populations in bulk soil (Blum and Shafer, 1988), but repeated applications of these compounds at concentrations previously used caused few obvious or detectable changes in the rhizosphere. Phenolic acids stimulated some microbial populations in the rhizosphere in experiment 1, but numbers were variable. Thus, apparent increases in mean populations that were often not significantly different (e.g., suggested by trends in Figure 2) could have achieved significance with increased replication. Nevertheless, significant increases in fast-growing bacteria on BMS in the rhizosphere occurred after only two applications of ferulic acid, and these increases were followed by a stabilization of the population to a level similar to that of controls. This result is consistent with changes that occurred with bulk soil (Blum and Shafer, 1988). Furthermore, increases

in numbers of fungal propagules occurred after increases in bacterial populations in these studies, as in the earlier bulk-soil studies (Blum and Shafer, 1988). However, other aspects of fungal responses were inconsistent in comparison of rhizosphere and bulk-soil studies. In bulk soil (Blum and Shafer, 1988), numbers of fungal propagules were increased by ferulic acid but not by *p*-coumaric acid, but the reverse occurred in the rhizosphere in experiment 1. The root presents a major disruptive influence on the soil as a source of carbon, other nutrients, and chemicals and of physical forces (Curl and Truelove, 1986). Although leaf expansion was inhibited by the compounds in the expected manner, the influence of the root apparently obscured the effects of even high concentrations of phenolic acids applied to the soil.

In two subsequent experiments, the repeated application of a low-concentration solution of ferulic acid did not modify growth responses of leaves or roots to the one-time application of a high-concentration solution. The significant suppression of leaf growth by the combination of a no-chronic (water)/acute treatment in each experiment again verified that the plant–soil system functioned typically with respect to that type of application. These observations supported prior experiments in which chronic exposure of plants to ferulic acid in a nonsterile solution culture system did not influence growth response to a later acute exposure (Blum and Dalton, 1985). In the first chronic/acute experiment, leaf growth was suppressed by both chronic and acute treatments, although effects were independent. In the second chronic/acute experiment, when treatment concentrations were lowered further, chronic treatments did not affect leaf growth, but the suppressive effect of acute treatments on the leaves was still evident. An improved understanding of plant responses to these allelotoxins under field conditions may require development of continuous low-input systems to replace the use of repeated applications that maintain availability of the compounds but also cause fluctuations (Blum and Shafer, 1988; Blum et al., 1987).

As anticipated, acute treatments suppressed root growth. However, the stimulation of root growth (final length and dry weight) that was observed for chronic treatments in the first chronic/acute experiment and suggested ($P < 0.09$, despite the low concentration) in the second was unexpected. In another experiment (Blum and Rebbeck, 1989), ferulic acid treatments caused a consistent suppression of elongation of primary + secondary roots. Furthermore, root growth improved rapidly after ferulic acid was removed from solution culture. However, that study was conducted with plants grown in a nonsterile, aerated nutrient solution that was completely replaced at 48-h intervals. Such liquid culture conditions do not permit establishment of the physical, chemical, and microbial gradients typical of the rhizosphere. The more variable effect of phenolic acids on root elongation in soil versus solution culture probably reflects major differences in the root environment, including differences in mobility of

the compounds, between the two methods. Although root growth responses to ferulic acid cannot be generalized yet, chronic exposure of roots to ferulic acid in the field may elicit plant responses that would not be expected from studies conducted with highly fluctuating concentrations.

The increase of bacterial populations in the rhizosphere induced by ferulic acid in both chronic/acute experiments was not uniform across all populations assayed, as reflected by significant changes in some populations but not in others. Applications of ferulic acid stimulated the proportion of bacteria in the rhizosphere that grew quickly on a medium amended with ferulic acid as the major source of carbon. This is consistent with results from bulk soil. When Portsmouth B_1 bulk soil material was treated with a plant nutrient solution only (Blum and Shafer, 1988), 45% of all bacterial isolates tested were classified as ferulic-acid metabolizers. When bulk soil material was treated with nutrient solution plus ferulic acid, ferulic-acid-metabolizing bacteria increased to 92% of all isolates tested. In the present study, nutrients were incorporated into the soil and periodically added to sustain plant growth, so mineral nutrients were provided for microorganisms as well. Recovery of fast-growing bacteria on FAM from control plants in the first or second chronic/acute experiment was only 7% or 24%, respectively, of the total recovered on BMS. Although the reason for the difference in this proportion between the first and second chronic/acute experiments is unclear, acute ferulic acid treatments approximately doubled the proportion in each experiment. These results suggest that ferulic-acid-utilizing bacteria represent a smaller proportion of the total community in the rhizosphere than in bulk soil.

Ferulic acid (chronic or acute) failed to change significantly the numbers of fungal propagules in the rhizosphere in the first chronic/acute experiment. Different species composition of the fungal communities in the rhizosphere versus bulk soil could explain differences in changes observed for ferulic versus *p*-coumaric acid, but fungi recovered in the assays were not identified. Although acute treatments had no effect on numbers of fungal propagules in the first chronic/acute experiment, acute treatments increased fungi in the second. This inconsistency remains unexplained, unless the significance for fungi in the second chronic/acute experiment occurred by chance alone ($P < 0.05$).

The chronic/acute experiments were intended in part to test the hypothesis that small quantities of ferulic acid added to the soil solution would increase microbial populations that could metabolize the compound and therefore lessen the impact of a subsequent high concentration on the plants. Increases in rhizosphere populations in response to chronic applications, which would have provided evidence for this mechanism, did not occur. Plant responses in the first chronic/acute experiment support the assumption that ferulic acid solution applied in chronic treatments to the soil surface was available in the rhizosphere. In the second, however, neither plants nor microorganisms reponded to

the very low concentrations applied in chronic treatments, so the presence of the compound in the rhizosphere was not confirmed in that case. Acute treatments caused changes of plant growth and rhizosphere populations in both chronic/acute experiments independent of chronic treatments, so the potential for acclimation of the plant-rhizosphere system to phenolic acids remains uncertain.

Effects of allelopathic compounds on rhizosphere microorganisms in these experiments were detected in several broadly defined types of microorganisms. Pathogens in the rhizosphere that infect roots cause diseases that can have major impact on plant growth, development, and mortality. However, noninfectious rhizosphere microorganisms also influence plant growth and function through competition for nutrients, production of toxins, stimulation or suppression of parasites, release of growth regulators, or improvement of nutrient relations (Curl and Truelove, 1986). One example of an effect of ferulic acid on a potentially growth-promoting population occurred in the chronic/acute experiments, when acute treatments increased populations of bacteria that release phosphate from organic complexes. Responses of plants to allelopathic compounds in nonsterile systems probably represent a balance of direct effects on the plant, indirect effects caused by microbial populations that have been suppressed, and indirect effects caused by other populations that have been stimulated. Such interactions are extremely complex but should be carefully studied for a thorough understanding of the importance of allelopathic substances in the field.

In summary, these experiments demonstrated that allelopathic phenolic acids in soil after rhizosphere ecology in terms of both plant response (root growth) and changes in population densities of bacteria and fungi in adjacent soil. Some changes in bacterial populations in the rhizosphere were consistent with changes induced by phenolic acids in earlier studies with bulk soil, but population responses of fungi to specific phenolic acids in the rhizosphere were different from responses in bulk soil (Blum and Shafer, 1988). The physical and chemical influences of the root itself may explain the differences in effects on bulk soil versus the rhizosphere. A major increase in microbial populations associated with roots was rejected as an explanation of the failure of chronic concentrations of ferulic acid to acclimate cucumber plants to subsequent high concentrations in liquid culture (Blum and Dalton, 1985) or soil. The magnitude of microbial mediation of plant responses to allelopathic compounds in nonsterile systems may remain uncertain until techniques can be developed to simulate a balance of concentrations, inputs, and sustained availability typical of the compounds in field soils.

Acknowledgments—We thank Lisa Sutphin for technical assistance and Susan Spruill for advice on statistical analyses.

REFERENCES

BALKE, N.E. 1985. Effects of allelochemicals on mineral uptake and associated physiological processes, pp. 161–173, *in* A.C. Thompson (ed.). The Chemistry of Allelopathy. American Chemical Society Monograph Series 268. American Chemical Society, Washington, D.C.

BLUM, U., and DALTON, B.R. 1985. Effects of ferulic acid, an allelopathic compound, on leaf expansion of cucumber seedlings grown in nutrient culture. *J. Chem. Ecol.* 11:279–301.

BLUM, U., and REBBECK, J. 1989. Inhibition and recovery of cucumber roots given multiple treatments of ferulic acid in nutrient culture. *J. Chem. Ecol.* 15:917–928.

BLUM, U., and SHAFER, S.R. 1988. Microbial populations and phenolic acids in soil. *Soil Biol. Biochem.* 20:793–800.

BLUM, U., DALTON, B.R., and RAWLINGS, J.O. 1984. Effects of ferulic acid and some of its microbial metabolic products on radicle growth of cucumber. *J. Chem. Ecol.* 10:1169–1191.

BLUM, U., DALTON, B.R., and SHANN, J.R. 1985. Effects of various mixtures of ferulic acid and some of its microbial metabolic products on cucumber leaf expansion and dry matter in nutrient culture. *J. Chem. Ecol.* 11:619–641.

BLUM, U., WEED, S.B., and DALTON, B.R. 1987. Influence of various soil factors on the effects of ferulic acid on leaf expansion of cucumber seedlings. *Plant Soil* 98:111–130.

CURL, E.A., and TRUELOVE, B. 1986. The Rhizosphere. Springer-Verlag, Berlin.

CUTLER, H.G. 1986. Isolating, characterizing, and screening mycotoxins for herbicidal activity, pp. 147–170, *in* A.R. Putnam and C.-S. Tang (eds.). Science of Allelopathy, Wiley-Interscience, New York.

DAGLEY, S. 1971. Catabolism of aromatic compounds by microorganisms, pp. 1–46, *in* A.H. Rose and J.F. Wilkinson (eds.). Advances in Microbial Physiology, Vol. 6. Academic Press, London.

DALTON, B.R., BLUM, U., and WEED, S.B. 1983. Allelopathic substances in ecosystems: Effectiveness of sterile soil components in altering recovery of ferulic acid. *J. Chem. Ecol.* 9:1185–1201.

DEL MORAL, R., and MULLER, C.H. 1970. The allelopathic effects of *Eucalyptus camaldulensis*. *Am. Midl. Nat.* 83:254–282.

EINHELLIG, F.A., and ECKRICH, P.C. 1984. Interactions of temperature and ferulic acid stress on grain sorghum and soybeans. *J. Chem. Ecol.* 10:161–169.

EINHELLIG, F.A., STILLE MUTH, M., and SCHON, M.K. 1985. Effects of allelochemicals on plant-water relationships, pp. 179–196, *in* A.C. Thompson (ed.). The Chemistry of Allelopathy. American Chemical Society Monograph Series 268. American Chemical Society, Washington, D.C.

EVANS, W.C. 1963. The microbial degradation of aromatic compounds. *J. Gen. Microbiol.* 32:177–185.

FLAIG, W. 1964. Effects of microorganisms in the transformation of lignin to humic substances. *Geochem. Cosmochim.* 28:1523–1533.

GREB, B.W., BLACK, A.L., and SMIKA, D.E. 1967. Effect of straw mulch rates on soil water storage during summer fallow in the Great Plains. *Soil Sci. Soc. Am. Proc.* 31:556–559.

GREB, B.W., BLACK, A.L., and SMIKA, D.E. 1974. Straw buildup in soil with stubble mulch fallow in the semiarid Great Plains. *Soil. Sci. Soc. Am. Proc.* 38:135–136.

HARRIS, R.F., and SOMMERS, L.E. 1968. Plate-dilution frequency technique for assay of microbial ecology. *Appl. Microbiol.* 16:330–334.

HOAGLAND, R.E., and WILLIAMS, R.D. 1985. The influence of secondary plant compounds on the association of soil microorganisms and plant roots, pp. 301–325, *in* A.C. Thompson (ed.). The Chemistry of Allelopathy. American Chemical Society Monograph Series 268. American Chemical Society, Washington, D.C.

LIEBL, R.A., and WORSHAM, A.D. 1983. Inhibition of pitted morning glory (*Ipomoea lacunosa* L.) and certain other weed species by phytotoxic components of wheat (*Triticum aestivum* L.) straw. *J. Chem. Ecol.* 9:1027–1043.

MARTIN, J.P., and HAIDER, K. 1976. Decomposition of specifically carbon-14-labeled ferulic acid: Free and linked into humic acid-type polymers. *Soil Sci. Soc. Am. J.* 40:377–380.

PATTERSON, D.T. 1981. Effects of allelopathic chemicals on growth and physiological responses of soybean (*Glycine max*). *Weed Sci.* 29:53–59.

PUTNAM, A.R., and TANG, C.-S. 1986. Science of Allelopathy. Wiley-Interscience, New York.

RICE, E.L. 1984. Allelopathy. Academic Press, Orlando.

SAS INSTITUTE. 1988. SAS/STAT User's Guide: Release 6.03 Edition. SAS Institute Inc., Cary, North Carolina.

SCHMIDT, S.K. 1988. Degradation of juglone by soil bacteria. *J. Chem. Ecol.* 14:1561–1571.

SHAFER, S.R. 1988. Influence of ozone and simulated acidic rain on microorganisms in the rhizosphere of *Sorghum*. *Environ. Pollut.* 51:131–152.

SPARLING, G.P., ORD, B.G., and VAUGHAN, D. 1981. Changes in microbial biomass and activity in soils amended with phenolic acids. *Soil Biol. Biochem.* 13:455–460.

STEEL, R.G.D., and TORRIE, J.H. 1980. Principles and Procedures of Statistics, 2nd ed. McGraw-Hill, New York. 633 pp.

THELANDER, O. 1985. Review of straw carbohydrate research, pp. 217–230, *in* R.D. Hill and L. Munck (eds.). New Approaches to Research on Cereal Carbohydrates, Progress in Biotechnology, Vol. 1. Elsevier, New York.

THOMPSON, A.C. 1985. The Chemistry of Allelopathy: Biochemical Interactions among Plants. American Chemical Society Monograph Series 268. American Chemical Society, Washington, D.C.

TURNER, J.A., and RICE, E.L. 1975. Microbial decomposition of ferulic acid in soil. *J. Chem. Ecol.* 1:41–58.

VAUGHAN, D., SPARLING, G.P., and ORD, B.G. 1983. Amelioration of the phytotoxicity of phenolic acids by some soil microbes. *Soil Biol. Biochem.* 15:613–614.

WANG, T.S.C., YANG, T., and CHANG, T. 1967. Some phenolic acids as plant growth inhibitors. *Soil Sci.* 103:239–246.

WILLIAMSON, G.B., and WEIDENHAMER, J.D. 1990. Bacterial degradation of juglone: Evidence against allelopathy? *J. Chem. Ecol.* 16:1739–1741.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

RESPONSES OF TWO HYMENOPTERAN PREDATORS TO SURFACE CHEMISTRY OF THEIR PREY: SIGNIFICANCE FOR AN ALKALOID-SEQUESTERING CATERPILLAR

C. B. MONTLLOR,* E. A. BERNAYS,[1] and M. L. CORNELIUS

Division of Biological Control
University of California
Berkeley, CA 94720

(Received July 17, 1990; accepted October 1, 1990)

Abstract—Larvae of *Uresiphita reversalis* (Guenée) (Lepidoptera: Pyralidae) sequester quinolizidine alkaloids from their leguminous hosts and store them primarily in the cuticle. Stored alkaloids are lost with the last larval molt. Extracts of late-instar larvae and of pupae were applied to larvae of the potato tuber moth, *Phthorimaea operculella* (Zeller) (Gelichiidae), which are normally palatable to two hymenopteran predators, the Argentine ant, *Iridomyrmex humilis* (Mayr) (Formicidae), and the paper wasp, *Mischocyttarus flavitarsus* (Sauss.) (Vespidae). Larvae of *P. operculella* treated with alkaloid extracts of *U. reversalis* larval exuviae, or with surface extracts of whole larvae, were deterrent to both predators, compared to untreated prey. Extracts of pupal exuviae added to *P. operculella*, however, were not deterrent. *P. operculella* larvae treated with the authentic alkaloids sparteine and cytisine were also deterrent to these hymenopteran predators. Storage of small but concentrated amounts of plant secondary compounds in the cuticle appears to be an efficacious means of defense against at least two common predators of lepidopteran larvae.

Key Words—Lepidoptera, Hymenoptera, predation, sequestration, chemical defense, cuticle.

INTRODUCTION

Many Lepidoptera have evolved mechanisms that serve defensive functions against vertebrate and invertebrate predators. Examples of chemical defense are

*To whom correspondence should be addressed.
[1]Present address: Department of Entomology, University of Arizona, Tucson, Arizona 85721.

0098-0331/91/0200-0391$06.50/0

well known (Blum, 1981; Brower, 1984; Witz, 1990), including defense derived by sequestration of host-plant secondary compounds, reinforced by aposematic characteristics of larvae or adults. Of 35 studies on chemical defense of Lepidoptera appearing in the literature in the last 20 years (Witz, 1990), only seven specifically dealt with invertebrate predators, in spite of the fact that invertebrates have been implicated as major mortality factors in lepidopteran populations in natural and experimental systems (e.g., Dempster, 1984; Feeny et al., 1985; Jones, 1987; Shaw et al., 1987; Steward et al., 1988; Watt, 1989) and also may have indirect negative effects on larval fitness (Stamp and Bowers, 1988). Consequently, invertebrate predators potentially exert a large selection pressure on lepidopteran populations.

It is assumed that aposematism evolved in response to vertebrate predators such as birds because they are visual hunters capable of learning to avoid distasteful prey. In addition, aposematic Lepidoptera often store plant compounds that are known vertebrate toxins, such as cardenolides (e.g., Brower, 1984), alkaloids (e.g., Rothschild et al., 1979; Kelly et al., 1987; Boppré, 1990; Montllor et al., 1990), cyanogens (e.g., Jones et al., 1962; Nahrstedt and Davis, 1983; Franzl et al., 1988), or the more unusual azoxyglycosides (Bowers and Larin, 1989). As a result of the association between aposematism and vertebrate predation, the importance of invertebrate (arthropod) predation on aposematic species is seldom studied. Although the responses of many invertebrate predators may not be affected by visual aposematic characteristics, it is possible that chemical protection of asposematic species may be effective against invertebrates as well as vertebrates (e.g., Berenbaum and Miliczky, 1984; Bowers and Larin, 1989). Moreover, invertebrate predators can learn to avoid chemically defended prey. For example, an insect predator (Mantidae) of the aposematic milkweed bug, *Oncopeltus fasciatus*, learned to avoid palatable *O. fasciatus* after encountering cardenolide-containing conspecifics (Berenbaum and Miliczky, 1984); whether aposematism facilitated learning in this case was not clear.

Larvae of *Uresiphita reversalis* have typically aposematic features. They are brown with white, black, and yellow patches laterally on each segment, feed conspicuously on terminal branches of their hosts (Bernays and Montllor, 1989), and sequester quinolizidine alkaloids (QAs) from *Genista monspessulana* (Papilionaceae), a major host plant in California (Montllor et al., 1990). Although the larvae excrete most of the QAs ingested, about 1% is retained and deposited in the larval cuticle, which contains about 2.5% dry weight QAs in the last larval instar (Montllor et al., 1990). Two hymenopteran predators, the Argentine ant, *Iridomyrmex humilis*, and the paper wasp *Mischocyttarus flavitarsus*, have been shown previously to strongly prefer other, more palatable, lepidopteran larvae over *U. reversalis* in choice tests (Bernays, 1988; Bernays and Cornelius, 1989). Because observations indicated that these two predators rejected *U. reversalis* larvae after only superficial contact with them, we thought

that these predators might be responding to chemicals in the larval cuticle. Specifically, we sought to determine whether the alkaloids that *U. reversalis* sequesters from its host plant and deposits in the larval cuticle might influence the behavior of invertebrate predators, apart from the other characteristics of these larvae (e.g., hairness and conspicuous coloration). We performed several experiments to test the response of ants and wasps to alkaloids and alkaloid extracts from *U. reversalis* on the surface of otherwise palatable prey, larvae of *Phthorimaea operculella*.

METHODS AND MATERIALS

Prey and Predators. Late-instar larvae of *P. operculella*, reared on potatoes in an insectary, were used as palatable prey. These larvae could be treated with various *U. reversalis* extracts and alkaloid solutions, in order to determine whether such treatments affected their acceptance to the Argentine ant *I. humilis*, and the paper wasp *M. flavitarsus*. *U. reversalis* were reared in an insectary on cut branches of French broom, *G. monspessulana*.

Treatments of Experimental Prey. Alkaloidal extracts (to be added to experimental prey) were made from dried material in methanol, taken up in HCl, basified, and extracted with chloroform or dichloromethane as previously described (Montllor et al., 1990), yielding alkaloids in the free base form. Three types of alkaloidal extracts were made from *U. reversalis* larvae. The first was an extract of cast exuviae (total of 112 mg dry weight) from approx. 90 last-instar larvae. The dried extract was dissolved in 400 μl of methanol, and 10 μl was added to each of 20 *P. operculella* larvae recently frozen on Dry Ice. This gave an extract equivalent of approx. two *U. reversalis* exuviae per test larva (assuming 100% efficiency of extraction, which is not likely). Control *P. operculella* larvae were treated with 10 μl of methanol alone. Pairs of treatment and control larvae then were presented to foraging ants as described below (ant experiment 1).

Second, pupal exuviae, which contain little or no alkaloid (Montllor et al., 1990), were collected when adults eclosed and were extracted by the same method as larval exuviae. An extract of 105 mg of pupal exuviae (from approx. 200 pupae) was taken up in 400 μl of methanol, and 10 μl was applied to each of 15 *P. operculella* larvae. Another group of 15 *P. operculella* larvae were treated with the larval exuviae extract described above. Pairs of larvae, one treated with larval exuviae extract, and the other with pupal exuviae extract were presented to foraging ants (ant experiment 2). A separate extract of 80 mg of pupal exuviae was taken up in 152 μl of methanol, and 5 μl was applied to each of 20 test larvae of *P. operculella*, giving the same concentration of extract per test larva as in ant experiment 2. These were paired with solvent-treated controls and presented to ants in choice tests (ant experiment 3).

Third, and finally, a surface extract of 100 late-instar *U. reversalis* larvae (freshly killed by freezing on Dry Ice) was made by swirling them in chloroform for 1 min. The dried extract was redissolved in 200 μl of chloroform, and 5 μl was added to *P. operculella* larvae (an extract equivalent of approx. 2.5 *U. reversalis* larvae per test larva). Pairs of extract-treated and solvent-treated larvae were presented to ants (ant experiment 4) and wasps as explained below.

In order to confirm that QAs in larval extracts might account for predator reactions, authentic quinolizidine alkaloids, sparteine (obtained from R. Molyneux, USDA, Albany, CA) and cytisine (Sigma Chemical Co.), were also used to treat *P. operculella* for choice tests. These are similar to, although not the same as, the QAs found in the plant and insect (dehydroaphylline and *N*-methylcytisine have been identified tentatively as the major alkaloids in the host plant) and have a positive effect on feeding and growth of larvae (Montllor et al., 1990). Larvae of *U. reversalis* contain approximately 17 μg total QAs per insect, derived from the host plant (Montllor et al., 1990). Alkaloid solutions were added to *P. operculella* larvae to give concentrations of 25 or 100 μg cytisine/test insect, and 25, 50 (wasp experiments only), or 100 μg sparteine/insect. Pairs of larvae, one alkaloid-treated and one solvent-treated, were presented to ants (ant experiments 5–8) and wasps in choice tests.

Predator Choice Tests. For ant trials, experimental larvae were placed on a 9-cm-diam. filter paper, one pair at a time, under a small potted citrus bush where large numbers of ants were foraging for homopteran honeydew in the greenhouse. For every pair of control and treated larvae, the number of ants contacting each larva was counted at 10-sec intervals (trials were videotaped) and summed over the duration of each trial. A trial lasted 1 min, unless ant activity was low; in such a case, the trial was allowed to continue up to a maximum of 2 min. The total number of ants at control and treated larvae were averaged for all the trials in an experiment (N = 11–20), and the means compared with a paired t-test.

The response of wasps to treated larvae was determined by placing a Petri dish containing a pair of larvae in the greenhouse where several nests of foraging wasps had been established (see Bernays, 1988). As soon as one of the pair was removed, a new pair of larvae was set out. Between each trial, the position of the control and treated larva was reversed to avoid any positional bias. There were at least 15 trials for each experiment. The numbers of treatment or control larvae taken first by the wasps was compared using a sign test.

RESULTS

Ant Experiments. P. operculella larvae that had been treated with extracts of *U. reversalis* fifth-instar exuviae or with chloroform surface extracts of fourth- and fifth-instar larvae were contacted by significantly fewer, and/or were con-

tacted for shorter times by foraging ants in greenhouse choice tests, compared to solvent controls (experiments 1 and 4, Table 1). Because of the nature of the assay, it is not possible to determine whether ants were deterred or repelled by these extracts; we will only assume that ants were deterred. Similar extracts of larval exuviae had been shown previously to contain 2.5% (dry weight) alkaloid (Montllor et al., 1990), presumed to be sequestered from the host plant *G. monspessulana*. The surface extracts of *U. reversalis* gave a positive indication for the presence of alkaloids (Montllor, personal observation) by forming a precipitate when mixed with a solution of potassium iodide and iodine (Montllor et al., 1990), although levels were not quantified.

Extracts of larval exuviae were significantly more deterrent than pupal exuviae of *U. reversalis*, and pupal exuviae did not significantly deter ants when paired with solvent controls (experiments 2 and 3, Table 1). Extracts of whole pupae previously had been shown to contain little or no alkaloid (Montllor et al., 1990), and pupae have been attacked by *Iridomyrmex humilis* and other (unidentified) species of ants in the insectary and the field (Montllor, personal observation).

Finally, ants were deterred by *P. operculella* larvae that had been treated with 25 or 100 μg of cytisine, or with 100 μg of sparteine, compared to solvent controls. Ants did not discriminate between control and test larvae treated with 25 μg of sparteine (Table 1). Thus, cytisine is a more effective deterrent, and in this context, it is interesting that cytisine was shown to be more phagostimulatory to *U. reversalis* than sparteine when added to an artificial substrate (Montllor et al., 1990).

TABLE 1. MEAN TOTAL NUMBER OF ANTS CONTACTING TREATED (*T*) AND CONTROL (C) *P. operculella* LARVAE IN PAIRED CHOICE TESTS[a]

Ant exp.	Treatment/control	*N*	Ants (*N*)		*P*
			T	C	
1	Larv exuv/Solv	15	2.7	17.8	0.002
2	Larv exuv/Pup exuv	13	1.7	5.9	0.03
3	Pup exuv/Solv	19	14.4	16.5	ns
4	Surface/Solv	11	6.5	30.5	<0.0001
5	Cytisine (25 μg)/Solv	17	10.8	18.2	0.01
6	Cytisine (100 μg)/Solv	16	6.9	17.7	<0.0001
7	Sparteine (25 μg)/Solv	14	7.1	9.2	ns
8	Sparteine (100 μg)/Solv	20	7.7	11.8	0.01

[a] *N* = number of trials. *U. reversalis* extracts: Larv exuv = fifth-instar exuviae; Pup exuv = pupal exuviae; surface = late-instar surface. Solv = solvent control (MeOH or $CHCl_3$). *P* value derived from *t* test (one-tailed); ns, $P > 0.08$.

Wasp Experiments. M. flavitarsus, given a choice between a *P. operculella* larva treated with a surface extract of *U. reversalis* or a solvent-treated control larva, took control larvae first in 75% (15/20) of the trials (Figure 1). *P. operculella* treated with 100 μg of sparteine or cytisine, or 50 μg of sparteine, were also deterrent to wasps relative to controls (wasps took control larvae first in 13–14 of 15 trials in each experiment), but alkaloids at the lowest concentration (25 μg/larva) had no statistically significant effect on wasp choice (Figure 1).

DISCUSSION

Extracts of larval cuticle or surface extracts of whole larvae of *U. reversalis* were highly deterrent to ants and wasps, as were authentic alkaloids, when added to the surface of the normally palatable *P. operculella* larvae. These two hymenopteran predators were shown previously to be deterred by *U. reversalis* larvae: the paper wasp *M. flavitarsus* avoided larvae of *U. reversalis* as prey (Bernays, 1988), and Argentine ants spent significantly more time contacting *P. operculella* compared to *U. reversalis* larvae in choice tests in which larvae were freshly killed (Bernays and Cornelius, 1989). Our results suggest that at

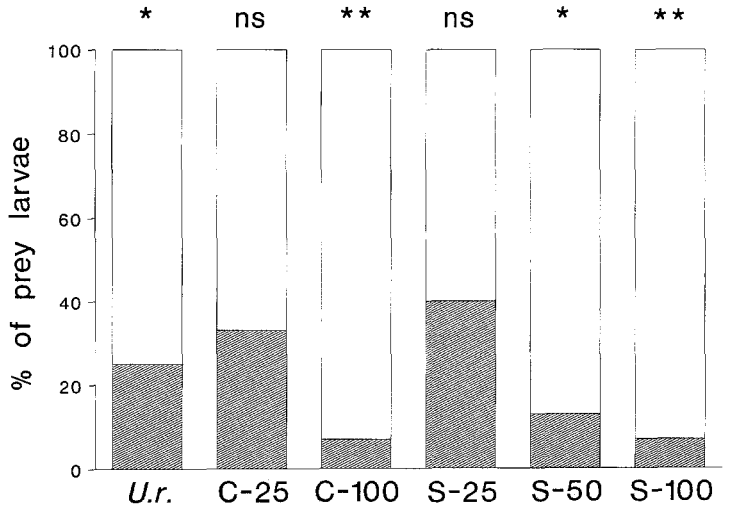


Fig. 1. Proportion of treated (hatched portion) and solvent control (open portion) *P. operculella* larvae taken first by foraging wasps (*M. flavitarsus*) in six experiments. Extract or chemicals added to dead larvae: *U.r.* = surface extract of late-instar *U. reversalis*; C-25 = 25 μg cytisine; C-100 = 100 μg cytisine; S-25, -50, and -100 = 25, 50, or 100 μg sparteine. $N = 15$ trials for each experiment, except *U.r.* ($N = 20$) and C-25 ($N = 27$). Sign tests within experiments: $*P < 0.02$; $**P < 0.001$; ns = $P < 0.05$.

least part of the deterrence of *U. reversalis* for these predators is due to the alkaloids sequestered from the host plant and deposited in the cuticle. Preliminary results suggest that cocoon silk also contains QAs (Wink, personal communication); if larval silk also contains QAs, this may add to the protection of larvae in the natural environment, since larvae feed and rest on leaves sparsely spun with silk.

Sparteine and cytisine only deterred wasps at the higher concentrations used, and the same was true for sparteine and ants, showing that deterrence of alkaloids to ants and wasps can be dosage-dependent. The high levels of QAs required to elicit a response from wasps (probably three times that found in the larval cuticle) and the differential effects of the two authentic alkaloids for ants indicate that the composition of the sequestered alkaloids may be important to predator deterrence or that other larval factors, such as hairiness or silk production, may be involved. The *Genista* host plant contains up to 27 alkaloids (Montllor et al., 1990), and we were not able to reflect the composition of *U. reversalis* cuticle with authentic alkaloids.

Deposition of plant compounds in the integument of larval Lepidoptera has not been studied extensively, but several examples are known. Included are pyrrolizidine alkaloids in *Creatonotos transiens* (Egelhaaf et al., 1990; von Nickisch-Rosenegk et al., 1990b; Boppré, 1990); cannabinoids in *Arctia caja* (Rothschild et al., 1977); and cardiac glycosides in *Syntomeida epilais* (von Nickisch-Rosenegk et al., 1990a). Larvae of *Zygaena trifolii* store cyanoglycosides in cuticular cavities (Franzl et al., 1988). Storage of plant secondary compounds in the integument appears to be an efficacious means of deterring potential predators: only a small portion of plant compounds ingested (about 1% in the case of *Uresiphita*) needs to be sequestered, if they are concentrated in the cuticle, in order to reach concentrations that are potentially deterrent to predators. Sequestration of larger amounts may pose potential autotoxicity problems, although little is known of the effects of most alkaloids on insects (Brattsten, 1986). Alternatively, or in addition, uptake may be physiologically difficult or "expensive" to accomplish; there is evidence that uptake of QAs is facilitated by a carrier in the gut of *U. reversalis* (M. Wink, unpublished), as has been found in *Creatonotos* for pyrrolizidine alkaloids (Wink and Schneider, 1988), and in a ctenuchid for cardiac glycosides (von Nickisch-Rosenegk et al., 1990a).

The significance of predators to *U. reversalis* in the natural habitat was estimated in long-term observations in the field (Bernays and Montllor, 1989). Predation was estimated to account for between 15 and 40% of mortality of young larvae on *Genista monspessulana*, and mortality was reduced when predators were excluded. In the field, wasps (Vespidae) and hemipterans (Anthocoridae) were observed preying on larvae. In addition, *Chrysoperla carnea* (Chrysopidae) and unidentified spiders (Thomisidae and Salticidae) did con-

sume larvae in no-choice experiments in the laboratory (Bernays and Montllor, 1989). An examination of responses of nine species of invertebrate predators to larvae of *U. reversalis* suggests that larvae are unpalatable to all but three (Montllor, Cornelius, and Bernays, unpublished). Therefore, the deterrence of *U. reversalis* to potential arthropod predators, without being absolute, is still impressive. Considering particularly the potential impact of such predators as ants and wasps on lepidopteran larvae (e.g., Gould and Jeanne, 1984; Jones, 1987; Laine and Niemela, 1980; Risch and Carroll, 1982; Skinner and Whittaker, 1981; Weseloh, 1989), the degree of deterrence documented here is probably a significant advantage.

Acknowledgments—Thanks to R. Barbehenn and J. Hamai for help with experiments and to K. Hagen, M. Graham, and members of the Insect-Plant Group (UC Berkeley) for helpful criticisms of an earlier draft. This work was supported in part by USDA-CSRS grant 88-38300-3628 to EAB.

REFERENCES

BERENBAUM, M. R., and MILICZKY, E., 1984. Mantids and milkweed bugs: efficacy of aposematic coloration against invertebrate predators. *Am. Midl. Nat.* 111:64–68.

BERNAYS, E. A. 1988. Host specificity in phytophagous insects: Selection pressure from generalist predators. *Entomol. Exp. Appl.* 49:131–140.

BERNAYS, E. A., and CORNELIUS, M. L. 1989. Relative acceptability of caterpillars with different host range to the generalist predator *Iridomyrmex humilis*. *Oecologia* 79:427–430.

BERNAYS, E. A., and MONTLLOR, C. B. 1989. Aposematism of *Uresiphita reversalis* larvae (Pyralidae). *J. Lep. Soc.* 43:261–273.

BLUM, M. S. 1981. Chemical Defenses of Arthropods. Academic Press, New York. 562 pp.

BOPPRÉ, M. 1990. Lepidoptera and pyrrolizidine alkaloids. *J. Chem. Ecol.* 16:165–186.

BOWERS, M. D., and LARIN, Z. 1989. Acquired chemical defense in the lycaenid butterfly, *Elimaeus atala*. *J. Chem. Ecol.* 15:1133–1146.

BRATTSTEN, L. B. 1986. Fate of ingested plant allelochemicals in herbivorous insects, pp. 211–255, *in* L. B. Brattsten and S. Ahmad (eds.). Molecular Aspects of Insect–Plant Associations. Plenum Press, New York.

BROWER, L. P. 1984. Chemical defense in butterflies, pp. 109–133, *in* R. I. Vane-Wright and P. R. Ackery (eds.). The Biology of Butterflies. Symposium of the Royal Entomological Society, London, Vol. VII. Princeton University Press, Princeton, New Jersey.

DEMPSTER, J. P. 1984. The natural enemies of butterflies, pp. 97–104, *in* R. I. Vane-Wright and P. R. Ackery (eds.). The Biology of Butterflies. Symposium of the Royal Entomological Society, London, Vol. VII. Princeton University Press, Princeton, New Jersey.

EGELHAAF, A., CÖLLIN, K., SCHMITZ, B., BUCK, M., WINK, M., and SCHNEIDER, D. 1990. Organ specific storage of dietary pyrrolizidine alkaloids. *Z. Naturforsch.* 45c:115–120.

FEENY, P., BLAU, W. S., and KAREIVA, P. M. 1985. Larval growth and survivorship of the black swallowtail butterfly in central New York. *Ecol. Monogr.* 52:167–187.

FRANZL, S., NAUMANN, C. M., and NAHRSTEDT, A. 1988. Cyanoglycoside storing cuticle of *Zygaena* larvae. *Zoomorphology* 108:183–190.

GOULD, W. P., and JEANNE, R. L. 1984. *Polistes* wasps (Hymenoptera: Vespidae) as control agents for lepidopteran cabbage pests. *Environ. Entomol.* 13:150–156.

JONES, D. A., PARSONS, J., and ROTHSCHILD, M. 1962. Released hydrocyanic acid from crushed

tissues of all stages of the life cycle of species of Zygaeninae (Lepidoptera). *Nature* 193:52–53.

JONES, R. E., 1987. Ants, parasitoids, and the cabbage butterfly *Pieris rapae*. *J. Anim. Ecol.* 56:739–749.

KELLY, R. B., SEIBER, J. N., JONES, D. D., SEGALL, H. J., and BROWER, L. P. 1987. Pyrrolizidine alkaloids in overwintering monarch butterflies (*Danaus plexippus*) from Mexico. *Experientia* 43:943–946.

LAINE, K. J., and NIEMELA, P. 1980. The influence of ants on the survival of mountain birches during an *Oporinia autumnata* (Lep., Geometridae) outbreak. *Oecologia* 47:39–42.

MONTLLOR, C. B., BERNAYS, E. A., and BARBEHENN, R. V. 1990. Importance of quinolizidine alkaloids in the relationship between larvae of *Uresiphita reversalis* (Lepidoptera: Pyralidae) and a host plant, *Genista monspessulana*. *J. Chem. Ecol.* 16:1853–1865.

NAHRSTEDT, A., and DAVIS, R. H. 1983. Occurence, variation and biosynthesis of the cyanogenic glycosides linamarin and lotuaustralin in species of the Heliconini (Insecta: Lepidoptera). *Comp. Biochem. Physiol.* 758:65–73.

RISCH, S. J., and CARROLL, C. R. 1982. Effect of a keystone predaceous ant, *Solenopsis geminata*, on arthropods in a tropical agroecosystem. *Ecology* 63:1979–1983.

ROTHSCHILD, M., ROWAN, M. G., and FAIRBAIRN, J. W. 1977. Storage of cannabinoids by *Arctia caja* and *Zonocerus elegans* fed on chemically distinct strains of *Cannabis sativa*. *Nature* 266:650–651.

ROTHCHILD, M., APLIN, R. T., COCKRUM, P. A., EDGAR, J. A., FAIRWEATHER, P. and LEES, R. 1979. Pyrrolizidine alkaloids in arctiid moths (Lep.) with a discussion on host plant relationships and the role of these secondary plant substances in the Arctiidae. *Biol. J. Linn. Soc.* 12:305–326.

SHAW, P. B., OWENS, J. C., HUDDLESTON, E. W., and RICHMAN, D. B. 1987. Role of arthropod predators in mortality of early instars of the range caterpillar, *Hemileuca oliviae* (Lepidoptera: Saturniidae). *Environ. Entomol.* 16:814–820.

SKINNER, G. J., and WHITTAKER, J. B. 1981. An experimental investigation of interrelationships between the wood-ant (*Formica rufa*) and some tree-canopy herbivores. *J. Anim. Ecol.* 50:313–326.

STAMP, N. E., and BOWERS, M. D. 1988. Direct and indirect effects of predatory wasps (*Polistes* sp.: Vespidae) on gregarious caterpillars (*Hemileuca lucina*: Saturniidae). *Oecologia* 75:619–624.

STEWARD, V. B., SMITH, K. G., and STEPHEN, F. M. 1988. Predation by wasps on lepidopteran larvae in an Ozark forest canopy. *Ecol. Entomol.* 13:81–86.

von NICKISCH-ROSENEGK, E., DETZEL, A., WINK, M, and SCHNEIDER, D. 1990a. Carrier-mediated uptake of digoxin by larvae of the cardenolide sequestering moth, *Syntomeida epilais*. *Naturwissenchaften* 77:336–338.

von NICKISCH-ROSENEGK, E., SCHEIDER, D., and WINK, M. 1990b. Time-course of pyrrolizidine alkaloid processing in the alkaloid exploiting arctiid moth, *Creatonotos transiens*. *Z. Naturforsch.* 45c:881–894.

WATT, A. D. 1989. The growth and survival of *Panolis flammea* larvae in the absence of predators on Scots pine and lodgepole pine. *Ecol. Entomol.* 14:225–234.

WESELOH, R. M. 1989. Simulation of predation by ants based on direct observations of attacks on gypsy moth larvae. *Can. Entomol.* 121:1069–1076.

WINK, M., and SCHNEIDER, D. 1988. Carrier-mediated uptake of pyrrolizidine alkaloids in larvae of the aposematic and alkaloid-exploiting moth *Creatonotos*. *Naturwissenschaften* 75:524–525.

WITZ, B. W. 1990. Antipredator mechanisms in arthropods: a twenty-year literature survey. *Fl. Entomol.* 73:71–99.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

COVER AND EFFICACY OF PREDATOR-BASED REPELLENTS FOR TOWNSEND'S VOLE, *Microtus townsendii*

MARKUS MERKENS,[1] ALTON S. HARESTAD,[1,*] and THOMAS P. SULLIVAN[2]

[1]*Department of Biological Sciences*
Simon Fraser University
Burnaby, B.C., Canada V5A 1S6

[2]*Department of Forest Sciences*
University of British Columbia
Vancouver, B.C., Canada V6T 1W5

(Received May 31, 1990; accepted October 9, 1990)

Abstract—Predator-based repellents have been used experimentally to control wildlife damage in both agriculture and forestry, but they have not always been effective. We examined the relative importance of cover and predator odors in forage patch selection by Townsend's vole, *Microtus townsendii*, and its behavior related to cover and predator cues. Experiments were conducted in which forage patch and area choices were related to available habitat alternatives. Outdoor enclosures were divided into halves: one side was treated and the other used as a control. Treatments consisting of "cover," "repellent," and "cover plus repellent" were compared to controls (no cover, no repellent). In the absence of cover, voles preferred to feed on the side without repellents. When cover was present, voles preferred to feed on the side with cover, regardless of whether or not repellents were present. Voles visited more feeding stations on the side without cover when repellents and cover were present than they visited during cover-only treatments. These additional feeding stations, visited outside of cover, were used only lightly as food sources. The amounts of oats eaten by voles decreased with increasing distance from cover. This inverse relationship had a steeper slope in cover-only treatments compared to cover plus repellent treatments. A selection model based on forage patch selection and a habitat preference hierarchy is proposed. We conclude that predator odors are effective as repellents, but their efficacy depends on habitat conditions. Managers intending to use

*To whom correspondence should be addressed.

0098-0331/91/0200-0401$06.50/0

predator-based repellents must ensure that alternative sites available to pests are better quality habitat than that in areas to be protected.

Key Words—Wildlife damage, Townsend's vole, *Microtus townsendii*, predator odors, repellents, cover.

INTRODUCTION

As intensive reforestation becomes more widely practiced in North America, wildlife pest problems are becoming more evident, especially in young forest plantations. Damage to forest regeneration projects caused by deer, snowshoe hares (*Lepus americanus*), and rodents can be considerable (Crouch, 1969; Black et al., 1979; Maguire, 1989). Seedlings can be killed by clipping, and established trees can be damaged through debarking and girdling of their stems (Sullivan and Sullivan, 1982; Harper and Harestad, 1986; Sullivan and Sullivan, 1986).

Predator odors affect behavior of various wildlife species (Stoddart, 1976; Stoddart, 1980; Gorman, 1984), and their use as repellents can reduce feeding damage (Sullivan et al., 1985a,b; 1988a,b). Initially, crude predator-based repellents were made using extracts from feces, urine, and scent glands of predators (Sullivan et al., 1985a,b). The major sulfur-containing compounds in these materials were identified as 2-propylthietane, 2-pentylthietane, and 3-propyl-1,2-dithiolane (Crump, 1978, 1980; Crump and Moors, 1985). These compounds were tested for their repellency to voles (Sullivan et al., 1988a) and a mixture of 2-propylthietane and 3-propyl-1,2-dithiolane has been suggested for commercial repellents. These compounds can be synthetically produced in large quantities and incorporated into controlled-release devices.

Predator-based repellents depend on an innate avoidance of predator cues by prey species. Gorman (1984) showed that this response is innate by demonstrating that common voles, *Microtus arvalis*, that have been isolated for at least 5000 years on an island devoid of mammalian predators, strongly avoided the odor of anal gland preparations from ermine, *Mustela erminea.*

Predator-based repellents are not always effective in field trials because environmental factors apparently influence their efficacy. If predator-based repellents are to become effective tools for controlling wildlife damage in forests and agricultural crops, the constraints imposed by environmental factors must be examined.

Field experiments involving animal behavior are difficult to design and conduct because many variables cannot be controlled. For this reason our experimental trials were conducted in bioassay pens. Although pen trials allow variables to be controlled, animals in pen trials are not subjected to all environmental influences encountered in the field, and their behavior may

deviate from that in the wild. Given this potential constraint, the purpose of our experiment was to examine use of cover and food in the presence or absence of predator semiochemicals. Cover, food, and predator odors are basic requirements or stimuli of animals. Even if animals are penned, they need to feed and should continue to be vigilant. It is reasonable to assume that if test animals are given the appropriate stimuli in captivity, then their actions should reveal behavioral processes concerning trade-offs in response to these stimuli.

Animals living in a patchy environment must travel to find food and meet their other requirements. During periods of travel, they are exposed to many risks. Individuals unsuccessful in their search for food or spending too much time and energy doing so are liable to starve, fail to reproduce, or risk predation. Animals should behave so as to minimize these combined risks (Holmes, 1984; Lima et al., 1985; Anderson, 1986; Lima and Valone, 1986).

Prey can avoid predators by moving about under or near cover (Rose and Birney, 1985; Getz, 1985). Another method of predator avoidance is linked to predator cue recognition. Many mammalian predators have specialized scent glands that are used to mark their territories. Although these secretions are of adaptive significance as pheromones to the emitter, they can be a detriment to the emitter by acting as kairomones to prey species. Prey species avoid these odors or reduce their activity in the presence of them (Stoddart, 1976, 1980; Gorman, 1984; Sullivan, 1986; Sullivan et al., 1988b). Recognition of and response to predator odors by prey is of adaptive significance because it reduces predation risk.

Given that voles are attracted to cover and repelled by predator cues, some process of balancing the risks of predation while meeting energy requirements must occur when both cover and predator cues are encountered simultaneously. Our objectives were to examine behavioral processes and to determine the relative importance of cover and predator cues as they relate to forage patch selection by Townsend's vole, *Microtus townsendii*.

METHODS AND MATERIALS

Test Animals. Townsend's voles were live-trapped from the George C. Reifel Migratory Bird Sanctuary in Delta, British Columbia, between September 1988 and September 1989. In British Columbia, this species can be found in the alluvial areas of the Fraser River Delta and similar areas on Vancouver Island. Its prefered habitat consists of moist fields and sedge meadows. The voles were held individually in wire-top animal cages and fed a diet of Purina Laboratory Rat Chow and whole oats ad libitum until needed for experiments.

Bioassay Pen. An outdoor enclosure was divided into three 6 × 11-m pens and vole-proofed by plugging holes and constructing a wire mesh fence (6-mm

mesh size) to a depth of 50 cm below ground between pens. Within each pen, four 20 × 30 × 15-cm plastic nest boxes filled with raw cotton were buried flush to the ground along the transverse midline of each pen (Figure 1). Each nest box was covered with a 50 × 100-cm galvanized pan. To provide cover near the nest boxes, a strip of straw, 5–15 cm deep, was placed along the line of nest boxes. Fifteen feeding stations were established at a 2 × 2-m spacing in a diagonal grid pattern in each half of the three pens. Feeding stations were constructed from two 1-liter plasticized cardboard milk cartons slid one into the other to form a cross. A 5-ml plastic Petri dish was placed in the center of each feeding station to hold oats. Four water bottles were placed in each pen.

Experimental Procedure. In each of four 15-day-long replicates, between September 1 and November 9, 1989, there were three experimental treatments, each paired with a control. Experimental treatments, consisting of "cover," "repellent," or "cover plus repellent" were in one side of the pen and the control, (no cover plus no repellent) in the other side. Treatments were assigned randomly to pen and side. The same treatment was not applied to the same pen twice in a row. Cover was provided by dispersing straw from one 11.4-kg bale to a depth of 5–15 cm over the treatment side of the pen. Repellents, comprised of a 1 : 1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane, were released from a 4-cm-long piece of 4-mm-diameter PVC in which they were impregnated at 1% by weight (Phero Tech Inc., Delta, British Columbia, Canada). The PVC

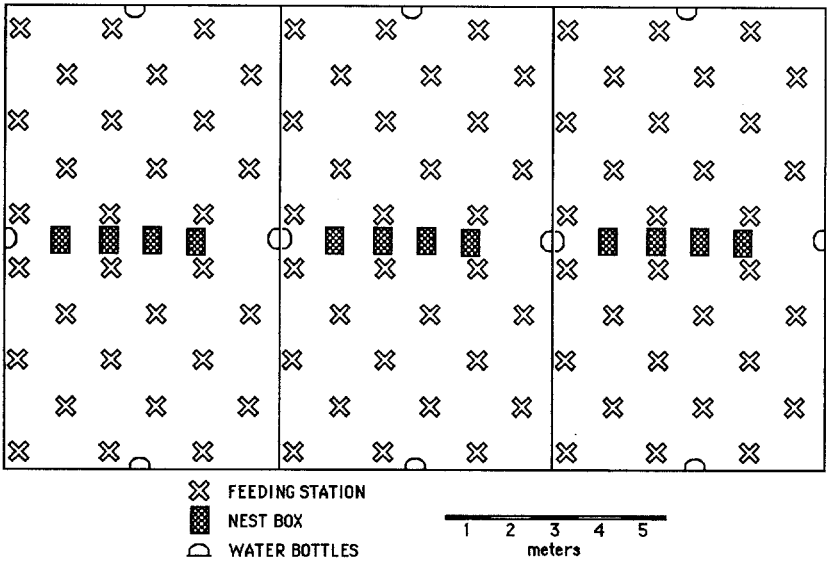


FIG. 1. Three bioassay pens showing nest box and feeding station placement. During each replicate half the pen was treated and the other half used as a control.

release devices were secured by a pin to the inside of each feeding station on the treatment side of the pen. Nonimpregnated release devices were secured to the insides of feeding stations not containing repellents. When treatments were repositioned at the start of each replicate, whole feeding stations with the repellent still attached were moved. The same repellent devices were used through all four replicates.

Before each replicate, the vegetation in each pen was mowed to 1 cm above ground level, burrows and runways previously constructed by voles were buried, and clean dishes filled to capacity were placed in each feeding station. Four female voles then were released directly into the nest boxes in each pen and left undisturbed for four days. On the fifth day, 10 g of oats were placed in each dish. Oats were removed and replaced with 10 g of fresh oats every other day for 10 days. Oats remaining in the feeding dishes were weighed to the nearest 0.1 g.

RESULTS

In the absence of cover, more oats were removed from feeding dishes on sides of pens not treated with repellents than from feeding dishes on sides with repellents (Figure 2). Most feeding from repellent-treated stations occurred

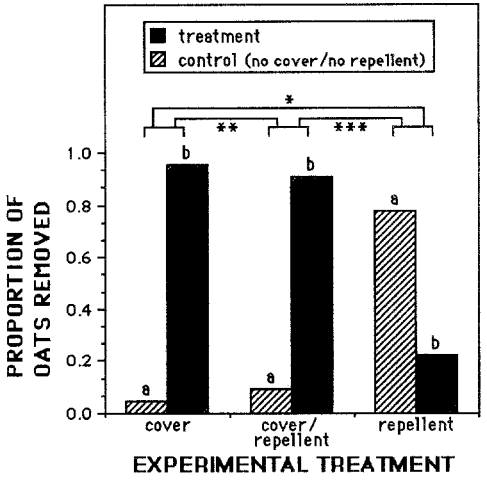


FIG. 2. Proportion of total weight of oats removed by voles from treatment and control sides of pens. Paired bars topped by different letters indicate significant difference between treatment and control, Wilcoxon signed rank test (Sokal and Rohlf, 1981), $P < 0.01$, $N = 4$. Asterisks indicate differences in consumption between treatments, von Mises distribution analysis (Stephens, 1982), $*F_{1,38} = 115.13$, $P < 0.005$; $**F_{1,38} = 5.75$, $P < 0.05$; $***F_{1,38} = 106.96$, $P < 0.005$.

around the periphery of the pens or in close proximity to the nest boxes. When cover was present, voles preferred to feed on the side with cover, regardless of whether or not repellents were present, with a slight, but significant difference between proportions in cover and cover plus repellent treatments (Figure 2). No trend in these proportions over time occurred for repellent and cover plus repellent experimental treatments (Spearman's rank correlation; repellent, $r_s = -0.2$, $P > 0.05$; cover plus repellent, $r_s = -0.8$, $P > 0.05$).

Oats were removed from more stations by voles on the control side in cover plus repellent treatment pens than in cover-only pens (Figure 3). Further evidence for this increased frequency of feeding station visitation was found when we examined the amount of oats removed from feeding stations (Figure 4). More feeding stations were visited on both sides of the pens when the experimental treatment was cover plus repellent. The greatest difference between cover and cover plus repellent treatments is a shift from stations never fed at (0.0 g) to stations fed at low levels (0.1–1.0 g). There is also a slight shift from high-use stations (9.1–10.0 g) to lower levels of use. More feeding stations were visited on the control side of the pen when the experimental treatment was predator odors plus cover than were visited on the control side when the experimental treatment was cover only. Although more feeding stations were visited, most of these were only slightly used. When the experimental treatment was repellent with no cover, stations that were visited on the repellent side were used less intensively than those on the control side (Figure 4). These differences

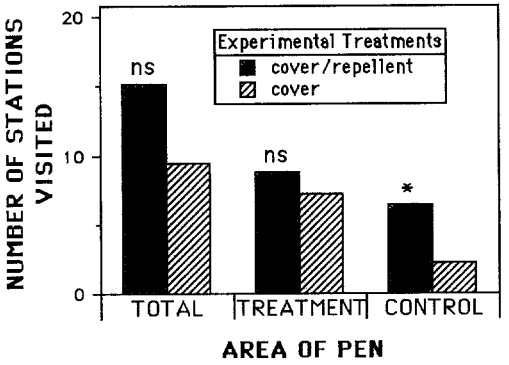


FIG. 3. Average number of stations visited per sampling period in the total, treatment, and control area of the pen for cover and cover plus repellent experimental treatments. Total number of stations visited was not significantly different between treatments, t test with equal variances, $t_6 = 1.05$, $P > 0.05$. Number of stations visited on the control side of the pen were significantly different, t test with equal variances, $t_6 = 3.79$, $P < 0.005$. No significant difference in means for treatment side was found, modified t test for means with unequal variances (Sokal and Rohlf, 1981), $t_3 = 0.625$, $P > 0.05$.

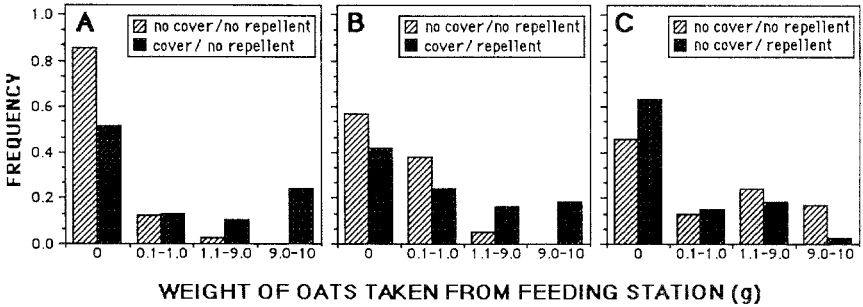


FIG. 4. Distributions of use for (A) cover, (B) cover plus repellent, and (C) repellent treatments. Differences between control and treatment distributions are significant for all treatments; (A) $\chi^2 = 113.96$, $df = 3$, $P < 0.0005$; (B) $\chi^2 = 86.98$, $df = 3$, $P < 0.0005$; (C) $\chi^2 = 41.34$, $df = 3$, $P < 0.0005$. Differences between the overall distributions of A and B are significant, von Mises distribution analysis (Stephens, 1982), $F_{7,210} = 9.33$, $P < 0.005$. This test is designed for use with continuous variables but can be used for discrete variables (as here) providing that there is high significance. Given the level of significance, the test is valid (Micheal Stephens, personal communication, Department of Mathematics and Statistics, Simon Fraser University, Burnaby, British Columbia, Canada).

suggest that in the absence of cover, voles may spend less time at repellent-treated feeding stations and consume a smaller fraction of the available food than at repellent-free stations.

When weights of oats taken from feeding stations (excluding those that were not visited) were compared to relative distance from cover for cover and cover plus repellent experimental treatments (Figure 5), the weight of oats removed decreased with increasing distance from cover. For this analysis, cover was considered to be that supplied by straw or afforded by the pen barriers or nest boxes. When the weights were $\log_{10}$-transformed and linear regressions calculated (Table 1), the r^2 values were significant, and the slopes of the two regression lines were different. Because the slope of the relationship between weight of oats removed and distance was steeper for cover treatments than for cover plus repellent treatments (Table 1), the effect of cover was reduced by the presence of repellents.

DISCUSSION

Although foraging theory predicts that animals maximize their net rate of energy intake, they should do so only within the constraints of predation risk (Krebs and Davies, 1981). Animals balance risks of predation and starvation both in laboratory and field studies (Milinski and Heller, 1978; Sih, 1980;

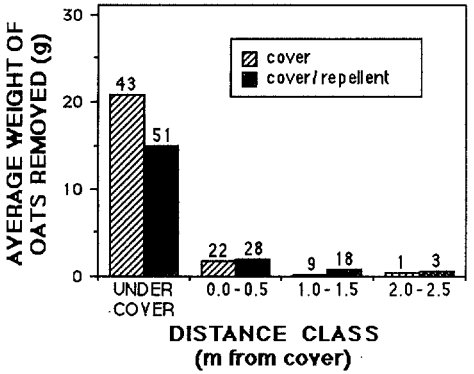


FIG. 5. Weights of oats taken from feeding stations over 10 days compared to distance from cover for cover and cover plus repellent experimental treatments. *N* is given above bars for each distance class and weight combination and is the sum of all stations over the entire experiment. See Table 1 for analysis.

TABLE 1. REGRESSION COEFFICIENTS AND STATISTICS OF LOG_{10} (WEIGHT OF OATS REMOVED) AND DISTANCE FROM COVER FOR COVER AND COVER PLUS REPELLENT TREATMENTS

Treatment	Intercept (SE)	Slope (SE)[a]	r^2	Significance
Cover	1.672 (0.197)	−0.861 (0.113)	0.442	$P < 0.001$
Cover plus repellent	0.986 (0.156)	−0.421 (0.081)	0.217	$P < 0.001$

[a] Analysis of covariance revealed that the treatment by distance interaction is significant, $F_{1,171} = 10.31$, $P = 0.002$.

Holmes, 1984). For animals to balance these risks, choices must be available to them. Food is often patchily distributed, and animals will move from patch to patch to sample individual patches for quality. Once they determine which patches have the "highest" quality, they should feed from those patches first until the patches are depleted or the quality of the patch is reduced. The animal should then move on to the next most favorable patch.

Patch quality is likely affected by many parameters, e.g., food quality and predation risk. Food quality at the feeding stations remained constant, or at least nearly constant, being equalized every second day of each replicate. Only parameters relating to perception of predation risk were altered. Given equal food quality, voles should have preferred low-predation risk patches over high-predation risk patches (Holmes, 1984).

Preferences in patch choice (Figure 2) suggest that cover is more important for security from predators than is avoidance of mammalian predator odors. However, when predator odors and cover were both present, voles visited more food patches on the sides of pens without cover (Figures 3 and 4) and consumed greater amounts of food further from cover (Figure 5, Table 1) compared to when predator odors were not present under cover. The reduction in perceived patch quality due to the presence of predator odors may have caused voles to search more actively for better quality patches, including the feeding stations on the sides without cover, which were used only slightly as food sources. These differences in intensity of feeding station use suggest that voles are either sampling food quality on sides without cover or trying to correct an energy deficit. The latter hypothesis is unlikely given that patterns of intensive patch use are similar for both cover and cover plus repellent treatments (Figure 4). Voles are likely visiting these extra feeding stations to sample food quality and may accept greater exposure for patches of higher food value. They would be balancing the risks of predation associated with both habitat types with varying food levels. They are more likely to search for high-quality food patches outside of cover when perceived predation risk under cover is increased through the presence of predator odors. This type of behavior has been described as "risk-balancing" (Fraser and Huntingford, 1986). In our experiment there was no difference in patch food quality between sides of the pens with and without cover; therefore, voles still preferred feeding under cover, accepting the increased risk of predation imposed by predator odors.

An alternate explanation for these results could be that voles habituated to the predator-based repellents during the first four days of the replicates. This hypothesis can not be tested directly with our experimental results. We hypothesize that such learning behavior would be selected against because any mistake in habituating to the odor when warnings should be taken seriously would be maladaptive.Given that voles isolated from predators for many generations were still repelled by predator odors (Gorman, 1984), this response is innate and clearly of high evolutionary significance. Furthermore, if voles could habituate to predator odors, then the consumption of oats in the repellent-only experimental treatments would have been uniform between treatment and control sides of the pen.

Risk of predation increases with distance from cover (Holmes, 1984). The hypothesis that animals should prefer to forage as close to cover as possible, balancing patch quality with predation risk was upheld by our results (Figure 5). However, when predator cues were present under cover, voles consumed a greater weight of oats as a function of distance from cover than they did when predator odors were not present under cover (Table 1). This result suggests that when predator odors were present under cover, voles sample for patch quality more frequently at greater distances from cover.

Voles are repelled by predator odors (Figure 2) (Sullivan et al., 1988a) and attracted to cover (Taitt et al., 1981; Getz, 1985), and their feeding behavior is affected by both predator odors and cover (Figures 2–4). As voles move about in their environment, they likely acquire information related to cover, food, and predator odors (information variables) and use it to maximize the benefit of their actions. Based on this assumption, a selection model outlining habitat preferences of voles with respect to information variables was developed (Figure 6). When voles are hungry, they begin searching for food, encountering habitats with varying levels of cover, food, and predator odors. If an encountered patch has no cover or contains predator odors, then voles should leave the patch and search for more suitable patches. If better patches are not available, then voles should return to and feed from the highest-value patch available. The selection model is based on a hierarchy of habitat preferences for voles. In our experiment, voles preferred patches with cover and no predator cues over all other patches given equal food quality. The least favored habitats smelled of predators and offered no cover.

Our results support the hypothesis that herbivore behavior is linked to habitat characteristics (Taitt et al., 1981; Holmes, 1984) and that manipulation of these habitat characteristics will cause shifts in areas used by voles (Taitt et al.,

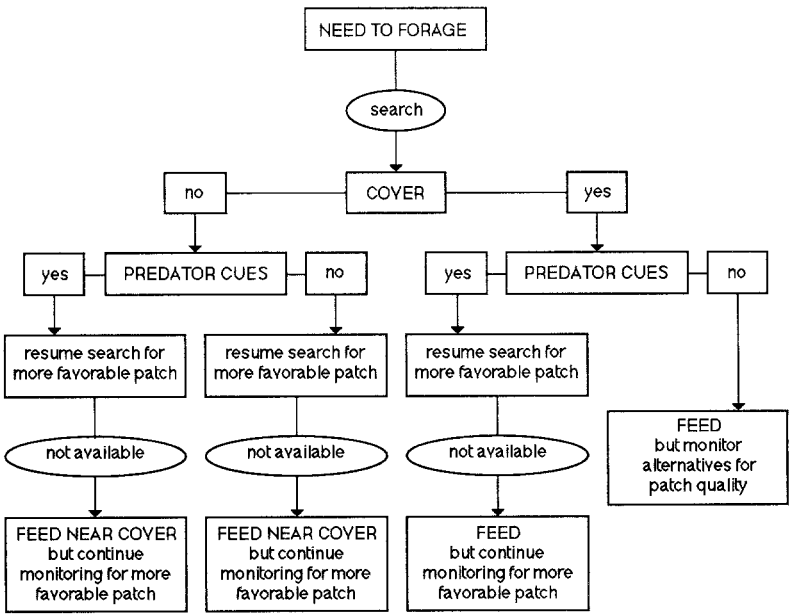


FIG. 6. Selection model outlining forage patch preference hierarchy based on cover, food, and predator cues.

1981). In our model, voles should consider habitat information and internal motivational states to determine which forage patch will be most beneficial to them. Application of predator-based repellents is intended to alter both the habitat information, and internal motivational states of target animals, thereby reducing the perceived quality of a treated forage patch, by increasing the apparent predation risk. If better alternatives are not available to searching voles, then they will be forced to feed in the repellent-treated patches. If voles are able to habituate to predator odors, then they would continue to feed in repellent treated areas until the repellents are reinforced by an encounter with a predator. Paramount to the control of vole damage is the existence or provision of better-quality patches in the vicinity of repellent-treated areas. This is a key factor in the success of any repellent-based management technique. By enhancing preferred browse in plantations, this principle has been used to reduce damage to seedlings by ungulates (Campbell, 1974). The challenge to pest managers is to examine vertebrate pest situations in the context of habitat conditions, not only on site but also adjacent to it, and to ensure that habitat alternatives are better than the areas to be protected.

Acknowledgments—We thank Phero Tech Inc. and The Science Council of British Columbia for providing financial support and the Canadian Wildlife Service and the Canadian Coast Guard for permission to trap animals on land under their jurisdiction.

REFERENCES

ANDERSON, P.K. 1986. Foraging range in mice and voles: The role of risk. *Can. J. Zool.* 64:2645–2653.

BLACK, H.C., DIMOCK, E.J., EVANS, J., and ROCHELLE, J.A. 1979. Animal damage to coniferous plantations in Oregon and Washington. Part 1: A survey 1963–1975. Oregon State University Forest Research Laboratory, Research Bulletin 25. Corvallis, Oregon.

CAMPBELL, D.L. 1974. Establishing preferred browse to reduce damage to douglas-fir seedlings by deer and elk. pp. 187–192, *in* H.C. Black (ed.). Proceedings, Symposium on Wildlife and Forest Management in the Pacific Northwest. Corvalis, Oregon.

CROUCH, G.L. 1969. Animal damage to conifers in national forests in the Pacific Northwest region. U.S.D.A. Forestry Service Research Bulletin PNW-28.

CRUMP, D.R. 1978. 2-Propylthietane, the major malodorous substance from the anal gland of the stoat. *Tetrahedron Lett.* 1978:5233–5234.

CRUMP, D.R. 1980. Thietanes and dithiolanes from the anal gland of stoat (*Mustela erminea*). *J. Chem. Ecol.* 6:341–347.

CRUMP, D.R., and MOORS, P.J. 1985. Anal gland secretions from the stoat (*Mustela erminea*) and the ferret (*Mustela putorius* forma *furo*): Some additional thietane components. *J. Chem. Ecol.* 11:1037–1043.

FRASER, D.F., and HUNTINGFORD, F.A. 1986. Feeding and avoiding predation hazard: The behavioural response of prey. *Ethology* 73:56–68.

GETZ, L.L. 1985. Habitats, pp. 286–309, *in* R.H. Tamarin (ed.). Biology of New World *Microtus*. Special Publication No. 8. The American Society of Mammalogists, Shippensburg, Pennsylvania.

GORMAN, M.L. 1984. The response of prey to stoat (*Mustela erminea*) scent. *J. Zool. London* 202:419–423.

HARPER, P.A., and HARESTAD, A.S. 1986. Vole damage to coniferous trees on Texada Island. *For. Chron.* 62:429–432.

HOLMES, W.G. 1984. Predation risk and foraging behavior of the hoary marmot in Alaska. *Behav. Ecol. Sociobiol.* 15:293–301.

KREBS, J.R., and DAVIES, N.B. 1981. An Introduction to Behavioral Ecology. Blackwell Scientific Publications, Oxford, U.K.

LIMA, S.L., and VALONE, T.J. 1986. Influence of predation risk on diet selection: A simple example in the grey squirrel. *Anim. Behav.* 34:536-544.

LIMA, S.L., VALONE, T.J., and CARACO, T. 1985. Foraging-efficiency-predation-risk trade-off in the grey squirrel. *Anim. Behav.* 33:155–165.

MAGUIRE, C.C. 1989. Small mammal predation on Douglas-fir seedlings in northwestern California. *Wildl. Soc. Bull.* 17:175–178.

MILINSKI, M., and HELLER, R. 1978. Influence of a predator on the optimal foraging behaviour of sticklebacks (*Gasterosteus aculeatus*). *Nature* 275:642–644.

ROSE, R.K., and BIRNEY, E.C. 1985. Community ecology, pp. 310–339, *in* R.H. Tamarin (ed.). Biology of New World *Microtus*. Special Publication No. 8. The American Society of Mammalogists.

SIH, A. 1980. Optimal behavior: Can foragers balance two conflicting demands? *Science* 210:1041–1043.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry, 2nd ed. W.H. Freeman, New York.

STEPHENS, M.A. 1982. Use of the von Mises distribution to analyze continuous proportions. *Biometrika* 69:197–203.

STODDART, D.M. 1976. Effect of the odour of weasels (*Mustela nivalis* L.) on trapped samples of their prey. *Oecologia* 22:439–445.

STODDART, D.M. 1980. Some responses of a free living community of rodents to the odours of predators, pp.1–10, *in* D. Mueller-Schwarz and R.M. Silverstein (eds.). Chemical Signals: Vertebrates and Aquatic Invertebrates. Plenum Press, New York.

SULLIVAN, T.P. 1986. Influence of wolverine (*Gulo gulo*) odor on feeding behaviour of snowshoe hares (*Lepus americanus*). *J. Mammal.* 67:385–388.

SULLIVAN, T.P., and SULLIVAN, D.S. 1982. Barking damage by snowshoe hares and red squirrels in lodgepole pine stands in central British Columbia. *Can. J. For. Res.* 12:443–448.

SULLIVAN, T.P., and SULLIVAN, D.S. 1986. Impact of feeding damage by snowshoe hares on growth of juvenile lodgepole pine in central British Columbia. *Can. J. For. Res.* 16:1145–1149.

SULLIVAN, T.P., NORDSTROM, L.O., and SULLIVAN, D.S. 1985a. Use of predator odors as repellents to reduce feeding damage by herbivores: I. Snowshoe hares (*Lepus americanus*). *J. Chem. Ecol.* 11:903–919.

SULLIVAN, T.P., NORDSTROM, L.O., and SULLIVAN, D.S. 1985b. Use of predator odors as repellents to reduce feeding damage by herbivores: II. Black-tailed deer (*Odocoileus hemionus columbianus*). *J. Chem. Ecol.* 11:921–935.

SULLIVAN, T.P., CRUMP, D.R., and SULLIVAN, D.S. 1988a. Use of predator odors as repellents to reduce feeding damage by herbivores: III. montane and meadow voles (*Microtus montanus* and *Microtus pennsylvanicus*). *J. Chem. Ecol.* 14:363–377.

SULLIVAN, T.P., CRUMP, D.R., and SULLIVAN, D.S. 1988b. Use of predator odors as repellents to reduce feeding damage by herbivores: IV. northern pocket gophers (*Thomomys talpoides*). *J. Chem. Ecol.* 14:379–389.

TAITT, M.J., GIPPS, J.H.W., KREBS, C.J., and DUNDJERSKI, J. 1981. The effect of extra food and cover on declining populations of *Microtus townsendii*. *Can. J. Zool.* 59:1593–1599.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

INTRASPECIFIC VARIATION AND INTERSPECIFIC DIFFERENCES IN SEX PHEROMONES OF SIBLING SPECIES IN *Ctenopseustis obliquana*[1] COMPLEX

J.R. CLEARWATER,[2,*] S.P. FOSTER,[2] S.J. MUGGLESTON,[2] J.S. DUGDALE,[2] and E. PRIESNER[3]

[2]*DSIR Plant Protection*
Mt. Albert Research Centre
Private Bag, Auckland, New Zealand

[3]*Max-Planck-Institute für Verhaltensphysiologie*
D-8131 Seewiesen, Germany

(Received August 9, 1990; accepted October 9, 1990)

Abstract—The specific status of *Ctenopseustis obliquana* pheromone-types I, II, and III has been more fully examined. Females of types I and III produce a mixture of (*Z*)-8- and (*Z*)-5-tetradecenyl acetates (Z8–14 : Ac, Z5–14 : Ac). The previously reported different ratios of these two components in females of the two types (type I = 80 : 20, III = 90 : 10) have been reinvestigated. The median ratios of each type differed significantly, although there was some overlap in the ranges of these ratios. A field cage trial showed that males of type III are attracted to females of type I, so the observed differences may be ascribed to intraspecific variation. In contrast to females of types I or III, females of type II produce Z5–14 : Ac but no Z8–14 : Ac. The electroantennogram (EAG) profile of antennae of type II males shows a maximum response to Z5–14 : Ac, while the EAG profiles of types I and III show a strong response to Z8–14 : Ac. In wind tunnel tests using mixtures of these two compounds, type II males prefer blends consisting of all or mostly Z5–14 : Ac, while type I males showed a preference to a mix of 70% Z8–14 : Ac plus 30% Z5–14 : Ac. We found that type I males are attracted to type I females when offered a choice between type I and type II females in a field cage test and that type II males similarly prefer type II females. Males of types I and II have specialist cells for Z8–14 : Ac and Z5–14 : Ac but differ with respect to relative densities of these cells and to further cell types responsive to other alkenyl acetates. Type II *C. obliquana* is considered therefore a sibling species of types I and III. In addition, the amount of Z5–14 : Ac produced by type II females varied geographically. Females from the North

*To whom correspondence should be addressed.

[1]Lepidoptera: Tortricidae: Tortricinae.

Island produced significantly less (median = 1.2 ng) Z5–14:Ac than females from the South Island (median = 2.2 ng). Type II populations in the North Island morphologically resembled sympatric type I, rather than type II from the South Island and are designated as type II (North Island). Type II (North Island) populations have so far been found only at Rukuhia (near Hamilton) and from Kerikeri. In contrast, type II populations are sympatric with type III populations over much of the South Island. In a field cage trial, males of types II and II (North Island) were attracted to females of both II and II (North Island). We tentatively ascribe the differences between type II and type II (North Island) to intraspecific variation.

Key Words—*Ctenopseustis obliquana*, Tortricinae, Lepidoptera, pheromones, (*Z*)-5-tetradecenyl acetate, (*Z*)-8-tetradecenyl acetate, sibling species, blend preferences, electrophysiology, intraspecific variation.

INTRODUCTION

In New Zealand, members of the endemic brownheaded leafroller (BHLR) complex, *Ctenopseustis obliquana* (of authors), are primary pests of horticultural crops (Wearing et al., 1990). Prior to 1984, *C. obliquana* was regarded as a single species (Green and Dugdale, 1982). Studies of the female-produced pheromone of a population from Mt. Albert (Auckland) had identified two components, (*Z*)-8-tetradecenyl acetate and (*Z*)-5-tetradecenyl acetate (*Z*8–14:Ac, *Z*5–14:Ac) in a 4:1 ratio (Young et al., 1985).

Between 1983 and late 1985, BHLR populations from 30 localities from Northland (ND) to Stewart Island (Figure 1) were sampled. Sex pheromone analyses of laboratory-reared females showed that BHLR included "at least three and possibly four sibling species" (Foster et al., 1986) that could be differentiated by sex pheromone components or ratios in the pheromone glands of female moths. These sibling species were called *Ctenopseustis obliquana* types I, II, III, and IV (Foster et al., 1986). The initial separation into four types was based on differences in ratio between *Z*8- and *Z*5–14:Ac (types I, III); presence of *Z*5–14:Ac (type II), and presence of a large quantity of (*Z*)-10-hexadecenyl acetate (*Z*10–16:Ac) (type IV) (Foster et al., 1986; Foster and Roelofs, 1987). It was later shown that the less common type II populations from the North Island differ morphologically from the South Island type II populations (Dugdale, 1990).

The differences perceptible to a human observer may not necessarily be important to male and female moths during courtship and mating. In this paper we examine the pheromone systems of types I, II, II(North Island), and III of BHLR. The components of the pheromone system investigated include female pheromone production, responses of male antennal receptor cells, blend preferences of males flying in a wind tunnel, and the preferences for females by

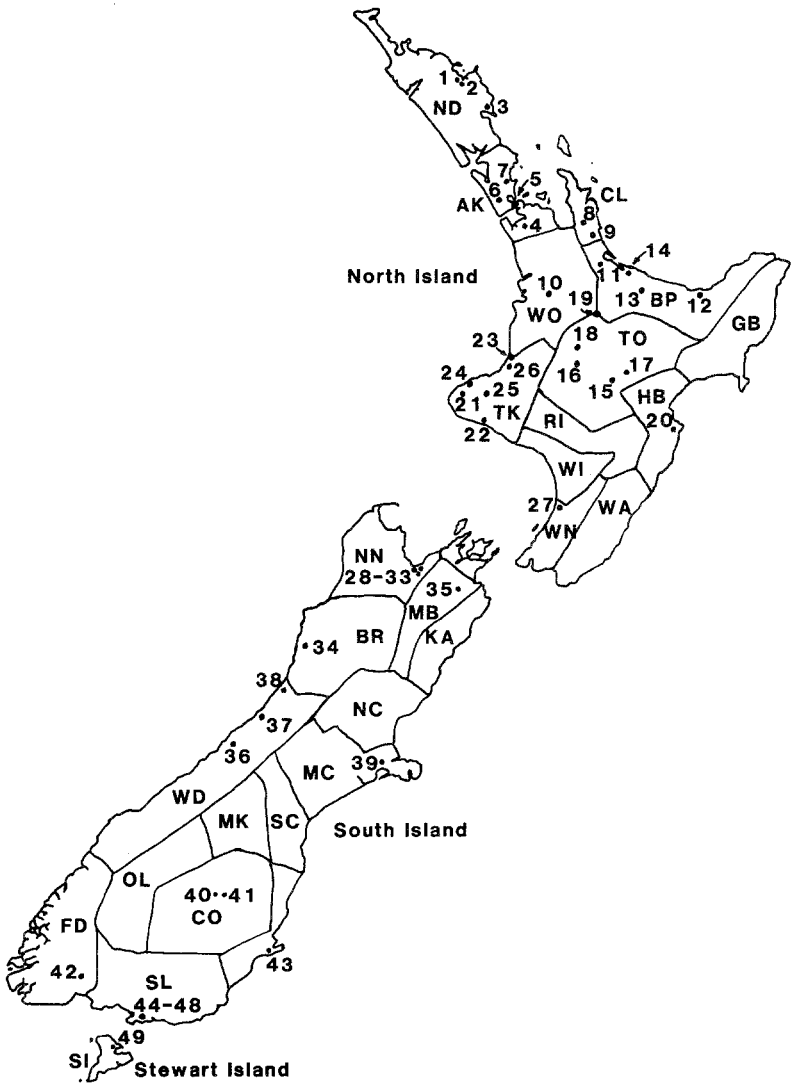


FIG. 1. Map of New Zealand with regional collection codes as defined by Crosby et al. (1976). Collection sites listed in Table 1 are numbered 1–49.

males flying freely in a field cage. These tests have enabled us to distinguish between differences that greatly affect mate choice and differences that do not appear to significantly affect mate choice.

METHODS AND MATERIALS

Collection and Rearing of Insects. Larvae were collected from localities listed in Table 1 or taken from laboratory colonies. Our main laboratory colonies of type I, originally from Mt. Albert, Auckland, of type II originally from Tai Tapu (near Christchurch), of type II(North Island), originally from Rukuhia

TABLE 1. LOCALITIES AT WHICH BROWNHEADED LEAFROLLER TYPES WERE COLLECTED, 1983–1986[a]

Area		Locality	Type	Area		Locality	Type
ND	1	Kerikeri°	I, II	TK	26	Tainui Reserve°	I
	2	Mangināngā°	I	WN	27	Levin°	I
	3	Whangarei°	I	NN	28	Appleby°	II, III
AK	4	Manurewa	I		29	Clarke River°	III
	5	Mt Albert	I		30	Hope Saddle°	III
	6	Mt Auckland°	I		31	Moutere Hill°	III
	7	Wenderholm°	I		32	Richmond°	II, III
CL	8	Kauaeranga	I		33	Wairoa Gorge	III
	9	Mt Te Aroha	I	BR	34	Bullock Creek°	III
WO	10	Rukuhia	II	MB	35	Onamalutu	III
BP	11	Katikati°	I	WD	36	Franz Josef°	II, III
	12	Opotiki°	I		37	Lake Ianthe°	II, III
	13	Otaramarae°	I		38	Nelson Creek°	II, III
	14	Te Puke°	I	MC	39	Tai Tapu	II
	15	Pouakanui N.	I	CO	40	Earnscleugh	III
TO	16	Burgess' Bog	I		41	Dunstan Road°	III
	17	Kaimanawa N	I	FD	42	Lake Hauroki°	III
	18	Pureora	I	DN	43	Dunedin°	II, III
	19	Tokoroa	I	SL	44	Bluff Hill (summit)	II
HB	20	Havelock N°	I		45	Bluff Hill (forest)	II, III, IV
TK	21	Lucy's Gully°	I		46	Forest Hill	II
	22	Manaia°	I		47	Lake Wilkie	II, III
	23	Mokau°	I		48	Tautuku°	II, III, IV
	24	New Plymouth	I	SI	49	Horseshoe Bay, Little River Oban°	II, III, IV
	25	N. Egmont	I				

[a]Two-letter area codes, e.g., AK, as in Crosby et al. (1976); ° = localities relevant to Foster et al. (1986).

(near Hamilton), and of type III, from Alexandra, were the sources of insects used in the various bioassays unless otherwise specified. Larvae were reared on a semisynthetic diet (Young et al., 1985).

Male and female pupae were incubated separately and underwent different temperature regimes depending on their planned use. Females for use in gas–liquid chromatographic (GLC) analyses of the sex pheromone gland and males for use in wind-tunnel experiments were kept under a reverse-phase light cycle (16:18 light–dark) at 18°C. Insects used in the field cage experiments were maintained on a normal phase light cycle (16:18 light–dark). Temperatures were manipulated in order to synchronize the emergence of adults for use in these field cage experiments. For example, for the comparison of types I and III, a group of larvae from the type I culture were reared at 22°C. The larvae for the type III group were reared at 20°C during early development, followed by periods at 15°C and 10°C. For all field cage experiments, male pupae and the resulting adults were conditioned outdoors in a screen cage sheltered by shrubs; the adults were allowed to feed on 10% honey solution during the conditioning period. Males were released into the field cage when they were 2–3 days old. For the experiment comparing responses of males of types I, II, and II(North Island) to caged females, female pupae underwent the same conditioning as the male pupae. Female pupae used in the earlier type I vs. type III experiment were taken from stocks held under ambient laboratory conditions. Females were fed with 10% honey solution applied to cotton wool plugging the entrance hole of the females container. During the type I vs. II vs. II(North Island) comparison, the honey solution and the feeding substrate were replaced daily to preclude the possibility that fermented food was affecting female behavior.

Capillary Gas Chromatographic Analyses. Pheromone gland epithelia were dissected from 3- to 6-day-old females, approximately 1 hr into the scotophase. Epithelia were individually extracted in 5–10 μl of distilled *n*-pentane for approximately 24 h at ambient temperature. Extracts were analyzed using a Pye Unicam 4500 gas chromatograph with splitless injector and a flame ionization detector. Capillary columns and temperature programs used were: a Quadrex Corporation (New Haven, Connecticut) 50 m × 0.25 mm ID CPS-1 (bonded SP2340 equivalent), at a program of 100–180°C/min after an initial delay of 1 min, and a 50 m × 0.32 mm ID (SGE Ltd., Victoria, Australia) BP-20 (cross-linked polyethylene glycol), at a program of 100–200°C at 4°C/min (delay of 1 min). Nitrogen was used as carrier gas, at a linear flow velocity of 20 cm/sec. Tridecanyl acetate was added to the extracts as an internal standard. Wilcoxon rank-sum tests were used to compare differences in pheromone production between the various groups.

Electrophysiology. The EAG responses of antennae of adult male *C. obliquana* to synthetic samples of monounsaturated acetates and alcohols (10 μg

on filter paper) were determined according to the procedures of Baker and Roelofs (1976). A normalized response profile was produced by comparison of each test response with the response to (*E*)-11-tetradecenyl acetate (*E*11–14:Ac) (10 μg on filter paper) presented alternately. Single cell analyses were carried out at Seewiesen according to the method of Priesner (1983).

Type I and II males were tested by the EAG and single cell analysis techniques. Type III males (Onamalutu Reserve, Marlborough) were tested with the EAG technique only.

Wind-Tunnel Experiments. Wind-tunnel design and techniques used are as described by Clearwater and Triggs (1991). Pheromone sources were rubber septa (Arthur H. Thomas Co., Philadelphia, Cat. No. 1780-B10) each loaded with a total of 1 μg of *Z*5–14:Ac and *Z*8–14:Ac in different ratios (see Figure 7 below). The two chemicals were synthesized by M.H. Benn (Department of Chemistry, University of Calgary, Alberta) and were greater than 98% isomerically pure. The numbers of type I and type II males flying in the plume of the chemicals and landing near the chemical source were recorded.

Field Cage Experiments. If the observed differences in pheromone production of the females from different regions are significant to males, then this may be detected by experiments in which males flying freely in a field cage are offered a choice of females. Females were placed in labeled wood/mesh containers (5 × 5 × 5 cm). The transfers were accomplished without the use of cold immobilization or CO_2 anesthetization. Each container was positioned in the center of a sticky tap (Pherocon IC, Zoecon Corporation, Palo Alto, California) and set out in a 10-m × 10-m × 2.4-m-high field cage. Males from the different localities were lightly dusted with contrasting dye powder, which could be discerned under UV illumination (long wavelength setting, "Chromato vue," Ultra-violet Products, California). The dyes used were from Dayglo Color Division, Switzer Bros. (Cleveland, Ohio). Traps were rerandomized in position daily. At the completion of each trial, the virgin status of each female was confirmed by examination of the bursa copulatrix.

For the type I vs. type III experiment, four containers each with three 2-day-old type III females, and four containers each with the same number of 2- to 3-day-old type I females were set out in sticky traps in a 3 × 3 array, with a single empty trap as a blank. Over the next four days, males were released into the field cage in the late afternoon. Males caught in the study traps were removed for identification (under UV light; to prevent transfer of dye powder between captured males, a fresh wooden spatula was used for each individual). For the type I vs. II vs. II(North Island) experiment, freshly emerged females were used. Females were caged singly, with five containers per type. Traps were hung in a 4 × 4 array (five of each population and a single blank trap). Other details were as for the preceding experiment. Each set of females in con-

tainers was tested for four nights, before being replaced with a fresh set of females for a further four nights.

RESULTS

Pheromone Gland Analyses. Of the 268 individually analyzed females originally collected as larvae from the field, 190 had both Z8- and Z5-14:Ac present, i.e., were either type I or type III of Foster et al. (1986); the remaining 78 had only Z5-14:Ac, i.e., were type II in the sense of Foster et al. (1986). Data from the group of females producing Z5- and Z8-14:Ac were divided into North (type I) and South and Stewart Island (type III) subgroups. Pheromone quantity (i.e., total amount of pheromone in the gland) did not differ between the two subgroups, the median quantities produced being 1.7 ng for both groups. Median pheromone quantity of the type I laboratory colony was the same, 1.7 ng ($N = 35$ females).

The frequency of ratios of Z8-14:Ac is presented in Figure 2. The two groups of females classified as type I and type III have significantly different median ratios ($P < 0.01$) of 84:16 ($N = 114$) and 93:7 ($N = 76$), respectively. However, there is some overlap of the respective ranges of ratios (Figure 2). Females of type III with the consistently lowest proportion of Z5-14:Ac (95:5, Z8:Z5) were collected from Bullock Creek (BR), Nelson Creek, Lake Ianthe, and Franz Josef (WD) ($N = 20$) (see Table 1, Figure 1). The 35 type I females from the laboratory colony (Figure 2C) gave a median ratio of 76:24 (range 94:6–69:31), i.e., a slightly higher Z5-14:Ac content in the pheromone than in the feral type I (North Island) samples.

The data from the type II females were divided into North Island and South and Stewart Island subgroups, types II(North Island) and II, respectively (Figure 3). Pheromone quantity from the two subgroups differed significantly ($P < 0.01$). Type II(North Island) females produced a median of 1.2 ng ($N = 16$), and type II females produced a median of 2.2 ng ($N = 62$).

Electrophysiology. The EAG response profiles of antennae from type I males (Figure 4) could easily be distinguished from those from type II males (Figure 5). The greatest responses of type I antennae stimulated by tetradecenyl acetates were observed to Z8-14:Ac and (*E*)-8-tetradecenyl acetate (*E*8-14:Ac). The response to Z5-14:Ac was lower than that to (*Z*)-7-tetradecenyl acetate (Z7-14:Ac), (*Z*)-11-tetradecenyl acetate and the internal standard, *E*11-14:Ac. Hexadecenyl acetates were generally less stimulatory; (*Z*)-8-hexadecenyl acetate and to a lesser extent Z10-16:Ac elicited the greatest EAG responses of this series.

In the type II population, the greatest EAG responses observed were to

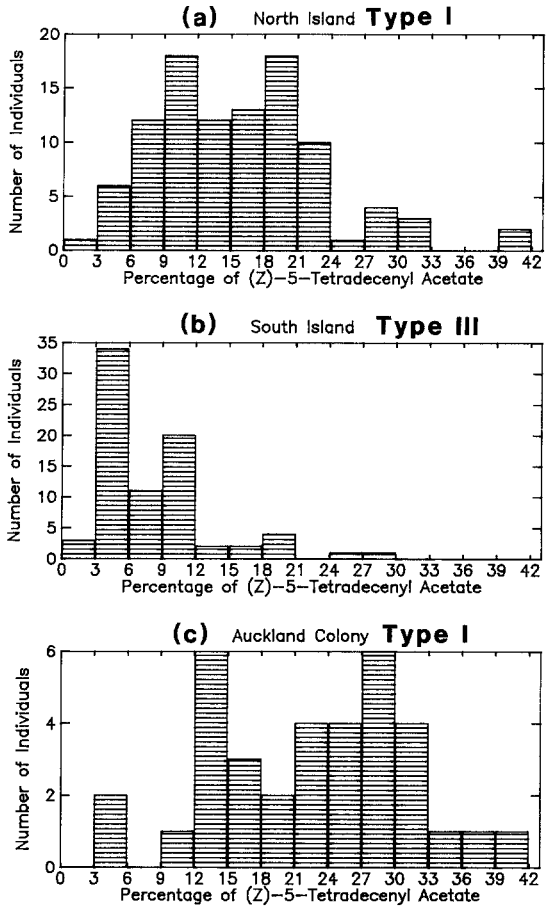


FIG. 2. Distribution of percentages of (*Z*)-5-tetradecenyl acetate in sex pheromone glands of *C. obliquana* [remaining percentage is (*Z*)-8-tetradecenyl acetate], (a) from the North Island (type I) (b) South Island (type III), and (c) the Auckland laboratory colony (type I).

(*Z*)-5-unsaturation in both the tetradecenyl and hexadecenyl acetate series. The response to *Z*8–14 : Ac was always low, indistinguishable from the response to most of the other *Z* isomers, and much smaller than the corresponding response observed from type I antennae.

The observed antennal responses (Figure 6) of type III males collected as larvae from Onamalutu Reserve (Marlborough) resembled the responses of type I (Figure 4), rather than type II males (Figure 5).

Single-cell analysis of sensilla trichodea on type I and type II male anten-

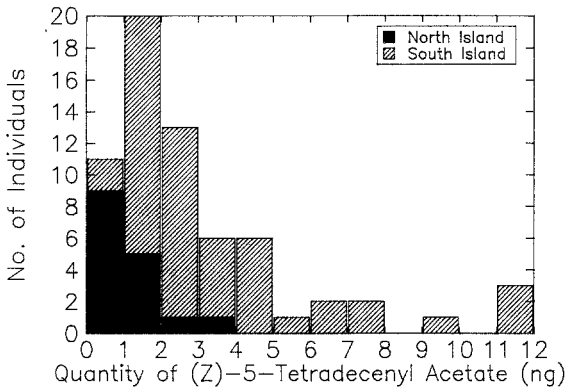


FIG. 3. Distribution of quantity of (*Z*)-5-tetradecenyl acetate in sex pheromone glands of individual females of type II *C. obliquana* from the South Island (shaded bars) and type II from the North Island (solid bars). Median values for the type II(South Island) sample = 2.2 ng, for type II(North Island) = 1.2 ng.

nae revealed the presence on both of specialist cells for *Z*8–14:Ac and *Z*5–14:Ac. The *Z*8–14:Ac cells of type I and the *Z*5–14:Ac cells of type II males consistently showed large amplitude spikes; the *Z*5–14:Ac cells of type I and *Z*8–14:Ac cells of type II males were recorded less frequently and showed spikes of small amplitude. In males of both types, three additional, less common, cell types, responsive to other alkenyl acetates were also discovered. The additional cells found on antennae of type I males responded specifically to *E*8–14:Ac, (*Z*)-8-dodecenyl acetate and (*E*)-8-dodecenyl acetate, while the additional cells on antennae of type II males responded to *Z*7–14:Ac, *E*8–14:Ac, and (*Z*)-10-tetradecenyl acetate.

Wind-Tunnel Experiments. Males from the type I and type II populations responded differently to a range of blends of *Z*5–14:Ac plus *Z*8–14:Ac presented in the wind tunnel (Figure 7). Sources with 100% or 80% *Z*5–14:Ac were significantly more attractive to type II males than sources with 10, 20, or 30% *Z*5–14:Ac ($P < 0.05$, contingency χ^2 analysis). The converse was true for type I males. The response profiles overlapped, with a small number of males capable of flying and landing in response to the optimal blend of the other type.

Field Cage Experiments. In experiments of type I vs. type III, traps baited with type I females caught more type I and III males than traps baited with type III females ($P < 0.05$, ANOVA followed by Fisher's protected LSD test on data transformed to their $\sqrt{x} + 0.5$ values) (Table 2). The catches of the type III females were not significantly different ($P > 0.05$) from the catch of the blank trap. The rate of recapture was low (39% of the type I, 18% of the type

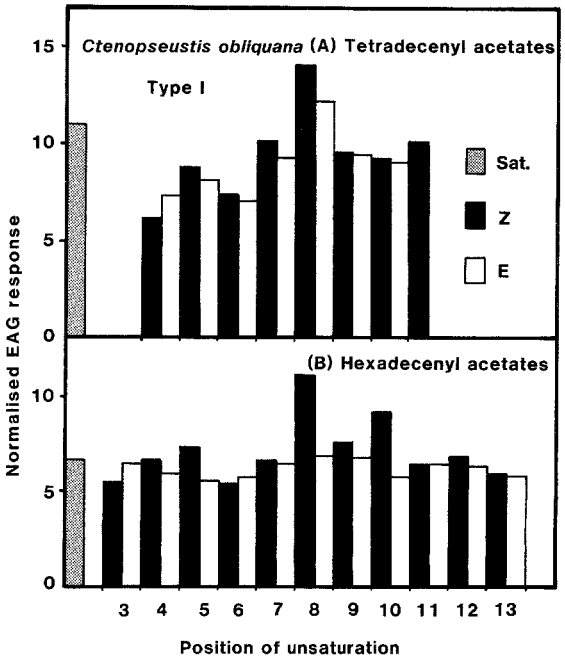


FIG. 4. Electroantennogram responses of antennae from male *C. obliquana* (type I). The profiles are each the average of 10 replicates and show the response to tetradecyl acetate or hexadecyl acetate (Sat) and various tetradecenyl and hexadecenyl acetate isomers. The moths were obtained from a culture of larvae collected from Mt. Albert, Auckland (AK).

III males). The catches of the traps were variable; three of the eight groups of three type I females caught 96% (44/46) of the males.

For type I vs. type II vs. type II(North Island) experiments, type I males were mostly caught in traps baited with type I females (Table 3). Traps baited with type II or II(North Island) females each caught nine [6 ± 2.4% (SE)] of the type I males compared with 125 (87 ± 3.4%) captured by traps baited with the type I females. The catches of type I males in traps baited with the type II and II(North Island) females were not significantly different ($P > 0.05$) from the blank trap catch. The number of males caught in each type I trap varied from 1 to 51.

Traps baited with type II(North Island) females caught very similar numbers of type II(North Island) (50) and type II (53) males. The traps with type II females also caught type II and type II(North Island) males in an almost 1 : 1 ratio (Table 3). However, the traps baited with type II(North Island) females

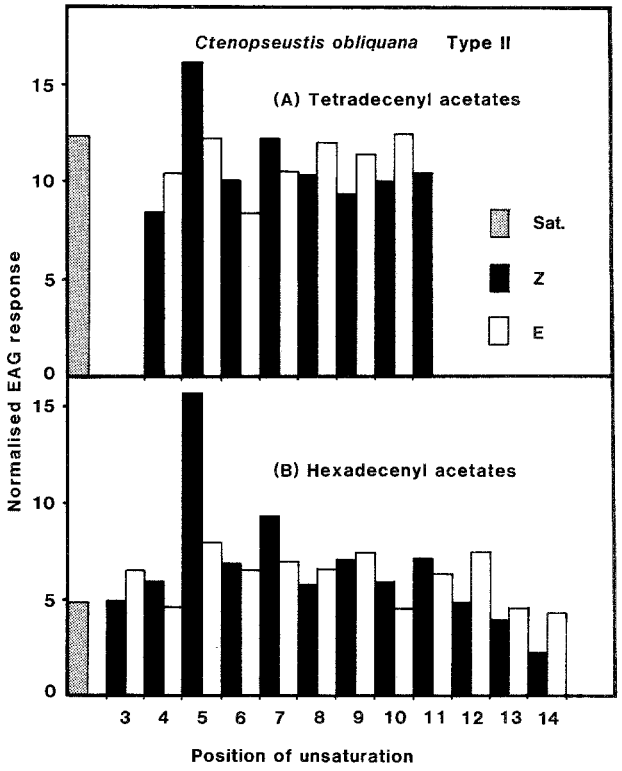


FIG. 5. Electroantennogram responses (as in Figure 4) of antennae of male *C. obliquana* (type II). The profiles are the average of (A) 10 and (B) two replicates. The moths were obtained from larvae collected at Tai Tapu near Christchurch (MC).

captured significantly more males (103) of these two groups than those with type II females (47) ($P < 0.05$, fitted log-linear model).

Of the total number of males released, greater than half were recaptured. Type I males were recovered in greatest numbers (73%) with type II and type II(North Island) males recovered in similar proportions (46% and 49%, respectively).

Distribution. We obtained collections from 49 localities around New Zealand (Table 1, Figure 1). While these are insufficient to establish the boundaries of the populations studied, they suggest that the degree of contact between Z8–14:Ac plus Z5–14:Ac populations (types I/III) and Z5–14:Ac populations [types II, II(North Island)] differed in the North and South Island. In the South Island almost half the collection sites provided individuals of type III and type II, while in the North Island, type II populations were found only in two sites,

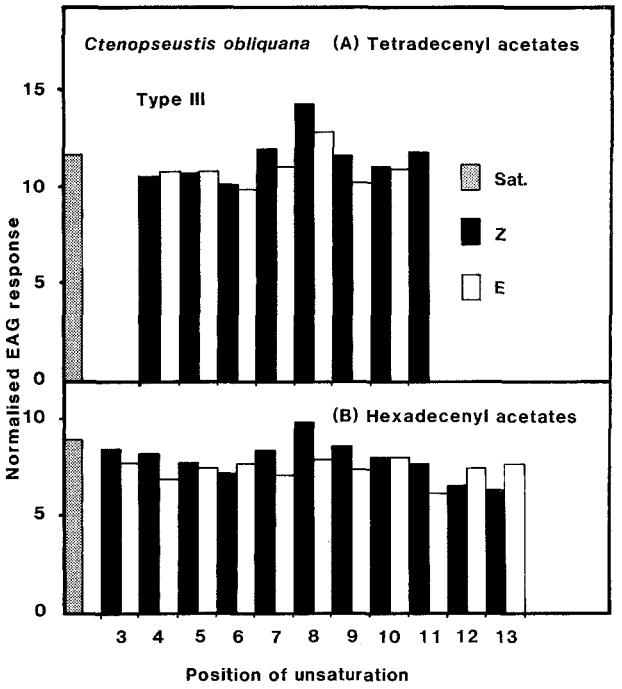


FIG. 6. Electroantennogram response (as in Figure 4) of antennae of male *C. obliquana* (type III). The profiles are the average of (A) 10 and (B) five replicates. The moths were obtained from larvae collected at Onamalutu Reserve (MB).

and only at Kerikeri were they sympatric with type I. Individuals of both types were collected on the same host at the same time of year at Kerikeri.

DISCUSSION

Our preliminary investigation divided *C. obliquana* (sensu Green and Dugdale, 1982) into types I, II and III, primarily on the basis of differing chemical content of the female pheromone gland (Foster et al., 1986). The additional data reported here support the earlier separation of type II from types I and III. Males and females of type II can be distinguished from their types I and III counterparts on the basis of content of female pheromone glands, EAG response profiles of male antennae, spike amplitudes of Z8–14 : Ac and Z5–14 : Ac specialist cells on the antennae of males, preferences for specific blends in wind-tunnel tests and preferences of males for females of their own type in field-cage tests. We conclude that these differences indicate that type II is a sibling species

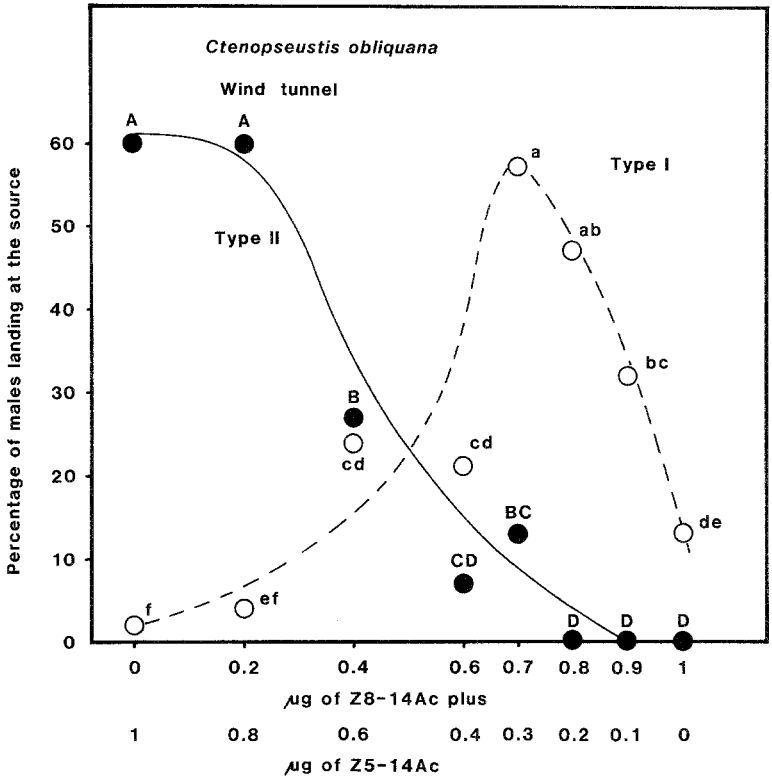


FIG. 7. Landing response profiles of type I (○) (Auckland) and type II (●) (Tai Tapu) to mixtures of Z5–14 : Ac plus Z8–14 : Ac (total 1 µg). Forty-seven type I males were flown to each septum. Thirty type II males were flown to each septum. Treatments accompanied by the same letter are not significantly different ($P < 0.05$). $\chi^2 = 72.94$ (type I) and = 101.471 (type II).

of types I and III. Nomenclatural changes consequent to this are dealt with elsewhere (Dugdale, 1990).

Greater sampling of populations has shown that type I and type III *C. obliquana*, while not as different as indicated in Foster et al. (1986), remain distinguishable on the basis of the median ratio of Z5–14 : Ac to Z8–14 : Ac. As the ranges of this ratio overlap, it would not be appropriate to distinguish between them nomenclaturally as a given idividual could not be assigned with confidence to either type I or III. Our field-cage experiments indicated that males both from Alexandra (type III) and from Auckland (type I) are attracted to Auckland females. However, males of neither type were attracted to Alexandra females, suggesting that either males of both types preferred the type I

TABLE 2. FIELD CAGE TEST: MALE *Ctenopseustis obliquana* CAUGHT IN TRAPS, BAITED WITH THREE VIRGIN FEMALES FROM AUCKLAND (TYPE I) OR ALEXANDRA (TYPE III) LABORATORY CULTURES[a]

	No. of Auckland males caught (type I)	No. of Alexandra males caught (type III)
Auckland females (type I)	23 a	23 a
Alexandra females (type III)	2 b	1 b
Blank	0 b	0 b
No. of males released	59	125

[a] Sum of two trials conducted January 11–17, 1986, at Mt. Albert Research Centre, Auckland. Numbers followed by the same letter are not significantly different at $P < 0.05$ (ANOVA followed by Fisher's protected lsd test on data transformed to their $\sqrt{x} + 0.5$ value).

TABLE 3. FIELD CAGE TEST: MALE *Ctenopseustis obliquana* CAUGHT IN TRAPS BAITED WITH SINGLE VIRGIN FEMALE FROM AUCKLAND (TYPE I), RUKUHIA (TYPE II, NORTH ISLAND), OR TAU TAPU (TYPE II) LABORATORY CULTURES[a]

	No. of Auckland males caught (type I)	No. of Rukuhia males caught (type II North Island)	No. of Tai Tapu males caught (type II)
Auckland females (type I)	125 a (87% ± 3.4%)	15 c,d (17% ± 4.9%)	4 d (5% ± 3.0%)
Rukuhia females (type II North Island)	9 d (6% ± 2.4%)	50 b (57% ± 6.5%)	53 b (65% ± 6.5%)
Tai Tapu females (type II)	9 d (6% ± 2.4%)	23 c (26% ± 5.7%)	24 c (30% ± 6.2%)
Blank	5 d	0 d	3 d
Numbers of males released	202	178	182

[a] Sum of two trials conducted February 27, 1986, and March 7, 1986, at Mt. Albert Research Centre, Auckland. Numbers followed by the same letter are not significantly different at $P < 0.05$ (fitted log-linear model).

females or that the Alexandra females were not releasing pheromone. If these females did not call, the artificial temperature manipulation conducted in order to synchronize populations could have been responsible. The two cultures also differed in the number of generations that had experienced laboratory conditions

(23 generations for type I, one for type III). Despite these reservations, the data suggest that types I and III are variant populations of a single species.

South Island type II populations are distinguishable from the sympatric type III populations on details of morphology, in addition to the above differences in the pheromone. However, type II(North Island) populations cannot be distinguished morphologically from type I populations (Dugdale, 1990). Differences were detected between attractiveness of the type II and II(North Island) females in the field cage bioassays, and this is paralleled by a difference in the pheromone content of the gland. These differences do not appear to be sufficient for specific status for both II and II(North Island) populations. Our conclusion that these two populations are variant populations of the same species is, however, tentative.

Specialist cells for Z8–14 : Ac are very common in the sensilla trichodea on the antennae of type I males, while specialist cells for Z5–14 : Ac are common in type II males. The large numbers of each specialist and their large spike amplitude determine the shape of the EAG profile for each type. This relationship between the number of specialist cells and the shape of an EAG profile has been demonstrated previously by Van der Pers and Löfstedt (1986). Z8–14 : Ac specialist cells in type II males and Z5–14 : Ac specialist cells in type I males are insufficiently common to clearly influence the EAG profile of their types. Although uncommon, the Z5–14 : Ac specialist cells of type I males clearly have an important role in mediating flight behavior. Sources lacking Z5–14 : Ac are much less attractive to males of type I (Figure 7). No function has yet been discovered for the Z8–14 : Ac specialists in type II males or for the other alkenyl acetate receptor cells found in both type I and type II males. Single-cell recordings have not yet been made from type III males, although the similarity of types I and III in EAG profiles (Figures 4 and 6) suggests that males of these two populations may share a similar set of antennal receptor cells.

Previous researchers have had little success when using laboratory-reared females of some New Zealand species to capture feral males. R. Galbreath and D. Catt (personal communication) recorded very low catches (0–4) of males when *Ctenopseustis obliquana* (sensu lato) and *Planotortrix excessana* (sensu lato) females were placed in gauze cages in sticky traps. Galbreath et al. (1985) consequently chose to use female extracts when comparing the responses of males from populations of *Planotortrix excessana* from Tokoroa and Lincoln. Our first experiment using live females in cages (Table 2) substantiated the difficulties encountered by Galbreath and Catt (personal communication). Very few of the traps baited with females caught males, even though GLC analyses of females handled similarly showed that these females produced the two pheromone components. Other authors have similarly reported failure of laboratory-reared females to attract males into traps, e.g. *Paralobesia viteana* (Clemens), the grape berry moth (Taschenberg et al., 1974). Our care in keeping the female

pupae in a cool shaded position outside, daily replacing of cotton wool and honey solution, and using newly emerged females without a one- or two-day maturing period probably contributed to the production of a much more attractive group of females for our second experiment.

We aim to study the differences in the pheromone systems of these sibling species more fully in order to investigate possible evolutionary processes.

Acknowledgments—We thank our colleagues and friends for providing collections of leafroller larvae from many parts of New Zealand; Dr. Wendell Roelofs (Cornell University, Geneva, New York) for advice and encouragement, and Dr. Yoshio Tamaki, Department of Sericulture, MAFF, Tsukuba, Japan, for helpful discussions. We thank Michael Benn of University of Calgary, Canada, for supplying Z5-14 : Ac and Z8-14 : Ac. Pritam Singh and David Roger (Rearing Group, DSIR) provided pupae of *C. obliquana* type I. Statistical analyses or advice were provided by John Maindonald and Chris Triggs, AMD Auckland, and W.C. Black, Kansas State University, Kansas.

REFERENCES

BAKER, T.C., and ROELOFS, W.L. 1976. Electroantennogram responses of the male moth *Argyrotaenia velutinana* to mixtures of sex pheromone components of the female. *J. Insect Physiol.* 22:1357-1364.

CLEARWATER, J.R., and TRIGGS, C.M. 1991. Pheromone blend discrimination by sexually active male *Ctenopseustis obliquana* is dependent on bioassay conditions. In preparation.

CROSBY, T.K., DUGDALE, J.S., and WATT, J.C. 1976. Recording specimen localities in New Zealand. *N.Z. J. Zool.* 3:69.

DUGDALE, J.S. 1990. Reassessment of *Ctenopseustis* Meyrick and *Planotortrix* Dugdale with descriptions of two new genera (Lepidoptera: Tortricidae). *N.Z. J. Zool.* 18:3.

FOSTER, S.P., and ROELOFS, W.L. 1987. Sex pheromone differences in populations of the brownheaded leafroller *Ctenopseustis obliquana*. *J. Chem. Ecol.* 13:623-629.

FOSTER, S.P., CLEARWATER, J.R., MUGGLESTON, S.J., DUGDALE, J.S., and ROELOFS, W.L. 1986. Probable sibling species complexes within two described New Zealand leafroller moths. *Naturwissenschaften* 73:S.156.

GALBREATH, R.A., BENN, M.H., YOUNG, H., and HOLT, V.A. 1985. Sex pheromone components in New Zealand greenheaded leafroller *Planotortrix excessana* (Lepidoptera: Tortricidae). *Z. Naturforsch.* 40c:266-271.

GREEN, C.J., and DUGDALE, J.S. 1982. Review of the genus *Ctenopseustis* Meyrick (Lepidoptera: Tortricidae), with reinstatement of two species. *N.Z. J. Zool.* 9:427-436.

PRIESNER, E. 1983. Receptors for di-unsaturated pheromone analogues in the male summerfruit tortrix moth. *Z. Naturforsch.* 38c:874-877.

TASCHENBERG, E.F., CARDÉ, R.T., HILL, A., TETTE, J.P., and ROELOFS, W.L. Sex pheromone trapping of the grapeberry moth. *Environ. Entomol.* 3:192-194.

VAN DER PERS, J.N.C., and LÖFSTEDT, C. 1986. Chap. 27 Signal-response relationships in sex pheromone communication in mechanisms, pp. 235-241, *in* Insect Olfaction. T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Clarendon Press, Oxford.

WEARING, C.H., THOMAS, W.P., DUGDALE, J.S., and DANTHANARAYANA, W. 1990. Australian and New Zealand species, *in* Tortricoid Pests, Their Biology, Natural Enemies and Control. Elsevier, Amsterdam. In press.

YOUNG, H., GALBREATH, R.A., BENN, M.H., HOLT, V.A., and STRUBLE, D.L. 1985. Sex pheromone components in the New Zealand brownheaded leafroller *Ctenopseustis obliquana* (Lepidoptera: Tortricidae). *Z. Naturforsch* 40c:262–265.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

EXPERIENCE EARLY IN LIFE AFFECTS VOLUNTARY INTAKE OF BLACKBRUSH BY GOATS

R.A. DISTEL and F.D. PROVENZA*

Range Science Department
Utah State University
Logan, Utah 84322-5230

(Received July 23, 1990; accepted October 10, 1990)

Abstract—Low nutritional quality and high levels of condensed tannins adversely affect voluntary intake of blackbrush (*Coleogyne ramosissima* Torr.) by goats. We studied: (1) how consumption of blackbrush or alfalfa pellets by young goats affected their consumption of those foods later in life, and (2) whether previous ingestion of blackbrush or alfalfa pellets affected the excretion of condensed tannins and total phenols from blackbrush in urine and feces, production of proline-rich proteins in saliva, excretion of nitrogen in feces, and mass of the liver, kidneys, parotid glands, and reticulorumen in goats. From 6 to 26 weeks of age, experienced goats were exposed to blackbrush, while inexperienced goats ate alfalfa pellets. Following exposure, both groups were offered older-growth blackbrush twigs (OG) or a choice between OG and current season's blackbrush twigs (CSG). A similar feeding trial was repeated nine months after exposure, and, in addition, both groups were offered a choice between OG ad libitum and alfalfa pellets at six levels of availability. Immediately after exposure, experienced goats ingested 95% more ($P < 0.01$) OG per unit of body weight than inexperienced goats, but both groups rejected CSG. Nine months after exposure, experienced goats ingested 27% more ($P < 0.01$) OG than inexperienced goats. Experienced goats ingested 30% more OG than inexperienced goats at every level of alfalfa pellet availability. The fate of condensed tannins and total phenols was similar for both groups, but experienced goats excreted 63% more ($P < 0.05$) uronic acids per unit of body weight. Neither experienced nor inexperienced goats produced proline-rich proteins in saliva. Experienced goats excreted 32% more ($P < 0.01$) nitrogen in feces per unit of nitrogen ingested than did inexperienced goats. The mass of the reticulorumen was 30% greater ($P < 0.05$) for experienced than for inexperienced goats one month after exposure, but did not differ 10 months after exposure. The mass of the liver, kidneys, and parotid glands did not differ between treatments. The results

*To whom correspondence should be addressed.

0098-0331/91/0200-0431$06.50/0

show that experience early in life can have profound and persistent effects on consumption of diets high in chemical defenses and low in nutritional quality. The results also suggest that several physiological and morphological factors are involved.

Key Words—Experience, condensed tannins, plant defense, intake, goats, blackbrush, *Coleogyne ramosissima*.

INTRODUCTION

Empirical evidence indicates that prior experience ingesting foods increases preferences for those foods by animals (Arnold, 1964; Leuthold, 1971; Arnold and Maller, 1977; Martin, 1978; Frost, 1981; Narjisse, 1981; Bartmann and Carpenter, 1982; Otsyina, 1983). Moreover, experiences early in life apparently have a more lasting effect than those later in life (reviewed by Arnold and Dudzinski, 1978; Matthews and Kilgour, 1980; Provenza and Balph, 1988; Squibb et al., 1990). Neurological, physiological, and morphological processes are amenable to change early in life and can be altered permanently so animals can better forage in the environment where they are reared (Provenza and Balph, 1987, 1988, 1990). Some authors suggest that previous experience, particularly in early life, may counter chemical defenses of plants, thus attenuating their negative effects on voluntary intake (Provenza and Balph, 1987, 1988; Lindroth, 1988).

Low nutritional quality and high levels of condensed tannins adversely affect voluntary intake of blackbrush by goats (Provenza et al., 1983, 1990; Provenza and Malechek, 1984). However, consumption of blackbrush by young goats could influence the physiological processes that deter consumption later in life. Mammals have evolved physiological (i.e., reduced uptake or transport of toxins) and biochemical (i.e., enzymatic transformation of toxins) adaptations to cope with plant chemical defenses such as tannins (Lindroth, 1988). For example, deer (Robbins et al., 1987) and rats (Mehansho et al., 1983) produce proline-rich proteins in saliva that bind tannins. In rats, the ingestion of tannins induces a marked increase in the size of the parotid salivary glands (Mehansho et al., 1983). Voles (Lindroth and Batzli, 1983) and snowshoe hares (Bryant et al., 1985) possess enzyme systems that detoxify phenolic compounds through conjugation with glucuronic acid. Such enzymes are located primarily in the liver and kidneys (Freeland and Janzen, 1974), but also occur in visceral tissues such as the rumen wall (Smith, 1986).

Experience early in life could also influence morphological processes. For example, the alimentary canal of red deer (Milne et al., 1978) and mule deer (Baker and Hobbs, 1987) may enlarge when they ingest poor-quality forages. Because distension of the reticulorumen wall may limit intake of roughage that

is low in nutrients (Grovum, 1988), young goats may adapt to a low-nutrient, high-fiber diet such as blackbrush by increasing gut size.

The objectives of this study were to determine the effects of experience early in life with blackbrush or alfalfa pellets on: (1) voluntary intake of those foods by goats; (2) the fate of blackbrush condensed tannins and total phenols, production of proline-rich proteins in saliva, and excretion of nitrogen in feces of goats; and (3) the mass of the liver, kidneys, parotid glands, and reticulorumen of goats.

METHODS AND MATERIALS

Plant Material. Blackbrush is a small (< 1 m) shrub that grows in nearly monospecific stands on millions of hectares in the southwestern United States (Bowns and West, 1976). Blackbrush is low in crude protein (4–7%) and low in digestible dry matter (38–48%) (Provenza et al., 1983). In addition, blackbrush twigs are high in phenols, which are composed primarily of condensed tannins (T. Clausen, personal communication). The allocation of tannins varies according to plant part (Provenza and Malechek, 1983). Current season's growth (CSG) from previously browsed plants contains 2.4 times more tannins than does older growth (OG) from unbrowsed plants. Even though CSG is higher than OG in crude protein (7% vs. 5%) and digestible dry matter (48% vs. 38%), goats prefer OG to CSG (Provenza and Malechek, 1984, 1986). Condensed tannins cause goats to avoid CSG (Provenza et al., 1990; Clausen et al., 1990).

Exposure. Thirty Spanish goats 6 weeks old were randomly assigned to either a treatment (experienced) or a control (inexperienced) group. Inexperienced goats ate alfalfa pellets in Logan in northeastern Utah, while experienced goats browsed blackbrush near Gunlock in southwestern Utah. Goats were exposed to blackbrush or alfalfa pellets from 6 to 26 weeks of age. This period covered the transition from monogastric to ruminant, which may be a "sensitive period" in the development of food preferences in ruminants (Squibb et al., 1990). The young goats remained with their mothers during exposure to enhance the establishment and persistence of their dietary habits (Thorhallsdottir et al., 1990a,b; Nolte et al., 1990; Mirza and Provenza, 1991).

Experienced goats browsed blackbrush from November 10, 1989, to February 10, 1990, on a 5-ha pasture that had been heavily browsed by another group of goats during the winter of 1988–1989. Vegetation on the site was a monospecific understory of blackbrush shrubs with an overstory of scattered juniper (*Juniperus osteosperma*) trees. The removal of terminal buds by herbivores during winter, when blackbrush is dormant, stimulates production of CSG during spring (Provenza et al., 1983). Thus, the blackbrush plants were composed of both OG and CSG. We observed young goats browsing on both

OG and CSG, and virtually all OG and CSG terminal branches had been browsed by the end of exposure. Experienced and inexperienced goats were weaned 10 days before feeding trials began to minimize the effects of weaning stress on the results.

Feeding Trial 1. The objective of the first trial was to determine the intake of blackbrush by experienced and inexperienced goats immediately after exposure. The goats were penned individually in southwestern Utah and offered alfalfa pellets ad libitum for four days. They then were offered chopped blackbrush OG ad libitum for the next seven days (period 1), a choice between OG and chopped blackbrush CSG ad libitum for the next seven days (period 2), and a choice between half of their ad libitum intake of OG in period 2 and CSG ad libitum for the final five days (period 3). Goats had access to the food from 0900 to 1700 hr daily. All the animals were weighed at the beginning and at the end of each period. Intake of blackbrush was expressed as grams of oven-dry tissue weight ingested per kilogram of body weight.

Both blackbrush OG and CSG were harvested by hand daily and chopped, in a mechanical chipper, into segments 1–3 cm in length. When a choice was involved (periods 2 and 3), OG and CSG were offered in separate food boxes. The position of the food boxes containing OG or CSG was changed each day to avoid bias. All animals had ad libitum access to water and salt. At 1700 hr goats were given enough Calfmanna, a concentrate-based pellet high in protein (25%) and energy, to cover 30% of their estimated energy requirements (NRC, 1981). The amount of Calfmanna offered was adjusted according to the body weight of each individual.

Feeding Trial 2. The objective of the second trial was to determine the intake of blackbrush by goats nine months after initial exposure. After the first feeding trial, both groups of goats grazed the same grass–legume (*Dactylis glomerata–Medicago* spp.) pasture for eight months. The procedures described in trial 1 were repeated, except that the length of period 3 was reduced to two days; the production of CSG was low as a result of drought, which precluded feeding CSG for five days as in trial 1.

Feeding Trial 3. The objective of the third trial was to determine the intake of blackbrush OG by goats when offered OG ad libitum and alfalfa pellets at six levels of availability. After trial 2, we determined the ad libitum intake of alfalfa pellets for each individual. Experienced and inexperienced goats then were offered a choice between blackbrush OG ad libitum and alfalfa pellets ad libitum on day 1. Subsequently, the amount of alfalfa pellets offered was reduced by 20% each day, whereas OG was offered ad libitum. On the last day of the trial, only OG was offered ad libitum. The intake of OG was measured throughout the trial.

Fate of Condensed Tannins and Total Phenols. During trial 1, five goats from each group were penned individually in metabolism stalls to collect feces

and urine. Urine samples were collected in a bucket containing 1 ml of concentrated mercuric chloride to prevent microbial growth. All samples were frozen at −20°C. Before the chemical analyses, blackbrush and fecal samples were freeze-dried and ground to pass a 1-mm screen. Blackbrush, fecal, and urine samples were analyzed for days 3 and 5 (representative of period 1), 11 and 13 (representative of period 2), and 17 and 19 (representative of period 3).

Phenols were extracted from blackbrush and feces according to a procedure developed by A.E. Hagerman (personal communication). Blackbrush and feces were extracted with 10% acetic acid in ether to remove pigments, and then with 1% sodium dodecyl sulfate in 70% acetone to remove phenols. A 150-mg sample was mixed with 5 ml of acetic acid in ether, extracted for 10 min, centrifuged (2000 rpm), and the supernatant was discarded. This procedure was repeated four times. The sample then was mixed with 5 ml of sodium dodecyl sulfate in acetone, centrifuged for 10 min, and the supernatant was collected. This procedure was repeated four times. Finally, the samples were analyzed for condensed tannins (Porter et al., 1986) and total phenols (Price and Butler, 1977). Condensed tannins and total phenols were expressed as blackbrush condensed tannin equivalents per unit organic matter, using standard curves prepared daily for the conditions used in the analysis. Condensed tannins used as standards were extracted from blackbrush CSG and purified according to the method of Hagerman and Butler (1980).

Urine samples were analyzed for total phenols (Price and Butler, 1977) and uronic acids (Bitter and Muir, 1962). The presence of uronic acids is an index of phenol detoxification in animals (Lindroth and Batzli, 1983). Total phenols in urine were expressed as blackbrush condensed tannin equivalents, whereas uronic acids in urine were expressed as glucuronolactone equivalents.

Excretion of Nitrogen in Feces. Blackbrush and fecal samples were analyzed for nitrogen using the Digesdahl procedure (Hach Company, 1987).

Production of Proline-Rich Proteins in Saliva. Saliva samples from five goats in each group were analyzed for days when fecal and urine samples were collected. Samples were collected in the morning before feeding. A sponge, placed in the mouth of each animal, was removed after the animal had chewed on it for 2–3 min, which enabled us to collect total mixed saliva (some salivary glands secrete only when the animal is chewing). The sponge was squeezed to collect saliva in a small tube containing 100 μl of phenylmethyl-sulfonyl fluoride, an antiproteolytic agent. One milliliter of saliva was collected per individual. The samples were frozen immediately in Dry Ice and later placed in a freezer at −20°C. Saliva samples were analyzed for proline-rich proteins according to the method of Austin et al. (1989).

Mass of Reticulorumen, Liver, Kidneys, and Parotid Glands. One month after exposure, four goats from each group that was in trial 1 were sacrificed and the fresh mass of the liver, kidneys, parotid glands, and reticulorumen was

determined. The contents of the reticulorumen were removed and the reticulorumen was washed and dried with a towel before weighing. The mass of the reticulorumen was measured in four goats from each group again 10 months after exposure.

After goats had ingested blackbrush OG, we determined how many alfalfa pellets they would ingest voluntarily to ascertain whether physical limitation in rumen volume might limit intake. Both groups of goats were offered blackbrush OG from 0900 to 1300 hr and then alfalfa pellets from 1300 to 1700 hr on the day following trial 1.

Statistical Analyses. The statistical design for the analyses of variance was a randomized block with experience as the main effect and individual goats as blocks. Periods and foods within periods were analyzed separately in trials 1 and 2. When the same measurement was repeated for more than one day, a repeated measures analysis was used (Winer, 1971).

RESULTS

Feeding Trial 1. Experienced goats ingested 12 g/kg of body weight or 95% more ($P < 0.01$) OG than inexperienced goats in periods 1 and 2 (Figure 1). On average, experienced goats ingested 332 g of OG, whereas inexperienced goats ingested 232 g of OG. In period 2, both groups rejected CSG when allowed to choose between OG and CSG. However, when OG was restricted and CSG was offered ad libitum in period 3, both groups ingested all the OG and experienced goats ingested 6 g/kg of body weight or 46% more ($P < 0.01$) CSG than did inexperienced goats. The average intake of CSG was 245 g and 225 g for experienced and inexperienced goats, respectively.

Feeding Trial 2. Experienced goats ingested 7 g/kg of body weight or 27% more ($P < 0.01$) OG than inexperienced goats nine months after exposure (Figure 2). On average, experienced goats ingested 716 g of OG, whereas inexperienced goats ingested 618 g of OG. When allowed to choose between OG and CSG in period 2, both groups rejected CSG. In period 3 when OG was restricted and CSG was offered ad libitum, goats did not differ ($P > 0.05$) in the amount of CSG they ingested.

Feeding Trial 3. When allowed to choose between OG and alfalfa pellets, experienced goats ingested more ($P < 0.05$) OG than inexperienced goats at every level of alfalfa pellet availability (Table 1). On average, experienced goats ingested 6 g/kg of body weight or 30% more than inexperienced goats.

Body Weight. In trial 1, there was no change in the weight of experienced goats (13 kg), but inexperienced goats lost an average of 4 kg, from 21 to 17 kg. Differences in the nutrient and energy contents of blackbrush OG and alfalfa pellets caused the differences in weight at the beginning of trial 1. There were

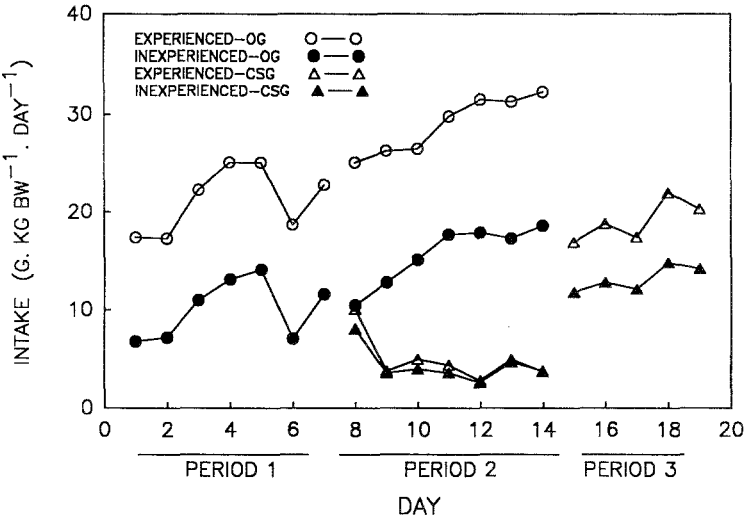


FIG. 1. Intake of blackbrush by goats immediately after exposure. Each value represents the mean of 14 (experienced) or 16 (inexperienced) goats. Goats were offered OG in period 1, a choice between OG and CSG in period 2, and a choice between half their ad libitum intake of OG in period 2 and CSG ad libitum in period 3. Differences between treatments are significant ($P < 0.01$), except for intake of CSG in period 2. There was no treatment by day interaction ($P > 0.05$). The standard errors of the means for periods 1, 2, and 3 are 1.0, 1.0 (OG) and 0.5 (CSG), and 0.7, respectively.

no changes in the weights of experienced (25 kg) or inexperienced (27 kg) goats during trial 2.

Fate of Condensed Tannins and Total Phenols. Experienced goats ingested more ($P < 0.05$) condensed tannins and total phenols than inexperienced goats in trial 1 (Table 2). Experienced and inexperienced goats did not differ ($P > 0.05$) either in the amount (milligrams excreted per gram ingested) of condensed tannins and total phenols in feces or in the amount of total phenols and uronic acids in urine (Table 3). However, experienced goats excreted more ($P < 0.05$) uronic acids than inexperienced goats during the trial when expressed on a per unit body weight basis (Table 3).

Excretion of Nitrogen in Feces. Experienced goats excreted 32% more ($P < 0.01$) nitrogen in feces, per unit of nitrogen ingested, than inexperienced goats and ingested a higher ($P < 0.01$) proportion of nitrogen as blackbrush protein (45% vs. 30%) throughout trial 1 (Table 4).

Production of Proline-Rich Proteins in Saliva. No proline-rich proteins were detected by electrophoresis in the saliva of either experienced or inexperienced goats.

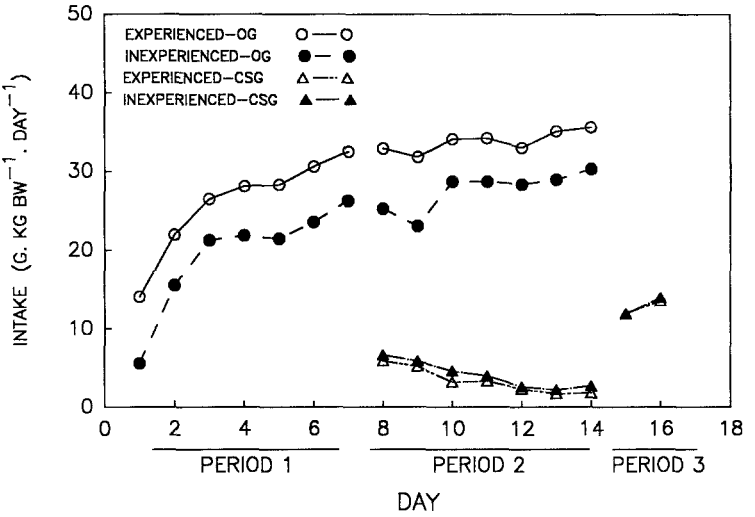


FIG. 2. Intake of blackbrush by goats nine months after exposure. Each value represents the mean of 10 (experienced) or 12 (inexperienced) goats. Blackbrush twigs offered were as described in the legend for Figure 1. Differences between treatments in intake of OG, but not CSG, are significant ($P < 0.01$). There was no treatment by day interaction ($P > 0.05$). The standard errors of the means for periods 1, 2, and 3 are 1.4, 1.2 (OG) and 0.5 (CSG), and 0.3, respectively.

TABLE 1. INTAKE OF BLACKBRUSH OG BY GOATS[a]

Alfalfa pellet availability (Ad Libitum %)	Intake of blackbrush OG by goats (g/kg body weight)		
	Experienced	Inexperienced	SE
100	13a	9b	1.5
80	16a	9b	1.9
60	19a	13b	2.6
40	29a	22b	2.8
20	34a	28b	2.2
0	36a	32b	1.5

[a]Blackbrush OG was offered ad libitum throughout the trial, while alfalfa pellets were offered at six levels of availability. Each value represents the mean of 10 (experienced) or 12 (inexperienced) goats.
[b]ab, means within a row are different (LSD 0.05).

TABLE 2. INGESTION OF CONDENSED TANNINS AND TOTAL PHENOLS BY GOATS IN TRIAL 1[a]

	Ingestion (mg/kg body wt)			
	Condensed tannins		Total phenols	
Day	Experienced	Inexperienced	Experienced	Inexperienced
Period 1				
3	1400a	700b	1890a	940b
5	1240a	740b	1720a	1030b
Period 2				
11	1970a	1450b	2920a	2160b
13	2060a	1430b	3120a	2160b
Period 3				
17	2800a	1760b	4170a	2900b
19	2320a	1730b	3550a	2650b

[a] Each value represents the mean of five goats. The standard error of a mean for condensed tannins is 130 and for total phenols is 183.
[b] ab, means within a row, within a chemical class, are different (LSD 0.05).

TABLE 3. EXCRETION OF CONDENSED TANNINS, TOTAL PHENOLS, AND URONIC ACIDS IN TRIAL 1[a]

	Goats		
Excretion	Experienced	Inexperienced	SE
Tannins in feces (mg/g ingested)	60a	62a	13
Phenols in feces (mg/g ingested)	94a	86a	15
Phenols in urine (mg/g ingested)	11a	12a	3
Uronic acids (mg/g phenol ingested)	29a	26a	11
Uronic acids (mg/kg body weight)	85a	52b	21

[a] Each value represents the mean of five goats for days 3, 5, 11, 13, 17, and 19.
[b] ab, means within a row are different (LSD 0.05).

Mass of Reticulorumen, Liver, Kidneys, and Parotid Glands. One month after exposure, the mass of the reticulorumen was 30% higher ($P < 0.05$) in experienced than in inexperienced goats (Table 5). Treatments did not differ either in the thickness of the reticulorumen wall or in the development of rumen papillae. Thus, the larger reticulorumen mass meant experienced goats had a larger reticulorumen capacity. There were no differences ($P < 0.05$) between treatments in the mass of liver, kidneys, or parotid glands. Ten months after

TABLE 4. EXCRETION OF NITROGEN IN FECES OF GOATS IN TRIAL 1[a]

Day	Nitrogen excretion (mg/g ingested)	
	Experienced	Inexperienced
Period 1		
3	363a	209b
5	374a	313b
Period 2		
11	392a	293b
13	374a	325a
Period 3		
17	454a	348b
19	462a	347b

[a] Each value represents the mean of five goats. The standard error of a mean is 27.
[b] ab, means within a row are different (LSD 0.05).

TABLE 5. MASS OF RETICULO RUMEN, LIVER, KIDNEYS, AND PAROTID GLANDS 1 MONTH AFTER EXPOSURE AND MASS OF RETICULO RUMEN 10 MONTHS AFTER EXPOSURE[a]

Time after exposure	Mass (g/kg body wt)			
	Reticulo rumen	Liver	Kidneys	Parotid glands
One month				
Experienced	25a	22a	5a	1a
Inexperienced	18b	19a	4a	1a
Ten months				
Experienced	25a			
Inexperienced	23a			

[a] Each value represents the mean of four goats. The standard errors of the means for reticulo rumen mass at one and 10 months after exposure are 2 and 1, respectively. The standard errors of the means for liver, kidneys, and parotid glands are 2.4, 0.4, and 0.1, respectively.
[b] ab, means within a column are different (LSD 0.05).

exposure the mass of the reticulorumen did not differ ($P > 0.05$) between treatments.

Experienced and inexperienced goats ate nearly the same amount of OG from 0900 to 1300 hr (Table 6) as they ate from 0900 to 1700 hr on day 14 of trial 1 (Figure 1). The alfalfa pellets ingested from 1300 to 1700 hr were 83%

TABLE 6. INTAKE OF BLACKBRUSH OG (0900–1300 hr) AND ALFALFA PELLETS (1300–1700 hr)[a]

Food	Intake (g/kg body wt)		SE
	Experienced	Inexperienced	
Blackbrush OG	29a	19b	1
Alfalfa pellets	24a	21a	2
Total	53a	40b	3

[a] Each value represents the mean of 14 (experienced) or 16 (inexperienced) goats.
[b] ab, Means within a row are different (LSD 0.05).

and 111% of the OG ingested from 0900 to 1300 hr by experienced and inexperienced goats, respectively.

DISCUSSION

First we discuss the mechanisms that may underlie the responses of experienced and inexperienced goats, then we address artifacts of the experimental design that may have influenced those responses, and finally we consider implications of the findings for the biological manipulation of shrubs.

Mechanisms that May Underlie Responses of Goats

Experience early in life with blackbrush increased voluntary intake of blackbrush OG and CSG by goats in trial 1 and increased intake of and preference for blackbrush OG in trials 2 and 3. These results may reflect differences between experienced and inexperienced goats in: (1) preference for blackbrush OG and CSG, (2) ability to detoxify phenols in OG and CSG, and (3) distension of the reticulorumen.

Preference for Blackbrush OG and CSG. Preference was the relative amount of blackbrush OG ingested when goats could choose between blackbrush OG and alfalfa pellets or between blackbrush OG and CSG. Exposure to blackbrush increased preference for blackbrush OG when offered with alfalfa pellets (Table 1). Animals develop preferences for foods ingested early in life in the presence of social models such as the mother (reviewed by Immelmann 1975; Matthews and Kilgour, 1980; Provenza and Balph, 1987, 1988, 1990; Provenza et al., 1991). The enhanced ingestion of blackbrush OG shows that goats can develop a preference for a food high in tannins and low in nutritional quality. Grazing experience early in life also increases the preference of sheep for various unpalatable plants, and these differences persisted for at least two

years even when sheep were exposed to a wide range of forages (Arnold, 1964; Arnold and Maller, 1977).

When allowed to choose between blackbrush OG and CSG, goats always preferred OG. The fact that the concentration of condensed tannins in CSG is over twice that in OG may explain the preference. Alternatively, differences in the structure of condensed tannins in OG and CSG may have affected the goat's response and are known to cause snowshoe hares to prefer bitterbrush CSG to blackbrush CSG (Clausen et al., 1990). The ratio of epicatechin to catechin in condensed tannins from blackbrush CSG is five times higher than that of condensed tannins from bitterbrush CSG.

Condensed tannin structure can vary with the age of plant parts. The epicatechin–catechin ratio of condensed tannins of *Pinus taeda* differs between the phloem and the heartwood (Karchesy and Hemingway, 1980), and most condensed tannins from the ripe fruit of *Cydonia oblonga* are bound covalently with sugars whereas condensed tannins from unripe fruit have no carbohydrate substituents (Porter et al., 1985). Porter (1984) argues that branching increases with the molecular weight of condensed tannins. Thus, condensed tannins from OG, which form larger polymers, may branch more than newly formed condensed tannins from CSG and may be less aversive to goats than those in CSG (Provenza et al., 1990).

Inexperienced goats initially ingested more ($P < 0.01$) CSG than experienced goats (Figure 1, day 8). Intake of CSG by inexperienced goats subsequently declined and their consumption of OG increased. Goats associate the taste of CSG with postingestive distress caused by condensed tannins and quickly learn an aversion to blackbrush CSG, presumably when condensed tannins stimulate the emetic system of the midbrain and brain stem (Provenza et al., 1990, 1991). Toxins can stimulate the emetic system by afferent nerves from throughout the body (e.g., gastrointestinal tract, cardiovascular system, liver, kidneys) (Borison, 1986; David et al., 1986; Grahame-Smith, 1986; Kosten and Contreras, 1989). This explains why inexperienced goats ingested more ($P < 0.01$) CSG on day 8 than on subsequent days.

Experienced goats also ingested more ($P < 0.01$) CSG initially than in subsequent days, perhaps because chopping CSG changed cues such as sight, odor, and taste that goats use to recognize CSG (Provenza et al., 1990, 1991). This is a likely explanation for why experienced goats sampled more CSG on day 8 than on day 9 (Figure 1).

Detoxification of Phenols. Only a small proportion of blackbrush condensed tannins and total phenols were excreted in feces (6% and 9%, respectively) and urine (1% of phenols ingested) (Table 3). Condensed tannins form strong hydrogen bonds with proteins, polysaccharides, and other cell constituents (Swain, 1979), and the technique used in this study may not have recovered all of them (A.E. Hagerman, personal communication).

In both treatments, the most nitrogen was excreted in feces during period 3 of trial 1, the time when goats ingested the most tannins. Experienced goats excreted more nitrogen in the feces than inexperienced goats (Table 4), perhaps because they ingested more tannins and because a higher proportion of the nitrogen they ingested was from the poorly digestible twigs of blackbrush. Nastis and Malechek (1981) also found that goats' excretion of nitrogen in feces increased with the tannin content of an alfalfa–gambel oak diet.

An alternative explanation for the fact that only a small proportion of blackbrush condensed tannins was excreted in the feces and urine is that part of the condensed tannins were depolymerized, absorbed from the gastrointestinal tract, and then detoxified. Depolymerization of condensed tannins, particularly procyanidins (Hemingway and McGraw, 1983; Hemingway et al., 1983), occurs readily under acidic conditions similar to those in the digestive tract of mammalian herbivores (Butler et al., 1986; Clausen et al., 1990). Condensed tannin depolymerization products may be toxic (McLeod, 1974; Kumar and Singh, 1974; Lindroth and Batzli, 1984; Mehansho et al., 1985; Butler et al., 1986). For example, voles die when fed quebracho tannins (Lindroth and Batzli, 1984), and hamsters fail to sexually mature or die if condensed tannin levels in their diets are sufficiently high (Mehansho et al., 1985, 1987). Condensed tannins also increase mixed-function oxidase activity in the liver and levels of phenols in urine (Sell and Rogler, 1983), which suggests that they are absorbed and metabolized to some extent.

Experienced goats excreted 63% more uronic acid than inexperienced goats (85 vs. 52 mg/kg body weight; Table 3), perhaps because experienced goats ingested more condensed tannins and total phenols (Table 2), or because they were better able to detoxify phenols. A greater ability to detoxify condensed tannins would lower concentrations of phenols in blood and might affect intake. Phenols in blood may stimulate emetic receptors in the midbrain and brain stem, thereby causing a conditioned food aversion and reducing intake (Provenza et al., 1990, 1991). Thus, experienced goats may have ingested more blackbrush OG and CSG, and consequently more blackbrush condensed tannins and total phenols, because they were better able to detoxify them. If detoxification required time for induction, this lag could explain the difference in intake of CSG between experienced and inexperienced goats during period 3 of trial 1, and the lack of any difference during period 3 of trial 2.

Freeland and Janzen (1974) hypothesize that mammalian herbivores ingest toxins in amounts that they can detoxify. By the end of trial 1, average tannin intake by experienced and inexperienced goats was 2560 and 1745 mg/kg body weight, respectively (average of days 17 and 19 in Table 2), which may be the maximum amount of tannins goats could detoxify. Sheep (Burritt and Provenza, 1989) and goats (Provenza et al., 1990, 1991) regulate intake of foods that contain lithium chloride (LiCl), a potentially toxic compound. When LiCl is

mixed with an otherwise nutritious food, sheep and goats will voluntarily ingest between 25 and 50 mg/kg body weight (DuToit et al., 1991).

Our results do not support the hypothesis that mixed-feeders such as goats (Hofmann, 1988) produce proline-rich proteins in saliva as a mechanism for neutralizing tannins (Robbins et al., 1987). Different animal species may have evolved different mechanisms to cope with tannins. Species such as goats may have evolved the ability to detoxify tannins or their metabolites to some extent, while species such as deer apparently evolved proline-rich proteins as a partial defense against tannins (Robbins et al., 1987).

Distension of Reticulorumen. Our data support the hypothesis that the capacity of the reticulorumen can increase when ruminants ingest foods high in fiber and low in nutrients (Milne et al., 1978; Baker and Hobbs, 1987), but do not support the hypothesis that experience early in life causes a permanent increase in the capacity of the reticulorumen. One month after exposure experienced goats had greater reticulorumen capacity than did inexperienced goats, but after eight months of grazing on the same grass–legume pasture the size of the reticulorumen did not differ between treatments.

Grovum (1988) argues that intake of roughage can be limited by the distension of the reticulorumen. While experienced and inexperienced goats ingested a considerable amount of alfalfa pellets immediately after they ingested blackbrush OG (Table 6), distension of the reticulorumen may still have limited their intake of blackbrush OG. Difference in the density of blackbrush OG and alfalfa pellets may explain the results in Table 6. The blackbrush OG ingested by experienced and inexperienced goats (29 and 19 g/kg body weight) occupied volumes of 112 and 73 cm^3, while the alfalfa pellets ingested by experienced and inexperienced goats (26 and 22 g/kg body weight) occupied volumes of 34 and 29 cm^3. Furthermore, finely ground and pelleted alfalfa breaks down much more quickly when masticated than the coarse, fibrous chipped blackbrush OG. Thus, rumen volume and distension of the reticulorumen may partially explain the large differences in intake between experienced and inexperienced goats in trial 1, and the reduction in such differences in trial 2.

Experimental Artifacts That May Have Influenced Responses of Goats

The differences in intake of blackbrush OG and CSG between treatments were greater immediately after exposure (Figure 1) than nine months after exposure (Figure 2). The nutritional plane of goats during the experiment and the exposure of inexperienced goats to blackbrush during trial 1 may help explain this result.

Effects of Nutritional Plane on Responses of Goats. Experienced and inexperienced goats were on drastically different planes of nutrition during exposure and differed in fat reserves and body weight during trial 1. The difference in

the amount of back fat and kidney fat between groups was apparent when the animals were sacrificed to measure visceral mass. The difference in fat reserves between treatments was probably even greater at the beginning of trial 1 when inexperienced goats weighed 21 kg and experienced goats weighed 13 kg. During trial 1, inexperienced goats lost 430 g/day during the first seven days (3 kg) and 70 g/day during the next 14 days (1 kg). By the end of trial 1, inexperienced goats weighed 17 kg while experienced goats weighed 13 kg. Inexperienced goats probably lost weight when they mobilized fat reserves to compensate for their low intake of blackbrush. There were no differences between treatments in fat reserves and body weight (26 kg vs. 24 kg for inexperienced vs. experienced goats) after both groups of goats grazed a grass–legume pasture for eight months.

The disparity in nutritional plane between experienced and inexperienced goats prior to trial 1 and the similarity in nutritional plane for the eight months prior to trial 2 may partially explain the differences in intake immediately after exposure and nine months later. Rats deprived of protein early in life ingest more food, and utilize protein more efficiently, than normal rats (Beck et al., 1983). Protein levels in blackbrush are not adequate to meet requirements of young goats (Provenza et al., 1983; NRC, 1981), and long-term protein deprivation may have caused experienced goats to ingest more blackbrush than experienced goats during trial 1. Unfortunately, we know of no data concerning the role of protein deprivation early in life on intake in ruminants. In addition, levels of body fat affect insulin levels in the blood, which may also affect intake and could explain why inexperienced goats consumed less food immediately following exposure than experienced goats. Evidence supports this hypothesis in rats, but there is no evidence to support this hypothesis in ruminants (Weston, 1982; deJong, 1985; Grovum, 1988).

Experience of the Control Group. Exposing inexperienced goats to blackbrush during the first trial also may help explain why the differences in intake of blackbrush OG between treatments were larger immediately after exposure than nine months later. In the feeding trial nine months after exposure, young goats in the control group had been exposed to blackbrush for 20 days. This duration of exposure relatively early in life, which represented 20% of the exposure of the treatment group, may have increased inexperienced goats' intake of blackbrush OG.

Implications

Goats are an alternative to chemical or mechanical control of undesirable plant species (Wood, 1987). Goats also can be used to modify the growth form of shrubs to increase their productivity and nutritional value (Provenza et al., 1983). Our results show that young goats exposed to blackbrush consequently

ate more of the shrub, findings which could enhance biological manipulation efforts. For example, if goats (average weight of 30 kg) forage on blackbrush-dominated rangeland for 90 days during the dormant season, as was typical of the studies of Provenza et al. (1983), 15 experienced goats would ingest 284 kg more blackbrush than 15 inexperienced goats based on the data in Figure 2 (0.007 kg more ingested/kg body weight/day × 30 kg body weight/goat × 15 goats × 90 days). These differences would be much larger using the intakes shown in Figure 1: experienced goats would ingest 486 kg more blackbrush than inexperienced goats (0.012 kg more ingested/kg body weight/day × 30 kg body weight/goat × 15 goats × 90 days). The results of Flores et al. (1989a–c) indicate that the differences in intake of blackbrush between experienced and inexperienced goats would be even greater under free-ranging conditions because herbivores learn foraging skills. In our studies, goats were offered chopped blackbrush twigs in food boxes so that plant form and foraging skills would not confound the results.

CONCLUSIONS

Results from our study show that experience early in life with different diets can have profound and persistent effects on intake. The results also suggest that several physiological and morphological factors are involved. Clearly, more research is needed to understand the relationship between preference and the ability to detoxify condensed tannins. It is also important to study the interrelationships among intake, rumen volume, rate of passage of particles through the gastrointestinal tract, and factors such as rumination efficiency, which are influenced by experience and could affect intake (Welch and Hooper, 1988). Moreover, the role of nutrient deficiency on intake of blackbrush by young animals must be understood. Finally, it is important to determine how age at exposure and duration of exposure to blackbrush influence physiological and morphological processes that affect intake of and preference for blackbrush by goats.

Acknowledgments—Financial support of the Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET) de la Republica Argentina through a fellowship to R.A. Distel is acknowledged. Additional support was provided by a grant from National Science Foundation (BSR-8614856) and a grant from the United States Agency for International Development. We thank Dr. Ann E. Hagerman for assistance with the chemical analyses and the analysis of goat's saliva for proline-rich proteins, and Johan and Mary Jane DuToit for assistance with some of the field trials. This is journal paper No. 4035, Utah Agric. Exp. Sta., Utah State Univ., Logan, Utah 84322-4845.

REFERENCES

ARNOLD, G.W. 1964. Some principles in the investigation of selective grazing. *Proc. Aust. Soc. Anim. Prod.* 5:258–271.

ARNOLD, G.W., and DUDZINSKI, M.L. 1978. Ethology of Free-Ranging Domestic Animals. Elsevier/North Holland, New York.

ARNOLD, G.W., and MALLER, R.A. 1977. Effects of nutritional experience in early and adult life on the performance and dietary habits of sheep. *Appl. Anim. Ethol.* 3:5–26.

AUSTIN, P.J., SUCHAR, L.A., ROBBINS, C.T., and HAGERMAN, A.E. 1989. Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. *J. Chem. Ecol.* 15:1335–1347.

BAKER, D.L., and HOBBS, T. 1987. Strategies of digestion: Digestive efficiency and retention time of forage diets in montane ungulates. *Can. J. Zool.* 65:1978–1984.

BARTMANN, R.M., and CARPENTER, L.H. 1982. Effects of foraging experience on food selectivity of tame mule deer. *J. Wildl. Manage.* 46:813–818.

BECK, B., DOLLET, J.M., MAX, J.P., and DEBRY, G. 1983. Relations among weight deficit, food intake and early deprivation in long-term protein malnourished rats. *Nutr. Res.* 3:743–748.

BITTER, T., and MUIR, H.M. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* 4:330–334.

BORISON, H.L. 1986. Anatomy and physiology of the chemoreceptor trigger zone and area postrema, pp. 10–17, *in* C.J. Davis, G.V. Lake-Bakaar, and D.G. Grahame-Smith (eds.). Nausea and Vomiting: Mechanisms and Treatment. Springer-Verlag, New York.

BOWNS, J.E., and WEST, N.E. 1976. An autecological study of blackbrush (*Coleogyne ramosissima* Torr.) on southwestern Utah rangelands. Utah Agric. Exp. Sta. Res. Rep. 27.

BRYANT, J.P., WIELAND, G.D., CLAUSEN, T., and KUROPAT, P. 1985. Interactions of snowshoe hare and feltleaf willow in Alaska. *Ecology* 66:1564–1573.

BURRITT, E.A., and PROVENZA, F.D. 1989. Food aversion learning: ability of lambs to distinguish safe from harmful foods. *J. Anim. Sci.* 67:1732–1739.

BUTLER, L.G., ROGLER, J.C., MEHANSHO, H., and CARLSON, D.M. 1986. Dietary effects of tannins, pp. 141–157, *in* V. Cody, J.B. Harborne, and E. Middleton (eds.). Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure–Activity Relationships. Alan R. Liss, New York.

CLAUSEN, T.P., PROVENZA, F.D., BURRITT, E.A., BRYANT, J.P., and REICHARDT, P.B. 1990. Ecological implications of condensed tannin structure: a case study. *J. Chem. Ecol.* 16:2381–2392.

DAVIS, C.J., HARDING, R.K., LESLIE, R.A., and ANDREWS, P.L.R. 1986. The organization of vomiting as a protective reflex: a commentary on the first day's discussion, pp. 65–75 *in* C.J. Davis, G.V. Lake-Bakaar, and D.G. Grahame-Smith (eds.). Nausea and Vomiting: Mechanisms and Treatment. Springer-Verlag, New York.

DEJONG, A. 1985. The role of metabolites and hormones as feedbacks in the control of food intake in ruminants, pp. 459–478, *in* L.P. Milligen, W.L. Grovum, and A. Dobson (eds.). Control of Digestion and Metabolism in Ruminants. Prentice-Hall, Englewood Cliffs, New Jersey.

DUTOIT, J.T., PROVENZA, F.D., and NASTIS, A. 1991. Conditioned taste aversions: How sick must a ruminant get before it learns about toxicity in foods? *Appl. Anim. Behav. Sci.* In press.

FLORES, E.R., PROVENZA, F.D., and BALPH, D.F. 1989a. Role of experience in the development of foraging skills of lambs browsing the shrub serviceberry. *Appl. Anim. Behav. Sci.* 23:271–278.

FLORES, E.R., PROVENZA, F.D., and BALPH, D.F. 1989b. The effect of experience on the foraging skill of lambs: Importance of plant form. *Appl. Anim. Behav. Sci.* 23:285–291.

FLORES, E.R., PROVENZA, F.D., and BALPH, D.F. 1989c. Relationship between plant maturity and

foraging experience of lambs grazing hycrest crested wheatgrass. *Appl. Anim. Behav. Sci.* 23:279–284.

FREELAND, W.J., and JANZEN, D.H. 1974. Strategies in herbivory by mammals: The role of plant secondary compounds. *Am. Nat.* 108:269–289.

FROST, S.K. 1981. Food selection in young naive impala *Aepyceros melampus*. *S. Afr. J. Zool.* 16:123–124.

GRAHAME-SMITH, D.G. 1986. The multiple causes of vomiting: Is there a common mechanism? pp. 1–8, *in* C.J. Davis, G.V. Lake-Bakaar, and D.G. Grahame-Smith (eds.). Nausea and Vomiting: Mechanisms and Treatment. Springer-Verlag, New York.

GROVUM, W.L. 1988. Appetite, palatability and control of feed intake, pp. 202–216, *in* D.C. Church (ed.). The Ruminant Animal. Prentice Hall, Englewood Cliffs, New Jersey.

HACH COMPANY. 1987. Feed Analysis Manual, pp. 12–16. Hach Company, Ames, Iowa.

HAGERMAN, A.E., and BUTLER, L.G. 1980. Condensed tannin purification and characterization of tannin associated proteins. *J. Agric. Food Chem.* 28:947–952.

HEMINGWAY, R.W., and MCGRAW, G.W. 1983. Kinetics of acid-catalyzed cleavage of procyanidins. *J. Wood Chem. Technol.* 3:421–435.

HEMINGWAY, R.W., MCGRAW, G.W., KARCHESY, J.J., FOO, L.Y., and PORTER, L.J. 1983. Recent advances in the chemistry of condensed tannins. *J. Appl. Polym. Sci.* 37:967–977.

HOFMANN, R.R. 1988. Anatomy of the gastro-intestinal tract, pp. 14–43, *in* D.C. Church (ed.). The Ruminant Animal. Prentice Hill, Englewood Cliffs, New Jersey.

IMMELMANN, K. 1975. Ecological significance of imprinting and early learning. *Annu. Rev. Ecol. Syst.* 6:15–37.

KARCHESY, J.J., and HEMINGWAY, R.W. 1980. Loblolly pine bark polyflavanoids. *J. Agric. Food Chem.* 28:222–228.

KOSTEN, T., and CONTRERAS, R.J. 1989. Deficits in conditioned heart rate and taste aversion in area postrema-lesioned rats. *Behav. Brain Res.* 35:9–21.

KUMAR, R., and SINGH, M. 1974. Tannins: Their adverse role in ruminant nutrition. *J. Agric. Food Chem.* 32:447–453.

LEUTHOLD, W. 1971. A note on the formation of food habits in young antelopes. *E. Afr. Wild. J.* 9:154–156.

LINDROTH, R.L. 1988. Adaptations of mammalian herbivores to plant chemical defenses, pp. 415–445, *in* K.C. Spencer (ed.). Chemical Mediation of Coevolution. Academic Press, San Diego, California.

LINDROTH, R.L., and BATZLI, G.O. 1983. Detoxication of some naturally occurring phenolics by prairie voles: A rapid assay of glucuronidation metabolism. *Biochem. Syst. Ecol.* 11:405–409.

LINDROTH, R.L., and BATZLI, G.O. 1984. Plant phenolics as chemical defenses: effects of natural products on survival and growth of prairie voles. *J. Chem. Ecol.* 10:229–244.

MARTIN, G.C. 1978. The animal-plant complex in forage palatability phenomena. *J. Anim. Sci.* 46:1470–1477.

MATTHEWS, L.R., and KILGOUR, R. 1980. Learning and associated factors in ruminant feeding behaviour, pp. 123–144, *in* Y. Ruckebusch and P. Thivend (eds.). Digestive Physiology and Metabolism in Ruminants. AVI Publishing Co., Westport, Connecticut.

MCLEOD, M.N. 1974. Plant tannins—their role on forage quality. *Nutr. Abst. Rev.* 44:803–815.

MEHANSHO, H., HAGERMAN, A., CLEMENTS, S., BUTLER, L., and CARLSON, D.M. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. *Proc. Natl. Acad. Sci. U.S.A.* 80:3948–3952.

MEHANSHO, H., CLEMENTS, S., SHEARES, B.T., SMITH, S., and CARLSON, D.M. 1985. Induction of proline-rich glycoprotein synthesis in mouse salivary glands by isoproterenol and by tannins. *J. Biol. Chem.* 260:4418–4423.

MEHANSHO, H., BUTLER, L.G., and CARLSON, D.M. 1987. Dietary tannins and salivary proline-rich proteins: interactions, induction, and defense mechanisms. *Annu. Rev. Nutr.* 7:423–440.

MILNE, J.A., MACRAE, J.C., SPENCE, A.M., and WILSON, S. 1978. A comparison of the voluntary intake and digestion of a range of forages at different times of the year by the sheep and the red deer (*Cervus elaphus*). *Br. J. Nutr.* 40:347–357.

MIRZA, S.N., and PROVENZA, F.D. 1991. Preference of the mother affects selection and avoidance of foods by lambs differing in age. *Appl. Anim. Behav. Sci.* In press.

NARJISSE, H. 1981. Acceptability of big sagebrush to sheep and goats: Role of monoterpenes. PhD thesis. Utah State University, Logan. 124 pp.

NASTIS, A.S., and MALECHEK, J.C. 1981. Digestion and utilization of nutrients in oak browse by goats. *J. Anim. Sci.* 53:283–289.

NOLTE, D.L., PROVENZA, F.D., and BALPH, D.F. 1990. The establishment and persistence of food preferences in lambs exposed to selected foods. *J. Anim. Sci.* 68:998–1002.

NRC. 1981. Nutrient requirements of domestic animals. No. 15. Nutrient requirement of goats. National Academy Press, Washington.

OTSYINA, R.M. 1983. Evaluation of shrubs as native supplements to cured crested wheatgrass (*Agropyron desertorum*) pasture for sheep. PhD thesis. Utah State University, Logan. 117 pp.

PORTER, L.J. 1984. Recent advances in chemistry of proantocyanidin polymers. *Rev. Latinoam. Quim.* 15:43–49.

PORTER, L.J., FOO, L.Y., and FURNEAUX, R.H. 1985. Isolation of three naturally occurring O-B-glucopyranosides of procyanidin polymers. *Phytochemistry* 24:567–569.

PORTER, L.J., HRSTICH, L.N., and CHAN, B.G. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* 25:223–230.

PRICE, M.L., and BUTLER, L.G. 1977. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *J. Agric. Food Chem.* 25:1268–1273.

PROVENZA, F.D., and BALPH, D.F. 1987. Diet learning by domestic ruminants: Theory, evidence, and practical implications. *Appl. Anim. Behav. Sci.* 18:211–232.

PROVENZA, F.D., and BALPH, D.F. 1988. The development of dietary choice in livestock on rangelands and its implications for management. *J. Anim. Sci.* 66:2356–2368.

PROVENZA, F.D., and BALPH, D.F. 1990. Applicability of five diet-selection models to various foraging challenges ruminants encounter, pp. 423–460, *in* R.N. Hughes (ed.). Behavioural Mechanisms of Food Selection. Springer-Verlag, Berlin.

PROVENZA, F.D., and MALECHEK, J.C. 1983. Tannin allocation in blackbrush (*Coleogyne ramosissima*). *Biochem. Syst. Ecol.* 11:233–238.

PROVENZA, F.D., and MALECHEK, J.C. 1984. Diet selection by domestic goats in relation to blackbrush twig chemistry. *J. Appl. Ecol.* 21:831–841.

PROVENZA, F.D., and MALECHEK, J.C. 1986. A comparison of food selection and foraging behavior in juvenile and adult goats. *Appl. Anim. Behav. Sci.* 16:49–61.

PROVENZA, F.D., BOWNS, J.E., URNESS, P.J., MALECHEK, J.C., and BUTCHER, J.E. 1983. Biological manipulation of blackbrush by goat browsing. *J. Range Manage.* 36:513–518.

PROVENZA, F.D., BURRITT, E.A., CLAUSEN, T.P., BRYANT, J.P., REICHARDT, P.B., and DISTEL, R.A. 1990. Conditioned flavor aversion: a mechanism for goats to avoid condensed tannins in blackbrush. *Am. Nat.* 136:810–828.

PROVENZA, F.D., PFISTER, J.A., and CHENEY, C.D. 1991. Mechanisms of learning in diet selection with reference to phytoxicosis in herbivores. *J. Range Manage.* In press.

ROBBINS, C.T., MOLE, S., HAGERMAN, A.E., and HANLEY, T.A. 1987. Role of tannins in defending plants against ruminants: Reduction in dry matter digestion? *Ecology* 68:1606–1615.

SELL, D.R., and ROGLER, J.C. 1983. Effects of sorghum grain tannins and dietary protein on activity of liver UDP-glucuronyltronsferase. *Proc. Soc. Exp. Biol. Med.* 174:93–101.

SMITH, G.S. 1986. Gastrointestinal toxifications and detoxifications in ruminants in relation to resource management, pp. 514–542, *in* K. Rozman and O. Hanninen (eds.). Gastrointestinal Toxicology. Elsevier, New York.

SQUIBB, R.C., PROVENZA, F.D., and BALPH, D.F. 1990. Effect of age of exposure on consumption of a shrub by sheep. *J. Anim. Sci.* 68:987–997.

SWAIN, T. 1979. Tannins and lignins, pp. 657–682, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, New York.

THORHALLSDOTTIR, A.G., PROVENZA, F.D., and BALPH, D.F. 1990a. Ability of lambs to learn about novel foods while observing or participating with social models. *Appl. Anim. Behav. Sci.* 25:25–33.

THORHALLSDOTTIR, A.G., PROVENZA, F.D., and BALPH, D.F. 1990b. The role of the mother in the intake of harmful foods by lambs. *Appl. Anim. Behav. Sci.* 25:35–44.

WELCH, J.G., and HOOPER, A.P. 1988. Ingestion of feed and water, pp. 108–124, *in* D.C. Church (ed.). The Ruminant Animal. Prentice Hall, Englewood Cliffs, New Jersey.

WESTON, R.H. 1982. Animal factors affecting feed intake, pp. 183–198, *in* J.B. Hacker (ed.). Nutritional Limits to Animal Production from Pastures. Commonwealth Agricultural Bureau, Farnham Royal, U.K.

WINER, B.V. 1971. Statistical Principles in Experimental Design. McGraw-Hill, New York.

WOOD, G.M. 1987. Controlling brush with animals. University of Vermont Agric. Exp. Sta. Res. Rep. 51.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

IDENTIFICATION OF VOLATILE COMPONENTS OF BOBCAT (*Lynx rufus*) URINE

M.J.I. MATTINA,* J.J. PIGNATELLO, and R.K. SWIHART

Connecticut Agricultural Experiment Station
123 Huntington Street
New Haven, Connecticut 0650

(Received July 23, 1990; accepted October 11, 1990)

Abstract—Bobcat (*Lynx rufus*) urine reduces scent-marking activity of woodchucks (*Marmota monax*) and feeding activity of snowshoe hares (*Lepus americanus*) and deer (*Odocoileus virginianus*, *O. hemionus*). In order to identify the semiochemicals responsible for these behavior modifications, a dichloromethane extract of the bobcat urine was analyzed by GC-MS. Among the known compounds identified in the extract are phenol, indole, dimethyl sulfone, and 3-mercapto-3-methylbutanol. Compounds for which spectroscopic data are presented for the first time include one sulfide, two disulfides, and two trisulfides. The sulfur compounds are derived from an amino acid, *S*-(1,1-dimethyl-3-hydroxypropyl)cysteine ("felinine"), which was identified several years ago in the urine of the domestic cat (*Felis domesticus*).

Key Words—Behavior modification, bobcat, *Lynx rufus*, urine components, crop protection, deer, *Odocoileus*, felinine, predator odors, repellent, volatile sulfur compounds, woodchuck, *Marmota monax*.

INTRODUCTION

Mammalian predator odors are effective in altering the behavior and activity of the corresponding mammalian prey (Fulk, 1972; Müller-Schwarze, 1972; Gorman, 1984; Sullivan and Crump, 1986). Recent reports focus on the reduction of herbivore damage to agricultural crops through the application of compounds produced by the natural predators of the herbivores (Sullivan and Crump, 1984; Sullivan et al., 1985a,b, 1988a,b; Swihart, 1990; Swihart et al., 1990). Specifically, sulfur-containing semiochemicals, which reduce feeding damage of

*To whom correspondence should be addressed.

0098-0331/91/0200-0451$06.50/0

snowshoe hares (*Lepus americanus*) on coniferous tree seedlings, have been identified in the glandular secretions of mustelids, a natural predator of hare (Sullivan and Crump, 1984).

Several studies have documented the repellent effect of the urine and/or feces of felids on felid prey, such as deer (*Odocoileus hemionus*, *O. virginianus*) (Müller-Schwarze, 1972; Sullivan et al., 1985b), snowshoe hare (*L. americanus*) (Sullivan et al., 1985a), and woodchuck (*Marmota monax*) (Swihart, 1990). Although a search of *Chemical Abstracts* uncovered no reports detailing the chemical composition of felid urine or feces, three volatile sulfur compounds, which may function as pheromones, have been identified in the urine of red fox (*Vulpes vulpes*): Δ^3-isopentenyl methyl sulfide, 2-phenylethyl methyl sulfide (Jorgenson et al., 1978; Wilson et al., 1978), and 3-methylbutyl methyl sulfide (Bailey et al., 1979). Since volatility is an important property of compounds identified as semiochemicals (Wheeler, 1976), we attempted to identify the volatile components in bobcat urine as the first step in the characterization of the semiochemicals in the urine responsible for the suppression of agricultural damage inflicted by woodchuck and deer.

METHODS AND MATERIALS

The bobcat urine was purchased from Hoosier Trapper Supply (Greenwood, Indiana). Information from the supplier indicated that the urine is sold as collected from caged animals. The material is subject only to screening through cheesecloth or nylon mesh at the time of collection and is supplied in 1-gallon plastic jugs. One liter of the urine was transferred to a 2-liter separatory funnel and extracted with three 50-ml portions of dichloromethane (Optima grade, Fisher Scientific). The extracts were combined and concentrated on a steam bath using a Kuderna-Danish apparatus fitted with a three-ball Snyder column and a 10-ml concentrator tube.

Gas chromatographic (GC) fractionation of the dichloromethane extract was used to obtain relatively pure solutions of several compounds of interest for [^{1}H]NMR and high resolution mass spectrometric (MS) characterization. Fractionation was carried out on a Hewlett-Packard 5840A gas chromatograph fitted with a 2-m × 2-mm glass column packed with 10% SP2100 on 100/120 Supelcoport (Supelco, Bellefonte, Pennsylvania). Helium was used as the carrier gas and chromatograms were recorded with flame ionization detection to establish retention times (*RT*). Fraction collection was accomplished by disconnecting the column from the detector and reconnecting it to 50 cm of uncoated, 0.53-mm-ID fused silica tubing. The approximately 15 cm of tubing that exited the GC oven wall was passed through a 1/8-in.-ID copper tube wrapped in glass wool and heating tape in order to maintain the temperature of

the tubing at 250°C. The end of the fused silica tubing was fitted to a 1/16-in. zero-dead-volume connector (Supelco). A 9-cm length of 1/16-in. stainless-steel tubing, which was attached to the connector with a Fingertight II threaded fitting (Rainin, Woburn, Massachusetts), was used to convey each effluent fraction into 3 ml of deuterochloroform ($CDCl_3$) or methanol contained in a 10-ml culture tube fitted with a septum screw cap. The end of the 1/16-in. stainless-steel conveyance tubing was positioned well below the surface of the $CDCl_3$ or the methanol. The septum was also pierced with a short length of stainless tubing to provide ventilation of the carrier gas. The culture tubes were chilled in an ice bath prior to, during, and following fraction collection. Some condensation of effluent occurred in the conveyance tube, which was at ambient temperature, necessitating a final rinse of the tube into the trap with the appropriate solvent. The GC temperature zones were programmed as follows: injection temperature, 300°C; initial oven temperature, 40°C, ramped at 5°C/min for 20 min then at 3°C to 310°C and held for 20 min.

The GC-MS studies were carried out by injecting the dichloromethane extract in splitless mode on a 30-m × 0.25-mm SPB-608 capillary column (Supelco). An HP 5890 GC was connected via a capillary direct interface to an HP 5988A quadrupole mass spectrometer. The temperature zones were set as follows: injector temperature, 220°C; interface temperature, 250°C; initial oven temperature, 40°C for 3 min, then ramped at 5°C/min to 100°C and held for 5 min, finally ramped at 20°C/min to 280°C, and held for 5 min. The mass spectrometer was operated in electron impact (EI) mode with electron energy set at 70 eV and source temperature at 200°C. Instrument tuning and acquisition were under HP 59970C ChemStation software control (version 3.2). Spectra were acquired in full scan operation from m/z 40 to m/z 500.

High-resolution accurate mass data were acquired either on a Kratos MS80 magnetic sector instrument under DS90 software control or on a VG 70SEQ under OPUS software control. [^{1}H]NMR data were acquired at 500 MHz.

RESULTS AND DISCUSSION

The total ion chromatogram (TIC) for the GC-MS analysis of the dichloromethane extract is shown Figure 1. The TIC peaks are assigned to the compounds illustrated in Figure 2. 3-Methyl-3-buten-1-ol (II), pyrazine (III), methylpyrazine (IV), 2,6-dimethylpyrazine (VII), phenol (IX), trimethylpyrazine (X), dimethyl sulfone (XI), δ-valerolactam (XIII), indole (XVI), 1-methylhydantoin (XVII), and phenylacetamide (XVIII) were positively identified by matching the *RT* and the mass spectrum of the urine extract peak with authentic material. Phenol and dimethyl sulfone (Williams et al., 1966) have been reported to be constituents of mammalian urine. δ-Valerolactam has been

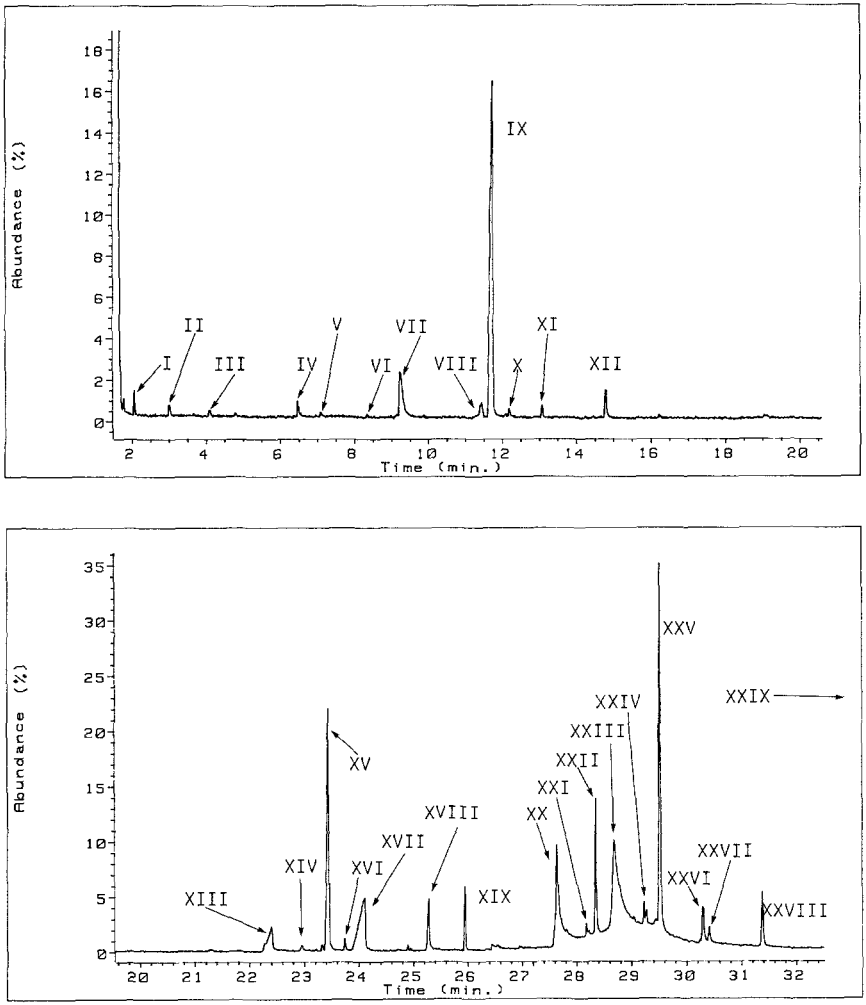
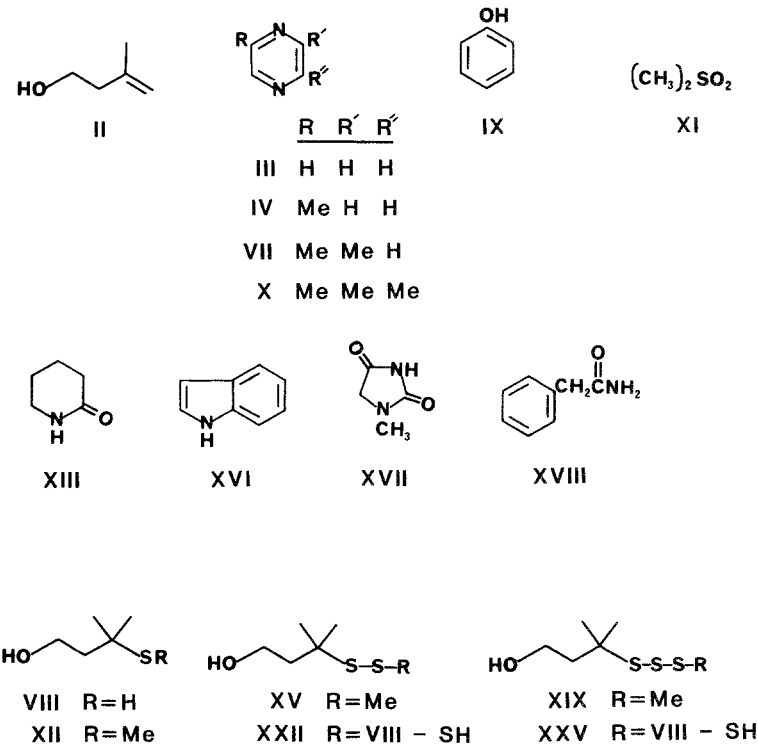


Fig. 1. TIC from the GC-MS of the dichloromethane extract of bobcat urine. I is an artifact from the CH_2Cl_2 solvent. V, VI, XIV, XXI, and XXVII are unidentified compounds.

found in the anal sac secretions of *Felis domesticus* (Preti et al., 1976). Indole has been identified in the anal sac secretions of several mustelids (Brinck et al., 1983).

Fatty acids (XX and XXIII), compounds XXIV, XXVI, bis-(2-ethylhexyl) phthalate (XXVIII), and cholesterol (XXIX) were identified by the probability-based matching algorithm in the HP software. It is not surprising to find fatty



XX	palmitic acid	XXVI	octadecanamide
XXIII	9-octadecenoic acid	XXVIII	phthalate ester
XXIV	nonanamide	XXIX	cholesterol

Fig. 2. Compounds identified in the dichloromethane extract of bobcat urine.

acids and cholesterol as urinary constituents. The phthalate ester (XXVIII) may be derived from laboratory contamination or from plasticizers frequently used in plastic containers such as those in which the urine is supplied. It should be noted that the identification of these high-boiling compounds based solely on the software matching algorithm must be considered tentative. Positive identification awaits spectral and *RT* matching with the authentic materials.

The mass spectra of compounds VIII, XII, XV, XIX, XXII, and XXV are shown in Figures 3–8. None of these spectra matched library entries in the 42,000-entry NBS Library supplied with the HP ChemStation software. All of the spectra are characterized by a base peak at m/z 69. The mass spectra of

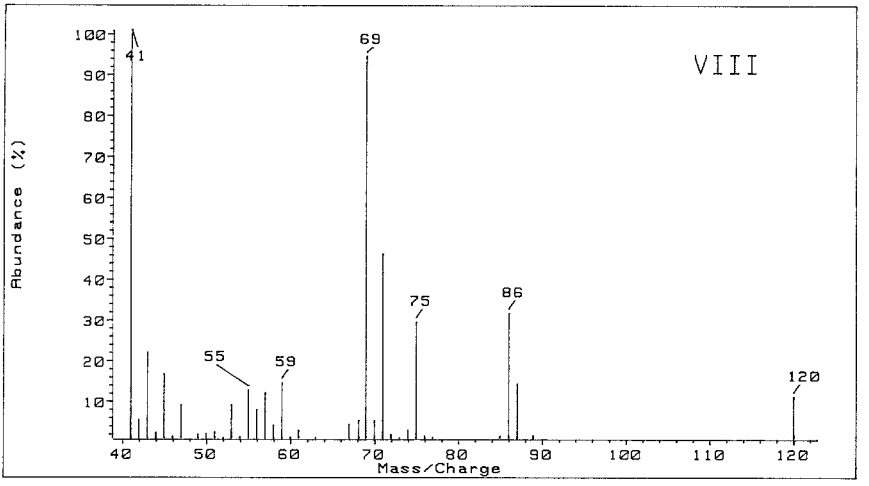


Fig. 3. Mass spectrum of compound VIII.

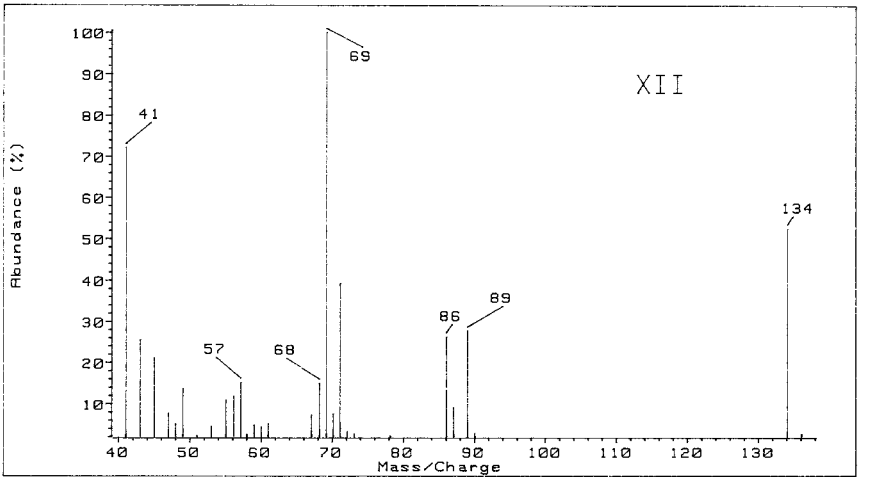


Fig. 4. Mass spectrum of compound XII.

several of the sulfur-containing compounds identified by Crump (1980a,b) and Brinck et al. (1983) in the anal gland secretions of mustelids have a base peak at m/z 69 due to $C_5H_9^+$. The similarity between our mass spectral data and that of Crump and Brinck prompted us to hypothesize that compounds VIII, XII, XV, XIX, XXII, and XXV incorporate sulfur and the isopentyl backbone. The molecular formula for these compounds, as well as the formula for a fragment ion, have been confirmed by high-resolution accurate mass measurements.

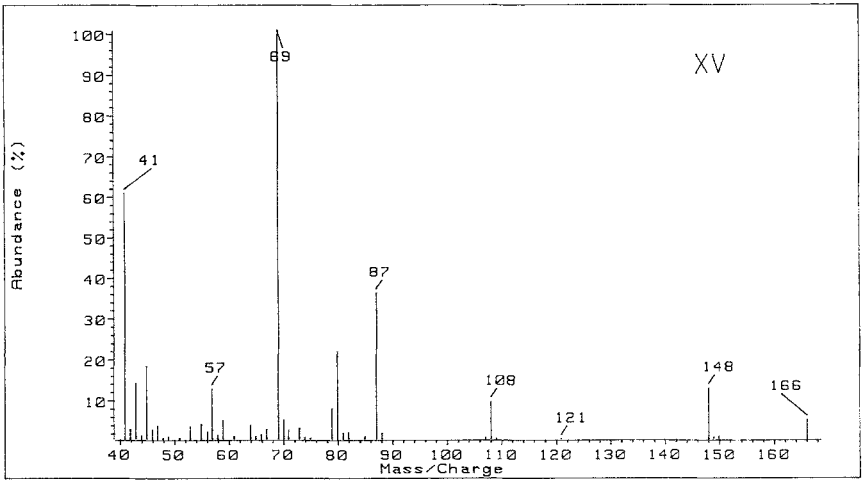


Fig. 5. Mass spectrum of compound XV.

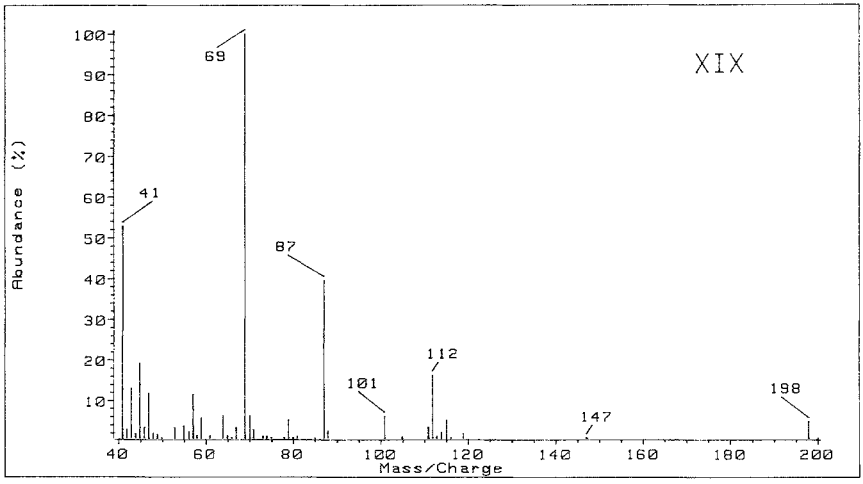


Fig. 6. Mass spectrum of compound XIX.

These data are summarized in Table 1. The 500 MHz [^{1}H]NMR spectral data for compounds VIII, XV, XXII, and XXV in $CDCl_3$ are summarized in Table 2. Compounds XII and XIX could not be obtained pure enough for NMR characterization.

On the basis of the high-resolution accurate mass data, the mass spectral fragmentation pattern (see Figure 3), and the [^{1}H]NMR data, compound VIII is identified as 3-mercapto-3-methylbutanol. This compound has been synthe-

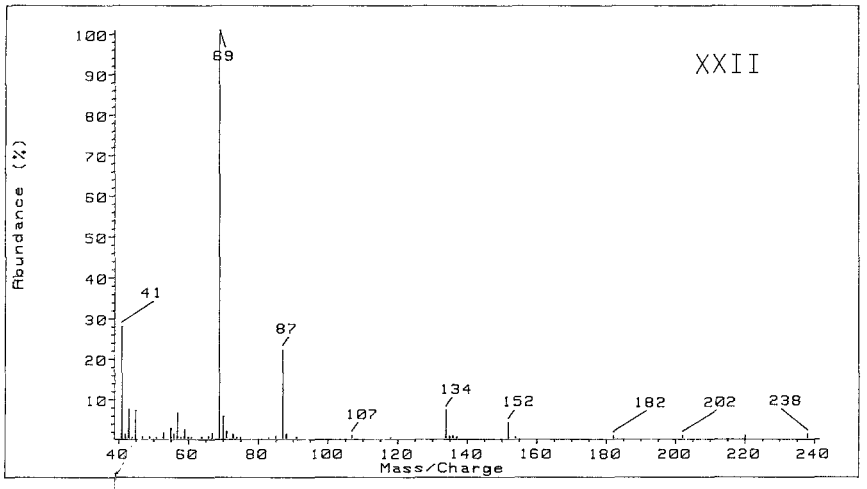


Fig. 7. Mass spectrum of compound XXII.

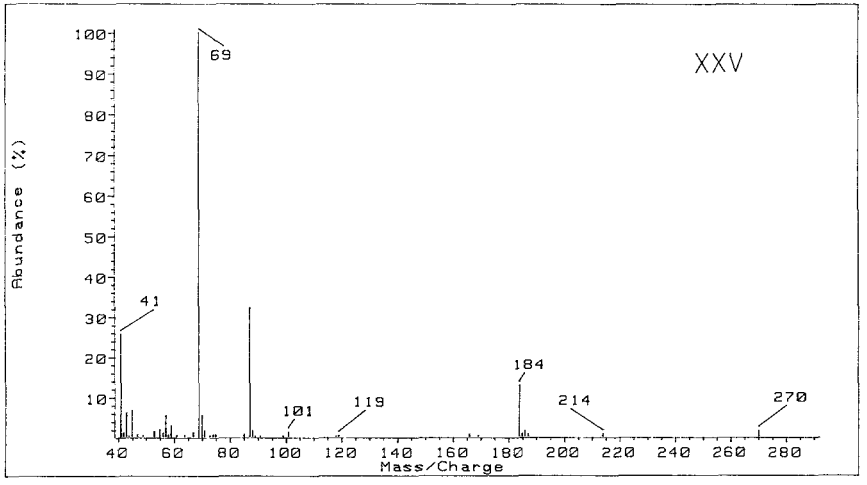


Fig. 8. Mass spectrum of compound XXV.

sized previously and its NMR and IR spectra obtained but not reported (Sweetman et al., 1971). Our [^{1}H]NMR data for compound VIII in $CDCl_3$ at 500 MHz agree with that of the previously synthesized 3-mercapto-3-methylbutanol obtained in CCl_4 at 60 MHz (L. Field, personal communication, 1990), allowing for solvent effects. The singlet (relative intensity 6) at 1.43 ppm and the two triplets (relative intensities 2) at 1.89 ppm and 3.87 ppm confirm the presence of an isopentyl backbone. The chemical shifts of the triplets indicate that

TABLE 1. COMPARISON OF THEORETICAL AND OBSERVED MASSES OF MOLECULAR AND FRAGMENT IONS PROPOSED FOR COMPOUNDS VIII, XII, XV, XIX, XXII, AND XXV

Compound	Formula[a]	Theoretical	Observed
VIII	$C_5H_{12}OS$	120.0606	120.0614
	$C_5H_{10}O$	86.0729	86.0756
XII	$C_6H_{14}OS$	134.0762	134.0765
	C_5H_9	69.0704	69.0702
XV	$C_6H_{14}OS_2$	166.0483	166.0479
	$C_6H_{12}S_2$	148.0378	148.0377
XIX	$C_6H_{14}OS_3$	198.0207	198.0210
	CH_4S_3	111.9475	111.9469
XXII	$C_{10}H_{22}O_2S_2$	238.1056	238.1041
	$C_5H_{10}S_2$	134.0222	134.0222
XXV	$C_{10}H_{22}O_2S_3$	270.0777	270.0781
	$C_5H_{12}OS_3$	184.0048	184.0056

[a]The first ion listed for each compound is the molecular ion.

the hydroxyl group is attached to the primary carbon and the sulfhydryl group is attached to the tertiary carbon (Jackman and Sternhell, 1969).

The structures for compounds XV (an unsymmetrical disulfide), XIX (an unsymmetrical trisulfide), XXII (a symmetrical disulfide), and XXV (a symmetrical trisulfide) are variations of the basis structure of compound VIII. The proposed structures are consistent with the high-resolution accurate masses of the molecular and fragment ions of each compound (see Table 1), the MS fragmentation patterns observed for the compounds (see Figures 5, 6, 7, and 8) and the [^{1}H]NMR data for the compounds (see Table 2), all reported here for the first time. Trisulfide compounds have been identified in nature, both in plants (Kjaer, 1977) and in animals (Singer et al., 1976). The identification of compounds VIII, XV, XIX, XXII, and XXV in the urine of *Felis catus* has been reported without additional experimental detail (Joulain and Laurent, 1989).

The presence of 3-methyl-3-buten-1-ol (compound II), as well as a very small amount of 3-methyl-2-buten-1-ol (RT = 3.4 min, Figure 1), in the dichloromethane extract should be noted. The structural similarity of the alcohol and the compounds VIII, XV, XIX, XXII, and XXV is apparent.

The most probable source of the 3-mercapto-3-methyl-butanol and its di- and trisulfide derivatives, which we have identified in bobcat urine, is the amino acid felinine. Felinine, an *S*-substituted-cysteine, was identified as a major constituent of the urine of another felid, the domestic cat (Westall, 1953). *C*-*S*-lyases are known, which catalyze the fragmentation of *S*-substituted cysteine into mercaptans. This pathway is discussed by Kjaer (1977) as the principal route for the biosynthesis of mercaptans, sulfides, disulfides, and trisulfides.

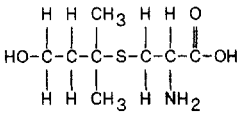
TABLE 2. [^{1}H]NMR CHEMICAL SHIFTS AND COUPLING CONSTANTS FOR COMPOUNDS VIII, XV, XXII, AND XXV

Compound	Chemical shift	Relative Intensity	Proton assignment
VIII[a]	1.43(s)	6	geminal methyl
	1.8 (S)	1	sulfhydryl
	1.89(T), J = 6.5[b]	2	methylene (secondary carbon)
	3.87(T),[c] J = 6.4	2	methylene (primary carbon)
	1.40(S)	6	geminal methyl
	1.75(S)	1	sulfhydryl
	1.82(T), J = 7	2	methylene (secondary carbon)
	3.76(T),[c] J = 7	2	methylene (primary carbon)
XV	1.35(S)	6	geminal methyl
	1.91(T), J = 6.9	2	methylene (secondary carbon)
	2.43(S)	3	methyl sulfide
	3.81(T),[c] J = 6.9	2	methylene (primary carbon)
XXII	1.32(S)	6	geminal methyl
	1.86(T)	2	methylene (secondary carbon)
	3.81(T)[c]	2	methylene (primary carbon)
XXV	1.38(S)	6	geminal methyl
	1.95(T), J = 6.9	2	methylene (secondary carbon)
	3.82(T),[c] J = 6.9	2	methylene (primary carbon)

[a] The first set of chemical shifts and coupling constants are data obtained in the current study. The second set of data were obtained for the previously synthesized compound VIII (L. Field, personal communication).

[b] Typical uncertainty in the J values is ± 0.7 Hz.

[c] Additional fine structure in the methylene multiplet attributed to coupling to the hydroxyl proton is eliminated upon shaking in D_2O. A well-defined triplet at 3.8 ppm results.



Felinine

SCHEME 1.

A synthetic deer repellent based on felinine and the acidic component of lion (*Felis leo*) feces has been patented (Baines et al., 1988). The report does not discuss the presence of sulfur compounds other than felinine in the composition. The repellent is formulated with phenol, indole, δ-valerolactam, and palmitic acid, all of which we have identified as constituents of bobcat urine.

Both Brinck (1983) and Crump (1980a,b) have identified 3,3-dimethyl-1,2-dithiolane ($C_5H_{10}S_2$, molecular weight 134 daltons) in the

anal gland secretions of mustelids. We observe what we believe to be a very small amount of this dithiolane (RT = 14.3 min, Figure 1), identified by its mass spectral fragmentation pattern and the high resolution accurate mass measurement of the molecular ion (m/z = 134.0224), in the extract.

The low-resolution mass spectrum for compound XII (see Figure 4) indicates that the nominal molecular weight of this compound is also 134 daltons. The high-resolution accurate mass of the molecular ion of compound XII, however, is not consistent with the molecular formula of the dithiolane ($C_5H_{10}S_2$), but rather with that of 1,1-dimethyl-3-hydroxypropyl methyl sulfide, $C_6H_{14}OS$ (see Table 1). This methyl sulfide also can be derived from felinine. Further investigations on the structure of compound XII are in progress.

Field trials indicate that the dichloromethane extract of bobcat urine significantly reduces feeding damage to eastern hemlock (*Tsuga canadensis*) by white-tailed deer (Swihart et al., 1990). Similar but less dramatic reductions in damage to woody plants caused by scent-marking activity of woodchucks in apple orchards were noted. Reports on these field studies are forthcoming.

Acknowledgments—We are indebted to Dr. Lamar Field for generously supplying us with the original IR and NMR spectra from his 1971 publication. We acknowledge the work done at the Yale University Instrumentation Center by D. Pentek and P. Demeu in acquiring high-resolution mass spectrometric data and NMR data, respectively. High-resolution GC-MS data also were acquired at the USEPA Environmental Research Laboratory in Athens, Georgia, by Dr. Susan Richardson. We would like to acknowledge the technical assistance of Areej Al-Mudamgha, Marta Day, and Peter Picone in the experimental work described in this study. We are indebted to a reviewer for making us aware of the work of Joulain and Laurent at the Research Laboratories of Robertet S.A., B.P. 100, F-06333 Grasse Cedex, France.

REFERENCES

BAILEY, S., BUNYAN, P.J., and PAGE, J.M.J. 1979. Variation in the levels of some components of the volatile fraction of urine from capative red foxes (*Vulpes vulpes*) and its relationships to the state of the animal, pp. 391–403, *in* D. Müller-Schwarze and R. M. Silverstein (eds.). Chemical Signals: Vertebrates and Aquatic Invertebrates. Plenum, New York.

BAINES, D.A., FAULKES, C. G., TOMLINSON, A.J.,and NING, P.C.Y.K. 1988. Eur. Pat. Appl. EP 280, 443. August 31, 1988.

BRINCK, C., ERLINGE, S., and SANDELL, M. 1983. Anal sac secretion in mustelids. *J. Chem. Ecol.* 9:727–745.

CRUMP, D.R. 1980a. Thietanes and dithiolanes from the anal gland of the stoat (*Mustela erminea*). *J. Chem. Ecol.* 6:341–347.

CRUMP, D.R. 1980b. Anal gland secretion of the ferret (*Mustela putorius* forma *furo*). *J. Chem. Ecol.* 6:837–844.

FULK, G.W. 1972. The effect of shrews on the space utilization of voles. *J. Mammal.* 53:461–478.

GORMAN, M.L. 1984. The responses of prey to stoat (*Mustela erminea*) scent. *J. Zool. (London)* 202:419–423.

JACKMAN, L.M., and STERNHELL, S. 1969. Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry, 2nd ed. Pergamon Press, Oxford, England.

JORGENSON, J.W., NOVOTNY, M., CARMACK, M., COPLAND, G.B., WILSON, S.R., and WHITTEN, W.K. 1978. Chemical scent constituents in the urine of the red fox (*Vulpes vulpes* L.) during the winter season. *Science* 199:796–798.

JOULAIN, D., and LAURENT, R. 1989. The catty odour in black-currant extracts versus the black-currant odour in cat's urine?, p. 87, *in* S.C. Bhattacharyya, N. Sen, and K.L. Sethi (eds.). 11th International Congress of Essential Oils, Fragrances and Flavours, New Delhi, India, November 12–16, 1989. Oxford & IBH Publishing Co., New Delhi.

KJAER, A. 1977. Low molecular weight sulphur-containing compounds in nature: A survey, *Pure Appl. Chem.* 49:137–152.

MÜLLER-SCHWARZE, D. 1972. Responses of young black-tailed deer to predator odors. *J. Mammal.* 53:393–394.

PRETI, G., MUTTERTIES, E.L., FURMAN, J.M., KENNELLY, J.J. and JOHNS, B.E. 1976. Volatile constituents of dog (*Canis familiaries*) and coyote (*Canis latrans*) anal sacs. *J. Chem. Ecol.* 2:177–186.

SINGER, A.G., AGOSTA, W.C., O'CONNELL, R.J., PFAFFMAN, C., BOWEN, D.V., and FIELD, F.H. 1976. Dimethyl disulfide: An attractant pheromone in hamster vaginal secretion. *Science* 191:948–950.

SULLIVAN, T.P. and CRUMP, D.R. 1984. Influence of mustelid scent gland compounds on suppression of feeding by snowshoe hares (*Lepus americanus*). *J. Chem. Ecol.* 10:1809–1821.

SULLIVAN, T.P., and CRUMP, D.R. 1986. Avoidance response of pocket gophers (*Thomomys talpoides*) to mustelid anal gland compounds, pp. 519–531, *in* D. Duval, D. Müller-Schwarze, and R.M. Silverstein (eds.). Chemical Signals in Vertebrates 4, Plenum, New York.

SULLIVAN, T.P., NORDSTROM, L.O., and SULLIVAN, D.S. 1985a. Use of predator odors as repellents to reduce feeding damage by herbivores. I. Snowshoe hares (*Lepus americanus*). *J. Chem. Ecol.* 11:903–920.

SULLIVAN, T.P., NORDSTROM, L.O., and SULLIVAN, D.S. 1985b. Use of predator odors as repellents to reduce feeding damage by herbivores. II. Black-tailed deer (*Odocoileus hemionus columbianus*). *J. Chem. Ecol.* 11:921–935.

SULLIVAN, T.P., CRUMP, D.R., and SULLIVAN, D.S. 1988a. Use of predator odors as repellents to reduce feeding damage by herbivores. III. Montane and meadow voles (*Microtus montanus* and *Microtus pennsylvanicus*). *J. Chem. Ecol.* 14:363–377.

SULLIVAN, T.P., CRUMP, D.R., and SULLIVAN, D.S. 1988b. Use of predator odors as repellents to reduce feeding damage by herbivores. IV. Northern pocket gophers (*Thomomys talpoides*) *J. Chem. Ecol.* 14:379–389.

SWEETMAN, B.J., VESTLING, M.M., TICARIC, S.T., KELLY, P.L., FIELD, L., MERRYMAN, P., and JAFFE, I.A. 1971. Biologically oriented organic sulfur chemistry. 8. Structure–activity relationships of penicillamine analogs and derivatives. *J. Med. Chem.* 14:868–872.

SWIHART, R.K., 1991. Modifying scent-marking behavior to reduce woodchuck damage to fruit trees. *Ecol. Appl.* 1:98–105.

SWIHART, R.K., PIGNATELLO, J.J., MATTINA, M.J.I. 1991. Predator urines reduce browsing damage by white-tailed deer. *J. Chem. Ecol.* Accepted.

WESTALL, R.G. 1953. The amino acids and other ampholytes of urine. *Biochem J.* 55:244–248.

WHEELER, J.W. 1976. Properties of compounds used as chemical signals, pp. 61–70, *in* D. Müller-Schwarze and M. Mozell (eds.). Chemical Signals in Vertebrates. Plenum, New York.

WILLIAMS, K.I.H., BURSTEIN, S.H., and LAYNE, D.S. 1966. Dimethyl sulfone: Isolation from human urine. *Arch. Biochem. Biophys.* 113:251–252.

WILSON, S.R., CARMACK, M., NOVOTNY, M., JORGENSON, J.W., and WHITTEN, W.K. 1978. Δ^3-Isopentenyl methyl sulfide. A new terpenoid in the scent mark in the red fox. (*Vulpes vulpes*). *J. Org. Chem.* 43:4675–4676.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

MEDIATION OF HOST SELECTION BY CUTICULAR HYDROCARBONS IN THE HONEYBEE TRACHEAL MITE *Acarapis woodi* (RENNIE)

P. LARRY PHELAN,[1,*] ALAN W. SMITH,[2] and GLEN R. NEEDHAM[2]

[1]*Ohio Agricultural Research & Development Center*
The Ohio State University
Department of Entomology
Wooster, Ohio 44691

[2]*Acarology Laboratory, Department of Entomology*
The Ohio State University
Columbus, Ohio 43210

(Received August 20, 1990; accepted October 15, 1990)

Abstract—Using a simple two-choice bioassay and video analysis of individual locomotory tracks, it was determined that a preference for young-bee hosts over old-bee hosts in female honeybee tracheal mites, *Acarapis woodi* (Rennie), is chemically mediated. When presented with a choice of cuticular extracts from 5-day-old and < 1-day-old adult bees, mites showed a significant preference for the young-bee extract in three of four bee colony sources. This discrimination was due apparently to a greater positive response elicited by the young-bee extract rather than a deterrent effect of old-bee extract, as the extract of old bees evoked a significantly higher response than a hexane control. A bioassay of silica-gel fractions of young-bee extract indicated that cuticular hydrocarbons alone were responsible for tracheal mite response. Further fractionation of a hydrocarbon fraction by argentation chromatography demonstrated that both saturated and unsaturated hydrocarbons were involved in the mite response, but when presented in opposition, mites showed a stronger response to the saturated than to the unsaturated components. Mites placed in zones treated with cuticular extract of young bees exhibited higher angular velocities than those placed on hexane, causing them to remain in the extract-treated zones for extended periods. These results point to a possible control strategy whereby migration of tracheal mites to young-bee hosts could be disrupted by artificially altering the chemical profile of the honeybee cuticle.

*To whom correspondence should be addressed.

0098-0331/91/0200-0463$06.50/0

Key Words—*Acarapis woodi*, tracheal mite, Tarsonemidae, *Apis mellifera*, Hymenoptera, Apidae, honeybee, hydrocarbons, host selection, cuticular lipids, kairomones.

INTRODUCTION

The honeybee tracheal mite, *Acarapis woodi* (Rennie), spends its entire life cycle primarily inside the first thoracic tracheae of adult European honeybees, *Apis mellifera*, as well as in other bees in the genus *Apis* (Delfinado-Baker and Baker, 1982). Both larval and adult stages pierce the tracheal walls with their mouthparts to feed on the hemolymph. Fertile adult mites transfer from the hair tips of infested bees to new host bees inside the hive. The axillary wing areas are initially sought by migrating mites, where they are protected from host grooming and can move safely to the tracheae of the new host (Royce et al., 1988); once inside the tracheae, females feed and lay several eggs. The economic importance of tracheal mites on the honeybee industry is difficult to assess at this time, with conflicting reports found in the literature (Bailey, 1989; Kjer et al., 1989a,b). This is due partially to the complexities of measuring the effect of the mites on honeybee colonies under controlled experimental conditions; however, numerous studies report that high infestation rates do engender detrimental effects on honeybee colonies, including decreased honey and brood production, shortened life-span, and increased winter mortality (Delfinado-Baker, 1988, and references therein).

Dispersing females rarely infest adult bees older than 4 days, and the susceptibility of bees to infestation declines precipitously from the first day of an adult bee's life (Morgenthaler, 1931; Bailey, 1958; Lee, 1963; Giordani, 1977; Gary and Page, 1987). Hirschfelder and Sachs (1952) suggested that decreasing susceptibility of adult bees to mite infestation was a consequence of the increasing stiffness of setae at the entrance of the thoracic spiracle of older bees; however, Lee (1963) found that neither these guard hairs, the muscles controlling the spiracle, nor the bee's second pair of legs were important in preventing dispersing mites from entering the tracheae of older bees. More recently, Gary and Page (1988) proposed that chemical cues were the most important factor determining host selection by the tracheal mite. In the present study, we: (1) demonstrate for the first time that tracheal mite host finding, as well as the preference for young bees, is chemically modulated; (2) present evidence concerning the chemical functionalities responsible for this preference; and (3) suggest how these chemicals affect the behavior of the mite through a preliminary video analysis of locomotory tracks.

METHODS AND MATERIALS

Combs containing emerging brood from four wild-type colonies (queens inseminated by several drones) were collected from the Ohio State University apiary and were placed in an incubator at 33°C, 50% relative humidity. The following morning, newly emerged worker bees (<24 hr) were collected in polystyrene cups and half of these were frozen immediately at −70°C, while the other half were provisioned with food (sucrose with 8% invert sugars, mixed with honey) and water. These bees were aged five days in the incubator (33°C, 50% relative humidity) and then frozen.

Cuticular extracts of 1- and 5-day-old bees obtained from each of the four colony sources were obtained by immersing 30 bees in 60 ml of hexane for 10 min and then concentrating the resulting extract to 6 ml under a stream of nitrogen (0.005 bee equivalents/μl). Young-bee extracts from colony 1 were fractionated by liquid chromatography using columns made from disposable pipets (70 × 5 mm), packed with silica gel (40–140 mesh). Fifteen bee-equivalents of extract (3 ml) were further concentrated under nitrogen to 0.5 ml and then loaded on the head of the column, which had been conditioned first with methylene chloride and then with hexane. Hydrocarbons were separated from non-hydrocarbon components of the extract by eluting the former with two 1-ml fractions of hexane and the latter with two 1-ml fractions of methylene chloride. Hydrocarbons were rechromatographed on silica gel impregnated with 20% silver nitrate (w/w); saturated hydrocarbons were eluted with two 1-ml fractions of hexane, while the unsaturated hydrocarbons were eluted by methylene chloride.

Gas-chromatographic analyses were carried out on a Hewlett-Packard 5890 capillary gas chromatograph equipped with a flame ionization detector and a Hewlett-Packard 5970 mass selective detector. Extracts or fractions (0.05 bee equivalents) were chromatographed using a splitless injection at 280°C onto a 0.25-mm × 30-m RSL-150 column (0.2 μm film thickness; Alltech Assoc., Deerfield, Illinois) held at 120°C for 1 min, then programmed to 300°C at 15°C/min, and a helium carrier flow of 0.9 ml/min. GC profiles of hydrocarbon silica-gel fractions from young and old bees of each colony were compared using a correlation matrix based on peak areas of the 20 most prominent hydrocarbon components.

Cuticular extracts and chromatographic fractions were tested for behavioral activity using a two-choice bioassay design. Glass cover slips (18 × 18 mm) were divided in half by drawing a line on the underside with an ink pen. A small ink dot was placed in the center of this line to designate the release zone for the mites. Treatments were applied to opposite halves of the top of the cover slip with a 20-μl micropipet (0.1 bee equivalents), and the solvent was allowed

to evaporate. Mites were collected using the dissection technique described by Smith et al. (1987) and Smith and Needham (1988), and transferred to the release zone of the cover slip with a human eyelash affixed to the end of a wooden dowel rod. Cover slips then were put in a glass Petri dish inside a transparent, plastic humidity chamber, held at ca. 93% relative humidity and 23°C by using a saturated salt solution of potassium nitrate. The influence of light was minimized by draping an opaque cloth over the entire system; the system was rotated 180° between tests. After 5 min, the response of the female mite was scored by locating her position on the cover slip with a Wild Heerbrugg® dissecting microscope (model M5A). The sequence of treatment presentation was randomized, with 50 female mites tested for each treatment; mites were tested individually and were only used once. The ratio of response in each bioassay was analyzed by chi-square ($P < 0.05$) to test for deviation from the expected ratio of 25:25 for 50 mites.

The locomotory behavior of individual mites was analyzed by videorecording the tracks of female mites placed on a 3-mm-diam. zone treated with either 5 μl of young-bee extract or an equivalent volume of hexane (five mites per treatment). Tracks were recorded using a Panasonic Solid State Color Videocamera (model WV-CD110A) attached to the microscope and then transcribed to clear acetate sheets placed over the video monitor. Lengths of tracks at 15-sec intervals were measured using an Alvin Curvameter, a hand-held instrument designed for measuring distances on maps.

RESULTS

Young- vs. Old-Bee Extracts. Female tracheal mites showed a significant preference for the cuticular extract of young bees over that of old bees in three of the four bee colonies tested (Table 1). Only in colony 2 was no significant discrimination between the extracts of young and old bees detected. When the extract of young bees from colony 2 was compared to old-bee extract from colony 1, mites showed a significant preference for the former (43:7, $\chi^2 = 25.92$). In addition, mites also preferred the extract of colony 2 old bees over that of colony 1 old bees (34:16, $\chi^2 = 6.48$). When extracts from young and old bees of colony 1 each were compared to a hexane blank, both elicited a significant positive response (Table 1).

In a comparison of hydrocarbon profiles from old bees of each colony with that from young bees averaged across the four colonies, profile similarity was inversely related to the degree of discrimination exhibited by females in the bioassay. Colony 2 old-bee profiles were most similar to young-bee profiles ($r = 0.82$), while old-bee profiles from colony 1 showed the greatest difference

($r = 0.47$). Similarity indices for old-bee hydrocarbon profiles from colonies 3 and 4 relative to young-bee profiles were 0.56 and 0.70, respectively.

Hydrocarbon vs. Nonhydrocarbon Components of Young-Bee Extract. The response of female mites to young-bee extract appeared to be due solely to the hydrocarbon lipids in that extract (Table 2). When the hydrocarbon-containing silica-gel fraction (hexane-eluted) was tested against the original extract, mites showed a significant preference for the former, while the nonhydrocarbon lipid fraction (methylene chloride-eluted) was significantly less active than the unfractionated extract. There was no difference in the response elicited by the recombined fractions and the crude extract.

Saturated vs. Unsaturated Hydrocarbons of Young-Bee Extract. When the above hydrocarbon fraction was refractionated on $AgNO_3$-impregnated silica gel, and these fractions were presented against the crude young-bee extract,

TABLE 1. DISCRIMINATION BETWEEN CUTICULAR EXTRACTS OF YOUNG (< 1-DAY-OLD) AND OLD (5-DAY-OLD) BEES FROM FOUR WILD-TYPE COLONIES BY FEMALE TRACHEAL MITES

Source	Young–Old	χ^{2a}
Colony 1	41:9	20.48*
Colony 2	28:22	0.72
Colony 3	35:15	8.00*
Colony 4	32:18	3.92*
Colony 1		
Young–hexane	46:4	35.28*
Old–hexane	44:6	28.88*

[a] Chi-square values followed by an asterisk are significant at $P < 0.05$.

TABLE 2. RESPONSE OF FEMALE TRACHEAL MITES TO CRUDE YOUNG-BEE CUTICULAR EXTRACTS VERSUS HYDROCARBON (HC) OR NONHYDROCARBON (NON-HC) CUTICULAR COMPONENTS

Treatment		χ^{2a}
Crude–HC lipids[b]	11:39	15.68*
Crude–non-HC lipids[c]	40:10	18.00*
Crude–HC + non-HC lipids	27:23	0.32
Crude–hexane	45:5	32.00*

[a] Chi-square values followed by an asterisk are significant at $P < 0.05$.
[b] Eluted from silica gel by hexane.
[c] Eluted from silica gel by methylene chloride.

neither fraction 2 nor fraction 4 elicited a response significantly different from the extract, while fractions 1 and 3 were significantly less active than the extract (Table 3). GC-MS analysis of these fractions showed that fraction 2 contained saturated hydrocarbons, while fraction 4 contained unsaturated hydrocarbons. Furthermore, mites showed no preference for fraction 3 over a hexane control (Table 3), thus confirming the complete separation of the saturated and unsaturated hydrocarbons. When allowed to choose between saturated hydrocarbons (fraction 2) and unsaturated hydrocarbons (fraction 4), mites showed a strong preference for the former. In total, these results suggest that of the honeybee cuticular lipids, saturated and unsaturated hydrocarbons both possess positive behavioral activity, but saturated hydrocarbons may play the greater role in tracheal-mite host selection.

Analysis of Tracheal-Mite Locomotory Tracks. Preliminary observations of female mites walking in the vicinity of honeybee extract revealed no orientation of the mites toward treated areas; however, when placed in contact with a zone treated with young-bee extract, the locomotory tracks of the mites were dramatically different from those of mites placed in hexane-treated zones (Figure 1). Mites showed a much higher angular velocity in the presence of bee extract, exhibiting tracks that circled within the treated zone and causing them to remain in the zone for extended periods. Mean ($\pm$SEM) track lengths within the zones were 36.5 $\pm$ 4.8 mm for young-bee extract and 5.6 $\pm$ 1.7 mm for hexane ($t = 6.06$, $P = 0.004$, $df = 4$). Rates of locomotion within a track showed very low variance, with mites in extract-treated zones appearing to move slightly faster than those on hexane, although this difference was not significant, possibly due to the low number of tracks analyzed (0.14 $\pm$ 0.01 mm/sec in extract-treated zones and 0.11 $\pm$ 0.002 mm/sec in hexane, $t = 2.41$, $P = 0.07$, $df = 4$).

TABLE 3. RESPONSE OF FEMALE TRACHEAL MITES TO $AgNO_3$-IMPREGNATED SILICA-GEL FRACTIONS OF YOUNG-BEE CUTICULAR HYDROCARBONS

Treatment		χ^{2a}
Crude–fraction 1	38:12	13.52*
Crude–fraction 2	28:22	0.72
Crude–fraction 3	42:8	23.12*
Crude–fraction 4	29:21	1.28
Crude–all fractions	27:23	0.32
Fraction 3–hexane	27:23	0.32
Fraction 2–fraction 4	35:15	8.00*
Crude–hexane	44:6	28.88*

[a] Chi-square values followed by an asterisk are significant at $P < 0.05$.

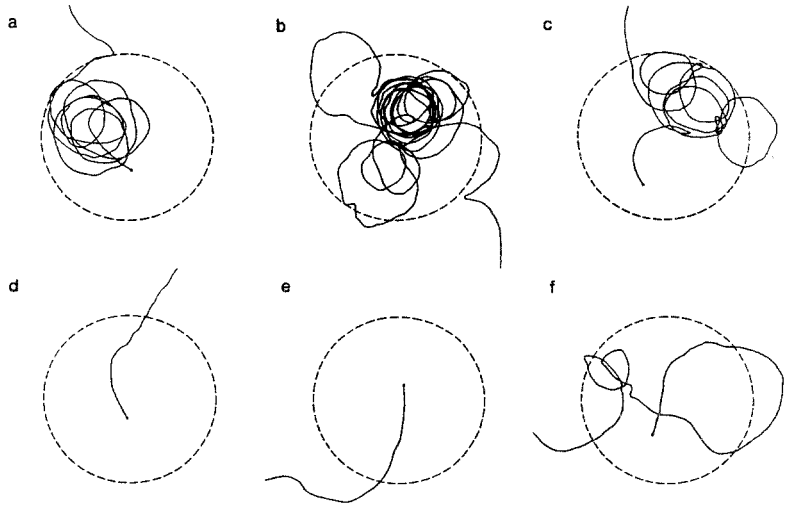


FIG. 1. Representative locomotory tracks of female tracheal mites in a 3-mm-diam. zone treated with young-bee extract (a–c) or hexane (d–f). The width of the female body was approximately four times the width of the line.

DISCUSSION

Several authors have documented the preference of tracheal mites for younger-bee hosts (Morgenthaler, 1931; Bailey, 1958; Lee, 1963; Giordani, 1977; Gary and Page, 1987). Although we do not know the adaptive function, if any, of this preference for young bees, there are several possibilities. First, the life cycle of the tracheal mite requires approximately 20 days (Bailey, 1963, Royce et al., 1988), while the average life-span of the honeybee is approximately 30 days during the peak flowering season (Wilson, 1971). Thus, the selection of young hosts may enhance the probability of completing development before host death. Alternatively, the preference may have originally arisen as a means to reduce competition with other mites, since, in the absence of any preference, the young bees would be less likely to have been colonized already by other mites. Finally, young bees stay in the colony, engaging in tasks such as brood care, while older bees assume the riskier roles such as foraging. By spending more of their developmental time inside the hive, these parasitic mites would enhance their survival.

Whatever its evolutionary significance, on a proximal level it is clear that the age-related preference of honeybee tracheal mites is mediated in some measure by chemoreception. Our preliminary video analysis suggests that the preference measured in this study is due to an increased angular velocity caused by cuticular lipids of young bees. Although we did not see any evidence for ori-

entation toward an extract-treated zone, once in contact with the extract, mites engaged in an extended series of circus movements that caused them to remain in that area for a longer period than when they were in contact with a hexane-treated zone. When mites that were walking on the extract came to the edge of the treatment, they generally turned back; however, most of the turning occurred well before they reached the border of the treated area. It also is interesting to note that despite the high angular velocity, turning was almost entirely in one direction, with little change from clockwise to anticlockwise.

Throughout this study, female tracheal mites consistently showed a significantly higher probability of contact with extracts of young bees than with a hexane control in our two-choice bioassay ($\bar{x} = 90\%$ across all experiments). In only one of the bee colonies did the mites not show a preference for the extract of young bees over old bees. When extracts of either young or old bees from this colony (colony 2) were presented to the mites against extract of colony 1 old bees, both elicited a significantly higher level of response. Thus, the lack of mite differentiation between young and old bees of colony 2 appeared to be due to a greater level of mite response to old bees of this colony compared to that of other colonies, rather than a lack of activity elicited by the cuticular extract of young bees. This view was supported by the fact that the GC hydrocarbon profile of colony 2 old bees was more similar to young-bee profiles than were old-bee profiles from any of the other three colonies. Even among the other three colonies, there appeared to be considerable variation in the level of female discrimination, and this discrimination appeared to be correlated with the degree of difference between hydrocarbon profiles of old and young bees. For colony 1, there was a 4.5-fold higher response to young-bee extract, while in colony 4, this response was only 1.7 times greater. Thus, colony 2 apparently only represents a greater extreme of this variability in age-related changes in surface-extractable lipids. The fact that the old-bee extract from colony 1 elicited a strong positive response when presented with hexane indicates that the age-related preference was due to the greater activity of the young-bee extract rather than a deterrent effect by old bees.

The combined results of the bioassays of fractionated extract of young bees suggest a mixture of both saturated and unsaturated hydrocarbons as the chemical constituents responsible for mite response, with the greater role probably played by the saturated components. Although almost all studies of honeybee cuticle have focused on hydrocarbons, a large portion of extractable cuticular lipids are nonhydrocarbons (Francis et al., 1989), such as long-chain fatty acids, the probable biosynthetic precursors of cuticular hydrocarbons. Not only does the positive response to young-bee extract appear to be elicited solely by the hydrocarbon constituents, the isolated hydrocarbons actually were preferred to the unfractionated extract, a preference that was lost when the hydrocarbon

fraction was recombined with the nonhydrocarbon components of the extract (Table 2). Francis et al. (1989) determined that surface-extractable nonhydrocarbon lipids increase by more than 10-fold in European honeybee workers during the 12 days after emergence as adults. Thus, a possible adaptive hypothesis for the reduced response to hydrocarbons caused by nonhydrocarbon lipids is that the concentration of nonhydrocarbons is used by tracheal mites as one indicator of honeybee age.

In addition to an age-related increase in extractable nonhydrocarbon lipids, Francis et al. (1989) also found dramatic and complex shifts in the relative proportion of hydrocarbon components. For example, unsaturated hydrocarbons increased proportionally relative to saturated hydrocarbons from 8% in newly emerged bees to 51% by day 6, and methyl- and dimethylalkanes decreased relative to normal-chain hydrocarbons from 46% to 2% during this same time period. Thus, the higher response to a fraction containing saturated hydrocarbons over the fraction containing unsaturated hydrocarbons suggests that the preference for young-bee extract may be due to a preference for alkanes, and particularly branched alkanes, which are virtually absent in older bees. The comparable level of response of the unsaturated hydrocarbon fraction relative to the crude cuticular extract is more difficult to understand, since these constituents are found in higher proportions in older bees. However, whereas all branched alkanes show a decline in the cuticle with age, the quantitative change in unsaturated constituents is more complex, with the overall increase due largely to an increase in the absolute amount of C_{33} and C_{35} alkenes and alkadienes alone (Francis et al., 1989). Many shorter-chain alkenes show a concomitant decline in absolute quantity with age. Thus, tracheal-mite response to the unsaturated hydrocarbon fraction may be due to a positive response to specific unsaturated components, or this response simply may represent a very general response to hydrocarbons, at least as measured by our bioassay. A more detailed study of the chemical modulation of tracheal-mite behavioral mechanisms might clarify the role of each of these chemical groups.

Chemoreception also may play a significant role in the host selection of other mite species. Hunter and Rosario (1988) have reviewed the evidence for olfactory perception of hosts in phoretic Mesostigmata. Olfactory receptors on tarsi I are specifically implicated in this process, as three distantly related species are unable to locate their hosts after amputation of these tarsi. These researchers suggest that cuticular hydrocarbons may play an important role in host seeking by Mesostigmata as well, although they present no evidence to support this supposition and erroneously refer to them as acting as a potential "attractant allomone." *Varroa jacobsoni*, another mite parasitic on honeybees, also uses olfactory chemoreception to locate suitable drone larvae (Le Conte et al., 1989). In this case, host finding is mediated by a blend of methyl palmitate,

ethyl palmitate, and methyl linoleate. Each ester evoked a significant response when presented individually against a solvent blank, and the blend of three components was no more active than methyl palmitate alone.

The results presented here demonstrate that host selection by the female tracheal mite is modulated at least in part by age-related changes in the cuticular-lipid profiles of the honeybee. We hypothesize that the preference for young bees over old bees observed for this species is due to changes in the tracheal-mite locomotory behavior caused primarily by saturated, and most probably branched, hydrocarbons, a response that may be attenuated by the presence of nonhydrocarbon lipids. We have not addressed, however, the mechanism by which the hydrocarbons act. Although members of the tracheal mite family Tarsonemidae have chemoreceptors on the tarsi of their front legs and gnathosoma (Krantz, 1978), we do not know if the mites are perceiving the chemicals via olfaction or gustation. Also to be resolved is the relative importance of mite chemoreception in relation to other modalities that might operate in tracheal-mite host selection. Nevertheless, it appears that extractable cuticular components must play a major role inasmuch as female mites showed a strong discrimination for young hosts in the absence of other potential cues. These findings point to a possible control strategy whereby migration of tracheal mites to young-bee hosts could be disrupted by artificially altering their chemical profile. In fact, the presence of vegetable oil on the integument of bees, which would increase the proportion of nonhydrocarbon lipids, significantly reduced the colonization of young bees by tracheal mites (Gary and Page, 1987; Smith, 1990). This novel control strategy is currently being investigated for possible implementation on a larger scale.

Acknowledgments—We thank A.C. Wayadande, and Drs. D.L. Wrensch, R.E. Page, Jr., and R.E. Treece for their helpful comments on the manuscript. R. Hendrickson kindly provided infested bees for a portion of the study, and J.L. Blackmer assisted in preparation of graphics. Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University, as well as by a grant from the California State Beekeepers Association. Ohio Agricultural Research & Development Center Manuscript No. 223-90.

REFERENCES

BAILEY, L. 1958. The epidemiology of the infestation of the honeybee, *Apis mellifera* L., by the tracheal mite, *Acarapis woodi* Rennie and the mortality of infested bees. *Parasitology* 48:493–506.

BAILEY, L. 1963. Infectious Diseases of the Honey Bee. Land Books, London. 176 pp.

BAILEY, L. 1989. Some notes on *Acarapis woodi* (Rennie). *Am. Bee J.* 129:543–545.

DELFINADO-BAKER, M. 1988. The tracheal mite of honey bees: A crisis in beekeeping, pp. 493–497, *in* G.R. Needham, R.E. Page, Jr., M. Delfinado-Baker, and C.E. Bowman (eds.). Africanized Honey Bees and Bee Mites. Ellis Horwood, Chichester. 572 pp.

DELFINADO-BAKER, M., and BAKER, E.W. 1982. Notes on honey bee mites of the genus *Acarapis* Hirst (Acari: Tarsonemidae). *Int. J. Acarol.* 8(4):211–226.

FRANCIS, B.R., BLANTON, W.E., LITTLEFIELD, J.L., and NUNAMAKER, R.A. 1989. Hydrocarbons of the cuticle and hemolymph of the adult honey bee (Hymenoptera: Apidae). *Ann. Entomol. Soc. Am.* 82(4):486–494.

GARY, N.E., and PAGE, R.E., JR. 1987. Phenotypic variation in susceptibility of honey bees, *Apis mellifera*, to infestation by tracheal mites, *Acarapis woodi*. *Exp. Appl. Acarol.* 3:291–305.

GARY, N.E., and PAGE, R.E., JR. 1988. Factors that affect the infestation of worker honey bees by tracheal mites (*Acarapis woodi*), pp. 506–511, *in* G.R. Needham, R.E. Page, Jr., M. Delfinado-Baker, and C.E. Bowman (eds.). Africanized Honey Bees and Bee Mites. Ellis Horwood, Chichester. 572 pp.

GIORDANI, G. 1977. Facts about acarine mites, pp. 459–467, *in* C. Meletinov (ed.). Proceedings, XXVIth International Congress on Apiculture.

HIRSCHFELDER, H., and SACHS, H. 1952. Recent research on the acarine mite. *Bee World* 33:201–209.

HOWARD, R. W., and BLOMQUIST, G. J. 1982. Chemical ecology and biochemistry of insect hydrocarbons. *Annu. Rev. Entomol.* 27:149–172.

HUNTER, P.E., and ROSARIO, R.M.T. 1988. Associations of Mesostigmata with other arthropods. *Annu. Rev. Entomol.* 33:393–417.

KJER, D.M., RAGSDALE, D.W., and FURGALA, B. 1989a. A retrospective and prospective overview of the honey bee tracheal mite, *Acarapis woodi* R. *Am. Bee J.* 129:25–28.

KJER, D.M., RAGSDALE, D.W., and FURGALA, B. 1989b. A retrospective and prospective overview of the honey bee tracheal mite, *Acarapis woodi* R. *Am. Bee J.* 129:112–115.

KRANTZ, G.W. 1978. A Manual of Acarology, 2nd ed. Oregon State University Bookstores, Corvallis. pp. 27–29.

LE CONTE, Y., ARNOLD, G., TROUILLER, J., MASSON, C., CHAPPE, B., and OURISSON, G. 1989. Attraction of the parasitic mite *Varroa* to the drone larvae of honey bees by simple aliphatic esters. *Science* 245(4918):638–639.

LEE, D.C. 1963. The susceptibility of honey bees of different ages to infestation by *Acarapis woodi* (Rennie). *J. Insect Pathol.* 5:11–15.

MORGENTHALER, O. 1931. An acarine disease experimental apiary in the Bernese Lake district and some of the results obtained there. *Bee World.* 12:8–10.

ROYCE, L.A., KRANTZ, G.W., IBAY, L.A., and BURGETT, D.M. 1988. Some observations on the biology and behavior of *Acarapis woodi* and *Acarapis dorsalis* in Oregon, pp. 498–505, *in* G.R. Needham, R.E. Page, Jr., M. Delfinado-Baker, and C.E. Bowman (eds.). Africanized Honey Bees and Bee Mites. Ellis Horwood, Chichester. 572 pp.

SMITH, A.W. 1990. Population dynamics and chemical ecology of the honey bee tracheal mite, *Acarapis woodi* (Acari: Tarsonemidae) in Ohio. PhD dissertation. The Ohio State University, Columbus, Ohio.

SMITH, A.W., and NEEDHAM, G.R. 1988. A new technique for the rapid removal of tracheal mites from honey bees for biological studies and diagnosis, pp. 530–534, *in* G.R. Needham, R.E. Page, Jr., M. Delfinado-Baker, and C.E. Bowman (eds.). Africanized Honey Bees and Bee Mites. Ellis Horwood, Chichester. 572 pp.

SMITH, A.W., NEEDHAM, G.R., and PAGE, R.E., JR. 1987. A method for the detection and study of live honey bee tracheal mites (*Acarapis woodi* Rennie). *Am. Bee J.* 127:433–436.

WILSON, E.O. 1971. The Insect Societies. Harvard University Press, Cambridge, Massachusetts.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

TRIMEDLURE ENANTIOMERS: DIFFERENCES IN ATTRACTION FOR MEDITERRANEAN FRUIT FLY, *Ceratitis capitata* (WIED.) (DIPTERA: TEPHRITIDAE)

R.E. DOOLITTLE,* R.T. CUNNINGHAM,[1] T.P. McGOVERN,[2] and P.E. SONNET[3]

Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Gainesville, Florida 32604

(Received July 10, 1990; accepted October 22, 1990)

Abstract—The most biologically active enantiomer of trimedlure, a synthetic lure attractive to male Mediterranean fruit flies, is the 1*S*,2*S*,4*R* enantiomer of isomer C, the *tert*-butyl ester of *cis*-4-chloro-*trans*-2-methylcyclohexanecarboxylic acid. We also determined that the 1*R*,2*R*,5*S* enantiomer of isomer A is significantly more attractive than its optical antipode. Essential differences in the current synthetic work that are critical to possible commercialization of this preparation are detailed herein.

Key Words—Mediterranean fruit fly, *Ceratitis capitata* Diptera, Tephritidae, attractant, trimedlure, stereoisomers, enantiomers.

INTRODUCTION

The synthetic male lure, trimedlure, has been used for over 25 years as the best practical attractant for survey and detection traps for the Mediterranean fruit fly, *Ceratitis capitata* (Wied.). It is a mixture of the eight stereoisomers of the *tert*-butyl esters of the *cis*- and *trans*-4 and 5-chloro-*trans*-2-methylcyclohexanecarboxylic acids (Beroza et al., 1961; McGovern and Beroza, 1966; McGovern et al., 1986a) (Figure 1). The commercial lure also contains less

*To whom correspondence should be addressed.
[1] Present address: Tropical Fruit and Vegetable Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Honolulu, Hawaii 96804.
[2] Deceased.
[3] Present address: Chemical Modification Research, Agricultural Research Service, U.S. Department of Agriculture, Wyndmoor, Pennsylvania 19118.

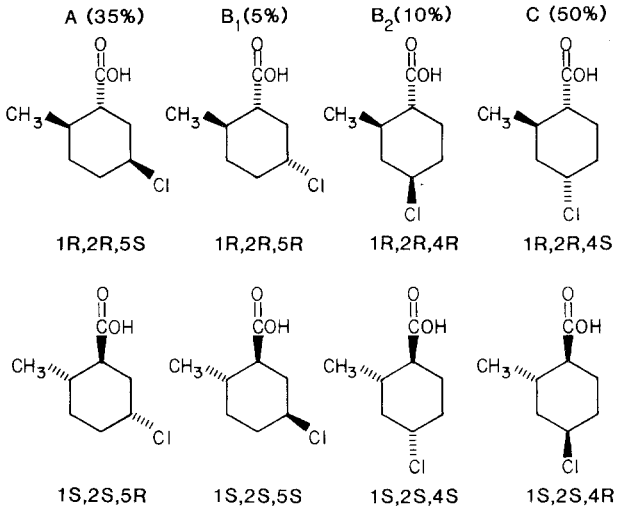


FIG. 1. Primary composition of commercial trimedlure (less than 5% 1,6-*cis*-isomers). Letter designators are those of McGovern and Beroza (1966).

than 5% of materials where the methyl and carboxyl groups are *cis*. It was recognized early through olfactometer tests that the four *trans* isomers (designated A, B_1, B_2, and C, each consisting of a pair of enantiomers) differed in biological activity (McGovern et al., 1966). These differences in biological activity recently have been confirmed and the relative rankings clarified in statistically analyzed field plot tests (McGovern et al., 1986a,b).

Sonnet et al. (1984) prepared the enantiomers of these structures and found differences in attraction between the enantiomers of A and C. However, the details of the bioassay results were not reported. This is the first report of the results of field tests on the relative attraction of the eight enantiomers of *trans*-trimedlure. It confirms the initial observations and includes the enantiomers of B_1 and B_2. In addition, sufficient materials were prepared for replicated testing of the A and C enantiomeric pairs to allow for greater statistical confidence in the results that had been the case in previous studies. Amplification of the synthetic details is provided herein.

METHODS AND MATERIALS

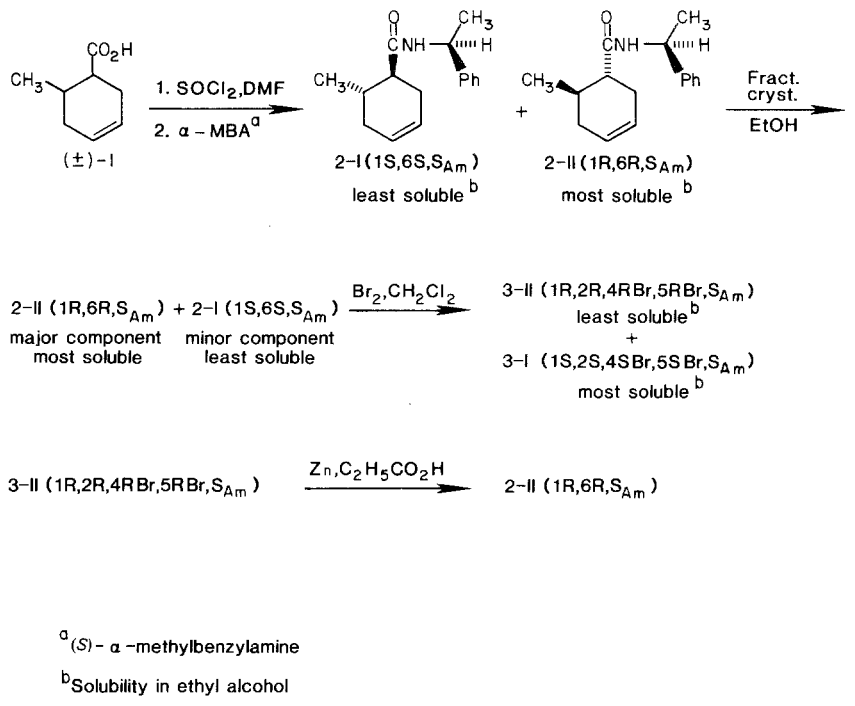
Chemical. Gas–liquid chromatography (GLC) was performed with Varian 1400, 1420, and 2100 instruments using the following capillary columns: column A, CPS-1 fused silica (0.25 mm × 34 m) (Quadrex Corp., New Haven, Connecticut); column B, BP-1 fused silica (0.22 mm × 12 m) (SGE, Inc.,

Austin, Texas); column C, OV-1701 fused silica (0.25 mm × 15 m) with helium carrier gas at 19 cm/sec and operated at temperatures as indicated. Infrared data were obtained with a Perkin-Elmer model 467 spectrophotometer using 3% solutions in CCl_4 or $CHCl_3$. A Nicolet 300-MHz FT NMR spectrometer was employed to obtain [^{1}H]- and [^{13}C]NMR data with 1 and 20% solutions in $CDCl_3$, respectively. Chemical ionization (methane) mass spectra were obtained with a Nermag model R10-10 spectrometer that was equipped with a chromatographic inlet served by a Varian 6000 instrument with a 3% OV-1 glass column (0.32 mm × 50 m). The HPLC column employed was a 2- to 10-μm BioSil-A (25 mm × 25 cm) operated with a Laboratory Data Control Constametric 2G pump and a Waters Associates model R401 differential refractometer detector with 1% diethyl ether in hexane as the eluting solvent. The α-methylbenzylamines were purchased from Hexcel Corp., Zeeland, Michigan. The (*S*)-α-methylbenzylamine was determined by the method described by Sonnet et al. (1984) to be 99.6% pure by GLC analysis of its methoxy (trifluoromethyl)phenyl acetamide. The *trans*-6-methyl-3-cyclohexene carboxylic acid (siglure acid) used as a starting material was obtained from Benzyl Products, Inc., Richmond, California. The commercially prepared acid had a mp of 46–57°C and contained approximately 3–5% of the *cis* isomer as determined by conversion to methyl esters with diazomethane and GLC analysis on column A at 100°C. One recrystallization from hexane at a ratio of 75 g of acid to 200–250 ml of solvent produced acid: mp 60–63°C. Green and Beroza (1959) reported mp 64–65°C for a sample of this acid that was 99+% isomerically pure by GLC analysis of the methyl ester.

The eight pure enantiomers of *trans*-trimedlure were prepared in a manner similar to the procedures described by Sonnet et al. (1984) (Figures 2 and 3). Only the essential and significant modifications are described at appropriate locations throughout this section. Authentic samples of intermediates and products were on hand from previous preparations for direct analytical comparison.

Synthesis and Partial Resolution of α-Methylbenzylamides 2-I and 2-II. The synthesis and resolution of the (*S*)-α-methylbenzylamide of *trans*-6-methyl-3-cyclohexenecarboxylic acid (±)-I was carried out as described previously, and the retention times were identical to those of authentic samples from the previous preparations. The 1*S*,6*S*,S_{Am} (Am = α-methylbenzylamine) amide (2-I) was produced in 50% yield of theoretical and 99.9% purity (as determined by GLC on column A at 230°C) by four to five recrystallizations from 95% ethanol using 5 ml/g of amide. The GLC κ's for the 1*S*,6*S*,S_{Am} and the 1*R*,6*R*,S_{Am} (2-II) were 14.22 and 14.59, respectively with α = 1.026. The [^{1}H]NMR and infrared spectra of these diastereomeric amides, as well as the mp were identical to those of authentic samples from the previous preparations.

Resolution of α-Methylbenzylamide 2-II. The 1*R*,6*R*,S_{Am} amide (2-II) [the more soluble diastereomer that was recovered from the mother liquors from



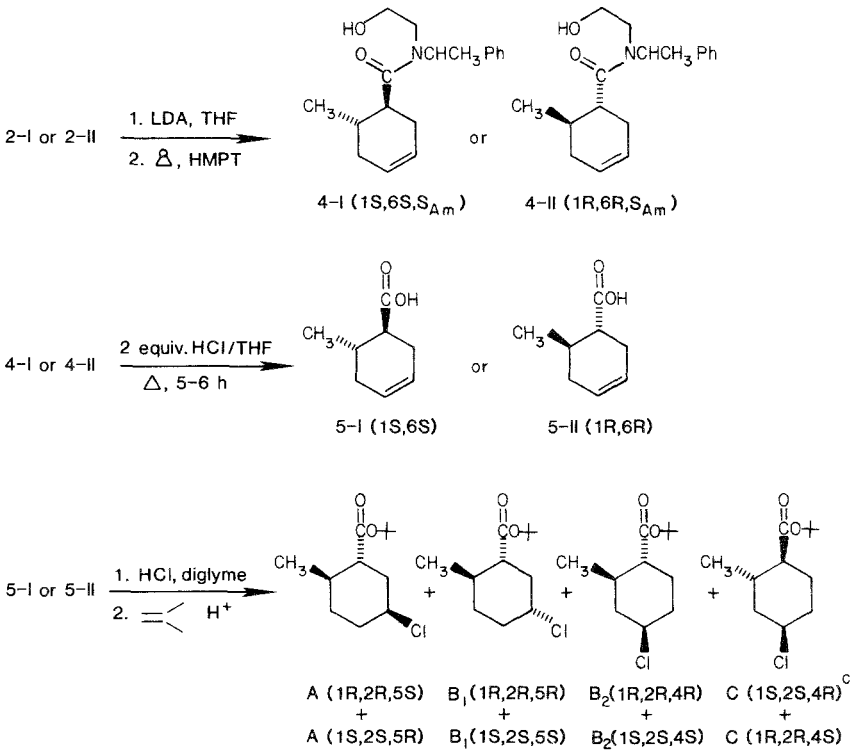
[a](*S*)-α-methylbenzylamine

[b]Solubility in ethyl alcohol

FIG. 2. Conversion of *trans*-6-methyl-3-cyclohexene carboxylic acid (siglure acid) to (*R*)- or (*S*)-α-methylbenzylamides and resolution of these amides.

which the $1S,6S,S_{Am}$ amide (2-I) had been crystallized] was purified by the bromination–debromination cycle described by Sonnet et al. (1984). Six to seven recrystallizations of the dibromide (3-II) from 95% ethanol using 10 ml/g of dibromoamide were required to produce a product of 99.9% purity [determined by reducing aliquots (100 mg) of dibromamide to the unsaturated amide (2-II) followed by GLC analysis]. The debromination procedure was modified slightly in that after the removal of the unreacted zinc by filtration, the propionic acid solution was diluted with water and extracted with methylene chloride (it was not possible to filter the debrominated amides as described because of the very fine particle size of the precipitate). The methylene chloride extract was washed thoroughly with water and cautiously with saturated sodium bicarbonate, then dried over sodium sulfate. The use of diethyl ether as extraction solvent is to be avoided as it extracts large quantities of acetic acid that makes washing with satd. sodium bicarbonate difficult.

N-*Hydroxyethylation of Diastereomerically Pure Amides 2-I and 2-II.* Labilization of the purified amides to hydrolytic conditions was accomplished



[c] Most attractive (Table1.)

FIG. 3. Recovery of the configurationally pure isomers of *trans*-6-methyl-3-cyclohexane carboxylic acid from the resolved (*R*)- or (*S*)-α-methylbenzylamides and conversion to the enantiomers of trimedlure.

by *N*-hydroxyethylation of the diastereomerically pure amides (2-I or 2-II) as described. Particular attention should be given to the careful drying of the amides under vacuum over P_2O_5 prior to *N*-hydroxyethylation since the yield of the reaction is reduced drastically if this drying is not thorough. The yield of *N*-hydroxyethylated amides (4-I or 4-II) from this procedure was typicaly 80–90%. The [^{1}H]NMR spectra of the products were identical to those of authentic samples and the GLC on columns B and C at 200°C indicated purities of ≥99.9%.

(1S,6S)-(+)-, and (1R,6R)-(−)-6-Methyl-3-Cyclohexenecarboxylic Acid,

5-I and 5-II. The conversion of the *N*-hydroxyethylated amides to (1*S*,6*S*)-(−)- and (1*R*,6*R*)-(+)-6-methyl-3-cyclohexenecarboxylic acids (5-I and 5-II, respectively) was accomplished most conveniently by acid hydrolysis to a mixture of the acid and the amino ester rearrangement product (see Sonnet et al., 1984) with aqueous HCl in THF under reflux (using a ratio of 3 ml of 6 N HCl/g amide) until all the starting *N*-hydroxyethylated amide had been consumed as determined by GLC on column B at 200°C. The GLC κs of the *N*-hydroxyethylated amides and the amino ester rearrangement product were 12.73 and 6.64, respectively with $\alpha = 1.92$. These products were separated by dilution of the hydrolysis mixture with water and extraction with ether. The ether solution then was extracted several times with 15% NaOH, the alkaline extracts were combined and acidified with 6 N HCl, and extracted several times with ether. The ether extracts were washed with H_2O, saturated NaCl, and dried over Na_2SO_4. Removal of the drying agent, evaporation of the solvent, and distillation (bp 70–75°C, 0.2 mm) gave the two enantiomerically pure acids in about 40% yield. The enantiomeric purity of the acids could be verified by reconversion to the amides with (*S*)-α-methylbenzylamine and GLC analysis on column A. Both acids were obtained in $\geq$99.99% ee.

The amino ester rearrangement products were set aside for possible conversion to additional quantities of the two acids either via reduction with lithium aluminum hydride followed by oxidation as was reported originally (Sonnet et al., 1984) or via further acidic hydrolysis. The [^{1}H]NMR and mass spectra of the two enantiomeric acids were in complete agreement with those of authentic samples.

Preparation of Eight Isomerically Pure Chloro Acids of Figure 1 from 5-I and 5-II. The addition of HCl to the two enantiomerically pure 6-methyl-3-cyclohexenecarboxylic acids was accomplished most conveniently by a slight modification of the published procedure (Beroza et al., 1961) in that the substitution of diglyme for the dioxane originally used resulted in a much cleaner reaction without the formation of the dark purple color that results when dioxane is used. Each pure acid (5-1 and 5-11) undergoes addition of HCl to produce a mixture of A, B_1, B_2, and C (Figure 1) in which positions 1 and 6 are configurationally determined, i.e., instead of a mixture of four racemates, one now deals with two mixtures of four individual isomers that are chromatographically separable. The reaction is worked up by ether extraction and acid–base manipulation to remove neutral by-products. The four isomeric hydrochlorinated acids (A, B_1, B_2, C) produced from each enantiomerically pure unsaturated acid were resolved by GLC analysis on column A at 185°C. The κ' values are A = 3.8, $B_1 = 4.35$, $B_2 = 4.1$, C = 4.75. The assignment of the order of elution of the isomers was based on the reported ratios (McGovern et al., 1966) of these isomers A, 35%; $B_1 + B_2$, 15% (with B_1 in lesser quantity) and C, 50% produced in the hydrochlorination reaction. The [^{1}H]NMR and mass spectra of the

isomeric mixture of each enantiomeric pair of acids was in complete agreement with the structures. The yield from this reaction was typically about 85%. Attempts to partially separate this mixture of isomeric acids by conversion to salts with α-methylbenzylamine followed by fractional crystallization as reported by Sonnet et al. (1984) were unsuccessful.

Preparation of Eight Isomers of trans*-Trimedlure from Two Acid Mixtures.* The *tert*-butyl esters (Figure 2) were prepared from the enantiomerically pure hydrochlorinated acids by the procedure (reaction with isobutylene plus acid catalyst under pressure) reported by McGovern et al. (1966) and Rabjohn (1963) in 70% yield. This procedure is superior to that of Sonnet et al. (1984), involving conversion to the mixture of acid chlorides and reaction with *tert*-butyl alcohol and *N,N*-dimethylaniline in refluxing ether which led to numerous by-products and gave low yields. The [^{1}H]NMR and mass spectra of the two mixtures of *tert*-butyl esters was in complete agreement with their structures. The HCl adducts of the esters (configurationally pure at positions 1 and 6) were separated by a combination of HPLC and low-temperature crystallization. The elution order on HPLC is B_1, B_2, A, and C. It is possible to separate directly pure A, B_1, B_2, and a portion of the C isomers by HPLC. Samples of C collected by HPLC always contained residual amounts of A (even after repeated HPLC) due to overlap of peaks A and C. Crystallization of the chromatographed C from pentane solutions at Dry Ice temperatures (leaving isomer A and some residual C in the pentane mother liquor) resolved this problem. This mother liquor could be reprocessed by HPLC to provide additional quantities of pure A and C. It was not possible to resolve completely the two sets of the four isomeric esters (A, B_1, B_2, and C) by GLC on column A at 150°C because B_1 and B_2 coelute. However, since these two isomers are separated so well by HPLC, and since Sonnet (1984) had reported that B_1 and B_2 purified by this same HPLC method were essentially 100% pure, when analyzed by GLC with a column that resolved all four isomers, we decided it was safe to assume that these samples were not cross contaminated. The isomeric purities as determined by GLC on column A at 150°C are: 1*S*,2*S*,5*R* (A), 98.2% (1.8% B_2); 1*S*,2*S*,5*S* (B_1) 100%; 1*S*,2*S*,4*S* (B_2) 100%; 1*S*,2*S*,4*R* (C) 100%; 1*R*,2*R*,5*S* (A) 97.4% (2.6% B_2); 1*R*,2*R*,5*R* (B_1) 100%; 1*R*,2*R*,4*R* (B_1) 100%; 1*R*,2*R*,4*R* (B_2) 100%; 1*R*,2*R*,4*S* (C) 94% (6% A). The enantiomeric purities are considered to be $\geq$99.9% based upon the measured enantiomeric purities of the resolved *trans*-6-methyl-3-cyclohexenecarboxylic acids in which no enantiomer could be detected by GLC analysis of the amides prepared with α-methylbenzylamines. It had been shown by Sonnet et al. (1984) that no epimerization occurred during the hydrochlorination procedure. Epimerization of the carboxyl group (which is thermodynamically disfavored) during the esterification procedure was considered highly unlikely since the procedure is quite mild (catalytic sulfuric acid and excess isobutylene in anhydrous diethyl ether at room temperature or slightly

above for 12 hr) compared to the acid hydrolysis procedure for the hydrolytic cleavage of the *N*-hydroxyethylated amides which proceeds with no epimerization.

Bioassay. In this recent series of tests, treatments were applied to small cotton wicks in Jackson sticky traps (Harris et al., 1971) at the rate of 10 mg of candidate chemical as a 10% solution in reagent grade acetone or as 10 μl of the neat chemical (isomer A preparations). In the original two tests in Sonnet et al. (1984), only 5 mg or less per wick was used, and it was to confirm the original results that the tests were repeated. The treatments were replicated five times. The traps were hung in trees in a large macadamia nut orchard (not a breeding host) near Hilo, Hawaii. Traps were 7.6 m or more from each other. Laboratory-reared (Tanaka et al., 1970), sterile (Ohinata et al., 1978) Mediterranean fruit fly males (3 days old) were released in an even distribution throughout the test plot. This test system, using artificially high populations in the field, has proven to be an effective means of testing relative attractiveness (McGovern et al., 1986b) and has higher precision (lower coefficient variation) than tests utilizing unevenly distributed wild populations. The sticky inserts were removed at each examination and the flies counted (two-day intervals). Data were transformed to the square root and analyzed by analysis of variance. Back transformation produced weighted means that were separated by Duncan's multiple range test at the $P \geq 0.05$ level (Duncan, 1951).

In the original study (Sonnet et al., 1984), we conducted two bioassays of the C and A enantiomers before exhausting the supply. In this current study, we conducted the experiment reported in Table 1 with both enantiomers of all four *trans* isomers and their racemic mixtures (except racemic B_1). Due to the limited quantities of B_1 and B_2, we ran a second experiment with the enantiomers of only A, C, and B_2 and a third experiment with the enantiomers of only A and C. The results of these partial tests are not reported here, but were in statistical agreement with the data reported in Table 1.

RESULTS AND DISCUSSION

The greater effectiveness of the 1*S*,2*S*,4*R* enantiomer of isomer C over its mirror image and all other isomeric forms and standard commercial trimedlure mixtures is shown clearly in Table 1. Furthermore, the 1*R*,2*R*,5*S* enantiomeric form of isomer A was significantly more attractive than its mirror image. The preliminary work failed to show significant differences between the isomer A enantiomers. The greater quantities of isomer A enantiomers available for this current study enabled us to double the dosage in the traps and to do repeat testing. In all of our tests, the 1*R*,2*R*,5*S* form was significantly superior.

There were no significant differences in the attractiveness of the *RR* forms

TABLE 1. WEIGHTED MEAN CATCH OF MALE MEDITERRANEAN FRUIT FLIES WITH ENANTIOMERS AND RACEMIC MIXTURES OF *trans* ISOMERS OF TRIMEDLURE (KEAAU, HAWAII)

Isomer[a]	Weighted mean catch[b]				
	A	B_1	B_2	C	
Racemic mixture	156.80c		20.41e	405.46ab	
Enantiomer *S, S, R*	78.25d			502.57a	
Enantiomer *S, S, S*		23.66e	23.37e		
Enantiomer *R, R, R*		50.01de	29.64de		
Enantiomer *R, R, S*	172.45c			46.79de	
Standard Trimedlure[c]					322.06b

[a] Isomers C and B_2 are the *tert*-butyl esters of *cis*- and *trans*-4-chloro-*trans*-2-methylcyclohexanecarboxylic acid, respectively; i.e., C = 1*S*,2*S*,4*R* and 1*R*,2*R*,4*S*. Isomers A and B_1 are the *tert*-butyl esters of *trans*- and *cis*-5-chloro *trans*-2-methylcyclohexanecarboxylic acid, respectively; i.e., A = 1*S*,2*S*,5*R* and 1*R*,2*R*,5*S*.

[b] Catches followed by the same letter are not significantly different [$P > 0.05$, Duncan's (1951) multiple range test; data were transformed to the square root for analysis and back-transformed to produce weighted means]. Two-day catch initial dosage 10 μl $\approx$ 10 mg. Randomized complete block field plot design with five replicates. F test = 26.95 with 11,44 degrees of freedom. $P < 0.01$. The flies in each trap were removed and counted every two days.

[c] Contains 27% A, 7% B_1, 18% B_2, 41% C *trans* isomer and 4% total *cis* isomers.

of the B_1 and B_2 isomers and their mirror images. However, because of the limited supply (i.e., small sample size) and the inherently low attraction of these isomers, such differences may exist and be demonstrated in larger-scale experiments.

Trimedlure exhibits in its attraction the same high specificity due to molecular configuration that we have come to expect from the natural attractants such as the sex pheromones (Brand et al., 1979), even though it would appear to be unrelated structurally to any of the components of the female produced attractant (Baker et al., 1985). It is clear from these results that the receptor system in the Mediterranean fruit fly is capable of discriminating between optical isomers even though the natural pheromone of this insect may or may not involve chemical chirality. This phenomenon has been observed in a lepidopteran system (Chapman et al., 1978). We would hope that these results emphasize the importance of considering the role of enantiomers when studying communication systems in insects and other biological systems.

Acknowledgments—We wish to acknowledge the valuable assistance of Tadao Urago, Vinnie Shishido, and Richard Mattos in these studies.

REFERENCES

BAKER, R., HERBERT, R.H., and GRANT, G.S. 1985. Isolation and identification of the sex pheromone of the Mediterranean fruit fly, *Ceratitis capitata* (Wied). *J. Chem. Soc., Chem. Commun.* 1985:824.

BEROZA, M., GREEN, N., GERTLER, S.I., STEINER, L.G., and MIYASHITA, D.H. 1961. Insect attractants: New attractants for the Mediterranean fruit fly. *J. Agric. Food Chem.* 9:361–365.

BRAND, J.M., YOUNG, J. C.H.R., and SILVERSTEIN, R.M. 1979. Insert pheromones: A critical review of recent advances in their chemistry, biology, and application, pp. 1–190, *in* W. Herz, H. Girsebach, and G. W. Kirby (eds.). Progress in the Chemistry of Organic National Products. Springer-Verlag, New York.

CHAPMAN, O.L., KLUN, J.A., MATTES, K.D., SHERIDAN, R.S., and MAINI, S. 1978. Chemoreceptors in Lepidoptera: Stereochemical differentiation of dual receptors for an achiral pheromone. *Science* 201:926–928.

DUNCAN, D.B. 1951. A significance test for differences between ranked treatments in an analysis of variance. *Va. J. Sci.* 12:171–189.

GREEN, N., and BEROZA, M. 1959. *cis,trans* Isomers of 6-methyl-3-cyclohexene-1-carboxylic acid and their *sec*-butyl esters. *J. Org. Chem.* 24:761–764.

HARRIS, E.J., NAKAGAWA, S., and URAGO, T. 1971. Sticky traps for detection and survey of three tephritids. *J. Econ. Entomol.* 64:62–65.

MCGOVERN, T.P., and BEROZA, M. 1966. Structure of the four isomers of the insect attractant trimedlure. *J. Org. Chem.* 31:1472–1477.

MCGOVERN, T.P., BEROZA, M., OHINATA, K., MIYASHITA, D., and STEINER, L.F. 1966. Volatility and attractiveness to the Mediterranean fruit fly of trimedlure and its isomers, and a comparison of its volatility with that of seven other insect attractants. *J. Econ. Entomol.* 59:1450–1455.

MCGOVERN, T.P., CUNNINGHAM, R.T., and LEONHARDT, B.A. 1986a. Attraction of *trans*-trimedlure and its four isomers in field tests with the Mediterranean fruit fly (Diptera: Tephritidae). *J. Econ. Entomol.* 80:617–620.

MCGOVERN, T.P., CUNNINGHAM, R.T., and LEONHARDT, B.A. 1986b. *cis*-Trimedlure: attraction for the Mediterranean fruit fly (Diptera: Tephritidae) and isomeric structural assignments. *J. Econ. Entomol.* 79:98–102.

OHINATA, K., FUJIMOTO, M., HIGA, H., TANAKA, N., and HARRIS, E.J. 1978. Mediterranean fruit fly: gamma irradiation in nitrogen and packaging for sterile insect release programs in Los Angeles. *J. Econ. Entomol.* 71:610–612.

RABJOHN, M. 1963. Di-*tert*-butyl malonate. Organic Synthesis. Coll. Vol. IV. John Wiley & Sons, New York. pp. 261–266.

SONNET, P.E., MCGOVERN, T.P., and CUNNINGHAM, R.T. 1984. Enantiomers of the biologically active components of the insect attractant trimedlure. *J. Org. Chem.* 49:4639-4643.

TANAKA, N., OKAMOTO, R., and CHAMBERS, D.L. 1970. Methods of mass rearing the Mediterranean fruit fly currently used by the U.S. Department of Agriculture, pp. 19–23, *in* Sterile Male Technique for Control of Fruit Flies. International Atomic Energy Agency, Vienna, Austria.

Journal of Chemical Ecology, Vol. 17, No. 2, 1991

CHEMISTRY OF FRUIT FLIES: NATURE OF GLANDULAR SECRETION AND VOLATILE EMISSION OF *Bactrocera (Bactrocera) cacuminatus* (HÉRING)

SABINE KROHN,[1] MARY T. FLETCHER,[2] WILLIAM KITCHING,[1,*] RICHARD A.I. DREW,[2] CHRISTOPHER J. MOORE,[2] and WITTKO FRANCKE[3]

[1]*Department of Chemistry*
The University of Queensland
Queensland, Australia, 4072

[2]*Department of Primary Industries*
Brisbane, Australia

[3]*Institut für Organische Chemie der Universität Hamburg*
D-2000 Hamburg 13, Germany

(Received November 14, 1990)

Abstract—The major component of the rectal glandular extract and volatile emission of male *Bactrocera cacuminatus* is racemic 1,7-dioxaspiro[5.5]undecane. 1-Hydroxy-5-nonanone as its open chain form, together with 6-*n*-butyl-3,4-dihydro-2H-pyran are minor components. 1,7-Dioxaspiro[5.5]undecan-4-ol is present at a low level and is shown to be exclusively the diastereomer with an equatorial hydroxy group by comparison with synthesized samples of both epimers. Examination of the trifluoroacetate by chiral gas chromatography has established the (4*S*,6*S*) stereochemistry (ca. 80% ee). The presence of 1,7-dioxaspiro[5.5]undecan-3-ol, or its isomerization product, 1,6-dioxaspiro[4.5]decan-2-ylmethanol, could not be confirmed. Trapping of the volatiles released by sexually mature male flies at dusk revealed that a number of the glandular components described above are released at mating time. Reexamination of the glandular secretion of sexually mature female olive flies (*B. oleae*) has failed to confirm the presence of any 1,7-dioxaspiro[5.5]undecanols, with the only volatile component (other than fatty acids) being 1,7-dioxaspiro[5.5]undecane.

Key Words—*Bactrocera cacuminatus*, *Bactrocera oleae*, Diptera, Tephritidae, olive fly, spiroacetal, ketoalcohol, chirality.

*To whom correspondence should be addressed.

INTRODUCTION

Of the 80 or so fruit-fly species located in Australia, five are major pests of horticulture (Drew et al., 1982). Several are minor or incidental pests, with the remainder lacking economic importance and more or less confined to their natural rainforest habitats. Although studies of certain economically important species may lead to chemically based monitoring and control measures, examination of other species also is justified from the perspective of general fruit-fly biology, taxonomy, and communication. The dorsalis complex of fruit flies (Perkins et al., 1990) is a major scourge throughout the Indo-Pacific region, and it was considered of interest to examine the chemistry of an "Australian" member of the complex. *Bactrocera (Bactrocera) cacuminatus* (Héring)[4] is distributed in the coastal regions of eastern Australia from Cape York in the north to East Gippsland in Victoria. This medium-sized species, which has been regarded as being a minor pest of tomatoes and capsicums in Queensland, is of interest in that it appears to have a single natural host, the wild tobacco plant (*Solanum mauritianum*), which flourishes in fringing rainforest vegetation and along small watercourses. Some years ago, we commented (Kitching et al., 1986) on some aspects of the chemistry of this species, which was intriguing because of its similarity with that of the female olive fly (*B. oleae*) (Baker and Herbert, 1987), a major pest in Mediterranean and North African areas. Male tephritid fruit flies store a pheromone in a reservoir and secrete it from a sac, and both organs are located in the lower abdominal (rectal) area. Only in one species, *B. oleae* (olive fruit fly), does the female release the sac pheromone. As part of a careful study of members of the doralis complex, more detailed studies of the chemistry of *B. cacuminatus* have been conducted and are reported below.

METHODS AND MATERIALS

Bactrocera cacuminatus specimens were supplied from the culture maintained at the Department of Primary Industries, Indooroopilly, Queensland. This culture was initially bred out from infested wild tobacco fruit (*Solanum mauritianum*).

Gland Secretion Analysis and Identification

Sexually mature adult male flies were briefly chilled (0°C) and their rectal glands excised and stored in spectrograde pentane (Kitching et al., 1989). Combined GC-MS examinations were performed using a Finnigan Mat 1020 or

[4] *Bactrocera* is the new genus name applied in the general taxonomical revision (Drew, 1989) to a large number of fruit-fly species previously classified in the genus *Dacus*.

Hewlett-Packard HP 5992 or 5970 MSD instruments with a nonpolar column. Low-resolution mass spectra refer to combined GC-MS measurements and high-resolution mass spectra were recorded on a Kratos MS-25 RFA spectrometer. Proton magnetic resonance spectra were recorded at 400 MHz (FT mode) on a JEOL JNM-GX 400 spectrometer. Deuterochloroform was employed as solvent, and chemical shifts (δ values) are relative to residual chloroform at δ7.24. [^{13}C]NMR spectra were recorded at 100 MHz, utilizing $CDCl_3$ as solvent and chemical shifts are relative to the central component of the $CDCl_3$ triplet at 77.00 ppm.

Synthesis of Certain Glandular Components

1,7-Dioxaspiro[5.5]undecane (**1**), 6-*n*-butyl-3,4-dihydro-2H-pyran (**2**), and *N*-3-methylbutylacetamide (**4**) are known compounds (Baker and Herbert, 1987).

*1-Hydroxy-5-nonanone (**5**).* *n*-Butyllithium (2.5 M in hexane, 20 ml) was added slowly to a cooled solution (−10°C) of 1,3-dithiane (6 g, 50 mmol) dissolved in dry THF (60 ml) (N_2). The reaction mixture was stirred for 2 hr at 0°C, and then 0.5 hr at 20°C. The solution then was recooled (−10°C), and *n*-butyl bromide (7 g, 51 mmol) was added. After stirring overnight at ca. 20°C, the reaction mixture was poured into a saturated NH_4Cl solution, and the aqueous layer extracted twice with ether. The combined organic fractions were washed with saturated $NaHCO_3$ solution, 1 M KOH, and then H_2O. Distillation of the dried (K_2CO_3) organic fraction provided 2-*n*-butyl-1,3-dithiane (bp 80–83°C, 0.2 mm; 8.36 g, 95%). [^{1}H]NMR ($CDCl_3$): 0.84 (t, 3H), 1.24–1.30 (m, 2H), 1.38–1.46 (m, 2H), 1.66–1.71 (m, 2H), 1.77–1.84 (m, 1H), 2.03–2.07 (m, 1H), 2.73–2.86 (m, 4H), 3.98 (t, 1H). [^{13}C]NMR ($CDCl_3$): 47.52, 35.05, 30.37, 28.66, 25.95, 22.21, 13.73. Mass spectrum: 176 (M^+, 29), 118 (100), 106 (4), 87 (9), 73 (5), 72 (3), 60 (16), 45 (35), 41 (38). This 1,3-dithiane derivative (8.36 g) was converted to the lithio derivative as described above and reacted with 4-bromo-1-butene to provide the dialkylated-1,3-dithiane (as shown in Scheme 1) bp 101°C (0.2 mm), 9.63 g (88%). [^{1}H]NMR ($CDCl_3$): 0.89 (t, 3H), 1.26–1.42 (m, 6H), 1.78–1.83 (m, 2H), 1.88–1.95 (m, 2H), 2.12–2.17 (m, 2H), 2.70–2.83 (m, 4H), 4.91–5.04 (m, 2H), 5.74–5.82 (m, 1H). [^{13}C]NMR ($CDCl_3$): 137.94, 114.70, 38.08, 37.13, 30.41, 28.56, 26.03, 25.92, 25.42, 22.83, 13.91. Mass spectrum: 230 (M^+, 16), 188 (13), 175 (70), 173 (46), 155 (42), 114 (39), 107 (48), 99 (65), 81 (40), 67 (43), 141 (100).

Hydroboration–Oxidation of 2-(3'-butenyl)-2-n-butyl-1,3-dithiane. The dialkylated-1,3-dithiane (9.63 g, 87 mmol) was dissolved in dry THF (120 ml) and cooled to 0°C, after which a solution of BH_3–THF complex (64.5 ml of 1 M solution) was added slowly. The reaction mixture was stored for 1 hr

at 0°C and for 12 hr at 20°C, and then treated with 3 M NaOH solution at 0°C (130 ml) and 30% w/v H_2O_2 solution (22 ml). After stirring at room temperature for 4 hr, the reaction mixture was extracted with ether several times and the combined organic layers were washed with H_2O, separated, and dried ($MgSO_4$). After solvent removal at reduced pressure (Rotvaporator) the product was purified by flash chromatography (hexane–ethyl acetate, 1:1) to provide 8.32 g (80%) of 2-(4′-hydroxybutyl)-2-*n*-butyl-1,3-dithiane. [^{1}H]NMR ($CDCl_3$): 0.86 (t, 3H), 1.17–1.56 (m, 7H), 1.79–1.91 (m, 8H), 2.74–2.77 (m, 4H), 3.62 (t, 2H). [^{13}C]NMR ($CDCl_3$): 62.56, 53.21, 37.93, 37.88, 32.74, 26.14, 25.93, 25.46, 22.83, 20.42, 13.93. Mass spectrum: 248 (M^+, 20), 191 (47), 175 (100), 141 (56), 117 (33), 107 (29), 106 (21), 101 (37), 81 (36), 55 (53), 41 (93). HRMS: calcd. for $C_{12}H_{24}OS_2$, 248.1268, observed 248.1271. Dethioketalization of the above 1,3-dithiane derivative utilized the recently reported method (Stork and Zao, 1989). Thus, the thioketal (2.48, 10 mmol) in MeOH–H_2O (10 ml, 9:1), was treated with [bis(trifluoroacetoxy)iodo]-benzene (15 mmol) at 20°C for about 3 min, and the reaction mixture was poured into water, extracted with ether, and dried ($MgSO_4$). After solvent removal under reduced pressure, the product was purified by flash chromatography (hexane) and then hexane–ethyl acetate (3:1). [^{1}H]NMR ($CDCl_3$): 0.87 (t, 3H), 1.25–1.31 (m, 2H), 1.48–1.56 (m, 3H), 1.56–1.66 (m, 4H), 2.38 (t, 2H), 2.43 (t, 2H), 3.60 (t, 2H). [^{13}C]NMR ($CDCl_3$): 211.51, 62.29, 42.54, 42.23, 32.16, 25.98, 22.34, 19.67, and 13.82. Mass spectrum: 158 (M^+, <1), 140 (14), 116 (20), 111 (10), 101 (24), 98 (74), 85 (85), 83 (79), 57 (98), 55 (100), 43 (59). HRMS: calcd. for $C_9H_{19}O_2$(M + 1), 159.1384; observed (CI) 159.1386; calcd. for $C_9H_{22}NO_2$(M + NH_4), 176.1649; observed (CI), 176.1666. The 1-hydroxy-5-nonanone (**5**) just described, generally coexisted with varying proportions of the cyclic hemiacetal (**6**), and these were easily resolved under capillary GC conditions. Compound **6** was characterized by its [^{13}C]NMR signal at δ98.8. In the CI mass spectrum (NH_3), **6** exhibited no molecular ion, with the base peak (M − OH) at *m/z* 141. HRMS: calcd. for $C_9H_{17}O$, 141.1279 (CI); observed, 141.1267.

1-Trifluoroacetoxy-5-nonanone (7). The dithiane derivative (0.6 g, 2.5 mmol) was dissolved in CH_2Cl_2 (10 ml) and treated with $(CF_3CO)_2O$ (2.1 g; 10 mmol) for 1 hr at 20°C. The reaction mixture was poured into saturated aqueous $NaHCO_3$ and extracted twice with ether and dried ($MgSO_4$). Removal of the ether provided an oil, which was purified by flash chromatography (hexane) to provide 0.3 g (47%) of **7**. [^{1}H]NMR: 0.87 (t, 3H), 1.25–1.31 (m, 2H), 1.48–1.54 (m, 2H), 1.62–1.72 (m, 4H), 2.37 (t, 2H), 2.44 (t, 2H), 4.32 (t, 2H). [^{13}C]NMR: 210.27, 157.50 (q, J = 41.7 Hz), 114.51 (q, J = 285.7 Hz), 67.87, 42.59, 41.63, 27.60, 25.94, 22.32, 19.68, 13.78. Mass spectrum: 254 (M^+, 1), 212 (4), 197 (11), 141 (4), 98 (23), 85 (91), 69 (31), 58 (25), 57 (94), 55 (100), 43 (33), 41 (60). HRMS: calcd. for $C_{11}H_{17}O_3F_3$, 254.1129;

(94), 55 (100), 43 (33), 41 (60). HRMS: calcd. for $C_{11}H_{17}O_3F_3$, 254.1129; observed, 254.1127; calcd. for (M + 1), $C_{11}H_{18}O_3F_3$, 255.1163; observed (CI), 255.1212.

1,7-Dioxaspiro[5.5]undecan-4-ol (17). This was prepared from 2-(benzene-sulfonyl)tetrahydro-2H-pyran and the tetrahydropyranyl ether of 3,4-epoxy-1-butanol in the reported manner (Ley et al., 1986). Purification by flash chromatography (ether–hexane 1:1) provided the spiroacetal (**17**) with little contamination with the axial epimer (**18**). [^{1}H]NMR: 4.04 (m, 1H), 3.40–3.55 (m, 4H), 2.01 (m, 1H), 1.83–1.99 (m, 1H), 1.45–1.60 (m, 2H), 1.12–1.43 (m, 7H). [^{13}C]NMR (C_6D_6): 18.94, 25.49, 35.63, 35.84, 45.80, 58.77, 60.19, 64.24, 97.25. These spectral data agree well with those reported (Baker and Herbert, 1987).

Chirality Determination of Natural 1,7-Dioxaspiro[5.5]undecan-4-ol. The synthesized, racemic sample of 4*SR*,6*SR* **17** was converted to the trifluoroacetate with $(CF_3CO)_2O$ in CH_2Cl_2 at 20°C. Analysis was conducted on a Lipodex A 50-m glass column (MH-408-5) at 100°C (isothermal). Under these conditions, the enantiomers of **17** were resolved nicely, and comparison with an authentic sample of very predominantly (4*S*,6*S*)-1,7-dioxaspiro[5.5]undecan-4-ol (as the trifluoroacetate) showed this latter enantiomer was the more slowly eluting of the pair. Similar treatement and analysis of the natural extract demonstrated that the 4*S*,6*S* enantiomer was the predominant natural isomer with an ee of at least 80%. The 4*R*,6*R* enantiomer was definitely presented in the natural material.

RESULTS AND DISCUSSION

Specimens were obtained from a culture bred from infested wild tobacco fruit, and excision of rectal glands from sexually mature males was conducted as previously described (Kitching et al., 1989). Gas chromatography and combined GC-MS examinations were conducted on pentane extracts of ca. 10 glands and revealed the presence of one dominant component (~90% of volatiles), estimated at 8 μg / fly. An apparent molecular ion [(*m* / *z* 156 (14)], corresponding to $C_9H_{16}O_2$, was accompanied by fragment ions at 101 (100), 100 (60), 98 (83), and 83 (32), such features suggesting a spiroacetal on the basis of known fragmentation patterns (Francke and Reith, 1979) and in particular 1,7-dioxaspiro[5.5]undecane (1). This conclusion was confirmed by GC and GC-MS comparisons with an authentic sample synthesised according to Erdmann's procedure (Baker et al., 1980). A minor, earlier eluting compound (~1%) exhibited ions at *m* / *z* 140 (20) ($C_9H_{16}O$) with the base peak at *m* / *z* 98 and other prominent ions at *m* / *z* 83 (27) and 70 (18). The volatility of this component, its mass spectrum and possible structural connection with **1**

were suggestive of 6-*n*-butyl-3,4-dihydro-2H-pyran (**2**), and this was confirmed by comparisons of the mass spectrum with that of an authentic sample (Francke, unpublished results, 1984). The base peak (*m* / *z* 98) is attributed to ion **3** (Figure 1).

The next eluting compound (~ 1.5%) with apparent M^+ at *m* / *z* 129 (5), ($C_7H_{15}NO$) showed prominent ions at *m* / *z* 73 (53), 72 (45), 60 (29), 55 (16), and 43 (100), and its identity as *N*-3-methylbutylacetamide (**4**) was confirmed by comparisons with a synthesized sample. Compound **4** is present in the rectal glandular secretion of other species, e.g., *Bactrocera tryoni* (Bellas and Fletcher, 1979).

Two significant, more slowly eluting components are of interest. The first (~2%) showed a weak, apparent M^+ at *m* / *z* 158 (~ 1) with a more prominent ion at *m* / *z* 140 (16), and the mass spectrum showed a general resemblance to that of **2**, suggesting some constitutional similarity. However, its retention time was much longer and was indicative of the corresponding keto alcohol (**5**) with the ions at *m* / *z* 140 possibly representing M^+-H_2O. The rearrangement ion at *m* / *z* 98 (23) (M^+-H_2O, McLafferty) and other ions at *m* / *z* 101 (17, $HO(CH_2)_4C{\equiv}O^+$), *m* / *z* 85 (62, C_4H_9-$C{\equiv}O^+$) and *m* / *z* 83 (47,

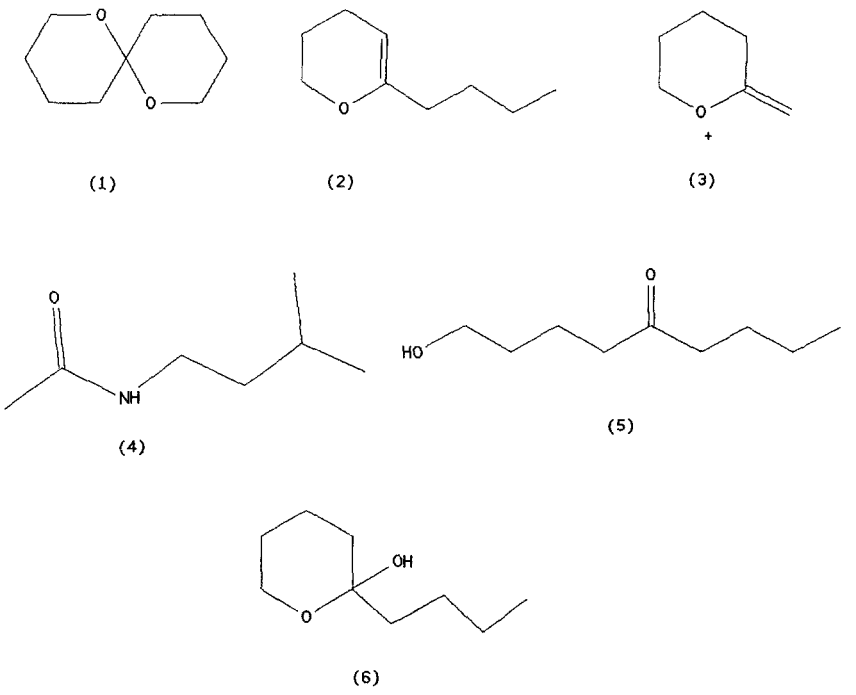
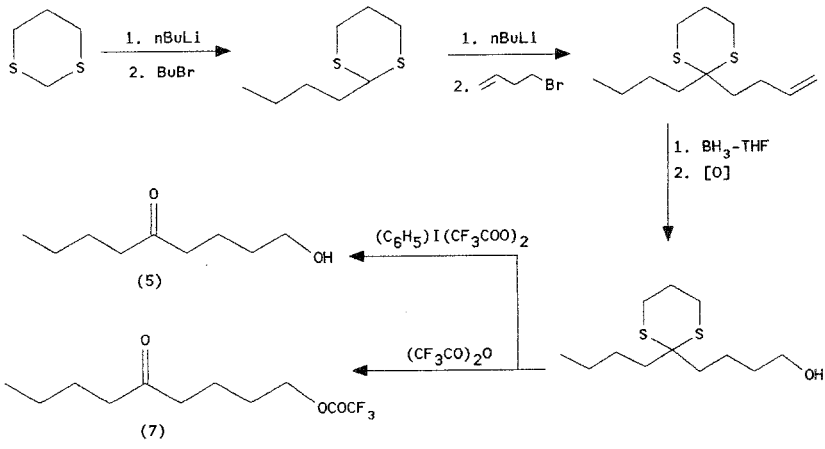


FIG. 1. Compounds associated with male *Bactrocera cacuminatus*.

$C_4H_7C{\equiv}O^+$), pointed to 1-hydroxy-5-nonanone (**5**) or its cyclic hemiacetal (**6**). Although **6** is probably the immediate precursor of **2** (dehydration) or even of **1** (hydroxylation–cyclization), retention time comparisons with **2** and of authentic **5** and **6** confirm **5** to be present in the extract with no detectable level of the hemiacetal **6**. In addition, treatment of the complete glandular sample with trifluoroacetic anhydride produced the trifluoroacetate of **5**, viz. **7**, which exhibited gas chromatographic behavior identical with that of a separately synthesized sample (Scheme 1). Key compounds **5** and **7** were acquired by standard procedure based on dithiane chemistry summarized in Scheme 1. Deprotection of the thioketal with trifluoroacetic anhydride led directly to **7**, whereas use of [bis(trifluoroacetoxy)iodo]benzene provided **5**, which, depending on solvent and conditions, coexisted with its cyclihemiacetal **6**. Compounds **5** and **6** were well separated under capillary gas chromatography conditions and both were characterized mass spectrometrically, as well as by [^{1}H] and [^{13}C]NMR spectroscopy for **5**. The ketal carbon in **6** resonated at δ98.8. Full spectroscopic details are in Methods and Materials.

A slightly slower eluting compound ($\sim$0.5%) with an apparent $M^+\cdot$ = 172 ($C_9H_{16}O_3$) also was observed. The most striking feature of the mass spectrum was the presence of pairs of ions at *m* / *z* 98 (91) and *m* / *z* 101 (100) (**8** and **9**), and also at 114 (50) and 117 (100) (**10** and **11**). Recalling that **1** exhibits intense ions at *m* / *z* 98 (83) and 101 (100), the above data indicated this new component was a hydroxylated derivative of **1**, and this was supported by the ion at *m* / *z* 155 (M-OH) and a more intense ion at *m* / *z* 127 (30%) considered to correspond to **12** (Figure 2).

Assessment of available mass spectral data (Baker and Herbert, 1987) indi-



SCHEME 1. Synthesis of 1-hydroxy-5-nonanone (**5**) and its trifluoroacetate (**7**).

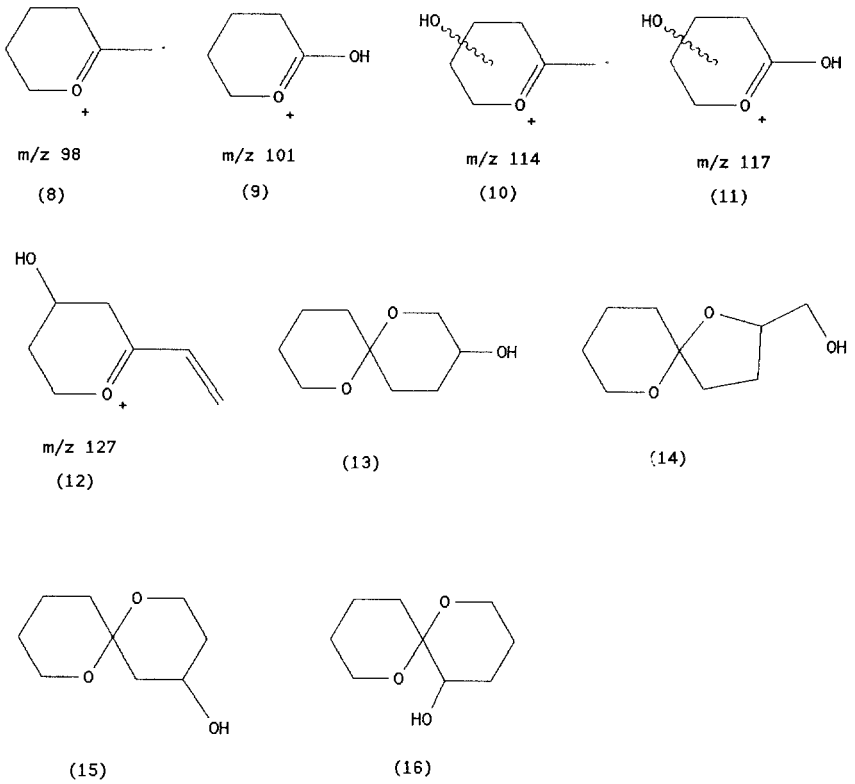
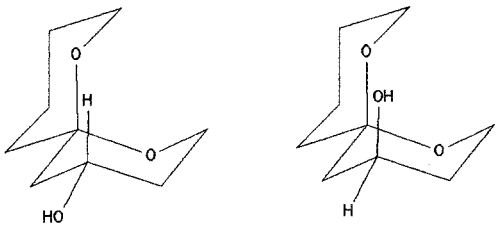


FIG. 2. Proposed structures of some ions and 1,7-dioxaspiro[5.5]undecanols.

cated that **13** and **14** could be ruled out and confirmation of the presence of **15** was obtained by examination of a synthesized sample that was an ca. 90:10 mixture of the epimeric alcohols **17** and **18** (based on [^{1}H] and [^{13}C]NMR analyses) from which pure **17** could be secured by flash chromatography (Figure 3).

Careful GC-MS comparisons established that the natural 1,7-dioxaspiro[5.5]undecan-4-ol was the equatorial epimer **17**, and none of the diastereomer **18** was detectable. Some fatty acids also were identified by mass spectral (library) comparisons and concluded to be the C_{14} saturated and C_{16} saturated and monounsaturated acids.

In our preliminary assessment of the chemistry of this species, there were indications from the GC-MS data that very small amounts of two isomers of another hydroxyspiroacetal were present. These were concluded to be isomers of **14** on the basis of a weak $M^{+}\cdot$ at 172 and the base peak at m/z 141 ($M\text{-}CH_2OH$), with other prominent ions at m/z 99 (30) and 101 (24). How-



(17) (4S,6S Shown) (18) (4R, 6S Shown)

FIG. 3. Diastereomers of 1,7-dioxaspiro[5.5]undecan-4-ol, with the 4*S*, 6*S* and 4*R*, 6*S* enantiomers drawn.

ever, subsequent examinations of glandular extracts derived from male *B. cacuminatus* of varying ages did not confirm the presence of these minor components or the 3-hydroxy derivative (**13**) with which **14** is known to equilibrate (Baker and Herbert, 1987). All examinations of glandular extracts did, however, show the presence of the 4-hydroxy derivative (**17**). 1,7-Dioxaspiro[5.5]undecan-5-ol (**16**) has not been identified from a natural source.

Chirality of 1,7-Dioxaspiro[5.5]undecane and 1,7-Dioxaspiro[5.5]undecan-4-ol

In our previous report (Kitching et al., 1986), we mentioned that **1**, when extracted from male *B. cacuminatus* rectal glands, was shown to be racemic by enantiomeric resolution on a metal chelate stationary phase. We have repeated this examination utilizing a per-*n*-hexylated β-cyclodextrin phase (Lipodex A) and confirm the previous result.

Conversion of the 90:10 epimeric mixture of **17** and **18** to their trifluoroacetates and examination with the Lipodex A column showed both diastereomers **17** and **18** were nicely resolved into their enantiomeric sets. A sample of the (4*S*,6*S*)-1,7-dioxaspiro[5.5]undecan-4-ol, kindly provided by Professor K. Mori, established that this enantiomer (as its trifluoroacetate) was the more slowly eluting enantiomer on the chiral column. Comparisons with the derivatized natural product showed that the natural 1,7-dioxaspiro[5.5]undecan-4-ol was predominantly the 4*S*,6*S* stereoisomer **17** (ee = ca. 80%).

Nature of Volatile Compounds Emitted by Male B. cacuminatus

From the point of view of intraspecific communication, the nature of the volatile components emitted by males at mating time (dusk) is of most relevance. For this determination, air was drawn (by a small battery-driven portable

pump) over a group of ca. 12–15 virgin male flies housed in a glass chamber, which exited via a glass tube in which was set a fine disk (1 mm thick) of highly active charcoal. After ca. 1 hr, the tube was removed and the disk washed with carbon disulfide and methanol, and examined by GC-MS methods. Compounds identified as present in the glandular secretion were found to be emitted into the atmosphere in roughly the same relative proportions except that **4** and **5** were now absent. A similar experiment in which the volatile components were adsorbed on Tenax and thermally desorbed into the Finnigan GC-MS system provided the same results.

Comparison with Chemistry of Female Olive Flies (B. oleae)

Extensive studies of the glandular chemistry of female olive flies have been conducted, as it is the female of this species that secretes and releases a blend attractive to conspecific males (Mazomenos and Haniotakis, 1981). The presence of **1** in female glands and volatiles has been confirmed by a number of studies, and it was shown to be racemic, although the enantiomers of **1** elicit different responses under some conditions from male and female flies (Haniotakis et al., 1986). In addition to **1**, Baker and Herbert (1987) have reported that both the 3- and 4-hydroxy derivatives of **1**, i.e., **13** and **15** were present in the rectal glands of female olive flies. Although in different subgenera of the genus *Bactrocera*, male *B. cacuminatus* and female *B. oleae* show a strong chemical resemblance, in that both secrete racemic **1** (~90%) as the major volatile component with minor amounts of other oxygenated compounds. Having established that 4*S*,6*S* **17** was the predominant stereoisomer of 1,7-dioxaspiro[5.5]undecan-4-ol present in *B. cacuminatus* males, we decided to conduct a similar examination of this component reported as being present in female olive flies. A collection of glands excised from sexually mature female *B. oleae* was kindly provided by Dr. G. Haniotakis (Demokritos Research Centre, Biology Institute, Athens). Although **1** was present in substantial amounts in these glandular extracts, we could not detect any hydroxy spiroacetals and we were thus thwarted in our original endeavor. Indeed **1** was the only component of low to medium volatility present, and the results show that various collections of female olive flies may be different chemically, and this may be a function of location, maturity, diet, and history. There is the possibility that the taxonomy of olive flies requires attention. After all, the species has a wide geographical distribution and sibling species may have developed.

Acknowledgments—The authors are grateful to Professor K. Mori, University of Tokyo, for a sample of the 4*S*,6*S* stereoisomer of 1,7-dioxaspiro[5.5]undecan-4-ol, and to Dr. G. Haniotakis, Demokritos Research Centre, Athens, for excised glands of female *B. oleae*. The expert technical assistance of Mr. E. Hamacek is gratefully acknowledged. Dr. Krohn is grateful to Deutsche Forschungsgemeinschaft for the receipt of a fellowship.

REFERENCES

BAKER, R., and HERBERT, R.H. 1987. Isolation and synthesis of 1,7-dioxaspiro[5.5]undecane and 1,7-dioxaspior[5.5]undecan-3- and 4-ols from the olive fly (*Dacus olease*). *J. Chem. Soc. Perkins Trans.* 1:1123.

BAKER, R., HERBERT, R., HOUSE, P.E., JONES, O.T., FRANCKE, W., and RUTH, W. 1980. Identification and synthesis of the major sex pheromone of the olive fly (*Dacus oleae*). *J. Chem. Soc. Chem. Commun.* p. 52.

BELLAS, T.E. and FLETCHER, B.E. 1979. Identification of the major components in the secretion from the rectal pheromone glands of the Queensland fruit flies *Dacus tryoni* and *Dacus neohumeralis*. *J. Chem. Ecol.* 5:795.

DREW, R.A.I. 1989. The tropical fruit flies (Diptera Tephritidae: Dacinae) of the Australasian and Oceanian Regions. *Mem. Qld. Mus.* 26:1–521. Brisbane ISSN OOA-8835.

DREW, R.A.I., HOOPER, G.H.S., and BATEMAN, M.A. 1982. Economic fruit flies of the South Pacific region. Queensland Department of Primary Industries, Brisbane).

FRANCKE, W., and REITH, W. 1979. Alkyl-1,6-dioxaspiro[4.4]nonane: A new class of pheromone. *Liebigs Ann. Chem.* 1979:1.

HANIOTAKIS, G., FRANCKE, W., MORI, K., REDLICH, H., and SCHURIG, V. 1986. Sex-specific activity of (*R*)-(−)-and (*S*)-(+)-1,7-Dioxaspiro[5.5]undecane, the major pheromone of *Dacus oleae*. *J. Chem. Ecol.* 12:11.

KITCHING, W., LEWIS, J.A., FLETCHER, M.T., DREW, R.A.I., MOORE, C.J., and FRANCKE, W. 1986. Spiroacetals in rectal gland secretions of Australasian fruit fly species. *J. Chem. Soc. Chem. Commun.* p. 853.

KITCHING, W., LEWIS, J.A., PERKINS, M.V., DREW, R.A.I., MOORE, C.J., SCHURIG, V., KONIG, W.A., and FRANCKE, W. 1989. Chemistry of fruit-flies composition of the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus halfordiae*. Characterisation of (*Z,Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. *J. Org. Chem.* 54:3893.

LEY, S.V., LYGO, B., STERNFELD, F., and WONNACOTT, A. 1986. Alkylation reactions of anions derived from 2-benzenesulfonyltetrahydropyran and their application to spiroketal synthesis. *Tetrahedron* 42:4333.

MAZOMENOS, B.E., and HANIOTAKIS, G.E. 1981. A multicomponent female sex pheromone of *Dacus oleae*. *J. Chem. Ecol.* 7:437.

PERKINS, M.V., FLETCHER, M.T., KITCHING, W., DREW, R.A.I., and MOORE, C.J. 1990. Chemical studies of the rectal gland secretions of some species of the *Bactrocera dorsalis* complex of fruit flies (Diptera: Tephritidae). *J. Chem. Ecol.* 16:2475.

STORK, G., and ZHAO, K. 1989. A simple method of dethioacetalisation. *Tetrahedron Lett.* 30(3):287.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

ANNOUNCEMENT

EIGHTH INTERNATIONAL ANNUAL MEETING OF INTERNATIONAL SOCIETY OF CHEMICAL ECOLOGY

The International Society of Chemical Ecology will hold its Annual Meeting in Dijon, France, July 3–7, 1991, at the University of Dijon.

Symposia have been arranged under the headings: Plant-Insect Interactions; Semiochemicals in Vertebrates; Evolutionary Perspectives on Semiochemicals in Insects; and Human Pheromones and Perfumes.

Contributed papers (approximately 15 minutes) and poster presentations throughout the range of Chemical Ecology are invited. Questions regarding presentations, registration, housing, and other logistics should be addressed to:

Dr. Rémy Brossut
Laboratoire de Zoologie
Université de Dijon
Boulevard Gabriel
21000 Dijon, France

A full package includes registration, meals, a single room in a student residence, and the banquet. Tentative fees are $350.00 U.S. for full members, $250.00 U.S. for students, and $400.00 U.S. for non-members. Nearby hotels are available (30 minute walk, 10 minutes by bus).

0098-0331/91/0300-0497$06.50/0

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

FUMIGANT TOXICITY OF ESSENTIAL OILS AGAINST FOUR MAJOR STORED-PRODUCT INSECTS

ELI SHAAYA,[1,*] UZI RAVID,[2] NACHMAN PASTER,[1] BENJAMIN JUVEN,[3] UZI ZISMAN,[1] and VLADIMIR PISSAREV [1]

[1]*Department of Stored Products, ARO*
[2]*Newe Ya'ar Experiment Station, ARO*
[3]*Department of Food Science, ARO*
Bet Dagan 50250, Israel

(Received August 29, 1990; accepted October 24, 1990)

Abstract—The fumigant toxicity of 28 essential oils extracted from various spice and herb plants and some of their major constituents were assessed for adult coleopterans *Rhyzopertha dominica*, *Oryzaephilus surinamensis*, *Tribolium castaneum*, and *Sitophilus oryzae*. Three groups of active materials were distinguished: (1) The compounds terpinen 4-ol, 1,8-cineole, and the essential oils of three-lobed sage, sage, bay laurel, rosemary, and lavender were most active against *R. dominica;* (2) The compounds linalool, α-terpineol, and carvacrol and the essential oils of oregano, basil, Syrian marjoram, and thyme were most active against *O. surinamensis*; and (3) the compound 1,8-cineole and the essential oils anise and peppermint were active against *T. castaneum*.

Key Words—*Sitophilus oryzae*, Curculionidae, *Rhyzopertha dominica*, Bostrichidae, *Oryzaephilus surinamensis*, Cucujidae, *Tribolium castaneum*, Tenebrionidae, Coleoptera, essential oils, fumigant toxicity.

INTRODUCTION

Insect control in stored-food products at present relies heavily upon the use of gaseous fumigants and residual insecticides, both of which pose serious hazards to warm-blooded animals and the environment. Fumigation is still one of the most effective methods for the prevention of storage losses. However, the availability of fumigants for insect control has dwindled drastically lately. As of

*To whom correspondence should be addressed.

0098-0331/91/0300-0499$06.50/0

now, only two fumigants are still in use, namely, methyl bromide and phosphine. The former is suspected of leaving residues that are harmful to warm-blooded animals (Dansi et al., 1984), while the latter, which is in wide use, has shown alarming indications of development of insect resistance to it (Mills, 1983; Tyler et al., 1983). Thus, it would be of marked benefit for the preservation of stored products to discover and develop new compounds that have the potential to replace the toxic fumigants, are less harmful, yet are easy, simple, and convenient to use. The present study was conducted to investigate further the potential use of essential oils, extracted from various spice and herb plants in Israel, and some of their major constituents as fumigants for the control of stored product insect pests.

METHODS AND MATERIALS

The beetles were bred at 27°C and 70% relative humidity. *Sitophilus oryzae* (Curculionidae) and *Rhyzopertha dominica* (Bostrichidae) were reared on soft wheat, *Oryzaephilus surinamensis* (Cucujidae), on ground wheat with the addition of glycerin and yeast, and *Tribolium castaneum* (Tenebrionidae) on wheat flour. The essential oils were obtained from fresh plants by steam distillation (Marcus and Lichtenstein, 1979). Major oil constituents were purchased commercially. The English name of the essential oil is used; in case no English name was available, the Latin name is given.

The fumigation chambers were 3.4-liter glass flasks with round bottoms, closed with a glass stopper fitted with a hook. The test materials were applied on a small square piece of filter paper, which was suspended by the hook, together with the insect cages, in the fumigation chamber. To obtain even distribution of the oil during the treatment, a magnetic stirrer was placed on the bottom of each flask. Twenty insects of each species, aged 5–10 days, were placed in each of the four cages (4 cm in length and 1.5 cm in diam), which were perforated with small holes enabling penetration of the gas. Small amounts of ground wheat were placed in each cage. Treatments were carried out for 24 hr, and percent mortality was recorded.

RESULTS

The fumigant toxicity of 28 essential oils and 10 of their principal components (Table 1) was tested against four major stored-product insect pests: *S. oryzae*, *R. dominica*, *O. surinamensis*, and *T. castaneum*. At a concentration of 5–15 μl/liter of air, only 11 oils and five of their major components were found to be highly active (Table 1). Among the active materials, it was possible to distinguish three groups according to their activity: Group 1 compounds terpinen 4-ol, 1,8-cineole, and the essential oils of three-lobed sage, sage, bay

TABLE 1. ESSENTIAL OILS AND SOME MAJOR CONSTITUENTS TESTED AS FUMIGANTS AGAINST FOUR MAJOR STORED-PRODUCT INSECTS

Group A[a]	The essential oils of oregano, basil, Syrian marjoran, thyme, three-lobed sage, sage, bay laurel, rosemary, lavender, anise, peppermint. The compounds linalool, α-terpineol, carvacrol, terpinen 4-ol, 1,8-cineole
Group B	The essential oils of celery, savory, lemon grass, rue, clary sage, cumin, caraway, lemon, grapefruit, orange, coriander, *Salvia dominica*, fennel, chamomile, *Artemisia arboresence*, parsley, *Artemisia judaica*. The compounds β-caryophyllen, *p*-cymene, α-terpinene, sabinene, myrcene

[a] Group A was found to be very active, and group B had low or no activity.

laurel, rosemary and lavender were found most active against *R. dominica*. All of them gave 100% mortality at a concentration of 15 μl/liter of air. *O. surinamensis* was less susceptible than *R. dominica* and at a concentration of 15 μl/liter of air, only terpinen 4-ol, 1,8-cineole, and rosemary gave 100% mortality (Figure 1). Group 2 compounds linalool, α-terpineol, and carvacrol and the essential oils of oregano, basil, Syrian marjoram, and thyme were found most active against *O. surinamensis*. One hundred percent mortality was achieved by linalool, α-terpineol, oregano, basil, and Syrian marjoram at a concentration of 15 μl/liter, and by linalool and oregano at 10 μl/liter (Figure 2). *S. oryzae* was much more resistant than *O. surinamensis* and *R. dominica* to all the tested essential oils and their major components. For *S. oryzae*, 100% mortality was obtained only with sage oil at a concentration of 15 μl/liter (Figure 1, see also Figures 2 and 3). In group 3 the compound 1,8-cineole and the essential oils of anise and peppermint were the only active fumigants against *T. castaneum* of all the materials tested. Anise and cineole gave 100% mortality at concentrations of 10 and 15 μl/liter, respectively.

Among the other oils of low activity, *Artemisia judaica*, parsley, *Salvia dominica*, and clary sage were found to be active mainly against *O. surinamensis;* at a concentration of 15 μl/liter, mortalities of 80, 75, 55, and 45%, respectively, was recorded. On the other hand, fennel oil and the compound γ-terpinene were active only against *R. dominica*, at a concentration of 15 μl/liter with 83% and 49% mortality, respectively (data not shown).

DISCUSSION

Three groups could be distinguished of the 28 essential oils and some of their major constituents as active fumigants against four major stored-product insects: group 1, most active against *R. dominica*, followed by *O. surinamensis*

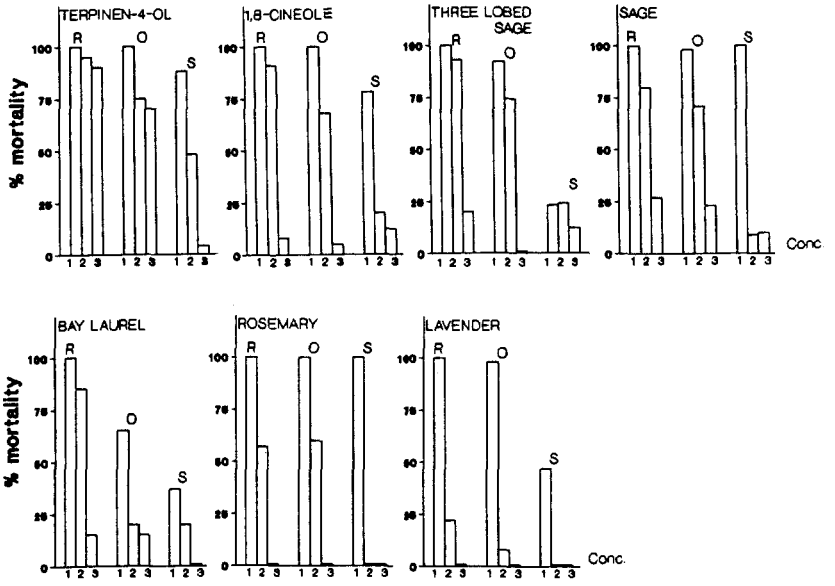


FIG. 1. Essential oils and compounds most active against *R. dominica*. Percent mortality of R = *R. dominica;* O = *O. surinamensis*, and S = *S. oryzae*, using various concentrations (1 = 15, 2 = 10, 3 = 5 μl/liter of air) of the test material. The results are the average of 5–10 replicates.

and *S. oryzae*, group 2, most active against *O. surinamensis*, followed by *R. dominica* and *S. oryzae;* and group 3, against *T. castaneum*, followed by *R. dominica*, O. surinamensis and *S. oryzae*.

From the activity of the various essential oils and their major constituents, it can be seen that alcohol and phenol, such as linalool and carvacrol, show greater activity against *O. surinamensis*. In the case of *R. dominica*, alcohol and ether, e.g., terpinen 4-ol and 1,8-cineole, are the most active, and ethers, such as 1,8-cineole, against *T. castaneum*. These differences in activity of the various compounds were used in the search for more potent compounds for the control of these insects. Recently, we were able to select a compound, ZP51, with much higher potency than all the essential oils and the various compounds tested. A concentration of 3 μl/liter was enough to obtain 100% mortality of the four test insects (Shaaya et al., in preparation). This should be compared with 20–30 mg/liter of methyl bromide recommended for control in the field of stored-product insects. In addition, this compound was most active against *S. oryzae*, a concentration of 1,5 μl/liter being enough to obtain 100% mortality.

The fumigant toxicity of a number of essential oils was also tested against *Sitotroga cerealella* and *Acanthoscelides obtectus* (Klingauf et al., 1983) and

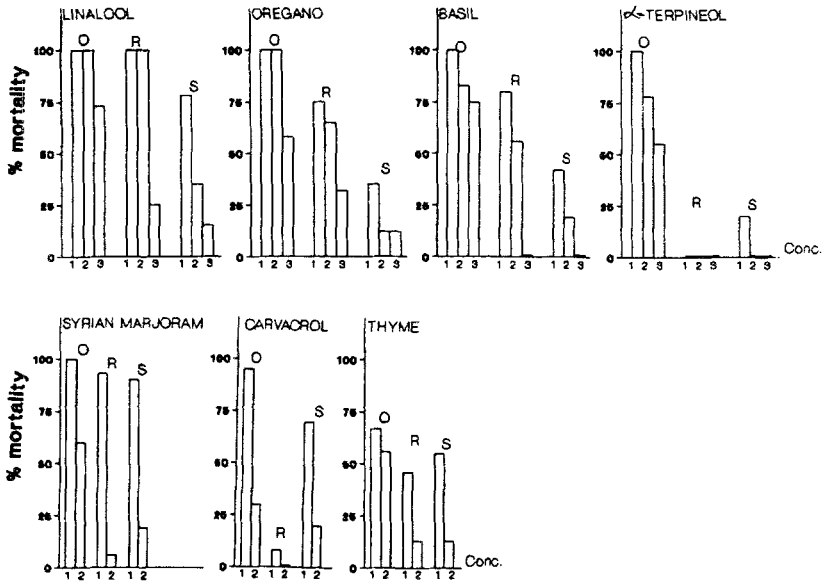


FIG. 2. Essential oils and compounds most active against *O. surinamensis*. Percent mortality of R = *R. dominica;* O = *O. surinamensis*, and S = *S. oryzae*, using various concentrations (1 = 15, 2 = 10, 3 = 5 μl/liter of air) of the test material. The results are the average of 5–10 replicates.

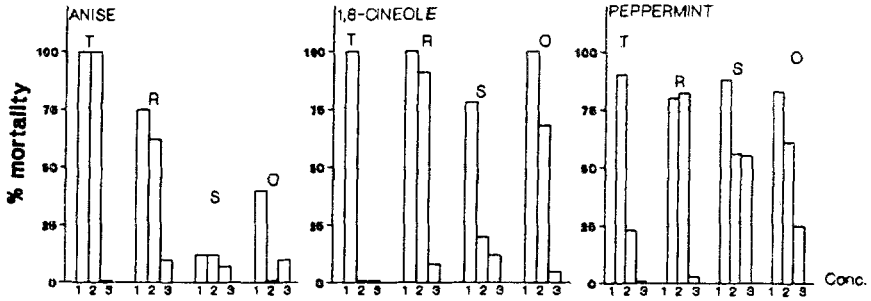


FIG. 3. Essential oils and compounds active against *T. castenaum*. Percent mortality of R = *R. dominica;* O = *O. surinamensis;* S = *S. oryzae*, and T = *T. castenaum*, using various concentrations (1 = 15, 2 = 10, 3 = 5 μl/liter of air) of the test material. The results are the average of 5–10 replicates.

of *Mentha piperita* against *T. castaneum* (Misra and Jitender, 1983). The various studies were done using different fumigation methods and exposure times, so it is not possible to correlate our results with those reported in the literature.

The existence of naturally occurring insecticidal plant components has been known for centuries. However, relatively few of these compounds are actually used in crop protection today (Marcus and Lichtenstein, 1979). Increasing problems concerning the use of modern synthetic insecticides, such as persistence of residues, resistance, and damage to the environment and human health have generated interest in naturally occurring compounds. It should be noted that biologically active compounds of food plants are assumed to be environmentally more acceptable and less hazardous than others to humans. The results presented in this study suggest that some oils or their major constituents could be efficient fumigants and also could be integrated with other pest management procedures.

REFERENCES

DANSI, L., VAN VELSEN, F.L., and VANDER HEUDEN, C.A. 1984. Methyl bromide: carcinogenic effects in the rat fore stomach. *Toxicol. Appl. Pharmacol.* 72:262–271.

KLINGAUF, F., BESTMANN, H.J., VOSTROWSKY, O. and MICHAELIS, K. 1983. Wirkung von ätherischen Ölen auf Schadinsekten. *Mitt. Dtsch. Ges. Allg. Angew. Entomol.* 4:123–126.

MARCUS, C., and LICHTENSTEIN, P. 1979. Biologically active components of anise: Toxicity and interaction with insecticides in insects. *J. Agric. Food Chem.* 27:1217–1223.

MILLS, K.M. 1983. Resistance to the fumigant hydrogen phosphide in some stored-product species associated with repeated inadequate treatments. *Mitt. Dtsch. Ges. Allg. Angew. Entomol.* 4:98–101.

MISHRA, R.C., and JITENDER, K. 1983. Evaluation of *Mentha piperita* L. oil as fumigant against the red flour beetle *Tribolium castaneum* (Herbst.). *Indian Performer* 27:73–76.

TYLER, P.S., TAYLOR, R.W., and REES, D.P. 1983. Insect resistance to phosphine fumigation in food warehouses in Bangladesh. *Int. Pest Control* 25:10–13, 21.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

RESPONSE OF TOTAL TANNINS AND PHENOLICS IN LOBLOLLY PINE FOLIAGE EXPOSED TO OZONE AND ACID RAIN[1]

D.N. JORDAN,[2] T.H. GREEN, A.H. CHAPPELKA,*
B.G. LOCKABY, R.S. MELDAHL, and
D.H. GJERSTAD

School of Forestry
Auburn University
Auburn, Alabama 36849-5418

(Received August 6, 1990; accepted October 29, 1990)

Abstract—Tannin and total phenolic levels in the foliage of loblolly pine (*Pinus taeda* L.) were examined in order to evaluate the effect of atmospheric pollution on secondary plant metabolism. The trees were exposed to four ozone concentrations and three levels of simulated acid rain. Tannin concentration (quantity per gram) and content (quantity per fascicle) were increased in foliage exposed to high concentrations of ozone in both ozone-sensitive and ozone-tolerant families. No effect of acid rain on tannins was observed. Neither total phenolic concentration nor content was significantly affected by any treatment, indicating that the ozone-related increase in foliar tannins was due to changes in allocation within the phenolic group rather than to increases in total phenolics. The change in allocation of resources in the production of secondary metabolites may have implications in herbivore defense, as well as for the overall energy balance of the plant.

Key Words—Plant defense, air pollution, acidic deposition, biological indicators, plant polyphenols, total phenolics, proanthocyanidins, condensed tannins, secondary metabolites, *Pinus taeda*.

INTRODUCTION

Trees possess both chemical and structural defenses against pathogens and herbivory (Loehle, 1988; McLaughlin and Shriner, 1980; Stafford, 1988). While

*To whom correspondence should be addressed.
[1] AAES Journal no. 9-902690P.
[2] Current address: Botany Department, University of Wyoming, Laramie, Wyoming 82017.

0098-0331/91/0300-0505$06.50/0

not the only defense available to plants, tannins may contribute to plant defense by increasing tissue toughness (Stafford, 1988) or by reducing nutritive value of foliage (Mole and Waterman, 1988). Tannins consist of polymerized phenols and are divided into two groups, condensed and hydrolyzable tannins (Goodwin and Mercer, 1983). Haslam (1988) states that hydrolyzable tannins are confined to the dicotyledons, indicating that the tannins produced by loblolly pine are condensed tannins (CT).

Foliar tannin content is affected by various environmental factors. Schultz and Baldwin (1982) and Schultz (1988) found increased tannin content in red oak leaves (*Quercus rubra*) following insect defoliation. Walters and Stafford (1984) found increases in condensed tannin concentration of Douglas-fir (*Pseudotsuga menziesii*) needles related to insect defoliation and also to mechanical damage. Mole et al. (1988) showed changes in condensed tannins in the foliage of several rain-forest species positively correlated with light intensity. Coley et al. (1985) suggest that chemical defenses are inversely correlated with resource availability. Gershenzon (1984) reported that deficiencies in nitrogen, sulfur, and other nutrients lead to increased production of phenolics in many plant species.

Plant susceptibility to disease or herbivory may be mediated by atmospheric pollution. Manning et al. (1969, 1970) found that ozone damage increased disease rates and infection severities of potato (*Solanum tuberosum*) and geranium (*Pelargonium hortorum*) plants inoculated with *Botrytis cinerea*. Hain (1987) discusses ozone effects on ponderosa pine (*P. ponderosa*) in conjunction with attacks by the western pine beetle (*Dendroctonus brevicomis*) and mountain pine beetle (*D. ponderosae*). He suggests that increased susceptibility is offset by decreased suitability, preventing an increase in overall infestation rate.

Ozone has been shown to affect tannin and total phenolic content of several plant species (Howell, 1974). Tingey (1989) discusses the importance of discovering an unambiguous indicator of pollution stress. Loehle (1988) states that leaf defenses should be a good variable for assessing pollution impact, because the allocation of carbon to defensive compounds in the foliage should be reduced as a result of decreased vigor in pollution-stressed trees. Jones and Coleman (1989) suggest that phenolics, in conjunction with some other biochemical indices, might be useful as an indicator of pollution effects, but differentiate between the effects of direct damage to plant structure and carbon stress due to interference with normal plant function by the pollutant. These authors predicted an increase in polymerized carbon-based secondary metabolites with pollution damage. The current study was conducted to determine whether atmospheric pollutants could alter foliar levels of defensive compounds (tannins and phenolics) in loblolly pine trees and to establish the basis for further inves-

tigations into the utility of plant phenolics as indicators of pollution stress, as well as the impacts of these stresses on forest pests and/or pathogens.

METHODS AND MATERIALS

Exposure Facilities. The effects of ozone and acid rain on loblolly pine in central Alabama were investigated using cylindrical open-top chambers (4.5 m diam. × 4.8 m high). The site is located on the upper Coastal Plain of eastern Alabama. The soil series present is a Cowarts, a Typic Kanhapludult. Six-month-old seedlings from two families of loblolly pine were planted in 24 chambers in January 1988. The families used were GAKR 15-23, considered to be ozone-tolerant (Reinert et al., 1988) and GAKR 15-91, regarded as ozone-sensitive (McLaughlin et al., 1988). The chambers were constructed with rain exclusion covers and were exposed to three target pH levels of artificial rain (pH 3.3, 4.3, and 5.3). Rain solutions were prepared by adjusting the pH of deionized water containing selected background ions (Cogbill and Likens, 1974) with a 1 N mixture of H_2SO_4 and HNO_3 in a 3 : 1 ratio. Rain was applied twice weekly through stainless-steel cone nozzles mounted at the top of each chamber. Rain volumes were based on monthly averages for the preceding 30-year period (Chappelka et al., 1990a).

In addition to the acid rain treatments, four ozone treatments were applied as multiples of the ambient concentration. Treatments were attained by filtering ambient air through charcoal filters (CF), applying nonfiltered ambient air (NF), and supplementing ozone concentrations to 1.7 and 2.5 times ambient (1.7 × NF and 2.5 × NF) (Chappelka et al., 1990a). The experiment was established as a 4 × 3 factorial with two replications. Actual ozone concentrations and rain pH, as well as a more detailed description of the application system, are reported by Chappelka et al. (1990b).

Additional trees from both families, which had been grown on the site outside of chambers, were also sampled to evaluate chamber effect. These trees were planted in a similar configuration to those in chambers but were exposed to natural air and rainfall.

Sampling Technique. In November of the second growing season, four trees in each chamber were sampled. Six fascicles per tree were removed from the second flush of current-year (1989) growth on a south-facing primary branch in order to limit potential differences in tannins and phenolics due to light exposure or leaf age (Mole et al., 1988). Fascicles from two trees from each family from each chamber were composited, resulting in one sample per family per chamber.

Laboratory Analyses. The fascicles were weighed, stored on ice, and trans-

ported to the laboratory, where they were immediately cut into 0.5–2.0-mm sections. One-gram samples were ground in 5 ml of 70% acetone with a mortar and pestle and then centrifuged at 5000 rpm for 15 min. The resulting supernatants were stored in the dark at 4°C until analysis.

Tannin samples were analyzed in duplicate by a modification of the radial diffusion assay of Hagerman (1987). A gel containing 1% (w/v) agarose and 0.05% bovine serum albumin (BSA, fraction V) in 50 mM acetic acid and 60 μM ascorbic acid at pH 5.0 was formed by pouring 9.5-ml aliquots of warm solution into standard plastic Petri plates and allowing each to cool overnight at 4°C. Four 3-mm wells per plate then were punched in the gel. Four 8-μl aliquots of sample were applied to each well, with sufficient time allowed for absorption, but not for complete drying between applications. After incubation for 90–120 hr at 30°C, two perpendicular radii of each sample were measured with a binocular microscope and platform micrometer to the nearest 0.01 mm. The radii were averaged, and the area of binding was calculated. The areas of the duplicate samples were averaged for the statistical analysis. Results are presented in mm^2 per gram fresh weight for concentration data and mm^2 per fascicle for content data.

Phenolics in the extract were determined with a colorimetric assay using 2.0 N Folin and Ciocalteu's phenol reagent (Sigma Chemical Co., St. Louis, Missouri). This method yields molar absorptivity specific for each phenolic compound (Singleton and Rossi, 1965) and therefore gives results impossible to present in absolute concentration units. Phenolic results are presented in A_{760} per gram and A_{760} per fascicle for concentration and content respectively. Data were analyzed using SAS (SAS Institute, Cary, North Carolina), analysis of variance (ANOVA), and linear regression procedures. All significance was reported at the $P = 0.05$ level.

RESULTS

Ozone exposure caused an increase in concentration and content of foliar CT in loblolly pine trees (Table 1). Linear regression analysis showed a significant increase in CT concentration (CT/g = 33.4 + 5.3 × ozone, $r^2 = 0.37$, $P \leq 0.0001$) and content (CT/fascicle = 12.8 + 1.6 × ozone, $r^2 = 0.16$, $P = 0.005$) with increasing ozone concentration. There was no significant difference between families in foliar tannin levels. However, the ozone-sensitive family exhibited the highest tannin levels of the experiment in the high ozone treatment. No significant differences in CT were observed among acid rain treatments, nor were significant acid rain × ozone interactions observed in this study.

No significant differences were observed in the total phenolic (TP) con-

centration or content among either ozone or acid rain treatments or between families (Table 1). The overall ratio of CT to TP was significantly increased (CT/TP = 37.4 + 6.7 × ozone, $r^2 = 0.27$, $P \leq 0.0001$) with ozone exposure, indicating a redistribution within the phenolic group (Figure 1).

Chambers themselves had no effect on either foliar tannins or total phenolics. The trees growing outside the chambers had levels of tannins and phenolics comparable to the ambient (NF, pH 5.3) chambers. Phenolic concentrations of these trees were 1.022 A_{760}/g and 1.027 A_{760}/g, and tannin concentrations were 40.02 mm^2/g and 41.23 mm^2/g for the tolerant and sensitive families, respectively. These data were not used in statistical analyses for ozone or acid rain effects.

DISCUSSION

The increase in foliar CT with ozone exposure corresponds to an observed increase in visible injury and reduction in foliar biomass (Chappelka et al., 1990b). This is contrary to what is proposed by Loehle (1988), who predicted decreases in chemical defenses, including tannins, associated with reduced vigor

TABLE 1. TANNIN AND PHENOLIC CONCENTRATION (mm^2/g AND A_{760}/g, RESPECTIVELY) AND CONTENT (mm^2/FASCICLE AND A_{760}/FASCICLE, RESPECTIVELY) BY FAMILY, OZONE EXPOSURE, AND pH TREATMENT

	Treatments[a]						
	Ozone				Rain pH		
Variable	CF	NF	1.7 × NF	2.5 × NF	3.3	4.3	5.3
Tolerant family							
TC[b]	32.8 (1.3)	38.7 (1.4)	38.5 (1.1)	45.5 (4.2)	38.7 (2.4)	38.4 (1.7)	39.6 (3.4)
TCO	11.6 (1.2)	13.8 (1.3)	14.7 (0.3)	15.8 (1.3)	14.0 (0.9)	13.8 (1.2)	14.1 (1.2)
PC	0.86 (0.06)	1.02 (0.03)	0.89 (0.06)	0.84 (0.09)	0.88 (0.06)	0.90 (0.05)	0.94 (0.06)
PCO	0.31 (0.03)	0.36 (0.03)	0.34 (0.02)	0.29 (0.03)	0.32 (0.03)	0.33 (0.03)	0.33 (0.33)
Sensitive family							
TC	37.3 (2.2)	38.3 (1.9)	36.4 (1.7)	54.6 (1.8)	42.5 (3.8)	41.1 (3.2)	41.3 (2.8)
TCO	14.8 (1.8)	15.1 (1.4)	13.2 (1.2)	19.6 (1.3)	15.2 (1.6)	15.7 (1.6)	16.2 (1.2)
PC	0.86 (0.06)	0.97 (0.05)	0.79 (0.07)	0.93 (0.07)	0.89 (0.04)	0.81 (0.08)	0.97 (0.03)
PCO	0.34 (0.04)	0.38 (0.02)	0.29 (0.04)	0.33 (0.03)	0.32 (0.03)	0.30 (0.03)	0.38 (0.02)

[a] CF = charcoal-filtered air; NF = nonfiltered air; 1.7 × NF = nonfiltered air × 1.7 ambient ozone; 2.5 × NF = nonfiltered air × 2.5 ambient ozone.

[b] TC = tannin concentration; TCO = tannin content; PC = phenolic concentration; PCO = phenolic content; means are followed in parenthesis by ±SE.

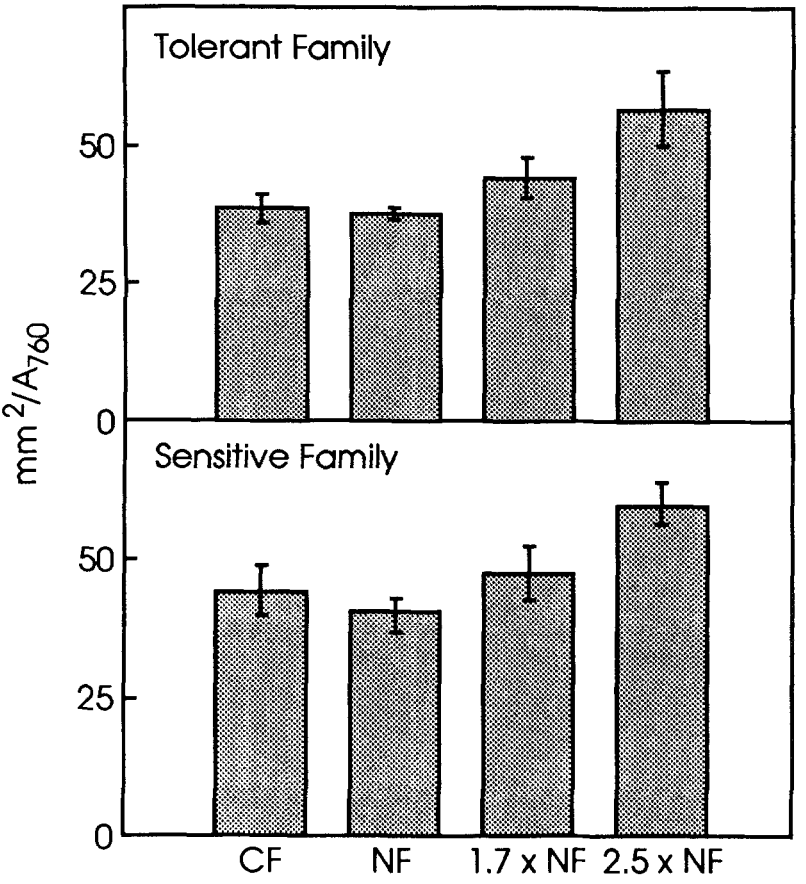


FIG. 1. Tannin–phenolic ratio by ozone exposure and family. Vertical bars represent standard error of the mean. CF = charcoal filtered air; NF = nonfiltered air; 1.7 × NF = 1.7 × ambient ozone; 2.5 × NF = 2.5 × ambient ozone.

resulting from pollution stress. Our data indicate that the increase in tannin production with increased ozone exposure is not a vigor-mediated response but is apparently a direct response to ozone-induced membrane damage. This interpretation agrees with Jones and Coleman (1989), who suggested that structural damage resulting from air pollution would cause an increase in polymerized carbon-based secondary metabolites.

The increases in secondary metabolites might have a substantial impact on the overall energy budget of the plant (McLaughlin and Shriner, 1980). Resources dedicated to production of defensive compounds cannot be utilized in growth and/or repair processes. From an energy or carbon allocation per-

spective, the ozone-treated trees have diverted a greater percentage of resources toward secondary metabolism. The qualitative correlation of visible injury, increased tannins, and high ozone exposure supports Howell's (1974) assertion that membrane damage and reallocation to secondary metabolites may contribute to ozone-related growth reductions.

Gershenzon (1984) reported increased phenolic production related to nutrient limitation in a number of species. In the current study, increased resource availability (nitrogen and sulfur supplied in pH treatments) did not alter the allocation of resources to defense, as represented by tannin content, although growth of the low pH-treated trees was significantly increased (Chappelka et al., 1990b). This is contrary to the carbon–nutrient balance hypothesis of Bryant et al. (1983), which suggests that at higher nutrient availability, less carbon is available for production of carbon-based defensive compounds. Based on this hypothesis, the trees exposed to low pH (higher nutrients) would have been expected to have lower foliar CT levels.

The ease of the radial diffusion assay of Hagerman (1987), the increase in tannins with ozone, and the lack of an acid rain effect indicate that foliar tannin levels would fit the criteria of Tingey (1989), who states that pollution indicators should: provide a readily detectable response to the pollutant, be easy to use and readily related to the response of interest, and have a distinctive syndrome not easily confused with other causes. Further studies are needed to establish whether foliar tannin levels are useful unambiguous chemical indicators of ozone stress, either alone or in combination with other measures.

If foliar tannin concentration is an important factor in deterring insect or pathogen attack, ozone and acid rain exposure at the applied rates does not appear likely to result in increased pest damage. Increased insect feeding has been associated with ozone by some researchers (Chappelka et al., 1988; Hain, 1987; Trumble et al., 1987). However, as Hain (1987) noted, increased host susceptibility does not imply increased infection or predation, provided that host suitability is also decreased. Decreased suitability may result from reduced foliar nutrient content or reduced digestibility of foliage (Coley, 1986). Increased attractiveness and decreased nutritive value would result in the type of susceptibility–suitability relationships described by Hain (1987). Further experiments are needed to determine whether tannin increases of this magnitude are effective against herbivory and if concurrent changes in foliar nutrient content or other defense mechanisms would further discourage attack or render the foliage more desirable.

Acknowledgments—The authors would like to thank Daureen Nesdill for her demonstration of tannin-binding evaluation; John Kush, Mark Bass, Karen Westin, and Efrem Robbins for their data collection; and Alan Tiarks, Bob Jones, Greg Somers, and Nancy Stumpff for their reviews of the manuscript. The study was partially supported by funds provided by the Southeastern Forest Experiment Station, Southern Commercial Forest Research Cooperative of the Forest Response

Program. The Forest Response Program, part of the National Acid Precipitation Assessment Program, is jointly sponsored by the USDA Forest Service, US Environmental Protection Agency, and the National Council of the Paper Industry for Air and Stream Improvement. This paper has not been subject to EPA or Forest Service policy/review and should not be construed to represent the policies of either agency or NCASI.

REFERENCES

BRYANT, J.P., CHAPIN, F.S., III, and KLEIN, D.R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

CHAPPELKA, A.H., KRAEMER, M.E., MEBRAHTU, T., RANGAPPA, M., and BENEPAL, P.S. 1988. Effects of ozone on soybean resistance to the Mexican bean beetle (*Epilachna varivestis* Mulsant). *Environ. Exp. Bot.* 28:53–60.

CHAPPELKA, A.H., KUSH, J.S., MELDAHL, R.S., and LOCKABY, B.G. 1990a. An ozone-low temperature interaction in loblolly pine (*Pinus taeda* L.). *N. Phytol.* 114:721–726.

CHAPPELKA, A.H., LOCKABY, B.G., MITCHELL, R.J., MELDAHL, R.S., KUSH, J.S., and JORDAN, D.N. 1990b. Growth and physiological responses of loblolly pine exposed to ozone and simulated acid rain in the field. *in* Proceedings of Air and Waste Management Association. June 24–29, 1990, Pittsburgh, Pennsylvania. 90–187.5.

COGBILL, C.V., and LIKENS, G.E. 1974. Acid precipitation in the northeastern United States. *Water Resour. Res.* 10:1133–1137.

COLEY, P.D. 1986. Costs and benefits of defense by tannins in a neotropical tree. *Oecologia.* 70:238–241.

COLEY, P.D., BYRANT, J.P., and CHAPIN, F.S., III. 1985. Resource availability and plant antiherbivore defense. *Science.* 230:895–899.

GERSHENZON, J. 1984. Changes in the levels of plant secondary metabolites under water and nutrient stress. *Recent Adv. Phytochem.* 18:273–320.

GOODWIN, T.W., and MERCER, E.I. 1983. Plant phenolics, pp. 567–626, *in* Introduction to Plant Biochemistry, 2nd ed. Pergamon Press, Oxford.

HAGERMAN, A.E. 1987. Radial diffusion method for determining tannin in plant extracts. *J. Chem. Ecol.* 13:437–446.

HAIN, F.P. 1987. Interactions of insects, trees and air pollutants. *Tree Physiol.* 3:93–102.

HASLAM, E. 1988. Plant polyphenols (syn. vegetable tannins) and chemical defense—a reappraisal. *J. Chem. Ecol.* 14:1789–1805.

HOWELL, R.K. 1974. Phenols, ozone, and their involvement in pigmentation and physiology of plant injury, pp. 94–105, *in* M. Dugger (ed.). Air Pollution Effects on Plant Growth. American Chemical Society, Washington, D.C.

JONES, C.G., and COLEMAN, J.S. 1989. Biochemical indicators of air pollution effects in trees: unambiguous signals based on secondary metabolites and nitrogen in fast-growing species? pp. 261–274, *in* Biologic Markers of Air-Pollution Stress and Damage in Forests. National Academy Press, Washington, D.C.

LOEHLE, C. 1988. Forest decline: Endogenous dynamics, tree defenses, and the elimination of spurious correlation. *Vegetatio* 77:65–78.

MANNING, W.J., FEDER, W.A., PERKINS, I., and GLICKMAN, M. 1969. Ozone injury and infection of potato leaves by *Botrytis cinerea. Plant Dis. Rep.* 53:691–693.

MANNING, W.J., FEDER, W.A., and PERKINS, I., 1970. Ozone injury increases infection of geranium leaves by *Botrytis cinerea. Phytopathology* 60:669–670.

MCLAUGHLIN, S.B., and SHRINER, D.S. 1980. Allocation of resources to defense and repair, p.

407-431, *in* J.G. Horsfall and E.B. Cowling (eds.). Plant Disease: An Advanced Treatise, Vol. V. How Plants Defend Themselves. Academic Press, New York.

McLaughlin, S.B., Adams, M.B., Edwards, N.T., Hanson, P.J., Layton, P.A., O'Neill, E.G., and Roy, W.K. 1988. Comparative sensitivity, mechanisms, and whole plant physiological implications of responses of loblolly pine genotypes to ozone and acid deposition. Environmental Sciences Division, Oak Ridge National Laboratory, Publication No. 3105. 301 pp.

Mole, S., and Waterman, P.G. 1988. Light-induced variation in phenolic levels in foliage of rain-forest plants. II. Potential significance to herbivores. *J. Chem. Ecol.* 14:23-34.

Mole, S., Ross, J.A.M., and Waterman, P.G. 1988. Light-induced variation in phenolic levels in foliage of rain-forest plants. I. Chemical changes. *J. Chem. Ecol.* 14:1-21.

Reinert, R.A., Schoeneberger, M.M., Shafer, S.R., Eason, G., Horton, S.J., and Wells, C. 1988. Responses of loblolly pine half-sib families to ozone. *in* Proceedings of the 81st Annual Meeting of the Air Pollution Control Association, Dallas, Texas. 88-125.2.

Schultz, J.C. 1988. Plant responses induced by herbivores. *Trends Ecol. Evol.* 3:45-49.

Schultz, J.C., and Baldwin, I.T. 1982. Oak leaf quality declines in response to defoliation by gypsy moth larvae. *Science* 217:149-151.

Singleton, V.L., and Rossi, J.A., Jr. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16:144-158.

Stafford, H.A. 1988. Proanthocyanidins and the lignin connection. *Phytochemistry* 27:1-6.

Tingey, D.T. 1989. Bioindicators in air pollution research—applications and constraints, pp. 73-80, *in* Biologic Markers of Air-Pollution Stress and Damage in Forests. National Academy Press, Washington, D.C.

Trumble, J.T., Hare, J.D., Musselman, R.C., and McCool, P.M. 1987. Ozone-induced changes in host-plant suitability: Interactions of *Keiferia lycopersicella* and *Lycopersicon esculentum*. *J. Chem. Ecol.* 13:203-218.

Walters, T., and Stafford, H.A. 1984. Variability in accumulation of proanthocyanidins (condensed tannins) in needles of Douglas fir (*Pseudotsuga menziesii*) following long-term budworm defoliation. *J. Chem. Ecol.* 10:1469-1476.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

VARIATION IN SEMIOCHEMICAL-MEDIATED PREY–PREDATOR INTERACTION: *Ips pini* (Scolytidae) AND *Thanasimus dubius* (Cleridae)

DANIEL A. HERMS,[1,*] ROBERT A. HAACK,[2] and BRUCE D. AYRES[3]

[1]*The Dow Gardens*
1018 W. Main St.,
Midland, Michigan 48640

[2]*USDA Forest Service, North Central Forest Experiment Station*
1407 S. Harrison Rd., East Lansing, Michigan 48823

[3]*Woods Run Forest Products*
Box 520, Colfax, Wisconsin 54730

(Received August 17, 1990, accepted October 29, 1990)

Abstract—The bark beetle *Ips pini* (Say) displays variation in its response to and production of enantiomeric blends of its pheromone ipsdienol. One of the principal predators of *Ips pini* is *Thanasimus dubius* (F.), which uses ipsdienol as a kairomone for prey location. During 1988 and 1989, in Wisconsin and Michigan, the response of both species to a range of enantiomeric blends of ipsdienol was investigated. Blends tested had the following ratios of the (*S*)-(+) to (*R*)-(−) enantiomers: 3%:97%, 25%:75%, 50%:50%, 75%:25%, and 97%:3%. Either 75% (+):25% (−) or 50% (+):50% (−) ipsdienol captured the most *Ips pini* in both years at both sites. The 25% (+):75% (−) blend also caught more *Ips pini* than the control during both years at both sites. All blends tested were attractive to *Thanasimus dubius* in both years at both locations. Blend preferences of both species were variable and labile at both sites. Response patterns of both species in Wisconsin were different from those in Michigan each year. Furthermore, response patterns of both species to the ipsdienol blends changed from 1988 to 1989 at both locations. A genetic component to this variation would permit predator-prey coevolution, as well as the development of resistance by *Ips pini* to management strategies based on mass-trapping with single blends.

Key Words—*Ips pini*, *Thanasimus dubius*, Coleoptera, Scolytidae, Cleridae,

*To whom correspondence should be addressed.

0098-0331/91/0300-0515$06.50/0

INTRODUCTION

Bark beetles (Coleoptera: Scolytidae) are the most destructive insect pests of North American forests. The pine engraver, *Ips pini* (Say), colonizes pine and spruce throughout much of North America (Lanier et al., 1972; Birch et al., 1980; Wood, 1982) and is the most common bark beetle in the Great Lakes region (Schenk and Benjamin, 1969). Adults and larvae feed on the phloem of weakened and recently killed trees. Upon colonization of a suitable host, adult males emit a pheromone containing primarily ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol), which attracts other males and females to the tree (Vité et al., 1972; Birch, 1978; Birch et al., 1980; Borden, 1982; Miller et al., 1989).

Ipsdienol occurs as (*R*)-(−) and (*S*)-(+) enantiomers, the relative proportion of which determines response specificity in *Ips pini*. There is geographic variation among North American populations of *Ips pini* in their response to and production of pheromones (Lanier et al., 1972; Birch, 1978; Miller et al., 1989). Western North American populations respond to 97% (−)-and are inhibited by (+)-ipsdienol (Birch et al., 1980). New York populations respond preferentially to a racemic mixture, i.e., 50% (+):50% (−) (Lanier et al., 1980). In Wisconsin, traps baited with 75% (+):25% (−) or 50% (+):50% (−)-ipsdienol caught significantly more beetles than 100% (−), 25% (+), or 100% (+) (Raffa and Klepzig, 1989). The 75% (+)-ipsdienol caught more than three times the number of beetles than did the racemic mixture during a two-week test, but differences were not significant.

Thanasimus dubius (F.) (Coleoptera: Cleridae) is a principal predator of *Ips pini* in the Great Lakes region (Schenk and Benjamin, 1969). *Thanasimus dubius* and other *Thanasimus* species use bark beetle pheromones as kairomones for prey location (Vité and Williamson, 1970; Bakke and Kvamme, 1980; Mizell et al., 1982; Hansen, 1983; Payne et al., 1984; Raffa and Klepzig, 1989). Enantiomeric specificity in their responses (Hansen, 1983; Payne et al., 1984; Raffa and Klepzig, 1989) is considered evidence that clerid predators have coevolved with their scolytid prey (Payne et al., 1984; Raffa and Klepzig, 1989). For example, predation may select for changes in the pheromone system of *Ips pini*, allowing temporary escape from its natural enemy. *Thanasimus dubius* then could be under strong selection to track these changes, permitting location of its primary prey (e.g., Price, 1981). Coevolution, however, requires heritable variation in the selected traits within populations of both species (Janzen, 1980). Miller et al. (1989) concluded that intrapopulation variation in the production of ipsdienol enantiomeric blends by *Ips pini* most likely had a genetic basis; however, this variation has not been shown to be heritable. Kairomonal response preferences by *Thanasimus dubius* to *Ips* and *Dendroctonus* pheromones have been observed to vary from year to year, depending on the relative abundances of the different species of prey (Billings and Cameron, 1984).

Bark beetle suppression through mass-trapping, using aggregation pheromones as bait, is receiving much attention, but requires detailed knowledge of the pheromone preferences of target populations (Bakke, 1982, 1989). A high level of genetic variation within populations of *Ips pini* would create the potential for rapid development of "resistance" to pheromone-based programs that rely on only one pheromone blend (Lanier et al., 1972; Slessor et al., 1985; Borden et al., 1986; Miller et al., 1989).

The objectives of this study were: (1) to further characterize the preferences of *Ips pini* and *Thanasimus dubius* to enantiomeric blends of ipsdienol in the Great Lakes region, (2) to determine the degree of intrapopulational variation in the response of both species to the ipsdienol mixtures, and (3) to compare preferences between populations of the two species within the Great Lakes Region.

METHODS AND MATERIALS

Blend Preferences. The response of *Ips pini* and *Thanasimus dubius* populations to enantiomeric mixtures of ipsdienol was monitored in the field during 1988 and 1989, near Colfax, Wisconsin, and in Midland, Michigan. The Wisconsin study was conducted in an even-aged, 18-ha, pure stand of red pine (*Pinus resinosa* Ait.) planted in 1958. The Michigan study was conducted in a mature (ca. 75-year-old), naturally regenerated, 8-ha, mixed stand of red pine and eastern white pine (*Pinus strobus* L.), with a deciduous understory.

Populations were sampled using eight-unit Lindgren funnel traps (Lindgren, 1983) baited with 8-mg, C-flex, ipsdienol dispensers provided by Phero Tech, Inc., Delta, British Columbia. The lures had a pheromone release rate of 0.2 mg/day, and were replaced every four weeks. The following enantiomeric blends of ipsdienol were tested during 1989 in Wisconsin and both years in Michigan: 3% (+):97% (−), 25% (+):75% (−), 50% (+):50% (−), 75% (+):25% (−), 97% (+):3% (−), and a blank control. Only the 25% (+):75% (−), 50% (+):50% (−), and 75% (+):25% (−) mixtures were tested in Wisconsin in 1988.

In Wisconsin during 1988, and in Michigan during both years, each treatment was randomly assigned to one trap. Traps were separated by 15–25 m. Captured insects were removed and counted weekly. Traps remained in place throughout the season, but the locations of the pheromone baits were rerandomized weekly to guard against spurious effects due to position. Thus, the experimental design was a randomized complete block, with blocks replicated in time. The 1989 Wisconsin experiment was designed as the previously described experiments but with traps deployed in three spatially separated blocks. Within each block, traps were separated by 15–25 m, and each treatment was represented once. The three blocks were equally spaced throughout the stand.

In Wisconsin, sampling was conducted from June 5 until October 16, during 1988, and April 9 until October 22, during 1989. In Michigan, sampling was conducted from April 13 until October 20, in 1988, and March 13 until November 20, in 1989.

Statistical Analysis. Data were analyzed separately by year and site using analysis of variance (PROC GLM; SAS Institute, 1988). Because variances were proportional to means, data were square-root transformed before analysis. Means were separated using the protected LSD test. Weeks with less than two insect captures were excluded from analyses.

Chi-square analyses were used to test for changes in pheromone preference between years. (PROC CATMOD, log-linear model; SAS Institute, 1988). Only the 25% (+), 50% (+), and 75% (+) blends were used in the chi-square analysis of the Wisconsin and Michigan *Ips* data, and the Wisconsin *Thanasimus* data. The other blends were not tested in Wisconsin in 1988. In Michigan, they did not capture enough *Ips* to affect the analysis. All blends were used in the chi-square analysis of Michigan *Thanasimus* data.

RESULTS

Ips pini *in Wisconsin.* There were differences in the 1988 blend preferences of *Ips pini* in Wisconsin ($F = 4.2$; $df = 2,36$; $P = 0.022$). The racemic blend tended to capture more insects than the 75% (+):25% (−) blend, but differences were not significant (Table 1). Both mixtures captured significantly more insects than did 25% (+):75% (−) ipsdienol (Table 1).

There were also differences in the number of *Ips* beetles captured by the ipsdienol blends in 1989. The analysis of variance indicated that a much greater proportion of the total variance was due to the pheromone blends than effects due to site, date, or any interactions ($F = 321.1$ for blend compared to $F = 19.7$ for date and $F \leq 4.4$ for all other effects) (Table 2). The significant blend × date interaction was due to a reduction in the absolute difference between blends in weeks when few beetles were captured. The relative proportion of beetles captured by the different blends tended to remain constant across the season. The 75% (+):25% (−)-ipsdienol captured significantly more *Ips* beetles than all other blends, followed by 50% (+):50% (−) and 25% (+):75% (−)-ipsdienol, respectively (Table 1). The 97% (+):3% (−) and 3% (+):97% (−) blends caught significantly more *Ips* than the control traps, but far fewer than the preferred blends.

Patterns of response by *Ips pini* to ipsdienol enantiomers changed from 1988 to 1989. The proportion of total beetles captured by 50% (+)-ipsdienol dropped from 52% in 1988 to 32% in 1989, while the proportion captured by the 75% (+) blend increased from 36% in 1988 to 56% in 1989 (Table 3).

TABLE 1. COMPARISON OF *Ips pini* AND *Thanasimus dubius* RESPONSE TO ENANTIOMERIC MIXTURES OF IPSDIENOL IN MICHIGAN AND WISCONSIN DURING 1988 AND 1989

Ipsdienol enantiomeric blend	Insects/trap/week ($\bar{X} \pm$ SEM)[a]			
	Ips pini			
	Wisconsin		Michigan	
	1988 (N = 19)	1989 (N = 75)	1988 (N = 22)	1989 (N = 22)
3%(+):97%(−)	not tested	0.9 ± 0.3d	0.1 ± 0.1c	0.0 ± 0.0c
25%(+):75%(−)	12.8 ± 4.5b	5.0 ± 0.5c	11.5 ± 7.8b	1.8 ± 0.5b
50%(+):50%(−)	56.6 ± 22.2a	17.3 ± 1.7b	46.5 ± 30.3a	6.5 ± 1.9a
75%(+):25%(−)	40.3 ± 20.6a	30.6 ± 3.0a	89.2 ± 60.4a	8.7 ± 3.8a
97%(+): 3%(−)	not tested	1.0 ± 0.2d	1.1 ± 0.8c	0.3 ± 0.1c
97%(+): 3%(−)	not tested	0.1 ± 0.0e	0.2 ± 0.2c	0.1 ± 0.1c
	Thanasimus dubius			
	1988 (N = 12)	1989 (N = 75)	1988 (N = 14)	1989 (N = 15)
3%(+):97%(−)	not tested	4.1 ± 0.6d	5.9 ± 2.7b	0.5 ± 0.4bc
25%(+):75%(−)	2.1 ± 0.7a	8.6 ± 1.0c	7.5 ± 2.1ab	1.1 ± 0.5b
50%(+):50%(−)	5.2 ± 1.9a	12.9 ± 1.5b	11.8 ± 4.9a	1.3 ± 0.5b
75%(+):25%(−)	2.3 ± 1.1a	18.7 ± 2.7a	9.9 ± 5.3ab	1.6 ± 1.0b
97%(+): 3%(−)	not tested	16.2 ± 1.8a	9.2 ± 2.7ab	4.8 ± 2.3a
Control	not tested	0.1 ± 0.1e	0.3 ± 0.3c	0.0 ± 0.0c

[a] Means within a column followed by the same letter are not significantly different ($P > 0.05$; protected LSD test applied to square-root transformed data).

Patterns of response by *Ips pini* to ipsdienol enantiomers changed from 1988 to 1989. The proportion of total beetles captured by 50% (+)-ipsdienol dropped from 52% in 1988 to 22% in 1989, while the proportion captured by the 75% (+) blend increased from 36% in 1988 to 56% in 1989 (Table 3). There was little change in the proportion of insects captured by 25% (+)-ipsdienol.

Ips pini *in Michigan.* Pheromone blends differed in *Ips* capture rates in 1988 ($F = 15.4$, $df = 5,105$; $P < 0.001$) and 1989 ($F = 10.3$; $df = 5,105$; $P < 0.001$). During both years, 75% (+)-ipsdienol captured more *Ips* than the racemic mixture, although not significantly more; the 25% (+) mixture captured significantly fewer insects than the two preferred blends, but significantly

TABLE 2. ANOVA OF *Ips pini* AND *Thanasimus dubius* TRAP CAPTURE DATA NEAR COLFAX, WISCONSIN, 1989

		Ips pini			*Thanasimus dubius*		
Source	*df*	MS	F	*P*	MS	F	*P*
Blend (B)	5	214.3	321.1	<0.001	97.1	105.4	<0.001
Site (S)	2	1.5	2.2	0.114	13.4	14.7	<0.001
Date (D)	25	13.1	19.7	<0.001	21.4	23.2	<0.001
B × S	10	1.2	1.7	0.074	1.2	1.3	0.207
B × D	125	2.9	4.4	<0.001	2.3	2.5	<0.001
S × D	50	1.1	1.6	0.013	1.7	1.8	0.002

[a]MS = Mean Square.

change was less than that observed in Wisconsin. The proportion of the total number of beetles captured by 75% (+)-ipsdienol dropped from 60% in 1988 to 50% in 1989, while the proportion captured by 50% (+)-ipsdienol increased from 31% in 1988 to 37% in 1989. There was little change in the proportion of *Ips* captured by the other pheromone blends (Table 3).

Thanasimus dubius *in Wisconsin.* In 1988, there also were apparent differences in the numbers of *Thanasimus* captured by different ipsdienol blends ($F = 3.0$; $df = 2,16$; $P = 0.079$). The racemic mixture captured more insects than the other two blends tested (Table 1).

There were highly significant differences among ipsdienol mixtures in numbers of *Thanasimus* beetles captured in Wisconsin in 1989 (Table 1). As with *Ips pini*, the variation accounted for by date, site, and all interactions was small compared with the variation due to pheromone blend (Table 2). The 75% (+)- and 97% (+)-ipsdienol blends were the most preferred; the racemic mixture captured significantly more *Thanasimus* than did 25% (+)-ipsdienol, which in turn was preferred over the 3% (+) blend (Table 1).

In Wisconsin, the pattern of response by *Thanasimus dubius* to ipsdienol enantiomers also changed significantly from 1988 to 1989 (Table 4), with the pattern of change similar to that of *Ips pini* at the same location. During 1988, the racemic mixture captured 54% of the total number of *Thanasimus*, compared to 24% and 22% for 75% (+)- and 25% (+)-ipsdienol, respectively. In 1989, however, the racemic mixture captured only 21% of the total *Thanasimus*, while the proportion captured by 75% (+)-ipsdienol increased to 31%. The 97% (+) blend, which was not tested in 1988, captured 27% of the total number of insects in 1989.

Thanasimus dubius *in Michigan.* In Michigan, all ipsdienol blends captured significantly more *Thanasimus* than the control during 1988 ($F = 8.2$; *df*

TABLE 3. PERCENT OF TOTAL NUMBER OF *Ips pini* AND *Thanasimus dubius* CAPTURED BY DIFFERENT IPSDIENOL ENANTIOMERS NEAR COLFAX, WISCONSIN, AND MIDLAND, MICHIGAN, IN 1988 AND 1989

		Percent (*S*) − (+)-Ipsdienol							
Location	Year	3%(+)	25%(+)	50%(+)	75%(+)	97%(+)	Control	Total insects	Trap weeks[a]
Ips pini									
Wisconsin	1988		11.6	51.6	35.8			2081	19
	1989	1.6	9.1	31.5	55.7	1.8	0.2	4118	75
Michigan	1988	0.1	7.7	31.3	60.0	0.7	0.1	3272	22
	1989	0.0	10.3	37.0	50.3	1.7	0.5	385	22
Thanasimus dubius									
Wisconsin	1988		22.0	54.0	24.0			115	12
	1989	6.7	14.3	21.4	30.8	26.6	0.2	4560	75
Michigan	1988	13.2	16.7	26.6	22.1	20.8	0.7	627	14
	1989	5.4	11.8	14.0	17.2	51.6	0.0	139	15

[a] Trap weeks = the number of weeks in which insects were captured × the number of traps baited with each pheromone blend.

mus, while the proportion captured by 75% (+)-ipsdienol increased to 31%. The 97% (+) blend, which was not tested in 1988, captured 27% of the total number of insects in 1989.

Thanasimus dubius *in Michigan.* In Michigan, all ipsdienol blends captured significantly more *Thanasimus* than the control during 1988 ($F = 8.2$; $df = 5,70$; $P < 0.001$) and 1989 ($F = 6.5$; $df = 5,60$; $P < 0.001$). The racemic blend captured significantly more *Thanasimus* than 3% (+)-ipsdienol, but there were no significant differences among the other blends (Table 1). In 1989, however, 97% (+)-ipsdienol was favored over all other blends with no differences observed among the remaining blends (Table 1).

As with the other three populations studied, the response pattern of *Thanasimus dubius* in Michigan changed significantly from 1988 to 1989 (Table 4). The proportion of the total number of *Thanasimus dubius* captured by 97% (+)-ipsdienol increased from 21% in 1988 to 52% in 1989; all other blends captured a greater proportion of the total number of *Thanasimus* beetles in 1988 than in 1989 (Table 3).

TABLE 4. CONTINGENCY ANALYSES COMPARING RRESPONSE PATTERNS OF *Ips pini* AND *Thanasimus dubius* TO IPSDIENOL ENANTIOMERIC BLENDS FOR 1988 AND 1989 NEAR COLFAX, WISCONSIN, AND MIDLAND, MICHIGAN[a]

Species	Location	*df*	chi-square	*P*
Ips pini[b]	Wisconsin	2	453.1	<0.001
Ips pini[b]	Michigan	2	11.9	0.003
Thanasimus dubius[b]	Wisconsin	2	20.3	<0.001
Thanasimus dubius[c]	Michigan	4	49.1	<0.001

[a] The null hypothesis tested was that relative attractiveness of ipsdienol blends did not differ between years.
[b] Compares 25%, 50%, and 75% (+)-ipsdienol.
[c] Compares 3%, 25%, 50%, 75%, and 97% (+)-ipsdienol.

more similar to New York populations, which respond preferentially to 50% (+)-ipsdienol (Lanier et al., 1980), than to western North American populations, which respond preferentially to the 97% (−) enantiomer (Birch et al., 1980). However, our experiment found substantial variation in response to ipsdienol within *Ips pini* populations from both Michigan and Wisconsin. Miller et al. (1989) demonstrated variation in the production of ipsdienol enantiomers among individual *Ips pini* within New York, California, and British Columbia populations. This indicates that variation exists among individuals in both the response and production components of the pheromone system of *Ips pini*. We also found substantial intrapopulation variation in the response of the clerid predator *Thanasimus dubius* to ipsdienol in both Michigan and Wisconsin.

The pheromone preferences within *Ips pini* and *Thanasimus dubius* populations in Wisconsin and Michigan were labile, changing from 1988 to 1989. Furthermore, these species exhibited geographic variation in their response to ipsdienol on a relatively local scale. The pattern of response by the Michigan *Ips pini* population to ipsdienol was different than that of the Wisconsin population both years. The same was true of the *Thanasimus dubius* populations. In fact, our 1988 data for *Thanasimus dubius* in Wisconsin differed from that collected by Raffa and Klepzig (1989) during the same year only 150 km away.

It is unknown to what degree, if any, intrapopulation variation in the response of *Thanasimus dubius* or *Ips pini* to ipsdienol is heritable. However, hybridization studies with *Ips pini* indicate a genetic basis to the interpopulation variation in response to ipsdienol (Piston and Lanier, 1974; Lanier et al., 1980). The spatial and temporal variation that we observed suggests a possible genetic component to the intrapopulation variation, as well. High genetic variation within both predator and prey species would provide the potential for coevolutionary change. *Thanasimus dubius* may select for changes in the pheromone

system of *Ips pini*, resulting in reduced predation (Raffa and Klepzig, 1989). The high variation observed in the response of *Thanasimus dubius* to ipsdienol may facilitate rapid counter adaptation to changes in *Ips pini* populations.

The potential capacity of *Ips pini* for evolutionary change in its pheromone system has important implications for pest management strategies. In particular, *Ips pini* may develop resistance to mass-trapping programs, adapting to ipsdienol blends other than those used as bait (Lanier et al., 1972; Slessor et al., 1985; Borden et al., 1986; Miller et al., 1989). Variation within populations of *Ips pini* may require that blend preferences of local populations be well characterized before and monitored during the course of mass-trapping programs. Several blends may have to be used simultaneously and/or rotated as part of a resistance management program.

Acknowledgments—We thank Herbert H. Dow for allowing us to conduct the Michigan experiments on his property. We are grateful to Matt Ayres for his substantial contributions to the analysis and interpretation of the data. Dave Petty and Tom Young assisted with field collections in Michigan. Cathy Infante and Dave Petty counted the insects. Earlier versions of the manuscript were criticized and improved by Matt Ayres, Ronald P. Billings, Kelly S. Johnson, Daniel R. Miller, James R. Miller, David G. Nielsen, James K. Nitao, Kenneth F. Raffa, and Stephen Teale. This study was conducted in cooperation with the Department of Entomology and Pesticide Research Center, Michigan State University.

REFERENCES

BAKKE, A. 1982. Mass trapping of the spruce bark beetle *Ips typographus* in Norway as part of an integrated control program, Vol. 2, pp. 17–25, *in* A.F. Kydonieus, M. Beroza, and G. Zweig (eds.). Insect Suppression with Controlled Release Pheromone Systems. CRC Press, Boca Raton, Florida.

BAKKE, A. 1989. The recent *Ips typographus* outbreak in Norway—experiences from a control program. *Holarc. Ecol.* 12:515-519.

BAKKE, A., and KVAMME, T. 1981. Kairomone response in *Thanasimus* predators to pheromone components of *Ips typographus*. *J. Chem. Ecol.* 7:305-312.

BILLINGS, R.F., and CAMERON, R.S. 1984. Kairomonal responses of Coleoptera, *Monochamus titillator* (Cerambycidae), *Thanasimus dubius* (Cleridae), and *Temnochila virescens* (Trogositidae), to behavioral chemicals of southern pine bark beetles (Coleoptera: Scolytidae). *Environ. Entomol.* 13:1542-1548.

BIRCH, M.C. 1978. Chemical communication in pine bark beetles. *Am. Sci.* 66:409-419.

BIRCH, M.C., LIGHT, D.M., WOOD, D.L., BROWNE, L.E., SILVERSTEIN, R.M., BERGOT, B.J., OHLOFF, G., WEST, J.R., and YOUNG, J.C. 1980. Pheromonal attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. *J. Chem. Ecol.* 6:703-717.

BORDEN, J.H. 1982. Aggregation pheromones, pp. 74–139, *in* J.B. Mitton and K.B. Sturgeon (eds.). Bark Beetles in North American Conifers: A System for the Study of Evolutionary Biology. University of Texas Press, Austin.

BORDEN, J.H., HUNT, D.W.A., MILLER, D.R., and SLESSOR, K.N. 1986. Orientation in forest Coleoptera: An uncertain outcome of responses by individual beetles to variable stimuli, pp.

97–109, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

HANSEN, K. 1983. Reception of bark beetle pheromone in the predaceous clerid beetle, *Thanasimus dubius* (Coleoptera: Cleridae). *J. Comp. Physiol.* 150:371–378.

JANZEN, D.H. 1980. When is it coevolution? *Evolution* 34:611–612.

LINDGREN, B.S. 1983. A multiple funnel trap for scolytid beetles (Coleoptera: Scolytidae). *Can. Entomol.* 115:299–302.

LANIER, G.N., BIRCH, M.C., SCHMITZ, R.F., and FURNISS, M.M. 1972. Pheromones of *Ips pini* (Coleoptera: Scolytidae): Variation in response among three populations. *Can. Entomol.* 104:1917–1923.

LANIER, G.N., CLASSON, A., STEWART, J.J., PISTON, J.J., and SILVERSTEIN, R.M. 1980. *Ips pini:* The basis for interpopulational differences in pheromone biology. *J. Chem. Ecol.* 6:677–687.

MILLER, D.R., BORDEN, J.H., and SLESSOR, K.N. 1989. Inter- and intrapopulation variation of the pheromone, ipsdienol produced by male pine engravers, *Ips pini* (Say) (Coleoptera: Scolytidae). *J. Chem. Ecol.* 15:233–247.

MIZELL, R.F., III, FRAZIER, J.L., and NEBEKER, T.E. 1982. Response of the clerid predator *Thanasimus dubius* (F.) to bark beetle pheromones and tree volatiles in a wind tunnel. *J. Chem. Ecol.* 10:177–187.

PAYNE, T.L., DICKENS, J.C., and RICHERSON, J.V. 1984. Insect predator-prey coevolution via enantiomeric specificity in a kairomone-pheromone system. *J. Chem. Ecol.* 10:487–492.

PISTON, J.J., and LANIER, G.N. 1974. Pheromones of *Ips pini* (Coleoptera: Scolytidae), response to interpopulational hybrids and relative attractiveness of males boring in two host species. *Can. Entomol.* 106:247–251.

PRICE, P.W. 1981. Semiochemicals in evolutionary time, pp. 251–279, *in* D.A. Nordlund, R.L. Jones, and W.J. Lewis (eds.). *Semiochemicals: Their Role in Pest Control.* Wiley, New York.

RAFFA, K.F., and KLEPZIG, K.D. 1989. Chiral escape of bark beetles from predators responding to a bark beetle pheromone. *Oecologia* 80:566–569.

SAS Institute. 1988. SAS/STAT User's Guide. Release 6.03. SAS Institute, Cary, North Carolina.

SCHENK, J.A., and BENJAMIN, D.M. 1969. Notes on the biology of *Ips pini* in central Wisconsin jack pine forests. *Ann. Entomol. Soc. Am.* 62:480–485.

SLESSOR, K.N., KING, G.G.S., MILLER, D.R., WINSTON, M.L., and CUTFORTH, T.L. 1985. Determination of chirality of alcohol or latent alcohol semiochemicals in individual insects. *J. Chem. Ecol.* 11:1659–1666.

VITÉ, J.P., and WILLIAMSON, D.L. 1970. *Thanasimus dubius:* Prey perception. *J. Insect. Physiol.* 16:233–239.

VITÉ, J.P., BAKKE, A. and J.A.A. RENWICK, 1972. Pheromones in *Ips* (Coleoptera: Scolytidae): Occurrence and production. *Can. Entomol.* 104:1967–1975.

WOOD, D.L. 1982. The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles. *Annu. Rev. Entomol.* 27:411–446.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

CHEMICAL CONSTITUENTS OF AN UNACCEPTABLE CRUCIFER, *Erysimum cheiranthoides*, DETER FEEDING BY *Pieris rapae*

M.B. DIMOCK,[1] J.A.A. RENWICK,* C.D. RADKE, and K. SACHDEV-GUPTA

Boyce Thompson Institute for Plant Research
Tower Road, Ithaca, New York 14853

(Received April 26, 1990; accepted November 5, 1990)

Abstract—The wild crucifer *Erysimum cheiranthoides* was found to contain extractable constituents that deterred feeding by larvae of the crucifer specialist *Pieris rapae* when applied to cabbage leaf disks in both choice and no-choice bioassays. High-performance liquid chromatography was used to separate the extract into several fractions, two of which retained the feeding deterrent activity of the extract. UV-absorption spectra of the fractions suggested that one contained cardenolides similar or identical to those reported to deter oviposition by *P. rapae* on *E. cheiranthoides*. The other active fraction evidently contains a compound that deters larval feeding but not adult oviposition. The results suggest that the chemical defense of *E. cheiranthoides* depends on two types of compounds acting on separate developmental stages of the insect.

Key Words—Feeding deterrents, Cruciferae, *Erysimum cheiranthoides*, cardenolides, *Pieris rapae*, cabbage butterfly, Lepidoptera, Pieridae.

INTRODUCTION

For many years, students of insect-plant relationships have been interested in the neurophysiological and behavioral mechanisms by which insect herbivores discriminate between host and nonhost plants. One hypothesis, advanced by Dethier (1982) and elaborated upon by Miller and Strickler (1984), suggests that host selection by phytophagous insects is mediated by both positive and

*To whom correspondence should be addressed.
[1] Current address: Crop Genetics International, 7249 National Drive, Hanover, Maryland 21076.

0098-0331/91/0300-0525$06.50/0

negative stimuli from potential host plants. When combined with other environmental factors, as well as internal excitatory and inhibitory inputs (i.e., the physiological condition of the insect itself), the balance of positive and negative plant stimuli determines whether or not the insect will attempt to feed or oviposit on that plant.

This hypothesis may explain why some specialist herbivores avoid certain plant species closely related to their preferred hosts, even when the host plant and its close relative share similar compounds that are known or presumed to stimulate oviposition or feeding. For example, certain flea beetles in the genus *Phyllotreta* feed only on plants containing glucosinolates, the mustard oil glycosides that characterize plants of the Cruciferae and a few related families (Feeny et al., 1970; Hicks, 1974; Nielsen, 1978a). However, crucifers in the genera *Iberis, Cheiranthus*, and *Erysimum* were rejected by flea beetles because these plants produced potent feeding deterrents in addition to the stimulatory glucosinolates. The phagostimulatory activity of the glucosinolates was apparently negated by the deterrent cucurbitacins of *Iberis* and the cardenolides of *Erysimum* and *Cheiranthus* (Nielsen, 1978b).

The cabbage butterfly, *Pieris rapae*, is another crucifer specialist that appears to select host plants on the basis of positive and negative chemical signals. Its larva, the imported cabbageworm, is stimulated to feed by the presence of glucosinolates (Verschaffelt, 1911), but will usually starve rather than feed on *Erysimum cheiranthoides*, a wild mustard found in Europe and temperate North America (F.S. Chew, personal communication and unpublished observations). *P. rapae* adults reject *E. cheiranthoides* as an oviposition substrate because the presence of deterrent compounds negates any positive response to oviposition stimulants, which are also present in extracts of the plant (Renwick and Radke, 1985, 1987). Our objective in these experiments was to determine if the reaction of *P. rapae* larvae to *E. cheiranthoides* can, like oviposition deterrence, be attributed to extractable chemical constituents of the plant.

METHODS AND MATERIALS

Extraction of Plant Material. E. cheiranthoides was grown from seed in a greenhouse at Ithaca, New York, under a combination of natural and artificial illumination. Mature foliage was harvested approximately four weeks after planting, plunged into boiling ethanol for 5 min, cooled in an ice bath, then homogenized in a blender. After filtration of the macerate through glass wool, the ethanol extract was dried under vacuum and the residue washed three times with hexane to remove lipids. The hexane-insoluble residue was dissolved in water, filtered, and extracted three times with *n*-butanol. The initial water extract, as well as the butanol and postbutanol water extracts were evaporated

to dryness under vacuum and stored at 4°C. Samples of each extract were later redissolved in water for bioassay against *P. rapae* larvae. Concentrations were expressed in gram leaf equivalents (gle) based on foliage fresh weight.

Feeding Preference Tests. Binary choice assays were conducted in a manner similar to the "disk test" of Jermy (1961) and Jermy et al. (1968). Leaf disks (176 mm^2) were cut from greenhouse-grown cabbage (cv. Golden Acre) with a cork borer and soaked 1 hr in different concentrations (log_2 dilution series) of each extract in aqueous solution. Control disks were soaked in distilled water. The leaf disks were patted dry on paper towels and fastened with No. 3 insect pins to a layer of paraffin wax on the bottom of a 250 ml waxed paper cup (75 mm diameter at base). Each disk was sandwiched between a pair of 16 mm^2 disks of dental dam rubber to support it on the pin about 5 mm above the bottom of the cup. The paraffin wax layer was covered with moist filter paper and a fine nylon screen that prevented larvae from feeding on the paper. Twelve disks (six experimental and six control) were pinned in an alternating circle around the periphery of each cup. The six experimental disks in each cup were treated with the same extract concentration. Ten fourth-instar larvae, from a laboratory colony maintained on greenhouse-grown cabbage, were released in the center of each arena. The cups were then covered with translucent polystyrene lids (perforated for ventilation) and placed on water-saturated paper towels in a large (40 × 27 × 9 cm) transparent polystyrene box. The box was covered to maintain high humidity and placed in a growth chamber at 25°C under fluorescent and incandescent lights. After 6 hr, larvae were removed and the box was refrigerated at 4°C overnight.

The following day, the leaf disks were removed, and the area consumed from each was measured to the nearest mm^2 using a graph paper template and a stereoscopic dissecting microscope. The template was made by stamping a circle on a piece of 1-mm^2 grid graph paper using the same cork borer used to punch leaf disks and an ink pad. This method allowed us to verify that disk shrinkage was minimal in undamaged disks as well as those fed upon by larvae. A feeding deterrent index (*FDI*) was calculated for each bioassay cup using the formula: $FDI = 100\ (C - T)/(C + T)$, where C and T represent the area consumed from control and treated disks, respectively. *FDI* thus reflects the difference in feeding rate due to treatment, expressed as a percentage of the total amount of feeding in each cup.

Fractionation of Extracts. Samples of extracts exhibiting strong feeding deterrent activity in the initial round of bioassays were redissolved in water, subjected to reverse-phase high-performance liquid chromatography (HPLC) using a Varian model 5000 liquid chromatograph equipped with a C-18 column, and eluted in a programmed gradient (3 ml/min) from 100% water to 100% acetonitrile (Figure 1, top). UV absorption by the column eluent was monitored at 219 nm and 254 nm with a Hewlett-Packard model 1040A diode array detec-

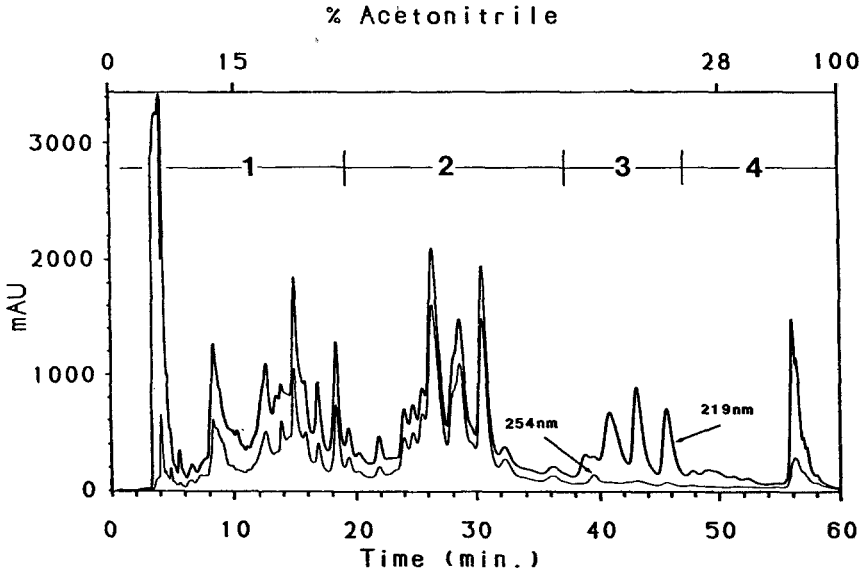


FIG. 1. Liquid chromatogram of *Erysimum cheiranthoides* butanol extract, with fractions 1–4 indicated. The injected sample (2 ml) contained the extracted equivalent of 20 g of fresh foliage. Elution gradient (100% water to 100% acetonitrile) is indicated at top.

tor. Several injections of 2 ml each (10 g leaf equivalent/ml) were performed in order to ensure sufficient material for bioassay. The eluted extract was collected as four fractions representing distinct but contiguous time intervals on the resulting chromatogram (Figure 1). Each fraction was dried under vacuum and redissolved in water for bioassay by the method described above.

No-Choice Tests. Cabbage leaf disks were soaked for 1 hr in serial dilutions (0.0625, 0.25, 1, and 4 gle/ml) of the butanol extract in water, patted dry, then placed on moist filter paper in 60 × 15-mm polystyrene Petri dishes, one leaf disk per dish. Control disks were soaked in distilled water and placed individually in dishes separate from the test disks. A young fourth-instar larva (collected from the laboratory colony within 12 hr of ecdysis) of known weight was placed in each dish. All dishes were placed on moist paper towels in a polystyrene box and held in a growth chamber at 25°C under fluorescent and incandescent lights. The number of larvae in contact with the disks and the number feeding were recorded at 2, 4, and 6 hr. At 6 hr the larvae were removed, and feeding rates were assessed visually as above. Relative consumption rate (RCR) was calculated for each larva by dividing the disk area consumed (mm^2)

by the initial larval weight. Two trials were conducted, the first using 15 and the second 10 replicate dishes per concentration.

RESULTS

The initial water extract of *E. cheiranthoides* was a strong deterrent of larval feeding, even at relatively low concentrations. This activity remained with the butanol-soluble portion of the extract, but the postbutanol water extract was inactive (Figure 2).

Despite the presence of numerous UV-absorbing compounds in HPLC fractions 1 and 2 of the butanol extract (Figure 1), these fractions did not deter larval feeding nearly as effectively as did fractions 3 and 4 (Figure 3). All four fractions were bioassayed again in duplicate (1 and 2) or triplicate (3 and 4) at a single concentration (0.25 gle/ml), and the results confirmed the previous finding that all deterrent activity resided in fractions 3 and 4 (Table 1). Fraction 4 was somewhat more active than fraction 3, but neither fraction alone was as deterrent at low concentrations as the butanol extract prior to HPLC fractionation (Figure 3, Table 1). The results of the choice bioassays for feeding deterrent activity are summarized in an extraction flowchart in Figure 4.

The butanol extract of *E. cheiranthoides* deterred larval feeding on treated disks even when no other food was available. In both trials, no larva was observed feeding at any time on any disk soaked in 4 gle/ml of the extract, and consumption rates were reduced by 50% or more (relative to controls) even at 1/16 of that concentration (Figure 5).

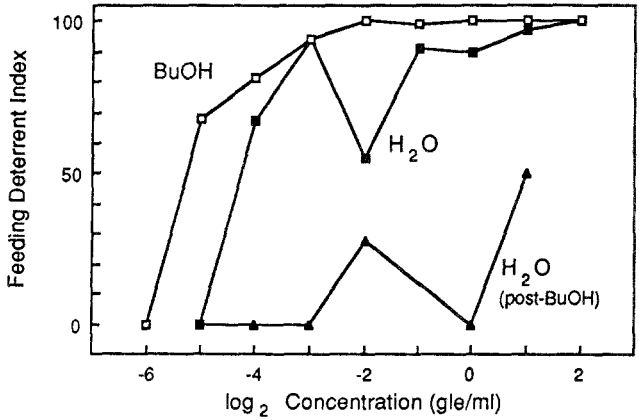


FIG. 2. Effect of *Erysimum cheiranthoides* extracts on feeding by *Pieris rapae* larvae in 6-hr choice bioassays.

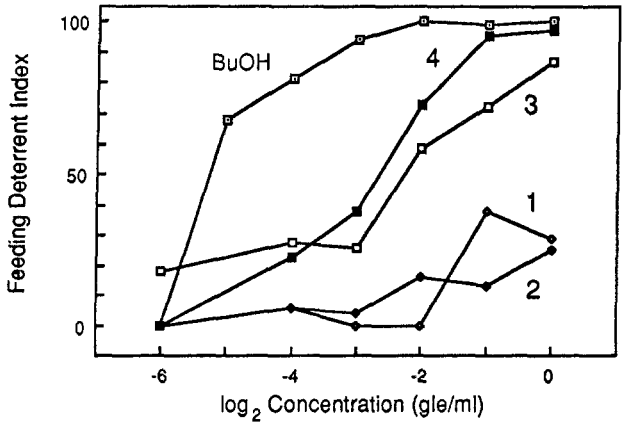


FIG. 3. Effect of *Erysimum cheiranthoides* butanol extract and HPLC fractions 1–4 on feeding by *Pieris rapae* larvae in 6-hr choice bioassays.

DISCUSSION

Our results indicate that rejection of *E. cheiranthoides* as food by *P. rapae* larvae involves deterrent chemical stimuli that can be extracted and transferred experimentally to otherwise acceptable host plant tissue. In combination with previous findings (Renwick et al., 1989; Sachdev-Gupta et al., 1990), the results also suggest that cardenolides are at least partly responsible for feeding deterrence. HPLC fraction 3 of the *Erysimum* butanol extract was highly active against larvae (Figure 3). The compounds represented by the three major peaks in this region of the chromatogram exhibited relatively high absorbance at 219 nm, but very little at 254 nm (Figure 1), a pattern characteristic of cardenolides (due to UV absorption by the butenolide ring). The specific cardenolides represented by the peaks of fraction 3 have been determined by Renwick et al. (1989) to be potent deterrents of oviposition by cabbage butterflies, and the most active constituents have been identified as the strophanthidin-based erysimoside and erychroside (Sachdev-Gupta et al., 1990).

Interestingly, fraction 4, although somewhat more active than fraction 3 as a larval feeding deterrent (Figure 3), is only a weak deterrent of oviposition (Renwick et al., 1989). The presence of an isolated, strongly UV-absorbing peak in fraction 4 (Figure 1) encourages speculation concerning the presence of other deterrents active against larvae, but not against adults. The large peak in fraction 4 may represent one or more additional cardenolides, but the strong absorbance at 254 nm relative to the fraction 3 peaks (Figure 1) suggests the presence of noncardenolide components that may or may not play a role in discrimination by larvae. We are currently addressing this question through

TABLE 1. FEEDING RATES OF *Pieris rapae* LARVAE ON CABBAGE LEAF DISCS SOAKED IN EXTRACS OF *Erysimum cheiranthoides*[a]

Treatment	Total area consumed (mm^2)		Feeding deterrent index[b]
	Control	Treated	
Initial H_2O extract	240	70	55 *
BuOH extract	267	1	99 **
Fraction 1[c]	882	1048	−9 ns
Fraction 2[c]	1097	909	9 ns
Fraction 3[d]	1802	410	63 ***
Fraction 4[d]	2023	310	73 ***
H_2O (post-BuOH)	424	237	28 ns

[a] 0.25 gram leaf equivalent per ml distilled water. Control disks were soaked in distilled water.
[b] * $P \leq 0.05$; ** $P \leq 0.025$; *** $P \leq 0.001$; ns, $P > 0.05$; Mann-Whitney U test (Sokal and Rohlf, 1981).
[c] Sum of two bioassay cups.
[d] Sum of three bioassay cups.

structural analyses of isolated pure compounds and concomitant bioassays similar to those described above. Preliminary results suggest the presence in fraction 4 of at least two active cardenolides with aglycones that differ from those of the oviposition deterrents of fraction 3.

Until positive identification of these feeding deterrent compounds is achieved, it is impossible to determine how closely our bioassay concentrations approached those occurring naturally in *E. cheiranthoides* foliage. For this reason we made no attempt to quantify uptake or surface retention of test solutions by the leaf disks, nor did we determine the amount of extract ingested by test larvae. However, the binary choice provided to larvae in our test chambers and the relative measure of activity provided by FDI were sufficient to separate deterrent from nondeterrent components of the *Erysimum* extract. It will likely prove more difficult to separate naturally active feeding deterrents from those with deterrent activity at unnaturally high concentrations without first identifying all deterrents, then relating the results of insect feeding assays of pure compounds to the quantities normally present in foliage.

The results suggest that *E. cheiranthoides* is protected from *P. rapae* by two distinct groups of compounds that may affect adult and larval behavior differently. The strophanthidin-based cardenolides act to deter adult oviposition (Renwick et al., 1989; Sachdev-Gupta et al., 1990), whereas a second group of putative cardenolides also serves to deter feeding by the larvae. The chemical identification of the most active feeding deterrents is currently underway.

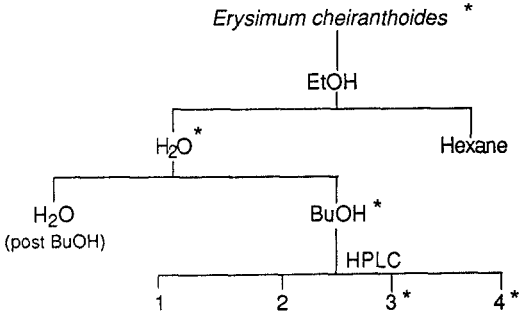


FIG. 4. Procedure for extraction of feeding deterrents from foliage of *Erysimum cheiranthoides*. Asterisks indicate feeding deterrent activity in choice bioassays against *Pieris rapae* larvae.

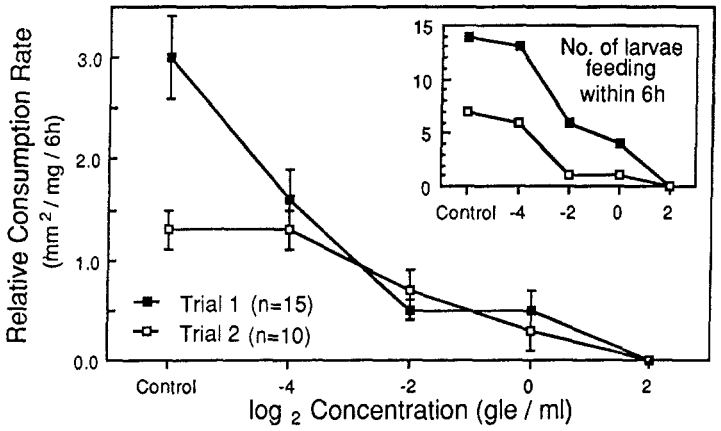


FIG. 5. Effect of *Erysimum cheiranthoides* butanol extract on feeding by *Pieris rapae* larvae in 6-hr no-choice bioassays. Vertical bars represent 95% confidence limits (based on *t* statistic).

Acknowledgments—Supported in part by USDA contract no. 86CRCR-1-2007.

REFERENCES

DETHIER, V.G. 1982. Mechanism of host-plant recognition. *Entomol. Exp. Appl.* 31:49–56.

FEENY, P., PAAUWE, K.L., and DEMONG, N.J. 1970. Flea beetles and mustard oils: host plant specificity of *Phyllotreta cruciferae* and *P. striolata* adults (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 63:832–841.

HICKS, K.L. 1974. Mustard oil glycosides: Feeding stimulants for adult cabbage flea beetles, *Phyl-*

JERMY, T. 1961. On the nature of oligophagy in *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). *Acta Zool. Acad. Sci. Hung.* 7:119–132.

JERMY, T., HANSON, F.E., and DETHIER, V.G. 1968. Induction of specific food preference in lepidopterous larvae. *Entomol. Exp. Appl* 11:211–230.

MILLER, J.R., and STRICKLER, K.L. 1984. Finding and accepting host plants, pp. 127–155, *in* W. Bell and R. Carde (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

NIELSEN, J.K. 1978a. Host plant discrimination within Cruciferae: Feeding responses of four leaf beetles to glucosinolates, cucurbitacins and cardenolides. *Entomol. Exp. Appl.* 24:41–54.

NIELSON, J.K. 1978b. Host plant selection of monophagous and oligophagous flea beetles feeding on crucifers. *Entomol. Exp. Appl.* 24:362–369.

RENWICK, J.A.A., and RADKE, C.D. 1985. Constituents of host and non-host plants deterring oviposition by the cabbage butterfly, *Pieris rapae. Entomol. Exp. Appl.* 39:21–26.

RENWICK, J.A.A., and RADKE, C.D. 1987. Chemical stimulants and deterrents regulating acceptance or rejection of crucifers by cabbage butterflies. *J. Chem. Ecol.* 13:1771–1776.

RENWICK, J.A.A., RADKE, C.D., and SACHDEV-GUPTA, K. 1989. Chemical constituents of *Erysimum cheiranthoides* deterring oviposition by the cabbage butterfly, *Pieris rapae. J. Chem. Ecol.* 15:2161–2169.

SACHDEV-GUPTA, K., RENWICK, J.A.A., and RADKE, C.D. 1990. Isolation and identification of oviposition deterrents to the cabbage butterfly, *Pieris rapae*, from *Erysimum cheiranthoides. J. Chem. Ecol.* 16:1059–1067.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry, 2nd ed. Freeman, San Francisco.

VERSCHAFFELT, E. 1911. The cause determining the selection of food in some herbivorous insects. *Proc. R. Acad. Amsterdam* 13:536–542.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

(Z)-OXACYCLOTRIDEC-10-EN-2-ONE DOES NOT APPEAR TO BE RESPONSIBLE FOR RESISTANCE TO ADULT ALFALFA WEEVIL FEEDING EXHIBITED BY *Medicago rugosa*[1,2]

ROBERT P. DOSS[3,*] and KATHLEEN J.R. JOHNSON[4]

[3]*USDA-ARS Horticultural Crops Research Unit*
3420 N.W. Orchard Ave.
Corvallis, Oregon 97330
Department of Horticulture
Oregon State University
Corvallis, Oregon 97331

[4]*Oregon Department of Agriculture*
635 Capitol Street, N.E.
Salem, Oregon 97310-0110

(Received August 29, 1990; accepted November 5, 1990)

Abstract—Steam distillate prepared from leaves of *Medicago rugosa* Desr. could inhibit feeding by adult alfalfa weevils (*Hypera postica* (Gyll.) on membrane filters, whereas an equivalent amount of steam distillate prepared using *M. sativa* L. had no effect on weevil feeding. Earlier work established that a 12-carbon lactone, (*Z*)-oxacyclotridec-10-en-2-one, was responsible for the feeding deterrent properties of the steam distillate from *M. rugosa*. The concentration of this volatile varied with plant age and leaf position. The maximum concentration in leaves of *M. rugosa* (area basis) was estimated, on the basis of tests using an inert substrate, to be too low to cause a detectable inhibition of feeding. No correlation between lactone content and weevil feeding was detected when leaves from similar positions on plants of different ages were used in a multiple choice bioassay. Although small amounts (< 12.5 μg) of (*Z*)-oxacyclotridec-10-en-2-one can inhibit adult alfalfa weevil feeding on 13-mm-diameter membrane filters, because leaf concentrations (area basis) of this compound are lower than required to cause a detectable inhi-

*To whom correspondence should be addressed.

[1] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the USDA and does not imply approval to the exclusion of other products or vendors that may also be suitable.

[2] Technical paper No. 9416 of the Agricultural Experiment Station, Oregon State University, Corvallis, Oregon 97331.

0098-0331/91/0300-0535$06.50/0

bition of feeding on the inert substrate, and because concentrations do not correlate with weevil feeding, it is unlikely that this lactone is responsible for resistance to adult alfalfa weevil feeding exhibited by *M. rugosa*.

Key Words—Coleoptera, Curculionidae, *Hypera postica*, alfalfa weevil, *Medicago rugosa*, Medicago sativa, insect resistance, (*Z*)-oxacyclotridec-10-en-2-one, *cis*-9-dodecen-12-olide, (*Z*)-12-hydroxydodec-9-enoic acid lactone.

INTRODUCTION

The alfalfa weevil, *Hypera postica* Gyll., is an important pest of alfalfa, *Medicago sativa* L. (Barnes and Ratcliffe, 1969; Pandey and Singh, 1984; Shade et al., 1975). Some *Medicago* species are resistant to alfalfa weevil feeding (Barnes and Ratcliff, 1969; Danielson et al., 1987; Johnson et al., 1980a, b; Pandey and Singh, 1984).

M. rugosa, like other resistant, glandular-haired species (Shade et al., 1975), apparently owes its resistance to larval feeding to entrapment of larvae in exudate secreted by the glandular hairs (Johnson et al., 1980a). This species is also very resistant (but not immune) to adult feeding (Barnes and Ratcliffe, 1969; Johnson et al., 1980b; Pandey and Singh, 1984), although entrapment of adults does not occur (Johnson et al., 1980b). Johnson et al. (1980b) observed that weevil adults avoided contact with *M. rugosa* foliage and concluded that olfactory cues were probably responsible for resistance to adult feeding.

Following these observations, Doss et al. (1989) isolated a volatile lactone from leaves of *M. rugosa*, (*Z*)-oxacyclotridec-10-en-2-one [(*Z*)-12-hydroxydodec-9-enoic acid lactone], that could inhibit feeding by adult alfalfa weevils on phagostimulant-treated membrane filters. Results of studies undertaken to determine whether or not the resistance of *M. rugosa* to feeding by adult alfalfa weevils could be attributed to the presence of this lactone are described below.

METHODS AND MATERIALS

Plant Material. Medicago rugosa Desr. and *M. sativa* L. cv. Ranger were grown from seeds using the methods described earlier (Doss et al., 1989).

Insects. Insects were collected and maintained as described previously (Doss et al., 1989). Newly eclosed adults were used for all bioassays with membrane filters. Bioassays with leaflets were carried out with insects that had been maintained in continuous darkness at 5 $\pm$ 2°C on fresh sprigs of *M. sativa* cv. Ranger and supplied with water using moist dental wicks. These weevils were removed from cold storage and placed under the bioassay conditions 10 days before the bioassays.

Bioassays. Multiple choice bioassays with membrane filters (Doss and Shanks, 1986; Doss et al., 1989) were used to examine the influence of crude steam distillate and purified (*Z*)-oxacyclotridec-10-en-2-one on feeding by adult alfalfa weevils. In order to promote feeding, the 13-mm-diameter membrane filters were pretreated with either 400 μg sucrose (Shanks and Doss, 1987) or with 50-mg-equivalents of an exhaustive ethanolic extract prepared using leaves of *M. sativa* cv. Ranger.

Bioassays with terminal leaflets were conducted using the same conditions as those used with membrane filters, except leaflets were not treated with sucrose or any plant extract. Petiolules were inserted through a Parafilm membrane into water-filled vials.

Additional details for individual bioassays are noted in the text and in figure and table legends.

Extraction, Purification, and Analysis of (Z)-Oxacyclotridec-10-en-2-one. Steam distillate was extracted (into hexane) using a modified Nielsen-Kryger apparatus (Veith and Kiwus, 1977). (*Z*)-Oxacyclotridec-10-en-2-one was purified from the steam distillate using solvent partitioning and liquid chromatography as described previously (Doss et al., 1989).

Estimates of the amount of (*Z*)-oxacyclotridec-10-en-2-one were made by soaking samples of 30 leaflets of *M. rugosa* in 10 ml diethyl ether (redistilled to remove butylated hydroxytoluene) to which *cis* nerolidol had been added as an internal standard. The ether was removed after 10 minutes, combined with two 5 ml washings of the leaves, and extracts and washings were partitioned 2× against equal volumes of saturated NaCl. The ether was carefully removed in vacuo, and the extract was reconstituted in hexane.

The hexane solutions were subjected to gas–liquid chromatography on a packed SP 2100 column as described previously (Doss et al., 1989). (*Z*)-Oxacyclotridec-10-en-2-one concentrations were estimated based on peak height. Under these conditions, retention times for *cis*-nerolidol and (*Z*)-oxacyclotridec-10-en-2-one were 13.8 and 14.2, minutes, respectively. Estimates were not corrected for relative detector response (1.039 ± 0.008, $\bar{X} \pm$ SE for five determinations). Estimation of the loss of the lactone from 13-mm-diameter membrane filters was made using procedures similar to those used with leaves.

Data were analyzed using analysis of variance. Duncan's multiple-range test was used to compare means. Results of statistical analyses are given in figure and table legends.

RESULTS

In a three-choice bioassay (Figure 1), application of 0.31 g equivalents of steam distillate from *M. rugosa* significantly reduced adult alfalfa weevil feeding on 13-mm-diameter membrane filters relative to feeding on filters treated

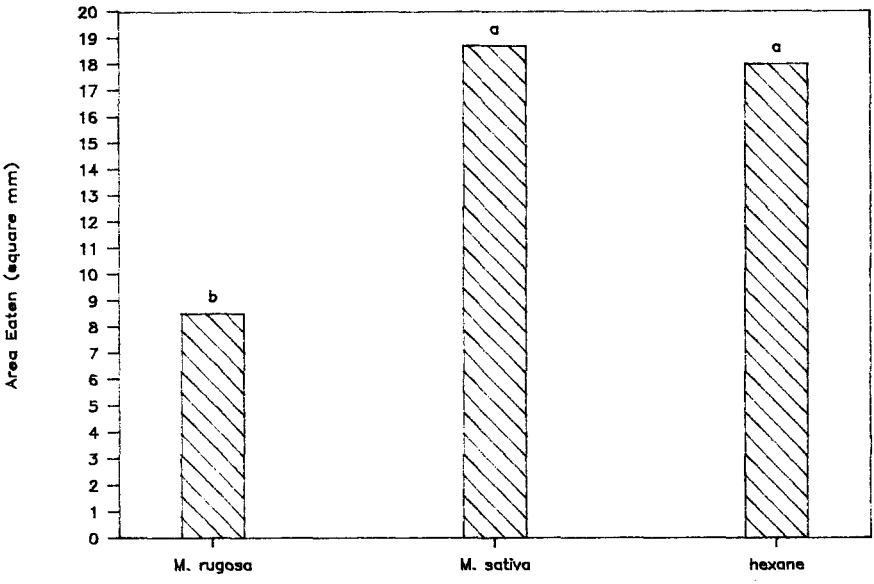


FIG. 1. Influence of steam distillate on feeding of adult alfalfa weevils on membrane filters. The bioassay was conducted for 24 hr with 12 weevils per bioassay arena. In order to stimulate feeding, filters were pretreated with 400 μg sucrose. Bars bearing different letters represent significantly different feeding ($P = 0.05$) as determined using Duncan's multiple-range test (F treatment = 8.9, $P = 0.0021$).

with hexane alone. A similar amount of extract from *M. sativa* cv. Ranger did not inhibit feeding. With the steam distillates used for this study the yield from *M. rugosa* leaflets was 0.16 mg/g fresh wt versus 0.02 mg/g fresh wt for *M. sativa* cv. Ranger. [Note that in other studies (Doss et al., 1989) exhaustive steam distillation of leaflets, petioles, and stems of *M. rugosa* yielded 0.23 mg/g fresh wt.] Leaflet masses per unit area were 0.024 $\pm$ 0.001 and 0.022 $\pm$ 0.001 g/cm^2 for *M. rugosa* and *M. sativa*, respectively ($\bar{X} \pm$ SE for $N = 5$).

(*Z*)-Oxacyclotridec-10-en-2-one, a prominent component of the steam distillate from *M. rugosa* (Doss et al., 1989), but not detected in steam distillate from *M. sativa* (data not shown) could inhibit adult alfalfa weevil feeding on membrane filters (Figure 2). About 70% of the lactone was lost from the membrane filters during the course of a 6-hr bioassay (Figure 3).

The amount of (*Z*)-oxacyclotridec-10-en-2-one extracted from leaves of *M. rugosa* varied significantly with plant age (Table 1) and with leaf position (data not shown). Adult alfalfa weevil feeding on leaves from plants of different ages did not correlate well with levels of extractable (*Z*)-oxacyclotridec-10-en-2-one (Table 1).

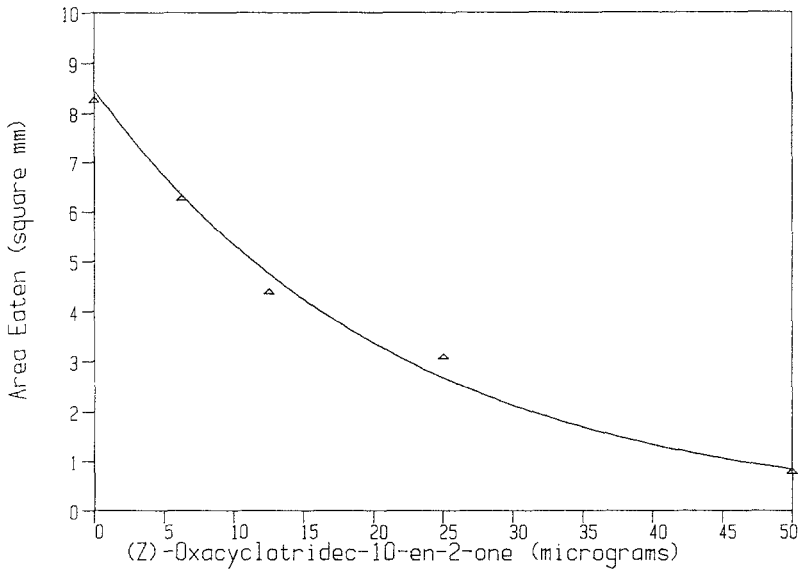


FIG. 2. Alfalfa weevil feeding on 13-mm-diameter membrane filters treated with several levels of (*Z*)-oxacyclotridec-10-en-2-one. The bioassay was conducted for 6 hr with 40 weevils per bioassay arena and 10 arenas (blocks). In order to stimulate feeding, filters were pretreated with 50-mg-equivalents of an exhaustive ethanolic extract of *M. sativa* cv. Ranger leaves. Data were analyzed using analysis of variance (minimum significant difference = 3.62, $F = 10.46$, $P < 0.001$) and were fitted using a straight-line semilog-type equation ($Y = ab^x$) where $a = 8.47$, $b = 0.955$, $r^2 = 0.99$, and $df = 3$.

DISCUSSION

M. rugosa has consistently shown resistance to adult alfalfa weevil feeding. In no-choice tests carried out by three separate groups of investigators, feeding on *M. rugosa* varied from 60% to 74% of feeding on *M. sativa* (Barnes and Ratcliffe, 1969; Johnson et al., 1980b; Pandey and Singh, 1984). Differences were much larger in choice tests (Pandey and Singh, 1984; Doss and Johnson, unpublished).

Search for a volatile feeding deterrent from *M. rugosa* was stimulated by the observation that adult alfalfa weevils avoided contact with this resistant annual species (Johnson et al., 1980b). Isolation of (*Z*)-oxacyclotridec-10-en-2-one from a steam volatile extract was guided by membrane filter bioassays (Doss et al., 1989). Results of these bioassays suggested that the lactone was the only material in the steam distillate that possessed feeding deterrent activity.

Leaflets of a susceptible alfalfa cultivar, *M. sativa* Ranger, yielded considerably less steam volatile materials than leaflets of *M. rugosa*, and no (*Z*)-

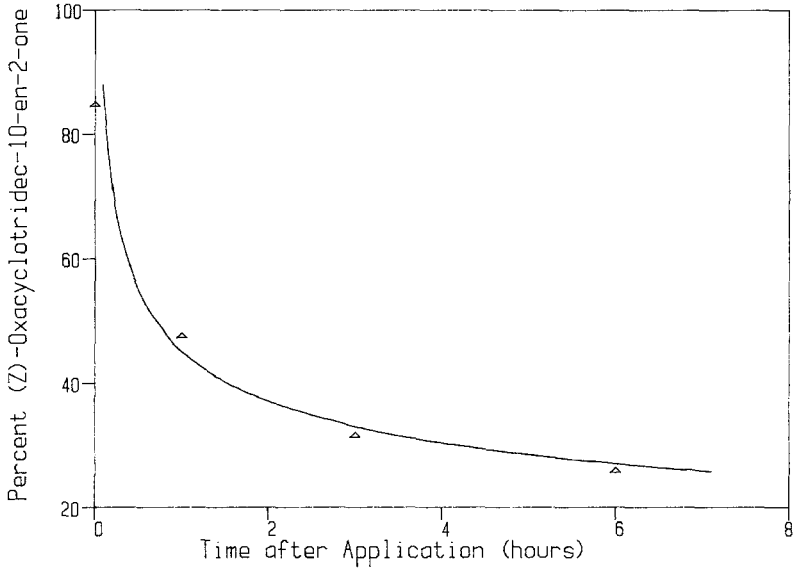


FIG. 3. The loss of (Z)-oxacyclotridec-10-en-2-one from 13-mm-diameter membrane filters. (Z)-Oxacyclotridec-10-en-2-one, 25 μg, was applied to each of 20 filters. Filters were placed under normal bioassay conditions and recovery of the added lactone was estimated gas chromatographically after extraction into hexane (See Methods and Material). Percent remaining is plotted on the Y axis. Each datum represents the mean for five determinations (standard errors ranged from 1 to 5 percent). The data were fitted with a straight-line log-type equation $Y = ax^b$ where $a = 45.3$, $b = -0.29$, $r^2 = 0.99$ and $df = 3$.

oxacyclotridec-10-en-2-one could be detected in the extract. Roughly similar yields of essential oils from leaves and stems (0.005–0.06 mg/g fresh wt) (Buttery and Kamm, 1980), or from aerial parts (0.09 mg/g fresh wt) (Kami, 1983) of alfalfa have been reported, but (Z)-oxacyclotridec-10-en-2-one has not been noted as a constituent of either of these volatile fractions.

Application of relatively small amounts of (Z)-oxacyclotride-10-en-2-one could inhibit adult alfalfa weevil feeding on phagostimulant treated membrane filters (Figure 2). Considering loss of material from the filters (Figure 3), the average concentration on the filters over the 6-hr bioassay period was about one half of the starting concentration.

The maximum concentration of (Z)-oxycyclotridec-10-en-2-one found in leaves of *M. rugosa* was 20.5 μg/g, or, on an areas basis, 0.49 μg/cm^2. A concentration of 1 μg/cm^2 would be expected on the basis of the relationship shown in Figure 2 to cause about a 5% reduction in area eaten from phagostimulant treated membrane filters. This is near the detection limits of the mea-

TABLE 1. (Z)-OXACYCLOTRIDEC-10-EN-2-ONE CONTENT OF AND ADULT ALFALFA FEEDING ON YOUNGEST FULLY EXPANDED LEAVES (TERMINAL LEAFLETS) OF *M. rugosa* PLANTS OF DIFFERENT AGES[a]

Plant age (weeks after sowing)	Sowing date	Condition of plants	(Z)-Oxacyclotridec-10-en-2-one ($\mu g/g$)	Area eaten (mm^2)
8	April 27	100% flowering 86% with seed pods early stages of senescence	0.5 ± 0.06[b]	12.8 ± 4.1[c]
6	May 11	59% flowering no seed pods green leaves	12.0 ± 0.8	59.3 ± 1.2
4	May 25	preanthesis	20.5 ± 0.8	47.8 ± 8.4
2	June 8	preanthesis no visible buds small plants	17.5 ± 3.8	26.6 ± 6.7

[a] Bioassay conducted June 22, 1988, analysis carried out June 23, 1988.
[b] Values are means ± SE for three determinations ($F_{age} = 13.4$, $P = 0.0046$).
[c] Values are means ± SE for 10 multiple choice bioassays. Each bioassay arena contained one terminal leaflet from a plant of each age (i.e., four leaflets per arena) ($F_{age} = 5.95$, $P = 0.0031$).

suring system used (about 0.5 mm^2), and it is substantially less than would be expected if (Z)-oxycyclotridec-10-en-2-one were the major factor in conferring the resistance to adult weevil feeding exhibited by *M. rugosa*.

It is possible that (Z)-oxacyclotridec-10-en-2-one could be more effective in inhibiting alfalfa weevil feeding when present at locally high concentrations, for example, in the glandular hairs of a leaf, than when uniformly distributed across a membrane filter. However, additional evidence that (Z)-oxacyclotridec-10-en-2-one is not responsible for the resistance to alfalfa weevil feeding exhibited by *M. rugosa* is derived from data presented in Table 1. There was no correlation between the concentration of the lactone and adult alfalfa weevil feeding even though the concentration varied about 40-fold. In fact, the least feeding occurred on leaves with the lowest concentration of the volatile. Notably, these plants were more mature than plants normally subject to infestation in the field would be. Weevils normally feed only on preanthesis plants. Thus, it remains possible that some other aspect of the physiology of these more mature plants inhibited weevil feeding and masked the effect of variation of (Z)-oxacyclotridec-10-en-2-one concentration.

The amount of the (Z)-oxacyclotridec-10-en-2-one obtained from young

leaves by extraction into diethyl ether (0.5–20μg/g fresh) (Table 1) was roughly comparable to the amount (8 μg/g fresh) obtained from an exhaustive steam distillate of similar leaves in an earlier study (Doss et al., 1989). It had been noted that the composition of the steam volatile fraction varied markedly with plant age, with much less lactone recoverable from postanthesis plants (Doss, unpublished).

The fact that this material was removed by short exposure to the extracting solvent suggests that it may have been sequestered in the glandular hairs. However, repeated efforts to detect the lactone in extracts made using detached (upright) glandular hairs did not yield conclusive results.

Although (*Z*)-oxacyclotridec-10-en-2-one, a prominent volatile component from *M. rugosa* leaves, can inhibit adult alfalfa weevil feeding on an inert substrate, evidence suggests that it does not play an important role in conferring resistance to weevil feeding possessed by this annual *Medicago* species. Because olfactory cues appear to be involved in this resistance (Johnson et al., 1980b), it is possible that a more volatile material, not detected in these studies, is responsible.

REFERENCES

BARNES, D.K., and RATCLIFF, R.H. 1969. Evaluation of annual species of *Medicago* as sources of alfalfa weevil resistance. *Crop Sci.* 9:640–642.

BUTTERY, R.G., and KAMM, J.A. 1980. Volatile components of alfalfa: Possible insect host plant attractants. *J. Agric. Food Chem.* 28:978–981.

DANIELSON, S.D., MANGLITZ, G.R., and SORENSEN, E.L. 1987. Resistance in perennial glandular-haired *Medicago* species to feeding by adult alfalfa weevils (Coleoptera:Curculionidae). *Environ. Entomol.* 16:708–711.

DOSS, R.P., and SHANKS, C.H., JR. 1986. Use of membrane filters as a substrate in insect feeding bioassays. *Bull. Entomol. Soc. Am.* 32:248–249.

DOSS, R.P., GOULD, S.J., JOHNSON, K.J.R., FLATH, R.A., and KOHNERT, R.L. 1989. (Z)-Oxacyclotridec-10-en-2-one, an alfalfa weevil feeding deterrent from *Medicago rugosa*. *Phytochemistry* 28:3311–3315.

JOHNSON, K.J.R., SORENSEN, E.L., and HACKER, E.K. 1980a. Effect of temperature and glandular-haired *Medicago* species on development of alfalfa weevil larvae. *Crop Sci.* 20:631–633.

JOHNSON, K.J.R., SORENSEN, E.L.,and HACKER, E.K. 1980b. Resistance in glandular-haired annual *Medicago* species to feeding by adult alfalfa weevils (*Hypera postica*). *Environ. Entomol.* 9:133–136.

KAMI, T. 1983. Composition of the essential oil of alfalfa. *J. Agric. Food Chem.* 31:38–41.

PANDEY, K.C., and SINGH, A. 1984. Laboratory evaluation of medics for resistance to lucerne weevil. *Indian J. Genet.* 44:253–258.

SHADE, R.E., THOMPSON, T.E., and CAMPBELL, W.R. 1975. An alfalfa weevil larvae resistance mechanism detected in *Medicago J. Econ. Entomol.* 68:399–404.

SHANKS, C.H., JR., and DOSS, R.P. 1987. Feeding responses by adults of five species of weevils (Coleoptera:Curculionidae) to sucrose and sterols. *Ann. Entomol. Soc. Am.* 80:41–46.

VEITH, G.D., and KIWUS, L.M. 1977. An exhaustive steam-distillation and solvent-extraction unit for pesticides and industrial chemicals. *Bull. Environ. Contam. Toxicol.* 17:631–636.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

BEHAVIORAL RESPONSE OF *Trichogramma pretiosum* RILEY AND *Telenomus sphingis* (ASHMEAD) TO TRICHOME/METHYL KETONE MEDIATED RESISTANCE IN TOMATO

R.K. KASHYAP,[1] G.G. KENNEDY,[2,*] and R.R. FARRAR, JR.[2]

[1]*Department of Entomology*
Haryana Agricultural University
Hisar 125004, India

[2]*Department of Entomology*
North Carolina State University
Raleigh, North Carolina 27695-7630

(Received September 6, 1990; accepted November 5, 1990)

Abstract—Effects of methyl ketone (2-tridecanone and 2-undecanone)/glandular trichome-based resistance to insects of *Lycopersicon hirsutum* f. *glabratum* C.H. Mull, PI 134417, on the behavioral responses of *Trichogramma pretiosum* Riley and *Telenomus sphingis* (Ashmead) were observed in the laboratory. For *T. pretiosum*, these effects included (1) longer time required by the wasps to cross a leaf disk (diam. 16.3 mm), (2) higher percentage of wasps initiating flight from disks before reaching the edge, and (3) entrapment of wasps in trichome exudates. In tomato genotypes with varying densities of type VI glandular trichomes (3.1–9.2/mm^2) but with no methyl ketones in the trichome tips, no such adverse effects were observed. The time taken to cross a disk or initiate flight by *T. sphingis*, a larger wasp than *T. pretiosum*, was not significantly affected on 16.3-mm leaf disks, although the percentage of *T. sphingis* initiating flight was directly correlated with trichome density among lines with no ketones. When placed on whole leaflets of plant lines with or without methyl ketones, *T. sphingis* was entangled by trichome exudates. However, unlike *T. pretiosum*, *T. sphingis* was eventually able to free itself from entanglement. Landing rates by *T. sphingis* on PI 134417 foliage contained in dialysis tubes were also reduced. Data indicate that suppression of egg parasitism by *T. sphingis* on resistant foliage is due to both repellent action of its foliar volatiles and reduced searching rates by the parasitoids. Results indicate that a significant portion of the reduction of egg parasitism by *T. pretiosum* and *T. sphingis* is attributable to the effects

*To whom correspondence should be addressed.

0098-0331/91/0300-0543$06.50/0

of 2-tridecanone and/or 2-undecanone present in the tips of type VI glandular trichomes on PI 134417 foliage, although the trichomes also adversely affect the wasps even in plant lines without ketones.

Key Words—*Lycopersicon hirsutum* f. *glabratum*, glandular trichomes, *Trichogramma pretiosum*, Hymenoptera, Trichogrammatidae, *Telenomus sphingis*, Scelionidae, plant-insect interaction, 2-tridecanone, 2-undecanone, parasitism.

INTRODUCTION

There have been many studies in which rates of parasitism by parasitic arthropods have been shown to be influenced by characteristics of their hosts' food plants, such as foliar morphology (Obrycki and Tauber, 1984; Keller, 1987) and plant volatiles (Altieri et al., 1981; Boethal and Eikenbary, 1986). On a finer scale, foliar trichomes (Rabb and Bradley, 1968; Obrycki, 1986; Kauffman and Kennedy, 1989a) or other plant surface characteristics may affect parasitoids (Hulspas-Jordaan and van Lenteren, 1978; Obrycki and Tauber, 1984) through repellency, alteration of searching behavior, physical entanglement, or toxicity.

The wild tomato, *Lycopersicon hirsutum* f. *glabratum* C.H. Mull, accession PI 134417, is highly resistant to the tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae) (Kennedy and Henderson, 1978) and Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Kennedy and Sorenson, 1985; Kennedy and Farrar, 1987). This resistance is conditioned by the 13-carbon methyl ketone, 2-tridecanone, that occurs in type VI (Luckwill, 1943) glandular trichomes on the foliage. 2-Tridecanone also confers a limited degree of resistance to tomato fruitworm, *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae), although higher levels of resistance to *H. zea* are conferred by factors associated with the foliar lamellae (Kennedy and Dimock, 1983; Dimock and Kennedy, 1983; Kennedy et al., 1985; Kennedy, 1986; Farrar and Kennedy, 1987a). The 11-carbon methyl-ketone, 2-undecanone, has been reported in the type VI trichomes of PI 134417 by Kennedy (1986) and Lin et al. (1987) and of other *L. hirsutum* f. *glabratum* accessions by Soost et al. (1968) and Lundgren et al. (1985). It is less abundant in PI 134417 trichomes and is less acutely toxic than 2-tridecanone against neonate *H. zea* (Dimock et al., 1982). However, in combination, 2-tridecanone and 2-undecanone are synergistic in their toxicity to several insect species, including *H. zea* (Farrar and Kennedy, 1987b, 1988; Lin et al., 1987). At levels frequently found in PI 134417, 2-undecanone also causes extensive mortality of *H. zea* pupae when ingested by fifth-instar larvae (Farrar and Kennedy, 1987b, 1988).

In North Carolina, eggs of *H. zea* on tomato are frequently parasitized by

Trichogramma spp. (Hymenoptera: Trichogrammatidae), while *M. sexta* eggs are parasitized by both *Trichogramma* spp. and *Telenomus sphingis* (Ashmead) (Hymenoptera: Scelionidae) (Kauffman and Kennedy, 1989a, Farrar and Kennedy, unpublished data). In the field, lower rates of parasitism by both species have been documented on tomato lines possessing high levels of glandular trichomes and 2-tridecanone (Farrar and Kennedy, unpublished data). Through trichome removal experiments, Farrar and Kennedy (unpublished data) demonstrated that differences among plant lines in rates of parasitism by *T. sphingis* were attributable to differences in density and composition of type VI glandular trichomes. In their experiments, they did not observe high levels of mortality among *T. sphingis* adults on foliage of PI 134417 or related plant lines, despite the occurrence of potentially toxic levels of 2-tridecanone associated with foliage of some of the plant lines. Since, in *T. sphingis* at least, reduction in rates of parasitism is evidently not due to wasp mortality, the present study was undertaken to investigate possible effects on wasp behavior as an explanation for the observed reduction in parasitism by both *Trichogramma* and *T. sphingis*. In addition, by comparing the two species, that have similar life histories but differ in size (*T. sphingis* is ca. 2.0 mm long; *Trichogramma* is ca. 0.5 mm), we hoped to gain insight into how such differences influence the interactions between trichome-mediated host plant resistance and biological control agents.

Because the trichome-based resistance of PI 134417 is well understood, it provides an ideal system for studying effects of host-plant resistance on organisms of the third trophic level. The results of this study should be useful in understanding host plant resistance and biological control interactions not only in tomato but in other crops as well.

METHODS AND MATERIALS

Colonies of *Trichogramma pretiosum* Riley and *T. sphingis* were maintained on *H. zea* and *M. sexta* eggs, respectively. All tomato plants were grown in a greenhouse using a commercial soil mix (Metro Mix 220) and were fertilized weekly (Peter's Professional 20-20-20 at 2.8 g/plant). Photoperiod was supplemented to 16 hr with metal halide lamps.

The following plant lines were included in this study: PI 134417, the susceptible *L. esculentum* Mill. cultivar Better Boy, F_1 (*L. esculentum* × PI 134417), BC-2 [a selection from an F_1 backcross family (F_1 × PI 134417)], BCS-9 and BCS-10 [selections from another F_1 backcross family (F_1 × *L. esculentum*)], and F_2-16 [a selection from an F_2 family (F_1 × F_1)]. The *L. esculentum* cultivar Walter was used to make crosses since Better Boy, a hybrid cultivar, does not cross readily with PI 134417. Insects respond similarly to Walter and Better Boy, and neither contains significant levels of 2-tridecanone (Farrar and Kennedy, unpublished data). The hybrid plants (except for F_1) were selected

based on the density of type VI trichomes on their foliage and the level of mortality of *M. sexta* in bioassays with excised foliage (Kennedy and Henderson, 1978).

To determine the levels of methyl ketones (2-tridecanone and 2-undecanone) associated with foliage of each plant line, the exudate of 1200 type VI trichome tips per plant line was collected using glass microcapillary tubes. The exudate was dissolved in 200 μl of methanol. The samples were analyzed using a Hewlett-Packard 5880 gas chromatograph with a 15-m Durabond DB5 capillary column, helium carrier gas, and temperature program analysis (100°C for 1 min, then to 275°C at 5°C/min, then 275°C for 5 min). 2-Tetradecanone (Wiley Organics, 4654 Kenny Rd., Columbus, Ohio 43220) was used as an internal standard. Collections of glandular trichome tips were made from disks (5.8 mm^2 in diameter) cut from each of four leaflets per plant line (one disk per leaflet). All type VI glandular trichomes on each disk were counted, and the exudate was collected from 300 trichomes per disk. These data were used to calculate the concentration of 2-tridecanone and 2-undecanone in tomato genotypes as weight per centimeter squared of foliage.

A test was conducted to evaluate the effect of methyl ketone-and/or trichome-mediated resistance on (1) rates of *H. zea* egg parasitism by *T. pretiosum* and (2) wasp entrapment (in the trichome exudates such that they could neither move nor fly and would ultimately die) on selected plant lines in laboratory cages. *H. zea* eggs were glued on the lower side of young fully expanded foliage of each plant line at a constant density of ca. 0.5 egg/cm^2. To obtain this density, a wire screen with a 0.4-cm^2 grid was supported above the leaf, and one egg was glued in each fifth square. A diluted (1 : 1) water-based white glue (Elmer's) was used. In preliminary tests, more than 70% of eggs on Better Boy foliage were parasitized when the ratio of female wasps to host eggs was 1 : 1.6. Thus, this ratio was maintained in all replicates of this test. After eggs were glued on each leaf, the petiole was placed in a Water-Pic (water-filled, rubber-capped plastic tube with tapering end). These tubes were then placed in empty flasks in clear Plexiglas cages (30 × 30 × 30 cm). One leaf of each plant line was placed in the cage. The parasitoids were collected from the colony in shell vials, anesthetized with carbon dioxide, sexed, counted, and, following their recovery, released in the cage. A small amount of honey was streaked on the inside top of the cage as food for the wasps. Only 0- to 24-hr-old wasps were used in the test. The cages were held at room temperature (25 ± 2°C) and the test was replicated seven times.

After 24 hr, the leaves were removed from the Water-Pics and scanned under a stereomicroscope to observe the entrapment of *T. pretiosum* in the trichome exudates. The eggs were removed from the foliage, placed in individual No. 0 gelatin capsules (Eli Lilly and Co., Indianapolis, Indiana 46285), and held at 27°C for 10 days. *T. pretiosum* requires seven to eight days to complete

development to adult emergence in *H. zea* eggs at this temperature (Kashyap, unpublished data). On the tenth day, eggs were categorized as parasitized or nonparasitized. Only those *H. zea* eggs from which adult *T. pretiosum* emerged successfully were categorized as parasitized. Our earlier data revealed that, at least on PI 134417 foliage, some *Heliothis* eggs that are stung by *T. pretiosum* do not yield adult parasitoids, presumably because of 2-tridecanone-mediated mortality of developing parasitoids inside the host eggs (Kashyap, Kennedy, and Farrar, unpublished data). Data on the percent *H. zea* egg parasitization as well as wasp entrapment on various tomato plant lines were normalized by arcsine$\sqrt{\%}$ transformation, analyzed by analysis of variance (ANOVA) with plant line means separated by least significant difference (LSD).

In another experiment, behavioral responses (walking and initiation of flight) of individual *T. pretiosum* and *T. sphingis* wasps were studied in insects placed on excised leaf disks of the six tomato lines. The leaf disks (diam. 16.3 mm) were cut from the first fully expanded leaf as described above. In preliminary tests, disks of this size were found adequate to observe the behavior of *T. pretiosum* adults on PI 134417 foliage. One hour prior to the start of the test, 1-day-old wasps, without any prior oviposition experience, were placed in a Petri dish (diam. 5.5 cm) with host eggs. The females were allowed to oviposit in the host eggs. An individual ovipositing wasp then was transferred to the center of the leaf disk by moving the egg with forceps. The egg was removed from the leaf-disk as soon as the wasp left it to walk on the leaf disk. The time (seconds) taken by the wasp to cross the edge of the leaf disk arena or initiate flight was recorded with a stopwatch. This behavior was observed under a magnifying ($\times 1.77$) lens (diam. 19.5 cm) illuminated evenly from all sides with a circular 22-W fluorescent tube. The incidence of flight initiation was considered an index of propensity to fly away from the leaf surface due to physical and/or chemical barriers on the leaf surface. Insects that spent more than 5 min at one spot were examined under a stereomicroscope to determine if they were entrapped in the trichome exudates. Each leaf disk and adult wasp was tested only once. Tests were conducted at room temperature (ca. 25°C). Fifty wasps of each species were observed on each plant line. Data on time to cross the disk or initiate flight were subjected to ANOVA; means were separated by LSD. Data on the proportion of wasps entrapped in trichome exudates were subjected to chi-square analysis.

Results obtained in this test with *T. sphingis* suggested that the disk size used was inappropriate for studying the full repertoire of behavioral responses of *T. sphingis*, which is larger than *T. pretiosum*. Thus, the responses of *T. sphingis* also were investigated on whole excised leaflets held horizontally 10–15 cm above a table top. The cut end of the petiole was placed in a Water-Pic held in a plastic cup. An adult female *T. sphingis* was transferred to the middle of the leaflet as described above and observed under the magnifying lens. The

time spent (1) walking rapidly or (2) not walking (characterized by grooming of mouthparts, legs, or antennae, or keeping the mouthparts close to the leaf surface, etc.) was recorded until the wasp flew or dropped from the leaf, or for 6 min, whichever was less. If the wasp spent more than 6 min on the leaflet, the total time it remained on the leaflet was also recorded. Each adult *T. sphingis* and leaflet were used only once. Twenty-five adult *T. sphingis* were observed on each Better Boy, F_1, and PI 134417. Surface area was measured for 10 leaflets of each plant line using a Li-Cor LI3100 leaf area meter. Percentage of time spent by an adult *T. sphingis* walking on the leaf surface (within the first 6 min) was calculated, and normalized using the arcsine$\sqrt{\%}$ transformation. Similarly, total time (seconds) on the leaflet was normalized using the logarithmic transformation. Data were subjected to ANOVA and the means separated by LSD.

The technique of Obrycki and Tauber (1984) was used to determine the influence of trichome/methyl ketone-mediated resistance in tomato on the landing rates of *T. sphingis*. Sections of dialysis tubing (Spectra/Por, molecular weight cutoff 6000–8000), 16 cm long × 7.6 cm diameter, were fitted with 3-cm-thick foam rubber plugs forming chambers 10 cm long × 7.6 cm diameter. Leaves of approximately equal area were collected from each of three plant lines (Better Boy, F_1, and PI 134417). One *M. sexta* egg was glued to each leaflet (adaxial surface) of the leaf with Elmer's glue. A leaf was inserted into each dialysis tube through a slit in one of the plugs. The petiole extended outside the plug and was inserted into a Water-Pic. The tubes were held horizontally, and about 10 unsexed *T. sphingis* adults were released in the tube. Time (minutes) taken by the first wasp to land on the leaf was recorded with a stopwatch, and observations were terminated at 15 min. Each group of wasps and each leaf were used only once. The areas of 10 leaves of each line were measured as above. The test was replicated ca. 30 times for PI 134417 and ca. 20 times each for F_1 and Better Boy. The percentage of observations in which one or more wasps landed within 15 min, and the frequency distribution of times taken by the first *T. sphingis* adult to land on the foliage, were analyzed by χ^2.

RESULTS AND DISCUSSION

In the laboratory cage test, percent parasitism of *H. zea* eggs by *T. pretiosum* was significantly lower on PI 134417, BC-2, and F_2-16 foliage than on foliage of the other plant lines, whereas significant entrapment of wasps occurred only on PI 134417 and BC-2 foliage (Table 1). 2-Tridecanone and 2-undecanone were found in the trichome tips of PI 134417 and BC-2 but not in the trichome tips of the other plant lines. In addition, these two plant lines had the highest densities of type VI trichomes (Table 2).

TABLE 1. RATE OF *H. zea* EGG PARASITISM AND ENTRAPMENT OF *Trichogramma pretiosum* IN LABORATORY CAGES WHEN GIVEN CHOICE AMONG EGGS ON TOMATO GENOTYPES VARYING IN LEVELS OF 2-TRIDECANONE-MEDIATED RESISTANCE TO *M. sexta*

Treatment	*H. zea* egg parasitism (%)[a]	Wasp entrapment on leaf (%)[a]
PI 134417	4.0 A	7.9 A
BC-2	5.8 A	3.0 A
F_2-16	15.7 A	0.0 B
BCS-10	65.4 B	0.2 B
Better Boy	77.3 B	0.0 B
BCS-9	85.8 B	0.0 B

[a] Means separation vertical; means with the same letter are not significantly different at $P \leq 0.05$ (LSD).

When placed on leaf disks, the time taken by adult *T. pretiosum* to walk off the leaf disk varied significantly among plant lines ($F = 33.49$; $P = 0.0001$; treatment $df = 5$; error $df = 169$) (Table 2). There were no differences among plant lines lacking methyl ketones in their trichome tips, despite a range of type VI trichome densities among these lines of 3.1–9.2/mm^2. The wasps required significantly more time to walk from the leaf disks of BC2 and PI 134417, both of which contain methyl ketones, than from disks of plant lines lacking methyl ketones. Both BC-2 and PI 134417 also have much higher densities of type VI trichomes than the other plant lines, thus the reduced walking speed on these lines is likely due in part to the purely physical effects of trichome density (Keller, 1987). However, the methyl ketones very likely play a role in the observed effects of PI 134417 and BC-2 foliage as well. 2-Tridecanone and 2-undecanone are lethal toxins for *T. pretiosum* and direct contact of *T. pretiosum* adults with PI 134417 foliage, or exposure to its foliar volatiles, which are rich in 2-tridecanone (Dimock and Kennedy, 1983), causes some mortality within 4 hr (Kennedy et al., 1991). A comparison of the response to BC-2 and PI 134417 supports this hypothesis. Although there was little difference in type VI trichome densities between these two plant lines, PI 134417 foliage had a much higher concentration of methyl ketones than BC-2 foliage, and the wasps took over twice as long to walk from leaf disks of PI 134417 than from those of BC-2. In addition, entanglement of *T. pretiosum* in type VI trichome tips was significantly greater on PI 134417 than on all other plant lines including BC-2, whereas, although entrapment on BC-2 tended to be greater than on the

TABLE 2. TIME REQUIRED BY *Trichogramma pretiosum* AND *Telenomus sphingis* TO CROSS OR INITIATE FLIGHT FROM TOMATO LEAF DISKS VARYING IN TRICHOME DENSITIES AND METHYL KETONE LEVELS

Treatments	Type VI trichome density (N/mm^2)	Concentration (μg//cm^2)		Time (sec) taken to[a]		Adult wasp entrapped in trichome exudates (%)[c]
		2-Tridecanone	2-Undecanone	Walk-off leaf disk[b]	Initiate flight	
Trichogramma pretiosum						
Better Boy	3.1	0	0	5.8 C	5.0 B	0 A
BCS-9	7.7	0	0	8.3 C	5.9 B	0 A
BCS-10	8.5	0	0	13.6 C	6.7 AB	0 A
F_2-16	9.2	0	0	10.3 C	3.8 B	2.0 A
BC-2	15.2	19.71	5.83	41.5 B	10.4 AB	8.0 A
PI 134417	17.0	31.66	9.42	96.6 A	15.3 A	34.0 B
F value				33.49	3.30	
Treatment df				5	5	
Error df				169	87	
$P > F$				0.0001	0.0089	
Telenomus sphingis						
Better Boy	5.1	0	0	18.2 A	2.2 A	0 A
BCS-9	6.2	0	0	30.6 A	3.4 A	0 A
F_2-16	8.4	0	0	32.7 A	41. A	0 A
BCS-10	10.2	0	0	34.2 A	3.8 A	0 A
BC-2	15.3	31.66	8.98	28.4 A	5.0 A	0 A
PI 134417	23.8	49.64	12.17	31.3 A	6.8 A	0 A
F value				1.72	1.98	
Treatment df				5	5	
Error df				222	65	
$P > F$				0.1319	0.0927	

[a]Means separation vertical by LSD; means with the same letter are not significantly different ($P > 0.05$).
[b]Diameter: 16.3 mm
[c]Means separation vertical by X^2; means with the same letter are not significantly different ($P > 0.05$).

plant lines lacking methyl ketones, the differences were not significant ($P > 0.05$).

Differences among plant lines in the time to initiation of flight by those *T. pretiosum* that did so were significant ($F = 3.30$; treatment $df = 5$; error $df = 87$; $P = 0.0089$) but did not reflect differences in either type VI trichome density or methyl ketone levels (Table 2). Although these wasps tended to take longer to initiate flight from PI 134417 than from any other plant line, the time to initiate flight from PI 134417 did not differ significantly from either BC-2, which also contained methyl ketones, or BCS-10, which did not.

In contrast to *T. pretiosum*, when *T. sphingis* were tested on leaf disks, there were no significant effects of plant line on either the time taken to walk from the leaf disk ($F = 1.72$; $P = 0.1319$; treatment $df = 5$; error $df = 222$), or to initiate flight ($F = 1.98$; $P = 0.0927$; treatment $df = 5$; error $df = 65$) (Table 2). Further, no adult *T. sphingis* were entrapped by the glandular trichomes.

In tests with *T. sphingis* on whole leaflets, the larger test arenas (4.26, 3.89, and 4.35 cm^2 for Better Boy, F_1, and PI 134417, respectively vs. 2.08 cm^2 for leaf disks) provided more opportunity for parasitoid contact with trichomes before the edge of the foliage was reached. A consequence of this was that there was significantly more time spent on PI 134417 and F_1 than on Better Boy foliage and significantly more time spent on PI 134417 than on F_1 foliage (Table 3). Of the time spent on Better Boy foliage, most (78%) was spent walking (searching). In contrast, a significantly lower percentage of the total time spent on PI 134417 and F_1 foliage was spent walking, and a correspondingly greater percentage of time was spent standing still or actively grooming (41% and 57%, respectively, Table 3). During the course of these experiments, we observed that the tip contents of the type VI trichomes on PI 134417, and to a lesser extent F_1, adhered to the appendages (legs, wings, antennae) of *T. sphingis*, hindering its movement and stimulating grooming behavior. In many cases, *T. sphingis* exhibited grooming behavior for prolonged periods (>60 min) after it walked from a leaflet, before it would fly.

Landing rates of *T. sphingis* also were influenced by plant line ($\chi^2 = 6.789$; $P = 0.0336$; $df = 2$). The percentage of observations in which one or more wasps landed within 15 min was lower (69%) on PI 134417 than on F_1 and Better Boy (100% each). The frequency distributions of time (minutes) taken by the first adult *T. sphingis* to land on foliage also were influenced by plant line ($\chi^2 = 19.443$; $P = 0.0035$; $df = 6$) (Figure 1). The distributions on F_1 and Better Boy did not differ significantly ($\chi^2 = 0.2859$; $P = 0.9627$; $df = 3$) but the distribution on PI 134417 differed significantly from those on Better Boy ($\chi^2 = 10.1416$; $P = 0.0174$; $df = 3$) and on F_1 ($\chi^2 = 8.298$; $P = 0.0402$; $df = 3$). In both cases, the time taken for the first wasps to land was greater on PI 134417. The mean areas of leaves used in these tests were 17.25, 15.72,

TABLE 3. PERCENT TIME SPENT WALKING BY *Telenomus sphingis* ON *M. sexta*-RESISTANT AND -SUSCEPTIBLE TOMATO LEAFLETS

Treatment	Trichome density (N/mm^2)	Concentration ($\mu g/cm^2$)		Time spent walking (%)[a,b]	Total time (sec) spent on leaflet[b]
		2-Tridecanone	2-Undecanone		
L. esculentum					
cv. Better Boy	2.9	0	0	78.3 A	156.5 A
F_1	11.6	0	0	57.0 B	1525.0 B
L. hirsutum f. *glabratum*, PI 134417	18.4	34.63	8.51	41.3 C	4952.9 C
F value				17.10	10.84
Treatment *df*				2	2
Error *df*				88	88
$P > F$				0.0001	0.0001

[a] Time spent by adult *Telenomus* on walking or not walking was observed for a 6-min period.
[b] Means separation vertical by LSD; means with the same letter are not significantly different ($P > 0.05$).

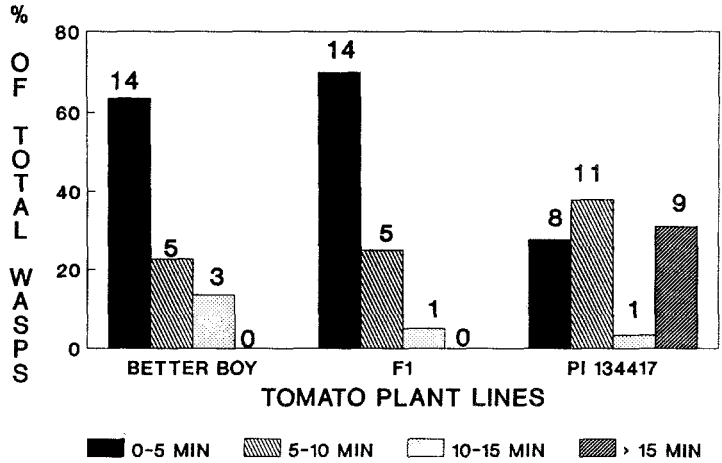


FIG. 1. Frequency distributions of times taken for the first *Telenomus sphingis* adult to land on foliage of Better Boy, F_1, and PI 134417. Numbers above bars indicate the number of wasps in that time interval.

and 14.69 cm^2 for Better Boy, F_1, and PI 134417, respectively. Farrar and Kennedy (unpublished data) found that *T. sphingis* adults avoided parasitizing *M. sexta* eggs in the presence of 2-tridecanone vapors, thus it is possible that the reduced landing rates on PI 134417 foliage observed in this study resulted from an avoidance response to the 2-tridecanone-rich volatiles associated with the foliage (Dimock and Kennedy, 1983), although effects of other foliar volatiles cannot be ruled out.

Results of these experiments help to explain previous observations that parasitism by both *T. pretiosum* and *T. sphingis* is greatly reduced on PI 134417 and related tomato lines possessing glandular trichome and/or 2-tridecanone-mediated resistance to *M. sexta*, relative to levels on susceptible *L. esculentum* cultivars. The type VI trichomes of PI 134417 act to impede movement and physically entrap adult *T. pretiosum*, as has been reported for several other species of small hymenopterans on hirsute plant types (Rabb and Bradley, 1968; Kantanyukul and Thurston, 1973; Elsey and Chaplin, 1978; Shah, 1982). On PI 134417, the adverse effects of type VI trichomes on *T. pretiosum* appear to be enhanced by the 2-tridecanone in their tips.

T. sphingis, which is larger and more robust than *T. pretiosum*, was less drastically affected by high type VI trichome densities and did not become permanently entrapped in their glandular tips. This is consistent with the observations of others that large-bodied parasitoid species were less affected by glandular trichomes on potato and tobacco than small-bodied species (Obrycki and Tauber, 1984; Belcher and Thurston, 1982). However, in the case of *T.*

sphingis, contact with the contents of the type VI trichome tips of both F_1 and PI 134417 prompted prolonged grooming and, hence, reduced searching efficiency. Factors other than the methyl ketones were responsible for stimulating the grooming behavior because the type VI trichomes of F_1 plants contain no detectable quantities of either 2-tridecanone or 2-undecanone. In addition to reduced searching efficiency on the foliage, adult *T. sphingis* also landed on PI 134417 foliage less frequently than on F_1 and Better Boy foliage, possibly in response to the repellent qualities of 2-tridecanone vapors associated with PI 134417 foliage. However, given the presence of other volatile compounds, including 2-undecanone in the trichomes of PI 134417, this repellency cannot be attributed with certainty to 2-tridecanone.

The type VI glandular trichomes that confer or contribute to the resistance of PI 134417 to a number of insect herbivores (Kennedy et al., 1986; Lin et al., 1987) also adversely affect an array of egg and larval parasitoids and predators of lepidopterous species (Kauffman and Kennedy, 1989a,b; Kennedy et al., 1990). A significant portion of the resistance to herbivores and, apparently, of the adverse effects on at least some species of natural enemies is due to the methyl ketones, 2-tridecanone and 2-undecanone, present in the trichome tips. In that a portion of the effects on natural enemies also is due to high densities of type VI trichomes, it may be possible to reduce the effects on natural enemies by selecting for plant lines with lower trichome densities but more 2-tridecanone per trichome tip. However, considering the toxic and/or repellent effects apparently attributable to 2-tridecanone, the effectiveness of this approach may be limited.

Acknowledgments—Research supported by USDA Competitive Grants Program in Biological Stress under grant 88-37252-4017 and by the North Carolina Agricultural Research Service. Use of trade names does not imply endorsement of products named nor criticism of similar ones not mentioned.

We thank M. Woodall for typing; V.B. Covington, D. Bumgarner, and B. O'Brien for technical assistance; L.A. Jones for assistance with gas chromatography; W.H. Swallow for assistance with statistical analyses; and J.R. Bradley, G.C. Rock, and J.F. Walgenbach for comments.

REFERENCES

ALTIERI, M.A., LEWIS, W.J., NORDLUND, D.A., GUELDNER, R.C., and TODD, J.W. 1981. Chemical interactions between plants and *Trichogramma* wasps in Georgia soybean fields. *Prot. Ecol.* 3:259–263.

BELCHER, D.W., and THURSTON, R. 1982. Inhibition of movement of larvae of the convergent lady beetle *Hippodamia convergens* by leaf trichomes of tobacco. *Environ. Entomol.* 11(1):19–94.

BOETHEL, D.J., and EIKENBARY, R.D. (eds.). 1986. Interactions of Plant Resistance and Parasitoids and Predators of Insects. Ellis Horwood, Chichester. 224 pp.

BURTON, R.L. 1970. A low-cost artificial diet for the corn earworm. *J. Econ. Entomol.* 63:1969–1970.

DIMOCK, M.B., and KENNEDY, G.G. 1983. The role of glandular trichomes in the resistance of *Lycopersicon hirsutum* f. *glabratum* to *Heliothis zea*. *Entomol. Exp. Appl.* 33:263–265.

DIMOCK, M.B., KENNEDY, G.G., and WILLIAMS, W.G. 1982. Toxicity studies of analogs of 2-tridecanone, a naturally-occurring toxicant from a wild tomato. *J. Chem. Ecol.* 8:837–842.

ELSEY, K.D., and CHAPLIN, J.F. 1978. Resistance of tobacco introduction 1112 to the tobacco budworm and green peach aphid. *J. Econ. Entomol.* 71:723–725.

FARRAR, R.R., JR., and KENNEDY, G.G. 1987a. Growth, food consumption and mortality of *Heliothis zea* larvae on the foliage of the wild tomato *Lycopersicon hirsutum* f. *glabratum* and the cultivated tomato, *L. esculentum*. *Entomol. Exp. Appl.* 44:213–219.

FARRAR, R.R., JR., and KENNEDY, G.G. 1987b. 2-Undecanone, a constituent of the glandular trichomes of *Lycopersicon hirsutum* f. *glabratum:* Effects on *Heliothis zea* and *Manduca sexta* growth and survival. *Entomol. Exp. Appl.* 43:17–23.

FARRAR, R.R., JR., and KENNEDY, G.G. 1988. 2-Undecanone, a pupal mortality factor in *Heliothis zea:* sensitive larval stage and *in planta* activity in *Lycopersicon hirsutum* f. *glabratum*. *Entomol. Exp. Appl.* 47:205–210.

FERY, R.L., and KENNEDY, G.G. 1987. Genetic analysis of 2-tridecanone concentration, leaf trichome characteristics and tobacco hornworm resistance in *Lycopersicon*. *J. Am. Soc. Hortic. Sci.* 112:886–891.

HULSPAS-JORDAAN, P.M., and VAN LENTEREN, J.C. 1978. The relationship between host-plant leaf structure and parasitization efficiency of the parasitic wasp *Encarsia formosa* Grahm (Hymenoptera: Aphelinidae). *Meded. Rijksfac. Landbouwwet. Gent* 43:431–440.

KANTANYUKUL, W., and THURSTON, R. 1973. Seasonal parasitism and predation of eggs in the tobacco hornworm on various host plants in Kentucky. *Environ. Entomol.* 2:939–945.

KAUFFMAN, W.C., and KENNEDY, G.G. 1989a. Relationship between trichome density in tomato and parasitism of *Heliothis* spp. (Lepidoptera: Noctuidae) eggs by *Trichogramma* spp. (Hymenoptera: Trichogrammatidae). *Environ. Entomol.* 18:698–704.

KAUFFMAN, W.C., and KENNEDY, G.G. 1989b. Inhibition of *Campoletis sonorensis* parasitism of *Heliothis zea* and of parasitoid development by 2-tridecanone-mediated insect resistance of wild tomato. *J. Chem. Ecol.* 15:1919–1930.

KELLER, M. 1987. Influence of leaf surface on movement by the hymenopterous parasitoid, *Trichogramma exiguum*. *Entomol. Exp. Appl.* 43:55–59.

KENNEDY, G.G. 1986. Consequences of modifying biochemically mediated insect resistance in *Lycopersicon* species, pp. 130–141, *in* M.R. Green and P.A. Hedin (eds.). Natural Resistance of Plants to Pests—Roles of Allelochemicals. ACS Symposium Series 296, Washington, D.C. 243 pp.

KENNEDY, G.G., and DIMOCK, M.B. 1983. 2-Tridecanone: A natural toxicant in a wild tomato responsible for insect resistance, pp. 123–128, *in* J. Miyamoto and P.C. Kearney (eds.). Pesticide Chemistry, Human Welfare and the Environment, Vol. 2. Pergamon Press, Tokyo.

KENNEDY, G.G., and FARRAR, R.R., JR. 1987. Response of insecticide-resistance and susceptible Colorado potato beetles *Leptinotarsa decemlineata* to 2-tridecanone and resistant tomato foliage: The absence of cross resistance. *Entomol. Exp. Appl.* 45:187–192.

KENNEDY, G.G., and HENDERSON, W.R. 1978. A laboratory assay for resistance to the tobacco hornworm in *Lycopersicon* and *Solanum* spp. *J. Am. Soc. Hortic. Sci.* 103:334–336.

KENNEDY, G.G., and SORENSON, C.E. 1985. Role of glandular trichomes in the resistance of *Lycopersicon hirsutum* f.*glabratum* to Colorado potato beetle (Coleoptera:Chrysomelidae). *J. Econ. Entomol.* 78:547–551.

KENNEDY, G.G., SORENSON, C.E., and FERY, R.L. 1985. Mechanisms of resistance to Colorado potato beetle in tomato, pp. 107–116, *in* D.N. Ferro and R.H. Voss (eds.). Proceedings of the Symposium on the Colorado Potato Beetle, XVII International Congress of Entomology. Massachusetts Agriculture Experiment Station Research Bulletin 704. 144 pp.

KENNEDY, G.G., NIENHUIS, J., and HELENTJARIS, T. 1986. Mechanisms of arthropod resistance in tomatoes, pp. 145–154, *in* D.J. Nevins and R.A. Jones (eds.). Tomato Biotechnology, Plant Biology, Vol. 4. Alan R. Liss, New York. 339 pp.

KENNEDY, G.G., FARRAR, R.R., JR., and KASHYAP, R.K. 1991. 2-Tridecanone/glandular trichome mediated insect resistance in tomato: Effect on parasitoids and predators of *Heliothis zea*, pp. 150–165, *in* P. Hedin (ed.). Naturally Occurring Pest Bioregulators, ACS Symposium Series. American Chemical Society, Washington, D.C.

LIN, S.Y.H., TRUMBLE, J.T., and KUMAMOTO, J. 1987. Activity of volatile compounds in glandular trichomes of *Lycopersicon* species against two herbivores. *J. Chem. Ecol.* 13:837–850.

LUCKWILL, L.C. 1943. The genus *Lycopersicon:* An historical, biological, and taxonomic survey of the wild and cultivated tomatoes. Aberdeen University Studies No. 120, Aberdeen University Press, Aberdeen, Scotland.

LUNDGREN, L., NORELIUS, G., and STENHAGEN, G. 1985. Leaf volatiles from some wild tomato species. *Nord. J. Bot.* 5:315–320.

OBRYCKI, J.J. 1986. The influence of foliar pubescence on entomophagous species, pp. 61–83, *in* D. Boethal and R.D. Eikenbary (eds.). Interactions of Plant Resistance and Parasitoids and Predators of Insects. Wiley & Sons, New York.

OBRYCKI, J.J., and TAUBER, M.J. 1984. Natural enemy activity on glandular pubescent potato plants in the greenhouse: An unreliable predictor of effects in the field. *Environ. Entomol.* 13:679–683.

RABB, R.L., and BRADLEY, J.R., JR. 1968. The influence of host plants on parasitism of eggs of the tobacco hornworm. *J. Econ. Entomol.* 61(5):1249–1252.

SHAH, M.A. 1982. The influence of plant surfaces on the searching behavior of *Coccinellid* larvae. *Entomol. Exp. Appl.* 31(4):377–380.

SOOST, R.K., SCORA, R.W., and SIMS, J.J. 1968. Contribution to the chromatographic analysis of leaf oils in the genus *Lycopersicon. Proc. Am. Soc. Hortic. Sci.* 92:568–571.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

IDENTIFICATION OF NEW SESQUITERPENOIDS IN CEPHALIC SECRETION OF CUCKOO BEE, *Nomada lathburiana* (APOIDEA, ANTHOPHORIDAE)

W. FRANCKE,[1,*] S. KROHN,[1] and J. TENGÖ[2]

[1]*Department of Organic Chemistry*
Hamburg University
Martin-Luther-King-Platz 6, D-2000 Hamburg, Germany
[2]*Ecological Research Station of Uppsala University*
S-386 00 Färjestaden, Sweden

(Received June 8, 1990, accepted November 14, 1990)

Abstract—A series of new sesquiterpene ketones and norsesquiterpene ketones could be identified from the cephalic secretion of females of the cuckoo bee, *Nomada lathburiana* (K.). The major component proved to be 2,6,10-trimethylundeca-(5*E*)-2,5,9-trien-4-one. Large amounts of high-boiling-point straight-chain hydrocarbons serve as solvents for the volatile, unstable ketones.

Key Words—Bee, *Nomada lathburiana*, nest parasite, cephalic secretion, terpenoids, Hymenoptera, Anthophoridae, cuckoo.

INTRODUCTION

Bees of the genus *Nomada* are nest parasites, "cuckoo bees." The female oviposits in the nests of host bees. The provision, stored in the nest as food for the host brood, is appropriated for the rearing of the parasite larva, which also kills and consumes the host larva (Stephen et al., 1969).

A correspondence in odor production has been shown to occur in the relationships between several *Nomada* species and their hosts (Tengö and Bergström, 1977). In parasite–host pairs involving *Nomada* and species of the genus *Andrena*, the compounds in common are farnesyl hexanoate or geranyl octanoate (Tengö and Bergström, 1975), while in *Nomada–Melitta* pairs octadecyl

*To whom correspondence should be addressed.

0098-0331/91/0300-0557$06.50/0

butyrate is shared (Tengö and Bergström, 1976). In the host these compounds are produced in the females' Dufour's gland, while males of the kleptoparasite secrete them from cephalic glands. The host female utilizes the Dufour's gland secretion in lining the brood cell (Hefetz, 1987, and references therein). In addition, similar to *Evylaeus* species (Hefetz et al., 1986), the volatile components contribute to nest odor specificity (Tengö, unpublished). It has been suggested (Tengö and Bergström, 1977), that the odoriferous secretion of the parasite, on the other hand, is transferred from the male to the female during copulation, as is indicated by the presence of farnesyl hexanoate on the cuticula of the mated *Nomada* female thorax (Tengö and Bergström, 1977). The biological significance of 3,7-dimethyldeca-2,6-dien-1,10-diol, which was identified as a male-specific component of the mandibular gland secretion of *Nomada annulata* (Duffield et al., 1990) is yet unknown. The role of this bishomoterpene, which was previously known from the androconial organs of danaid butterflies (Meinwald et al., 1969), in host–parasite interactions has been discussed.

The *Nomada* females emit a sex-specific odor from cephalic glands. They seem to be territorial, defending host nest(s), which are a resource for oviposition (Tengö, 1984). Aggressive fights, comprising face-to-face flights, head-to-head butts, and pounces (Linsley and MacSwain, 1955; Tengö, 1984) occur between competing females outside the host nests. It is likely that female volatile compounds produced in cephalic glands play a role in these combats, since the characteristic smell, actively emitted by the *Nomada* females, is particularly intense during the antagonistic encounters between conspecific females (Tengö, unpublished). Preliminary investigations revealed the female cephalic odor to be species specific (Tengö and Bergström, unpublished).

The objective of the present study was to identify the volatile compounds emitted by females of the species *Nomada lathburiana* (K.).

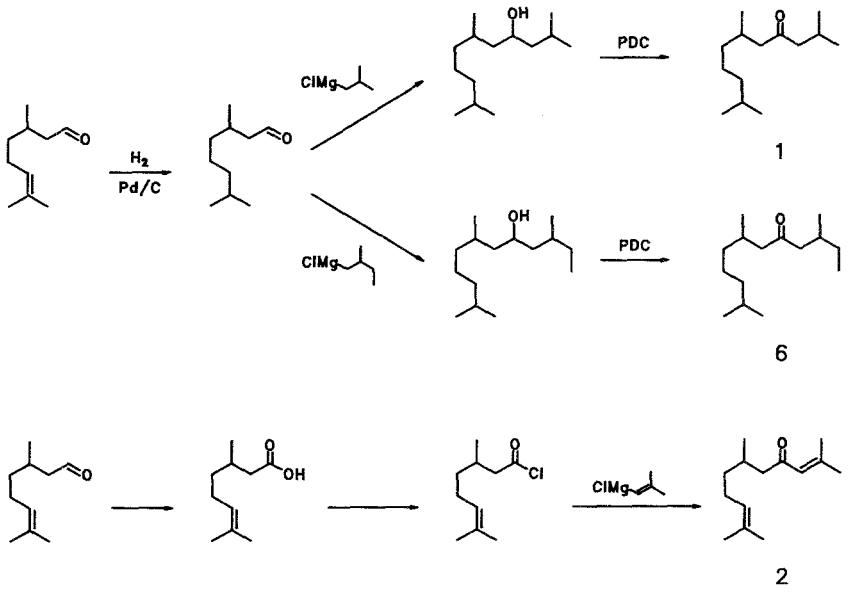
METHODS AND MATERIALS

Bees were collected during April and May in Sweden, outside Vallentuna, north of Stockholm. After being killed on Dry Ice in the field, the animals were decapitated. Subsequently, the mandibles were loosened from their attachments and, together with the heads, extracted with pentane. The extracts were concentrated in microvials (Klimetzek et al., 1989) and submitted to chemical analyses.

Gas chromatographic separation was carried out on a Carlo Erba Fractovap 2101 AC using fused silica columns (50 m, 0.25 mm ID) coated with WG 11 or FFAP, programmed from 60 to 200°C at a rate of 3°C/min. For identification of single components, the gas chromatograph was coupled to a Finnigan 311 A mass spectrometer, operated at 70 eV. Structural proof of natural com-

pounds was achieved by comparison of retention times and spectroscopic data with those of synthetic reference samples.

Syntheses of the new terpenoides are outlined in Schemes 1–3. Compounds **1, 6,** and **2** were prepared by Grignard coupling (Scheme 1). Dihydrocitronellal, 3,7-dimethyloctanal, obtained by hydrogenation (hexane, 5% PD/C) of citronellal, was reacted with 2-methylpropyl magnesium chloride (diethylether, usual procedure, reflux) to yield 2,6,10-trimethylundecan-4-ol or, with the Grignard reagent of 2-methylbutyl chloride, to yield 3,7,11-trimethyldodecan-5-ol. These secondary alcohols were oxidized according to the method of Corey and Smith (1979) to the respective ketones **1** and **6**. For the synthesis of compound **3**, citronellal was oxidized to citronellic acid (Corey and Smith, 1979), which was subsequently converted to citronellyl chloride following the method of Barnard and Bateman (1950). To get the target ketone 3, a Grignard reagent was prepared (THF, 50°C, N) from magnesium and 1-bromo-2-methylpropene (Braude and Evans, 1955) and, during an inverse procedure, added to the citronellic acid chloride (THF, −78°C, N). After complete addition, the reaction mixture was stirred at room temperature for 12 hr before work up in the usual manner. [^{1}H]NMR (C_6D_6) of **1:** 0.75–0.90 (15H); 0.95–1.30 (6H); 1.40–1.55 (m, 1H); 1.83–1.98 (3H); 2.0–2.2 (3H). [^{1}H]NMR (C_6D_6) of **6:** 0.8–0.92 (15H); 1.05–1.40 (8H); 1.45–1.52 (m, 1H); 1.85–2.04 (2H); 2.13–2.23 (2H); 2.3–2.41 (2H). [^{1}H]NMR (C_6D_6) of **2:** 0.90 (d, J = 7.3 Hz, 3H); 1.01–1.45 (m, 3H);

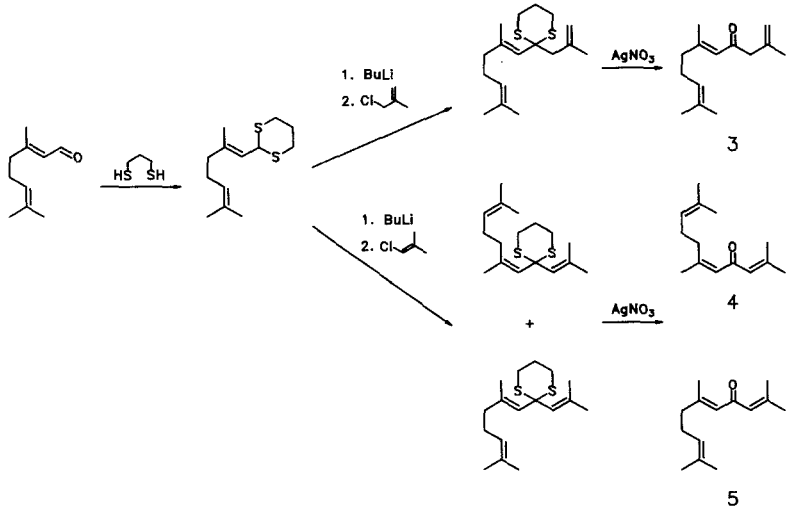


SCHEME 1. Syntheses of compounds **1, 6,** and **2.**

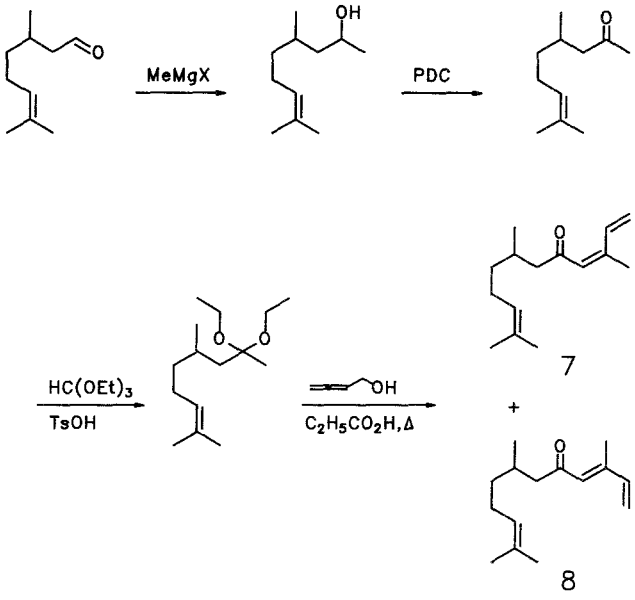
1.47 (s, 3H); 1.67 (d, J = 1.1 Hz, 3H); 1.85–2.32 (m, 4H); 2.15 (d, J = 1.1 Hz, 3H); 5.2 (t, J = 5.4 Hz, 1H); 5.46 (d, J = 1.1 Hz, 1H).

The syntheses of compounds **3, 4,** and **5** were carried out by Corey-Seebach coupling (Seebach, 1969) of the 1,3-dithiane derivative of geranial (Scheme 2). Similar to the method of Hoppmann and Weyerstahl (1978), the dithiane was dissolved in THF, cooled to −40°C, deprotonated with *n*-butyl lithium, held for 5 hr at −20°C, again cooled to −40°C, and subsequently coupled to 1-bromo-2-methyl-2-propene. The resulting 2,2-disubstituted 1,3-dithiane was converted to the corresponding ketone **3** by decomposition with silver nitrate in ethanol following the procedure of Reece et al. (1968). In the same way as described above, a mixture of the *E, Z* isomers of 2,6,10-trimethyl-2,5,9-undecatrien-4-one, **4** and **5**, was prepared from the anion of the dithiane derivative of geranial and 1-bromo-2-methyl-1-propene. [^{1}H]MNR (C_6D_6) of **3:** 1.46 (s, 3H); 1.6 (s, 3H); 1.68 (d, J = 1.1 Hz, 3H); 1.84–1.94 (m, 2H), 2.10 (t, 2H); 2.16 (d, J = 1.2 Hz, 3H); 2.94 (s, 2H); 4.74 (s, 1H); 4.84 (s, 1H); 5.06 (t, J = 5.6 Hz, 1H); 5.98 (s, 1H). [^{1}H]NMR (C_6D_6) of **4:** 1.48 (s, 3H); 1.54 (d, 3H); 1.62 (d, J = 1.1 Hz, 3H); 1.90–2.01 (m, 2H); 2.06 (t, 2H); 2.15 (d, 3H); 2.21 (d, J = 1.1 Hz, 3H); 5.06 (t, J = 5.6 Hz, 1H); 5.90 (d, J = 1.1 Hz, 1H); 5.97 (d, J = 1.1 Hz, 1H). [^{1}H]NMR (C_6D_6) of **5:** 1.48 (s, 3H); 1.54 (d, 3H); 1.62 (d, J = 1.1 Hz, 3H); 1.90–2.01 (m, 2H); 2.06 (t, 2H); 2.21 (d, J = 1.1 Hz, 3H); 2.28 (d, J = 1.1 Hz, 3H); 5.06 (t, J = 5.6 Hz, 1H); 5.93 (d, J = 1.1 Hz, 1H); 5.97 (d, J = 1.1 Hz, 1H).

The synthesis of the terpenes **7** and **8** (Scheme 3) started from citronellal



SCHEME 2. Syntheses of compounds **3, 4,** and **5.**



SCHEME 3. Syntheses of compounds **7** and **8.**

and proceeded via 2,2-diethoxy-4,8-dimethyl-7-nonene. In a manner resembling the synthesis of monoterpenes bearing a vinyl ketone moiety (Bäckström et al., 1987), the latter acetal was reacted with 2,4-pentadien-1-ol to yield **7** and **8** in a Claisen rearrangement. [^{1}H]NMR (C_6D_6) of **8:** 0.9 (d, J = 0.58 Hz, 3H); 1.56 (s, 3H); 1.68 (s, 3H); 1.9–2.5 (m, 7H); 2.27 (s, 3H), 5.12 (d, J = 10.6 Hz, 1H); 5.18 (t, J = 5.3 Hz, 1H); 5.32 (d, J = 17.4 Hz, 1H); 5.89 (s, 1H); 6.15 (dd, J = 10.6 Hz, J = 17.4 Hz, 1H).

Crude products were purified either by HPLC (Waters; Whatman column; 0.5 × 9.4 mm ID; Partisil 10 Pac, hexane–ethyl acetate) or by preparative gas chromatography (Carlo Erba 2400V; stainless-steel column 6 m × 6 mm ID; 10% OV17 on chromosorb G/AWDMCS; 60–80 mesh; helium).

RESULTS AND DISCUSSION

The cephalic secretion of *Nomada lathburiana* females is a complex mixture of minute amounts of some new mevalogenins, largely dominated by acetogenines, which are represented by straight-chain hydrocarbons. The latter contain uneven numbers of carbon atoms; the series starts at nonadecane and shows increasing concentration to tricosane, after which it decreases. The identified terpenoids are listed in Table 1; chemical structures are shown in Figure 1;

TABLE 1. SESQUITERPENOIDS IN CEPALIC SECRETION OF FEMALE *Nomada lathburiana*

1. 2,6,10-Trimethylundecan-4-one
2. 2,6,10-Trimethylundecan-2,9-dien-4-one
3. 2,6,10-Trimethylundecan-(5*E*)-1,5,9-trien-4-one
4. 2,6,10-Trimethylundecan-(5*Z*)-2,5,9-trien-4-one
5. 2,6,10-Trimethylundecan-(5*E*)-2,5,9-trien-4-one
6. 3,7,11-Trimethyldodecan-5-one
7. 3,7,11-Trimethyldodecan-(3*Z*)-1,3,10-trien-5-one
8. 3,7,11-Trimethyldodecan-(3*E*)-1,3,10-trien-5-one

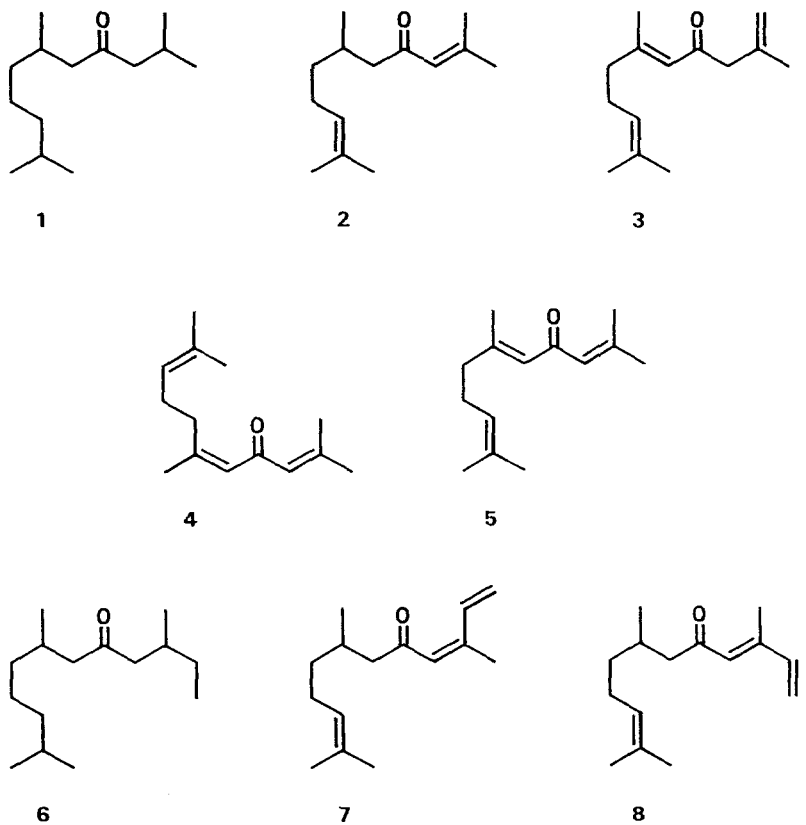


FIG. 1. Structural formulas of sesquiterpenoids and norsesquiterpenoids occurring in the cephalic secretion of *Nomada lathburiana* females.

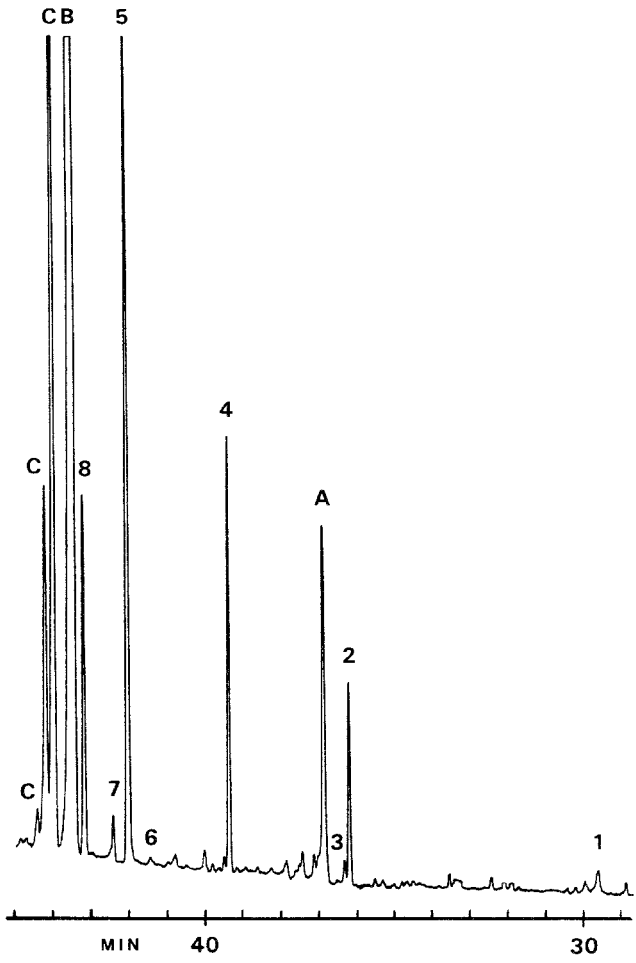


FIG. 2. Gas chromatogram of the terpenoid region of *Nomada lathburiana* female cephalic secretion, numbering according to Table 1 A = nonadecane, B = heneicosane, C = heneicosene (conditions, see text).

relative proportions can be seen in the gas chromatogram (Figure 2). Numbering in the table and in the figures are the same. Mass spectra of the major terpenoids **2, 4, 5,** and **8** are shown in Figure 3.

The characteristic strong odor, perceivable by the human nose, is represented by oxygenated sesquiterpenoids. The components are acyclic, showing a typical branching pattern and a carbonyl group at an unusual position. The vinylketone moiety present in six of these rather volatile compounds renders them highly unstable. Attempts to determine the absolute configurations of the chiral components are presently under way.

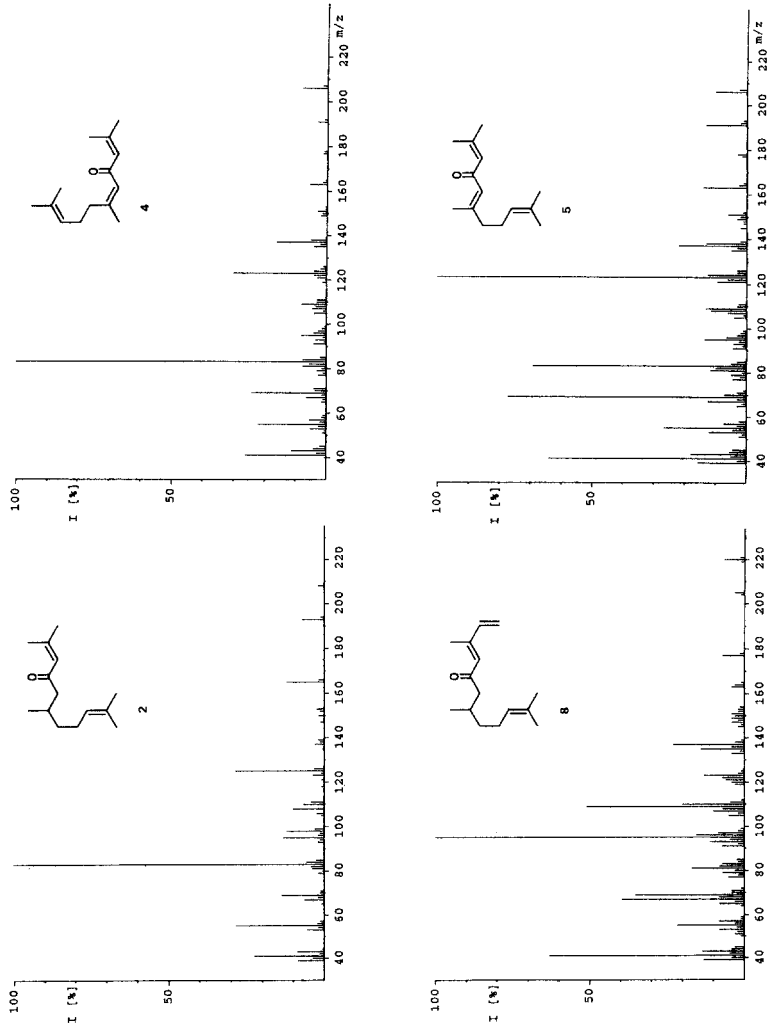


FIG. 3. Mass spectra (70 eV) of compounds **2**, **4**, **5**, and **8**.

Compounds **4** and **5** correspond to the known 2-methylpseudodamascone (Schulte-Elte et al., 1975), which may give an idea of the rose-like character of the intense smell produced by the *Nomada* females.

As for the biological function of the compounds, one may speculate that electrophilic vinylketones, which represent ideal Michael acceptors, are used as repellents or defense compounds. In some species of *Ips* bark beetles, 2-methyl-6-methylene-2,7-octadien-4-one (ipsdienone), which shows the same vinylketone substructure as some of the *Nomada* ketones reported here, acts as a strong repellent (J.P. Vité, U. Kohnle, W. Francke, unpublished). It is interesting to note that several unbranched vinylketones of acetogenetic origin are reported as defense compounds of termites (Spanton and Prestwich, 1982). Another vinylketone, 1-nonen-3-one, is the main compound in the cephalic secretion of females of the cleptoparasitic megachilid bee, *Coelioxys mandibularis* Nyl. (Tengö et al., 1982); however, its biological function remains unknown.

Acknowledgments—The authors wish to thank Yngve Sjögren for assisting in collecting the bees, and the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the Swedish Natural Research Council for financial support.

REFERENCES

BÄCKSTRÖM, P., STRIDH, K., LI, L., and NORIN, T. 1987. Claisen rearrangements with mesityl oxid dimethyl ketal. Synthesis of ipsdienone, *E*-and *Z*-ocimenone, 2,6-dimethyl-2,7-octadien-4-one and 2,6-dimethyl-2,7-octadien-4-ol. *Acta Chem. Scand. B.* 41:442–447.

BARNARD, D., and BATEMAN, L. 1950. Experiments relating to the synthesis of homogeranic acid. *J. Chem. Soc.* pp. 926–932.

BRAUDE, E.A., and EVANS, E.A. 1955. Alkenylation with lithium alkenyls. Part IX. Synthesis of dimethylbutadiene carboxylic acid. *J. Chem. Soc.* pp. 3325–3331.

COREY, E.J., and SMITH, G. 1979. Useful procedures for the oxidation of alkohols involving pyridinium dichromate in aprotic media. *Tetrahedron Lett.* pp. 399–402.

DUFFIELD, R.M., SIMON-JORDAN , C., RIDDICK, E.W., and WHEELER, J.W. 1990. Exocrine secretions of bees. X. 3,7-Dimethyldeca-2,6-dien-1,10-diol: A sex-specific compound from *Nomada annulata* (Hymenoptera: Anthophoridae). *J. Chem. Ecol.* 16:1069–1076.

HEFETZ, A. 1987. The role of Dufour's gland secretions in bees. *Physiol. Entomol.* 12:243–253.

HEFETZ, A., BERGSTRÖM, G., and TENGÖ, J. 1986. Species, individual and kin specific blends in Dufour's gland secretions of halictine bees. Chemical evidence. *J. Chem. Ecol.* 12:197–208.

HOPPMANN, A., and WEYERSTAHL, P. 1978. Terpene und Terpen-Derivate. VII: Terpene aus C_5- und C_{10}-Bausteinen durch Alkylierung von 1,3-Dithianen. *Tetrahedron* 34:1723–1728.

KLIMETZEK, D., KAUOHLER, J., KROHN, S., and FRANCKE, W. 1989. Das Pheromon-System des Waldreben-Borkenkäfers, *Xylocleptes bispinus* Duft. (Col., Scolytidae). *J. Appl. Entomol.* 107:304–309.

LINSLEY, E.G., and MACSWAIN, J.W. 1955. The habits of *Nomada opacella* Timberlake with notes on other species (Hymenoptera: Anthophoridae). *Wasmann J. Biol.* 13:253–276.

MEINWALD, J., MEINWALD, Y.C., and MAZZOCCHI, P.H. 1969. Sex pheromone of the queen butterfly: Chemistry. *Science* 164:1174–1175.

REECE, C.A., RODIN, J.O., BROWNLEE, R.G., DUNCAN, W.G., and SILVERSTEIN, R.M. 1968. Synthesis of the principle components of the sex attractant from male *Ips confusus* frass: 2-Methyl-6-methylene-7-octen-4-ol, 2-methyl-6-methylene-2,7-octadiene-4-ol and (+)-*cis* verbenol. *Tetrahedron* 24:4249–4256.

SCHULTE-ELTE, K.-H., STRICKLER, H., GAUTCHI, F., PICKENHAGEN, W., GADOLA, M., LIMACHER, J., MÜLLER, B., WUFFLI, F., and OHLOFF, G. 1975. Synthesen und Cyclisierungsreaktionen der Pseudodamascone. *Liebig Ann. Chem.* pp. 484–508.

SEEBACH, D. 1969. Nucleophile Acylierung mit 2-Lithium-1,3-dithianen bzw.1,3,5-Trithianen. *Synthesis* pp. 17–36.

SPANTON, S.G., and PRESTWICH, G.D. 1982. Chemical defence and self-defence. *Tetrahedron* 38:1921–1930.

STEPHEN, W.P., BOHART, G.E., and TORCHIO, P.F. 1969. The biology and external morphology of the bees. Agr. Exp. Stn., Oregon State University., Corvallis. 140 pp.

TENGÖ, J. 1984. Territorial behavior of the kleptoparasite reduces parasitic pressure in communally nesting bees. XVII Int. Congress of Entomology, Hamburg. p. 510.

TENGÖ, J., and BERGSTRÖM, G. 1975. All-*trans*-farnesyl hexanoate and geranyl octanoate in the Dufour's gland secretion of *Andrena* (Hymenoptera: Apidae). *J. Chem. Ecol.* 1:253–268.

TENGÖ, J., and BERGSTRÖM, G. 1976. Odor correspondence between *Melitta* females and males of their nest parasite *Nomada flavopicta* K. (Hymenoptera: Apoidea). *J. Chem. Ecol.* 2:57–65.

TENGÖM, J., and BERGSTRÖM, G. 1977. Cleptoparasitism and odor mimetism in bees: Do *Nomada* males imitate the odor of *Andrena* females? *Science* 196:1117–1119.

TENGÖ, J., BERGSTRÖM, G., BORG-KARLSON, A.-K., GROTH, I., and FRANCKE, W. 1982. Volatile compounds from cephalic secretions of females in two cleptoparasitic bee genera, *Epeolus* (Hym., Anthophoridae) and *Coelioxys* (Hym., Megachilidae). *Z. Naturforsch.* 37c:376–380.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

1-OCTEN-3-OL, ATTRACTIVE SEMIOCHEMICAL FOR FOREIGN GRAIN BEETLE, *Ahasverus advena* (WALTL) (COLEOPTERA: CUCUJIDAE)[1]

A.M. PIERCE,[2,*] H.D. PIERCE, JR.,[2] A.C. OEHLSCHLAGER,[2] and J.H. BORDEN[3]

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

(Received November 28, 1989; accepted November 16, 1990)

Abstract—Volatiles were captured on Porapak Q from foreign grain beetles, *Ahasverus advena* (Waltl), feeding on rolled oats at various population densities. At low population density, males, females, and mixed-sex beetles four to six weeks posteclosion and older produced 1-octen-3-ol. Mixed-sex beetles emitted almost pure (*R*)-(−) enantiomer. Weekly production rates of 1-octen-3-ol by males were at least four times greater than those of females. Production of 1-octen-3-ol was barely detectable in volatiles from mixed-sex adults maintained at the highest population density. Laboratory bioassays in a two-choice, pitfall olfactometer modified to retain responding beetles revealed that 1-octen-3-ol serves as an aggregation pheromone for *A. advena*. Both racemic and chiral 1-octen-3-ols were good attractants for mixed-sex adults in the pitfall olfactometer.

Key Words—*Ahasverus advena* (Waltl), foreign grain beetle, Coleoptera, Cucujidae, 1-octen-3-ol, volatile attractant, aggregation pheromone, population density.

INTRODUCTION

The foreign grain beetle, *Ahasverus advena* (Waltl), is a cosmopolitan pest of stored products. Primarily fungivorous, *A. advena* usually infests damp and

*To whom correspondence should be addressed.
[1]Research supported by the Natural Sciences and Engineering Research Council of Canada, Strategic Grant G0958 and Operating Grants A3881 and A3785.

0098-0331/91/0300-0567$06.50/0

decaying, mold-damaged cereals, oilseeds, and other stored food (Sinha and Watters, 1985). The insect develops well on pure cultures of common storage fungi (David et al., 1974), and larvae and adults exhibit elevated levels of chitinolytic enzymes, indicating that fungal chitin can be utilized as a food source (Fukamizo et al., 1985). There are observations, however, confirming direct feeding on foods in the absence of visible mold (Woodroffe, 1962).

Identification of attractive semiochemicals for *A. advena* could lead to the development of integrated control programs (Levinson and Levinson, 1979; Burkholder, 1981). To date, we have found that males of six species of economically damaging, cucujid grain beetles produce aggregation pheromones (Oehlschlager et al., 1988). An additional aggregation pheromone, 1-octen-3-ol, was found to be produced by males and females of two of these species, *Oryzaephilus surinamensis* (L.) and *O. mercator* (Fauvel), when adults were maintained at low population densities in aeration cultures (A.M. Pierce et al., 1989).

The objective of the present study was to identify beetle-produced, attractive semiochemical(s) for adult *A. advena*.

METHODS AND MATERIALS

Insect Rearing. A. advena were reared at 28°C and 65–70% relative humidity in darkness. Stock cultures were set up in 3.8-liter glass jars containing 1 kg of large-flake, rolled oats and brewer's yeast (95:5, w/w) with an inoculum of 2500 adults. The medium initially had been sprayed with 80 ml of distilled water and mixed well. Numbers of adults were determined by mean weight (1 beetle = 0.42 mg, $N = 1600$).

Beetles for Aerations. Beetles were sexed as pupae by examination of the genital papillae (Halstead, 1963) using the following procedure. Late-stage larvae were removed from stock cultures and placed individually in 3.7-ml shell vials each containing a moistened oat flake. The open vials were kept at 28°C and 65–70% relative humidity in darkness until pupation occurred. Prior to sexing, pupae were placed on several layers of moistened filter paper in covered, 150-mm-diam. glass Petri dishes to loosen the posterior attachment of the last larval exuvia. After 30 min at approx. 24°C, the softened exuvia was gently removed from the tip of the abdomen with a fine brush and the sex determined. The segregated sexes were returned to jars of moistened rolled oats at approximately the same densities as used in aerations. Unless stated otherwise, for mixed-sex aerations, late-stage larvae were transferred directly to jars of moistened oats at the appropriate density. The beetles were kept at 28°C and 65–70% relative humidity until used in aerations as adults.

Collection of Volatiles. Volatiles from male, female, or mixed-sex beetles

were obtained by aeration of adults in vertically oriented, cylindrical glass chambers (18 cm × 10 cm OD) containing 50 g of large-flake, rolled oats (H.D. Pierce et al., 1984). Charcoal-filtered, humidified air was drawn by aspiration (1.65 liters/min) through a culture (held at 23–24°C in darkness) and then through a glass trap (150 mm × 14 mm OD) filled with conditioned Porapak Q (H.D. Pierce et al., 1984). At intervals of seven days, the culture was removed from the aeration chamber, the beetles separated from the oats and returned to a clean chamber with a fresh 50-g portion of oats, and the aeration continued using a fresh Porapak Q trap. Volatiles were recovered by extraction of the Porapak Q with purified pentane in a Soxhlet extractor for 24 hr and concentrated by distillation of the pentane through a Dufton column.

Two experiments were conducted using mixed-sex and single-sex beetles, respectively. For mixed-sex beetles, simultaneous aerations were set up one each at population densities of 2, 5, 50, and 200 beetles/g oats, along with a rolled oat control. The aeration with 2 beetles/g oats used virgin males and virgin females, which were combined in equal amounts immediately prior to commencement of aeration. For single-sex beetles, simultaneous aerations were set up with segregated virgin males and virgin females at a population density of 5 beetles/g oats, along with a rolled oat control.

The amounts of volatiles collected were expressed as beetle hours (bh) or gram hours (gh) where 1 bh = the volatiles from one adult aerated for 1 hr, and gh = the volatiles from 1 g of rolled oats aerated for 1 hr.

Moisture Content of Aeration Medium. At the time of medium change for aeration cultures, a 25- to 30-g sample of used medium was placed in an open, 100-mm-diam. glass Petri dish, immediately weighed, and dried to constant weight in a small convection oven at 50–52°C for one week. Moisture content of the unused, rolled oat medium was periodically checked in the same manner.

Test Beetles for Bioassay. Mixed-sex *A. advena* were harvested from stock cultures and placed in fresh medium at a density of approx. 10,000 beetles/kg at least one week before bioassays commenced. Test beetles were 5–12 weeks posteclosion at the time of bioassay. Approximately 48 hr prior to a bioassay, 0.8 g of beetles were removed from the culture and preconditioned without food at 23°C in darkness in a 6-liter Erlenmeyer flask through which charcoal-filtered, humidified air was drawn at 1.5 liters/min.

Bioassay Procedures. Several hours prior to a bioassay, 12 conditioned beetles were aspirated into each of up to 96, 60-ml glass vials. Unless stated otherwise, bioassays of pentane solutions were conducted using a two-choice, pitfall olfactometer (A.M. Pierce et al., 1981) in which oat flakes were placed individually in the experimental and control vials. The vials of test beetles and vials for the olfactometers then were placed in an environmental chamber maintained at 23°C and 60–65% relative humidity for approx. 2 hr before the bioassay commenced. Filter paper disks treated with a 10-μl aliquot of either an

experimental stimulus in purified pentane or purified pentane as a control were put singly into the bottoms (on top of the oat flake) of two glass vials suspended from holes in the bottom of a plastic Petri dish arena. Twelve beetles were released into the dish, and the lid was replaced. Bioassays for each test stimulus were replicated 10 or 12 times, using 12 fresh beetles in each replicate. After 2 hr in darkness, the numbers of beetles in experimental and control vials were recorded. All doses for a particular test stimulus were done in the same bioassay session, beginning with 12 replicates of the lowest dose and proceeding in sequence to the highest dose. The responsiveness of the test beetles was checked by bioassay of a standard low dose of Porapak Q-captured beetle volatiles at the start of a bioassay session.

In the initial bioassay session, response by *A. advena* in the two-choice, pitfall olfactometer was determined at selected time intervals by counting the number of beetles in the experimental and control vials. Counting the beetles in the vials necessitated picking up and slightly tilting the olfactometer dish. This process did not disturb the responding beetles if done gently. The experimental stimuli for this bioassay were a measure of frass from a mature stock culture and a pentane extract thereof. The latter was prepared by placing 2.5 g of frass in a sintered glass funnel. Approximately 10 ml of pentane was added, and the mixture was stirred with a spatula. The solvent then was drawn off by application of a slight vacuum. This process was repeated three times. The combined pentane washes were concentrated under a stream of nitrogen to 1 ml.

The untransformed data were analyzed by the paired-sample t test, $\alpha = 0.05$ (Zar, 1984), and results were expressed as the mean percent response of the total number of beetles per treatment.

Alcohols. Racemic 1-octen-3-ol and (*Z*)-3-nonenol were purchased from Bedoukian Research, Inc. (Danbury, Connecticut). (*R*)-(−)- and (*S*)-(+)-1-Octen-3-ol with optical purities of 98% and 99.5%, respectively, were obtained from Dr. A. Mosandl (Mosandl et al., 1986). 1-Nonanol was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin). (*Z*) -2-Octenol was available from a previous study (Oehlschlager et al., 1984). Chemical purities were >99% in all cases.

The enantiomeric excess (ee) and chirality of beetle-produced 1-octen-3-ol were determined by the method of Slessor et al. (1985).

Instrumental Methods. A Hewlett-Packard 5830A gas chromatograph equipped with a 18835B capillary inlet system and a flame-ionization detector was employed for analyses by gas chromatography (GC). Samples were analyzed on an open-tubular glass column (40 m × 0.5 mm ID) coated with SP-1000 (Supelco Canada Ltd., Oakville, Ontario). The temperature program for analytical GC was 70°C for 2 min, then 4°C/min to 180°C, holding for 30 min or less. Helium was the carrier gas, and the injection port and detector temper-

atures were 260°C and 270°C, respectively. Quantification of 1-octen-3-ol in purified pentane was conducted using methyl laurate as an internal standard.

Coupled gas chromatography–mass spectroscopy (GC-MS) was carried out on a Hewlett-Packard 5895B GC/MS-DS fitted with a fused silica column (30 m × 0.25 mm ID) coated with Carbowax 20M (J&W Scientific, Folsom, California) with helium as the carrier gas.

RESULTS

Aerations. When aerated in low-density aeration cultures, mixed-sex and single-sex *A. advena* produced 1-octen-3-ol (Figures 1 and 2). The highest rate of production of 1-octen-3-ol was found for mixed-sex beetles at the lowest population density of 2 beetles/g oats, followed next in this order by male, mixed-sex, and female beetles each at a density of 5 beetles/g oats (Figure 1). 1-Octen-3-ol was not produced in significant amounts by mixed- or single-sex

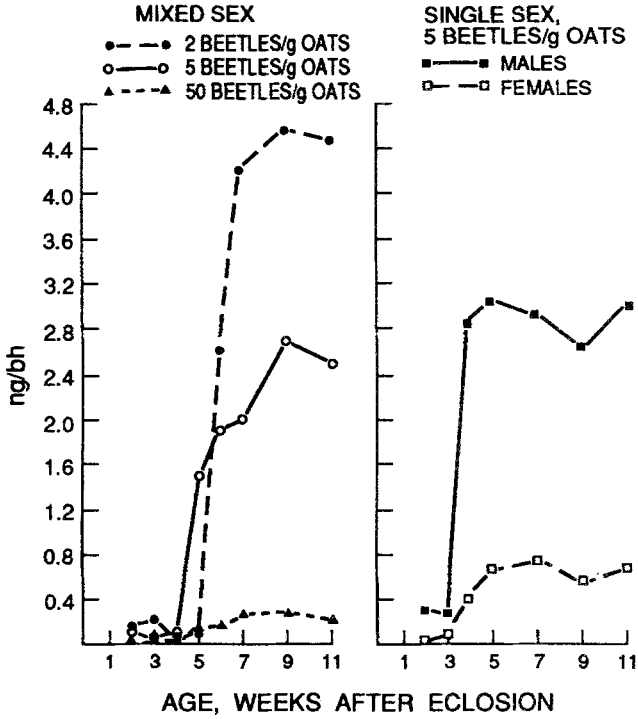


FIG. 1. Production of 1-octen-3-ol by *A. advena* in mixed- and single-sex aeration cultures as a function of adult age and population density.

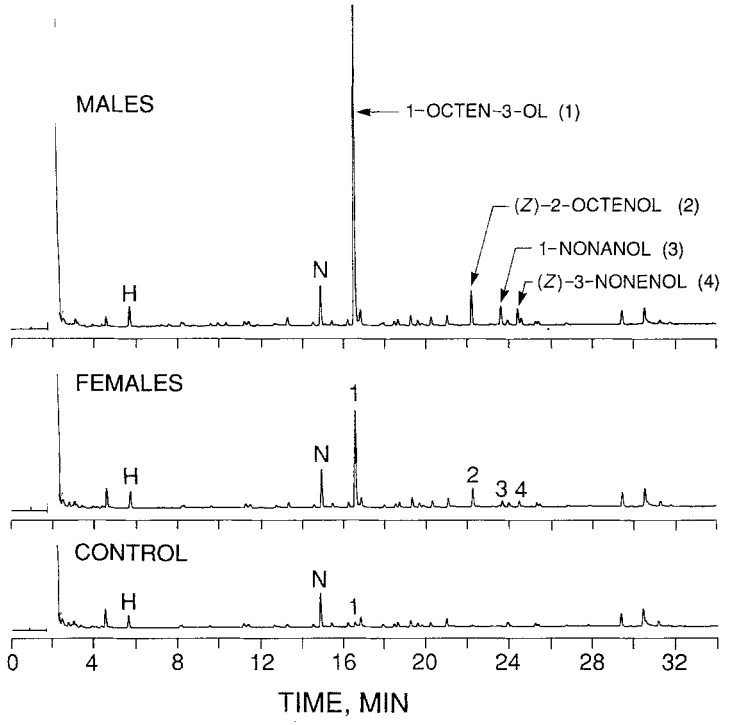


FIG. 2. Gas chromatograms of Porapak Q-captured volatiles from male and female *A. advena* (population density, 5 beetles/g oats) and the rolled oat control. Each chromatogram represents approximately the same amount of rolled oat volatiles. H = hexanal; N = nonanal.

beetles until four to six weeks posteclosion. Mixed-sex beetles at a density of 50 beetles/g oats produced low levels of 1-octen-3-ol at five weeks posteclosion and older. The elevated production rates of the alcohol at all of the above population densities were maintained throughout the 11 weeks of the experiments. Production of 1-octen-3-ol by mixed-sex adults at a density of 200 beetles/g oats was only slightly greater than the control values and ranged from 0.002 to 0.01 ng/bh. The amount of 1-octen-3-ol detected in the rolled oat control aerations was always <0.3 ng/gh.

Male and female *A. advena* produced small amounts of three additional alcohols, (Z)-2-octenol, 1-nonanol, and (Z)-3-nonenol, when production of 1-octen-3-ol was well established (Figure 2). These compounds were detected only when 1-octen-3-ol was present in significant amounts in beetle aeration volatiles. When expressed as a percent of the amount of 1-octen-3-ol present in these volatiles, the amounts of (Z)-2-octenol, 1-nonanol, and (Z)-3-nonenol

ranged from 4.1 to 11.4, 2.7 to 7.0, and 2.1 to 5.8% for males, and 9.7 to 18.7, 5.9 to 7.8, and 7.1 to 7.4% for females, respectively. The identities of the four alcohols were confirmed by GC-MS using authentic standards for comparison. Examination of the chromatograms from the beetle and rolled oat control aerations revealed no additional peaks in the beetle-aeration volatiles other than the four alcohols (Figure 2).

Mixed-sex *A. advena* aerated at a population density of 5 beetles/g rolled oats produced 1-octen-3-ol in the (*R*)-(−) configuration with an ee of 92%.

The mean moisture contents of the used aeration media were ≤10.7% for the single-sex aerations and controls, and ≤13.5% for the mixed-sex aerations and controls (Table 1). The moisture contents of the control media were similar to that of the respective single-sex or mixed-sex media. The moisture content of the unused medium was lower than that of any of the used aeration media.

Bioassays. The suitability of the two-choice, pitfall olfactometer for the identification of attractive, volatile semiochemicals for *A. advena* was determined by testing frass from mature stock cultures (Figures 3 and 4). Response to a measure of frass was quickly positive, and this response was maintained throughout the 2-hr test period (Figure 3). Response to an extract of frass was positive initially, but beetles immediately started climbing out of the test vials until no significant response was observed by 1–2 hr after the start of the bioassay (Figure 4). The addition of an oat flake in each pitfall vial retained the responding beetles in the vial of first choice until the end of the 2-hr test period (Figure 4), and this modification was used in all further bioassays.

To establish if the volatiles from low-density, older beetles contained

TABLE 1. MOISTURE CONTENT OF ROLLED OAT AERATION MEDIA (8 REPLICATES)

Culture type	Culture density (beetles/g medium)	Moisture content (%)	
		$\bar{X}$	Range
Unused medium		6.4	5.8–7.3
Male	5	10.0	9.0–10.9
Female	5	10.6	9.6–11.2
Single-sex control		10.7	9.9–12.2
Mixed-sex	2	13.1	12.5–13.6
Mixed-sex	5	13.4	12.8–13.9
Mixed-sex	50	13.5	12.6–13.9
Mixed-sex	200	12.9	12.1–13.4
Mixed-sex control		13.5	13.1–13.9

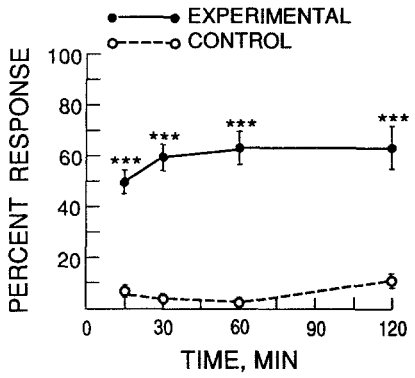


FIG. 3. Time-dependent response ($\bar{X} \pm$ SE) by mixed-sex *A. advena* in two-choice, pitfall bioassay to a 15-mg measure of frass (control = empty vial). Significant response (paired-sample *t* test) to experimental stimulus indicated by: ***$P < 0.001$ (10 replicates, 12 adults per replicate).

attractive semiochemicals, the Porapak Q-captured volatiles from mixed-sex and control aerations were bioassayed over a range of stimulus doses containing the same gh amounts of rolled oat volatiles (8.65–8650 gh) for both extracts (Figure 5). Mixed-sex *A. advena* responded well to beetle-aeration volatiles containing 1 ng or more of 1-octen-3-ol (Figure 5). Attraction to the rolled oat control, however, was obtained only at the highest test dose containing 2.6 ng of 1-octen-3-ol (Figure 5), suggesting that this alcohol was a major, beetle-produced attractant. Based on the shapes of the curves in Figure 5, the beetle-aeration volatiles were no better as an attractant than the rolled oat control at test doses containing similar, low amounts of 1-octen-3-ol.

Chiral and racemic 1-octen-3-ols were bioassayed at doses of 0.1 ng to 100 μg (Figure 6). The lower threshold for positive response for both (*R*)-(−)- and racemic 1-octen-3-ol was around 1 ng, while significant positive response was first demonstrated towards the (*S*)-(+) enantiomer at a 10 ng dose. Thus, all three 1-octen-3-ols were excellent attractants for mixed-sex *A. advena* over the experimental dose range of 10 ng to 10 μg, while the 100 μg doses elicited no significant response.

When (Z)-2-octenol, 1-nonanol, or (Z) -3-nonenol were bioassayed individually over an experimental dose range of 0.1 ng to 10 μg, no significant response was elicited from mixed-sex *A. advena* (data not shown). Preliminary bioassays of four-part mixtures of (Z)-2-octenol, 1-nonanol, (Z)-3-nonenol, and (*R*)-(−)-1-octen-3-ol showed no enhancement of the positive response to 1-octen-3-ol by the three minor alcohols (data not shown).

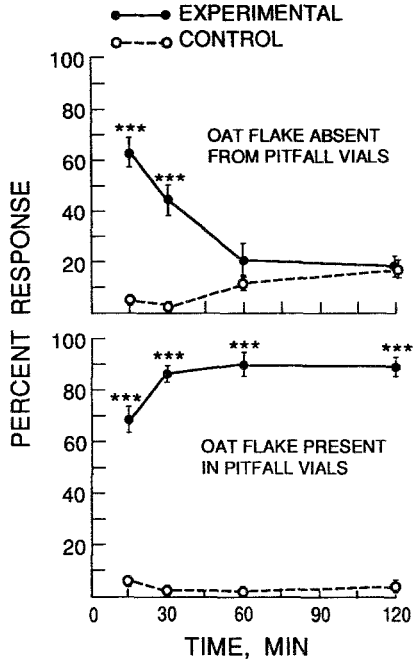


FIG. 4. Time-dependent response ($\bar{X} \pm$ SE) by mixed-sex *A. advena* in two-choice, pitfall bioassay to a pentane extract of 25 mg of frass, with oat flake absent from (top) or present in (bottom) experimental and control vials. Significant response (paired-sample *t* test) to experimental stimulus indicated by: ***$P < 0.001$ (10 replicates per treatment, 12 adults per replicate). Bioassays in this figure and in Figure 3 were performed in the same 2-hr session.

DISCUSSION

A. advena are excellent climbers of glass. In pitfall vials without oat flakes we determined that if, after initially responding to a volatile stimulus, the beetles found no host- and/or beetle-related stimuli, they climbed out of the vials, resulting in no significant response at the end of the 2-hr bioassay (Figure 4). The oat flake evidently provided host-related stimuli (possibly thigmotactic) that served to retain the responding beetles (Figure 4), as did frass from mature cultures containing visible mold (Figure 3). The same modification of the two-choice bioassay was necessary for *C. quadricollis* (H.D. Pierce et al., 1988). As suggested for *C. quadricollis*, volatile attractants might function not only to draw responding insects to an attractive source, but also to stimulate other behaviors such as searching for food and mates. If the secondary stimuli are not found, the responding insect may leave the site (H.D. Pierce et al., 1988).

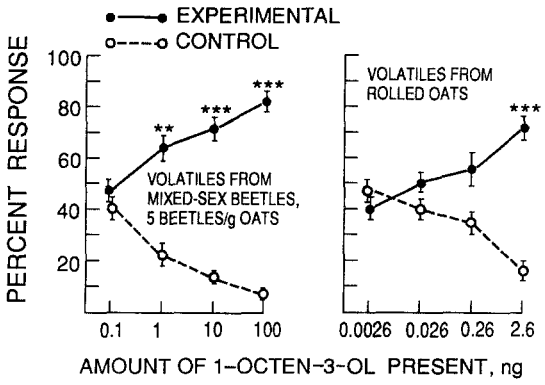


FIG. 5. Response ($\bar{X} \pm$ SE) by mixed-sex *A. advena* in two-choice, pitfall bioassay to Porapak Q-captured volatiles from (left) older, mixed-sex *A. advena* feeding on rolled oats at a population density of 5 beetles/g oats, and (right) response to the rolled oat control volatiles. The experimental stimuli in both series of treatments contained the same gh amounts of rolled oat volatiles. Significant response to experimental stimulus indicated by: $^{**}P < 0.01$, $^{***}P < 0.001$ (12 replicates per treatment, 12 adults per replicate). Bioassays in this figure were performed in the same 2-hr session.

(*Z*)-2-Octenol has been identified in the volatiles from mold (Kaminski et al., 1972) and mushrooms (Tressl et al., 1982). This alcohol also was found in low amounts in the volatiles from low-density *O. surinamensis* and *O. mercator* cultures of both sexes (A.M. Pierce et al., 1989). Although not attractive to *A. advena* or *O. mercator* (A.M. Pierce et al., 1989), (*Z*)-2-octenol was a weak attractant for *O. surinamensis* (A.M. Pierce et al., 1989).

As did *A. advena*, male and female *O. surinamensis* and *O. mercator* older than one month posteclosion produced large amounts of almost optically pure (*R*)-(−)-1-octen-3-ol when maintained in low-density aeration cultures (A.M. Pierce et al., 1989). As in the present study, both enantiomers of the alcohol were attractive to both *Oryzaephilus* spp. (A.M. Pierce et al., 1989). *O. surinamensis* is a primary pest of stored grain (Sinha and Watters, 1985). *A. advena*, as a secondary pest of stored grain, could be attracted to damaged grain stores lightly infested with *O. surinamensis* since both species produce and utilize 1-octen-3-ol.

1-Octen-3-ol is a well-known natural product produced in large amount by numerous species of molds (Kaminski et al., 1974) and mushrooms (Pyysalo, 1976). Mushroom-produced 1-octen-3-ol was found to be the (*R*)-(−) enantiomer (Dijkstra and Wikén, 1976). Since 1-octen-3-ol is known to be formed when grain is contaminated by molds (Kaminski et al., 1973), the alcohol may play a major role in the attraction of *A. advena* to deteriorating grain stores.

Our results suggest that 1-octen-3-ol is an aggregation pheromone, as well

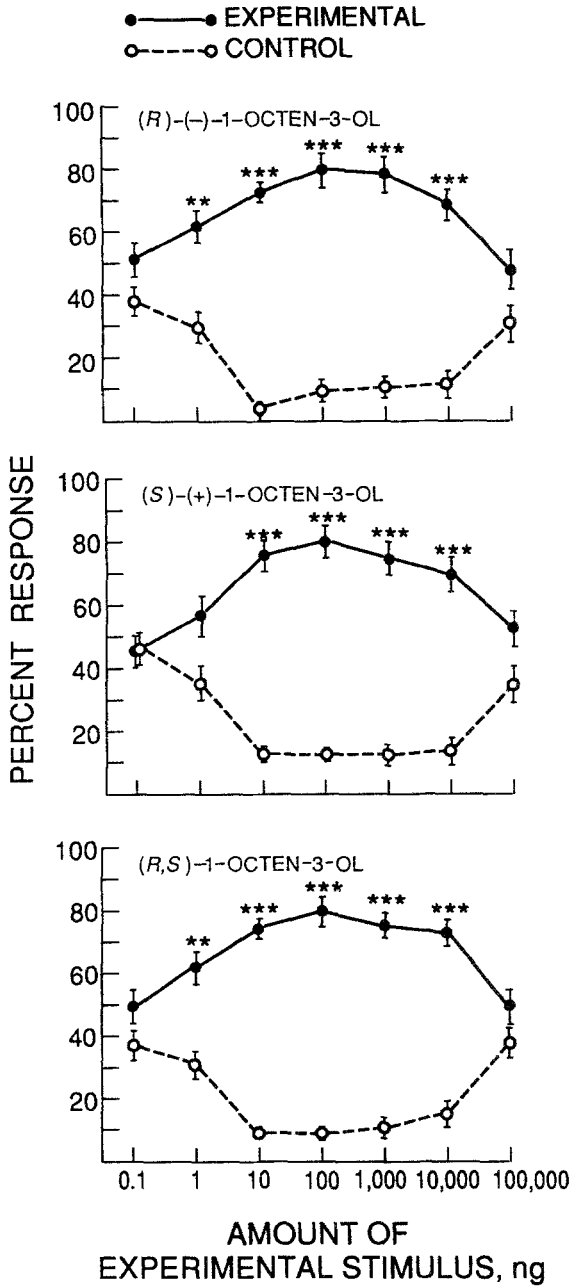


FIG. 6. Response ($\bar{X} \pm$ SE) by mixed-sex *A. advena* in two-choice, pitfall bioassay to (*R*)-(−)-, (*S*)-(+)-, and (*R*,*S*)-1-octen-3-ol. Significant response (paired-sample *t* test) to experimental stimulus indicated by: $^{**}P < 0.01$, $^{***}P < 0.001$ (12 replicates per treatment, 12 adults per replicate).

as a host kairomone, for *A. advena*, although the alcohol might be produced by associated symbiotic microorganisms. Symbiotic microorganisms have been implicated in pheromone biosynthesis for some bark beetles (Brand et al., 1976; Byers and Wood, 1981), and a fungal metabolite was identified as the wing gland pheromone of the moth, *Aphomia sociella* L. (Kunesch et al., 1987). A logical precursor of 1-octen-3-ol is linoleic acid (Wurzenberger and Grosch, 1982), which is found in abundance in the rolled oats fed to *A. advena*.

In contrast to the other cucujids studied to date (Oehlschlager et al., 1988), no other male-produced aggregation pheromones have been found for *A. advena*. 1-Octen-3-ol apparently was not derived in large amounts from the rolled oat medium since only trace amounts were detected in control aerations, and the moisture content of the medium both before and after aeration was too low for effective microbial growth (Milner et al., 1947). Had 1-octen-3-ol been produced primarily by fungal contamination of oats, there should have been equal or greater amounts produced as culture density increased. Rather, production of 1-octen-3-ol decreased as culture density increased (Figure 1). Moreover, production rates by males were at least four times greater than those by females. As found for *Oryzaephilus* spp. (A.M. Pierce et al., 1989), the onset of production of significant amounts of 1-octen-3-ol at the lower population densities occurred in older adults. The data indicate, therefore, that 1-octen-3-ol is an aggregation pheromone for *A. advena*, which functions to draw beetles into a lightly infested host. When population density is high, some unknown feedback mechanism evidently suppresses pheromone production (Figure 1), ensuring against overcrowding.

Acknowledgments—We thank Dr. A. Mosandl for gifts of the chiral 1-octen-3-ols and E. Merletti for technical assistance.

REFERENCES

BRAND, J.M., BRACKE, J.W., BRITTON, L.N., MARKOVETZ, A.J., and BARRAS, S.J. 1976. Bark beetle pheromones: production of verbenone by a mycangial fungus of *Dendroctonus frontalis*. *J. Chem. Ecol.* 2:195–199.

BURKHOLDER, W.E. 1981. Biomonitoring for stored-product insects, pp. 29–40, *in* E.R. Mitchell (ed.). Management of Insect Pests with Semiochemicals. Plenum, New York.

BYERS, J.A., and WOOD, D.L. 1981. Antibiotic-induced inhibition of pheromone synthesis in a bark beetle. *Science* 213:763–764.

DAVID, M.H., MILLS, R.B., and SAUER, D.B. 1974. Development and oviposition of *Ahasverus advena* (Waltl) (Coleoptera, Silvanidae) on seven species of fungi. *J. Stored Prod. Res.* 10:17–22.

DIJKSTRA, F.Y., and WIKÉN, T.O. 1976. Studies on mushroom flavours 1. Organoleptic significance of constituents of the cultivated mushroom, *Agaricus bisporus*. *Z. Lebensm. Unters.-Forsch.* 160:255–262.

FUKAMIZO, T., SPEIRS, R.D., and KRAMER, K.J. 1985. Comparative biochemistry of mycophagous and non-mycophagous grain beetles. Chitinolytic activities of foreign and sawtoothed grain beetles. *Comp. Biochem. Physiol.* 81B:207–209.

HALSTEAD, D.G.H. 1963. External sex differences in stored-products Coleoptera. *Bull. Entomol. Res.* 54:119–134.

KAMINSKI, E., LIBBEY, L.M., STAWICKI, S., and WASOWICZ, E. 1972. Identification of the predominant volatile compounds produced by *Aspergillus flavus*. *Appl. Microbiol.* 24:721–726.

KAMINSKI, E., STAWICKI, S., WASOWICZ, E., and PRZYBYLSKI, R. 1973. Detection of deterioration of grain by gas chromatography. *Ann. Technol. Agric.* 22:401–407.

KAMINSKI, E., STAWICKI, S., and WASOWICZ, E. 1974. Volatile flavor compounds produced by molds of *Aspergillus, Penicillium*, and *Fungi imperfecti*. *Appl. Microbiol.* 27:1001–1004.

KUNESCH, G., ZAGATTI, P., POUVREAU, A., and CASSINI, R. 1987. A fungal metabolite as the male wing gland pheromone of the bumble-bee wax moth, *Aphomia sociella* L. *Z. Naturforsch.* 42c:657–659.

LEVINSON, H.Z., and LEVINSON, A.R. 1979. Trapping of storage insects by sex and food attractants as a tool of integrated control, pp. 327–341, *in* F.J. Ritter (ed.). Chemical Ecology: Odour communication in Animals. Elsevier/North-Holland Biomedical Press, Amsterdam.

MILNER, M., CHRISTENSEN, C.M., and GEDDES, W.F. 1947. Grain storage studies. VI. Wheat respiration in relation to moisture content, mold growth, chemical deterioration and heating. *Cereal Chem.* 24:182–199.

MOSANDL, A., HEUSINGER, G., and GESSENER, M. 1986. Analytical and sensory differentiation of 1-octen-3-ol enantiomers. *J. Agric. Food Chem.* 34:119–122.

OEHLSCHLAGER, A.C., MISHRA, P., and DHAMI, S. 1984. Metal catalyzed rearrangements of allylic esters. *Can. J. Chem.* 62:791–797.

OEHLSCHLAGER, A.C., PIERCE, A.M., PIERCE, H.D., JR., and BORDEN, J.H. 1988. Chemical communication in cucujid grain beetles. *J. Chem. Ecol.* 14:2071–2098.

PIERCE, A.M., BORDEN, J.H., and OEHLSCHLAGER, A.C. 1981. Olfactory response to beetle-produced volatiles and host-food attractants by *Oryzaephilus surinamensis* and *O. mercator*. *Can. J. Zool.* 59:1980–1990.

PIERCE, A.M., PIERCE, H.D., JR., BORDEN, J.H., and OEHLSCHLAGER, A.C. 1989. Production dynamics of cucujolide pheromones and identification of 1-octen-3-ol as a new aggregation pheromone for *Oryzaephilus surinamensis* and *O. mercator* (Coleoptera: Cucujidae). *Environ. Entomol.* 18:747–755.

PIERCE, H.D., JR., PIERCE, A.M., MILLAR, J.G., WONG, J.W., VERIGIN, V.G., OEHLSCHLAGER, A.C., and BORDEN J.H. 1984. Methodology for isolation and analysis of aggregation pheromones in the genera *Cryptolestes* and *Oryzaephilus* (Coleoptera: Cucujidae), pp. 121–137, *in* Proceedings of the Third International Working Conference on Stored-Product Entomology. Kansas State University, Manhattan, Kansas.

PIERCE, H.D., JR., PIERCE, A.M., JOHNSTON, B.D., OEHLSCHLAGER, A.C., and BORDEN, J.H. 1988. Aggregation pheromone of square-necked grain beetle, *Cathartus quadricollis* (Guér.) *J. Chem. Ecol.* 14:2169–2184.

PYYSALO, H. 1976. Identification of volatile compounds in seven edible fresh mushrooms. *Acta Chem. Scand. B.* 30:235–244.

SINHA, R.N., and WATTERS, F.L. 1985. Insect Pests of Flour Mills, Grain Elevators, and Feed Mills and their Control. Research Branch Agriculture Canada, Publication 1776.

SLESSOR, K.N., KING, G.G.S., MILLER, D.R., WINSTON, M.L., and CUTFORTH, T.L. 1985. Determination of chirality of alcohol or latent alcohol semiochemicals in individual insects. *J. Chem. Ecol.* 11:1659–1667.

TRESSL, R.D., BAHRI, D., and ENGEL, K.-H. 1982. Formation of eight-carbon and ten-carbon components in mushrooms (*Agaricus campestris*). *J. Agric. Food. Chem.* 30:89–93.

WOODROFFE, G.E., 1962. The status of the foreign grain bettle, *Ahasverus advena* (Waltl) (Col., Silvanidae), as a pest of stored products. *Bull. Entomol. Res.* 53:537–540.

WURZENBERGER, M. and GROSCH, W. 1982. The enzymic oxidative breakdown of linoleic acid in mushrooms (*Psalliota bispora*). *Z. Lebensm. Unters.-Forsch.* 175:186–190.

ZAR, J.H. 1984. Biostatistical Analysis, 2nd ed. Prentice-Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

FUNGAL VOLATILES: SEMIOCHEMICALS FOR STORED-PRODUCT BEETLES (COLEOPTERA: CUCUJIDAE)[1]

A.M. PIERCE,[2,*] H.D. PIERCE, JR.,[2] J.H. BORDEN,[3] and A.C. OEHLSCHLAGER[2]

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

(Received November 28, 1989, accepted November 16, 1990)

Abstract—Responses by five species of cucujid grain beetles (mixed-sex adults) to various volatiles were assessed by means of a two-choice, pitfall olfactometer. The test volatiles were short-chain alcohols and ketones known to be produced by fungi. Both racemic and chiral 1-octen-3-ols were strong attractants for *Cryptolestes ferrugineus* (Stephens), as had been found previously for *Oryzaephilus surinamensis* (*L.*), *O. mercator* (Fauvel), and *Ahasverus advena* (Waltl). 3-Methylbutanol was another good attractant for these four cucujids, and it was the only test compound to which *Cathartus quadricollis* (Guér.) responded positively. 1-Octen-3-one, racemic 3-octanol, and 3-octanone showed various degrees of attractiveness for the former four species of cucujids. *O. surinamensis* was the only species of test beetle to show much positive response to 2-phenylethanol and ethanol. For *O. mercator* and *O. surinamensis*, 3-methylbutanol enhanced positive response to their respective cucujolide aggregation pheromones.

Key Words—Coleoptera, Cucujidae, grain beetles, *Oryzaephilus surinamensis* (L.), *Oryzaephilus mercator* (Fauvel), *Cryptolestes ferrugineus* (Stephens), *Ahasverus advena* (Waltl), *Cathartus quadricollis* (Guér.), fungal volatiles, attractant semiochemicals.

*To whom correspondence should be addressed.
[1]Research supported by the Natural Sciences and Engineering Research Council of Canada, Strategic Grant G0958 and Operating Grants A3881 and A3785.

0098-0331/91/0300-0581$06.50/0

INTRODUCTION

Many species of stored-product arthropods are found in damp, deteriorating grain and stored food in close association with fungi and bacteria (Sinha and Wallace, 1966; Sinha and Watters, 1985). Various cucujid grain beetles and other storage arthropods are known to feed and reproduce on some species of fungi associated with grain (Sinha, 1968).

There is evidence that fungal-derived volatiles may serve as attractants for storage pests (Vanhaelen et al., 1979). In laboratory tests, adult *C. ferrugineus* were attracted to volatiles from grain-borne fungi (Dolinski and Loschiavo, 1973). *Oryzaephilus surinamensis* (L.) and *O. mercator* (Fauvel) responded positively to volatiles from fungal-infested frass in laboratory bioassays (A.M. Pierce et al., 1981). Unpublished analyses of these frass volatiles by coupled gas chromatography-mass spectroscopy revealed the presence of 1-octen-3-ol, 3-octanone, 3-octanol, 2-phenylethanol, and 3-methylbutanol.

We have identified male-produced aggregation pheromones (cucujolides) for six species of the economically important Cucujidae (Oehlschlager et al., 1988). Preliminary studies of trapping cucujids from stored grain, however, suggested that trap effectiveness might be improved with the addition of host-produced volatiles to the pheromone baits (Javer et al., 1990).

Our objective here was to test various short-chain alcohols and ketones, known to be produced by fungi, as potential attractants for five species of cucujids. The test beetles were the sawtoothed grain beetle, *O. surinamensis;* merchant grain beetle, *O. mercator;* rusty grain beetle, *C. ferrugineus;* foreign grain beetle, *Ahasverus advena* (Waltl); and squarenecked grain beetle, *Cathartus quadricollis* (Guér.).

METHODS AND MATERIALS

Insect Rearing. Beetles were reared on large-flake, rolled oats and brewer's yeast (95:5, w/w) in 3.8-liter glass jars at 28°C and 60–70% relative humidity in darkness. For *A. advena* and *C. ferrugineus*, the medium initially had been moistened with distilled water and mixed well.

Alcohols and Ketones for Bioassay. Racemic 3-octanol and 3-octanone were purchased from Chem Samples Co. (Columbus, Ohio). 3-Methylbutanol was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin) and 2-phenylethanol from Matheson Coleman & Bell (East Rutherford, New Jersey). Racemic 1-octen-3-ol was purchased from Bedoukian Research, Inc. (Danbury, Connecticut). (*R*)-(−)- and (*S*)-(+)-1-Octen-3-ol with optical purities of 98% and 99.5%, respectively, were obtained from Dr. A. Mosandl (Institut f. Pharmazie u. Lebensmittelchemie, 8700 Würzburg, Germany). 1-Octen-3-one was prepared by oxidation of 1-octen-3-ol with active MnO_2 in pentane and

was 99.4% pure after purification by gas chromatography (GC). Reagent grade ethanol was distilled through a Dufton column before use. All compounds were checked by GC before use and found to be ≥99% pure.

Cucujolides for Bioassay. Macrocyclic lactones used for bioassay were racemic 3(Z)-dodecen-11-olide (cucujolide II), racemic 3(Z),6(Z)-dodecadien-11-olide (IV), 3(Z),6(Z)-dodecadienolide (IX), and racemic 5(Z),8(Z)-tetradecadien-13-olide (V) (Oehlschlager et al., 1983, 1988; Millar and Oehlschlager, 1984).

Instrumental Methods. A Hewlett-packard 5830A gas chromatograph equipped with a 18835B capillary inlet system and a flame-ionization detector was employed for analyses by GC. Helium was the carrier gas, and the injection port and detector temperatures were 260°C and 270°C, respectively. Samples were analyzed on an open-tubular glass column (40 m × 0.5 mm ID) coated with SP-1000 (Supelco Canada Ltd., Oakville, Ontario). The temperature program for analytical GC was 70°C for 2 min, then 4°C/min to 180°C and holding. A Varian 1700 gas chromatograph fitted with a stainless-steel column (3.05 m × 6.4 mm OD) packed with 25% Carbowax 20 M on Chromosorb A (60–80 mesh) was used for preparative GC at 145°C.

Test Beetles for Bioassay. At least one week before bioassays commenced, mixed-sex adults were harvested from stock cultures and placed in fresh medium at a density of approx. 10,000 beetles/kg for *A. advena* and *C. quadricollis*, and 15,000–20,000 beetles/kg for *O. mercator* and *C. ferrugineus*. Since response by *O. surinamensis* to olfactory stimuli is extremely sensitive to population density (A.M. Pierce et al., 1983), test beetles were maintained at a low density of 1000 beetles/kg medium for at least one week before bioassays began.

Test beetles were used in bioassays 5–12 weeks after eclosion. Prior to a bioassay, up to 1500 *A. advena, C. ferrugineus, C. quadricollis*, or *O. surinamensis* were preconditioned without food at 23°C in darkness in a 6-liter Erlenmeyer flask through which charcoal-filtered, humidified air was drawn at 1.5 liters/min. This preconditioning, which reduced variability in olfactory responses, was carried out for 72 hr for *C. ferrugineus* and 48 hr for the other three species listed above. For *O. mercator*, sufficient preconditioning was achieved by holding each replicate of 12 beetles in a 60-ml glass vial without food for 20 hr at 23°C in darkness prior to a bioassay.

Bioassay Procedures. Bioassays were done at 23°C and 60–65% relative humidity. A two-choice, pitfall olfactometer (A.M. Pierce et al., 1981) was used to test attractiveness of experimental stimuli. Filter paper disks treated with a 10-μl aliquot of either an experimental stimulus in purified pentane or purified pentane as a control were put singly into the bottoms of two glass vials suspended from holes in the bottom of a plastic Petri dish arena. For *C. quadricollis* and *A. advena*, which are excellent climbers of glass, the bioassay was

modified by first placing an oat flake in the bottoms of the experimental and control vials to retain the responding beetles in the vial of first choice (H.D. Pierce et al., 1988; A.M. Pierce et al., 1991). Twelve beetles were released into the dish, and the lid was replaced. Bioassays for each test stimulus were replicated 12 times, using 12 fresh beetles in each replicate. After 2 hr, the numbers of beetles in experimental and control vials were recorded. Unless indicated otherwise, all doses for a particular test compound were done in the same bioassay session for a particular species of beetle, beginning with 12 replicates of the lowest dose and proceeding in sequence to the highest dose. The responsiveness of the test beetles was checked by bioassaying a standard, low dose of Porapak Q-captured beetle volatiles (H.D. Pierce et al., 1984) at the start of a bioassay session.

1-Octen-3-one, racemic 3-octanol, 3-octanone, 3-methylbutanol, 2-phenylethanol, and ethanol were tested over a range of doses from 0.1 or 1 ng to 100 μg or 1 mg for all five species. Chiral and racemic 1-octen-3-ols were bioassayed over a range of doses from 0.1 ng to 100 μg only for *C. ferrugineus*, since olfactory responses to the 1-octen-3-ols by the other four species are reported elsewhere (see Discussion). For *O. surinamensis* and *O. mercator*, experiments were conducted to test the response to 3-methylbutanol, the respective cucujolide aggregation pheromones, and mixtures thereof to determine if response to the pheromones could be enhanced by the addition of the alcohol. 3- Methylbutanol was used in these latter experiments since preliminary bioassays (Figure 5 below) indicated that this alcohol was a good attractant for *Oryzaephilus* spp.

The untransformed data were analyzed by the paired-sample t test (Zar, 1984), and the results expressed as the mean percent response of the total number of beetles per treatment. For some experiments, the differences between experimental and control values (untransformed) were analyzed by a t test for unpaired data. For these latter experiments, results were expressed as mean percent response = 100 (experimental − control)/N where experimental and control were the number of beetles in the vials containing the experimental and control stimuli, respectively, and N was the total number of beetles released into the dishes.

RESULTS

Individual Alcohols and Ketones. Both chiral and racemic forms of 1-octen-3-ol were similar, excellent attractants for *C. ferrugineus* at doses ranging from 1 ng to 10 μg, while the 100-μg doses were highly repulsive (Figure 1). Olfactory responses to chiral and racemic 1- octen-3-ols by *O. mercator, O. surinamensis, A. advena*, and *C. quadricollis* are reported elsewhere (A.M. Pierce et al., 1989, 1991; H.D. Pierce et al., 1988).

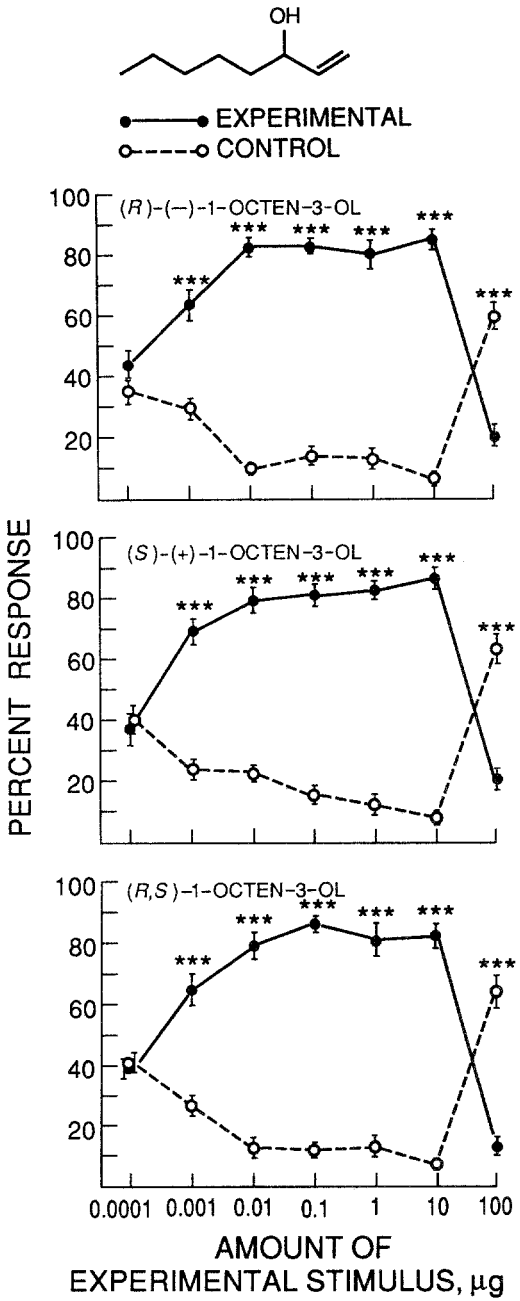


FIG. 1. Response ($\bar{X} \pm$ SE) by mixed-sex *C. ferrugineus* in two-choice, pitfall bioassay to (*R*)-(−)-, (*S*)-(+)-, and (*R*, *S*)-1-octen-3-ol. Significant response (paired-sample *t* test) to experimental stimulus indicated by: $***P < 0.001$ (12 replicates per treatment, 12 adults per replicate).

Positive responses were elicited from all species to 1-octen-3-one except from *C. quadricollis* (Figure 2). Of the four positively responding species, *O. mercator* had the lowest threshold for significant attraction at a 10-ng dose. For the four positive responders, the range of attractive doses was 100- or 1000-fold; at the 100 μg dose, positive response was either diminished or negative response was observed. The two highest doses of 1-octen-3-one, 10 μg and 100 μg, elicited negative response from *C. quadricollis*.

Racemic 3-octanol was an attractant for all species except *C. quadricollis* (Figure 3). Of the four positive responders, *A. advena* had the lowest threshold for positive response at the 10-ng dose and was positively attracted over the largest range of doses of 1000-fold. *C. quadricollis* was strongly repelled by doses of 1 μg and greater, while the other four species were repelled by a 100-μg dose.

The corresponding saturated ketone, 3-octanone (Figure 4), was a potent attractant for *C. ferrugineus* with high attraction at a 10-ng dose and a 10,000-fold range of attractive doses. *O. mercator* and *A. advena* also responded positively, but with a response threshold of 100 ng. Very slight positive response was elicited from *O. surinamensis*. *C. quadricollis* was strongly repelled by doses of 100 μg and 1 mg, and the other four species were repelled by the 1-mg dose.

3-Methylbutanol elicited significant positive responses from all five species at threshold doses of 1–10 ng (Figure 5). Positive attraction extended to the 10-μg dose for *O. surinamensis* and *A. advena*, and at least to 100 μg for *O. mercator* and *C. ferrugineus;* higher doses were not tested. Positive response by *C. quadricollis* declined at 10 μg of the alcohol.

O. surinamensis was the only species of the five tested to show much positive response to 2-phenylethanol (Figure 6). Except for *O. mercator*, all species were repelled by large amounts of this alcohol.

O. surinamensis was attracted to ethanol over an experimental range of 10 ng to 1 mg (Figure 7); higher doses were not tested. *O. mercator* and *A. advena* responded positively to 10 μg and 100 μg of ethanol, whereas no significant response was demonstrated by *C. ferrugineus* or *C. quadricollis* over a test range of 10 ng to 1 mg (data not shown).

3-Methylbutanol and Cucujolide Mixtures. Table 1 shows the response by *O. mercator* to 3-methylbutanol, cucujolides II + IV, and mixtures thereof. Cucujolides II + IV is the blend of macrolide aggregation pheromones produced by *O. mercator* (Oehlschlager et al., 1988). Positive response to 0.4 ng of II + IV was obtained with the addition of 1 ng of 3-methylbutanol. Positive response to 4 ng of II + IV was enhanced with the addition of 10 ng of the alcohol. No significant enhancement of response to the cucujolides was obtained by the addition of larger amounts of 3-methylbutanol in experiments 3, 4, 5, and 6 (Table 1). When 1 mg of 3-methylbutanol was added to 40 μg of II +

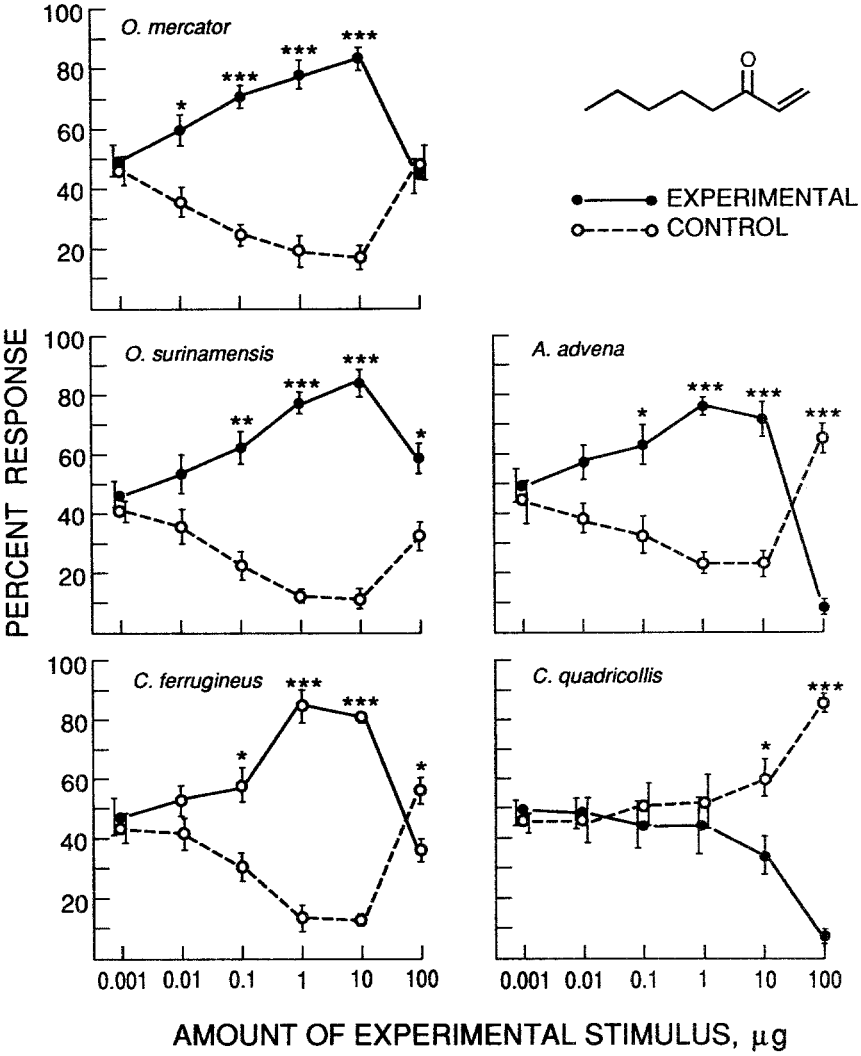


FIG. 2. Response ($\bar{X} \pm$ SE) by five species of cucujid grain beetles (mixed-sex) in two-choice, pitfall bioassay to 1-octen-3-one. Significant response (paired-sample t test) to experimental stimulus indicated by: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (12 replicates per treatment, 12 adults per replicate).

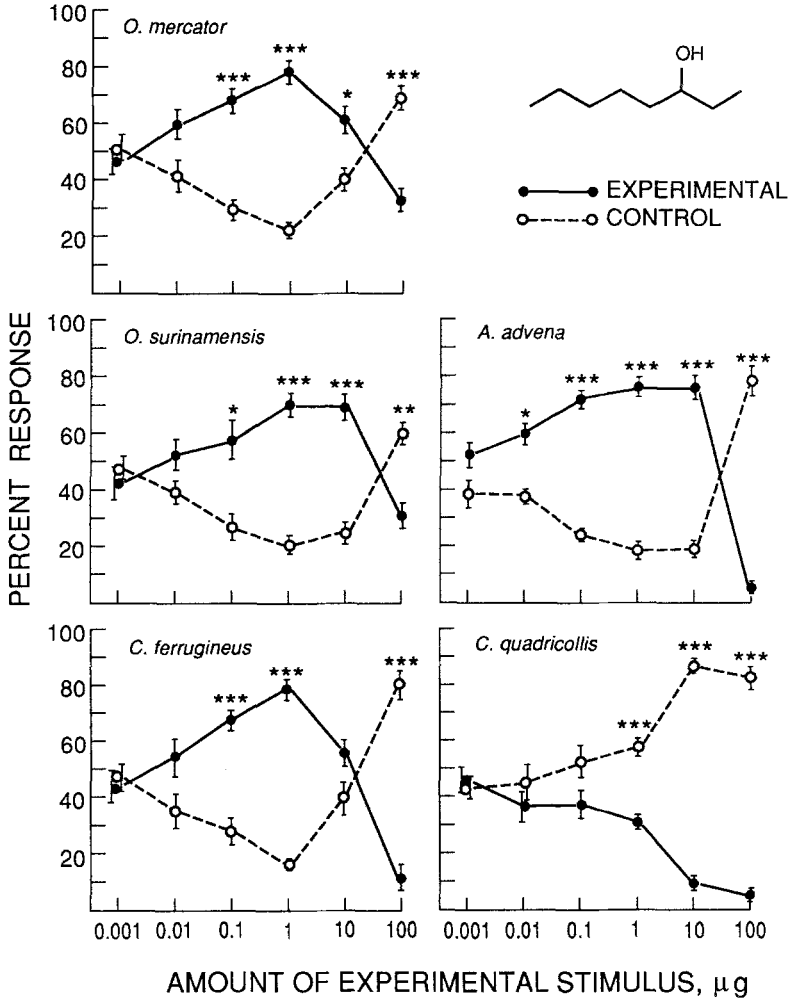


FIG. 3. Response ($\bar{X} \pm$ SE) by five species of cucujid grain beetles (mixed-sex) in two-choice, pitfall bioassay to (*R*,*S*)-3-octanol. Significant response (paired-sample *t* test) to experimental stimulus indicated by: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (12 replicates per treatment, 12 adults per replicate).

IV, the repulsiveness of the high alcohol dose was counteracted by the attractive cucujolides.

Table 2 gives the response of *O. surinamensis* to 3-methylbutanol, cucujolides IV + IX + V, and mixtures thereof. A combination of cucujolides IV + IX + V is the blend of macrolide aggregation pheromones produced by *O.*

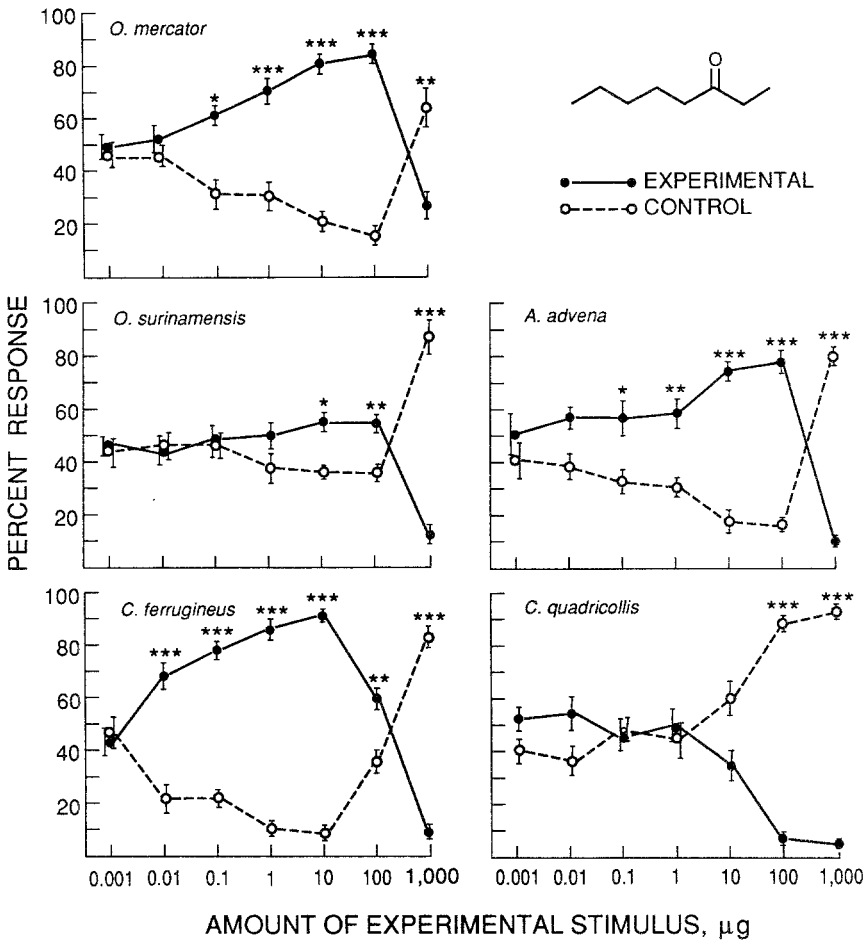


FIG. 4. Response ($\bar{X} \pm$ SE) by five species of cucujid grain beetles (mixed-sex) in two-choice, pitfall bioassay to 3-octanone. Significant response (paired-sample t test) to experimental stimulus indicated by: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (12 replicates per treatment, 12 adults per replicate).

surinamensis (Oehlschlager et al., 1988). Positive response to IV + IX + V was enhanced by the addition of 10 and 100 ng of 3-methylbutanol to 9 and 90 ng of the cucujolides, respectively. No significant enhancement of response to the cucujolides was obtained by the combination of larger amounts of alcohol and cucujolides in experiments 4 and 5 (Table 2). A repellent 100 µg dose of 3-methylbutanol was counteracted by the presence of the attractive cucujolides.

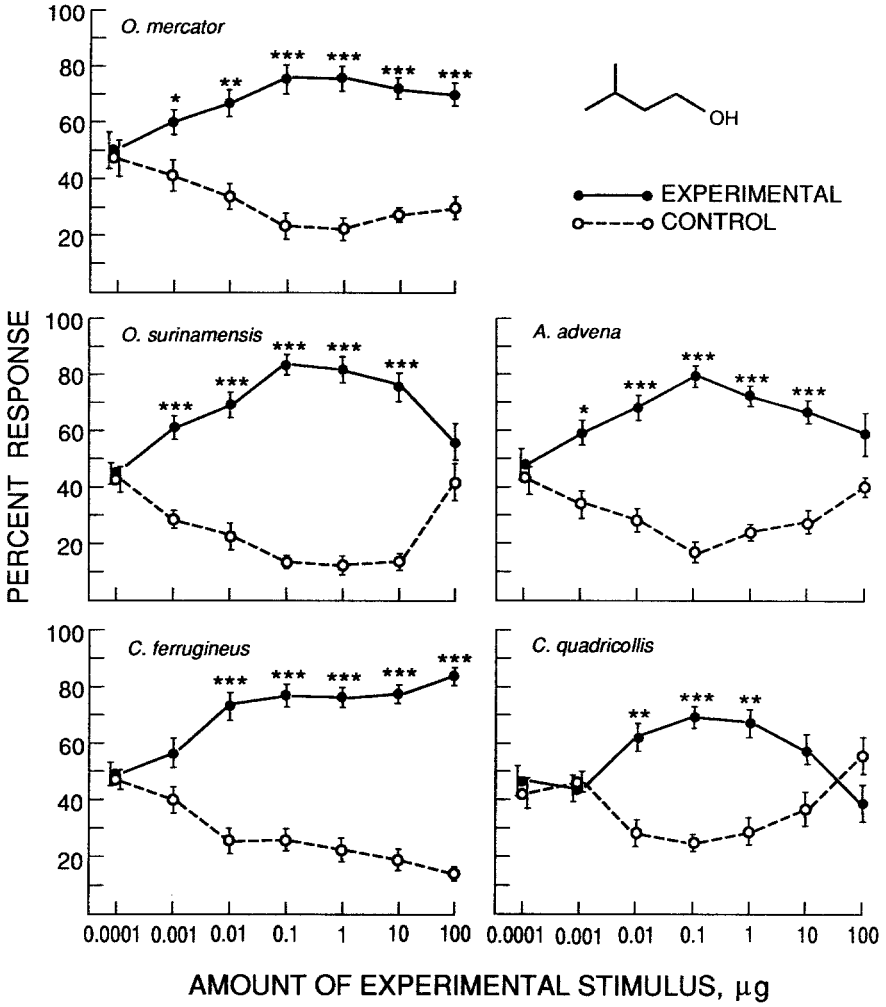


FIG. 5. Response ($\bar{X} \pm$ SE) by five species of cucujid grain beetles (mixed-sex) in two-choice, pitfall bioassay to 3-methylbutanol. Significant response (paired-sample t test) to experimental stimulus indicated by: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (12 replicates per treatment, 12 adults per replicate).

DISCUSSION

Biological Implications. We have shown previously that chiral and racemic 1-octen-3-ols are strong attractants for *Oryzaephilus* spp. and *A. advena* (A.M. Pierce et al., 1989, 1991), as demonstrated in the present study for *C. ferru-*

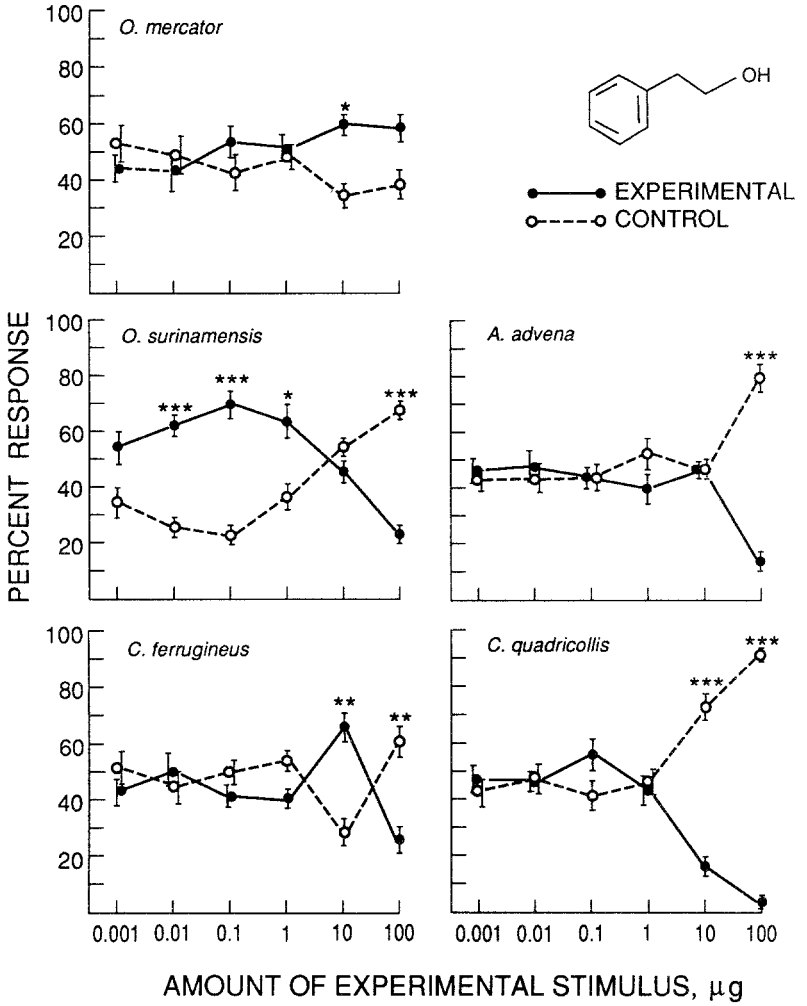


FIG. 6. Response ($\bar{X} \pm$ SE) by five species of cucujid grain beetles (mixed-sex) in two-choice, pitfall bioassay to 2-phenylethanol. Significant response (paired-sample t test) to experimental stimulus indicated by: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (12 replicates per treatment, 12 adults per replicate).

gineus. When maintained at low population densities, male and female *Oryzaephilus* spp. and *A. advena* older than 1–1.5 months posteclosion produced large amounts of almost optically pure (*R*)-(−)-1-octen-3-ol as aggregation pheromone (A.M. Pierce et al., 1989, 1991). Production of 1-octen-3-ol by *C. ferrugineus* was never observed in similar experiments (A.M. Pierce, personal

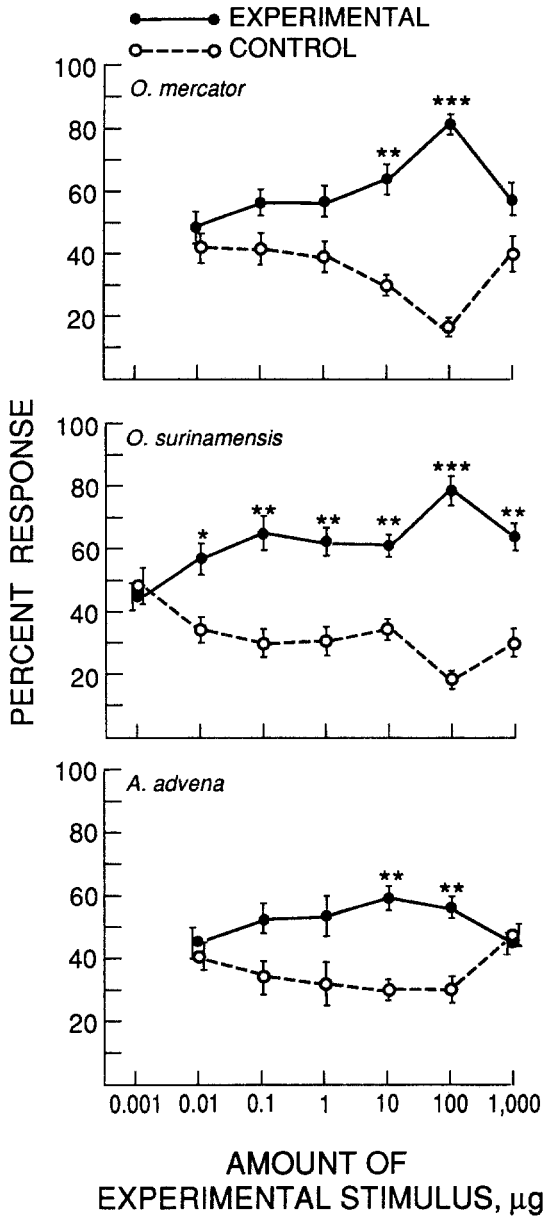


FIG. 7. Response ($\bar{X} \pm$ SE) by mixed-sex *O. mercator*, *O. surinamensis*, and *A. advena* in two-choice, pitfall bioassay to ethanol (CH_3CH_2OH). Significant response (paired-sample *t* test) to experimental stimulus indicated by: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (12 replicates per treatment, 12 adults per replicate).

TABLE 1. RESPONSE BY MIXED-SEX *O. mercator* IN TWO-CHOICE, PITFALL BIOSSAY TO 3-METHYLBUTANOL, RACEMIC CUCUJOLIDES II + IV, AND MITURES OF 3-METHYLBUTANOL AND II + IV

Exp.[a]	Dosage (μg) 3-Methyl-butanol	Dosage (μg) II + IV (1:1)	Response (%, $\bar{X} \pm$ SE) Experimental	Control[b]	Experimental–control[c]
1	0.001		59.4±5.5	34.3±4.5*	25.1±9.2 ab
		0.0004	53.0±4.4	44.4±3.9NS	8.6±7.7 a
	0.001	0.0004	67.8±4.2	28.0±4.5***	39.8±8.3 b
2	0.01		67.6±6.1	25.5±5.0**	42.1±10.8 a
		0.004	68.2±2.9	30.4±2.4***	37.8±5.2 a
	0.01	0.004	79.1±3.3	20.2±3.3***	58.9±6.3 b
3	0.1		73.4±3.4	25.2±3.4***	48.2±6.7 a
		0.04	81.2±2.1	18.1±2.0***	63.1±4.0 a
	0.1	0.04	80.4±3.9	17.5±3.5***	62.9±7.2 a
4	1		76.4±4.1	19.4±3.9***	57.0±7.8 a
		0.4	92.3±1.9	7.0±2.0***	85.3±3.7 b
	1	0.4	93.0±3.0	5.6±2.6***	87.4±5.4 b
5	10		69.1±4.0	23.7±3.7***	45.4±7.4 a
		4	90.3±2.8	8.3±2.7***	82.0±5.5 b
	10	4	93.1±2.1	6.9±1.7***	86.2±3.6 b
6	100		63.0±4.9	30.4±3.7***	32.6±8.1 b
	1000		20.8±4.8	74.3±5.1***	−53.5±10.0 a
		40	87.7±2.7	11.0±3.4***	76.7±5.7 c
	100	40	89.6±2.1	9.7±2.0***	79.9±4.0 c
	1000	40	76.2±5.8	18.2±5.3***	58.0±10.8 bc

[a] Each experiment was completed in a separate 2-hr session.

[b] Significant response (paired-sample t test) to experimental stimulus indicated by: ***$P < 0.001$; **$P < 0.001$; *$P < 0.05$; NS, not significant (12 replicates per treatment, 12 adults per replicate).

[c] Means within an experiment followed by the same letter are not significantly different, t test for independent samples, $P < 0.05$.

observations). In contrast, male and female *C. quadricollis* produced (*R*)-(−)-1-octen-3-ol, which was a repellent for this species (H.D. Pierce et al., 1988).

1-Octen-3-ol, 3-octanol, and 3-octanone are produced by numerous species of molds (Kaminski et al., 1980) and mushrooms (Pyysalo, 1976); in these studies, 1-octen-3-ol was a major volatile. 1-Octen-3-one, 3-methylbutanol, and 2-phenylethanol have been identified in the volatiles from some species of mushrooms (Pyysalo, 1976); 2-phenylethanol also has been characterized from molds (Collins, 1976). 3-Methylbutanol is known to be produced when grain

TABLE 2. RESPONSE BY MIXED-SEX *O. surinamensis* IN TWO-CHOICE, PITFALL BIOASSY TO 3-METHYLBUTANOL, CUCUJOLIDES (*R,S*)-IV + IX + (*R,S*)-V, AND MIXTURES OF 3-METHYLBUTANOL AND IV + IX + V

	Dosage (μg)		Response (%, $\bar{X} \pm$ SE)		
Exp.[a]	3-Methyl-butanol	IV + IX + V (2:1:6)	Experimental	Control[b]	Experimental–control[c]
1	0.001		59.7±4.7	33.1±3.8**	26.6±8.2 ab
		0.0009	50.7±4.2	43.7±3.4NS	7.0±7.3 a
	0.001	0.0009	66.7±5.0	29.8±4.2**	36.9±9.2 b
2	0.01		65.0±4.8	33.6±4.2**	31.4±9.2 a
		0.009	58.0±4.0	35.7±4.0*	22.3±7.7 a
	0.01	0.009	75.5±3.6	20.9±2.4***	54.6±5.5 b
3	0.1		75.0±3.2	23.6±3.6***	51.4±6.7 a
		0.09	71.3±3.5	21.0±3.3***	50.3±6.3 a
	0.1	0.09	82.6±3.0	13.9±2.6***	68.7±5.2 b
4	1		76.8±5.0	16.9±4.2***	59.9±9.2 a
		0.9	81.0±2.5	15.3±2.7***	65.7±4.7 a
	1	0.9	84.9±4.1	9.4±3.0***	75.5±6.7 a
5	10		68.1±3.5	29.2±4.2***	38.9±7.7 a
		9	80.4±3.0	16.1±2.4***	64.3±5.2 b
	10	9	81.8±2.7	14.0±3.2***	67.8±5.7 b
6	10		38.4±6.3	54.1±7.0NS	−15.7±12.5 a
		90	75.0±3.1	20.8±3.3***	54.2±6.1 b
	100	90	74.3±3.6	23.6±3.7***	50.7±7.2 b

[a] Each experiment was completed in a separate 2-hr session.
[b] Significant response (paired-sample *t* test) to experimental stimulus indicated by: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS, not significant (12 replicates per treatment, 12 adults per replicate).
[c] Means within an experiment followed by the same letter are not significantly different, *t* test for independent samples, $P < 0.05$.

is contaminated by bacteria as well as by molds (Kaminski et al., 1980). Although more commonly found as fungal metabolites, some of these compounds have been identified in the volatiles of stored cereals. For example, 1-octen-3-ol and 3-methylbutanol have been detected in the volatiles from oat groats (Heydanek and McGorrin, 1981), rice (Maga, 1984), and soybeans (del Rosario et al., 1984).

The positive responses to the alcohols and ketones in this study suggest that some of these volatiles might be used by the cucujids as host-finding kairomones in nature. The presence of aggregation pheromone-emitting cucujids on such an attractive host, in turn, further might enhance attraction of members

of the same species or, in the case of 1-octen-3-ol-emitting cucujids, of the same and different cucujid species. As an adaptive advantage for kairomone-producing microbes, the cucujids could serve as vectors for the microorganisms.

The repulsiveness of all the test compounds except 3-methylbutanol and ethanol to *C. quadricollis* in this study probably is not ecologically significant since negative responses occurred at high doses unlikely to be found in nature. While small amounts of 1-octen-3-ol were repellent to *C. quadricollis*, the presence of the male-produced aggregation pheromone overrode the repellent effect of 1-octen-3-ol and, hence, aggregation on a food source and mating would not be inhibited (H.D. Pierce et al., 1988). It was suggested that 1-octen-3-ol might function alone as a female-produced epideictic pheromone (Prokopy, 1981) to promote dispersion of female *C. quadricollis* (H.D. Pierce et al., 1988).

Some of the test alcohols and ketones have been implicated in the communication systems of other arthropods, which supports Blum's (1970) concept of pheromonal parsimony. For instance, 3-methylbutanol is a potent host-produced kairomone for cheese mite (Yoshizawa et al., 1972) and a pheromone for scolytid beetles (Borden, 1985). Ethanol and 2-phenylethanol have been identified as a kairomone and pheromone, respectively, for scolytid beetles (Borden, 1985). A number of myrmicine ants release 3-octanone and 3-octanol as major components of their alarm pheromone complex (Crewe et al., 1970). 1-Octen-3-ol is an oxen-produced kairomone that serves as a powerful attractant for tsetse flies (Hall et al., 1984).

Practical Implications. The data in Tables 1 and 2 suggest that, for *Oryzaephilus* spp., adding 3-methylbutanol to field traps containing cucujolide aggregation pheromones might be an economical way of extending the attractive range of the less volatile cucujolides. The addition of small amounts of attractive, rolled oat-produced aldehydes to small amounts of cucujolides lowered the response threshold by mixed-sex *O. mercator* to the pheromones in laboratory bioassays (A.M. Pierce et al., 1990). Adding (*R*, *S*)-1-octen-3-ol to cucujolide mixtures also enhanced response to the cucujolides in the two-choice, pitfall bioassay for *O. mercator* and *O. surinamensis* (A.M. Pierce et al., 1989). Field traps baited with a mixture of cucujolides and 1-octen-3-ol required 100-fold less of cucujolides than traps baited with only the cucujolides for equivalent performance in recapturing *O. surinamensis* (H.D. Pierce et al., 1990). As found with high amounts of 3-methylbutanol and cucujolides in the present study, the attractiveness of the cucujolides counteracted the repulsiveness of high doses of 1-octen-3-ol in laboratory bioassays (A.M. Pierce et al., 1989) and in field traps (H.D. Pierce et al., 1990).

Results to date for several cucujid species suggest that the most efficient and cost-effective approach for the use of semiochemicals in field traps might be to use a combination of pheromones plus appropriate host-produced coat-

tractants. Laboratory trapping studies utilizing mixtures of the most attractive volatiles identified for a particular species need to be conducted under controlled, but realistic, conditions to determine the efficacy of these mixtures as trap baits. Many pheromones or pheromone blends of stored-grain beetles, while species-specific, are not repellent to other species (Lindgren et al., 1985; Oehlschlager et al., 1988), and some host and fungal kairomones are attractive to more than one species. Studies need to be done, therefore, to design improved, complex baits that can be used in the smallest number of traps to attract the largest possible number of beetle species.

Acknowledgments—We thank Dr. A. Mosandl for gifts of the chiral 1-octen-3- ols and E. Merletti for technical assistance.

REFERENCES

BLUM, M.S. 1970. The chemical basis of insect sociality, pp. 61–94, *in* M. Beroza (ed.). Chemicals Controlling Insect Behavior. Academic Press, New York.

BORDEN, J.H. 1985. Aggregation pheromones, pp. 257–285, *in* G.A. Kerkut (ed.). Behavior, Vol. 9, *in* G.A. Kerkut and L.I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon Press, Oxford.

COLLINS, R.P. 1976. Terpenes and odiferous materials from microorganisms. *Lloydia* 39:20–24.

CREWE, R.M., BRAND, J.M., FLETCHER, D.J.C., and EGGERS, S.H. 1970. The mandibular gland chemistry of some South African species of *Crematogaster*. *J. Ga. Entomol. Soc.* 5:42–47.

del ROSARIO, R., de LUMEN, B.O., HABU, T., FLATH, R.A., MON, T.R., and TERANISHI, R. 1984. Comparison of headspace volatiles from winged beans and soybeans. *J. Agric. Food Chem.* 32:1011–1015.

DOLINSKI, M.G., and LOSCHIAVO, S.R. 1973. The effect of fungi and moisture on the locomotory behavior of the rusty grain beetle, *Cryptolestes ferrugineus* (Coleoptera: Cucujidae). *Can. Entomol.* 105:485–490.

HALL, D.R., BEEVOR, P.S., CORK, A., NESBITT, B.F., and VALE, G.A. 1984. 1-Octen-3-ol: A potent olfactory stimulant and attractant for tsetse isolated from cattle odours. *Insect Sci. Appl.* 5:335–339.

HEYDANEK, M.G., and MCGORRIN, R.J. 1981. Gas chromatography–mass spectroscopy investigations on the flavor chemistry of oat groats. *J. Agric. Food Chem.* 29:950–954.

JAVER, A., BORDEN, J.H., PIERCE, H.D., JR., and PIERCE, A.M. 1990. Evaluation of pheromone-baited traps for monitoring of cucujid and tenebrionid beetles in stored grain. *J. Econ. Entomol.* 83:268–272.

KAMINSKI, E., STAWICKI, S., WASOWICZ, E., and KASPEREK, M. 1980. Volatile odour substances produced by microflora. *Nahrung* 24:103–113.

LINDGREN, B.S., BORDEN, J.H., PIERCE, A.M., PIERCE, H.D., JR., OEHLSCHLAGER, A.C., and WONG, J.W. 1985. A potential method for simultaneous, semiochemical-based monitoring of *Cryptolestes ferrugineus* and *Tribolium castaneum* (Coleoptera: Cucujidae and Tenebrionidae). *J. Stored Prod. Res.* 21:83–87.

MAGA, J.A. 1984. Rice product volatiles: A review. *J. Agric. Food Chem.* 32:964–970.

MILLAR, J.G., and OEHLSCHLAGER, A.C. 1984. Synthesis of *Z,Z*-skipped diene macrolide pheromones for *Cryptolestes* and *Oryzaephilus* grain beetles (Coleoptera Cucujidae). *J. Org. Chem.* 49:2332–2338.

OEHLSCHLAGER, A.C., WONG, J.W., VERIGIN, V.G., and PIERCE, H.D., JR. 1983. Synthesis of

two macrolide pheromones of the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens). *J. Org. Chem.* 48:5009–5017.

OEHLSCHLAGER, A.C., PIERCE, A.M., PIERCE, H.D., JR., and BORDEN, J.H. 1988. Chemical communication in cucujid grain beetles. *J. Chem. Ecol.* 14:2071–2098.

PIERCE, A.M., BORDEN, J.H., and OEHLSCHLAGER, A.C. 1981. Olfactory response to beetle-produced volatiles and host-food attractants by *Oryzaephilus surinamensis* and *O. mercator*. *Can. J. Zool.* 59:1980–1990.

PIERCE, A.M., BORDEN, J.H., and A.C. OEHLSCHLAGER. 1983. Effects of age and population density on response to beetle and food volatiles by *Oryzaephilus surinamensis* and *O. mercator* (Coleoptera: Cucujidae). *Environ. Entomol.* 12:1367–1374.

PIERCE, A.M., PIERCE, H.D., JR., BORDEN, J.H., and OEHLSCHLAGER, A.C. 1989. Production dynamics of cucujolide pheromones and identification of 1-octen-3-ol as a new aggregation pheromone for *Oryzaephilus surinamensis* and *O. mercator* (Coleoptera: Cucujidae). *Environ. Entomol.* 18:747–755.

PIERCE, A.M., PIERCE, H.D., JR., OEHLSCHLAGER, A.C., and BORDEN, J.H. 1990. Attraction of *Oryzaephilus surinamensis* (L.) and *O. mercator* (Fauvel) (Coleoptera: Cucujidae) to some common volatiles of food. *J. Chem. Ecol.* 16:465–475.

PIERCE, A.M., PIERCE, H.D., JR., OEHLSCHLAGER, A.C., and BORDEN, J.H. 1991. 1-Octen-3-ol, an attractive semiochemical for the foreign grain beetle, *Ahasverus advena*, (Waltl) (Coleoptera: Cucujidae). *J. Chem. Ecol.* 17:567–580.

PIERCE, H.D., JR., PIERCE, A.M., MILLAR, J.G., WONG, J.W., VERIGIN, V.G., OEHLSCHLAGER, A.C., and BORDEN J.H. 1984. Methodology for isolation and analysis of aggregation pheromones in the genera *Cryptolestes* and *Oryzaephilus* (Coleoptera: Cucujidae), pp. 121–137, *in* Proceedings of the Third International Working Conference on Stored-Product Entomology. Kansas State University, Manhattan, Kansas.

PIERCE, H.D., JR., PIERCE, A.M., JOHNSTON, B.D., OEHLSCHLAGER, A.C., and BORDEN, J.H. 1988. Aggregation pheromone of square-necked grain beetle, *Cathartus quadricollis* (Guér.) *J. Chem. Ecol.* 14:2169–2184.

PIERCE, H.D., JR., PIERCE, A.M., BORDEN, J.H., and OEHLSCHLAGER, A.C. 1990. The effect of aggregation pheromones on efficacy of cardboard traps for sawtoothed grain beetle (Coleoptera: Cucujidae). *J. Econ. Entomol.* 83:273–276.

PROKOPY, R.J. 1981. Epideictic pheromones that influence spacing patterns of phytophagus insects, pp. 181–213, *in* D.A. Nordlund, R.L. Jones, and W.J. Lewis (eds.). Semiochemicals: Their Role in Pest Control. John Wiley & Sons, New York.

PYYSALO, H. 1976. Identification of volatile compounds in seven edible fresh mushrooms. *Acta Chem. Scand. B* 30:235–244.

SINHA, R.N. 1968. Adaptive significance of mycophagy in stored-product arthropoda. *Evolution* 22:785–798.

SINHA, R.N., and WALLACE, H.A.H. 1966. Ecology of insect-induced hot spots in stored grain in western Canada. *Res. Popul. Ecol.* 8:107–132.

SINHA, R.N., and WATTERS, F.L. 1985. Insect Pests of Flour Mills, Grain Elevators, and Feed Mills and their Control. Research Branch Agriculture Canada, Publication 1776.

VANHAELEN, M., VANHAELEN-FASTRÉ, R., GEERAERTS, J., and WIRTHLIN, T. 1979. *cis-* and *trans-*Octa-1,5-dien-3-ol, new attractants to the cheese mite *Tyrophagus putrescentiae* (Schrank) (Acarina, Acaridae) identified in *Trichothecium roseum* (Fungi Imperfecti). *Microbios* 23:199–212.

YOSHIZAWA, T., YAMAMOTO, I., and YAMAMOTO, R. 1972. Isolation and structural elucidation of cheese components, which attract the cheese mite, *Tyrophagus putrescentiae*. *Mem. Tokyo Univ. Agric.* 15:1–29.

ZAR, J.H. 1984. Biostatistical Analysis, 2nd ed. Prentice-Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

CHARACTERIZATION OF CHEMICALS MEDIATING OVIPOSITIONAL HOST-PLANT FINDING BY *Amyelois transitella* FEMALES

P. LARRY PHELAN,[1,*] CARYN J. ROELOFS,[1]
ROGER R. YOUNGMAN,[2,3] and
THOMAS C. BAKER[2]

[1]*Department of Entomology*
Ohio Agricultural Research & Development Center
Ohio State University
Wooster, Ohio 44691

[2]*Department of Entomology*
University of California
Riverside, California 92521

(Received August 6, 1990, accepted November 16, 1990)

Abstract—Ovipositional host-finding in the navel orangeworm, *Amyelois transitella* (Walker), is brought about by an in-flight response to host odors. Wind-tunnel studies of the response of gravid females to almonds showed that this response is mediated primarily by long-chain fatty acids, particularly oleic acid and linoleic acid. Evidence for the behavioral activity of fatty acids is based on the fact that: (1) behavioral activity of almond oil was concentrated in a single liquid chromatographic fraction whose composition was predominantly long-chain fatty acids, (2) behavioral activity was lost when either almond oil or the active fraction of that oil was treated with diazomethane, (3) full activity was elicited by a selective extraction of free fatty acids from crude almond oil, and (4) upwind response by females was elicited by a blend of synthetic oleic and linoleic acids, albeit at a level less than that elicited by almond oil. Five fatty acids identified from the almond oil were: myristic acid (1%), palmitic acid (16%), stearic acid (3%), oleic acid (58%), and linoleic (22%). Attraction to various combinations of synthetic acids was observed only when oleic acid was present, and oleic acid elicited upwind flights to the source when presented alone; however, short-range responses were enhanced by the addition of linoleic acid, which elicited no long-range

*To whom correspondence should be addressed.
[3]Present address: Department of Entomology, Virginia Polytechnic Institute & State University, Blacksburg, Virginia 20461.

0098-0331/91/0300-0599$06.50/0

orientation by itself. Despite significant levels of attraction to synthetic blends, the percentage of females flying to the source was lower than that flying to acidulated almond oil, the best natural attractant tested. Thus, although long-range response may be mediated primarily by a blend of oleic and linoleic acids, additional and as yet unidentified components must also play an important role. Long-range chemically modulated host finding in this and other generalist plant feeders is discussed with respect to current models of the evolution of host finding, and it is argued that suggestions that long-range host finding should be correlated with narrowness of host utilization are logically flawed and are not supported by our current understanding of specific examples of host finding.

Key Words—Host finding, *Amyelois transitella*, navel orangeworm, Lepidoptera, Pyralidae, flight behavior, chemoorientation, attraction, fatty acids, oleic, linoleic, palmitic

INTRODUCTION

Amyelois transitella (Walker), the navel orangeworm, was first detected in the United States in rotting fruit and its initial presence elicited little concern because of its apparent preference for fallen or diseased fruits (Wade, 1961); however, since that time, this insect has become the key pest of almonds, as well as a serious problem in pistachios and walnuts, causing nut growers in the western United States many millions of dollars in damage each year. Previous work based on field trapping suggested that host finding by the navel orangeworm was mediated by odors of almond nuts and/or volatiles from frass of navel orangeworm larvae (Curtis and Clark, 1979; Andrews and Barnes, 1982; Rice et al., 1984). A long-range response to almond odors was confirmed by Phelan and Baker (1987) in studies of flight by navel orangeworm females in a laboratory wind tunnel. This work demonstrated that mated females, but not males or virgin females could be induced to take off and fly more than 1.5 m up an odor plume of almond by-products. Unrefined oil from almonds was determined to be the most potent source of flight activity on a per-weight basis, with 100 mg of this oil eliciting a response several times greater than mummified almonds, the preferred sites of oviposition in the field. Recently, the zigzagging flight track of navel orangeworm females flying to host odors was analyzed in further detail by Haynes and Baker (1989), who describe track angles for females in contact with a host-odor plume and after loss of the odor plume. Comparison of various angular and velocity components of the flight pattern revealed a counterturning program very similar to that of navel orangeworm males flying to female sex pheromone.

In the present study, we examined the chemical basis of host finding in navel orangeworm females with the objective of characterizing the constituents of almond odor that are responsible for ovipositional attraction.

METHODS AND MATERIALS

Navel orangeworm larvae were reared on a honey–bran–glycerol diet according to the methods of Strong et al. (1968). Pupae were collected from rearing containers and were placed in cups with moist vermiculite held in mating cages. The behavioral characterization of almond-odor components responsible for ovipositional attraction of navel orangeworm females was carried out in wind tunnels at both institutions. The construction of the Riverside tunnel, which measured 0.6 × 0.6 × 1.8 m, has been described previously (Willis and Baker, 1984). The tunnel at the OARDC is similar in operation although somewhat larger. The working portion of this tunnel measured 2.5 m long × 1 m wide × 0.5 m high and was constructed of 6-mm Plexiglas. Airflow was generated by a 3/4-horsepower Dayton model 3C152 fan with a 64-cm-diam. six-wing blade and was made laminar as it was forced through three muslin screens spaced 10 cm apart. The wind speed was maintained at 0.4 m/sec by a 61 × 61-cm adjustable-louver box mounted on the upwind side of the fan box, which was equipped with standard fiberglass furnace filters to reduce the ambient dust reaching the muslin screens. The odor plume was exhausted from the downwind end of the wind tunnel and was directed outside the building by a 30-cm-diam. flexible exhaust duct and a 25-cm-diam. 1/15-horsepower exhaust fan. The tunnel was illuminated by three 60-W incandescent white bulbs mounted 1.2 m above the tunnel floor and operated at 30 V to provide 0.2 lx at floor level during bioassays. Females 3 to 6 days old were selected from the mating cages and were acclimated to experimental conditions by holding them in the wind without odors for 0.5 hr prior to testing, which was conducted 2.5–0 hr before lights-out. Odor sources were applied to a 5.5-cm-diam. filter-paper disk held 20 cm above the tunnel floor at the upwind end of the tunnel, and the female moths were released at the downwind end in groups of 10 individuals from 5.5-cm-diam. × 9.0-cm-long screen release cylinders placed on a 15-cm-tall platform. Four categories of female behaviors were recorded: takeoff, flight up the plume, flight to the odor source (those approaching to within 5 cm of the source), and landing on the source. Treatments were presented individually in a randomized complete-block design. Percent response results were analyzed by two-way ANOVA after $\sin^{-1}(x + .005)^{1/2}$ and means were separated using Duncan's new multiple-range test (SAS, 1985), except for response to different quantities of acidulated almond oil, which was subjected to regression analysis.

Crude almond oil and almond soapstock were obtained from Liberty Vegetable Oil Co. (Sante Fe Springs, California). Soapstock is composed of salts of free fatty acids removed during the refining of vegetable oils by treating the oils with potassium or sodium hydroxide. The free fatty acids were reconstituted by making the soapstock strongly acidic (pH 3) with H_2SO_4. The resulting oil, termed acidulated almond oil, was separated from the aqueous layer and an

unidentified intermediate layer in a separatory funnel. High-purity authentic samples (>99% by capillary GC) of constituents identified from almond oil were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin) and were stored at −20°C under nitrogen. Acidulated oils from various other commodities also were tested for their ability to evoke orientation by navel orangeworm females; these acidulated oils included peanut, corn, soybean, and palm kernel.

A preliminary fractionation of 0.5 ml crude almond oil was carried out using a silica-gel SepPak (Millipore Corp., Milford, Massachusetts), eluting with 2 ml hexane, 2 ml ether–hexane (5:95), 2 ml ether, and 2 ml methylene chloride. Fractions were bioassayed for behavioral activity by presenting them individually and in combination to females in the wind tunnel. Characterization of active constituents was carried out on a Hewlett-Packard 5890 capillary gas chromatograph interfaced with a Hewlett-Packard 5970 Mass Selective Detector (GC-MS); electron-impact mass spectra were generated at 70 eV. Splitless injections were made onto either DB-1 (30 m × 0.25 mm ID, 0.25-μm film thickness) or DB-23 (30 m × 0.25 mm ID, 0.25-μm film thickness) fused-silica capillary columns (J & W Scientific, Folsum, California). Fatty acids were extracted from 1 ml of crude almond oil by washing the oil three times with 4 ml of an aqueous 10% $NaHCO_3$ solution. The aqueous fractions were combined and brought to pH 3 with H_2SO_4. This solution then was extracted three times with 10 ml hexane, and the hexane fractions were combined and concentrated to 1 ml under N_2. Fatty acids were derivatized to their methyl esters with diazomethane generated from 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) using a micro-diazomethane-generation apparatus (Aldrich Chemical Co.). Presence and position of double bonds were confirmed using the epoxidation method of Bierl-Leonhardt et al. (1980). After derivatization of fatty acids to methyl esters, double bonds were epoxidized and the epoxides were submitted to hydrogenation to reduce remaining double bonds in diunsaturated constituents. The resulting products were analyzed using GC-MS.

RESULTS

Wind-tunnel bioassay of the SepPak fractionation of crude almond oil demonstrated full activity to be contained in the ether fractions. Upwind flights to the source were 15/50 (30%) for crude almond oil, 17/50 (34%) for the ether fractions, and 14/50 (28%) for all fractions combined. No response was elicited by the hexane, hexane–ether, or methylene chloride fractions. GC analysis of the active fractions suggested the primary constituents to be fatty acids. Treatment of the fraction with diazomethane followed by GC-MS supported this initial supposition, as the original peaks, which tailed excessively, were now replaced with earlier-eluting peaks of much greater symmetrical shape (Figure

1). Mass spectrometry indicated methyl esters of myristic acid (1%), palmitic acid (16%), stearic acid (3%), oleic acid (58%), and linoleic (22%). The position of double bonds in each was confirmed by epoxidation, in which oleic acid produced the single compound 9,10-epoxyoctadecanoic acid, palmitoleic acid gave rise to 9,10-epoxyhexadecanoic acid, while linoleic acid produced two peaks, 9,10-epoxyoctadecanoic acid and 12,13-epoxyoctadecanoic acid. Diagnostic MS ions used for confirmation of double-bond position were *m/z* 155 and *m/z* 199 for 9,10-epoxyoctadecanoic acid, *m/z* 113 and *m/z* 241 for 12,13-epoxyoctadecanoic acid, and *m/z* 127 and *m/z* 199 for 9,10-epoxyhexadecanoic acid.

That almond-oil fatty acids played a role in navel orangeworm attraction was verified by treating crude almond oil with diazomethane. Only about a quarter as many females were attracted to the diazomethane-treated oil compared to the untreated oil (Figure 2). This level of response to diazomethane-treated oil was not significantly different from that to blank filter paper, which attracted no females. The reduction in the number of females landing on the treated oil was even greater, with a 24-fold higher response to the untreated oil. Navel orangeworm attraction to free fatty acids removed from crude almond oil was compared with that to crude oil using the amount of free fatty acids found in 100 mg of oil (3 mg for this batch of crude oil). Female navel orangeworm flew to the acidulated almond oil at levels significantly higher than to the crude oil (45 $\pm$ 3.4% vs. 30 $\pm$ 3.7%, respectively; $P < 0.05$, six groups of 10

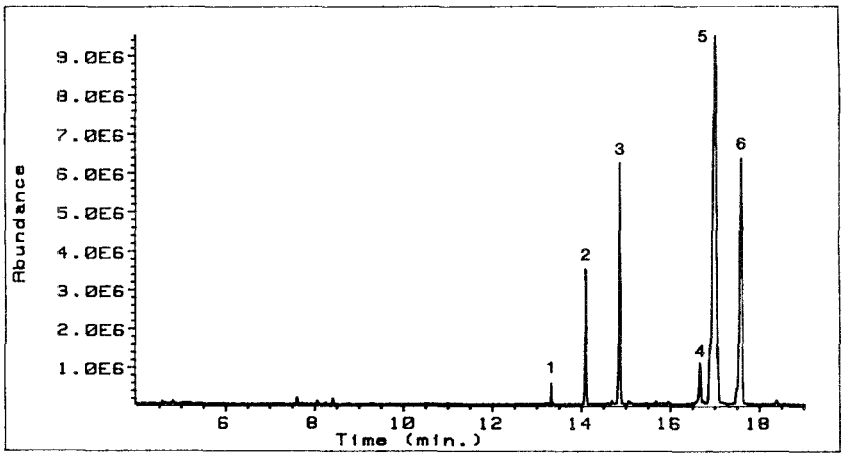


FIG. 1. Capillary gas chromatogram of the behaviorally active silica-gel fraction of crude almond oil after treatment with diazomethane. Column: DB-23 (30 m $\times$ 0.25 mm). 1 = methyl myristate, 2 = 20 ng methyl pentadecanoate (internal standard), 3 = methyl palmitate, 4 = methyl stearate, 5 = methyl oleate, and 6 = methyl linoleate. Temperature program: 30°C increased to 200°C at a rate of 12°C/min.

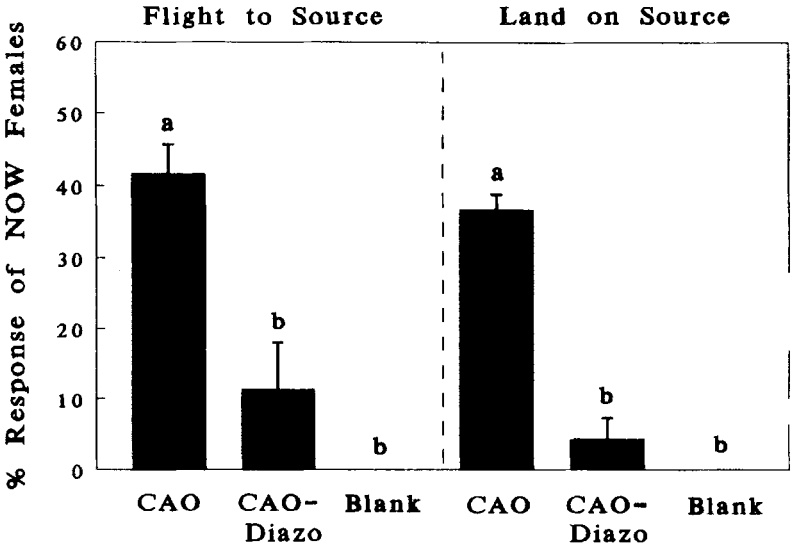


FIG. 2. Response ($\bar{X} \pm$ SEM) of navel orangeworm (NOW) females to 100 mg crude almond oil (CAO), 100 mg CAO treated with diazomethane, or blank filter paper. Bars marked by the same letter are not significantly different ($P < 0.05$, six replicates of 10 females).

females). Furthermore, of the females that approached the acidulated oil, 93% of those females landed, which was comparable to the 94% landing rate found with the crude oil.

Since the behavioral activity appeared to be concentrated in the fatty-acid components of the crude almond oil, subsequent work focused on acidulated almond oil. A dose–response curve was generated to determine the quantity of acidulated almond oil evoking maximal upwind orientation and landing by navel orangeworm females. Flights to the source were analyzed as a fourth-order polynomial. The highest-order significant polynomial we found was a quadratic relationship, with the resulting model: $y = 0.057x - 0.001x^2$, $r^2 = 0.81$. Thus, within the range of 0.5–50 mg, the probability of female flight to the source increased with increasing amount of oil, with the negative quadratic indicating a slight leveling off of response at the highest dose (Figure 3). A regression analysis of landing response and the quantity of almond oil yielded a similar model: $y = 0.054x - 0.001x^2$, $r^2 = 0.81$. Furthermore, there was no evidence of premature arrestment at the higher levels of oil, as landing rates were similar in all quantities for which comparisons could be made. At 5 mg, 10 mg, and 50 mg of acidulated almond oil, landing rates were 92%, 90%, and

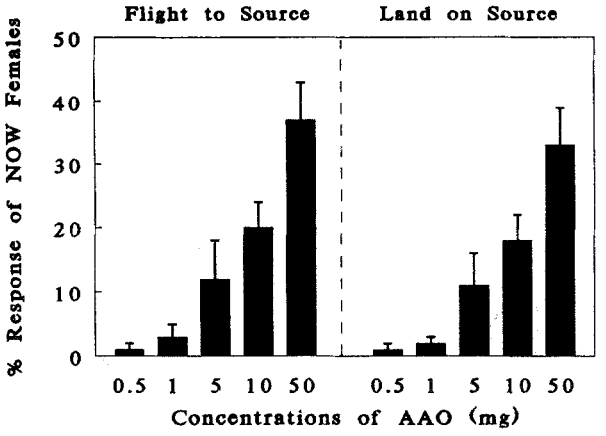


FIG. 3. Response ($\bar{X} \pm$ SEM) of NOW females to different quantities of acidulated almond oil (AAO) (nine replicates of 10 females).

91%, respectively, for those females approaching the source, while percent approaches to the source at the lower concentrations were too low for informative comparisons. Since 50 mg of acidulated oil evoked the greatest long-range response without premature arrestment, this became the positive control in subsequent bioassays to which other treatments were compared.

A wind-tunnel bioassay of acidulated oils from commodities other than almonds indicated that the behaviorally active constituents were not unique to almonds (Figure 4). Acidulated peanut oil evoked the second highest response, with the number of females flying up to and the percentage landing on the source slightly lower than that to acidulated almond oil. The number of flights to acidulated corn oil was lower still, with the number of landings much lower. Responses to acidulated soybean and palm kernel oils were significantly lower than other oils tested, with no females flying to palm kernel oil.

Binary combinations of synthetic samples of either myristic and palmitic acids or palmitic and linoleic acids elicited no flights to the source when presented at the amounts found in 50 mg acidulated oil (Figure 5). Although the combination of oleic and palmitic acids did evoke some flights to source, this activity was not significantly higher than zero. The highest activity elicited among binary combinations was that to oleic plus linoleic acids. This blend attracted 40% of females to the source, not significantly different from that to the five- acid blend. Acidulated almond oil evoked more flights to the source than any combination of synthetic fatty acids tested, 59% of females, although this was not significantly higher than the response to the oleic–linoleic acid blend. The difference in response to natural compared to synthetic odor sources

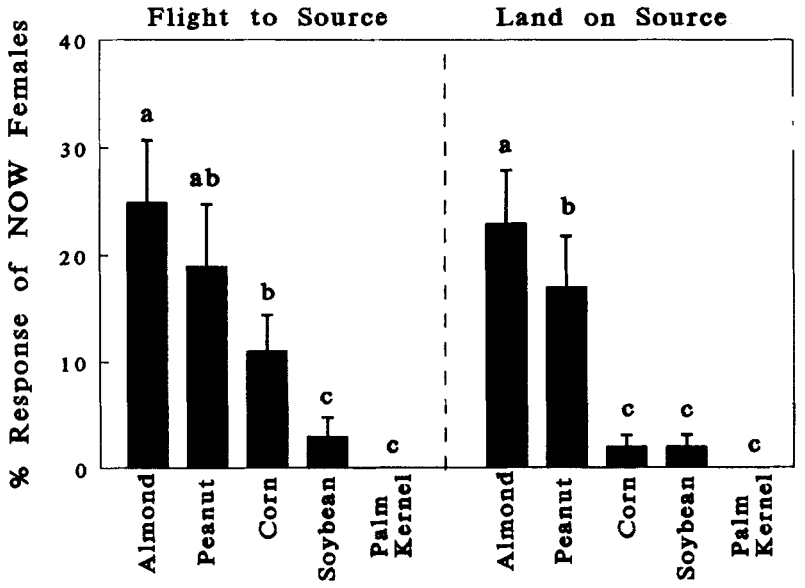


FIG. 4. Response ($\bar{X} \pm$ SEM) of NOW females to 50 mg of various acidulated oils. Bars marked by the same letter are not significantly different ($P < 0.05$, 12 replicates of 10 females).

was much more pronounced when the number of landings were measured. The most active synthetic blend, oleic acid plus linoleic acid, only evoked landing in 14% of females, which represented 35% of those approaching the source, whereas 86% of females approaching acidulated almond oil landed on the source. The proportion of females landing on the oleic–linoleic acid blend was not significantly different from the proportion landing on the total-fatty-acid blend (10% of all females, 32% of source-approaching females). Finally, oleic acid and linoleic acid were tested individually to determine the relative contribution of these two components to navel orangeworm female attraction. When comparing flights to the source, oleic acid elicited a response comparable to that of the synthetic total fatty-acid blend (Figure 6), while linoleic acid elicited no flights to the source. However, the number of females landing on oleic acid was significantly lower than the number landing on the four-acid blend. These overall female landing rates corresponded to 36% of females approaching oleic acid and 65% of females approaching the five-acid mixture. As in the previous experiment, more females approached the acidulated almond oil than approached the synthetic fatty acids, with the difference in response amplified when landing rates were compared; of females approaching the acidulated oil, 81% landed.

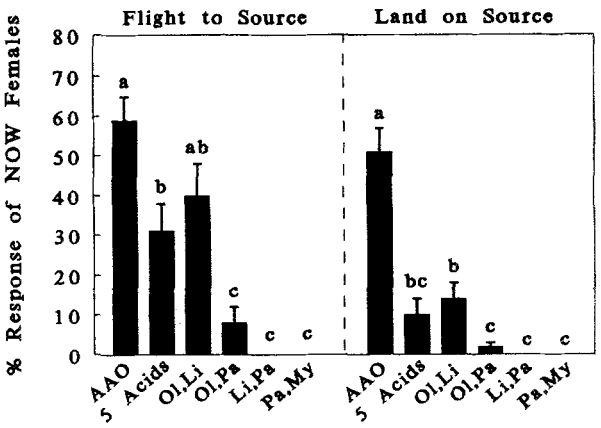


FIG. 5. Response ($\bar{X} \pm$ SEM) of NOW females to 50 mg acidulated almond oil (AAO), a blend of five synthetic fatty acids: 29 mg oleic (Ol), 11 mg linoleic (Li), 8 mg palmitic (Pa), 1.5 mg stearic, and 0.5 mg myristic (My), or various binary combinations of these acids. Bars marked by the same letter are not significantly different ($P < 0.05$, nine replicates of 10 females).

DISCUSSION

The role of volatiles in navel orangeworm ovipositional behavior was first implicated by Ortega (1950), who observed greater navel orangeworm egg-laying in walnuts previously damaged by navel orangeworm larval feeding; Caltagirone et al. (1968) made similar observations in almonds. Curtis and Clark (1979) confirmed this increased egg-laying to be chemically mediated by demonstrating that it also could be elicited by extracts of the larval frass. The results of our bioassay-guided identification of navel orangeworm ovipositional attractants strongly suggest a primary role played by long-chain fatty acids in eliciting this behavior. Our wind-tunnel observations demonstrated that despite the relatively low volatility of these compounds, gravid navel orangeworm females can detect and respond to them from a distance of at least 2 m. Evidence for the behavioral activity of fatty acids derives from our observations: (1) that the behavioral activity of almond oil was concentrated in a single liquid chromatographic fraction whose composition was predominantly long-chain fatty acids; (2) that behavioral activity was lost when either almond oil or the active fraction of that oil was treated with diazomethane, a reagent narrowly reactive with acidic compounds; (3) that full activity could be elicited by a selective extraction of free fatty acids from crude almond oil; and (4) that upwind response by females could be elicited by a blend of synthetic oleic and linoleic acids, albeit at a level less than that elicited by almond oils. Although treatment of the almond

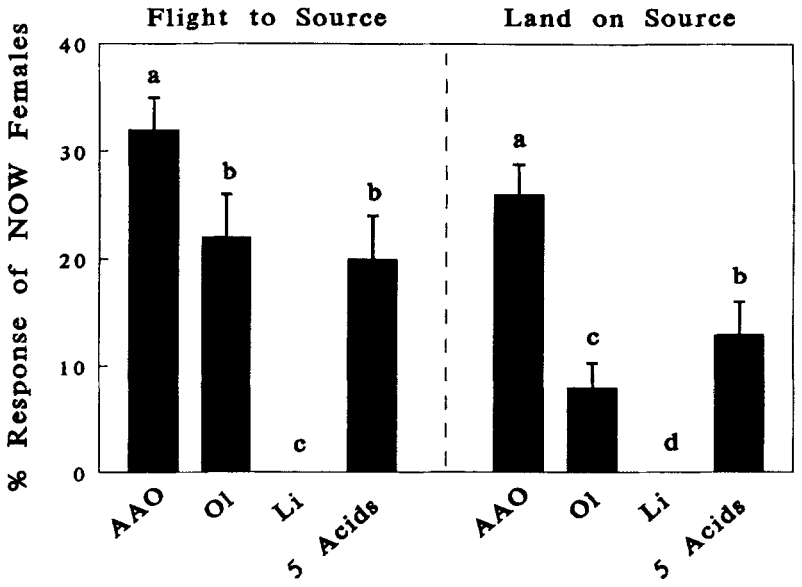


FIG. 6. Response ($\bar{X} \pm$ SEM) of NOW females to acidulated almond oil (AAO), 29 mg oleic acid (Ol), 11 mg linoleic acid (Li), or a blend of five synthetic fatty acids: 29 mg oleic, 11 mg linoleic, 8 mg palmitic, 1.5 mg stearic, and 0.5 mg myristic. Bars marked by the same letter are not significantly different ($P < 0.05$, 22 replicates of 10 females).

oil with diazomethane greatly diminished response, some upwind orientation persisted. This may have been due to the methyl esters acting as fatty-acid analogs, evoking a low level of response in females, or it may indicate that additional non-fatty-acid components of almond odor play a secondary role in navel orangeworm attraction. The fact that acidulated oils from other commodities can elicit a long-range response in navel orangeworm females also is suggestive that ubiquitous long-chain fatty acids are the key components modulating host finding, rather than volatile compounds unique to almonds. Moreover, the level of response to the acidulated oils was correlated with the relative proportion of oleic acid in those oils. Acidulated peanut oil, which we determined to be composed of 57% oleic acid, 18% linoleic acid, and 18% palmitic acid, had the fatty-acid composition most similar to acidulated almond oil and elicited the highest level of response. Acidulated corn oil, which is 26% oleic acid, 59% linoleic acid, and 12% palmitic acid, elicited a lower navel orangeworm response. Acidulated soybean oil is only 22% oleic acid, 55% linoleic acid, 11% palmitic acid, and in addition contains 8% linolenic acid. Acidulated palm kernel oil is predominantly lauric acid (51%) and contains only 15% oleic acid and only 1% linoleic acid. This oil evoked no upwind flights to the source.

In an early attempt to discover chemical attractants for the navel orangeworm, Price et al. (1967) screened over 200 compounds with a variety of chemical functionalities placed in water-bucket field traps. Those compounds consistently eliciting the highest trap catch were all phenyl-containing constituents, with phenyl propionate being the most active compound. The trap catch by all chemicals was strongly female-biased. It is not known what function if any these compounds serve in the chemical ecology of the navel orangeworm; however, they do not appear to be important in ovipositional behavior, as Curtis and Clark (1979) later reported that phenyl propionate actually inhibited navel orangeworm egg-laying. One possibility is that they elicit food-finding behavior in adults foraging for nectar sources. Later, Lieu et al. (1982a,b) identified 42 components from a steam distillation extraction of wheat bran, predominantly aldehydes, and almost 50 components from an ether extract of larval frass, including the same long-chain fatty acids found to be behaviorally active in the present study; however, although both of the original substrates were known to elicit oviposition and presumably attraction in the field (Rice et al., 1976; Curtis and Clark, 1979), none of the identified constituents evoked a significant navel orangeworm response in a laboratory ovipositional bioassay. This was true even when a blend of the four principal long-chain fatty acids was presented. The discrepancy in results between these earlier studies and our own probably are due to the difference in bioassay methods. While Lieu et al. (1982a,b) were measuring an ovipositional response, our use of the wind-tunnel bioassay allowed us to observe the long-range orientation of navel orangeworm females. Since we found a lower landing response to the synthetic fatty acids relative to the almond oils, this probably explains why Lieu et al. (1982a,b) did not measure a significant ovipositional response to these compounds.

Despite the importance of understanding how insects locate their host plants, surprisingly few systems have been characterized for the chemicals responsible in long-range host finding. In fact, this paucity of well-studied examples has led Jermy et al. (1988) to suggest that "distant olfactory attraction to host plants in nature may be much rarer than has been thought." We use the term "long-range response" to chemicals *sensu* Kennedy (1977), who defined it mechanistically as chemically modulated orientation far enough from an odor source to preclude chemotactic response to a longitudinal chemical gradient. In practice, this usually represents a distance greater than a few decimeters. Many studies have suggested a role played by volatiles in host finding and/or selection by phytophagous insects (reviewed by Ramaswamy, 1988). Unfortunately, the evidence typically is circumstantial or at best confounds short-range acceptance behaviors with long- distance orientation to host odors, since conclusions are usually based on "end-result" observations or nondiscriminating bioassays (Kennedy, 1977). Furthermore, tests of synthetic host-plant compounds rarely utilize release rates comparable to a natural odor source. Some of our best doc-

umented cases for the role of volatile chemicals in long-distance host-finding are found in phytophagous flies. In a series of wind-tunnel studies, Hawkes and Coaker (1979, *et ante*) documented host-odor-modulated anemotaxis in the cabbage fly, *Delia brassicae*. In response to volatiles from crucifers, these flies landed in the plume, faced upwind, and walked or made a series of hopping flights to the odor source. The same behaviors also were observed in the field, and it was concluded that response to host odors can occur from greater than 15 m (Hawkes, 1974). This orientation behavior has been characterized more recently in greater detail by measuring flight track-angles and insect airspeed in response to discrete plumes and dispersed clouds of allylisothiocyanate, a major volatile constituent of crucifer odors (Nottingham and Coaker, 1985). Similar behavioral responses have been documented for the onion fly (*Delia antiqua*) as reviewed by Miller and Strickler (1984), and the apple maggot fly (*Rhagoletis pomonella*). The latter, whose host-finding behavior also has been examined in the wind tunnel, shows the same chemically modulated response to a blend of synthetic 8- to 10-carbon esters as to an extract of apple volatiles (Fein et al., 1982).

In concurrent and complementary discussions by May and Ahmad (1983) and Lance (1983) on the proximal mechanisms of host finding in oligophages and polyphages, respectively, it is argued that breadth of host utilization by an insect herbivore (among other factors) will be a major determinant in the evolution of long-range response to host odors. The basic premise underlying this argument is that since the host of the specialist is more likely to be rarer and interspersed among nonhosts, there is a more intense selection pressure to be able to recognize and locate the host from a distance. Correspondingly, generalist feeders are less likely to "require accurate, specific, long-distance orientation mechanisms" (Lance, 1983). It is postulated that once the generalist is in contact with a plant, the variation in suitability of plant material is better assessed by gustatory stimuli and/or cues from other modalities. However, although the selection may be less intense for herbivores with broad dietary ranges than for the specialist feeders, nonetheless there is an adaptive advantage in the use of more efficient mechanisms for generalists as well. Thus, while the nature of the chemical cues utilized and the narrowness of the tuning of the herbivore's receptor system would be expected to differ between two species with different breadths of host range, there is no reason to believe that a broad host range would obviate host-plant finding by a long-range response. Furthermore, as pointed out by May and Ahmad (1983), evidence to support the specialist feeder–long-range host-finding model is not only lacking but actually runs counter. For example, the highly polyphagous *Schistocerca gregaria* can use odor-modulated anemotaxis to locate its host from a distance (Kennedy and Moorehouse, 1969), and the Japanese beetle appears to use a diverse group of compounds in finding its hosts, based on observations of beetles flying into

baited traps from a distance (Fleming, 1969; Ladd and McGovern, 1984 and references therein). Long-range chemoorientation to host odors also has been demonstrated recently in another polyphagous feeder, *Trichoplusia ni* (Landolt, 1989), as well as in the broadly feeding nitidulid beetles, *Carpophilus hemipterus* and *C. lugubris* (Blackmer and Phelan, in press; Phelan and Lin, in press), all of which display zigzagging flight up a laboratory wind tunnel to a variety of host odors and/or synthetic blends. The navel orangeworm is extremely broad in its host diet, feeding on the nuts, fruits, and seed pods from plants as diverse as *Acacia*, citrus, almonds, and *Yucca* (Wade, 1961). The detailed behavioral studies on the navel orangeworm (Phelan and Baker, 1987; Haynes and Baker, 1989; and this study) leave little doubt that host finding in this insect also is based primarily on a chemically modulated long-distance response.

In conclusion, we would submit that the proposed relationship between narrower host-plant utilization and increased powers of long-range host location is not supported by the evidence of well-studied examples, including the navel orangeworm. Here we have demonstrated that ovipositional host finding by the broadly feeding navel orangeworm is mediated at some distance from the host by means of a long-range response to a blend of long-chain fatty acids, primarily oleic and linoleic acids. However, although a synthetic blend of these chemicals can elicit upwind flights to the odor source, it does not reproduce the same level of navel orangeworm response as either crude or acidulated almond oils. This difference between response to natural compared to synthetic sources of odor was even greater when close-range behaviors such as landing were considered. Although the behaviorally active acidulated almond oil is composed almost solely of long-chain fatty acids, the relatively low vapor pressure of these components would make their relative abundance in the volatile profile greatly reduced. Thus, minor components of these oils, such as products of fatty-acid breakdown probably represent a significant contribution to almond odors. Work presently is being carried out to characterize the potential behavioral role played by these more volatile constituents in eliciting close-range response to host odors.

Acknowledgments—We thank R.S. Vetter and B.A. Nault for technical assistance, and Liberty Vegetable Oil Co., Sante Fe Springs, California, for supplying the crude almond oil and the almond soapstock. Thanks also to J.L. Blackmer and Drs. L.R. Nault and R.E. Treece for critical review of the manuscript. Salaries and support were provided by state and federal funds as well as by grants from the Almond Board of California appropriated to both institutions; Ohio Agricultural Research & Development Center Manuscript No. 214-90.

REFERENCES

ANDREWS, K.L., and BARNES, M.M. 1982. Differential attractiveness of infested and uninfested mummy almonds to navel orangeworm moths. *Environ. Entomol.* 11:280–282.

BIERL-LEONHARDT, B.A., DEVILBISS, E.D., and PLIMMER, J.R. 1980. Location of double-bond

position in long-chain aldehydes and acetates by mass spectral analysis of epoxide derivatives. *J. Chromatogr. Sci.* 18:364–367.

BLACKMER, J.L., and PHELAN, P.L. Effect of physiological state and fungal inoculation on chemically modulated host-plant finding by *Carpophilus hemipterus* and *Carpophilus lugubris*. *Entomol. Exp. Appl.* In press.

CALTAGIRONE, L.E., MEALS, D.W., and SHEA, K.P. 1968. Almond sticktights contribute to navel orangeworm infestations. *Calif. Agric.* 22:2–3.

CURTIS, C.E., and CLARK, J.D. 1979. Responses of navel orangeworm moths to attractants evaluated as oviposition stimulants in an almond orchard. *Environ. Entomol.* 8:330–333.

FEIN, B.L., REISSIG, W.H., and ROELOFS, W.L. 1982. Identification of apple volatiles attractive to the apple maggot, *Rhagoletis pomonella*. *J. Chem. Ecol.* 8:1473–1487.

FLEMING, W.E. 1969. Attractants for the Japanese beetle. *USDA Tech. Bull.* 1399:1–87.

HAWKES, C. 1974. Dispersal of adult cabbage fly [*Erioischia brassicae* (Bouche)] in relation to a brassica crop. *J. Appl. Ecol.* 11:83–93.

HAWKES, C., and COAKER, T.H. 1979. Factors effecting the behavioral response of adult cabbage root fly, *Delia brassicae*, to host plant odor. *Entomol. Exp. Appl.* 25:45–58.

HAYNES, K.F., and BAKER, T.C. 1989. An analysis of anemotactic flight in female moths stimulated by host odour and comparison with the males' response to sex pheromone. *Physiol. Entomol.* 14:279–289.

JERMY, T., SZENTESI, Á., and HORVÁTH, J. 1988. Host plant finding in phytophagous insects: the case of the Colorado potato beetle. *Entomol. Exp. Appl.* 49:83–98.

KENNEDY, J.S. 1977. Behaviorally discriminating bioassays of attractants and repellents, pp. 215–229, *in* H.H. Shorey and J.J. McKelvey (eds.). Chemical Control of Behavior. John Wiley & Sons, New York.

KENNEDY, J.S., and MOOREHOUSE, J.E. 1969. Laboratory observations on locust responses to windborne grass odour. *Entomol. Exp. Appl.* 12:487–503.

LADD, T.L., and MCGOVERN, T.P. 1984. 2-Methoxy-4-propylphenol, a potent new enhancer of lures for the Japanese beetle (Coleoptera: Scarabaeidae). *J. Econ. Entomol.* 77:957–959.

LANCE, D.R. 1983. Host-seeking behavior of the gypsy moth: The influence of polyphagy and highly apparent host plants, pp. 201–224 *in* S. Ahmad (ed.). Herbivorous Insects: Host-Seeking Behavior and Mechanisms. Academic Press, New York.

LANDOLT, P.J. 1989. Attraction of the cabbage looper to host plants and host plant odor in the laboratory. *Entomol. Exp. Appl.* 53:117–124.

LIEU, F.-Y., RICE, R.E., and JENNINGS, W.G. 1982a. Volatile components of navel orangeworm attractants: I. Wheat bran baits. *Chem. Mikrobiol. Technol. Lebensm.* 7:151–153.

LIEU, F.-Y., RICE, R.E., and JENNINGS, W.G. 1982b. Volatile components of navel orangeworm attractants: II. Volatile constituents of larval frass from infested almonds. *Chem. Mikrobiol. Technol. Lebensm.* 7:154–160.

MAY, M.L., and AHMAD, S. 1983. Host location in the Colorado Potato Beetle: Searching mechanisms in relation to oligophagy, pp. 173–199, *in* S. Ahmad (ed.). Herbivorous Insects: Host-Seeking Behavior and Mechanisms. Academic Press, New York.

MILLER, J.R., and STRICKLER, K.L. 1984. Finding and accepting plants, pp. 127–157, *in* W.J. Bell and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, New York.

NOTTINGHAM, S.F., and COAKER, T.H. 1985. The olfactory response of cabbage root fly, *Delia radicum*, to the host plant volatile allylisothiocyanate. *Entomol. Exp. Appl.* 39:307–316.

ORTEGA, J.C. 1950. The navel orangeworm on walnuts in southern California. *Diamond Walnut News* 32:6–7.

PHELAN, P.L., and BAKER, T.C. 1987. An attracticide for control of *Amyelois transitella* (Lepidoptera: Pyralidae) in almonds. *J. Econ. Entomol.* 80:779–783.

PHELAN, P.L., and LIN, H. Chemical characterization of fruit and fungal volatiles attractive to the

driedfruit beetle, *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae). *J. Chem. Ecol.* In press.

PRICE, D.W., MAZRIMAS, J.A., and SUMMERS, F.M. 1967. Chemical attractants for navel orangeworm moths. *Calif. Agric.* 21:10–11.

RAMASWAMY, S.B. 1988. Host finding by moths: sensory modalities and behaviors. *J. Insect Physiol.* 34:235–249.

RICE, R.E., SADLER, L.L., HOFFMAN, M.L., and JONES, R.A. 1976. Egg traps for the navel orangeworm, *Paramyelois transitella* (Walker). *Environ. Entomol.* 5:697–700.

RICE, R.E., JOHNSON, T.W., PROFITA, J.C., and JONES, R.A. 1984. Improved attractant for navel orangeworm (Lepidoptera: Pyralidae) egg traps in almonds. *J. Econ. Entomol.* 77:1352–1353.

SAS, 1985. SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina.

STRONG, R.G., PARTIDA, G.J., and WARNER, D.N. 1968. Rearing stored-product insects for laboratory studies: six species of moths. *J. Econ. Entomol.* 61:1237–1249.

WADE, W.H. 1961. Biology of the navel orangeworm, *Paramyelois transitella* (Walker), on almonds and walnuts in northern California. *Hilgardia* 31:129–171.

WILLIS, M.A., and BAKER, T.C. 1984. The effects of intermittent and continuous pheromone stimulation on the flight behavior of the oriental fruit moth, *Grapholitha molesta*. *Physiol. Entomol.* 9:341–358.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

trans-2-NONENAL INSECT REPELLENT, INSECTICIDE, AND FLAVOR COMPOUND IN CARROT ROOTS, CELL SUSPENSIONS, AND "HAIRY" ROOT CULTURES

D.A. CHAMBERLAIN,[1,2] G. WILSON,[1] and M.F. RYAN[2,*]

Departments of [1]Botany and [2]Zoology
University College Dublin
Belfield, Dublin 4, Ireland

(Received May 31, 1990; accepted November 19, 1990)

Abstract—As *trans*-2-nonenal (T2N) acts as an insect repellent, an insecticide, and a flavor compound, it would be desirable to manipulate its concentration in plants. Simple, rapid , and accurate trapping on cartridges of activated charcoal detected T2N in headspace over carrot roots and regenerants. Concentrations ranged from 0.07 to 0.43 ppm total volatiles over the homogenized roots of a range of European carrot cultivars with the following statistically significant differences: Long Chantenay > Sytan > Vertou > Touchon Prima. This suggests that the expression of T2N is determined genetically. Given the potential for genetic manipulation by *in vitro* techniques, the release of T2N and other terpenoids was monitored in carrot cell suspensions and in "hairy" root cultures of cv. Gelbe Rheinische. T2N concentrations were 6.23 ppm and 0.005 ppm, respectively, as compared with 0.15 ppm over the homogenized root of the same cultivar. Two terpenoids additional to T2N were detected from cell suspension cultures as compared with 14 from "hairy" root cultures. The ready detection in regenerants of this significant aldehyde indicates that its presence could be monitored in derivatives of other *in vitro* manipulations.

Key Words—*trans*-2-Nonenal, insecticide, carrot root, root regenerants, genetic differences, genetic manipulation.

*To whom correspondence should be addressed.

INTRODUCTION

The side effects of synthetic pesticides are leading to a reappraisal of natural compounds for use in pest control. There is both a theoretical basis, related to coevolution, and practical evidence for the occurrence of insecticides in plants (e.g., Ryan et al., 1978; Ryan and Byrne, 1988). The application of plant tissue culture techniques facilitates two new approaches in the exploitation of natural plant products in crop protection. First, cultures of plant cells provide an industrial biosynthetic source of natural compounds having insecticidal properties (Zieg et al., 1983). Second, plant tissue culture methods enable the cloning, by somatic embryogenesis, of an elite individual plant having desirable chemical properties. Obvious preliminary steps are the screening of plant populations for cultivars and individual plants having elevated levels of a natural plant defense compound, followed by the screening of *in vitro* regenerants for satisfactory synthesis of the compound.

Unusually, perhaps, the naturally occurring aldehyde *trans*-2-nonenal (hereafter T2N) is an insect repellent, insecticide, and a flavor compound. Bioassays of 36 components of carrot root oil indicated that T2N repelled and killed the larva of the parasitic, and thus adapted, insect, *Psila rosae* (F.), the carrot fly: the lethal concentration (LD_{50}) of the vapor in a non-contact bioassay was 4000 ppm (Guerin and Ryan, 1980). T2N at 50 ppm elicited 98% repellency from the American cockroach, *Periplaneta americana* (Scriven and Meloan, 1984). More recently, T2N proved effective against a broad range of 11 insect and three acarine pests (Taha, 1987; Awde, 1988).

Results from a human test panel indicated that T2N, representing 0.3% of carrot root oil obtained by steam distillation, had the lowest odor threshold of all carrot root volatiles tested (Buttery et al., 1968). Subsequently, use of multidimensional scaling of odor compounds derived from cold solvent extraction led to the claim that T2N was the single compound closest to carrot odor (Alabran et al., 1975). However, this compound with *trans*-2,*cis*-6-nonadienal is generally recognized as producing the characteristic aroma of cucumber (Forss et al., 1962).

The foregoing spectrum of effects suggests that it would be appropriate to manipulate the concentration of T2N in carrot root. Accordingly, we examined intrinsic variability in the production of this compound by comparing its concentration in the headspaces of roots from eight carrot cultivars with a European provenance. Our use of small charcoal traps enabled rapid, simple, and sufficient absorption to provide, from a single root, a concentration detectable by GC-MS. Given the potential for the derivation of new cultivars by *in vitro* techniques, we also sought this compound in the headspaces of undifferentiated cell suspension cultures and of differentiated "hairy" root cultures. The latter are profuse, quick-growing, regenerated roots induced *in vitro* by plasmid-

transformed cells. Such cultures may represent an industrial source of secondary metabolites (Rhodes, 1988).

METHODS AND MATERIALS

Collection and Analysis of volatiles. Collection and analysis of volatiles employed microtraps containing 2 mg of washed and sieved activated charcoal in a glass capillary tube (ID 2 mm). Traps were precisely matched into groups of three, with the same resistance to airflow, by use of a bubble flowmeter attached to each individual trap outlet on the sampling apparatus. Thus, the headspace was sampled in triplicate (Figure 1). Control experiments showed that such traps absorbed more than 93% of total test volatiles from a headspace stream flowing at 60 ml/min over 2.5 hr. Volatiles from carrot material, thus aerated and maintained in a water bath at 40°C, were swept onto the charcoal traps (Figure 1) for subsequent identification by GC-MS.

Entrapped volatiles were desorbed at the head of the gas- chromatographic column using a modification of the method of Clarke and Cronin (1975) (Figure 2). Essentially, each charcoal trap was oriented in the injection apparatus such that depressing the plunger placed the trap in the heating block governing the temperature of the injection port (280°C). Thus, for 100 sec desorbed volatiles were swept directly by the carrier gas into the GC capillary column. Modifications to the original injection/desorbtion apparatus were as follows: (1) the trap was attached tightly to the central plunger by a detachable silicone rubber collar facilitating trap removal and replacement; (2) the injection apparatus was

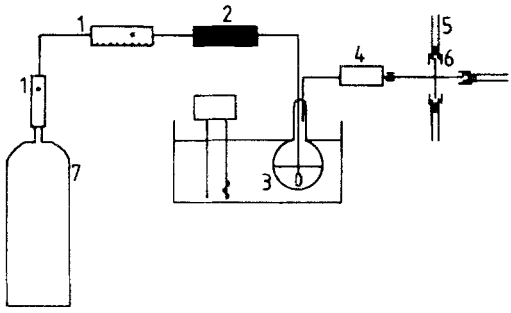


FIG. 1. Schematic diagram of the volatile collection apparatus. Air passed from cylinder (7) through flowmeters (1), was cleaned by passage over activated charcoal (2), and entered the sample flask (3) through a 10-cm × 0.6-cm-ID teflon tube and a sintered glass sparger. Volatile-enriched air then passed over anhydrous calcium chloride, packed in a 12-cm Pasteur pipet (4), and into a three-armed glass manifold (6), ID 0.2 cm, attached by Si-rubber collars to three charcoal capillary traps (5).

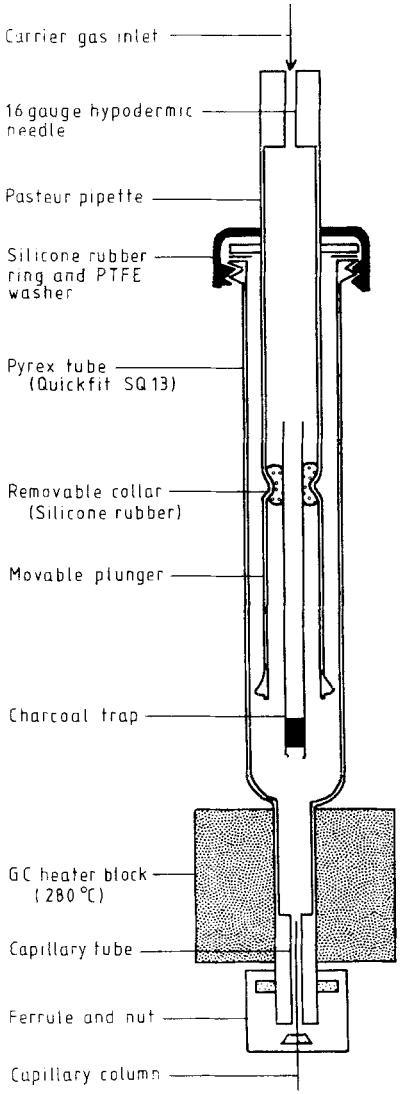


FIG. 2. Desorption device for transfer of adsorbed volatiles from the charcoal capillary trap to the head of the GC-column. Modified after Clarke and Cronin (1975), see text for other details.

attached to the column using a ferrule fitting to eliminate leakages; (3) the procedure was simplified by the use of a prerun purge (2 min), thus obviating the need to purge the outer jacket (e.g., no carrier gas bypass) (Figure 2). For GC conditions see the legend to Figure 3 (below).

Carrot Plants, Cell Suspensions, and "Hairy" Root Cultures. Carrot seeds of seven cultivars representing a spectrum of resistance to the carrot root fly, *Psila rosae* (F.) (Maki and Ryan, 1989) were obtained from The Institute of Horticultural Research, Wellesbourne, Warwick, U.K. These were sown in a cold glasshouse in April 1987 and the roots harvested in September of that year.

Undifferentiated cell suspension cultures derived from cv. Gelbe Rheinische were maintained on Murashige and Skoog (1962) medium (1 μM 2,4-dichlorophenoxy acetic acid; 1 μM naphthalene acetic acid) and subcultured at four-week intervals using a 1 : 10 inoculum ratio. Cells were harvested by filtration for headspace analysis 14 days after inoculation.

Transformed root cultures ("hairy" roots) of cv. Gelbe Rheinische were established according to the method of Leemans et al. (1982) using *Agrobacterium rhizogenes*, strain LBA 9402, containing the binary vector pBin 19. Roots grown in hormone-free Murashige and Skoog liquid medium were subcultured every two weeks. Both root cultures and cell suspension cultures were grown in an orbital shaker (100 rpm, 5 cm throw) in low fluorescent light at 23°C.

All plant material was washed free of either soil or culture medium, frozen in liquid nitrogen, and ground vigorously in a hand-held coffee grinder (Braun 120 W). The resultant powder, still frozen, was sieved through a 10-gauge mesh, and a 10-g batch was weighed into a 100-ml long-necked flask. To this was added 10-ml distilled water containing 12.5 ng hexanol, as internal standard, prior to aeration.

RESULTS AND DISCUSSION

Aeration of the same carrot root, intact and then homogenized,gave qualitatively similar GC profiles. *trans*-2-Nonenal (T2N) was identified in the headspace over the homogenized roots of all cultivars studied, in concentrations from 0.07 to 0.43 ppm of total volatiles entrapped. Significant differences in concentrations were: Long Chantenay > Sytan > Vertou > Danvers > Clauses Jeune Obtuse > Touchon Prima (Figure 3). These results suggest that the concentration of T2N in carrot root is determined genetically, a conclusion further indicated by the similar values obtained for the related cultivars Long Chantenay and Royal Chantenay. Therefore, the concentration of T2N in carrot and other plant species may be amenable to genetic manipulation through the application of plant tissue culture methods.

T2N headspace concentrations over the homogenates of cell suspension

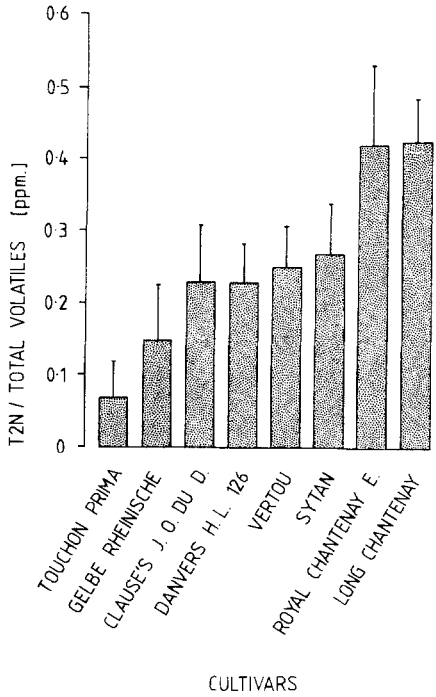


FIG. 3. Relative proportion of *trans*-2-nonenal in the total headspace volatile component of homogenized whole root from eight carrot cultivars (ppm) as judged from GC using the internal standard method (hexanol as internal standard). Vertical bars representing standard errors indicate the precision of the method. Values obtained from cell suspension and "hairy" root cultures (cv. Gelbe Rheinische) were 6.23 and 0.005 ppm, respectively. GC conditions: Hewlett Packard 5890A chromatograph linked to automatic computing integrator 3380A; glass capillary Supelcowax column, 530 μM ID, 30m long; carrier gas, helium 2 ml/min; injection port temperature 280°C; column temperature, 22°C for 100 sec, then 5°C/sec rise to 52°C maintained for 100 sec, then 2°C/min rise to 210°C.

cultures and "hairy" root were 6.23 ppm and 0.005 ppm total volatiles, respectively, a 125-fold difference. The former is equivalent to 14-fold more than over homogenized root, whereas the latter concentration is comparable to that over whole root (Figure 4). The enhanced concentration in cell suspension culture reflects the relatively low level of total volatiles present because T2N peak areas, per unit fresh weight of tissue, were roughly similar from cell suspensions and whole root. It is relevant that selection and screening of plant cell cultures for secondary metabolites has led to the isolation of some 20 cell lines, from other plant species, capable of accumulating secondary plant products at levels exceeding that in the plant (Wilson, 1988).

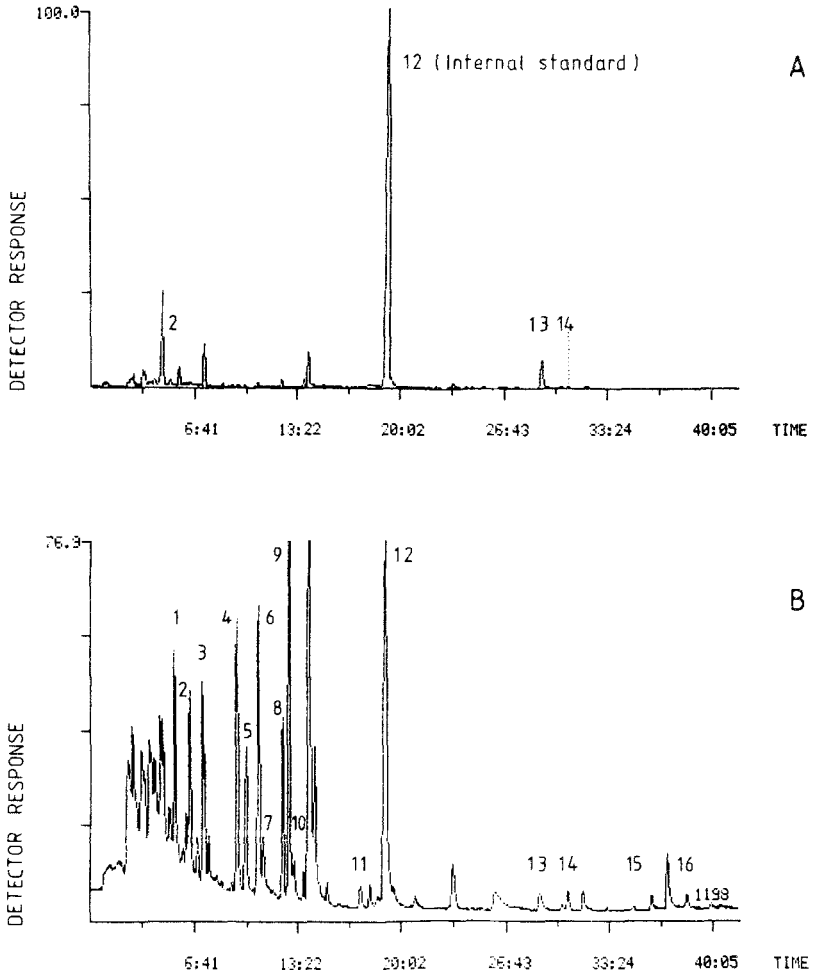


FIG. 4. GC traces from GC-MS analysis of headspace volatiles of homogenized cells from carrot cell suspension cultures (A) and from cultured "hairy" roots (B) (cv. Gelbe Rheinische): VG Analytical 7070E mass spectrometer, PYE 204 gas chromatograph, Finnigan-Mat INCOS 2400 data system. For peak identifications, see Table 1.

Only camphene and bornyl acetate, in addition to T2N, were confirmed from cell suspensions, whereas 14 additional compounds were from "hairy" root cultures (Figure 4, Table 1). All except *trans*-ocimene, *cis*-ocimene, β-himachalene, and β-selinene have been identified from carrot root in previous investigations.

These carrot cell suspension cultures, therefore, resemble cultures of several other plants in which the range of mono- and sesquiterpenes, synthesized

TABLE 1. PEAK IDENTIFICATIONS OF CARROT CELL AND "HAIRY" ROOT SUSPENSIONS[a]

Constituents	Cell suspension	Hairy root	Peak No.	Previous identification in carrot root
Camphene	+	+	2	+abc
trans-2-Nonenal	+	+	13	+ac
Bornyl acetate	+	+	14	+abc
trans-2-Heptanol	−	+	11	+a
trans-Ocimene	−	+	10	−
cis-Ocimene	−	+	8	−
β-Himachalene	−	+	16	−
β-Selinene	−	+	15	−
Terpinolene	−	+	9	−
Sabinene	−	+	7	+abc
Limonene	−	+	6	+ab
Myrcene	−	+	4	+abc
α-Pinene	−	+	1	+abc
β-Pinene	−	+	3	+abc
γ-Terpinene	−	+	5	+abc

[a] +: present; −: not detected; a: Buttery et al. (1968) in atmospheric steam distilled carrot root oil; b: Heatherbell and Wrolstad (1971) in an aqueous extract of fresh carrot; c: Alabran et al. (1975) in a cold solvent extract of fresh carrot.

and accumulated by undifferentiated cells in culture, is markedly less than in the whole plant (Charlewood et al., 1988). As some free monoterpenes are toxic to plant cells, it was suggested that isoprenoid synthesis was severely limited by end-product inhibition. Accordingly, it is interesting that two of the three compounds identified from cell suspension cultures have entomological significance: T2N is an insecticide and insect repellent, and bornyl acetate is employed as a host-location cue by the larva of the carrot fly (Guerin and Ryan, 1980). In cucumber, T2N is synthesized through the oxidation of linoleic acid by lipoxygenase followed by hydroperoxide cleavage (Galliard et al., 1976). However, most terpenes are synthesized through the acetate–mevalonate pathway which, apart from the synthesis of low levels of camphene and bornyl acetate, appears to be suppressed in the present cell suspensions.

By contrast, once morphological differentiation has become established, either as a callus or in "hairy" root formation (Figure 4), additional secondary metabolic pathways become functional. The GC profile of carrot "hairy" root cultures contains many terpenes characteristic of the whole carrot root, which is consistent with the accumulation of secondary metabolites such as nicotine

and atropine in "hairy" roots of other plant species (Rhodes et al., 1986; Kamada et al., 1986).

The sensitivity and simplicity of the present method of charcoal trapping, combined with direct GC injection, facilitates the sampling of relatively small amounts of tissue and thus the screening of individual plants. Our present detection of significant differences in T2N concentrations between different cultivars should lead to the identification of an elite plant. Cloning by somatic embryogenesis from such a parent could provide a basis for accelerated crop improvement through the development of cultivars having enhanced levels of the insecticide. The present methodology will allow the monitoring of T2N from such regenerants.

Acknowledgments—We thank the National Board for Science and Technology (*EOLAS*), Dublin, for funding a proposal from Michael F. Ryan and Graham Wilson.

REFERENCES

ALABRAN, D.M., MOSKOVITZ, H.R., and MABROUK, F. 1975. Carrot root oil components and their dimensional characterisation of aroma. *J. Agric. Food Chem.* 23:229–232.

AWDE, J. 1988. Naturally occurring compounds and analogues as prototype insecticides and flukicides. PhD thesis. National University of Ireland, Dublin.

BUTTERY, R.G., SEIFERT, R.M., GUADAGNI, D.G., BLACK, D.R., and LING, L.C. 1968. Characterisation of some volatile constituents of carrots. *J. Agric. Food Chem.* 16:1009–1015.

CHARLEWOOD, B.V., BROWN, J.T., MOUSTAN, C., and CHARLEWOOD, K.A. 1988. Pelagoniums: Flavours, fragrances and the new technology. *Plants Today*. 1:42–46.

CLARKE, R.G., and CRONIN, D.A. 1975. The use of activated charcoal for the concentration and analysis of headspace vapours containing food aroma volatiles. *J. Sci. Food Agric.* 26:1615–1624.

FORSS, A., DUNSTONE, A.R., RANSHAW, E.H., and STARK, K.W. 1962. The flavour of cucumbers. *J. Food Sci.* 27:90–93.

GALLIARD, T., PHILLIPS, D.R., and REYNOLDS, J. 1976. The formation of *cis*-3-nonenal, *trans*-2-nonenal and hexanal from linoleic acid hydroperoxide isomers by a hydroperoxide cleavage enzyme system in cucumber (*Cucumis sativus*) fruits. *Biochim. Biophys. Acta* 441:181–192.

GUERIN, P.M., and RYAN, M.F. 1980. Insecticidal effect of *trans*-2-nonenal, a constituent of carrot root. *Experientia* 36:1387–1388.

HEATHERBELL, D.A., and WROLSTAD, R.E. 1971. Carrot volatiles. 1. Characterisation and effects of canning and freeze drying. *J. Food Sci.* 36:219–224.

KAMADA, H., OKAMURA, N., MOTOYOSHI, S., HARADA, H., and SHIMOMURA, K. 1986. Alkaloid production by "hairy" root cultures in *Atropa belladonna*. *Plant Cell Rep.* 5:239–242.

LEEMANS, J., DEBLAERE, R., WILLMITZER, L., DE GREVE, H., HERNALSTEENS, J.P., VAN MONTAGUE, M., and SCHELL, J. 1982. Genetic identification of functions of T1-DNA transcripts in octopine crown galls. *EMBO J.* 1:147–152.

MAKI, A., and RYAN, M.F. 1989. Root-mediated effects in carrot resistance to the carrot fly, *Psila rosae*. *J. Chem. Ecol.* 15:1867–1882.

MURASHIGE, T., and SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Pl.* 15:473–497.

RHODES, M.J.C., HILTON, M., PARR, A.J., HAMILL, J.D., and ROBINS, R.J. 1986. Nicotine pro-

duction by "hairy" root cultures of *Nicotiana rustica:* fermentation and product recovery. *Biotech. Lett.* 8:415–420.

RYAN, M.F., and BYRNE, O. 1988. Plant–insect coevolution and inhibition of acetylcholinesterase. *J. Chem. Ecol.* 14:1965–1975.

RYAN, M.F., and GUERIN, P.M. 1982. Behavioural responses of the carrot fly larva, *Psila rosae*, to carrot root volatiles. *Physiol. Entomol.* 7:315–324.

RYAN, M.F., GUERIN, P.M., and BEHAN, M. 1978. Possible roles for naturally occurring chemicals in the biological control of the carrot fly, pp. 130–153 *in* J.J. Duggan (ed.). Biological Control Symposium of the Royal Irish Academy, Dublin.

SCRIVEN, R., and MELOAN, C.E. 1984. (*E,Z*)-2,6-Nonadien-1-al and (*E*)-2-nonen-1-al present in crushed cucumbers are natural repellents for the American cockroach (*Periplaneta americana*). *Ohio J. Sci.* 84:82–85.

TAHA, H.A. 1987. Studies on *trans*-2-nonen-1- al and *trans*-2,*cis*-6, nonadien-1-al as naturally occurring insecticides. PhD thesis. National University of Ireland, Dublin.

WILSON, G. 1988. Growing compounds from plants. *Technol. Ireland* 20:20–24.

ZIEG, R.G., ZITO, S.W., and STABA, E.J. 1983. Selection of pyrethrin producing tissue cultures. *Planta Med.* 48:88–91.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

DEFENSIVE STRATEGY OF TWO *Hypselodoris* NUDIBRANCHS FROM ITALIAN AND SPANISH COASTS

C. AVILA,[1,3] G. CIMINO,[1,*] A. FONTANA, M. GAVAGNIN,[1] J. ORTEA,[2] and E. TRIVELLONE[1]

[1]*Istituto per la Chimica di Molecole di Interesse Biologico, CNR via Toiano 6, 80072, Arco Felice, Naples, Italy*

[2]*Department of Biology of Organisms and Systems University of Oviedo, Spain*

(Received June 18, 1990; accepted November 19, 1990)

Abstract—*Hypselodoris* nudibranchs from different geographic areas (Spain and Italy) have been studied in order to investigate their general defensive strategy. Longifolin (**1**) and nakafuran-9 (**2**) are the main ichthyodeterrent allomones used by the mollusks to avoid predation. Evidence of their dietary origin is presented and the very effective strategy against predators, which includes secretion of allomones into the mucus and their storage into specific mantle dermal formations (MDFs), is also discussed.

Key Words—Opisthobranch, nudibranch, *Hypselodoris webbi, Hypselodoris villafranca*, sponge, *Dysidea fragilis*, chemical defense, longifolin, nakafuran-9, isotavacfuran .

INTRODUCTION

Nudibranchs are apparently unprotected shell-less mollusks. In spite of the absence of mechanical defense, only seldom they are victims of predators. In fact, nudibranchs have elaborated a series of very effective defensive strategies (Edmunds, 1966; Ros, 1977; Thompson, 1960), including the use of chemicals that generally derive from the diet and that sometimes are directly produced by the animal (Karuso, 1987).

The defensive allomones generally are localized in the mantle of the mol-

*To whom correspondence should be addressed.

[3]F.P.I.-M.E.C. Spanish fellowship (University of Barcelona) at the I.C.M.I.B.

0098-0331/91/0300-0625$06.50/0

lusks, and they can display either ichthyotoxic or feeding-deterrent properties. A rigorous test to evaluate these activities should be directed against predators living in the same habitat as the nudibranch. However, as this experimental procedure is difficult, it is generally accepted tests are performed in aquaria with freshwater fishes to detect ichthyotoxicity (*Gambusia affinis*) (Coll et al., 1982; Gunthorpe and Cameron, 1987) and feeding deterrency (*Carassius auratus*) (Cimino et al., 1982; Thompson et al., 1982).

During our studies of the defensive strategies of Mediterranean opisthobranchs (Cimino et al., 1982, 1985, 1986; Cimino and Sodano, 1989), we have observed that longifolin (**1**), already known as a plant metabolite (Hayashi et al., 1972), is the defensive allomone of Tyrrhenian *Hypselodoris webbi* (Cimino et al., 1982), displaying both ichthyotoxic and ichthyodeterrent activities. A dietary origin was strongly suspected for longifolin on the basis of its presence among the metabolites of the sponge *Pleraplysilla spinifera* (Cimino et al., 1975). However, it is unlikely that *H. webbi* preys upon *P. spinifera*. In fact, **1** is present in *P. spinifera* only as a minor component along with two major sesquiterpenoids (Cimino et al., 1975, 1978) that are absent in *H. webbi*.

It is interesting to observe that , to our knowledge, there are no reports regarding the diet of *H. webbi*. Recently (García-Gómez et al., 1990), **1** has been found also in populations of *H. webbi* from the Straits of Gibraltar; in particular, it is located in mantle dermal formations (MDF's) strategically placed near gills and rhinophores.

In this paper, we aim to clarify: (1) the origin of longifolin in *H. webbi;* (2) the distribution of the defensive allomone, or related metabolites, in specific organs and in the mucous secretion of the nudibranch; and (3) by a comparative study, the defensive strategy exhibited by populations of *H. webbi* and *H. villafranca* from the Spanish and Italian coasts.

METHODS AND MATERIALS

General Experimental Procedures. [^{1}H]- and [^{13}C]NMR spectra were performed in $CDCl_3$ or C_6D_6 on a WM 500 Bruker spectrometer coupled to an Aspect 2000 computer system. Chemical shifts expressed in δ units are reported in ppm downfield from the internal standard TMS ($\delta = 0$). Mass spectra were recorded with AEI MS 30 and Kratos MS 50 mass spectrometers.

Commercial Merck silica gel 60 (70–230 mesh ASTM) was used for column chromatography. Analytical TLC was carried out using precoated silica gel Merck F254 plates. The chromatograms were sprayed with either Ehrlich's reagent or 0.1% $CeSO_4$–2 N H_2SO_4 and heated to detect the spots. The extract of *H. villafranca* from Asturias also was analyzed on silica gel TLC treated with a solution of $AgNO_3$ (8% in acetone–water 8:2) and fractionated on a silica gel–$AgNO_3$ (8%) column.

Anatomical dissections were made observing the animal through a Swift Microscope (20–40×).

Biological Material. The sponge *Dysidea fragilis* was collected in Taranto (Mediterranean Sea) during October 1988 and in Asturias (Atlantic Ocean) during December 1988.

Collections of *H. webbi* were made from: Blanes, March 1990; Castellammare di Stabia, December 1988; and Taranto, August 1989. Specimens of *H. villafranca* were collected from Taranto (June and October 1988 and March 1989) and Asturias (December 1988). The location of these stations is shown in Figure 1.

All the Mediterranean material was collected either snorkeling or scuba-diving between 0.2 m and 10 m depth, whereas the Atlantic material was collected in the tidal zone. Mucous secretions were obtained disturbing the animals with a glass stick.

Anatomical Dissection. Frozen animals were usually dissected in four parts: mantle dermal formations (MDFs), rest of mantle and foot, digestive gland, and the rest of the viscera (reproductive organs, digestive duct, etc.).

Isolation Procedure of Liposoluble Material. All the biological material (sponge, whole nudibranchs, dissected parts, mucous secretion) was treated with acetone. The acetone extract was evaporated at reduced pressure and the residue treated with diethyl ether. Generally, the ethereal extracts were analyzed by SiO_2 TLC (petroleum ether) and fractionated on SiO_2 column (petroleum ether). In particular, the MDFs of *H. webbi* collected in Castellammare were treated with C_6D_6, and the extract was dried on Na_2SO_4 and then submitted to NMR analysis without any purification. The diethyl ether soluble fraction from the

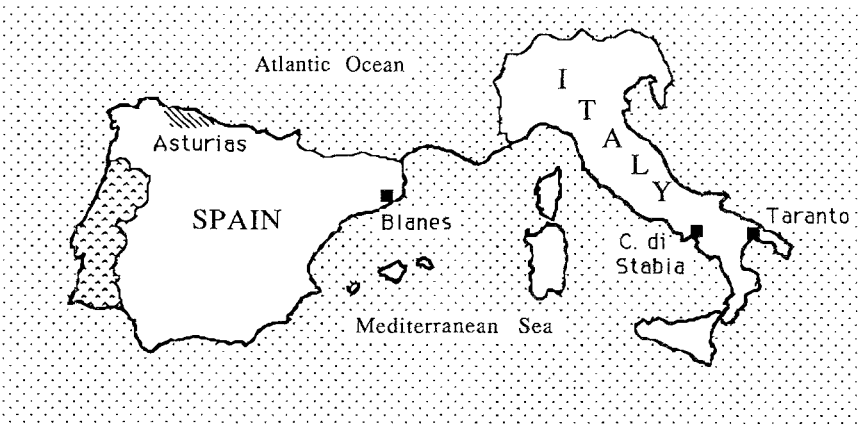


FIG. 1. Localities where *Hypselodoris* and sponges were collected.

acetone extract of Atlantic *H. villafranca* (24 specimens) was first eluted on silica gel column with *n*-heptane, obtaining longifolin (**1**) (6 mg) along with an unresolved mixture (15 mg) of less polar furanosesquiterpenoids. Further purification of the latter fraction on $AgNO_3$–SiO_2 (8%) column (*n*- heptane–benzene 9 : 1) gave nakafuran-9 (**2**) (4 mg). Furthermore, the highly unstable metabolite, isotavacfuran (**3**), of *H. webbi* (Blanes), was isolated, protecting the extracts from light during preparation for analysis.

RESULTS

Chemical Analysis. After extraction with acetone, dry weights of the dissected parts and amounts of the extracts from single specimens of *H. webbi* were determined (Table 1). Those of *H. villafranca* are summarized in Table 2.

All the specimens collected in a single area were extracted simultaneously. In the case of *H. webbi* from Castellammare, the mean value (obtained by halving the observed weights from two animals) was preferred for a better comparison with the other specimens. The observed differences are probably due to the different size of the mollusks. In particular, the amount of longifolin (**1**) in *H.*

TABLE 1. FURANOSEQUITERPENOIDS FROM DIFFERENT POPULATIONS OF *H. webbi*[a]

Geographic area	Sections	Dry weight[b]	Acetone extract	Longifolin (**1**)	Nakafuran-9 (**2**)	*Iso*tavacfuran (**3**)
Castellammare	MDF's	63.5	110.0	32.5		
(mean value of	Rest of mantle	564.0	184.0			
2 specimens)	Digestive gland	446.5	92.5	20.0		
	Rest of viscera	265.0	61.0			
Taranto	MDFs	152.0	98.0[c]	53.0	3.8	
(1 specimen)	Rest of mantle	647.2	156.8			
	Digestive gland	810.8	422.0	26.0	2.0	
	Rest of viscera	336.5	194.0			
Blanes	MDFs	46.5	29.1	10.0		14.0
(1 specimen)	Rest of mantle	244.6	83.4			
	Digestive gland	143.4	250.6	14.0		14.0
	Rest of viscera	56.4	21.1			

[a] All values are given in milligrams.
[b] After extraction with acetone.
[c] Diethyl ether extract.

TABLE 2. FURANOSESQUITERPENOIDS FROM DIFFERENT POPULATIONS OF *H. villafranca*[a]

Geographic area	Sections	Dry weight[b]	Acetone extract	Longifolin (1)	Nakafuran-9 (2)
Asturias	MDFs	4.0	11.5	6.0	4.0
(24 specimens)	Rest of mantle	115.0	35.0		
	Digestive gland	58.0	30.0	4.0	2.0
	Rest of viscera	95.0	13.0		
Taranto	MDFs	24.0	27.0	13.0	
(12 specimens)	Rest of mantle	53.0	18.0		
	Digestive gland	89.0	28.0	10.0	
	Rest of viscera	52.0	13.0		

[a] All values are given in milligrams.
[b] After extraction with acetone.

webbi from Blanes seems to be too scanty. However, the size of the animal was small, as supported by the dry weights, and another sesquiterpenoid, isotavacfuran (**3**), cooccurs in the same organism. Analogously, the main differences observed for *H. villafranca* can be explained by: (1) the anatomical dissections were made by different operators (in particular, the MDFs from Asturias were completely devoid of other tissues); (2) the minor amounts of **1** in population from Asturias are counterbalanced by the presence of other furanosesquiterpenoids; and (3) the specimens were collected in different seasons.

The [^{1}H]NMR spectrum of longifolin (**1**) from the MDFs of a specimen of *H. webbi* collected in Castellammare is reported in Figure 2. Moreover, 25 mg of longifolin (**1**) were also isolated from the acetone extract of the mucous secretion of the same animal. The [^{1}H]- and [^{13}C]NMR data for **1, 2** and isotavacfuran (**3**) are reported below. The assignments have been supported by a series of two-dimensional NMR experiments and confirmed by ^{1}H–^{1}H selected decouplings.

Moreover, we have obtained from Atlantic *Dysidea fragilis* (dry weight 19 g) 10 mg of nakafuran-9 (**2**), whereas *Dysidea fragilis* from Taranto (dry weight 1 g) yielded 6 mg of pure longifolin (**1**).

Spectral Data of 1, 2, and 3. Longifolin (**1**): (3-methyl-2-[(2′*E*)-3′-methyl-4′-(4″-methyl-2″-furyl)-2′-butenyl] furan; $C_{15}H_{18}O_2$ EI-MS *m/z:* 230 (M^+); [^{1}H]NMR (C_6D_6): 1.60 (3H, s, CH_3- 3′); 1.78 (3H, s, CH_3-4″); 1.81 (3H, s, CH_3-3); 3.16 (2H, s, H-4′); 3.22 (2H, d, J= 7.0 Hz, H-1′); 5.46 (1H, t, J= 7.0 Hz, H-2′); 5.77 (1H, bs, H-3″); 6.00 (1H, d, J = 1.5 Hz, H-4); 6.92 (1H, bs, H-5″); 7.07 (1H, d, J = 1.5 Hz, H-5). [^{13}C]NMR (C_6D_6): 9.7 (CH_3-4″ and CH_3-3); 15.8 (CH_3-3′); 25.5(C-1′); 38.6 (C-4′); 109.2 (C-3″); 113.0 (C-

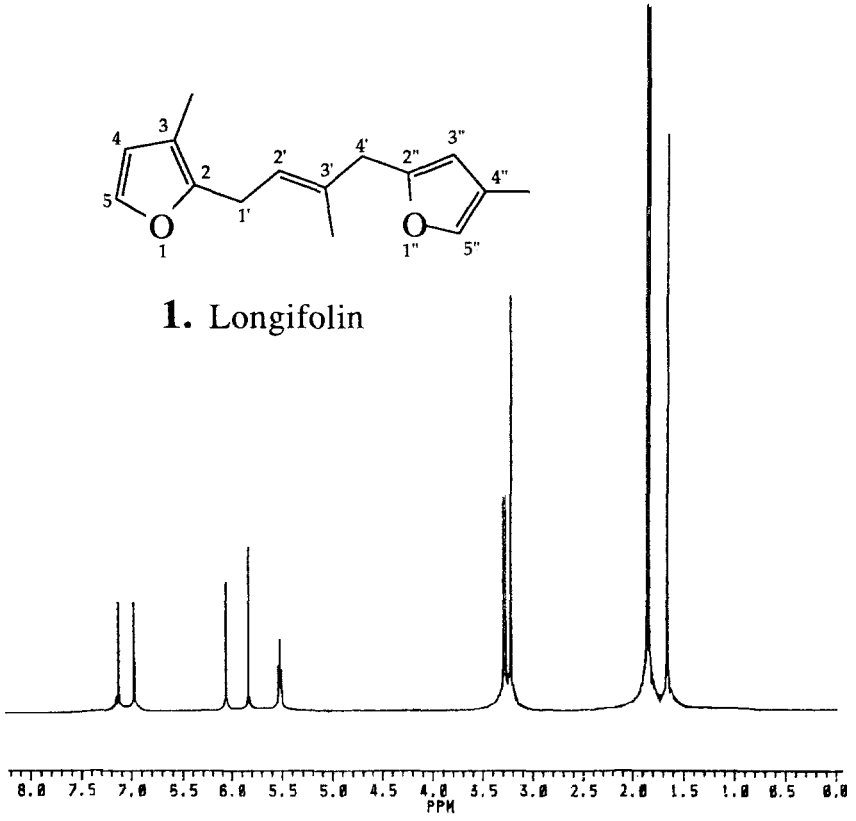


FIG. 2. [^{1}H]NMR spectrum of (**1**) (40 mg without any purification) from the MDFs of a single *H. webbi* from Castellammare di Stabia.

4); 113.7 (C-4″); 120.5 (C-3); 122.8 (C-2′); 133.2 (C-3′); 138.0 (C- 5″); 140.1 (C-5); 149.8 (C-2); 154.3 (C-2″).

The [^{1}H]NMR spectrum in C_6D_6 is reported in Figure 2, and numeration is according to Guella et al., (1985b). The assignments, already reported in the quoted paper, have been confirmed by two dimensional NMR experiments: ^{1}H-^{1}H COSY, ^{1}H-^{13}C HETCOR.

Nakafuran-9 (**2**): $[\alpha]_D = -107.5$ (c = 0,2 $CHCl_3$); C_{15} H_{18} O_2 EI-MS *m/z* (%): 216 (M^+, 100), 201 (55), 175 (35). [^{1}H]NMR ($CDCl_3$): 1.08 (3H, s, H-15's); 1.37 (1H, ddd, J = 4.1, 13.3 and 13.3 Hz, H-5a); 1.57 (3H, s, H-13's or H-14's); 1.59 (3H, s, H-14's or H-13's); 1.72 (1H, ddd, J = 2.7, 2.7 and 13.0 Hz, H-12a), 1.79 (1H, ddd, J = 1.8, 4.3 and 13.0 Hz, H-12b); 1.92 (1H, bddd, J = 4.1, 4.1 and 13.3 Hz, H-5b); 1.95 (1H, bd, J = 15.5 Hz H-9a); 2.25 (1H, ddd, J = 4,1, 13.3 and 16.0 Hz, H-4a); 2.33 (1H, ddd, J

= 4.1, 4.1 and 16.0 Hz, H-4b); 2.37 (1H, bdd, J = 5.2 and 15.5, H-9b); 3.17 (1H, m, H-10); 6.06 (1H, d, J = 1.7 Hz, H-2); 7.12 (1H, d, J = 1.7 Hz, H-1). [^{13}C]NMR ($CDCl_3$): 13.2 (C-14); 19.8 (C-13); 23.0 (C-4); 30.7 (C-15); 31.8 (C-10); 37.6 (C-6); 38.2 (C-9); 38.6 (C-5); 41.3 (C-12); 113.1 (C-2); 118.3 (C-3); 126.3 (C-7 or C-8); 129.5 (C-8 or C-7); 138.4 (C-1); 156.2 (C-11).

Numeration is according to Schulte et al. (1980). The assignments have been supported by ^{1}H-^{1}H decoupling experiments, ^{1}H-^{1}H COSY, and ^{1}H-^{13}C HETCOR. The comparison with the previous reported NMR data has revealed some minor differences, in particular for the [^{13}C]NMR resonances assigned to the methyls.

Isotavacfuran (**3**): (3-methyl-2-[(3′*E*) -3′-methyl-4′-(4″-methyl-2″-furyl)-3′-butenyl] furan C_{15} H_{18} O_2 EI-MS *m/z:* 230 (M^+); [^{1}H]NMR (C_6D_6): 1.77 (6H, s, CH_3-3 or CH_3-4″); 1.78 (3H, s, CH_3-4″ or CH_3-3); 1.88 (3H, s, CH_3-3′); 2.34 (2H, t, J = 7.5 Hz, H-2′); 2.60 (2H, t, J = 7.5 Hz, H-1′); 5.95 (1H, bs, H-3″); 5.98 (1H, d, J = 1.5 Hz, H-4); 6.11 (1H, bs, H-4′); 6.91 (1H, bs, H-5″); 7.08 (1H, d, J = 1.5 Hz, H-5).

Numeration and assignments are according to Guella et al. (1985b).

DISCUSSION

Hypselodoris webbi is a well-studied opisthobranch with a very broad distribution in Atlantic and Mediterranean waters. This animal has received the names of *Doris scacchi* Delle Chiaje, 1830; *Doris elegans* Cantraine, 1835; *Doris picta* Philippi, 1836; *Doris valenciennesi* Cantraine, 1840; *Doris nardi* Vérany, 1846; and *Doris calcarea* Vérany, 1846. However, this species should be named *Hypselodoris webbi* D'Orbigny, 1839, according to Bouchet and Ortea (1980). All the references to the specific names *elegans* or *valenciennesi* in the genus *Hypselodoris, Doris*, or *Glossodoris* found in Atlantic and Mediterranean literature should be attributed to *H. webbi*.

Surprisingly, in spite of being one of the better studied Chromodorididae, data regarding the diet of *H. webbi* are not reported in the literature. On the basis of the presence of large amounts of longifolin (**1**) in its digestive gland, predation of the nudibranch upon an unknown sponge was suspected (Cimino et al., 1982). However, the real origin of the ichthyotoxic and feeding deterrent **1** is uncertain. In fact, its finding in *H. webbi* specimens living in different geographical areas (García-Gómez et al., 1990) could support de novo biosynthesis (Faulkner, 1988b).

Aiming at clarification, we have extended our studies to possible prey living in both Italian and Spanish *H. webbi* habitats, with particular attention to *Dysidea* sponges, known to be the prey of Pacific *Hypselodoris* species (Faulkner, 1988a; Karuso, 1987).

Further studies also have been performed on the small *H. villafranca* (Risso, 1818) (=*Glossodoris gracilis*), which also contains longifolin (**1**) and which, on the basis of this evidence, was deemed to be a sponge predator (Cimino et al., 1980).

H. webbi was collected from three different stations (Castellammare di Stabia, Taranto, and Blanes). Large amounts of **1** were present in all the populations (Table 1). The anatomical dissections (according to García-Gómez et al., 1986, 1990) confirmed that the presence of **1** on the mantle is limited to the MDFs. In particular, the MDFs from a Tyrrhenian specimen were extracted with C_6D_6 and, without any purification, analyzed directly by NMR. The [^{1}H]NMR spectrum (Figure 2) revealed the presence of very abundant and pure **1**.

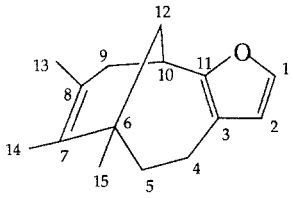
Large amounts of longifolin (**1**) also were found in the digestive gland (Table 1) and in the mucous secretion of the nudibranch (experimental).

The chemical analysis of the organic molecules from *H. webbi* populations (Taranto and Blanes) confirmed the presence of **1**, but also revealed the cooccurrence of other furanosesquiterpenoids (Table 1). More exactly, nakafuran-9 (**2**), the already known feeding deterrent allomone of the Hawaian nudibranchs *H. godeffroyana* and *Chromodoris maridadilus* (Schulte et al., 1980), was characterized as a minor component from the Italian nudibranchs, while, as already reported (García-Gómez et al., 1990), a second slightly less polar, Ehrlich-positive metabolite of **1**, was detected by TLC analysis of the ethereal extract of the Spanish mollusks. In spite of its high instability, it was characterized after a careful isolation procedure. The spectral data were identical to those of isotavacfuran (**3**) already described as the synthetic isomer of the sponge metabolite tavacfuran (**4**) (Guella et al., 1985b) (Figure 3).

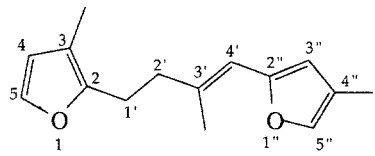
Substantially coherent results have been obtained studying *H. villafranca* from two different stations (Taranto and Asturias). From the Italian specimens, **1** was isolated as the main metabolite, while the organic extracts from the Spanish animals were characterized by the presence of a complex mixture of furanosesquiterpenoids, which includes **1** and nakafuran-9 (**2**) as major components (Table 2).

Analogously, in the anatomical dissection (Table 2) of *H. villafranca*, the allomones were found in the MDFs, in the digestive gland, and in the mucous secretion of the mollusk. On the contrary, they were completely absent in both the mantle devoid of MDFs and the rest of the viscera devoid of digestive glands. The number of MDFs seems to be related to the animal size: we observed three to five of them in the anterior part and two to five in the posterior part of *H. villafranca*, and 15–30 in the anterior part (sometimes continuously) and 14–20 in posterior part of *H. webbi*.

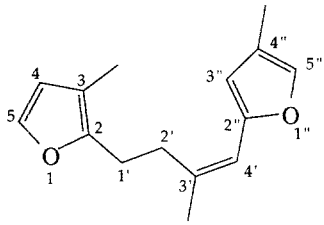
It seems that both these nudibranchs (Figure 4) have elaborated an artful defensive strategy. When molested, these mollusks secrete a white mucus full



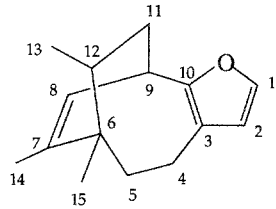
2. Nakafuran-9



3. iso-Tavacfuran



4. Tavacfuran



5. Nakafuran-8

FIG. 3. Furanosesquiterpenoid structures **2, 3, 4,** and **5.**

of ichthyodeterrent furanosesquiterpenoids. However, if the predator bites the prey, the MDFs explode, delivering further amounts of antifeedant compounds to defend gills and rhinophores, vital organs for the animals. However, the origin of the defensive allomones remains to be clarified.

During the collection of biological material in Taranto, *H. villafranca* was observed while it was feeding on a white sponge, identified as *Dysidea fragilis*. The chemical analysis revealed large amounts of **1** in the sponge, supporting a prey–predator relationship between *D. fragilis* and *H. villafranca*. Until now, in spite of the widespread presence of *H. webbi*, it was not possible to detect such relationships with any sponge. In fact, *H. webbi* usually was observed swimming or moving on bare rock. The lack of field observation could be due either to night diet or to other reasons. The above reported data on *H. villafranca* stimulated other direct observations on *H. webbi* (Mollo, unpublished results) in an aquarium. Specimens of *H. webbi* collected in Sorrento (20 km from Castellammare di Stabia) were observed grazing upon *D. fragilis*. Simultaneously, the levels of the allomone **1** sharply decreased in starved individuals maintained for some days in tanks without sponges.

Analogously, the probable sponge origin of nakafuran-9 (**2**) was supported by the chemical analysis of *D. fragilis* collected in Asturias. In fact, abundant **2** was detected in lipid extracts from the Atlantic sponge, which should be one of the prey of the Atlantic *H. villafranca*. It is worth noting that previous chem-

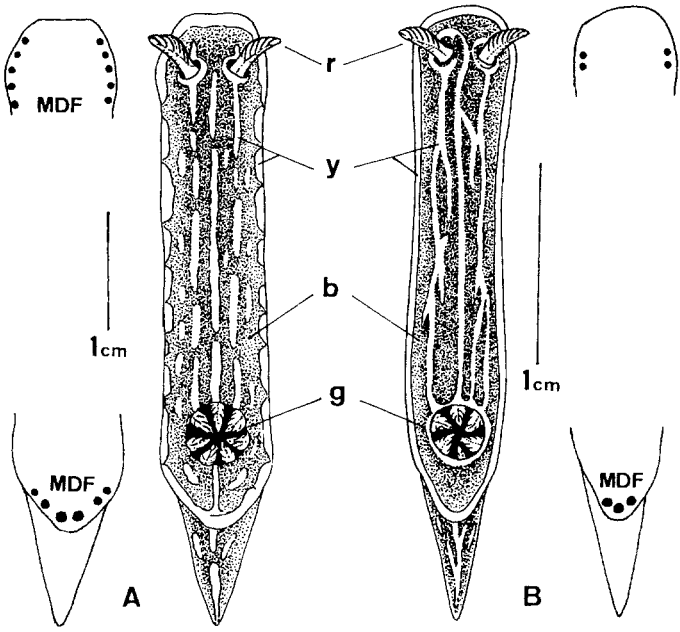


FIG. 4. Dorsal view of *H. webbi* (A) and *H. villafranca* (B) and schematic distribution: r = rhinophores, y = yellow, b = blue, g = gills.

ical studies on another specimen of Atlantic (North Brittany Sea) *D. fragilis* (Guella et al., 1983, 1985a) revealed a different pattern of furanosesquiterpenoids, while *D. fragilis* collected at Hawaii (Schulte et al., 1980) contains **2** along with its isomer nakafuran-8 (**5**).

According to these data, we can suggest both species of *Hypselodoris* nudibranchs from Italian and Spanish coasts live in communities where *Dysidea* (or related) sponges are not rare, and they sequester from the sponges some feeding deterrent metabolites that are helpful for their own defense against predators.

In particular, *H. webbi* is able to select in different geographical areas the variety of *Dysidea fragilis* very rich in longifolin (**1**). However, on the basis of chemical analysis, sometimes its diet may be extended to other *Dysidea* (or related) species. In fact, in both the Spanish and Italian populations of *H. webbi*, longifolin (**1**) cooccurs with other metabolites that are completely absent in the extracts of the Italian *D. fragilis*, but that are known to be characteristic (nakafuran-9) or closely related (isotavacfuran) to *Dysidea* metabolites. The extraordinary mechanism that brings the dietary defensive sesquiterpenoids into the MDFs remains to be clarified. Most likely, the allomones are stored in these protected formations to avoid the dangerous effects of its own weapon to the animal. Surprisingly, no openings were observed in the MDFs, as reported in

the paper of García-Gómez et al. (1986), the first to suggest a defensive role for the MDFs.

Both *Hypselodoris* species are conspicuous in appearance (Figure 4): they present a dark blue body or white–yellow body with some yellow longitudinal lines and points. MDFs are always camouflaged in the yellow border of the mantle. They are aposematic species, exhibiting Müllerian mimicry, together with other *Hypselodoris* species, as was predicted by Ros (1976) studying coloration. The presence of the same kind of defensive compounds in different species further supports this assumption. Actually, *H. cantabrica* and *H. tricolor* seem to be related in that they present the same kind of allomones.

Acknowledgments—We thank Miss D. Rosell (C.E.A.B.-C.S.I.C., Blanes, Spain) for identifying the sponges. We also thank Mr. A. Crispino, Mr. A. Trabucco, Mr. R. Turco, and Mr. G. Villani for their valuable technical assistance. Thanks are extended also to the staff of the ICMIB NMR Service. This work is part of a CSIC (Spain)-CNR (Italy) bilateral project, and it is partially supported by the "Progetto Finalizzato Chimica Fine II" CNR-Roma.

REFERENCES

BOUCHET, P., and ORTEA, J. 1980. Quelques Chromodorididae blues (Mollusca, Gastropoda, Nudibranchiata) de l'Atlantique Oriental. *Ann. Inst. Oceanogr. Paris*, 56(2):117–125.

CIMINO, G., DE ROSA, S., DE STEFANO, S., MORRONE, R., and SODANO, G. 1985. The chemical defense of nudibranch molluscs. *Tetrahedron* 41:1093–1100.

CIMINO, G., DE ROSA, S., DE STEFANO, S., and SODANO, G. 1982. The chemical defense of four Mediterranean nudibranchs. *Comp. Biochem. Physiol.* 73(B):471–474.

CIMINO, G., DE ROSA, S., DE STEFANO, S., and SODANO, G. 1986. Marine natural products: New results from Mediterranean invertebrates. *Pure Appl. Chem.* 58:375–386.

CIMINO, G., DE STEFANO, S., DE ROSA, S., SODANO, G., and VILLANI, G. 1980. Novel metabolites from some predator–prey pairs. *Bull . Soc. Chim. Bel.* 89:1069–1073.

CIMINO, G., DE STEFANO, S., MINALE, L., and TRIVELLONE, E. 1975. Furanosesquiterpenoids in sponges V. Spiniferins from *Pleraplysilla spinifera. Tetrahedron Lett.* 16:3727–3730.

CIMINO, G., DE STEFANO, S., MINALE, L., and TRIVELLONE, E. 1978. Furanosesquiterpenoids in sponges VI. Further structural studies for spiniferins, sesquiterpenes from *Pleraplysilla spinifera. Experientia* 34:1425–1427.

CIMINO, G., and SODANO, G. 1989. The chemical ecology of Mediterranean Opisthobranchs. *Chem. Scripta* 29:389–394.

COLL, J.C., LA BARRE, S., SAMMARCO, P.W., WILLIAMS, W.T., and BAKUS, G.T. 1982. Chemical defences in soft corals (Coelenterata: Octocorallia) of the Great Barrier Reef: A study of comparative toxicities. *Mar. Ecol. Prog. Ser.* 8:271–278.

EDMUNDS, M., 1966. Protective mechanisms in the Eolidacea (Mollusca Nudibranchia). *J. Linn. Soc. (Zool.)* 46:27–71.

FAULKNER, D.J. 1988a. Marine natural products. *Nat. Prod. Rep.* 5(6):613–663 (and previous reports in the same series).

FAULKNER, D.J. 1988b. Feeding deterrents in molluscs. *Mem. Calif. Acad. Sci.* 13:29–36.

GARCÍA-GÓMEZ, J.C., COVENAS, R., MEDINA, A., and LOPEZ-CAMPOS, J.L. 1986. Study of the anatomy and histology of the dermal formations (DFs) of chromodorid nudibrancs (Gastro-

poda: Opisthobranchia), Abstracts, 9th International Malacological Congress, Edinburgh. p. 28.

GARCÍA-GÓMEZ, J.C., CIMINO, G., and MEDINA, A. 1990. Studies on the defensive behaviour of *Hypselodoris* (Gastropoda, Nudubranchia): Ultrastructure and chemical analysis of mantle dermal formations (MDFs). *Mar. Biol.* 106:245–250.

GUELLA, G., GUERRIERO, A., TRALDI, P., and PIETRA, F. 1983. Penlanfuran, a new sesquiterpene from the marine sponge *Dysidea fragilis* (Mont) of Brittany. A striking difference with the same Hawaiian species. *Tetrahedron Lett.* 64:3897–3898.

GUELLA, G., GUERRIERO, A., and PIETRA, F. 1985a. Sesquiterpenoids of the sponge *Dysidea fragilis* of the North Brittany Sea. *Helv. Chim. Acta* 68:39–48.

GUELLA, G., MANCINI, I., GUERRIERO, A., and PIETRA, F. 1985b. New furano-sesquiterpenoids from Mediterranean Sponges. *Helv. Chim. Acta* 68:1276–1282.

GUNTHORPE, L., and CAMERON, A.M. 1987. Bioactive properties of extracts from Australian dorid nudibranchs. *Mar. Biol.* 94:39–43.

HAYASHI, N., KOMAE, H., EGUCHI, E., NAKAYAMA, N., HAYASHI, S., and SAKAO, T. 1972. Two new furanosesquiterpenes from *Actinodaphne longifolia. Chem. Ind.* 14:572–573.

KARUSO, P. 1987. Chemical ecology of the nudibranchs, pp.31–60, *in* P.J. Scheuer (ed.). Bioorganic Marine Chemistry, Vol. 1. Springer-Verlag, New York.

ROS, J. 1976. Sistemas de defensa en los opistobranquios. *Oecol. Aquat.* 2:41–77.

ROS, J. 1977. La defensa en los opistobranquios. *Invest. Cienc.* 12:48–60.

SCHULTE, G.R., SCHEUER, P.J., and MCCONNELL, O.J. 1980. Two furanosesquiterpene marine metabolites with antifeedant properties. *Helv. Chim. Acta* 63(8):2159–2167.

THOMPSON, T.E. 1960. Defensive adaptations in opistobranchs. *J. Mar. Biol. Assoc.* U.K. 39:123–134.

THOMPSON, T.E., WALKER, R.P., WRATTEN, S.J., and FAULKNER, D.J. 1982. A chemical defense mechanism for the nudibranch *Cadlina luteomarginata. Tetrahedron* 38:1865–1873.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

IDENTIFICATION OF FLORAL COMPOUNDS FROM *Abelia grandiflora* THAT STIMULATE UPWIND FLIGHT IN CABBAGE LOOPER MOTHS

KENNETH F. HAYNES,* JING ZHANG ZHAO, and ABDUL LATIF

Department of Entomology
University of Kentucky
Lexington, Kentucky 40546

(Received September 18, 1990, accepted November 19, 1990)

Abstract—Four major volatile components emitted from flowers of *Abelia grandiflora* were identified based on retention time using two capillary columns of different polarities and electron impact mass spectrometry. These are phenylacetaldehyde, benzaldehyde, 2-phenylethanol, and benzyl alcohol. A blend of these compounds was as effective as a cluster of flowers in stimulating upwind flight by male *Trichoplusia ni* to the source in a wind-tunnel test. Phenylacetaldehyde or 2-phenylethanol were each as effective as the complete blend in stimulating source location by male moths. Attraction to a source of the synthetic blend was demonstrated in virgin males and females and mated males and females, but virgin moths of both sexes were more likely than mated moths to complete the sequence of behavioral responses necessary to locate the odor source.

Key Words—Lepidoptera, Noctuidae, *Trichoplusia ni*, floral attractants, anemotaxis, wind tunnel.

INTRODUCTION

Volatile chemicals emitted from plants facilitate host-finding in a wide range of insects. In moths, such volatile chemicals often enable females to locate suitable oviposition sites (Phelan and Baker, 1987; Liu et al., 1988; Ramaswamy, 1988; Haynes and Baker, 1989; Landolt, 1989; Tingle et al., 1989) but may also allow male and female adults to find their own food resources (Wiesenborn and Baker, 1990). Feeding by adult female moths may improve reproductive suc-

*To whom correspondence should be addressed.

0098-0331/91/0300-0637$06.50/0

cess by increasing egg production, extending the ovipositional period, improving ovipositional host-finding success, and/or increasing resources allocated to each egg. Feeding by adult males may improve mate-finding success, increase longevity and the associated chances for multiple mating, or increase "nutritive" spermatophore size, thereby indirectly improving reproductive success. Adult cabbage looper moths, *Trichoplusia ni* (Hübner) and other Plusiinae feed on nectar from the ornamental shrub *Abelia grandiflora* (André), (honeysuckle family, Caprifoliaceae); both male and female moths are attracted to these flowers from sunset to 1 AM (Grant, 1971a). Olfactory stimuli play an important role in mediating nectar-finding by *T. ni*, but the chemical composition of these cues has not been defined.

METHODS AND MATERIALS

Abelia grandiflora plants were maintained in a greenhouse (maximum daytime temperature 35°C, minimum nighttime temperature 21°C). Volatile collections were conducted for 24 hr from flower clusters of six *A. grandiflora* plants. Volatile floral compounds were collected on 70 mg of Tenax-TA (Alltech, Deerfield, Illinois), which had been loaded into a glass tube (0.5 cm diam. × 5.5 cm long) plugged at both ends with glass wool. The large open ends of two glass funnels (10 cm diam.) were butted against one another to enclose a flower cluster containing 6–10 open flowers. The funnels were sealed together with Teflon tape and supported in position with a ringstand to avoid stress to the plant stem. A Tenax trap was attached to the 3.0-cm stems of both funnels with a 2-cm section of Tygon tubing. Air was drawn through the system at 175–200 ml/min via a vacuum pump attached to one of the traps. Approximately 400 μl of HPLC- grade hexane was added to the Tenax and was allowed to drip into a graduated vial. Additional hexane was added to the Tenax until exactly 600 μl was recovered in the vial. One hundred microliters of this solution was combined with 100 μl of a 100 ng/μl solution of (*R*)-(+)-1-phenylethanol (internal standard). One microliter of this solution was injected into a Hewlett-Packard (Avondale, Pennsylvania) 5890A gas chromatograph linked to a Hewlett-Packard 5970B mass selective detector (MSD). The gas chromatograph was equipped with a 30-m DB-Wax capillary column (J&W Scientific, Folsom, California, 0.25 μm film thickness, 0.25 mm diam.). The oven temperature was held at 40°C for 2 min and then increased by 10°C/min to a final temperature of 210°C. The MSD scanned from 40 to 400 *m/z*. Analyses were also conducted using 30-m DB-5 capillary column (J&W Scientific, 0.25 μm film-thickness, 0.25 mm diam.) with the same temperature program. Identifications were based on comparisons of retention times and mass spectra of the floral volatiles to authentic phenylacetaldehyde (Aldrich Chem. Co., Milwaukee, Wisconsin),

2-phenylethanol (Sigma Chem. Co., St. Louis, Missouri), benzaldehyde (Sigma) and benzyl alcohol (Sigma).

Cabbage looper larvae were reared on an artificial diet following the procedures described by Shorey and Hale (1965). Pupae were separated according to sex. Pupae and adults were held in 3.8-liter paper cartons with nylon screen lids. Males and females were maintained in separate environmental chambers under a photoperiod of 16:8 (light–dark) with temperature cycling from 25°C during the photophase to 22°C during the scotophase. Adults had access to a cotton pad saturated with 10% sucrose–water. In most of the bioassays virgin males were used, but when mated adults were required, 2- to 3-day-old virgin male and female moths were placed together 4 hr into the scotophase in a 40 × 46 × 40-cm (l × w × h) observation box with a clear Plexiglas top that allowed observations to be made under dim red light. Pairs in copula were transferred from the box to 0.5-liter cartons. At the initiation of the photophase, mated males and females were sorted into separate containers by observing the dorsal long abdominal scales found only on males (Grant, 1971b).

To conduct wind-tunnel tests for behavioral responses of *T. ni* to floral volatiles, 3- to 4-day-old adult moths were placed into hardware cloth cylinders (8 × 4.5 cm diam.) with aluminum lids before the start of the scotophase. A wind tunnel similar to that described by Miller and Roelofs (1978) was used. These behavioral assays were conducted under dim red light 0–2 hr after the initiation of a scotophase. Moths were released individually 1.5 m downwind of the stimulus source. Behavioral responses to the test stimulus were noted in the following categories: wing fanning in the release cage (WF), upwind flight in the odor plume (UWF, consisting of repeated counterturns directly downwind of the stimulus source), the distance of the moth's closest approach to the test stimulus, and source contact (SC).

A randomized complete block design was used for four experiments. An arcsine-square root transformation was used on percentage data before analyses of variance were performed. The first experiment included the following test stimuli: a cluster of 10 *A. grandiflora* flowers whose stem was placed in a 4-dram vial with water, and 0.1, 1.0, and 10.0 mg of phenylacetaldehyde in 100 μl of hexane. In all experiments, this volume was delivered onto ca. 35 mg of cotton sealed in the hollow interior of a polyethylene vial cap that had a removable lid (vial closure, 1.9 cm diam. × 1.2 cm high). A 3-mm hole was cut in the cap to increase the rate of volatilization from this source. This experiment consisted of nine blocks with five moths per treatment per block. In the second experiment, a blend of synthetic chemicals was prepared to approximate the blend ratios of compounds identified in volatile emissions from *A. grandiflora* flowers (1.0 mg phenylacetaldehyde, 0.77 mg 2- phenylethanol, 0.80 mg benzaldehyde, 0.13 mg benzyl alcohol in 100 μl of hexane). This synthetic blend and 100 μl of hexane (blank) were delivered to the plastic caps as above.

Again, 10 *A. grandiflora* flowers served as a positive control. Twelve blocks with five moths per treatment per block were used. In the third experiment, an evaluation of male moths' responses to the blend of synthetic compounds was compared to each compound individually (1 mg) (eight blocks, five moths per treatment per block). In the last experiment, an evaluation of the responses of virgin males and females and mated males and females to the complete synthetic blend vs. hexane blank was conducted (eight blocks, five moths per treatment for each sex and mating status per block).

RESULTS

Compounds with retention times (RT) identical to authentic phenylacetaldehyde, 2-phenylethanol, benzaldehyde, and benzyl alcohol were found in volatile collections from flower clusters using relatively polar (DB-Wax, Figure 1) and relatively nonpolar (DB-5) capillary columns. These identifications were supported by mass spectra. Authentic benzaldehyde and the first unknown were characterized by ions at *m/z* 51, 77 (base peak), 105, and 106 (molecular ion). Synthetic phenylacetaldehyde and an unknown peak with RT identical to this

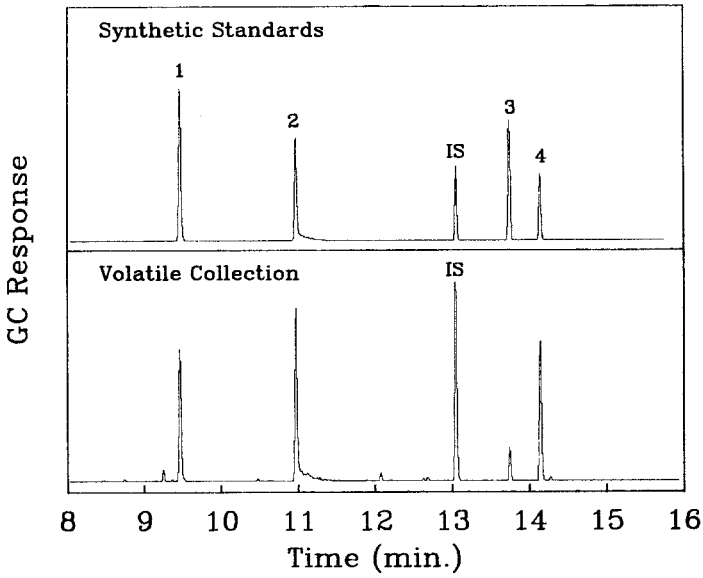


FIG. 1. Total ion chromatograms of synthetic standards and volatile collection from *Abelia grandiflora* flowers. The internal standard (IS) was (*R*)-(+)-1-phenylethanol.**1** = benzaldehyde, **2** = phenylacetaldehyde, **3** = benzyl alcohol, **4** = 2- phenylethanol.

compound were characterized by ions at *m/z* 65, 91 (base peak) and 120 (molecular ion). A third floral compound and authentic benzyl alcohol were characterized by ions at *m/z* 79 (base peak), 107, and 108 (molecular ion). Synthetic 2-phenylethanol and a fourth floral compound were characterized by ions at *m/z* 65, 91 (base peak), and 122 (molecular ion).

The quantity of phenylacetaldehyde, 2-phenylethanol, benzaldehyde, and benzyl alcohol emitted from *A. grandiflora* flowers in a 24-hr period was determined using two capillary columns (Table 1). Results using the DB-Wax column and MS detector indicated that phenylacetaldehyde was the most abundant compound recovered from these collections (37.4%), followed by benzaldehyde (33.3%), 2-phenylethanol (24.2%), and benzyl alcohol (5.1%). Results were similar using a DB-5 column and a flame ionization detector. The results from the DB-Wax column were used to formulate a blend of synthetic compounds that mimics the proportions of compounds found in volatile collections from flowers.

In the first behavioral experiment, 38% of male moths flew upwind and contacted a plastic cap containing 0.1 mg of phenylacetaldehyde, which was significantly lower than the response stimulated by a cluster of flowers (76%, overall $F = 7.7$, $P < 0.05$, analysis of variance, Scheffé's multiple comparison procedure). The percentage of males contacting sources containing 1.0 mg (60%) and 10.0 mg (67%) was not significantly different from the response to the flower cluster ($P > 0.05$). One milligram of phenylacetaldehyde was selected as the standard dose for additional experiments.

The synthetic blend of floral compounds stimulated wing fanning, upwind flight, flight to within 0.5 m, and source contact at levels not significantly different from levels stimulated by a cluster of *Abelia* flowers (Table 2). Of those

TABLE 1. QUANTITIES OF VOLATILE CHEMICALS EMITTED FROM *Abelia grandiflora* FLOWERS AND TRAPPED ON TENAX-TA[a].

Compound	Quantity per flower ± SEM (μg)[b]	
	DB-Wax[c]	DB-4[d]
Phenylacetaldehyde	4.04 ± 0.63	4.84 ± 0.65
2-Phenylethanol	2.62 ± 0.22	1.75 ± 0.45
Benzaldehyde	3.60 ± 0.78	4.27 ± 0.88
Benzyl alcohol	0.55 ± 0.17	0.64 ± 0.36

[a] Collections were run for 24 hr ($N = 6$) and were made from flower clusters on six different potted *Abelia grandiflora* plants.
[b] Flower clusters contained 6–10 flowers, but quantity per flower was calculated.
[c] 30-m DB-Wax capillary column.
[d] 30-m DB-5 capillary column.

TABLE 2. BEHAVIORAL RESPONSES OF MALE *Trichoplusia ni* TO HEXANE CONTROL (BLANK), CLUSTER OF *Abelia grandiflora* FLOWERS, AND BLEND OF SYNTHETIC COMPOUNDS IDENTIFIED FROM THESE FLOWERS ($N = 12$)[a]

Treatment	Behavioral response ± SEM (%)[b]			
	WF	UPW	≤0.5 m	SC
Blank	73.3 ± 4.5b[c]	1.7 ± 1.7b	0 b	0 b
Flowers	86.7 ± 3.8ab	56.7 ± 7.3a	53.3 ± 7.5a	53.3 ± 7.5a
Synthetic Blend[d]	91.7 ± 3.0a	65.0 ± 6.1a	56.7 ± 6.0a	56.7 ± 6.0a

[a] Twelve experimental blocks, each block consisted of five moths released individually to a specific test stimulus.

[b] WF = wing fanning in the release cage; UPW = upwind flight in odor plume; ≤0.5 m = moth progressed to within 0.5 m of the test stimulus source; SC = source contact.

[c] Means in the same column are significantly different if they do not share a letter in common ($P < 0.05$, ANOVA and Scheffé multiple comparison procedure).

[d] The synthetic blend consisted of 1 mg phenylacetaldehyde, 0.77 mg 2-phenylethanol, 0.80 mg benzaldehyde, and 0.13 mg benzyl alcohol applied to plastic cap.

moths that initiated upwind flight to flowers or the synthetic blend, most completed this flight by contacting the source (94.0 and 87.2%, respectively). Wing fanning in the release cage was not a good assay for activity of test stimuli, because the percentage of moths exhibiting this behavior was high even for the hexane (blank) treatment.

The synthetic blend was not significantly better in stimulating source contact than two of its components, phenylacetaldehyde or 2-phenylethanol (Table 3). While moderate numbers of male moths initiated upwind flight to benzaldehyde or benzyl alcohol, very few of these were able to maintain their upwind progress and contact the source.

The synthetic blend stimulated upwind flight to the source in virgin males and females and in mated males and females (Figure 2). There was a significant difference between the blank and synthetic blend regardless of mating status or sex of the moths in terms of the percentage of moths that fanned their wings, initiated upwind flight, progressed to within 0.5 m of the source, and contacted the source ($P < 0.05$, $F = 25$, 103, 140, 196, respectively). Interestingly, the mating status of the moth also had a significant effect on upwind flight, flying to within 0.5 m, and contacting the source ($P < 0.05$, $F = 11$, 7, 7, respectively). Mated moths were less likely to complete this sequence of behaviors than were virgins. Males and females were equally likely to exhibit each of the behaviors recorded ($P > 0.05$, $F = 0$, 0.1, 0.1, 0.3, in sequence).

TABLE 3. BEHAVIORAL RESPONSES OF MALE *Trichoplusia ni* TO FOUR VOLATILE CHEMICALS IDENTIFIED FROM *Abelia grandiflora* FLOWERS AND TO A BLEND OF THESE COMPOUNDS ($N = 8$)[a]

Treatment	Behavioral Response ± SEM (%)[b]			
	WF	UPW	≤0.5 m	SC
Synthetic blend[c]	95.0 ± 3.3a[d]	85.0 ± 5.0a	62.5 ± 4.5a	55.0 ± 5.0a
Phenylacetaldehyde (1 mg)	92.5 ± 3.6a	75.0 ± 6.3ab	52.5 ± 6.5ab	47.5 ± 6.5a
2-Phenylethanol (1 mg)	85.0 ± 6.3a	60.0 ± 11.0ab	42.5 ± 8.8abc	40.0 ± 8.4ab
Benzaldehyde (1 mg)	85.0 ± 7.3a	42.5 ± 11.0b	12.5 ± 5.3c	2.5 ± 2.5c
Benzyl alcohol (1 mg)	82.5 ± 8.0a	62.0 ± 14.1ab	25.5 ± 8.2bc	15.0 ± 8.2bc

[a] Eight experimental blocks, each block consisted of five moths released individually to a specific test stimulus.
[b] See Table 2 for behavioral categories.
[c] See Table 2 for chemical composition of this blend.
[d] Means in the same column are significantly different if they do not share a letter in common ($P < 0.05$, ANOVA and Scheffé multiple comparison procedure).

DISCUSSION

Phenylacetaldehyde is reported to be an attractant for Plusiinae moths (including *T. ni*) and other Noctuidae (Smith et al., 1943; Creighton et al., 1973). It has been identified as a volatile compound emitted by the bladder flower, *Araujia sericofera* Brothero, that attracts and traps many species of moths (Cantelo and Jacobson, 1979). From our study it appears to be a major constituent of the blend of volatile compounds emitted from flowers of *A. grandiflora*, an ornamental shrub that is frequently visited by *T. ni* moths foraging for nectar (Grant, 1971a).

Another component of the fragrant blend of compounds from these flowers is 2-phenylethanol. 2-Phenylethanol has been identified from male scent brushes of several species of moths (Aplin and Birch, 1970; Birch et al., 1990). Jacobson et al. (1976) isolated this compound from the genital scent brushes of male *T. ni*, but others have been unable to detect it (Hagan and Brady, 1981; Landolt and Heath, 1990). It is possible that the overt behavioral response of female *T. ni* to 2-phenylethanol documented by Jacobson et al. (1976) was due to its proposed role in stimulating nectar-finding behavior, rather than to their proposal that it mediated precopulatory behavior. Benzaldehyde and benzyl alco-

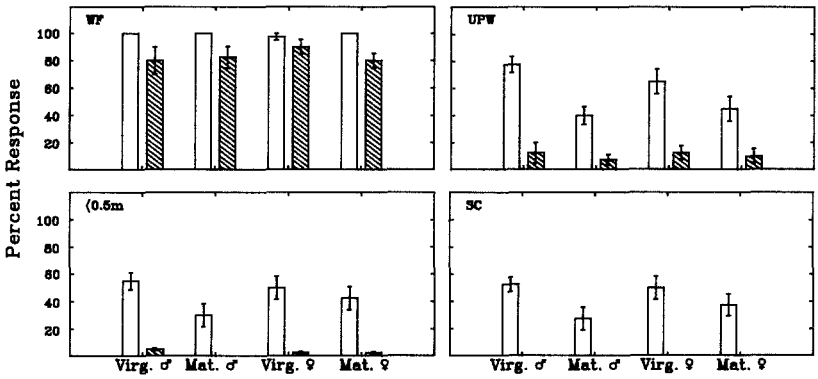


FIG. 2. Behavioral responses of virgin and mated, male and female *Trichoplusia ni* to hexane blank (cross-hatched bars) and a blend of phenylacetaldehyde, 2-phenylethanol, benzaldehyde, and benzyl alcohol (open bars). WF = wing fanning in the release cage, UPW = upwind flight at the level of the odor plume, <0.5 m = flying to within 0.5 m of the source, SC = source contact. Bars indicate ±SEM.

hol, two additional components of *Abelia's* volatile blend, have been identified from scent brushes of other noctuid moths (see Birch et al., 1990). While our wind tunnel assays have not identified an important role for these two compounds in mediating upwind flight to *Abelia* flowers, it is possible that their presence increases the specificity of the response. Grant (1971a) indicated that mainly plusiine moths were observed feeding on *A. grandiflora* flowers. The response to phenylacetaldehyde includes species from other subfamilies in Noctuidae (Cantelo and Jacobson, 1979). Benzaldehyde has been reported to have a repellent effect on several species of moths (Hirai, 1982). We did not observe any decrease in source location as a consequence of including benzaldehyde in the synthetic blend, and in fact benzaldehyde by itself did stimulate a few moths to fly upwind and contact the source.

The demonstration that a synthetic blend of floral chemicals stimulates upwind flight and source-finding in virgin and mated individuals of both sexes suggests the importance of nectar-finding for reproductive success in all adult *T. ni*. Our finding that mated adults were less successful than virgins in locating these odor sources may reflect a refractory period for foraging behavior after mating. In females, this change could indicate a shift towards ovipositional host-finding activity during the hours in which nectar-feeding usually occurs.

Acknowledgments—We thank J.D. Clark and J.T. Collins for technical assistance. D.M Campero, R.E. Hunt, P.J. Landolt, Y. Liu, D.A. Potter, and K.V. Yeargan reviewed and improved an earlier draft of this manuscript. We acknowledge support of the University of Kentucky Major Research Instrumentation Bond Program in the purchase of equipment used in this study (ID#'s

7E-8164-11, L- 250-8A080, L-251-8A080, and L-252-8A080). This investigation (paper No. 90-7-174) was conducted in connection with a project of the Kentucky Agricultural Experiment Station and is published with the approval of the director.

REFERENCES

APLIN , R.T., and BIRCH, M.C. 1970. Identification of odorous compounds from male Lepidoptera. *Experientia* 26:1193.

BIRCH, M.C., POPPY, G.M., and BAKER, T.C. 1990. Scents and eversible scent structures of male moths. *Annu. Rev. Entomol.* 35:25–58.

CANTELO, W.W. and JACOBSON, M. 1979. Phenylacetaldehyde attracts moths to bladder flower and to blacklight traps. *Environ. Entomol.* 8:444–447.

CREIGHTON, C.S., MCFADDEN, T.L., and CUTHBERT, E.R. 1973. Supplementary data on phenylacetaldehyde: An attractant for Lepidoptera. *J. Econ. Entomol.* 66:114–115.

GRANT, G.G. 1971a. Feeding activity of adult cabbage loopers on flowers with strong olfactory stimuli. *J. Econ. Entomol.* 64:315–316.

GRANT, G.G. 1971b. Scent apparatus of the male cabbage looper, *Trichoplusia ni*. *Ann. Entomol. Soc. Am.* 64:347–352.

HAGAN, D.V., and BRADY, U.E. 1981. Absence of detectable 2-phenylethanol in *Trichoplusia ni*, a reported pheromone of males. *J. Ga. Entomol. Soc.* 16:192–196.

HAYNES, K.F. and BAKER, T.C. 1989. An analysis of anemotactic flight in female moths stimulated by host odour and comparison with the males' response to sex pheromone. *Physiol. Entomol.* 14:279–289.

HIRAI, K. 1982. Directional flow of male scent released by *Pseudaletia separata* Walker (Lepidoptera: Noctuidae) and its repellent effect on adults and larvae of four noctuid and one phycitine moth. *J. Chem. Ecol.* 8:1263–1270.

JACOBSON, M., ADLER, V.E., KISHABA, A.N., and PRIESNER, E. 1976. 2-Phenylethanol, a presumed sexual stimulant produced by the male cabbage looper moth, *Trichoplusia ni*. *Experientia* 32:964–966.

LANDOLT, P.J. 1989. Attraction of the cabbage looper to host plants and host plant odor in the laboratory. *Entomol. Exp. Appl.* 53:117–124.

LANDOLT, P.J., and HEATH, R.R. 1990. Sexual role reversal in mate-finding strategies of cabbage looper moths. *Science* 249:1026–1028.

LIU, S.-H., NORRIS, D.M., and MARTI, E. 1988. Behavioral responses of female adult *Trichoplusia ni* to volatiles from soybeans versus a preferred host, lima bean. *Entomol. Exp. Appl.* 49:99–109.

MILLER, J.R., and ROELOFS, W.L. 1978. Sustained-flight tunnel for measuring insect responses to wind-borne sex pheromones. *J. Chem. Ecol.* 4:187–198.

PHELAN, P.L., and BAKER, T.C. 1987. An attracticide for control of *Amyelois transitella* (Lepidoptera: Pyralidae) in almonds. *J. Econ. Entomol.* 80:779–783.

RAMASWAMY, S.B. 1988. Host finding by moths: Sensory modalities and behaviours. *J. Insect Physiol.* 34:235–249.

SHOREY, H.H., and HALE, R.L. 1965. Mass-rearing of the larvae of nine noctuid species on a simple artificial medium. *J. Econ. Entomol.* 58:522–524.

SMITH, C.E., ALLEN, N., and NELSON, O.A. 1943. Some chemotropic studies with *Autographa* spp. *J. Econ. Entomol.* 36:619–621.

TINGLE, F.C., HEATH, R.R., and MITCHELL, E.R. 1989. Flight response of *Heliothis subflexa* (GN.) females (Lepidoptera: Noctuidae) to an attractant from groundcherry, *Physalis angulata* L. *J. Chem. Ecol.* 15:221–231.

WIESENBORN, W.D., and BAKER, T.C. 1990. Upwind flight to flowers by *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). *Environ. Entomol.* 19:490–493.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

FLIGHT BEHAVIOR OF SCOLYTID BEETLE IN RESPONSE TO SEMIOCHEMICALS AT DIFFERENT WIND SPEEDS

S.M. SALOM[1,*] and J.A. McLEAN

Faculty of Forestry
University of British Columbia
Vancouver, British Columbia V6T 1W5 Canada

(Received August 9, 1990; accepted November 20, 1990).

Abstract—The response of the striped ambrosia beetle, *Trypodendron lineatum* (Olivier) (Coleoptera: Scolytidae), to a semiochemical-baited funnel trap was studied in a wind tunnel in the first of two experiments. Wind speeds were tested over a range of 0.0–0.9 m/sec. Percent beetle capture decreased linearly with increasing wind speed within the range tested. A second experiment showed that in the presence of wind, beetles flew upwind to a semiochemical-baited substrate. In still air, they tended to fly randomly and erratically; yet in close proximity to the baited substrate, a greater proportion of the beetles were arrested in response to the chemical stimuli and landed on the substrate than when an airflow was present. These results suggest that *T. lineatum* are capable of responding to semiochemicals under varied wind conditions typically present in a forest where they use wind to orient to olfactory stimuli. However, greater numbers are arrested in response to the stimuli under relatively still conditions.

Key Words—*Trypodendron lineatum*, Coleoptera, Scolytidae, flight behavior, wind speed, semiochemicals, wind tunnel.

INTRODUCTION

Several species of scolytid beetles have been found to exhibit upwind flight to attractive material in the field (Chapman, 1962; McMullen and Atkins, 1962; Gara, 1963; Botterweg, 1982; Byers, 1988). It has been difficult to demonstrate

*To whom correspondence should be addressed.
[1]Current address: Department of Entomology, Virginia Polytechnic Institute & State University, Blacksburg, Virginia 24061-0319.

0098-0331/91/0300-0647$06.50/0

this behavior in a wind tunnel, where only two scolytid species (Choudhury and Kennedy, 1980; Salom and McLean, 1990a), one anobiid species (Birch and White, 1988), and one clerid species (Mizell et al., 1984) have exhibited upwind flight to semiochemicals.

The striped ambrosia beetle, *Trypodendron lineatum* (Olivier) (Coleoptera: Scolytidae), a xylomycetophagous pest of most coniferous tree species in western North America and northern Europe (Borden, 1988), uses semiochemicals to find suitable hosts for colonization, mating, and reproduction. These compounds include the attractive host kairomone, ethanol (Moeck, 1970, 1971), and the aggregation pheromone, lineatin, 3,3,7-trimethyl-2,9-dioxatricyclo[3.3.1.$0^{4,7}$]nonane (MacConnell et al., 1977; Borden et al., 1979). During spring flight dispersal, *T. lineatum* must detect and respond to suitable host material; mainly dead or dying coniferous trees or logs that have aged for at least two months (Mathers, 1935). Temperature (Chapman and Kinghorn, 1958), time of day (Rudinsky and Daterman, 1964), and wind direction (Chapman, 1962; Salom and McLean, 1989) are known to affect general flight activity, but little is known about close-range flight behavior in response to host material (Borden et al., 1986; Byers, 1988).

The wind tunnel studies presented here examined the effect of wind speed on close-range response of *T. lineatum* to a lineatin- and ethanol-baited trap and loglike substrate. The first study was designed to determine the wind speed at which the highest proportion of test beetles would respond positively to a semiochemical-baited funnel trap (Lindgren, 1983). Based on field data from Chapman (1962) and Rudinsky and Daterman (1964), it was hypothesized that the highest proportion of beetles would be captured at the lower end of the wind speed range tested (0.0–0.9 m/sec). A second study was designed to evaluate the flight characteristics of *T. lineatum* in the presence and absence of semiochemicals at different wind speeds. It was hypothesized that different flight behavior characteristics could be identified for beetles responding to semiochemicals under moving and still air conditions.

METHODS AND MATERIALS

Insects. Beetles were collected daily during May and June of 1986 and 1988 in lineatin-baited, multiple-funnel traps placed near a large log boom storage area at the mouth of the north arm of the Fraser River, British Columbia. Captured beetles were stored at 4°C, under a 14:10 hr (light–dark) photoperiod, in 1-liter plastic jars containing slightly moistened cloth towels. The jars were checked twice weekly to maintain appropriate moisture levels. Most beetles survived for 50–100 days under these conditions. Since the beetles were

captured in pheromone traps, they were judged to have had sufficient flight exercise to ensure subsequent response to semiochemicals (Graham, 1959; Bennett and Borden, 1971).

Beetles were sexed and scrutinized for presence of all tarsi, both antennae, and for an overall generally healthy appearance in the morning prior to flight in the wind tunnel. Only beetles that met these standards were tested further. The beetles were allowed to acclimate to the warmer room temperatures (22–25°C) for 10 min before release, a period sufficient to ensure maximal response to the trap (Salom, 1989).

Wind Tunnel. The studies were conducted in a wind tunnel (Angerilli and McLean, 1984), 3.6 m long and 1.2 m high and wide. Cool white fluorescent lights (72 W), set 1.5 m above the wind tunnel, simulated diurnal conditions and facilitated direct observations of flying beetles. A sheet of cellulose acetate was placed over the top of the tunnel to diffuse the incoming light and reduce glare. Vertical light measurements, made 0.8 m from the ceiling in the exhaust, middle, and wind entrance sections of the tunnel, averaged 127 lx (SD = 20 lx). Wind speed measurements were made using a Lambrecht hotwire anemometer (sensitivity = ± 2 cm/sec). No air movement was found to occur along the tunnel, across the tunnel, or vertically within the tunnel at 0.0 m/sec.

A release platform consisting of a circular, flat glass plate 13 cm diam., was suspended horizontally 35 cm above the tunnel floor and 1 m from the downwind screen. Preliminary trials suggested that optimal response by *T. lineatum* occurred when the release platform was placed at this height. It was high enough for beetles to be continuously exposed to the semiochemical plume prior to takeoff (Choudhury and Kennedy, 1980), and we assumed it was close enough to the striped floor to use optomotor cues if needed. The plate was covered with a white industrial paper towel to enhance traction for the insects. A 16-cm^2 $\times$ 0.7-cm-deep plastic weighing boat, covered by the same material, was taped to the upper side of the glass plate. This allowed the beetles two perching opportunities for flight takeoff: the first around the edge of the weighing boat and, if they fell off of that, a second opportunity around the edge of the glass plate.

Effect of Different Wind Speeds on Percent Beetle Capture in Funnel Traps. A multiple-funnel trap, placed in the center of the upwind section of the tunnel, ca. 0.6 m from the screen at the airflow entrance of the tunnel, was baited with two lineatin Biolure dispensers (Consep Membranes of Bend, Oregon), one in the upper and one in the lower portion of the trap, and a plastic bottle dispenser (Phero Tech. Inc., Delta, British Columbia, Canada) containing 95% ethanol in the middle. Release rates, determined gravimetrically at 20°C in the laboratory, were 100–120 μg/day for each lineatin dispenser and 75 mg/day for the ethanol dispenser. The dispensers were identical to those used in mass-trapping programs of *T. lineatum* throughout southwestern British Columbia, and the

release rates were well within the range of rates that elicited positive responses observed for *T. lineatum* in the wind tunnel (Salom and McLean, 1988).

A nine-replicate, randomized complete block design was used, in which the treatments were wind speeds of 0.00, 0.15, 0.30, 0.45, 0.60, 0.75, and 0.90 m/sec (0–3.2 km/hr). For each treatment, males and females were tested separately. Groups of 50 males and 30–50 females were placed on the release tray. All treatments within one block (replication) were run for each sex on the same day, and the experiment was run for nine days over a 21-day period. Each replication of a given treatment lasted for 20 min. Beetles were used only once.

An additional three-replicate, control experiment assessed male and female catches in unbaited traps in still air. Prior to these replicates, the funnel trap, tunnel, and release tray were cleaned with a 0.5% sodium hypochlorite solution.

All data were subjected to analysis of variance with the GLM procedure (SAS, 1985) to test for differences between sex. Since treatment values were set at equal intervals throughout the wind-speed range tested, the data were subjected to orthogonal polynomial analysis (SAS Institute, 1985). This allowed for the prediction of the model needed to "fit" the data for regression analysis (Hicks, 1982).

Flight Behavior at Different Wind Speeds. A preliminary study was conducted in the wind tunnel at 0.3 m/sec wind speed to determine the number of beetles landing in or on various semiochemical-baited substrates. The substrates tested were a 28-cm-diam. × 71-cm-long log of western hemlock, *Tsuga heterophylla* (Raf.) Sarg., oriented either vertically or horizontally across the width of the tunnel; a simulated log (SL) of the same dimensions and orientations as the real log, made of cardboard, cotton fiber tag paper, and construction paper; and a vertically oriented funnel trap. All substrates were baited with two lineatin and one ethanol dispenser. The beetles showed no preference for any substrate or orientation ($F_{4,50} = 0.75$; $P > 0.05$) with mean percent captures ranging from 12.2 to 15.8 (SE = 1.3–2.1). Therefore, a horizontal SL was used to test the effect of wind speed on flight behavior, because it allowed for control of released volatiles not possible with a real log and assumed the position of most infested material in the field. The SL was placed on two concrete blocks with holes to reduce the disruption of air flow. Two lineatin lures and an ethanol dispenser, identical to those used in study 1, were attached to the SL along the upwind side, providing a large, relatively even downwind plume, as verified by releasing $TiCl_4$ smoke at the different wind speeds. The release platform and its placement in the tunnel were the same as in the trap catch study above.

To study *T. lineatum* flight behavior, the overall experiment was divided into two parts. Part 1 examined beetle response to a SL baited with lineatin and ethanol. Part 2 examined beetle response to an unbaited SL, serving as a control. In each part, beetles were studied at wind speeds of 0.0, 0.3, 0.6, and 0.9

m/sec. Parts 1 and 2 were conducted on separate experimental days. The tunnel and the release platform were carefully cleaned with a 0.5% sodium hypochlorite solution prior to running part 2.

Two beetles of the same sex were released per replicate and observed for up to 8 min in the experiment. Fifty replicates were run for each level of wind speed and baiting treatment (lineatin and ethanol or none) tested. Beetles were used only once. Observation parameters included occurrence of flight, body orientation prior to and during flight takeoff (relative to the SL and the direction from which the wind was blowing), overall flight direction, type of flight, and lock-on behavior.

Overall flight direction was scored as uptunnel, downtunnel, or nondirectional, depending upon the predominant direction flown by the insect throughout the observation period. If beetles did not exhibit predominant up-or downtunnel flight, they were considered nondirectional fliers. These categories were based on the general amount of time a beetle spent in each section of the tunnel.

Type of flight was scored as: (1) erratic—quick, sharp changes in movement, with no specific direction of flight; (2) steady—slow and stable, with a narrow flight line that may have included more than one directional heading throughout the flight period; and (3) direct—a fast, straight-line flight to some endpoint, with only one directional heading. Sometimes beetles exhibited and were recorded having more than one type of flight behavior.

Lock-on behavior in this study was considered steady flight in a predominantly uptunnel direction, with the insect orienting specifically toward the SL, and finally hovering around or landing on the SL. This differs from the traditional definition used for Lepidoptera, where the moths narrow their flight track in a zigzag pattern during upwind flight (Sweeney and McLean, 1987).

All observations were recorded on a cassette tape and later transcribed onto data sheets. The data were summarized as frequencies and analyzed separately, for each behavioral category, as multidimensional contingency tables using the CATMOD procedure in SAS Institute (1985). This procedure uses a linear models approach to the analysis of categorical data, applying general weighted least-squares regression techniques to cell proportions in complex categorical data layouts (Kleinbaum and Kupper, 1978). This approach allowed us to test for first-and second-order interactions, main effects, and contrast between levels of main effects, for a specific response, using the chi- square (χ^2) statistic.

RESULTS

Effect of Different Wind Speeds on Percent Beetle Capture in Funnel Traps. The relationship between the numbers of *T. lineatum* caught at different wind speeds did not differ between sexes ($F_{1,102} = 2.42$; $P > 0.05$); therefore, the

data were pooled. A linear model was determined to be the best fit of the data for regression analysis ($F_{1,107} = 65.7$; $P < 0.001$). The percent of beetles captured in the traps decreased as wind speed increased within the range tested (Figure 1). However, much of the variation in these data is not explained by the regression ($r^2 = 0.33$). The high variation in the results may be due in part to the varied physiological states (e.g., varying energy reserves) of the beetles (Atkins, 1966; Borden, 1982), or perhaps the conditions in the wind tunnel were not optimal for *T. lineatum* response, or both. Beetle responses to the unbaited control treatment in still air averaged only 2.7% (SE = 1.0), indicating lack of contamination in the tunnel and little attraction to the trap alone.

Flight Behavior at Different Wind Speeds. The number of beetles taking flight was not influenced by wind speed. However, frequency of flight was analyzed by sex and bait separately because of a significant interaction occurring between these factors ($\chi^2_1 = 17.1$; $P \leq 0.01$), with less males responding than females to a baited SL (Table 1).

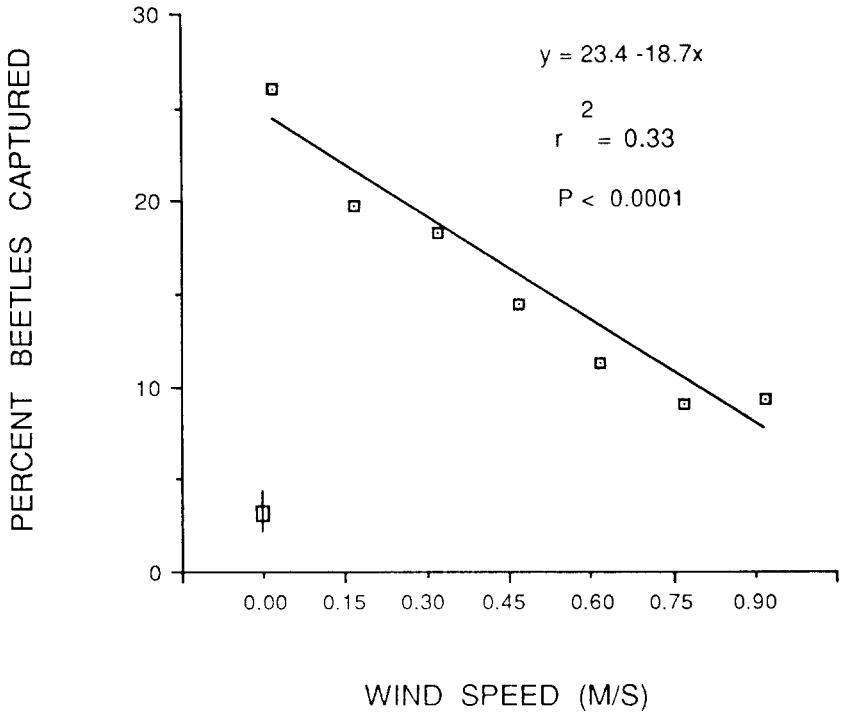


FIG. 1. The percent capture of *Trypodendron lineatum* in a semiochemical-baited funnel trap in a wind tunnel at different wind speeds. The box with a vertical line represents the mean percent ± SE response of the beetles at 0.0 m/sec to an unbaited funnel trap.

TABLE 1. FREQUENCY OF *Trypodendrom lineatum* FLIGHT IN WIND TUNNEL AT DIFFERENT WIND SPEEDS IN PRESENCE OF UNBAITED AND SEMIOCHEMICAL-BAITED SIMULATED LOG[a]

Wind speed (m/sec)	Percentage Flying	
	Males	Females
Unbaited		
0.0	61	67
0.3	70	74
0.6	77	68
0.9	70	80
Baited		
0.0	56	72
0.3	44	74
0.6	47	76
0.9	45	67

[a] One hundred beetles released for each wind speed of each bait treatment. No significant differences in number of beetles flying, where each sex within each bait treatment was analyzed separately.

Most beetles faced uptunnel prior to and during takeoff (Figure 2). Body orientation did not differ between males and females ($\chi^2_3 = 4.4$; $P > 0.05$); therefore, the data for both sexes were pooled. Uptunnel body orientation increased significantly in the presence of an airflow for both baited and unbaited treatments (Figure 2). The presence of semiochemicals did not result in increased uptunnel orientation, but it did result in a significant increase in the occurrence of nondirectional orientation at 0.3 m/sec ($\chi^2_4 = 9.6$; $P < 0.05$).

Overall flight direction did not differ between males and females ($\chi^2_2 = 4.7$; $P > 0.05$); therefore, the data were pooled. Upon exposure to wind, flight direction changed from uptunnel and nondirectional to downtunnel for both the baited and unbaited treatments (Figure 3). Increases in wind speed above 0.3 m/sec did not cause any further change in flight direction for the baited treatment, but it did cause an increase in downwind flight for the unbaited treatment. A higher frequency of beetles exhibited uptunnel flight in the presence of semiochemicals, with statistical differences occurring at wind speeds of 0.3 ($\chi^2_2 = 7.1$; $P < 0.05$), 0.6 ($\chi^2_2 = 10.9$; $P < 0.01$), and 0.9 m/sec ($\chi^2_2 = 8.8$; $P < 0.05$).

Data for type of flight showed significant speed by bait ($\chi^2_6 = 20.3$; $P < 0.005$) and by sex ($\chi^2_6 = 16.4$; $P < 0.05$) interactions, leading to a separate analysis for each factor and preventing statistical comparisons between the factors. However, in still air, steady flight of both sexes appeared to be more frequent in response to the baited treatment than to the unbaited treatment (Fig-

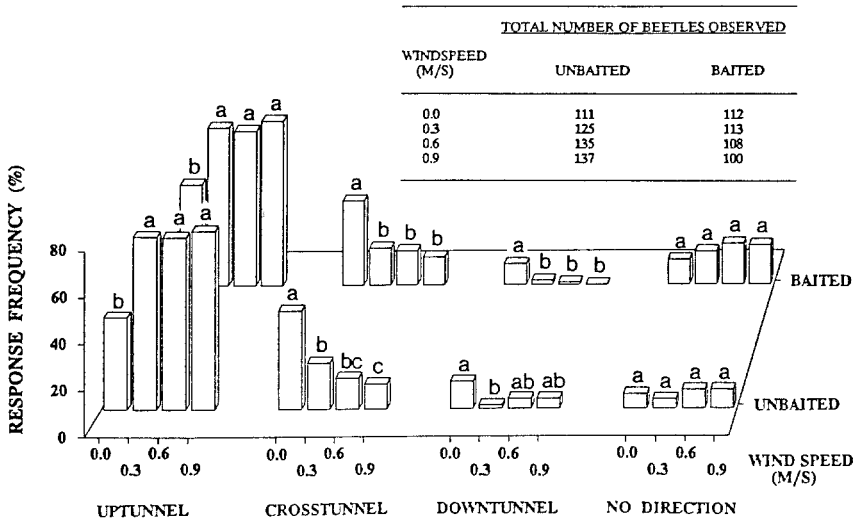


FIG. 2. Body orientation of *T. lineatum*, prior to and during flight takeoff, at different wind speeds and in the presence of an unbaited and semiochemical-baited simulated log. Male and female data were pooled. Bars with different letters for each orientation within a bait treatment category are significantly different (χ^2; $P \leq 0.05$), for individual comparisons. Orientation categories are relative to the simulated log and the direction from which the wind was blowing. Nondirectional orientation was tallied for beetles facing toward the floor from the edge or underneath the tray.

ure 4). Occurrence of steady flight decreased significantly in the presence of wind for the baited treatment, compared to no difference observed for beetles in the unbaited treatment. The frequency of erratic flight displayed by female beetles in response to both baited and unbaited SLs decreased significantly as wind speed increased from 0.6 to 0.9 m/sec (Figure 4). In response to the baited SL, the frequency of erratic flight of male beetles varied significantly, with no apparent pattern to changing wind speed, but was unchanged in response to the unbaited treatment (Figure 4). As wind speed increased from 0.6 to 0.9 m/sec, erratic and steady flight decreased for both males and females, while direct flight increased.

No differences in lock-on responses were observed between males and females ($\chi^2_2 = 1.94$; $P > 0.05$); therefore, the data were pooled. The frequency of *T. lineatum* exhibiting a lock-on response to a baited and unbaited SL interacted significantly with wind speed ($\chi^2_6 = 32.9$; $P < 0.001$), preventing statistical comparisons between the two treatments. However, the lock-on response was generally higher in the presence of semiochemicals at all wind speeds than when the semiochemicals were absent (Figure 5). The differences in response

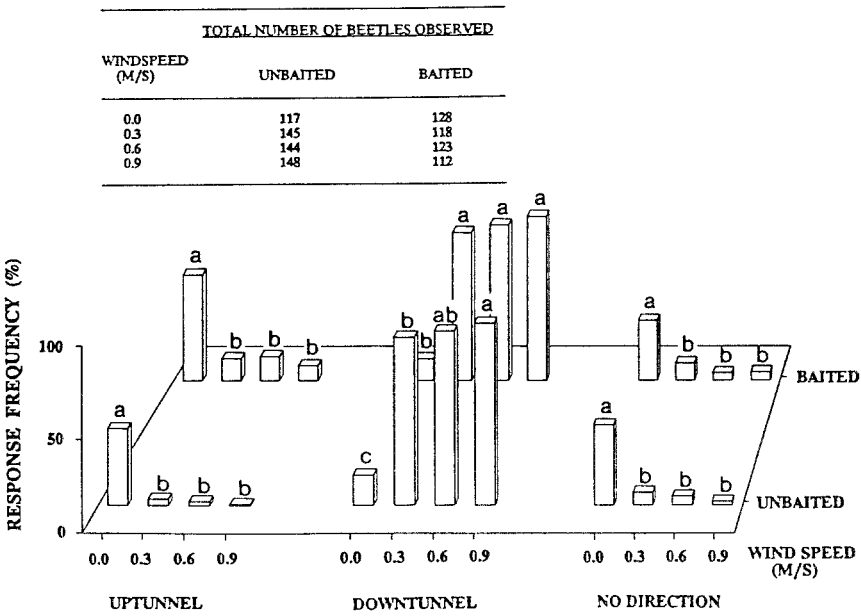


FIG. 3. Overall flight direction exhibited by *T. lineatum*, at different wind speeds, in the presence of an unbaited and semiochemical-baited, simulated log. Male and female data were pooled. Bars with different letters for each directional flight response within a bait treatment category are significantly different (χ^2; $P \leq 0.05$) for individual comparisons.

between these treatments were especially noticeable in the absence of wind, where 54% of the flying beetles showed lock-on behavior in the presence of a baited SL, with 29% landing on it, compared to only 8% locking on in the presence of an unbaited SL, of which less than 5% landed. The frequency of the responses decreased in the presence of an airflow, yet increasing wind speed from 0.3 to 0.9 m/sec did not result in further decreases.

DISCUSSION

In the first study, the decrease in the number of beetles caught in the funnel traps, with increased wind speed, was expected since *T. lineatum* prefer to fly under low wind speed conditions (Rudinsky and Daterman, 1964). The high frequency of beetle capture in still air was unanticipated. The data suggest that, depending on the presence or absence of wind, *T. lineatum* may use different mechanisms or flight patterns for orienting toward and landing on host material. This is not unlike the different mechanisms used by *Ips paraconfusus* Lanier

WINDSPEED (M/S)	TOTAL NUMBER OF BEETLES OBSERVED			
	MALES		FEMALES	
	UNBAITED	BAITED	UNBAITED	BAITED
0.0	61	56	66	69
0.3	64	40	72	74
0.6	75	44	64	73
0.9	65	41	74	67

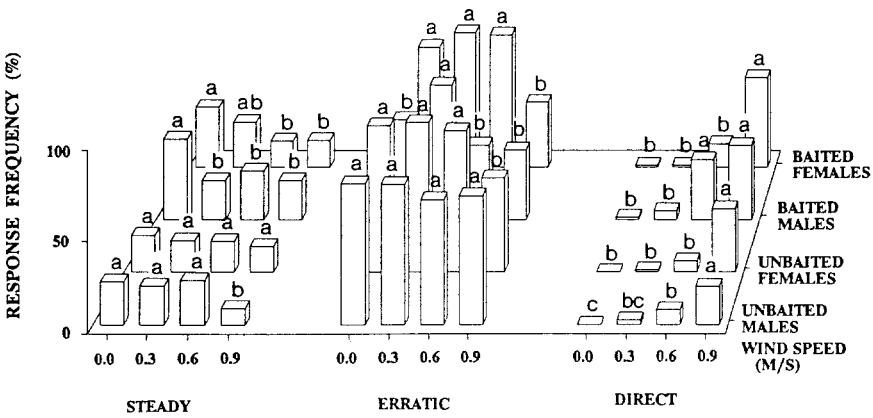


FIG. 4. Type of flight exhibited by *T. lineatum*, at different wind speeds, in the presence of an unbaited and semiochemical-baited simulated log. Bars with different letters for flight type response within each bait treatment and sex category are significantly different (χ^2; $P \leq 0.05$) for individual comparisons.

observed in pedestrian bioassays in the absence and presence of wind (Akers and Wood, 1989a,b).

In the second study, the occurrence of flight was unaffected by wind (Table 1), and may have been due to the range of wind speed we tested being below the threshold observed to inhibit beetle flight in the field (Chapman, 1962).

In still air, the higher frequencies of lock-on and landing response to a semiochemical-baited SL seem to have resulted from random flight bringing the beetles close to the SL by chance, followed by arrestment. This interpretation is based mostly on observation and could not be deduced from the categorical data collected, since we recorded up to three types of flight for each beetle, with none being mutually exclusive (Figure 4). Arrestment at the SL suggests chemotaxis as a possible mechanism. Akers and Wood (1989a) demonstrated that walking *I. paraconfusus* were capable of responding chemotactically from a distance of 16–18 cm away from a pheromone source in still air. Visual attraction to the silhouette also may have contributed to beetle arrestment. Further studies are needed to separate the effects of both olfactory and visual stimuli on *T. lineatum* arrestment.

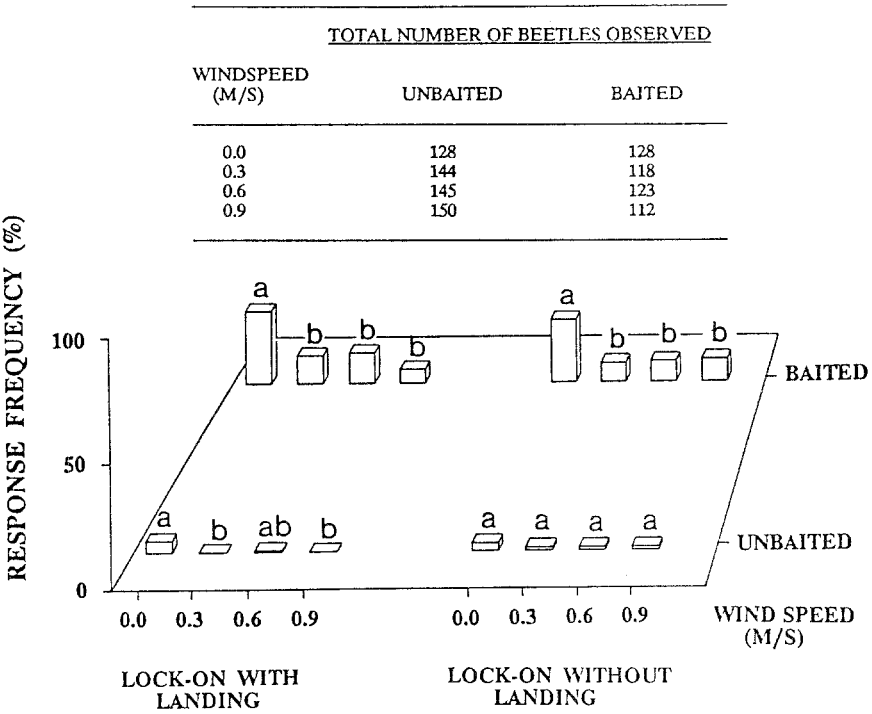


FIG. 5. Lock-on response exhibited by *T. lineatum*, at different wind speeds, in the presence of an unbaited and semiochemical-baited simulated log. Male and female data were pooled. Bars with different letters for each behavior within a bait treatment category are significantly different (χ^2; $P \leq 0.05$) for individual comparisons.

Compared to still air conditions, *T. lineatum* flight behavior changed in the presence of wind. Overall flight direction shifted substantially from uptunnel to downtunnel at a wind speed as low as 0.3 m/sec (Figure 3).

In the field, *T. lineatum* have been observed flying upwind to attractive sources (Chapman, 1962; Salom and McLean, 1989, 1990b), however close-range (i.e., < 1 m) orientation and landing on the host substrate does not appear to require air movement. The responses reported in wind-tunnel studies by Choudhury and Kennedy (1980) for *Scolytus multistriatus* (Marsh.), Birch and White (1988) for *Anobium punctatum* (Deg.), and Mizell et al. (1984) for *Thanasimus dubius* (F.) did not test insect response to a pheromone source in still air. In studies with Lepidoptera, Farkas and Shorey (1972) first demonstrated that the pink bollworm, *Pectinophora gossypiella* (Saund.), did not require moving air to fly toward a pheromone source. Baker and Kuenen (1982) confirmed these results with *Grapholita molesta* (Busk.) and showed that the zig-

zagging mechanism used by these moths to orient to the odor source would not change if wind was removed once the behavior had been initiated. Little evidence is currently available to indicate this behavior is also used by scolytids (Byers, 1989).

With wind present, beetles tended to fly downwind, away from the source of semiochemicals (Figure 3). Despite the constant exposure of the beetles to the semiochemical plume, only 20% of the beetles flew upwind. The high frequency of nonresponders may have been a result of the artificial conditions imposed on the beetles by the wind tunnel (i.e., lack of space for full flight behavior).

Another explanation for the low frequency of upwind flight to a baited substrate may be due to the variation in physiological state of individual beetles, considered an important factor in influencing orientation of bark beetles to olfactory stimuli. Borden (1982) and Wood (1982) hypothesized that some beetles respond immediately to semiochemicals and colonize suitable host trees nearby, while others fly for a period of time before responding and thus start new centers of attraction at greater distances. Atkins (1966, 1969) determined that there was a strong correlation between dispersal distance and fat content in *Dendroctonus pseudotsugae* Hopk. Physical state of the beetles, such as nematode or microorganism infection, also may play a role in this (Nickel, 1971). *Trypodendron lineatum* was not evaluated for the presence of such infections in this study. Although we are not sure of the physiological mechanism influencing *T. lineatum*, we believe the variability in response behavior within a population may account for the high variation in response for our study. Variation in scolytid response and difficulty in eliciting beetle response to semiochemicals in a wind tunnel have been reported for other scolytids (Byers, 1988; Akers and Wood; 1989b). Even in pedestrian bioassays, low frequency of ambrosia beetle response to semiochemicals is quite common, with 40–50% response being the highest observed (Borden et al., 1976; MacConnell et al., 1977).

The decreases in *T. lineatum* response to semiochemicals at increasing wind speeds correspond well with observations in field situations, where twice as many marked *T. lineatum* were recaptured in a forest when wind speed averaged less than half the normal speed (i.e., 0.18 m/sec compared to 0.39 m/sec) (Salom and McLean, 1990a). Other scolytids such as *D. frontalis* Zimm. (Vité et al., 1964) and *D. ponderosae* (Edson, 1978) have been captured in higher numbers during periods of calm air.

It may be argued that by increasing the wind speed in the tunnel, a decrease in the size of the semiochemical plume would result, reducing the chance that beetles will respond while in flight. Sanders (1985) showed that a plume of smoke released from a point source the size of a lure decreased in size with an increase in wind speed. However, in our studies, a test with $TiCl_4$ smoke showed

that the plume sizes did not decrease significantly between 0.25 and 0.75 m/sec from the simulated log.

In relating our wind-tunnel results to *T. lineatum* dispersal and subsequent attraction to host material, it is important to understand beetle response capabilities under varied environmental conditions as well as their own varied physiological states. In a mark–recapture study, Salom and McLean (1991) found that beetles were consistently recaptured in significantly higher numbers (ratio of 3:1) in the forest than in open settings, although the latter settings were closer to the beetle release sites. Wind speed was ca. 4× higher in the open than in the forest, supporting the hypothesis that low wind speed is critical in arresting beetle flight at sources of attraction. The field data, as well as the wind-tunnel study reported here, suggest that once *T. lineatum* reach within 1 or 2 m of the host, arrestment and subsequent landing on the host is best facilitated during periods of light or no wind. When wind is present, *T. lineatum* can orient to and land on a host, but will do so at lower frequencies. The differences in *T. lineatum* flight behavior in the absence and presence of wind, as they search for attractive host material, allow them to optimize their search by enabling them adapt to the varying environmental conditions encountered.

Acknowledgments—We gratefully thank J.H. Borden, B.S. Lindgren, T.L. Payne, P.L. Phelan, J.D. Sweeney, and D.L. Wood for reviewing earlier versions of this manuscript; F. Ho for providing statistical advice; A. Black for use of his anemometer; and S.M. Hasim-Salom for assistance in figure drafting. This research was supported by operating grant A0462 from the Natural Sciences and Engineering Research Council of Canada to J.A. McLean.

REFERENCES

AKERS, R.P., and WOOD, D.L. 1989a. Olfactory orientation responses by walking female *Ips paraconfusus* bark beetles: I. Chemotaxis assay. *J. Chem. Ecol.* 15:3–24.

AKERS, R.P., and WOOD, D.L. 1989b. Olfactory orientation responses by walking female *Ips paraconfusus* bark beetles: II. In an anemotaxis assay. *J. Chem. Ecol.* 15: 1147–1160.

ANGERILLI, N., and MCLEAN, J.A. 1984. Windtunnel and field observations of western spruce budworm responses to pheromone-baited traps. *J. Entomol. Soc. B.C.* 81:10–16.

ATKINS, M.D. 1966. Laboratory studies on the behavior of the Douglas-fir beetle, *Dendroctonus pseudotsugae* Hopk. *Can. Entomol.* 101: 164–165.

BAKER, T.C., and KUENEN, L.P.S. 1982. Pheromone source location in flying moths: A supplementary nonanemotactic mechanism. *Science* 216: 424–427.

BENNETT, R.B., and BORDEN, J.H. 1971. Flight arrestment of tethered *Dendroctonus pseudotsugae* and *Trypodendron lineatum* (Coleoptera: Scolytidae) in response to olfactory stimuli. *Ann. Entomol. Soc. Amr.* 64:1273–1286.

BIRCH, M.C. and WHITE, P.R. 1988. Responses of flying male *Anobium punctatum* (Coleoptera: Anobiidae) to female sex pheromone in a wind tunnel. *J. Insect Behav.* 1:111–115.

BORDEN, J.H. 1982. Aggregation pheromones, pp. 74–139, *in* J.B. Mitton and K.B. Sturgeon (eds.). Bark Beetles in North American Conifers. University of Texas Press, Austin.

BORDEN, J.H. 1988. The striped ambrosia beetle, *Trypodendron lineatum* (Olivier), pp. 579–596, *in* A.A. Berryman (ed.). Dynamics of Forest Insect Populations. Plenum Publ. New York.

BORDEN, J.H., CHONG, L., MCLEAN, J.A., SLESSOR, K.N., and MORI, K. 1976. *Gnathotrichus sulcatus:* Synergistic response to enantiomers of the aggregation pheromone sulcatol. *Science* 192: 894–896.

BORDEN, J.H., HANDLEY, J.R., JOHNSTON, B.D., MACCONNELL, J.G., SILVERSTEIN, R.M., SLESSOR, K.N., SWIGAR, A.A., and WONG, D.T.W. 1979. Synthesis and field testing of 4,6,6-lineatin, the aggregation pheromone of *Trypodendron lineatum* (Coleoptera, Scolytidae). *J. Chem. Ecol.* 5: 68¾81–689.

BORDEN, J.H., HUNT, D.W.A., MILLER, D.R., and SLESSOR, K.N. 1986. Orientation in forest Coleoptera: An uncertain outcome of responses by individual beetles to variable stimuli, pp. 97–110, *in* T.L. Payne, M.C. Birch, and C.J.E. Kennedy (eds.) Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

BOTTERWEG, P.F. 1982. Dispersal and flight behavior of the spruce bark beetle *Ips typographus* in relation to sex, size, and fat content. *Z. Angew. Entomol.* 94:466–489.

BYERS, J.A. 1988. Upwind flight orientation to pheromone in the western pine beetle tested with rotating windvane traps. *J. Chem. Ecol.* 14:189–198.

BYERS, J.A. 1989. Chemical ecology of bark beetles. *Experientia* 45:271–283.

CHAPMAN, J.A. 1962. Field studies on attack flight and log selection by the ambrosia, *Trypodendron lineatum* (Oli.) (Coleoptera:Scolytidae). *Can. Entomol.* 94:74–92.

CHAPMAN, J.A., and KINGHORN, J.M. 1958. Studies of flight and attack activity of the ambrosia beetle, *Trypodendron lineatum* (Oliv.) and other Scolytids. *Can. Entomol.* 90:362–372.

CHOUDHURY, J.H., and KENNEDY, J.S. 1980. Light versus pheromone-bearing wind in control of flight direction by bark beetles, *Scolytus multistriatus. Physiol. Entomol.* 5:207–214.

EDSON, L.J. 1978. Host colonization and the arrival sequence of the mountain pine beetle and its insectan associates. PhD dissertation, University of California, Berkeley. 196. pp.

FARKAS, S.R., and SHOREY, H.H. 1972. Chemical trail-following by flying insects: A mechanism for orientation to a distant odor source. *Science* 178:67–68.

GARA, R.I. 1963. Studies on the flight behavior of *Ips confusus* (Lec.) (Coleoptera: Scolytidae) in response to attractive material. *Contrib. Boyce Thompson Inst.* 21:51–66.

GRAHAM, K. 1959. Release by flight exercise of the chemotropic response from photopositive domination in a Scolytid beetle. *Nature* 184:283–284.

HICKS, C.R. 1982. Fundamental Concepts in the Design of Experiments. CBS College Publ., New York. 425 pp.

KLEINBAUM, D.G., and KUPPER, L.L. 1978. Applied Regression and Other Multivariable Methods. Duxbury Press, California. 486 pp.

LINDGREN, B.S. 1983. A multiple funnel trap for scolytid beetles (Coleoptera). *Can. Entomol.* 115:299–302.

MACCONNELL, J.G., BORDEN, J.H., SILVERSTEIN, R.M., and STOKKINK, E. 1977. Isolation and tentative identification of lineatin, a pheromone from the frass of *Trypodendron lineatum* (Coleoptera: Scolytidae), *J. Chem. Ecol.* 5:549–561.

MATHERS, W.G. 1935. Time of felling in relation to injury from ambrosia beetles or pine worms. *B.C. Lumberman* 19:14.

MCMULLEN, L.H., and ATKINS, M.D. 1962. On the flight and host selection of the Douglas-fir beetle, *Dendroctonus pseudotsugae* Hopk. (Coleoptera: Scolytidae). *Can. Entomol.* 94:1309–1325.

MIZELL, R.F., III, FRAZIER, J.L., and NEBEKER, T.E. 1984. Response of the clerid predator *Thanasimus dubius* (F.), to bark beetle pheromones and tree volatiles in a wind tunnel. *J. Chem. Ecol.* 10:177–187.

MOECK, H.A. 1970. Ethanol as the primary attractant for the ambrosia beetle *Trypodendron lineatum* (Coleoptera: Scolytidae). *Can. Entomol.* 102:985–995.

MOECK, H.A. 1971. Field test of ethanol as a Scolytid attractant. *Can. Dep. Fish. For. Bi-Mon. Res. Notes* 27:11–12.

NICKEL, W.R. 1971. Behavior of the shothole borer *Scolytus rugulosus* altered by the nematode parasite *Neoparasitylenchus ruglosi*. *Ann. Entomol. Soc. Amr.* 64:751.

RUDINSKY, J.A., and DATERMAN, G.E. 1964. Field studies on flight patterns and olfactory responses of ambrosia beetles on Douglas-fir forests of western Oregon. *Can. Entomol.* 96:1339–1352.

SALOM, S.M. 1989. Dispersal and flight behavior of *Trypodendron lineatum* (Coleoptera: Scolytidae) as influenced by semiochemical and environmental factors. PhD dissertation, University of British Columbia, Vancouver, B.C. 195 pp.

SALOM, S.M., and MCLEAN, J.A. 1988. Semiochemicals for capturing the ambrosia beetle, *Trypodendron lineatum* in multiple funnel traps in British Columbia. *J. Entomol. Soc. B.C.* 85:34–39.

SALOM, S.M., and MCLEAN, J.A., 1989. Influence of wind on the spring flight of *Trypodendron lineatum* (Olivier) (Coleoptera: Scolytidae) in a second growth coniferous forest. *Can. Entomol.* 121:109–119.

SALOM, S.M., and MCLEAN, J.A. 1990a. Flight and landing behavior of *Trypodendron lineatum* (Coleoptera: Scolytidae) in response to different semiochemicals. *J. Chem. Ecol.* 16:2589–2604.

SALOM, S.M., and MCLEAN, J.A. 1990b. Dispersal of *Trypodendron lineatum* within a valley setting. *Can. Entomol.* 122:43–58.

SALOM, S.M., and MCLEAN, J.A. 1991. Environmental influences on dispersal of *Trypodendron lineatum* (Coleoptera: Scolytidae). *Environ. Entomol.* In press.

SANDERS, C.J. 1985. Flight speed of male spruce budworm moths in a wind tunnel at different wind speeds and at different distances from a pheromone source. *Physiol. Entomol.* 10:83–88.

SAS Institute. 1985. SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina 956 pp.

SWEENEY, J.D., and MCLEAN, J.A. 1987. Effect of sublethal infection levels of *Nosema* sp. on the pheromone-mediated behavior of the western spruce budworm, *Choristoneura occidentalis* Freeman (Lepidoptera: Tortricidae) *Can. Entomol.* 119:587–594.

VITÉ, J.P., GARA, R.I., and VON SCHELLER, H.D. 1964. Field observations on the response to attractants of bark beetles infesting southern pines. *Contrib. Boyce Thompson Inst.* 22:461–470.

WOOD, D.L. 1982. The role of pheromones, kairomones, and allomones in the host selection behavior of bark beetles. *Annu. Rev. Entomol.* 27:411–446.

Journal of Chemical Ecology, Vol. 17, No. 3, 1991

IDENTIFICATION AND PREPARATION OF ANTIINSECTAN DIENOLS FROM *Dipterocarpus kerrii* TREE RESINS[1]

DAVID P. RICHARDSON,[2,*] ADAM C. MESSER,[3] BLAIR A. NEWTON,[2] and NEAL I. LINDEMAN[2]

[2]*Department of Chemistry*
Williams College
Williamstown, Massachusetts 01267-2092

[3]*School of Food and Nutritional Science*
University of Shizuoka
395 Yada, Shizuoka 422 Japan

(Received September 10, 1990; accepted November 20, 1990)

Abstract—Originally isolated from *Dipterocarpus kerrii*, the two previously uncharacterized sesquiterpenes, **1** and **20**, were synthesized from α-gurjunene. A novel process involving *m*-chloroperoxybenzoic acid oxidation of α-gurjunene produced **20** in one step. Spectroscopic studies determined that the diene moiety in **20** is nonconjugated and also found the C-4 tertiary alcohol center to have the α-configuration, while the other stereocenters have configurations matching the corresponding centers in α-gurjunene. Bioassays with termites demonstrated that **20** was more toxic than **1**, resulting in a 50% mortality in seven days when offered to *Neotermes ?dalbergiae* on filter papers. The chemicals appear to result from biotransformation of α-gurjunene. In view of its similarity to the known sesquiterpene γ-gurjunene, we suggest that **20** be referred to as γ-gurjunenol.

Key Words—*Dipterocarpus kerrii*, sesquiterpenes, chemical synthesis, dienol, tropical biology, termiticide, *Neotermes*, α-gurjunene, γ-gurjunene, γ-gurjunenol, bioassay, stereochemistry.

INTRODUCTION

Many tropical trees produce resins that possess a number of biologically active natural products. These provide chemical defense against insects and fungi

*To whom correspondence should be addressed.

[1] Presented at the ICSE/PSNA Joint Meeting, August 11–15, 1990, Université Laval, Québec City, Québec, Canada.

0098-0331/91/0300-0663$06.50/0

(Langenheim, 1990). In forests of Southeast Asia, trees of the plant family Dipterocarpaceae are well known for their production of resins. Often containing sesquiterpenes, these resins circulate throughout the tree.

In addition to several known sesquiterpenes isolated from the biologically active fractions of dipterocarp tree resins (Messer et al., 1990), we have discovered two novel sesquiterpenes (Richardson et al., 1989). Because these novel compounds could have contributed to the biological activity of impure fractions of resins of the Southeast Asian tree *Dipterocarpus kerrii* King, additional investigation was needed to determine whether the chemicals could be of ecological significance. Although structures for these novel compounds were proposed, the limited amounts of these compounds available prevented a definitive assignment of stereochemistry and toxicity (Richardson et al., 1989).

Refinement of stereochemical detail in the assigned structures and additional bioassays required preparation of gram quantities of the previously unknown compounds. Additionally, since these compounds are labile and are found only in fresh resins gathered from trees growing in the Malay Archipelago, a synthetic source of the compounds was required to permit further study. In this paper, we describe the preparation of the novel sesquiterpenes from their probable biosynthetic precursor, α-gurjunene, which is itself the major component of *D. kerrii* resin. We report the final and complete structure and stereochemistry of **20**, as well as a novel route for its preparation. Bioassays demonstrated pronounced termiticidal activity for **20**, but the related structure, **1**, was much less toxic.

METHODS AND MATERIALS

Solvents and Chemicals

Unless otherwise noted, all reaction solvents and reagents used were anhydrous commercial grade. Reactions were conducted under an atmosphere of anhydrous nitrogen in glassware that had been oven dried overnight at 130–140°C and allowed to cool under a stream of nitrogen.

α-Gurjunene. For the majority of these studies, enriched samples of α-gurjunene were obtained via purification of crude *D. kerrii* resin. By GLC analysis, the composition of crude resin was found to be approximately 85% α-gurjunene. Using fractional vacuum distillation, material of 89–92% purity could be obtained, and this was used as starting material in most of the studies directed at preparation of **1** and **20**. Samples of starting material obtained in this fashion were contaminated with other resin components with GLC retention times and TLC mobilities very similar to those for α- gurjunene, but further purification was not attempted. These contaminants did not complicate any of the chemical reactions described and were not found to interfere in the purifi-

cation of the desired reaction products. Experiments requiring very pure starting material utilized >97% pure (−)-α-gurjunene purchased from Fluka Chemie, AG. Our previous work established that (−)-α-gurjunene is the major volatile component in crude *D. kerrii* resin (Richardson et al., 1989).

γ-Gurjunene. Studies involving γ-gurjunene utilized >98% pure material from Fluka Chemie, AG.

Instrumentation

Natural and synthetic chemicals of interest were identified using a combination of analytical thin-layer chromatography (TLC), gas–liquid chromatography (GLC), gas chromatography–mass spectrometry (GC-MS), Fourier transform-nuclear magnetic resonance spectroscopy (both [^{1}H]- and [^{13}C]NMR), ultraviolet spectroscopy (UV), and infrared spectroscopy (IR).

Analytical TLC was performed on precoated plates (0.25 mm, silica gel, Kodak, Rochester, New York). Spots were visualized by examination under ultraviolet light and/or treatment with anisaldehyde spray reagent (90% of a 5% solution of *p*-anisaldehyde in ethanol–5% glacial acetic acid–5% concentrated sulfuric acid) followed by brief heating with a heat gun.

Analytical GLC was performed with a Varian model 1800 gas chromatograph equipped with a flame ionization detector and a fused silica NON-PAKD capillary column (SPB-1, dimethylpolysiloxane, 0.5 mm × 15 m, Supelco, Inc.) with a carrier gas (helium) flow rate of 30 ml/min. Samples were analyzed with oven temperature held at 150°C for 5 min followed by programming at 20°C/min to 250°C. Injector and detector temperatures were 250°C.

GC-mass spectra were measured with electron impact (EI) ionization at 70 eV using a Hewlett-Packard GC-MS system consisting of the following components: a model 5890 gas chromatograph fitted with a fused silica capillary column (HP-1, cross-linked methylsilicone, 12 m × 0.2 mm × 0.33 μm film thickness, helium carrier gas, 12 psi) coupled to a model 5971A mass selective detector. Capillary split injection (50:1) was used with an injector temperature of 250°C. Samples were analyzed with oven temperature held at 150°C for 5 min followed by programming at 20°C/min to 250°C.

NMR spectra were measured on an IBM-Bruker model WP200SY (200 MHz) NMR spectrometer. Spectra were measured in $CDCl_3$ or deuteriobenzene solution containing 0.05% v/v tetramethylsilane (TMS) as internal standard. [^{1}H]NMR data are presented as follows: chemical shift in ppm on the δ scale relative to internal TMS [multiplicity, number of hydrogens, coupling constant(s) in Hertz]. [^{13}C]NMR data were measured at 50.3 MHz using broadband ^{1}H decoupling and are presented in ppm on the δ scale relative to internal TMS, chloroform solvent (77.0 ppm), or benzene (128.0 ppm).

Ultraviolet spectra were measured on a Perkin-Elmer model Lambda 3A

UV/visible spectrophotometer. Infrared spectra were obtained on a Perkin-Elmer model 283B spectrophotometer with samples as thin films or in $CHCl_3$ solution. Optical rotations were measured on a Perkin-Elmer model 241 polarimeter in a 1-dm cell.

Purification of synthetic materials was performed using flash chromatography (Still et al., 1978) on silica gel (average particle size 40 μm, J.T. Baker, Phillipsburg, New Jersey) using the indicated solvent for elution.

Purification and Syntheses

α-Gurjunene, 3 (Figure 1). Vacuum distillation (0.7 mm) of crude *D. kerrii* resin (7.7 g; 85% α-gurjunene, GLC analysis) gave two fractions, each as a light yellow oil: 77–78°C (4.44 g; 89% α-gurjunene by GLC; 56% recovery) and 79–80°C (0.99 g; 92% α-gurjunene by GLC; 13% recovery). Pot residues from each vacuum distillation of crude resin were retained and combined. Vacuum distillation (2.5 mm) of this material (23.47 g; 70% α-gurjunene by GLC) yielded additional material (5.95 g; 25% recovery) of somewhat lower purity (80% α-gurjunene by GLC).

Conjugated Dienol, 1 (Figure 1). To a solution of partially purified **3** (2.79 g, 89% α-gurjunene, equivalent to 2.48 g, 12.1 mmol of pure **3**) in glacial acetic acid (6.9 ml) and freshly distilled acetic anhydride (8.3 ml) was added selenium dioxide (0.55 g, 4.97 mmol). The resulting reddish-brown mixture was stirred magnetically at room temperature for 24 hr, a second portion of selenium dioxide (0.56 g, 5.08 mmol) was added, and the solution was stirred at room temperature for an additional period of three days.

The mixture was diluted with methylene chloride and suction filtered through a pad of Celite. Water (20 ml) was added to the filtrate, which was stirred at room temperature for 2 hr and then concentrated in vacuo to remove water and acetic acid. The resulting crude product was dissolved in chloroform (50 ml), washed with water (3 × 30 ml) and saturated $NaHCO_3$, and dried over anhydrous Na_2SO_4. Removal of the drying agent and evaporation of the solvent gave 4.86 g of crude acetate **5** as a brown oil. TLC analysis (90:10 hexanes–ether) showed the major product as a spot with $R_f = 0.5$. Purification by flash chromatography (90:10 hexanes–ether) gave 1.11 g (40% yield) of pure acetate **5** as light yellow oil. IR and [^{1}H]NMR data (reported below) were in agreement with those of other workers (Streith and Ourisson, 1963).

Lithium aluminum hydride (0.48 g, 12.6 mmol) was weighed into a 500 ml three-necked round-bottom flask fitted with a 50-ml pressure-equalizing addition funnel and a reflux condenser. Anhydrous ether (38 ml) was added via the addition funnel and the resulting slurry was stirred magnetically at room temperature. A solution of **5** (2.57 g, 9.87 mmol) in anhydrous ether (27 ml) was introduced dropwise via the addition funnel over the course of 0.5 hr and

the funnel was rinsed with a second volume of ether (20 ml), which was added to the reaction mixture. After stirring at room temperature for 40 min, ethyl acetate (6.3 ml, 64.2 mmol) was added via the addition funnel to quench excess hydride, and the mixture was stirred for 10 min. Next, a solution of methanol and water (50:50 v/v, 25 ml) was added and the mixture was stirred for an additional 90 min. The mixture was washed with water (3 × 20 ml), dried over Na_2SO_4, and concentrated in vacuo to give 1.02 g of crude **1** as a brown oil with a single major spot at R_f = 0.44 by TLC (50:50 hexanes–ether). Purification by flash chromatography (50:50 hexanes–ether) gave 0.43 g (20% yield) of pure conjugated dienol **1**. [^{1}H]NMR and IR data (reported below) were in agreement with those previously reported (Streith and Ourisson, 1963); IR, [^{1}H]NMR, [^{13}C]NMR (see Table 1) and GC-MS data (reported below) were in agreement with data for natural **1** (Richardson et al., 1989).

Acetate ***5.*** IR (thin film): 2939, 1731, 1458, 1370 cm^{-1}; [^{1}H]NMR: δ 0.69 (d, 3H, J = 6.7), 1.22 (m, 1H), 1.42, (s, 3H), 1.45 (s, 3H), 1.5–1.75 (m, 4H), 1.76 (br. s, 3H), 1.97 (s, 3H), 2.10 (br. d, 1H, J = 20), 2.45 (br. dd, 1H, J = 7.5, 20), 2.90 (m, 1H), 3.30 (m, 1H), 5.26 (br. s, 1H), 5.64 (br. s, 1H); [^{13}C]NMR ($CDCl_3$): δ 170.26, 149.50, 141.43, 131.65, 115.86, 86.05, 50.07, 40.56, 35.18, 34.01, 32.95, 24.14, 23.63 (two overlapping peaks), 22.42, 16.08, 12.76; UV (cyclohexane): λ_{max} = 239 nm (ϵ = 4800).

Conjugated Dienol ***1.*** IR (thin film): 3401, 1706, 1459, 1380 cm^{-1}; [^{1}H]NMR: δ 0.68 (d, 3H, J = 7), 1.21 (two overlapping s, 6H total), 1.27 (m, 2H), 1.71 (br. s, 3H), 1.78 (br. d, 1H, J = 11), 1.9–2.1 (m, 3H), 2.12 (br. d, 1H, J = 18), 2.29 (m, 1H), 2.45 (br. dd, 1H, J = 8, 18), 3.34 (m, 1H), 5.4 (m, 1H), 5.67 (br. s, 1H); [^{13}C]NMR: (Table 1); UV (cyclohexane): λ_{max} = 244 nm (ϵ = 12,900); $[\alpha]_D$ = +55.1° (c = 1.67 × 10^{-3} g/ml, CCl_4); GC-MS *m/z* (rel. intensity): 220 (M^+, 0.8), 205 (1.0), 202 (M-H_2O, 23.5), 187 (15.8), 162 (16.2), 161 (9.8), 159 (58.4), 147 (19.5), 145 (46.6), 133 (22.3), 131 (57.2), 120 (27.2), 119 (40.5), 107 (24.2), 105 (76.3), 94 (27.0), 91 (88.2), 81 (35.1), 79 (38.3), 77 (44.7), 59 (43.4), 41 (100).

Nonconjugated Dienol ***20*** *(γ-Gurjunenol, see Figure 8) Epoxidation at 0°C.* To a solution of *m*-chloroperbenzoic acid (80% pure, 0.465 g, 2.15 mmol) in methylene chloride (20 ml) at 0°C was added, via syringe, α-gurjunene, **3** (80% pure, 0.50 g, 1.96 mmol) in methylene chloride (10 ml). The reaction mixture was stirred magnetically at 0°C for 10 min, diluted with methylene chloride (10 ml), and washed with saturated aqueous Na_2CO_3 (3 × 15 ml). After drying (Na_2SO_4), concentration in vacuo afforded 0.73 g of crude product as a colorless oil with a major product spot at R_f = 0.51 by TLC analysis (70:30 hexanes–ether). Purification by flash chromatography (70:30 hexanes–ether) gave pure **20** (0.173 g, 43% yield) as a colorless oil. Two minor products were also observed by TLC (R_f = 0.40 and 0.26). These were isolated as a mixture from flash chromatography of the crude epoxidation reaction mixture. Analysis

by IR and [^{1}H]NMR showed these materials to be the *m*-chlorobenzoic acid esters, **21**, epimeric at C-4 (see Figure 9). Present in roughly equal amounts ([^{1}H]NMR analysis), these materials were isolated in 24% yield (0.175 g) based on starting **3**. The more polar epimer was determined to have an α C-4 tertiary hydroxyl group by correlation with structure **20** using the following chemical modifications: benzoate ester elimination (KOtBu, DMF, 150 C, 1.5 hr) from the more polar epimer produced **20**, while the same conditions with the less polar epimer produced isomeric material.

20. IR, [^{1}H]NMR, [^{13}C]NMR (see Table 2) and GC-MS data were in agreement with data for natural **20** (Richardson et al., 1989). IR (thin film): 3453, 2965, 1645, 1448, 1378, 1187 cm^{-1}; [^{1}H]NMR: δ 0.83 (d, 3H, J = 7), 1.27 (s, 3H), 1.28–1.5 (br. m, 4H), 1.72 (br. s, 3H), 1.40–1.89 (br. m, 6H), 2.85 (m, 1H), 3.03 (m, 1H), 4.68 (br. s, 1H), 4.76, (br. s, 1H), 5.71 (dd, 1H, J = 3.2, 6.5); [^{13}C]NMR: (Table 2); UV (cyclohexane): no absorption; $[\alpha]_D$ = +143° (c = 3.5 × 10^{-4} g/ml, CCl_4); GC-MS *m/z* (rel. intensity): 220 (M^+, 14.1), 205 (M-CH_3, 30.8), 202 (9.0), 187 (14.8), 173 (5.5), 162 (100), 159 (24.6), 149 (35.5), 147 (29.5), 145 (22.5), 131 (20.4), 120 (17.3), 119 (46.1), 117 (12.3), 107 (43.3), 105 (58.6), 95 (23.7), 93 (48.1), 91 (60.4), 81 (22.3), 79 (53.1), 77 (36.4), 67 (22.2), 55 (31.7).

21. The following data are for a 50:50 mixture of epimers at C-4. IR (thin film): 3450, 2955, 1717, 1550, 1295, 1263, 1125; [^{1}H]NMR: δ 0.85 (d, 3H, J = 7), 0.91 (d, 3H, J = 7), 1.28 (s, 3H), 1.34 (s, 3H), 1.55 (s, 3H), 1.57 (s, 3H), 1.59 (s, 3H), 1.605 (s, 3H), 1.65–2.10 (overlapping multiplets, 20H, total for both epimers), 2.95–3.30 (overlapping multiplets, 2H, total for both epimers), 5.75 (m, 2H, total for both epimers), 7.36 (dd, 2H, total for both epimers, J = 7, 7), 7.50 (m, 2H, total for both epimers), 7.85 (m, 2H, total for both epimers), 7.95 (m, 2H, total for both epimers).

Epoxidation at −50°C. To a slurry of *m*-chloroperbenzoic acid (80% pure, 0.116 g, 0.538 mmol) in methylene chloride (3 ml) at −50°C was added, via syringe, α-gurjunene, **3** (Fluka, 0.100 g, 0.489 mmol) in methylene chloride (1 ml); a second portion of methylene chloride (1 ml) was used to rinse the syringe and was added to the reaction mixture. The reaction was stirred magnetically at −50°C for 10 min, was diluted with methylene chloride (10 ml) and was washed with saturated aqueous Na_2CO_3 (3 × 5 ml). After drying (Na_2SO_4), concentration in vacuo afforded 0.142 g of crude product as a colorless oil. TLC analysis showed the same product mixture as reaction at 0°C, but analysis by [^{1}H]NMR showed the crude mixture to contain approximately 50% of a new product (tentatively identified as epoxide **6**), along with the same mixture of products observed in epoxidations conducted at 0°C. Purification by flash chromatography (70:30 hexanes–ether) gave pure **20** (0.082 g, 76% yield) as a colorless oil. Epoxide **6** was not isolated and apparently rearranged to **20** during chromatography.

Dihydro-13,14-γ-gurjunenol ***14*** *(see Figure 5).* To γ-gurjunenol, **20** (0.104 g, 0.469 mmol) dissolved in 95% ethanol–benzene (50:50 v/v, 7.5 ml) in a 20 ml test tube was added $(Ph_3P)_3RhCl$ (Wilkinsons's catalyst, 50 mg), which slowly dissolved with swirling to give a deep orange solution. The test tube was placed in a Parr hydrogenation apparatus, which was sealed, evacuated with a water aspirator, and then filled with hydrogen (45 psi). The evacuation–hydrogen pressurization operations were repeated three times, and the reaction mixture was agitated under 45 psi of hydrogen. After 6 hr, the system was brought to atmospheric pressure and a 0.25-ml aliquot was removed and concentrated in vacuo. Analysis by [^{1}H]NMR showed complete consumption of starting material and the presence of single new product, **14**. TLC analysis (70:30 hexanes–ether) showed a single spot with the same R_f (0.51) as starting material. Flash chromatography (50:50 hexanes–ether) afforded pure **14** as a colorless oil (0.468 g, 45% yield). IR ($CHCl_3$, 0.07 M): 3600, 2960, 1465, 1382 cm^{-1}; [^{1}H]NMR: δ 0.79 (d, 3H, J = 7), 0.845 (d, 3H, J = 6.4), 0.850 (d, 3H, J = 6.4), 1.29 (s, 3H), 1.31–1.47 (overlapping multiplets, 3H), 1.51–1.98 (overlapping multiplets, 9H), 3.09 (m, 1H), 5.77 (d, 1H, J = 3.4, 6.1).

5,6-Epoxydihydro-13,14-γ-gurjunenol ***19*** *(see Figure 8).* To a solution of *m*-chloroperbenzoic acid (80%, 0.168 g, 0.077 mmol) in methylene chloride (1 ml) at 0°C was added **14** (0.0157 g, 0.0706 mmol) in methylene chloride (0.3 ml) via syringe. The syringe was rinsed with fresh methylene chloride (2 × 0.3 ml), which was added to the reaction mixture. After 2.5 hr, TLC analysis (50:50 hexanes–ether) showed complete consumption of starting **14** (R_f = 0.40) and a single new product spot (R_f = 0.56). The reaction mixture was diluted with methylene chloride (10 ml), washed with saturated Na_2CO_3 (10 ml), and dried (Na_2SO_4). Concentration in vacuo afforded crude **19** (0.0138 g) as a colorless oil. Analysis of this material by [^{1}H]NMR showed no starting material and a single epoxide product. Purification by flash chromatography (90:10 hexanes–ether) gave pure **19** as a colorless oil (0.0102 g, 61% yield). IR ($CHCl_3$, 0.07 M): 3495, 2965, 1468, 1386, 1188 cm^{-1}; [^{1}H]NMR: δ 0.97 (d, 6H, J = 6.7), 1.02 (d, 3H, J = 7.7), 1.22 (s, 3H), 1.23–1.57 (overlapping multiplets, 4H), 1.6–1.8 (overlapping multiplets, 2H), 1.81–1.93 (overlapping multiplets, 5H), 2.52 (br. t, 1H, J = 9.1), 2.86 (s, 1H, -OH), 3.08 (s, 1H, epoxide-H).

Termite Bioassays

Bioassays were conducted in Bogor, Indonesia (Messer et al., 1990). Solutions of test chemicals were prepared in dichloromethane to dispense 1-, 5-, and 10-mg quantities of the compounds onto 4.5-cm-diameter Whatman No. 1 filter paper circles. Control filter papers were treated with dichloromethane. After the solvent had been allowed to evaporate off at ambient temperature (25–31°C), the filter papers were placed in 5-cm Petri dishes. To each of the eight replicate

dishes prepared for each condition, 25 *Neotermes ?dalbergiae* termites were added. Thus at the beginning of a bioassay, $N = 200$ for each condition. At daily intervals the number of termites surviving was recorded, dead termites were removed, and the filter papers were moistened with water. Except when being counted, termites were kept covered, in a darkened room, at ambient laboratory temperatures and humidity (25–31°C, ca. 85% relative humidity). Bioassays were terminated after 14 days.

Statistical Testing

All statistical tests were performed on untransformed data with Data Desk (Velleman, 1989). A normal distribution was confirmed for all data prior to statistical testing. Statistical significance of treatment effects was determined by performing pooled *t* tests on counts of termites surviving at day 14 in each dish of a condition and in control dishes at the same time point. A one-way analysis of variance of counts of termites surviving at day 14 was used to determine if application rates were significant for each test chemical.

RESULTS AND DISCUSSION

Structural Analysis

Our initial investigation of the chemical ecology of the dipterocarp *D. kerrii* had located two novel sesquiterpene dienols in the termiticidal and fungicidal fractions of this tree's resin (Richardson et al., 1989). Following extensive spectroscopic work, these materials were assigned the structures **1** and **2** (Figure 1). Neither of these compounds had been isolated previously from a natural source, although **1** had been reported (Streith and Ourisson, 1963) as a product of synthetic manipulations of α-gurjunene, **3**. The need to extend the toxicity studies begun in our initial investigation, and the need to confirm and complete

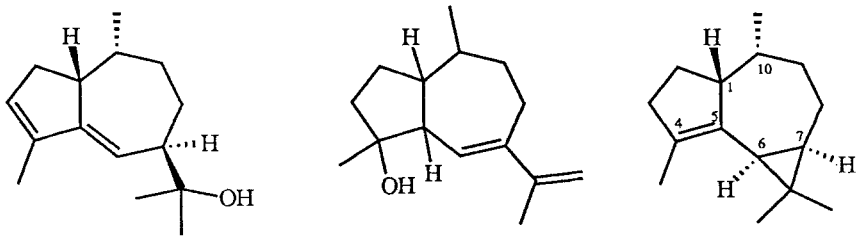


FIG. 1. Sesquiterpenes isolated from *D. kerrii* resin. Structure **2** has been revised to **20**; see text.

the structural assignments for **1** and **2** made in that work, required efficient, nonambiguous chemical syntheses of these sesquiterpenes.

Preparation of **1** was straightforward and followed the two-step process previously developed (Streith and Ourisson, 1963) in structural work with α-gurjunene (Palmade et al., 1963). In our hands, the first step, oxidation of **3** with selenium dioxide in acetic anhydride, produced the dienol acetate **5** in 40% yield. As shown in Figure 2, this process is believed (Streith and Ourisson, 1963) to proceed via initial allylic oxidation at C-3, followed by olefin isomerization with concomitant rupture of the cyclopropane, and is terminated by capture of the C-11 carbocation center in **4** by acetate. Subsequent reduction of **5** with lithium aluminum hydride produced the target dienol **1** in 20% yield (Newton, 1989).

Our spectroscopic data (IR and [^{1}H]NMR) for **5** and **1** matched those reported by Ourisson for these materials (Streith and Ourisson, 1963). More importantly, our data (GC-MS, [^{1}H]NMR, and [^{13}C]NMR) (Table 1) for synthetic **1** matched our previously measured data for naturally occurring **1** (Richardson et al., 1989). These data make it clear that our original structural assignment for the sesquiterpene dienol **1** was correct.

In contrast, we found our UV and optical rotation data for synthetic and natural **1** (Richardson et al., 1989) to differ somewhat: natural **1**: UV (cyclohexane) λ_{max} = 243 nm (ϵ = 2400); $[\alpha]_D$ = +50° (c ≈ 2 × 10^{-4} g/ml, CCl_4); synthetic **1:** UV (cyclohexane) λ_{max} = 244 nm (ϵ = 12,900); $[\alpha]_D$ = +55.1° (c = 1.67 × 10^{-3} g/ml, CCl_4). We feel this disagreement simply indicates that the very small sample of **1** (<0.2 mg) that we originally isolated was not pure. This is not surprising in view of the considerable lability of **1**, which we have reported previously (Richardson et al., 1989).

The lability of these materials posed a significant difficulty in our initial structural work, particularly in studies with the second sesquiterpene dienol. Due to the limited quantity of this dienol available for spectroscopic study and due to its slow but steady decomposition in NMR solvents, we were able to report only a preliminary structure (**2**) for this natural product in our previous publication (Richardson et al., 1989). As shown in Figure 1, we originally proposed that **2**, like **1**, was a member of the guiane sesquiterpene family and

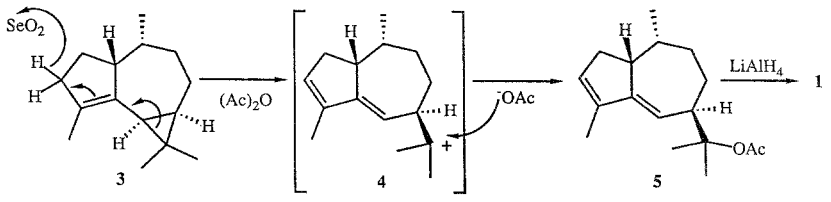


FIG. 2. Synthesis of dienol **1** from α-gurjunene, **3**.

TABLE 1. [^{13}C]NMR DATA FOR NATURAL AND SYNTHETIC **1**[a]

Natural **1**	Synthetic **1**
13.08	12.92
	15.96
24.00	24.10
27.28	27.41
28.30	28.26
33.14	33.35
34.21	34.32
35.42	35.46
40.98	41.18
53.08	53.36
73.46	73.62
117.25	117.28
132.04	131.95
141.67	141.73
149.82	149.86

[a]Spectra were measured in C_6D_6.

contained both tertiary alcohol and conjugated diene moieties. However, we were unable to assign the stereochemical configuration of C-1, C-4, C-5, and C-10, and the determination of these centers was one of the primary reasons for developing a synthetic route to **2**.

It can be seen that structure **2** is isomeric with dienol **1**. The ready availability of synthetic **1**, using the chemistry discussed above, initially made attractive a synthetic route to **2** via isomerization of **1**. Although conceptually straightforward, we decided that isomerization of **1** was impractical due to the marginal stability of the highly substituted dienols involved. Furthermore, this preparative approach would not be unambiguous with respect to construction of the C-4 and C-5 stereocenters. Instead, we pursued a more novel route for synthesis of this material, which would provide efficient access to synthetic **2** and which would also use α-gurjunene as starting material.

Our plan, as diagrammed in Figure 3, was to treat α-gurjunene with *m*-chloroperbenzoic acid (MCPBA) to prepare epoxide **6**. This material, either after isolation or *in situ* during the epoxidation process, would then undergo acid-promoted epoxide cleavage, via intermediate **7**, to produce carbocation **8** after rearrangement and cyclopropane cleavage. Ultimately, **8** would undergo deprotonation, under the action of exogenous base, to give either the conjugated dienol **9** or the nonconjugated dienol **10** as final product. (Of course, substitution, via direct capture of carbocation **8**, could compete with this process.) We anticipated that **10** would be the preferred product of this process since its for-

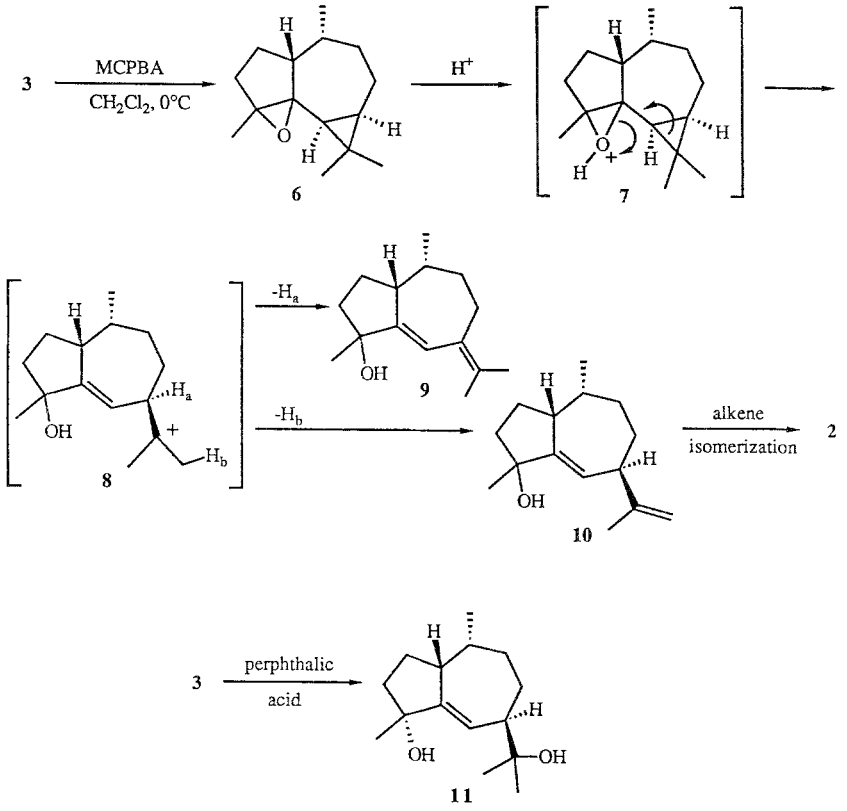


FIG. 3. Synthesis plan for preparation of dienol **2** from α-gurjunene, **3**.

mation via deprotonation is statistically more likely and because the stability of the conjugated diene **9** should be offset by steric factors since its C-7/C-11 double bond is both exocyclic and tetrasubstituted. Final conversion of **10** into the target dienol **2** would require isomerization of its C-5/C-6 double bond into conjugation with its isopropylidene unit. Although we anticipated that this process would require some experimentation, literature reports of this type of transformation using organometallic reagents (Curran et al., 1987; Whitesides and Neilan, 1976) gave us confidence that this overall approach was sound.

In addition, a general literature precedent existed for this epoxidative transformation of α-gurjunene. As depicted in Figure 3, a previous study reported preparation of diol **11** from treatment of α-gurjunene with the epoxidizing agent perphthalic acid (Palmade et al., 1963). This study did not establish the mechanism for formation of this material, but it is reasonable to suggest that it arises via trapping, by an oxygen-containing nucleophile, of an intermediate carbo-

cation like **8** in Figure 3. Ourisson established that the C-4 tertiary alcohol in this material had α-configuration, as shown in structure **11**, implying that the epoxidation process had proceeded on the α-face of the C-4/C-5 double bond in **3**. Similar stereochemical results have been observed in reactions of **3** with diborane (Pesnelle and Ourisson, 1965) and with osmium tetroxide (Palmade et al., 1963). Hence, it was anticipated that our approach to **2** would give material with the following stereochemical detail: (1) The C-1 and C-10 centers would have configurations identical to those in α-gurjunene. (Since dienol **1** was found to have this stereochemistry at C-1 and C-10, we anticipated that these centers would be the same in **2**.) (2) C-4 would carry an α-tertiary alcohol group. (Our prediction was that MCPBA, like other electrophiles studied, would attack α-gurjunene from the α-face of the alkene to provide a final product with an α-tertiary alcohol center.) (3) The C-5 configuration would require determination. (Prediction of the configuration at this center was difficult since it would depend upon the process used to isomerize **10** to **2**.) Thus, although it was uncertain if this route would provide material with stereochemical configuration exactly identical to natural **2**, we were confident that it would give rapid access to material which would have the correct connectivity and which would answer several of our configurational questions.

In the event, we were delighted to find that treatment of α-gurjunene with 1.1 equivalents of MCPBA (methylene chloride, 0°C) resulted in complete consumption of starting material in 10 min (GLC analysis) and gave a crude product whose [^{1}H]NMR spectrum was almost identical (Newton, 1989) to original spectra for natural **2**! Although this crude material contained several products, analysis by both TLC and GLC showed a single major product that could be isolated in pure form by flash chromatography. Both [^{1}H]NMR and [^{13}C]NMR (Table 2) data for this synthetic material matched those we originally measured for natural **2** (Richardson et al., 1989). We interpreted this result to mean that MCPBA-epoxidation of **3** had proceeded to intermediate diene **10** as depicted in Figure 3 and that this material had subsequently undergone acid-catalyzed isomerization to **2** *in situ*. The isolated yield for **2** under these conditions was 33–48%.

Although IR data for natural and synthetic **2** were also in good agreement (see above), two other measurements were not. First, the rotation for freshly purified synthetic **2** was higher than that measured for natural **2** (synthetic **2**: $[\alpha]_D = +143°$ c $= 3.5 \times 10^{-4}$ g/ml, CCl_4; natural **2**: $[\alpha]_D = +63°$ c $\approx 2 \times 10^{-3}$ g/ml CCl_4; Richardson et al., 1989). As with the comparison of synthetic and natural **1** above, we interpreted this result simply to mean that our original sample of natural **2** had not been pure. However, we also found that freshly purified samples of synthetic **2** showed no significant UV absorption, meaning that this substance did not contain a UV chromaphore, while natural material had shown a peak at $\lambda_{max} = 219$ nm ($\epsilon = 1400$). Since it was evident

TABLE 2. [^{13}C]NMR DATA FOR NATURAL AND SYNTHETIC **2**[a]

Natural **2**	Synthetic **2**
14.75	14.98
21.51	21.33
25.33	25.80
26.87	26.98
26.27	27.30
32.86 (overlapping peaks)	33.11, 33.19
41.33	41.44
45.39	45.22
46.65	47.09
80.40	80.35
110.96	110.87
123.85	123.98
145.83	146.23
152.09	151.96

[a]Structure **2** has been revised to **20**; see text. Spectra were measured in $CDCl_3$.

that the NMR data for the natural and synthetic materials matched, this result implied that our initial structure assignment for dienol **2** was in error. It was evident, however, that the nature of our error would have to be fairly minor and that the major details in our original structure had to be correct, since the structure of the starting material for our one-step synthetic process had so many parallels with the structure for **2** originally proposed.

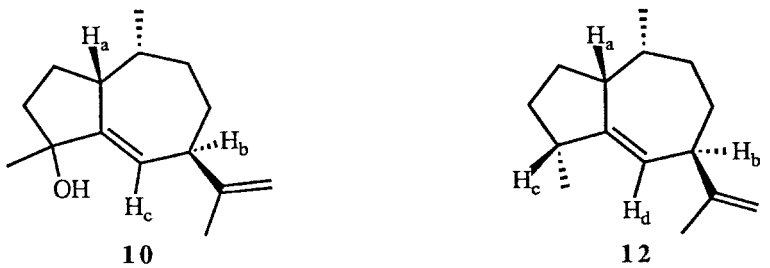
The simplest solution to this problem was to retain the guiane sesquiterpene framework for **2** and to hypothesize that the diene system in this material was nonconjugated. The alkene signals in NMR spectra (^{1}H and ^{13}C) of **2** clearly indicate the presence of an isopropylidene moiety and a second, trisubstituted double bond. Thus, the most straightforward modification of structure **2** meeting these requirements was to position the trisubstituted alkene at C-5/C-6 rather than C-6/C-7. This produced the nonconjugated dienol **10** (see Figure 9), the same structure which appeared as an intermediate in our original synthetic route to **2**, above (Figure 3).

Proving that **10** was in fact the correct structure for both our second *D. kerrii* dienol and the product of MCPBA epoxidation of α-gurjunene required: (1) establishing that the diene system was nonconjugated, with the trisubstituted double bond at C-5/C-6; and (2) determining the configuration of the C-4 tertiary alcohol center. The configurations of the centers at C-1, C-7 and C-10 in **10** were assigned to be identical with the configurations of the corresponding centers in α-gurjunene. This was logical since the epoxidation process produc-

ing **10** from α-gurjunene would not have affected the configurations of these centers.

Unfortunately, attempts to determine directly the stereochemical configuration of **10** using difference NOE and two-dimensional NOE (NOESY) techniques were inconclusive. As a result, it was necessary to employ an indirect approach, involving chemical modification, in order to prove and refine our new hypothetical structure for compound **10**. Fortunately, our structural work with **10** was aided by the availability of a naturally occurring sesquiterpene with marked structural similarities to **10**, namely γ-gurjunene, **12**. As shown in Figure 4, γ-gurjunene essentially duplicates hypothetical structure **10**, with the exception that C-4 in this material, which bears an α-methyl group, lacks a tertiary alcohol moiety. Hence, both literature data for γ-gurjunene (Ehret and Ourisson, 1969) and data acquired through our own measurements (Richardson and Lindeman, unpublished data) served as a useful reference for confirming and refining hypothetical structure **10**.

For example, [^{1}H]NMR spectra for γ-gurjunene and **10** show strong parallels. The allylic protons in **12** appear in two groups: H_a and H_b (C-1 and C-7, respectively, Figure 4) overlapping at δ 2.85, and H_c (C-4) at δ 2.50 (Richardson and Lindeman, unpublished data). As revealed in the spectroscopic data above, compound **10** shows two allylic signals at δ 3.03 and 2.85, which can be reasonably labeled, in analogy with the assignments in **12**, as H_a and H_b, respectively (Figure 4). Compound **12** shows an olefin proton signal at δ 5.40 (H_d) which appears as a double-double-doublet due to coupling with all three allylic protons H_a, H_b, and H_c. A similar double-doublet signal is present in the [^{1}H]NMR of **10** at δ 5.71. By analogy with **12**, it is reasonable to conclude that this signal in **10** corresponds to a trisubstituted olefin proton on C-6 (labeled H_c), which couples to the two allylic protons at C-1 (H_a) and C-7 (H_b). Finally, both compounds show the presence of isopropylidene olefin protons, but with slight differences: **12**: δ 4.72 and 4.73 (500 MHz, Messer et al., 1990; these signals are unresolved at 200 MHz, Richardson and Lindeman, unpublished data); **10**: δ 4.76 and 4.68 (200 MHz, Newton, 1989). These differences



10 **12**

FIG. 4. Structure of γ-gurjunene (**12**) and partial revised structure (**10**) for dienol **2**.

in chemical shift are probably due to subtle conformational effects that place the isopropylidene groups in **10** and **12** in slightly different magnetic environments.

Likewise, [^{13}C]NMR spectra for the two compounds have similar chemical shifts for the four olefinic carbons in each molecule: **10**: δ 152.09, 145.83, 123.85, 110.96 (Richardson et al., 1989); **12**: δ 150.48, 147.13, 122.28, 110.52 (Richardson and Lindeman, unpublished data). Furthermore, just as we established for compound **10**, γ-gurjunene is not UV-active (Ehret and Ourisson, 1969). Thus, all of the NMR data, and the lack of UV activity for these materials, suggest that the diene systems are similar in both sesquiterpenes (e.g., both contain an isopropylidene group and a trisubstituted double bond) and lend support to our hypothesis that **10** includes a nonconjugated diene. Since the olefinic proton H_c in **10** shows allylic coupling to both H_a and H_b, the only reasonable position for the trisubstituted double bond was at C-5/C-6.

Additional support for these conclusions was obtained by studying chemical modifications of **10** and **12**. Previous workers (Ehret and Ourisson, 1969) had shown that selective reduction of the isopropylidene double bond in **12** could be accomplished, using hydrogen in the presence of Wilkinson's catalyst (Figure 5), to give a 92% yield of dihydro-13,14-γ-gurjunene, **13**. Although somewhat slower (6 hr vs. 2.5), reduction of **10** under these conditions dis-

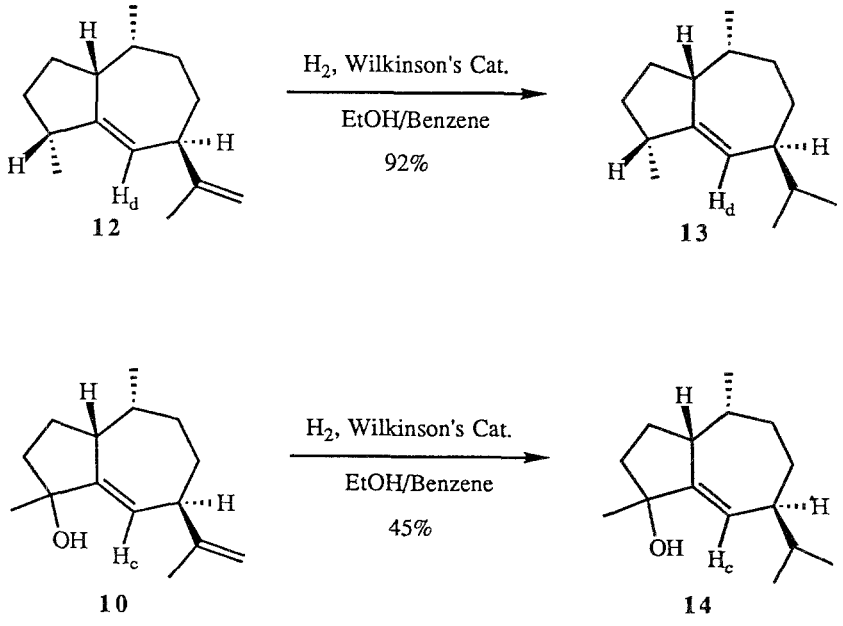


FIG. 5. Selective hydrogenations of **12** and **10**.

played the same selectivity for the disubstituted double bond, giving dihydro-13,14-**10** (**14**, Figure 5) in 45% yield.

The selectivity of these reductions could be verified by the following features in the corresponding [^{1}H]NMR spectra: (1) the disappearance of the signals for the isopropylidene olefin and methyl group protons; (2) the appearance of isopropyl methyl group doublets in **13** (δ 0.88 and 0.85, Richardson and Lindeman, unpublished data) and in **14** (δ 0.855 and 0.850); (3) the disappearance of one of the original allylic proton signals in both **10** and **12** [e.g., the signal for H_b (Figure 4) in both **10** and **12** is shifted upfield since this proton is no longer doubly allylic after reduction]; and (4) the multiplicity and chemical shift of trisubstituted olefin proton signals in both **10** and **12** (H_c and H_d, respectively, in Figures 5 and 6) were changed only slightly by the reduction [H_d: **12**, δ 5.40, (ddd); **13**, δ 5.42, (ddd); H_c: **10**, δ 5.71, (dd); **14**, δ 5.80, (dd)]. In both reactions, selective reduction of the disubstituted double bonds was good evidence that the diene systems were nonconjugated, since hydrogenation of conjugated dienes is usually complicated by poor selectivity and double-bond migration (e.g., 1,2- and 1,4-hydrogen addition; Cais, 1964). Hence, although the yield for reduction of **10** was somewhat lower than that for **12**, due to the inherent liability of the allylic alcohol system, this final piece of chemical evidence placed the nonconjugated diene structure we have assigned to compound **10** on a firm footing.

With the position of the trisubstituted double bond in **10** located at C-5/C-6, the final structural problem to solve was the configuration of the C-4 ter-

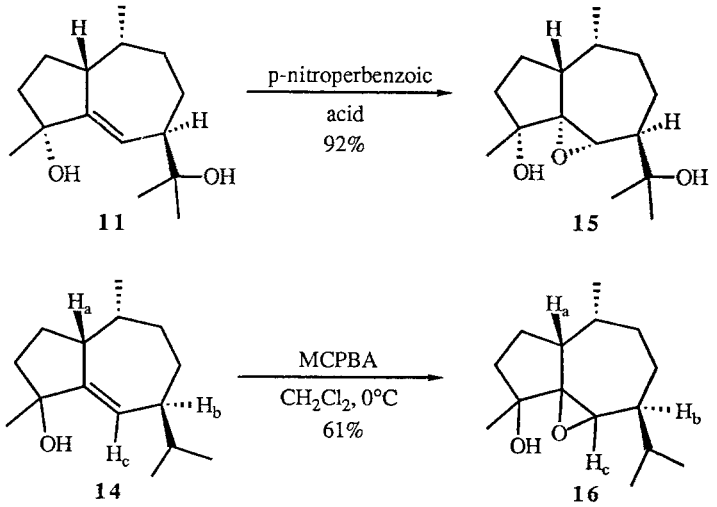


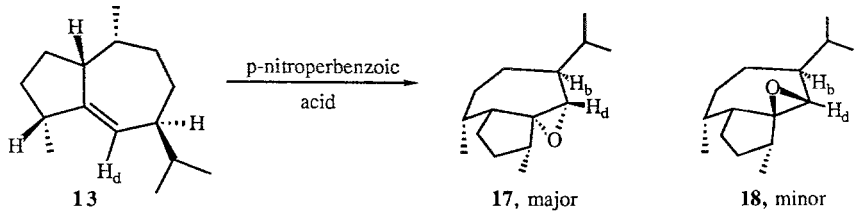
FIG. 6. Epoxidation of allylic alcohols **11** and **14**.

tiary alcohol center. We accomplished this assignment using a chemical modification approach that mirrored the original structural studies on both α- and γ-gurjunene (Streith and Ourisson, 1963). As shown in Figure 6, *p*-nitroperbenzoic acid epoxidation of the diol **11**, obtained from perphthalic acid oxidation of α-gurjunene (Figure 3), proceeded to give epoxide **15** (Palmade et al., 1963). Using IR spectroscopy, the stereochemical relationship between the C-4 hydroxyl center and the epoxide was deduced as follows: At high dilution, **15** showed two hydroxyl bands at 3615 cm^{-1} and 3510 cm^{-1} in its IR spectrum, which were assigned as free (C-13) and internally hydrogen-bonded hydroxyl groups (C-4), respectively. An intramolecular hydrogen bond at the C-4 hydroxyl group was interpreted to mean that this group was associated with the new epoxy group, which required that both groups be positioned on the same face of **15**. This interpretation was strengthened by the known tendency of allylic alcohol epoxidations to occur under the stereochemical direction of the hydroxyl group (Henbest and Wilson, 1957; Tanaka et al., 1974).

With this approach, treatment of our monohydroxy compound **14** with MCPBA produced a single epoxide product (TLC and [^{1}H]NMR analysis) in 61% yield. High-dilution (0.07 M) IR spectra for this epoxide product showed a single band in the hydroxyl region at 3495 cm^{-1}, while **14** had shown hydroxyl absorption at 3600 cm^{-1} at same concentration. This implied, as with compound **15**, that epoxidation of **14** had produced a product containing an intramolecular hydrogen bond between epoxide and hydroxyl groups. This result indicated that the epoxide and hydroxyl groups in this material were syn, but, as shown in structure **16** (Figure 6) for this product, this information alone did not enable us to assign the configuration of the C-4 hydroxyl group. As above, a syn relationship between these groups had been anticipated on the basis of the usual internal asymmetric induction observed in epoxidation of allylic alcohol systems.

A configurational assignment for C-4 could be made, however, using [^{1}H]NMR data for compound **16**. As with compound **10**, discussed above, the olefin proton (H_c, Figure 6) on the trisubstituted double bond in compound **14** appears as a double-doublet due to coupling with allylic protons H_a and H_b. Upon epoxidation, the signal for this proton is shifted to δ 3.08 and appears as a singlet, implying that in compound **16** this proton has adopted a new configuration that does not have the proper dihedral angles with respect to H_a and H_b to allow for coupling. This observation may be used to infer the stereochemical relationship between the known C-7 isopropyl group center and the C-5/C-6 epoxide centers, and, hence, can be used to deduce the configuration of the C-4 hydroxyl group.

Making these deductions relies upon reference to [^{1}H]NMR data measured in earlier γ-gurjunene structural studies (Ehret and Ourisson, 1969). As depicted in Figure 7, it was found that *p*-nitroperbenzoic acid epoxidation of dihydro-

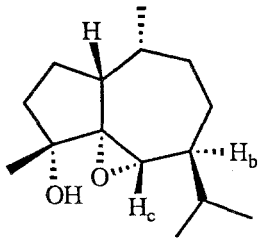


13 17, major 18, minor

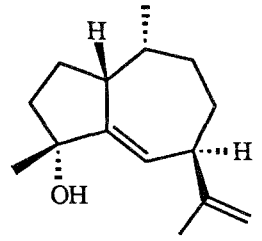
FIG. 7. Epoxidation of dihydro-13,14-γ-gurjunene (**13**) and stereochemistry of product epoxides.

13,14-γ-gurjunene, **13** (Figure 5), produced a pair of epoxide products, **17** and **18**, in yields of 52% and 5%, respectively. The signals for the epoxide protons (H_d) in the [^{1}H]NMR spectra of these materials appeared as follows: **17**, δ 3.12, (singlet); **18**, δ 3.00, (doublet, J = 4 Hz). Using conformational arguments, Ehret and Ourisson convincingly demonstrated that the only structures for **17** and **18** that were consistent with the measured NMR data were those shown in Figure 7. In these structures, both epoxides adopt a quasiboat conformation for the cycloheptane system, with **17** being the α-epoxide and **18** being the β-epoxide. In this conformation, the dihedral angles between H_d and H_b in **17** and **18** are 90° and 60°, respectively. Thus, as observed, and in agreement with the usual Karplus relationships, coupling between these protons would not be expected in **17**, while a small coupling would be expected in **18**.

Although we isolated only a single epoxide product, **16**, from epoxidation of our compound **14** (Figure 6), the structural similarity between our system and those in Ourisson's studies made the remaining configurational assignment in **16** straightforward. As previously discussed, the signal for epoxide proton H_c (on C-6) in **16** appears as a singlet (δ 3.08) in the [^{1}H]NMR spectrum of this material. Since it is reasonable to assume that the cycloheptane system in **16** would adopt a conformation similar to that in Ourisson's systems **17** and **18** (e.g., quasiboat), a singlet for H_c indicates that this proton does not couple with the C-7 proton (H_b). This result, in analogy to **17**, implies a *trans* relationship between these protons, with H_c being β (Figure 6). A β position for H_c requires the C-5/C-6 epoxide to be in the α position. Finally, since our previous IR measurements had determined a syn relationship between the epoxide and hydroxyl groups, an α-epoxide implied that the C-4 tertiary alcohol group must also be in the α position. Thus, structure **19** results (Figure 8) as a complete assignment for the epoxide, shown as partial structure **16**, above. This information for **19** implies that the C-4 hydroxyl group in the second sesquiterpene dienol that we originally isolated from *D. kerrii* resin (Richardson et al., 1989) is also α, and, thus, our final structure assignment for this material is **20**, depicted in Figure 8. Hence, the major direction of attack by MCPBA during



19



20

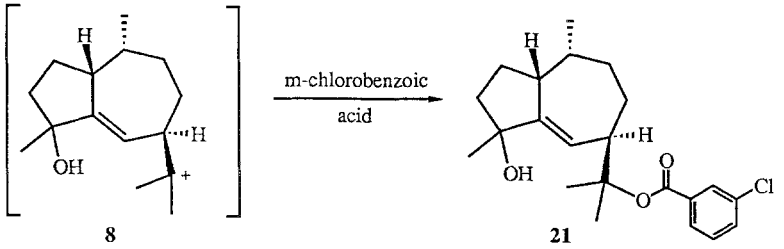
FIG. 8. Structure of epoxide from epoxidation of allylic alcohol **14** and final revised structure for dienol **20**, γ-gurjunenol (originally assigned structure **2**, see text).

epoxidation of α-gurjunene is on the α-face of the C-4/C-5 double bond, which agrees with observations recorded for various other electrophiles, as discussed above. In view of the marked structural similarities which exist between the second *D. kerrii* dienol and γ-gurjunene, we suggest that **20** be referred to as γ-gurjunenol. To the best of our knowledge, this is the first report of structure **20** in the literature.

In addition to **20**, crude epoxidation mixtures also showed the presence of two more polar products by TLC analysis (70:30 hexanes–ether; R_f = 0.40 and 0.26, respectively). These products could be isolated in pure form and were established, through chemical modification studies and analysis of IR, [^{1}H]NMR, and GC-MS spectra, to be the m-chlorobenzoic acid esters, **21**. Epimeric at the C-4 tertiary alcohol center, these side products were isolated in essentially equal amounts (10–14% yields) and presumably arise via trapping of the intermediate carbocation **8** (Figure 9) by m-chlorobenzoic acid present in epoxidation reaction mixtures.

Interestingly, when epoxidation of α-gurjunene was conducted at −50°C, this process was essentially just as rapid as at 0°C (complete in 10 min, GLC) but, after the same workup, it gave a crude product, which [^{1}H]NMR analysis showed to include approximately 50% of a previously unobserved material. This new product was quite labile and would disappear ([^{1}H]NMR analysis) if left standing in $CDCl_3$ solution at room temperature for 48 hr. Attempts to observe this new product using other analytical techniques (GC-MS, TLC) were unsuccessful. Finally, we were surprised to find that immediate column chromatography of the crude product from reaction at −50°C produced a 76% yield of **20**—a significantly higher yield than observed in epoxidations at 0°C.

We interpreted these results to mean that at both reaction temperatures epoxidation proceeds initially to give the intermediate epoxide **6**, as shown above in Figure 3. Apparently, at 0°C, this material undergoes fairly rapid protonation by the acid present to give the unstable intermediate **7**, which then solvolyzes to give **20**, along with the usual ester side products, **21**. At −50°C, however,



8 21

FIG. 9. Proposed mechanism for formation of m-chlorobenzoic acid ester side products, **21**, during epoxidation of α-gurjunene.

protonation and/or subsequent solvolysis appear to be much slower, and once the reagent-derived acids are removed in the workup, a sufficient amount of epoxide **6** then survives to be observed by [^{1}H]NMR. We conclude that due to its expected acid lability, epoxide **6** decomposes during chromatographic purification of the crude product on silica gel, producing a higher net yield of the desired product, **20**, since MCPBA and its reaction products are no longer present to complicate this process. The acid lability of this new product, our putative epoxide **6**, could be confirmed directly with simple [^{1}H]NMR experiments. For example, addition of 0.25 ml of 0.6 M $CHCl_3$ solution of trifluoroacetic acid to a freshly prepared [^{1}H]NMR sample of the crude material from epoxidation at −50°C resulted in the immediate disappearance of the new product and left only signals for **20** and the ester side products, **21**.

Bioassay Results

Bioassay results conducted at the 10-mg application rate are shown in Figure 10. At all dosages assayed, termites that were offered the test compounds showed greater mortality than those in control dishes (one-way ANOVA, $P < 0.05$, with 14 *df* in *t* tests of all conditions). The mortality caused by each of the test compounds was dose-dependent ($P < 0.0005$, 2 *df*). However, only compound **20** showed significant toxicity, at the 10-mg application rate, as judged by the criterion of achieving a 50% kill in seven days.

Identity of Toxic Components

In previous work we localized the toxicity of *D. kerrii* resin within two bioactive fractions, the first containing roughly equal amounts of spathulenol and **1**, and the second containing roughly equal amounts of epicyclocolorenone and **20** (originally assigned structure **2**). In conjunction with the results reported here, it is possible to infer the following: (1) **1** does not contribute substantially to the toxicity of the first fraction, implying that spathulenol is largely responsible for the bioactivity of this particular fraction. We conclude this because in

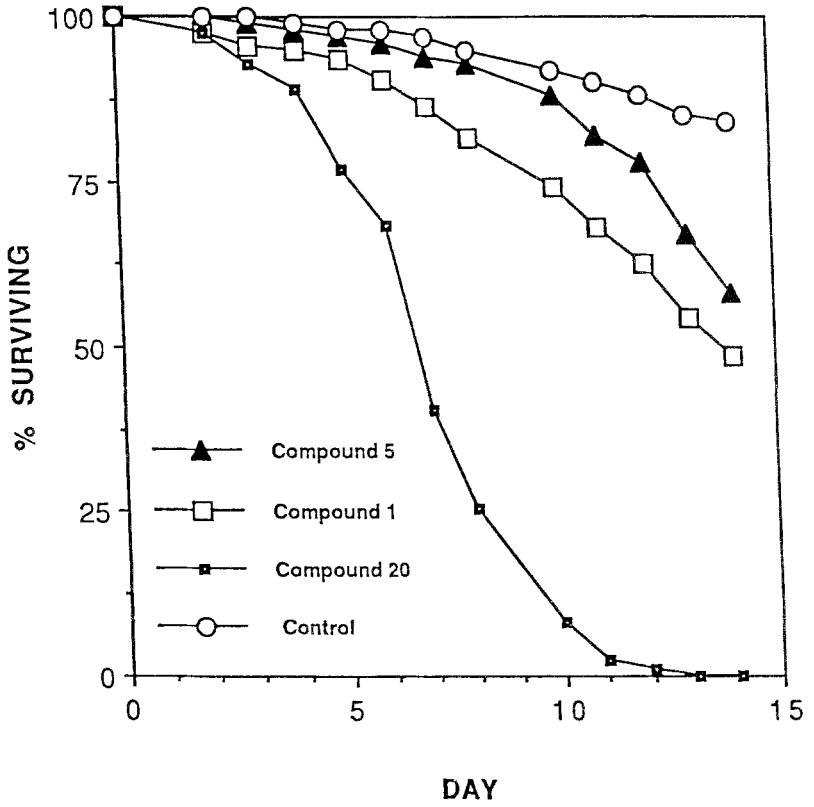


FIG. 10. Toxicity of synthetic compounds **1**, **5**, and **20** to *Neotermes ?dalbergiae* drywood termites; 10-mg application rate. Details in text.

pure form **1** causes very little mortality (<50% in two weeks), while in the mixture with spathulenol 50% mortality was observed after seven days. (2) In pure form, **20** causes 50% mortality within seven days. The same level of toxicity is observed in mixtures with epicyclocolorenone, suggesting that the toxic effects of this mixture are due primarily to **20** (Richardson, et al., 1989). It is unlikely that synergistic effects operate in these mixtures, since other studies did not find evidence for synergism, either between various resin sesquiterpenes or between purified components or crude resins and the insecticide synergist piperonyl butoxide (Messer, 1990; Messer et al., 1990).

We also examined toxicity of the synthetic precursor to **1**, the corresponding acetate **5**. This was of interest since the tertiary alcohol moiety in **1** was labile in dilute acid. Since it is known that acidic conditions are often present in insect alimentary tracts (Noirot and Noirot, 1969), we speculated that the

acetate group might enhance the toxicity of the compound **1** structure as it would render the oxygenated center at C-13 less susceptible to acid-catalyzed solvolysis. While **5** was found to be less sensitive than **1** to acidic conditions, it is only slightly more toxic, as shown in Figure 10.

In conclusion, along with completing and verifying their structures, we have found that sesquiterpenes **1** and **20** belong to a family of resin-derived components that play a role in defending dipterocarps from biological attack (Richardson et al., 1989; Messer, 1990). In comparison to other plant defensive compounds (Harborne, 1982), these sesquiterpenes are not very toxic. This may reflect that these compounds have evolved in response to chronic fungal and insectan insults, which are continuously present and which do not require an acute response. Fresh *D. kerrii* resin, which contains α-gurjunene as its principal component, contains roughly 4% of both **1** and **20** (Richardson et al., 1989). Because of their lability, the dienols are found only in fresh resins of *D. kerrii*, while α-gurjunene persists as resins age. In view of their structural similarities with α-gurjunene, this suggests that the dienols arise via biosynthetic modification of this parent structure as opposed to spontaneous degradation during the aging of resins.

Acknowledgments—Research in Indonesia was supported by the United States Agency for International Development, Program in Science and Technology Cooperation Contract DPE-5542-G-SS-6024-00. We thank K. Iskandar, BIOTROP-Bogor, for technical assistance; Dr. Rosemary Gianno for providing resin samples; and Dr. S. Bacchus for determining the termite specimens. We also thank Professor Jerrold Meinwald and Dr. Braden Roach of Cornell University for assistance with GC-MS measurements. Portions of this work were submitted by B.A.N. in partial fulfillment of the requirements for the BA degree with honors, Williams College.

REFERENCES

CAIS, M. 1964. Conjugated dienes, pp. 955–1024, *in* S. Patai (ed.). The Chemistry of Functional Groups; The Chemistry of Alkenes. Interscience Publishers, London.

CURRAN, D.P., JACOBS, P.B., ELLIOTT, R.L., and KIM, B.H. 1987. Total synthesis of (−)-specionin. *J. Am. Chem. Soc.* 109:5280–5282.

EHRET, C., and OURISSON, G. 1969. Le γ- gurjunene, structure et configuration. Isomerisation de l'α- gurjunene. *Tetrahedron* 25:1785–1799.

HARBORNE, J.B. 1982. Plant toxins and their effects on animals, pp. 66–97, *in* Introduction to Ecological Biochemistry, 2nd ed. Academic Press, New York.

HENBEST, H.B., and WILSON, R.A.L. 1957. Aspects of stereochemistry. Part I. Stereospecificity in formation of epoxides from cyclic allylic alcohols. *J. Chem. Soc.* pp. 1958–1965.

LANGENHEIM, J.H. 1990. Plant resins. *Am. Sci.* 78:16–24.

MESSER, A.C. 1990. Chemical ecology in an Indonesian context. University Microfilms, Ann Arbor, Michigan.

MESSER, A.C., MCCORMICK, K., SUNJAYA, HAGEDORN, H., TUMBEL, F., and MEINWALD, J. 1990. Defensive role of tropical tree resins: Antitermitic sesquiterpenes from Asian Dipterocarpaceae. *J. Chem. Ecol.* 16:3333–3352.

NEWTON, B.A. 1989. Synthesis of Anti-insectan Sesquiterpenes. Senior honors thesis. Department of Chemistry, Williams College, Williamstown, Massachusetts.

NOIROT, C., and NOIROT, T.C. 1969. The digestive system, pp. 49–84, *in* K. Krishna and F.M. Weesner (eds.). Biology of Termites. Academic Press, New York.

PALMADE, M., PESNELLE, P., STREITH, J., and OURISSON, G. 1963. L'α-gurjunene. I. Structure et stereochimie. *Bull. Soc. Chim. Fr.* pp. 1950–1959.

PESNELLE, P., and OURISSON, G. 1965. Hydroboration of α-gurjunene. A rational correlation with cyclocolorenone. *J. Org. Chem.* 30:1743–1747.

RICHARDSON, D.P., MESSER, A.C., GREENBERG, S., HAGEDORN, H.H., and MEINWALD, J. 1989. Defensive sesquiterpenoids from a dipterocarp (*Dipterocarpus kerrii*). *J. Chem. Ecol.* 15:731–747.

STILL, W.C., KAHN, M., and MITRA, A. 1978. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 43:2923–2925.

STREITH, J., and OURISSON, G. 1963. L'α-gurjunene. II. Quelques reactions de l'α-gurjunene. *Bull. Soc. Chim. Fr.* pp. 1960–1965.

TANAKA, S., YAMAMOTO, H., NOZAKI, H., SHARPLESS, K.B., MICHAELSON, R.C., and CUTTING, J.D. 1974. Stereoselective epoxidations of acyclic allylic alcohols by transition metal-hydroperoxide reagents. Synthesis of *dl*-C_{18} *Cecropia* juvenile hormone from farnesol. *J. Am. Chem. Soc.* 96:5254–5255.

VELLEMAN, P. 1989. Data Desk. Odesta Press, Northbrook, Illinois.

WHITESIDES, T.H., and NEILAN, J.P. 1976. Thermolysis of diene iron tricarbonyl complexes. *cis-trans* Isomerization and hydrogen scrambling reactions in cyclic and acyclic complexes. *J. Am. Chem. Soc.* 98:63–73.

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

ANNOUNCEMENT

The University of Pennsylvania will host the Chemical Signals in Vertebrates VI Symposium from June 16–22, 1991 at the Penn Tower Hotel in Philadelphia. Abstracts are solicited for a very wide variety of vertebrate chemical communications (odor perception mechanisms and systems) in aquatic and terrestrial environments. Presentations are 20 minutes plus 10 minute discussions before general sessions or may be submitted as a poster, the format to be ascertained by the abstract review committee. These were due by March 15, 1991.

For further information on program and housing, please write to:

Dr. Richard L. Doty
c/o Ms. Catherine M. Beinhauer
Conference Management Associates
127 Brook Hollow
Hanover, NH 03755
USA

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

PARENTALLY PROVIDED ALKALOID DOES NOT PROTECT EGGS OF *Utetheisa ornatrix* (LEPIDOPTERA: ARCTIIDAE) AGAINST ENTOMOPATHOGENIC FUNGI[1]

GREGGORY K. STOREY,[2] DANIEL J. ANESHANSLEY,[3] and THOMAS EISNER[4,*]

[2]*University of Florida, IFAS, Citrus Research & Education Center 700 Experiment Station Road, Lake Alfred, Florida 33850*

[3]*Cornell University, Agricultural and Biological Engineering 122 Riley-Robb Hall, Ithaca, New York 14853*

[4]*Cornell University, Section of Neurobiology & Behavior W347 Mudd Hall, Ithaca, New York 14853*

(Received June 11, 1990; accepted November 26, 1990)

Abstract—Eggs of *Utetheisa ornatrix* proved equally vulnerable to fungal infection (*Beauveria bassiana*, *Paecilomyces lilacinus*) whether they contained parentally provided pyrrolizidine alkaloid (monocrotaline) or were free of such alkaloid. In in vitro tests, monocrotaline, either as free base or N-oxide, had no inhibiting effect on fungal cultures.

Key Words—Entomopathogenic fungi, insect egg, *Utetheisa ornatrix*, Lepidoptera, Arctiidae, chemical defense, pyrrolizidine alkaloid, monocrotaline, parental investment.

INTRODUCTION

The arctiid moth *Utetheisa ornatrix* is chemically protected at all stages of development. As a larva, it feeds on plants of the genus *Crotalaria* (Leguminosae), containing pyrrolizidine alkaloids (Sharma et al., 1965; Johnson et al., 1985). It sequesters these alkaloids systemically, retains them through metamorphosis, and as an adult bestows them upon the eggs (Conner et al., 1981; Dussourd et al., 1988). The alkaloids are proven defensive agents. They render

*To whom correspondence should be addressed.

[1] Report No. 99 of the series Defense Mechanisms of Arthropods; No. 98 is Attygalle et al., *Experientia* (in press).

0098-0331/91/0400-0687$06.50/0

adults unpalatable to orb-weaving spiders (Eisner and Meinwald, 1987) and eggs to coccinellid beetles (Dussourd et al., 1988). Unanswered was the question of whether the alkaloids might also protect the eggs against entomopathogens. We present evidence here that one pyrrolizidine alkaloid, monocrotaline, the toxic principle of *Crotalaria spectabilis*, one of the primary food plants of *U. ornatrix* (Conner et al., 1981), fails to protect the eggs against entomopathogenic fungi.

Our experiments were of two kinds. First, we measured the infectivity of selected fungi to *U. ornatrix* eggs that were either parentally endowed with monocrotaline or free of the chemical. Second, we determined the inhibiting effect of monocrotaline, in either of its natural chemical forms (free base; N-oxide), upon fungal cultures.

METHODS AND MATERIALS

Utetheisa *Eggs.* The two types of eggs (with or without monocrotaline) were obtained from laboratory cultures of *U. ornatrix* raised, respectively, on monocrotaline-containing and monocrotaline-free diet. The two diets have been described (Conner et al., 1981; Dussourd et al., 1988). The monocrotaline-free diet is a semi-synthetic pinto bean-based mixture (PB diet); the monocrotaline-containing diet is made of the same mixture, with 10% of its pinto bean content replaced by *C. spectabilis* seeds (CS diet). Analyses had shown eggs from PB-raised parents to be totally devoid of alkaloid, while those from CS-raised parents contained in the order of 0.9 μg monocrotaline/egg, ca. 90% in N-oxide form (J. Kopecky, J. Meinwald, T. Eisner, unpublished).

Fungal Cultures. The entomopathogenic fungi used and the insects from which they were isolated, were as follows: *Beauveria bassiana*, from the little leaf-notcher beetle, *Artipus floridanus* (Curculionidae); *Paecilomyces lilacinus*, from Fuller's rose beetle eggs, *Pantomorus cervinus* (Curculionidae); *Metarhizium anisopliae*, from the mole cricket, *Scapteriscus vicinus* (Gryllidae); and *Paecilomyces fumosoroseus*, from the sweet potato whitefly, *Bemisia tabaci* (Aleyrodidae).

The fungi were obtained originally as monosporal isolates and kept at −70°C in the culture collection of the Citrus Research and Education Center, University of Florida.

Pure cultures of the fungi were grown on Sabouraud dextrose agar (SDA) in Petri dishes (15 × 100 mm) at 27°C. After 10 days, conidia were harvested by scraping the plates with a sterile microscope slide. Conidia then were placed in 10 ml sterile deionized water (SDW), vortexed 1 min, and washed twice in SDW using centrifugation (5000 *g*, 5 min) to remove agar residue. Conidial concentration in the suspension was determined by hemacytometer counts. Sus-

pensions were stored at −20°C. All experiments were conducted with conidial suspensions (and dilutions thereof) prepared by this method.

Prior to each experiment, viability of conidia was evaluated by inoculating 100 μl of Sabouraud dextrose broth (SDB) with 100 μl of the conidial suspension and incubating for 24 hr at 27°C. Conidia were checked (inverted phase microscopy, 400×) for incidence of germ tube formation, which was invariably at levels above 90%.

Monocrotaline. Monocrotaline free base was obtained commercially (Sigma Chemical Co., St. Louis, Missouri). Monocrotaline N-oxide was prepared from the free base by Robert K. Vander Meer (USDA-ARS, P.O. Box 14565, Gainesville, Florida 32604).

Effect of Fungi upon Eggs. U. ornatrix egg clusters [17.1 ± 9.1 (SD) eggs/cluster, 4–44 range, $N = 125$ clusters), either monocrotaline-laden (parents CS-diet raised) or monocrotaline-free (parents PB-diet raised), were dipped in conidial suspensions of *B. bassiana* or *P. lilacinus* and then placed on agar plates (15 g agar/liter) and incubated at 27°C for 96 hr. Each fungal suspension was tested at five concentrations [0 (= control), 4.5×10^4, 4.5×10^6, 4.5×10^7, 4.5×10^8 conidia/ml]. Viability per cluster was scored as percent eggs that hatched.

For each fungus, the experimental series was run three times, with one to three egg clusters of each type per concentration. Since replicate values did not differ statistically, they were lumped for calculation of mean viabilities. Total number of egg clusters per sample concentration ranged from five to nine.

Effect of Monocrotaline on Fungi. Due to differences in solubility, the N-oxide and free-base forms of monocrotaline were assessed by slightly different procedures.

1. Monocrotaline N-Oxide. Effect on the germination of four fungi was assessed: *B. bassiana*, *M. anisopliae*, *P. lilacinus*, and *P. fumosoroseus*. Eight aqueous dilutions [0 (= water), 0.08, 0.15, 0.31, 0.63, 1.25, 2.5, 5.0 μg/μl] of an aqueous monocrotaline N-oxide solution were filter-sterilized by passage through an HPLC solvent syringe filter (0.45 μm, MSI Cameo). Standard volumes of each dilution (100 μl), together with 100 μl of SDB conidial suspension (ca. 1×10^5 conidia/ml) of one of the fungi, were added to individual wells of a sterile multiwell microtiter plate. After 24 hr of incubation at 27°C, conidial germination was scored by checking (inverted phase microscopy, 400×) approximately 100 conidia per well for presence or absence of germ tube. Three replicates per conidial dilution were assayed, providing the basis for calculation of mean percent germination per sample.

2. Monocrotaline Free Base. The procedure was similar as under (1), except that only two fungi were used (*B. bassiana*, *M. anisopliae*) and the free base samples were added (5 μl) to the test wells in methanol solution and allowed to dry by solvent evaporation before addition (200 μl) of the SDB conidial

suspension. Since this essentially involved surface application of the test chemical, test dosages were expressed as quantity of chemical per unit of well surface area.

Four filter-sterilized dilutions of a methanol solution of the free base were tested [0 (= methanol), 0.15, 1.5, and 15 $\mu g/cm^2$]. Incubation was again at 27°C, with three replicates per dilution sample, and check for germ tube formation was at 24 hr. The three values again provided the basis for calculation of mean percent germination per sample.

RESULTS

Effect of Fungi upon Eggs. As is evident from Figure 1, the two fungi proved comparably infective to monocrotaline-containing (CS) and monocrotaline-free (PB) eggs. For both fungi, statistical comparison at the 4.5×10^6 conidia/ml concentration level, where viabilities were around 50%, indicates no statistical difference in the effect upon the two eggs ($t < 0.53$, $P > 0.5$, $df = 16$).

Effect of Monocrotaline upon Fungi. Neither the free base nor the N-oxide form of the compound had an inhibiting effect on the fungi tested. Conidial germination was consistently above 98%, even with the highest chemical concentrations assayed [for the $N = 28$ N-oxide samples and their controls ($N = 4$), mean germination was consistently 100.0%; for the $N = 6$ free-base samples, mean germination ranged from 98.6% to 99.0% and was 99.3% for the controls ($N = 2$)].

DISCUSSION

Our data indicate that *U. ornatrix* eggs are vulnerable to fungal infection and that one pyrrolizidine alkaloid, monocrotaline, provides no protection against such infection. Whether one can generalize from this single compound, and from the relatively few fungi against which it was tested, that pyrrolizidine alkaloids as a group are ineffective as antifungal agents remains uncertain. The only defensive function so far proven for pyrrolizidine alkaloids in *Utetheisa* concerns their deterrence to arthropodan predators (Eisner and Meinwald, 1987; Dussourd et al., 1988).

The concentration range at which we assessed monocrotaline for conidial inhibition (0–15 μg free base; 0–5 μg N-oxide) spans the level at which pyrrolizidine alkaloid occurs in *Utetheisa* eggs in nature. This level, while not determined for monocrotaline itself, but for the closely related pyrrolizidine alkaloid usaramine, sequestered by *Utetheisa* from another food plant (*Crota-*

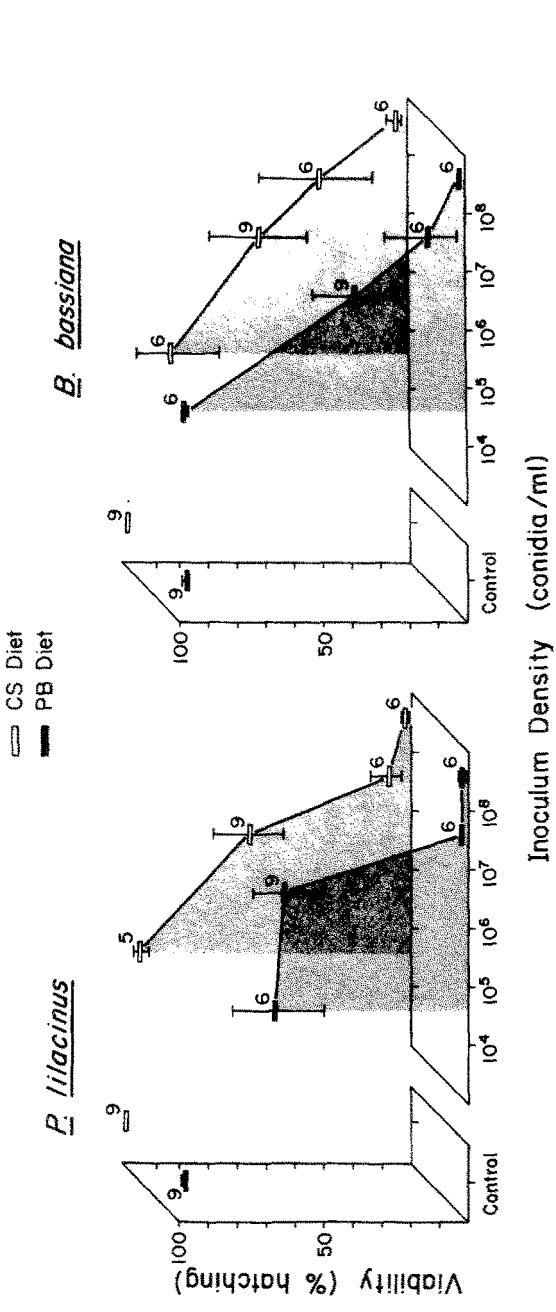


FIG. 1. Infectivity of *Paecilomyces lilacinus* and *Beauveria bassiana*, at various inoculum densities, to *Utetheisa ornatrix* eggs. Viability per egg cluster is expressed as percent (mean ± standard error) that hatched. Number of clusters per sample is given by numerals beside means. CS diet = monocrotaline-containing eggs from parents raised on CS diet; PB diet = monocrotaline-free eggs from parents raised on PB diet. For purposes of plotting, the square root of the viabilities (as probabilities) were transformed to arc sines to determine means and standard error of the means. Probabilities of 1 and 0 were changed (according to Snedecor and Cochran, 1980, pp. 327–328) to $0.25/N$, where N = number of eggs per cluster.

laria mucronata), was found to be on the order of 0.8 μg/egg, with upwards of 95% in N-oxide form (B.L. Roach, cited in Dussourd et al., 1988).

The fungi used in our study were conspecifics or congeners of species proven to be pathogenic to other insect eggs. Thus, *P. fumosoroseus* was shown to be infective to eggs of a noctuid moth and of two curculionid beetles (Rodriguez-Rueda and Fargues, 1980; Poprawski et al., 1985). The curculionid eggs were further shown to be vulnerable to infection by *B. bassiana* and *M. anisopliae*, as well as to a second species of *Paecilomyces* and *Metarhizium*.

Surprisingly little is known about the susceptibility of insect eggs to entomopathogenic infection (Steinhaus, 1963; Ferron, 1978), or about antibiotic mechanisms by which insect eggs might be protected against such infection. Our negative data notwithstanding, we are tempted to predict that such mechanisms exist and that interesting strategies will be uncovered by which antifungal or antibacterial agents are maternally, paternally, or biparentally transmitted to insect eggs.

Acknowledgments—Supported in part by grant AI 02908 from NIH. We thank Dr. Ben-Huai Lye for advice.

REFERENCES

CONNER, W.E., EISNER, T., VANDER MEER, R.K., GUERRERO, A., and MEINWALD, J. 1981. Precopulatory sexual interaction in an arctiid moth (*Utetheisa ornatrix*): Role of a pheromone derived from dietary alkaloids. *Behav. Ecol. Sociobiol.* 9:227–235.

DUSSOURD, D.E., UBIK, K., HARVIS, C., RESCH, J., MEINWALD, J., and EISNER, T. 1988. Biparental defensive endowment of eggs with acquired plant alkaloid in a moth (*Utetheisa ornatrix*). *Proc. Natl. Acad. Sci. U.S.A.* 85:5992–5996.

EISNER, T., and MEINWALD, J. 1987. Alkaloid-derived pheromones and sexual selection in Lepidoptera, pp. 251–269, *in* G.D. Prestwich and G.J. Bloomquist (eds.). Pheromone Biochemistry. Academic Press, Orlando, Florida.

FERRON, P. 1978. Biological control of insect pests by entomogenous fungi. *Annu. Rev. Entomol.* 23:409–442.

JOHNSON, A.E., MOLYNEUX, R.J., and MERRILL, G.B. 1985. Chemistry of toxic range plants. Variation in pyrrolizidine alkaloid content on *Senecio*, *Amsinckia*, and *Crotalaria* species. *J. Agric. Food Chem.* 33:50–55.

POPRAWSKI, T.J., MARCHAL, M., and ROBERT, P.H. 1985. Comparative susceptibility of *Otiorhyncus sulcatus* and *Sitona lineatus* (Coleoptera: Curculionidae) early stages to five entomopathogenic Hyphomycetes. *Environ. Entomol.* 14:247–253.

RODRIGUEZ-RUEDA, D. and FARGUES, J. 1980. Pathogenicity of entomopathogenic Hyphomycetes, *Paecilomyces fumosoroseus* and *Nomuraea rileyi*, to eggs of noctuids, *Mamestra brassicae* and *Spodoptera littoralis*. *J. Invertebr. Pathol.* 36:399–408.

SAS INSTITUTE. 1985. SAS User's Guide: Basics, 5th ed. SAS Institute, Cary, North Carolina.

SHARMA, R.K., KASTURE, A.V., KAPOOR, K.K., and ATAL, C.K. 1965. Phytochemical investi-

gation of the genus *Crotalaria*. Part V. Occurrence of tertiary bases and their *N*-oxides in Indian *Crotalaria*. *Lloydia* 28:209–211.

SNEDECOR, G.W., and COCHRAN, W.G. 1980. Statistical Methods. Iowa State University Press, Ames, Iowa.

STEINHAUS, E.A. 1963. Insect Pathology, An Advanced Treatise, Vol. 1. Academic Press, New York.

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

BEHAVIORAL AND ELECTROANTENNOGRAM RESPONSES OF PERIPLANONE ANALOGS

KENTARO OKADA,* MASATAKA MORI, KAZUKO SHIMAZAKI, and TATSUJI CHUMAN

Life Science Research Laboratory, Japan Tobacco Inc.
6-2 Umegaoka, Midori-ku, Yokohama 227, Japan

(Received July 26, 1990; accepted November 26, 1990)

Abstract—The biological activities of synthetic periplanone analogs, including four candidates of periplanone-A (P-A), were evaluated by behavioral and electroantennogram (EAG) assays. Among 16 periplanone analogs, six compounds evoked pheromonal activity from the male American cockroaches. The threshold dosages of these biological active analogs were 10–10^5 times lower than that of the known periplanone mimic, germacrene-D. The conformation required for eliciting the pheromonal activity is discussed in terms of the structure–activity relationship of these analogs. Hauptmann's P-A elicited the strongest pheromonal activity among four candidates of P-A in our bioassay, suggesting that Hauptmann's P-A is a natural P-A produced from female cockroaches.

Key Words—American cockroach, *Periplaneta americana*, Orthoptera, Blattidae, pheromone, mimics, analog, electroantennogram, EAG, bioassays, periplanone analogs.

INTRODUCTION

The study of pheromone mimics of the American cockroach, *Periplaneta americana*, were initiated after the discovery by Bowers and Bodenstein (1971) that the plant-derived terpenoids, (+)-bornyl acetate, α- and β-santalol caused sexual excitement in male American cockroaches.

Stimulated by this discovery, Nishino et al. (1977) examined many bicyclic monoterpenoids and found (+)-*trans*-verbenyl acetate elicited pheromonal activity. Structural factors for sex pheromonal activity of verbenyl acetate and

*To whom correspondence should be addressed.

0098-0331/91/0400-0695$06.50/0

related compounds have been investigated, and these demonstrated that (+)-verbanyl methyl carbonate expressed the strongest activity among the derivatives (Nishino and Takayanagi, 1981; Nishino et al., 1982; Manabe and Nishino, 1983, Manabe et al., 1985).

On the other hand, germacrene-D was found to be a sex pheromone mimic and its derivatives have been investigated (Tahara et al., 1975; Takahashi et al., 1978). So far, no decisive conclusion about the structure–activity relationship of the germacrene-type structure analogs has been drawn.

The first purpose of this paper is to determine the relative thresholds of periplanone analogs and offer valuable information for the elucidation of the structure–activity relationship among periplanone analogs.

The second purpose is to confirm the correct structure of periplanone-A (P-A) by behavioral and electroantennogram (EAG) assays. Persoons et al. (1974) first isolated P-A with P-B (**1**) (Figure 1), and proposed a tentative structure of P-A (**4**) (Figure 1) (Persoons et al., 1982). This proposed structure was examined during its synthesis. The spectroscopic data, however, did not coincide with those reported by Persoons and it was found to be biologically inactive (Shizuri et al., 1987a,b). The spectroscopic data of Persoons' P-A were reinvestigated and another structure (**5**) (Figure 1) was proposed (Shizuri et al., 1987c).

Hauptmann et al. (1986) isolated a new sex pheromone component having a germacrene skeleton (Hauptmann's P-A, **2**), contrary to the structure pro-

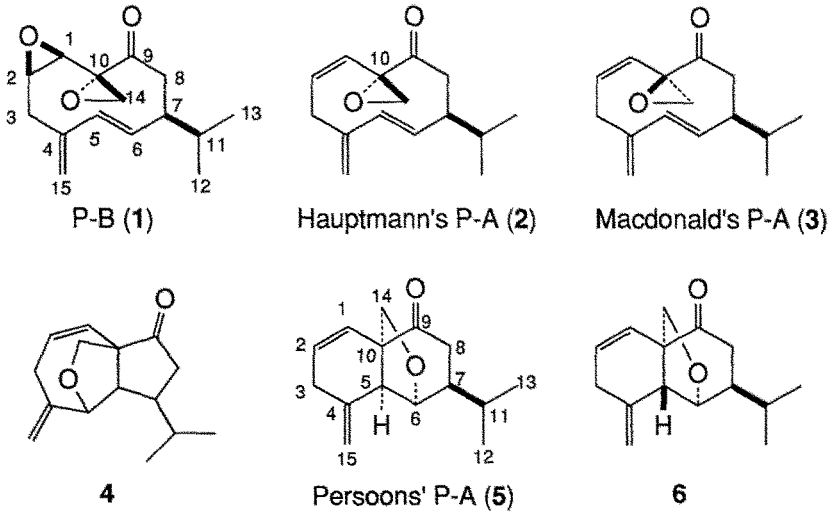


FIG. 1. Periplanone-B and four candidates of Periplanone-A. The numbering scheme for the carbon atoms of compounds **1**, **2**, and **5** are according to Persoons et al. (1982).

posed by Persoons. Meanwhile, Macdonald et al. (1987) synthesized the C-10 epimer of Hauptmann's P-A (**3**) and claimed that this was the correct structure of the P-A (Macdonald's P-A) (Figure 1).

Recently, Kuwahara and Mori (1991) synthesized both Hauptmann's and Macdonald's P-A. X-ray crystallographic analysis of the synthetic Hauptmann's P-A showed that Hauptmann's assumption about the structure of P-A was correct. It also was determined that thermal rearrangement of Hauptmann's P-A yielded a compound that had identical spectroscopic properties to those of Persoons P-A (Persoons et al., 1982). The structure of the rearrangement product from Hauptmann's P-A (**2**) was established to be **5** (Figure 1) by X-ray crystallographic analysis (Kuwahara and Mori, 1989). In this study, behavioral and EAG responses of four candidates of P-A are examined in order to provide biological evidence of the structural elucidation of P-A.

METHODS AND MATERIALS

Pheromones and Analogs. Synthetic pheromones and analogs, except for germacrene-D, were used for this assay. The chemical and optical purity of each test compound is summarized in Table 1. Eighteen compounds that we

TABLE 1. PURITY OF COMPOUNDS TESTED

Compound No.	Chemical purity	Optical purity
1	Crystal	100% ee
2	>99%	>98% ee
3	>99%	>98% ee
5	Crystal	>99% ee
6	Crystal	(±)
7	Crystal	>98% ee
8	>99%	>98% ee
9	>99%	>98% ee
10	>98%	
11	Crystal	>99% ee
12	>99%	(±)
13	>99%	(±)
14	>99%	(±)
15	>99%	(±)
16	>99%	(±)
17	>99%	(±)
18	>99%	(±)
19	>99%	Diastereomeric mixture

used in this assay were divided into four groups, based on their chemical structural features.

Group 1 includes P-B (Figure 1) and periplanone analogs that have a germacrene skeleton (Figure 2). (−)-P-B (**1**) (Persoons et al., 1976) was synthesized in optically active form (Kitahara et al., 1987). Analog **7** also was synthesized in optically active form (Mori et al., 1988). Analogs **8** and **9** were optically active intermediates in the synthesis of (−)-P-B (Mori et al., 1990a). Germacrene-D (**10**), isolated from ylang-ylang essential oil, was a gift from Dr. Y. Takagi of T. Hasegawa Inc.; the chemical purity was 98% as judged by GLC.

Group 2 includes analogs that have no *exo*-methylene and isopropyl groups on the 10-membered ring (Figure 3). Analog **11**, which has the same three oxygen-containing functionalities in the same positions as those of P-B, was prepared as a P-B analog, and **12** as a Hauptmann's P-A analog. Analogs **13**–**17** were the synthetic intermediates in the synthesis of analog **11** (Mori et al., 1990b).

Group 3 includes the chain analogs **18** and **19** (Figure 4), which have a partial structure of periplanones.

Group 4 includes the candidates of P-A, Hauptmann's P-A (**2**), Macdonald's P-A (**3**), Persoons' P-A (**5**) and the epimer of Persoons' P-A (**6**) (Figure 1). Persoons' P-A (**5**) was prepared from Hauptmann's P-A (**2**) by thermal rearrangement on gas chromatography (Kuwahara and Mori 1989). Hauptmann's

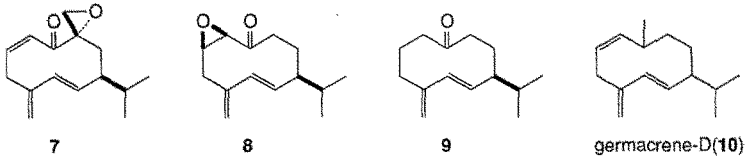


FIG. 2. Periplanone analogs, group 1.

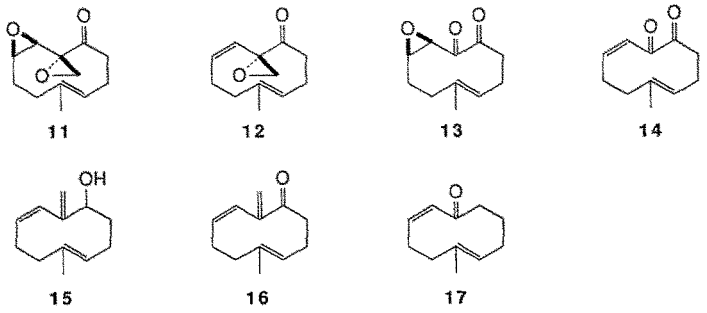
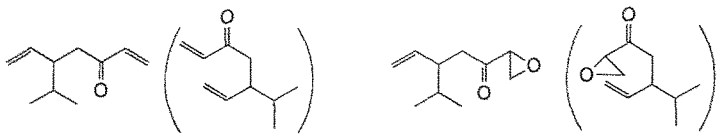


FIG. 3. Periplanone analogs, group 2.



18 19

FIG. 4. Periplanone analogs, group 3.

P-A (**2**) and Macdonald's P-A (**3**) were synthesized in optically active form (Kuwahara and Mori,1990). The analog **6**, which is the C-5 epimer of Persoons' P-A, was synthesized in racemic form (Mori and Igarashi, 1989). The decadal dilution series of each test compound, of which the concentrations were $10-10^{-7}$ $\mu g/\mu l$, were prepared in distilled *n*-hexane.

Behavioral Assay for Pheromonal Activity. Behavioral assay was performed using essentially the same procedure as described by Okada et al. (1990b). Five male cockroaches in a glass assay box were exposed to a stimulant introduced in an airstream for 5 min. Responses of the insects were recorded by a video tape recorder. Locomotory activity was measured by counting the number of times the cockroaches crossed a line drawn across the center of the bottom of the assay box. The number of wing-raising individuals per total test insects during the test period was expressed as a wing-raising rate in percentage. A male cockroach that raised his wing for both extended and momentary periods was counted in the rate.

EAG Recording. An antenna of the male cockroach was cut away from the head at the scape and placed on the plastic stage. EAGs were recorded with Ag–AgCl electrodes. The recording electrode was a glass capillary (ID = 0.7 mm) filled with TES Ringer solution, it was inserted in the apical end of the antenna, and the indifferent electrode was positioned into the pool of Ringer solution (ϕ = 10 mm, depth = 15 mm) where the proximal end was dipped. One microliter of the test solution was directly applied to the inner side of a disposable pipet (BDL 4642, Dickinson Co. Ltd.) and the solvent (*n*-hexane) was allowed to evaporate. Then the disposable pipet was attached to the pheromone application system. Pheromone vapor (total volume 2 cc, duration 0.5 sec, controlled by a glass syringe whose plunger was regulated by a speed-control motor) was introduced into an airstream (flow rate 1 liter/min) purified with a charcoal filter and water, and applied to the middle part of the antenna. Each antenna was stimulated by a series of test compounds at an interval of 30 sec. The EAG responses were automatically analyzed with our original measurement program on a microcomputer (PC9801XL4, NEC Co. Ltd.) equipped with an A-D converter (ADZ-2000, Dia-medical Co. Ltd.) after having been amplified with a microelectrode and a high gain amplifier (MEZ-8201, AVH-11, Nihon Kohden Co. Ltd.) by a factor of 10^3.

RESULTS

Dose responses of behavioral and EAG activities for the test compounds that elicited any behavioral activity are shown in Figure 5. The mean locomotory activity and SD of the control (*n*-hexane) was 2.1 ± 2.3. The upper value of 99% confidence intervals of locomotory activity was 3.4. The mean wing-raising rate and SD of the control was 0.0 ± 0.0. The mean EAG amplitude and SD of the control was 0.22 ± 0.09 mV. The upper value of 99% confidence intervals of the EAG amplitude was 0.32 mV.

The significant responses of locomotory activity and EAG amplitude are defined to be the responses that are greater than the upper values of the 99% confidence intervals.

All compounds of the group 1 evoked the typical pheromonal activity. P-B (**1**) elicited the strongest activity among the tested compounds. The dose that evoked a significant locomotory activity was 10^{-8} μg (Okada et al., 1990b). The dose that evoked a significant EAG amplitude was 10^{-7} μg. Compound **7** is an analog of Hauptmann's P-A (**2**), differing only in the position of the carbonyl and spiro epoxy groups on the ring. This analog elicited the strongest activity among all of the analogs except for the natural periplanones. Significant locomotory activity was elicited at 10^{-4} μg, significant EAG amplitude was 10^{-3} μg. Analog **8**, having a carbonyl and an epoxy group on the germacrene skeleton, elicited significant locomotory activity at 10^{-2} μg and significant EAG at 10^{-1} μg. Analog **9**, which has a carbonyl group on the germacrene skeleton, evoked significant locomotory activity at 10^{-1} μg. The threshold dosage of the EAG response was 10^{-1} μg. The wing-raising behavior was rather weak and the behavior lasted for a very short period even at 10 μg. Germacrene-D (**10**), which has no oxygen-containing functionality, elicited significant locomotory activity at 10 μg. Significant EAG amplitude was elicited at 1 μg. The wing raising activity at 10 μg was weak, as with the analog **9**.

In the group 2, analog **11**, having the same oxygen-containing functionalities at the same positions as those of P-B but no *exo*-methylene and isopropyl groups on the ten 10-membered ring system, evoked significant locomotory activity at 10^{-3} μg. Analog **12**, which has the same oxygen-containing functionalities at the same positions as those of Hauptmann's P-A (**2**), elicited no behavioral and EAG response. Compound **13**, an analog of **11**, also failed to elicit pheromonal activity. Analogs **14–17** elicited no significant behavioral and EAG responses even at the dose of 10 μg.

In group 3, analogs **18** and **19** elicited no significant behavioral and EAG responses even at a dosage of 10 μg.

In group 4, Hauptmann's P-A (**2**) elicited significant locomotory activity at 10^{-6} μg. Significant EAG response was recorded at 10^{-4} μg. The locomotory threshold dosage of Macdonald's P-A (**3**) was 10^{-2} μg, 10^4 times higher than

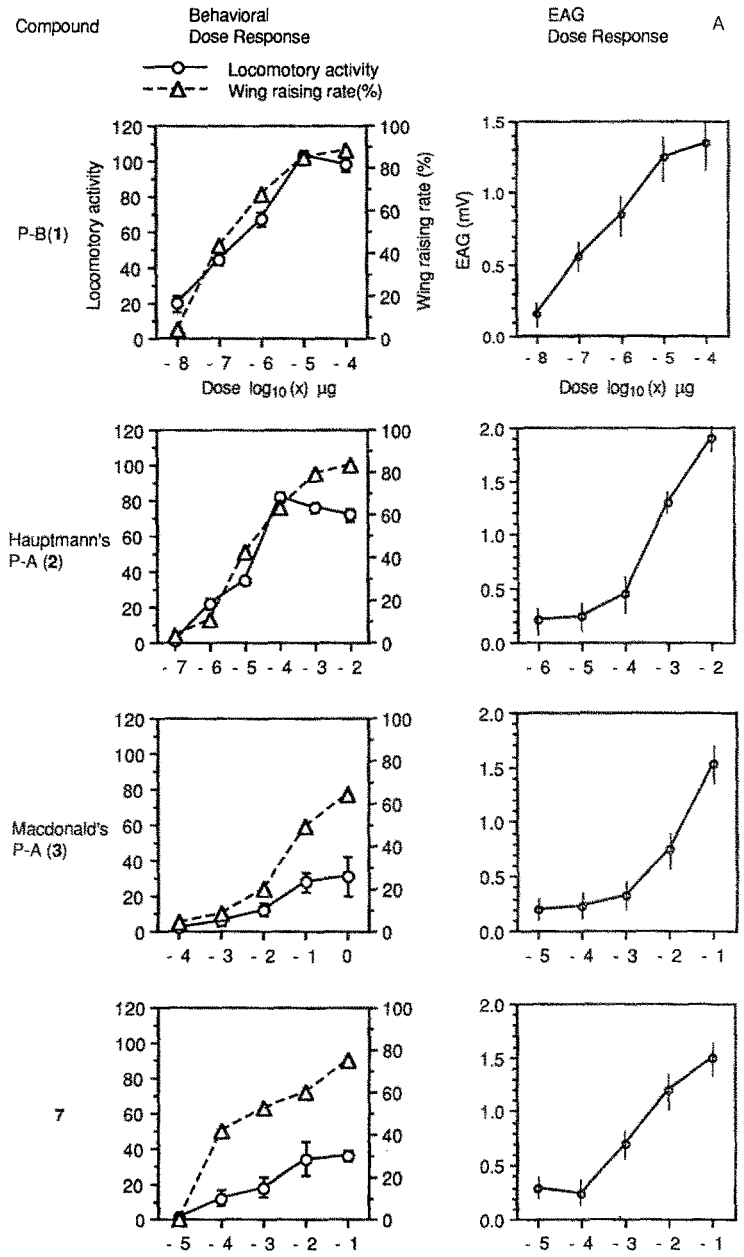


FIG. 5. Dose response of periplanone analogs. Measurements of behavioral and EAG response are repeated 10 times at each data point. (Replication in the case of P-B and P-A is 20 times). Vertical bars indicate standard errors.

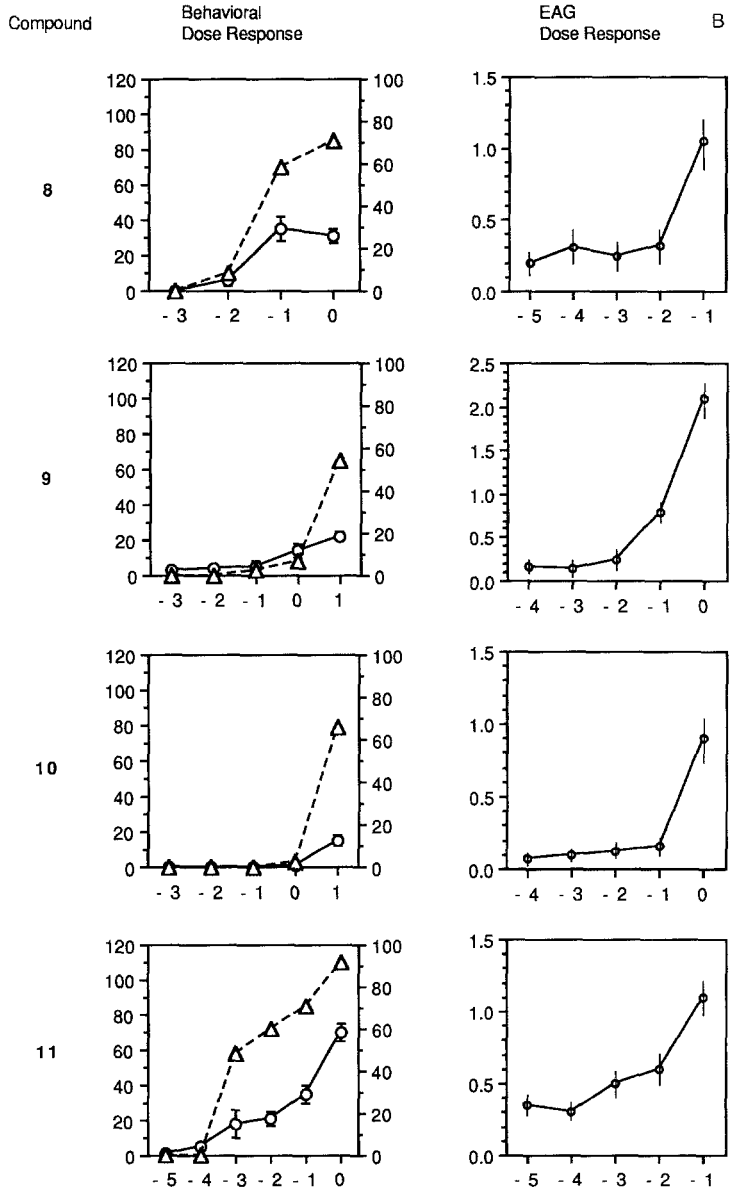


FIG. 5. Continued

that of Hauptmann's P-A (**2**). A significant EAG amplitude of analog **3** was recorded at 10^{-2} μg.

Behavioral and EAG assays indicate that Persoons' P-A (**5**) was inactive even at 10 μg. The epimer of Persoons' P-A (**6**) also was inactive even at 10 μg.

No inhibitory or synergistic effect of the biologically inactive analogs **5**, **6**, and **12–19**, to the pheromonal activity of P-B was observed even when 10 μg of these analogs were mixed with 10^{-6} μg of P-B. ($P > 0.05$)

DISCUSSION

Structure–Activity Relationship of Periplanone Analogs. Of the six analogs, **3**, **7**, **8**, **9**, **10**, and **11**, found to have pheromonal activity in our bioassay, five are germacrene derivatives, the exception being analog **11**. Other analogs, **5**, **6**, and **12–19**, have neither significant behavioral nor EAG activity. These inactive analogs show no significant inhibitory and/or synergistic effects to the pheromonal activity of P-B during their addition to P-B.

The structure–activity relationship of the germacrene analogs in the group 1 indicates that the number of the oxygen-containing functionalities on the ring system concerns the effectiveness of the pheromonal activity. The threshold dosages of locomotory activity and wing-raising became higher with the sequential removal of the oxygen-containing functionalities.

The chain analogs **18** and **19** had no pheromonal activity, suggesting that a specific conformation of the molecule resulting from the 10-membered ring system is important to elicit pheromonal activity.

The strong activity of analog **11** possessing the same oxygen-containing functionalities as those of P-B (**1**) indicates that the proper position of the functionalities is important to elicit the activity. But analog **12**, which has the same two oxygen containing functionalities at the same positions as those of P-A (**2**) had no activity. This suggests that the three-dimensional configuration of oxygen atoms and ring conformation of analog **12** are different from those of P-A.

The structure–activity relationship of Hauptmann's P-A (**2**) and Macdonald's P-A (**3**) also indicated that a specific conformation relating to the three-dimensional configuration of the oxygen atom in the spiro epoxy must be important for eliciting activity.

Stable conformations of natural periplanones (**1**, **2**), active analogs **7** and **11**, and Persoons' P-A (**5**) were examined by X-ray crystallographic analysis (Figure 6) (Okada et al., 1990a; Mori et al., 1990a,b; Kuwahara and Mori, 1989). The analysis indicates that all these active compounds (**1**, **2**, **7**, **11**) have a common conformational property on the 10-membered ring and the three-dimensional position of the oxygen atoms (Figure 6). It is noteworthy that the

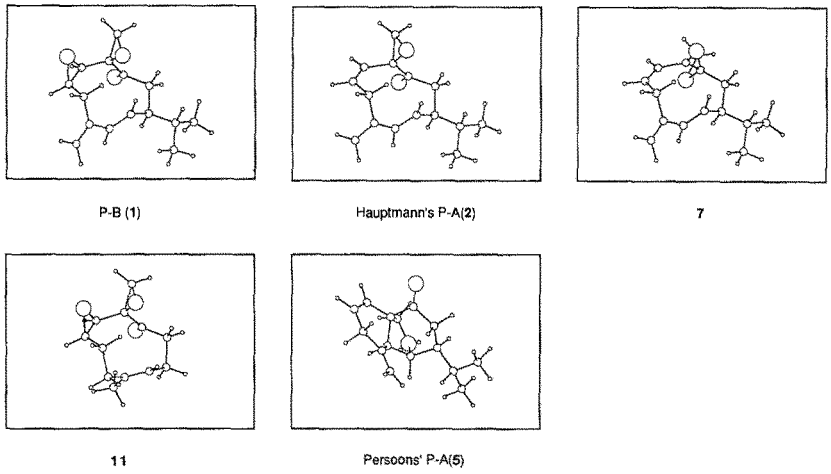


FIG. 6. Stereoscopic drawings of periplanones and their analogs. These conformations of P-B (**1**), Hauptmann's P-A (**2**), **7**, and **11** are drawn according to coordinates from X-ray crystallographic analyses (Okada et al., 1990a; M. Mori et al., 1990a,b). Persoons' P-A (**5**) is drawn according to the molecular mechanics calculation analysis (Program MM2). The conformation is superimposable on that of the corresponding alcohol of Persoons' P-A obtained from an X-ray crystallographic analysis (Kuwahara and Mori, 1989). Large, medium, and small circles indicate oxygen, carbon, and hydrogen atoms, respectively.

three-dimensional configuration of oxygen atoms in analog **7** is superimposable on those of natural periplanones (Mori et al., 1990a). The conformation of Persoons' P-A (**5**), which elicited no pheromonal activity, could not be superimposed on those of natural periplanones.

Both natural periplanones (**1**, **2**) and active analogs **3**, **7–11** could elicit typical male pheromonal behaviors such as antennal waving, rapid locomotion, wing-raising, and homosexual behavior. In the case of natural periplanones, the dose–response curve of the wing-raising rate increased in a manner parallel to that of the locomotory activities with ascent of sample dosages (Figure 5). On the contrary, analogs **3**, **7**, and **11** showed a slightly different response pattern from those of the natural periplanones. Dose–responses of the wing-raising and locomotory activities were not parallel. The wing-raising activity was greater than the locomotive activity.

Correct Structure of Natural P-A. There has been discussion as to which structure of P-A, Hauptmann's (**2**), Macdonald's (**3**) or Persoons' P-A (**5**), was correct. Nishino et al. isolated Hauptmann's P-A from the female's feces. However, they could not detect Persoons' P-A. Moreover synthetic Persoons' P-A elicited no behavioral and EAG responses in our assay. This strongly suggests

that Persoons' P-A is an artificial product derived from Hauptmann's P-A in the isolation procedure. (Sass, 1983; Nishino et al., 1988; Kuwahara and Mori, 1989). Takahashi et al. (1988) reported that the behavioral activity of Hauptmann's P-A (**2**) was 1000 times greater than Macdonald's P-A, and similar results could be obtained in our behavioral and EAG assay. The above results indicate that Hauptmann's P-A is a natural P-A produced from female American cockroaches.

Acknowledgments—The authors are indebted to Profs. K. Mori and T. Kitahara, and Dr. S. Kuwahara, The University of Tokyo, for generously supplying the samples of synthetic Macdonald's P-A, Persoons' P-A and its epimer, and to Dr. T. Takagi of T. Hasegawa Co. Ltd. for providing the germacrene-D.

REFERENCES

BOWERS, W.S., and BODENSTEIN, W.G. 1971. Sex pheromone mimics of the American cockroach. *Nature* 232:259–261.

HAUPTMANN, H., MÜHLBAUER, G., and SASS, H. 1986. Identifizierung und Synthese von Periplanon A. *Tetrahedron Lett.* 27:6189–6192.

KITAHARA, T., MORI, M., and MORI, K. 1987. Total synthesis of (−)-periplanone-B, natural major sex-excitant pheromone of the American cockroach, *Periplaneta americana*. *Tetrahedron* 43:2689–2699.

KUWAHARA, S., and MORI, K. 1989. Clarification of the structure of Persoons's periplanone-A, an artifact derived from Hauptmann's periplanone-A. *Tetrahedron Lett.* **30**:7447–7450.

KUWAHARA, S., and MORI, K. 1991. Synthesis of both the enantiomers of Hauptmann's periplanone-A and clarification of the structure of Persoons's periplanone-A. *Tetrahedron.* **46**:8083–8092.

MACDONALD, T.L., DELAHUNTY, C.M., and SAWYER, J.S., 1987. Synthetic studies of periplanone A, a sex pheromone of *Periplaneta americana*. *Heterocycles* 25:305–313.

MANABE, S., and NISHINO, C. 1983. Sex pheromonal activity of (+)-bornyl acetate and related compounds to the American cockroach. *J. Chem. Ecol.* 9:433–448.

MANABE, S., NISHINO, C., and MATSUSHITA, K. 1985. Studies on relationship between activity and electron density on carbonyl oxygen in sex pheromone mimics of the American cockroach, part XI. *J. Chem. Ecol.* 11:1275–1287.

MORI, K., and IGARASHI, Y., 1989. Synthetic confirmation of the structure of the rearrangement product of periplanone-A. *Tetrahedron Lett.* 30:5145–5148.

MORI, M., OKADA, K., KITAHARA, T., and MORI, K. 1988. Hauptmann's periplanone-A, characterization and elucidation of the formation. Abstr. 16th Int. Symp. Chem. Natl. Products. (IUPAC), Kyoto, p. 649.

MORI, M., OKADA, K., SHIMAZAKI, K., and CHUMAN, T., KUWAHARA, S., KITAHARA, T., and MORI, K., 1990a. X-ray crystallographic and NOE studies on the conformation of periplanones and their analogues. *J. Chem. Soc. Perkin. I.* 1990:1769–1777.

MORI, M., OKADA, K., SHIMAZAKI, K., and CHUMAN, T., 1990b. Synthesis of a simple analog of periplanone-B. *Tetrahedron Lett.* 31:4037–4040.

NISHINO, C., and TAKAYANAGI, H. 1981. Sex pheromonal activity of (+)-*trans*-verbenyl acetate and related compounds to the American cockroach, *Periplaneta americana* L. *J. Chem. Ecol.* 7:853–865.

NISHINO, C., TOBIN, T.R., and BOWERS, W.S. 1977. Sex pheromone mimics of the American cockroach (Orthoptera: Blattidae) in monoterpenoids. *Appl. Entomol. Zool.* 12:287–290.

NISHINO, C., TAKAYANAGI, H., and MANABE, S. 1982. Comparison of sex pheromonal activity on the American cockroach between acetates and propionates of verbenyl type alcohols. *Agric. Biol. Chem.* 46:2781–2785.

NISHINO, C., KOBAYASHI, K., FUKUSHIMA, M., IMANARI, M., NOJIMA, K., and KOHNO, S. 1988. Structure and receptor participation of periplanone A, the sex pheromone of the American cockroach. *Chem. Lett.* 1988:517–520.

OKADA, K., MORI, M., KUWAHARA, S., KITAHARA, T., MORI, K., SHIMAZAKI, K., and CHUMAN, T. 1990a. Behavioral and electroantennogram responses of male American cockroaches to periplanones and their analogs. *Agric. Biol. Chem.* 54:575–576.

OKADA, K., MORI, M., SHIMAZAKI, K., and CHUMAN, T. 1990b. Behavioral responses of male *Periplaneta americana* L. to the female sex pheromone components, periplanone-A and periplanone-B. *J. Chem. Ecol.* 16:2605–2614.

PERSOONS, C.J., RITTER, F.J., and LICHTENDONK, W.J. 1974. Sex pheromones of the American cockroach Periplaneta americana. Isolation and partial identification of two excitants. *Proc. Kon. Ned. Akad. Wetensch., Amsterdam, C* 77:201–204.

PERSOONS, C.J., VERWIEL, P.E.J., RITTER, F.J., TALMAN, E., NOOIJEN, P.J.F., and NOOIJEN, W.J. 1976. Sex pheromones of the American cockroach, *Periplaneta americana*: A tentative structure of periplanone-B. *Tetrahedron Lett.* 24:2055–2058.

PERSOONS, C.J., VERWIEL, P.E.J., RITTER, F.J., and NOOYEN, W.J. 1982. Studies on sex pheromone of American cockroach, with emphasis on structure elucidation of periplanone-A. *J. Chem. Ecol.* 8:439–451.

SASS, H. 1983. Production, release and effectiveness of two female sex pheromone components of *Periplaneta americana. J. Comp. Physiol. A* 152:309–317.

SHIZURI, Y., YAMAGUCHI, S., TERADA, Y., and YAMAMURA, S. 1987a. Biomimetic reaction of germacrene-D epoxides in connection with periplanone A. *Tetrahedron Lett.* 28:1791–1794.

SHIZURI, Y., YAMAGUCHI, S., YAMAMURA, S., ISHIHARA, M., OHBA, S., SAITO, Y., NIWA, M., TERADA, Y., and MIYAZAKI, M. 1987b. The synthesis of a tricyclic hydroazulenone from *exo*-epoxygermacrene-D in connection with periplanone A. *Tetrahedron Lett.* 28:3831–3834.

SHIZURI, Y., YAMAGUCHI, S., TERADA, Y., and YAMAMURA, S. 1987c. What is the correct structure for periplanone A? *Tetrahedron Lett.* 28:1795–1798.

TAHARA, S., YOSHIDA, M., MIZUTANI, J., KITAMURA, C., and TAKAHASHI, S. 1975. A sex stimulant to the male American cockroach in the Compositae plants. *Agric. Biol. Chem.* 39:1517–1518.

TAKAHASHI, S., KITAMURA, C., and HORIBE, I. 1978. Sex stimulant activity of sesquiterpenes to the males of the American cockroach. *Agric. Biol. Chem.* 42:79–82.

TAKAHASHI, S., TAKEGAWA, H., TAKAHASHI, T., and DOI, T. 1988. Sex pheromone activity of synthetic (±)-periplanone-A and (±)-epiperiplanone-A to males of *Periplaneta* and *Blatta*. *J. Pestic. Sci.* 13:501–503.

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

ALLELOPATHIC POTENTIAL OF *Nuphar lutea* (L.) SIBTH. & SM. (NYMPHAEACEAE)

STELLA D. ELAKOVICH* and JEAN W. WOOTEN

Department of Chemistry and Biochemistry and Department of Biological Sciences
University of Southern Mississippi
Hattiesburg, Mississippi 39406

(Received October 9, 1990; accepted November 26, 1990)

Abstract—Aqueous extracts of *Nuphar lutea* (L.) Sibth. & Sm. leaves (blades plus petioles) and roots plus rhizomes were tested for allelopathic activity using lettuce seedling and *Lemna minor* L. assay systems. The 12.5, 25, 125, and 250 parts per thousand (ppt) treatments of both extracts killed the lettuce seedlings. At 2.5 ppt of extract, radicle growth of lettuce was 29% of the control for leaves and 31% of the control for roots plus rhizomes. *Lemna minor* frond number was reduced to 34% of the control by the 25 ppt leaf extract and to 43% of the control by the 25 ppt roots plus rhizomes extract. *L. minor* was killed by concentrations of 125 ppt and above of both plant part extracts. As expected, the frond number and total chlorophyll content measured by the *L. minor* assay were highly correlated. Osmotic potentials below 143 mOsmol/kg had no influence on *L. minor* growth. Neither the osmotic potential nor the pH of the undiluted extracts of *N. lutea* were in the range known to influence the growth of either lettuce seedlings or *L. minor*. *Nuphar lutea* extracts were many times more inhibitory than 16 other hydrophytes we previously examined.

Key Words—Allelopathy, bioassay, duckweed, *Lemna minor*, lettuce seedling, *Nuphar lutea*, osmotic potential, *Lactuca sativa*.

INTRODUCTION

Examinations of the allelopathic potential of hydrophytes have accelerated since Oborn et al. (1954) first suggested that these plants could influence the growth and distribution of neighboring plants. Among hydrophytes, *Eleocharis coloradoensis* (Britt.) Gilley has been examined most frequently for its allelopathic

*To whom correspondence should be addressed.

0098-0331/91/0400-0707$06.50/0

potential. Field observations of Yeo (1980) over a 12-year period showed *E. coloradoensis* capable of displacing species of *Potamogeton* and *Elodea*. Aquaria experiments of Frank and Dechoretz (1980) supported Yeo's findings involving *Potamogeton*. Nichols and Shaw (1983) made field observations that suggested three species of *Eleocharis* can displace other aquatics. Ashton et al. (1985) bioassayed organics leached from the culture media in which axenically cultured *E. coloradoensis* was grown. They found these leachates inhibited *Hydrilla verticillata* (L.f.) Royle and *Potamogeton pectinatus* L. as well as tomato cell cultures and lettuce seedling roots. Aqueous extracts of *E. interstincta* (Vahl.) R.&S. and *E. cellulosa* Torr. inhibited the growth of the duckweed *Lemna paucicostata* Hegelm (Sutton and Portier, 1989). We recently reported the allelopathic potential of 16 aquatic and wetland plants as determined by two bioassays (Elakovich and Wooten, 1989). Continuation of this work led to the examination of the allelopathic potential of *N. lutea* by way of lettuce seedling and *L. minor* bioassays.

Both pH and osmotic potential can influence the outcome of allelopathy bioassays. Bell (1974) explored the influence of osmotic potential in assays for allelopathy that used the terrestrial grass *Bromus rigidus* Roth. He concluded that results from test solutions having osmotic concentrations in excess of 50 mOsmol/kg should be interpreted with care. Buchanan et al. (1978) found that osmotic potentials of -1.8 atm (approximately 148 mOsmol/kg for mannitol solutions) had no influence on germination of the two terrestrial grasses and three cereals he examined. Cheng and Riemer (1988) found that neither osmotic potentials of less than 70 mOsmol/kg nor pH differences affect lettuce germination or growth. Between pH 4 and 6 there is little influence on growth of *L. minor* under control conditions (Einhellig et al., 1985). The influence of osmotic potential on *L. minor* growth has not been examined previously.

Nuphar lutea is comprised of nine subspecies. We examined the subspecies *N. lutea* ssp. *macrophyllum* (Small) Beal, which occurs in ponds, lakes, sluggish streams, or backwaters, pools in marshes and swamps, and ditches and canals. It ranges from southern Maine to southern Wisconsin, southward to southern Florida and Texas (Godfrey and Wooten, 1981). *N. lutea* produces alkaloids having a regular sesquiterpenic skeleton incorporated into 3-furyl-substituted piperidines or quinolizidines as well as C_{30} alkaloids, which contain a sulfur atom along with two 3-furyl substituents (Wrobel and Ruszkowska, 1979; LaLonde and Wong, 1977; Iwanow et al., 1986). Salin and Bridges (1982) have characterized an iron-containing superoxide dismutase that is present in the leaves. Cullen et al. (1973) examined the antifungal activity of the rhizomes whose medicinal utility had been reported 20 years earlier (Tatarov, 1945).

This paper explores the allelopathic potential of *N. lutea* according to the results of two bioassays commonly applied to plant extracts. The influence of osmotic potential on *L. minor* growth is also examined.

METHODS AND MATERIALS

Field-collected, fresh *N. lutea* (L.) Sibth. & Sm. plants, growing in a quiescent pond, were washed free of debris and drained of excess water. Plants were divided into two portions: (1) leaves, including blades and petioles; and (2) rhizomes and roots. These two portions were treated as separate samples in all assays. A 200-g fresh-weight aliquot of each sample and 200 ml of distilled, deionized water were blended in a commercial Waring blender, and the resulting pulpy mixture was refrigerated for 24–72 hr. The mixture was filtered through cheese cloth and the filtrate centrifuged at 22,095*g* at 4°C for 20 min to remove particulate matter. It then was sterilized by filtration through a 0.45-μm Millipore filter. This extract, considered 100%, was diluted to achieve test concentration solutions expressed as parts per thousand (ppt). Since extracts were prepared by blending equal amounts of plant material and water, the actual plant concentration was smaller than the extract concentrations reported here.

Lettuce seedling bioassays on each sample were conducted using 0 (control), 25, 125, and 250 ppt of extract per test plate. Each test plate contained 30 ml of 1.0% agar and the appropriate amount of distilled water. The osmotic potential of each undiluted plant extract was measured with a Wescor 5100 Series Micro Osmometer; pH of each extract also was determined. Each extract was assayed under axenic conditions in two duplicate 9-cm disposable sterile Petri dishes. Twenty lettuce seeds (*Lactuca sativa* L. Black Seeded Simpson), germinated to the point of radicle appearance, were transferred to the prepared Petri dishes. After three to four days or when the control plate showed good growth, the radicle lengths were measured to the nearest millimeter. After results of the initial lettuce seedling bioassays were examined, the experiments were repeated using extract concentrations of 2.5, 12.5, and 25 ppt.

Bioassays using *L. minor* were performed under axenic conditions according to the method of Einhellig et al. (1985), modified as previously described (Elakovich and Wooten, 1989). Initial extract concentrations were identical to the lower concentrations used in the lettuce seedling assay, 2.5, 12.5, and 25 ppt. A second set of assays included concentrations of 22, 111, and 222 for the roots and rhizomes extract, and 22, 25, and 125 ppt for the leaf extract. Each concentration was tested twice (two duplicates) using six wells per trial (N = 6/duplicate). The osmotic potential and pH of each test solution were measured. Four fronds were added initially to each well. The assays were terminated and the fronds were counted when there were approximately 20 fronds in each control well. Counted fronds were placed in a small test tube and 1.5 ml of 95% ethanol was added to extract chlorophyll. After 24 hr at room temperature or 48–72 hr in a refrigerator, 1.5 ml of water was added to each tube. The absorbance of the chlorophyll extracts was read at 649 nm and 665 nm. Total chlorophyll concentration was calculated from the formula: μg chlorophyll/ml

solution = (5.10) × (A665nm) + (20.02) × (A664nm) (G.R. Leather, personal communication).

The influence of osmotic potential on *L. minor* frond growth was determined by addition of mannitol to the growth medium to achieve osmotic potentials of 74, 143, and 266 mOsmol/kg. The *L. minor* assay was carried out as described above with a sample containing growth medium with no added mannitol (60 mOsmol/kg) serving as the control.

Results were subjected to analyses of variance and either the *t* test or Duncan's multiple-range test, as appropriate. Pearson product–moment correlations were calculated from frond and chlorophyll data.

RESULTS AND DISCUSSION

Assumptions of the analysis of variance were valid for all data; no transformations were necessary. Mean radicle growth of the control in the initial lettuce seedling bioassay for the leaf extract was 2.26 cm and for roots plus rhizomes 2.35 cm. Seedlings exposed to all extract concentrations (25, 125, and 250 ppt) died. The experiments using lower concentrations of leaf extract resulted in control mean radicle growth of 1.95 cm and growth at 2.5 ppt of 0.57 cm, 29% of the control; seedlings died at 12.5 and 25 ppt of extract (Table 1). The 2.5 ppt of roots plus rhizomes extract also caused a significant reduction ($P \leq 0.001$) in radicle length with values at 31% of the control. Seedlings died at the two higher concentrations (Table 1). There was no difference in activity between the leaf and the roots plus rhizomes extracts (*t* test, $P \leq 0.05$).

In one experiment, *L. minor* mean frond number at 25 ppt of leaf extract was 34% of the control, 5.6 fronds as compared to 16.5 for the control. At 12.5 and 2.5 ppt, mean frond number was 53% and 78% of the control, respectively. In the second experiment, which tested concentrations of 22, 25, and 125 ppt, the original fronds died before any growth occurred at 125 ppt. All means were different (Duncan's multiple-range test, $P \leq 0.05$) except for those between 22 and 25 ppt in the second experiment. In tests with 22, 111, and 222 ppt of extract from roots plus rhizomes, growth was observed only at the lowest concentration. This growth was 50% of the control (significantly different, *t* test, two-tailed $P \leq 0.001$). *Lemna minor* died in treatments of 111 and 222 ppt, so a second experiment examined the effects of lower extract concentrations. At 2.5 ppt of roots plus rhizomes extract, mean frond number was reduced to 78% of control, whereas growth in 12.5 ppt was 65%, and at 25 ppt it was 43%. The observed difference between 2.5 and 12.5 ppt of extract was not significant ($P \leq 0.05$). Results of the 22 and 25 ppt extract concentrations from the two experiments agree well.

Lemna minor frond numbers and total chlorophyll content of extracts from

TABLE 1. EFFECTS OF AQUEOUS *Nuphar* EXTRACTS IN LETTUCE SEEDLING RADICLE, *Lemna* FROND GROWTH AND TOTAL CHLOROPHYLL BIOASSAYS

	Extract (ppt)	Radicle growth $\bar{X}$ cm	%	Frond growth[a] $\bar{X}$ No.	%	Chlorophyll[a] $\bar{X}$ μg/ml	%
Leaves + petioles							
Exp 1	control	1.95		16.5a		9.56a	
	2.5	0.57[b]	29	12.8b	78	7.49b	78
	12.5	0		8.7c	53	3.03c	32
	25	0		5.6d	34	1.51d	16
Exp 2	control	2.26		18.0a		9.52a	
	22	[c]		9.25b	51	2.27b	24
	25	0		7.7b	43	1.62c	17
	125	0		0		0	
Roots + rhizomes							
Exp 1	control	1.81		17.8a		8.88a	
	2.5	0.56[b]	31	14.0b	78	7.24b	82
	12.5	0		11.5c	65	3.23c	36
	25	0		7.8d	43	1.51d	17
Exp 2	control	2.35		23.6		12.54	
	22	[c]		11.7[b]	50	3.09[b]	25
	111	[c]		0		0	
	222	[c]		0		0	

[a] Values followed by the same letter within a given experiment are not significantly different according to the Duncan's multiple-range test at $P < 0.05$.
[b] Significantly different, T-test, 2-tailed, $p < = 0.001$.
[c] This assay included extract concentrations of 25, 125, and 250 ppt. All seedlings died.

both plant parts were highly correlated ranging from $r = 0.82$–0.91 ($P \leq 0.001$) except at 25 ppt of experiment 2 for leaf extract where $r = 0.63$ ($P = 0.029$). Results of leaves compared with roots plus rhizomes from experiment 1, were not different except for the 12.5 ppt treatment (t test, $P \leq 0.05$).

The influence of osmotic potential on *L. minor* frond growth is illustrated in Figure 1. The test solutions decreased slightly in osmotic potential over the course of the experiment; osmotic potentials measured at the beginning of the assay are shown in Figure 1. Mean frond numbers for plants grown in solutions of 60, 74, and 143 mOsmol/kg were not different (Duncan's multiple-range test, $P \leq 0.05$). Only at 266 mOsmol/kg was there growth inhibition.

The osmotic potential of the undiluted extract of leaves of *N. lutea* was 115 mOsmol/kg; that of the roots plus rhizomes was 107 mOsmol/kg. The osmotic potential of the diluted leaf extract at the end of the assay was

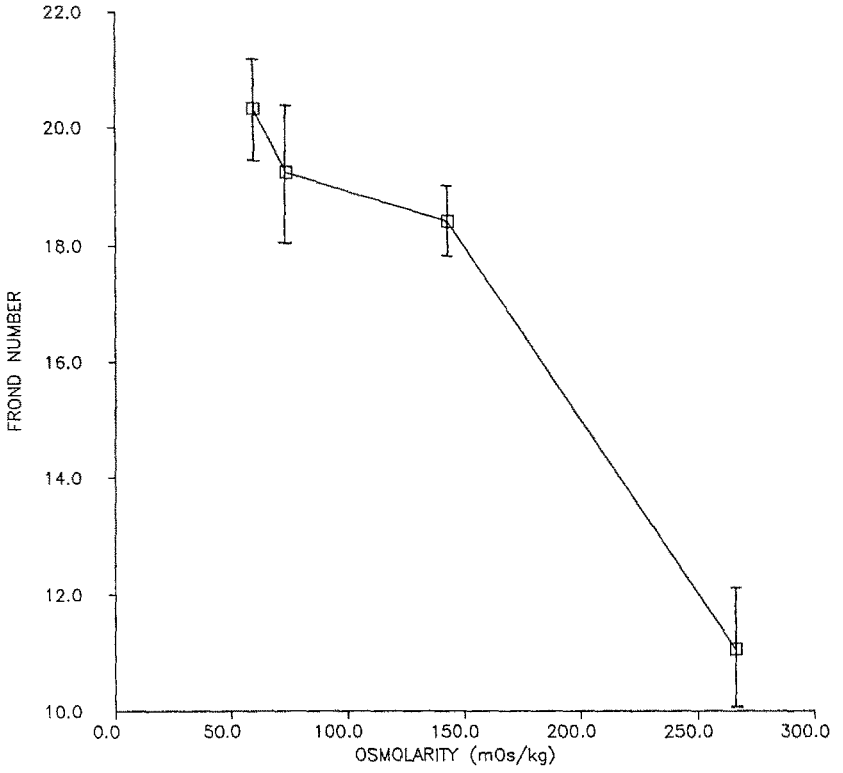


FIG. 1. Effect of osmotic potential (in milliosmoles per kilogram) on *L. minor* frond growth. Standard error is indicated with a vertical line.

33 mOsmol/kg at 25 ppt, and 52 mOsmol/kg at 125 ppt. The osmotic potential of the growth medium alone was 60 mOsmol/kg at the beginning of the assay. Extracts of both the leaves and roots plus rhizomes of *N. lutea* were strongly inhibitory to lettuce seedling growth, *L. minor* frond growth, and *L. minor* chlorophyll production. Our results (Figure 1) show that as long as the osmotic potential was below 143 mOsmol/kg, it did not contribute to growth inhibition of *L. minor*; Cheng and Riemer (1988) found that osmotic potentials of less than 70 mOsmol/kg do not affect lettuce seedling growth. Thus, neither the lettuce seedling nor the *L. minor* growth inhibition we observed can be attributed to osmotic potential.

Both extracts of *N. lutea* were acidic: the pH of the leaf extract was 4.05, that of the roots plus rhizomes was 4.00. Initial pH of the *L. minor* growth medium was 4.65. Not surprisingly, this unbuffered medium changed pH during the week or so of the assay. In one experiment the pH at the end of the

assay period was 6.8 in the control, 5.7 in the 25 ppt sample, and 4.55 in the 125 ppt sample. Although these are distinctly different pH values, they are still in the range (pH 4–6) where growth of *L. minor* is expected to be good (Einhellig et al., 1985). In fact, the final high pH of the control may slightly reduce *L. minor* growth, so that the observed growth inhibition of the extracts of lower pH may be a minimal expression of growth inhibition.

Elimination of pH and osmotic concentration as causative agents of growth inhibition does not prove that observed inhibition is due to allelopathy, but it certainly strengthens the case for allelopathy. There is no universal bioassay for the quantification of allelopathic potential. Seed germination tests appear to be less sensitive than seedling growth bioassays (Einhellig et al., 1985). The *L. minor* bioassay allows use of an aquatic plant as the target species and allows determination of both frond number and chlorophyll content. Frond number alone often adequately determines inhibitory activity, but would not be sufficient if the allelochemical reduced frond size but not number (Einhellig et al., 1985). The high correlation of frond number and chlorophyll content for *N. lutea* extracts indicates frond number alone is a sufficient measure of growth. Earlier work in our laboratories (Elakovich and Wooten, 1989) has shown that results of lettuce seedling assays are not completely correlated with results of *L. minor* assays, and the lettuce seedling assay is more sensitive. The results with *N. lutea* confirm these earlier results. Of the 16 selected hydrophytes we examined earlier, none showed inhibition (Dunnett's test) of *L. minor* at 20 ppt of extract, and only 12 of the 16 showed inhibition at 200 ppt of extract. All of these extracts inhibited growth of lettuce seedlings at 250 ppt of extract, but only six showed inhibition (Dunnett's test) at 25 ppt of extract. Thus *N. lutea* is many times more inhibitory than any of these 16 other hydrophytes!

As mentioned earlier, the alkaloid content of *N. lutea* has been extensively examined. Other alkaloids have been shown to be allelopathic (Rice, 1984), but the allelopathic activity of the *Nuphar* alkaloids has not been investigated. *Nuphar lutea* is thus a good candidate for further work to determine the nature of the compounds causing the growth inhibition reported here.

Acknowledgments—The technical assistance of Mr. Dale Suchy is gratefully acknowledged.

REFERENCES

ASHTON, F.M., DITOMASO, J.M., and ANDERSON, L.W.J. 1985. Spikerush (*Eleocharis* spp.): A source of allelopathics for the control of undesirable aquatic plants. *ACS Symp. Ser.* **268:** 401–414.

BELL, D.T. 1974. The influence of osmotic pressure in tests for allelopathy. *Trans. Ill. State Acad. Sci.* 67:312–317.

BUCHANAN, B.A., HARPER, K.T., and FRISCHKNECHT, N.C. 1978. Allelopathic effects of bur but-

tercup tissue on germination and growth of various grasses and forbs *in vitro* and in soil. *Great Basin Nat.* 38:90–96.

CHENG, T.-S., and RIEMER, D.N. 1988. Allelopathy in threesquare burreed (*Sparganium americanum*) and American eelgrass (*Vallisneria americana*). *J. Aquat. Plant Manage.* 26:50–55.

CULLEN, W.P., LALONDE, R.T., WANG, C.J., and WONG, C.F. 1973. Isolation and *in vitro* antifungal activity of 6,6′-dihydroxythiobinupharidine. *J. Pharm. Sci.* 62:826–827.

EINHELLIG, F.A., LEATHER, G.R., and HOBBS, L.L. 1985. Use of *Lemna minor* L. as a bioassay in allelopathy. *J. Chem. Ecol.* 11:65–72.

ELAKOVICH, S.D., and WOOTEN, J.W. 1989. Allelopathic potential of sixteen aquatic and wetland plants. *J. Aquat. Plant Manage.* 27:78–84.

FRANK, P.A., and DECHORETZ, N. 1980. Allelopathy in dwarf spikerush (*Eleocharis coloradoensis*). *Weed Sci.* 28:499–505.

GODFREY, R.K., and WOOTEN, J.W. 1981. Aquatic and Wetland Plants of Southeastern United States. University of Georgia Press, Athens. pp. 166–167.

IWANOW, A., WOJTASIEWICZ, K., and WROBEL, J. 1986. Sulphoxides of thiobinupharidine thiohemiaminals from *Nuphar lutea*. *Phytochemistry* 25:2227–2231.

LALONDE, R.T., and WONG, C. 1977. Properties of sulfur containing *Nuphar* alkaloids. *Pure Appl. Chem.* 49:169–181.

NICHOLS, S.A., and SHAW, B.H. 1983. Physical, chemical and biological control of aquatic macrophytes. Lake Restoration, Protection and Management. U.S. E.P.A., Washington, D.C. pp. 181–192.

OBORN, E.T., MORAN, W.T., GREENE, K.T., and BARTLEY, T.R. 1954. Weed control investigations on some important plants which impede flow of western irrigation waters. Joint Lab. Rep. SI-2, USDA, Bur. Reclam. Eng. Lab., and USDA, ARS Field Crops Branch. pp. 16–17.

RICE, E.L. 1984. Allelopathy, 2nd ed. Academic Press, New York. pp. 285–287.

SALIN, M.L., and BRIDGES, S.M. 1982. Isolation and characterization of an iron-containing superoxide dismutase from water lily, *Nuphar lutea*. *Plant Physiol.* 69:161–165.

SUTTON, D.L., and PORTIER, K.M. 1989. Influence of allelochemicals and aqueous plant extracts on growth of duckweed. *J. Aquat. Plant Manage.* 27:90–95.

TATAROV, A.P. 1945. White and yellow water lilies. *Farmatsiya* 8:29–31. *Chem. Abstr.* 41:2210i.

WROBEL, J.T., and RUSZKOWSKA, J. 1979. Chemistry of C_{30} alkaloids from yellow water lily—*Nuphar luteum* Sm. Collect Lect. 3rd Int. Symp. Furan Chem. pp. 157–160.

YEO, R.R. 1980. Life history and ecology of dwarf spikerush (*Eleocharis coloradoensis*). *Weed Sci.* 28:263–272.

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

PHEROMONAL ACTIVITY OF SINGLE CASTOREUM CONSTITUENTS IN BEAVER, *Castor canadensis*

D. MÜLLER-SCHWARZE* and PETER W. HOULIHAN

College of Environmental Science and Forestry
State University of New York
Syracuse, New York 13210

(Received May 22, 1990; accepted November 27, 1990)

Abstract—Behavioral activity of single components of beaver castoreum was demonstrated for the first time. In four experiments samples were presented to free-ranging beaver in their family territories. First, responses to whole castoreum and anal gland secretion (AGS) from males and females were tested. Second, 24 compounds, known to be constituents of beaver castoreum, were individually screened for activity. Four of these consistently released immediate responses during the observation periods. These are the phenols 4-ethylphenol and 1,2-dihydroxybenzene and the ketones acetophenone and 3-hydroxyacetophenone. In the most complete responses, the beaver sniffed from the water, were attracted to the odor, swam toward its source, went on land, and then approached, sniffed, pawed, and scent-marked the artificial scent mound. 4-Ethoxyphenol, a compound not yet found in castoreum, also released these responses. Five additional compounds resulted in a few delayed visits to the samples during the night following the observations, as evidenced by destroyed scent mounds. These are 4-methyl-1,2-dihydroxybenzene, 4-methoxyacetophenone, 5-methoxysalicylic acid, salicylaldehyde, and 3-hydroxybenzoic acid. Third, mixtures of 24 and six compounds were tested. Responses to these mixtures could be as strong as those to whole castoreum. Fourth, the four regularly active compounds were tested in two additional beaver populations and proved to be active there, too. The response was strongest in the densest beaver population.

Key Words—Adirondacks, beaver, bioassay, *Castor canadensis*, castoreum, field study, New York, phenols, pheromone, scent marks, territorial marking.

*To whom correspondence may be addressed.

0098-0331/91/0400-0715$06.50/0

INTRODUCTION

Mammals use mixtures of compounds as well as single compounds as intraspecific olfactory signals, most of them arguably termed pheromones.

Mixtures. Examples of active mixtures are the scent marks of marmosets and tamarins (Callitrichidae) (reviewed by Epple et al., 1986); the "maleness" (or "aggression") signal in mice (Novotny et al., 1985); and the puberty-delaying pheromone in the urine of female mice, *Mus musculus* (Novotny et al., 1986). Biological activity of several compounds of a mixture may interact in synergistic, redundant, or additive fashion. We discuss the first two.

Synergism. Aggressive behavior in male laboratory mice is intensified by a mixture of two compounds from the urine, dehydrobrevicomin and 2-*sec*-butyl-4,5-dihydrothiazole. Either compound alone is inactive. It is important to note that they have to be dissolved in (otherwise inactive) urine. An aqueous solution of the two compounds is not active (Novotny et al., 1985).

The scent marks of the saddle-back tamarin, *Saguinus fuscicollis*, from the secretion of the circumgenital–suprapubic glands, contain at least 16 volatile butyrate esters, squalene, and several other unidentified volatile acids, alcohols, aldehydes, alkanes, and esters. Both the butyrate esters and and the volatiles are necessary for coding information on gender and subspecies (reviewed in Epple et al., 1986). This synergism of many compounds also has been involved in the term *odor image* (Albone, 1984).

Three compounds have been identified in the chest gland secretion of the primate *Galago crassicaudatus*. These are benzyl cyanide, 2-(hydroxyphenyl)ethanol, and *p*-hydroxybenzyl cyanide. Individually, none of the three compounds released any response from free-ranging galagos. But a mixture of compounds **1** and **2** applied to pipes was sniffed as frequently as pipes carrying galago scent marks after having been left for one week in a galago cage. A mixture of all three compounds also elicited a high rate of sniffing. No specific pattern of scent marking was reported (Katsir and Crew, 1980).

Redundancy. The puberty-delaying pheromone in the urine of female mice consists of three compounds. These are *n*-pentyl acetate, *cis*-2-penten-1-yl-acetate, and 2,5-dimethyl pyrazine. The biological effect, puberty delay in young females by 1.5–3.7 days, can be brought about by the pyrazine only, the two acetate esters, or all three compounds combined (Novotny et al., 1985), amounting to redundancy. An earlier example of redundancy is the sex pheromone of the domestic boar (*Sus scrofa*). Boar saliva contains α-androstenol and α-androstenone, either of which increases the probability of a sow in estrus assuming the mating stance. Activity is not increased by mixing both compounds (Melrose et al., 1971).

Single-Component Pheromones. Some pheromones consist of only one compound. The best investigated examples are the two sex pheromones of the

golden hamster, *Mesocricetus auratus*. The male is attracted by dimethyl disulfide in the mature female's vaginal secretion (Singer et al., 1976). Actual mounting is stimulated by aphrodisin, a protein ($alpha_{2u}$-globulin; mol wt = 17,000 daltons) (Singer et al., 1987).

The beaver, *Castor canadensis*, is one of the few mammals that permit rigorous experiments in the field. It resides year-round in family territories; is large enough to be observed from a distance; often occurs in partially open habitat; can be conditioned to the presence of people; can be observed during the last hours of daylight when its typically nocturnal activity begins; exhibits simple, predictable, and easily quantifiable behavior; and sizable populations are reasonably accessible to researchers. Therefore, we chose the beaver as a model for chemical stimulus–response experiments in a free-ranging mammal.

Beavers scent mark their territories by building mud piles on the banks of their ponds and applying two secretions, castoreum from their castor sacs and anal gland secretion, to these mounds. These scent mounds have been described many times, starting with 19th-century naturalists. Here we concentrate on chemical studies, the composition of beaver castoreum and anal gland secretion having been well investigated. Table 1 lists all compounds identified in castoreum by Lederer (1946), Valenta and Khaleque (1959), and Maurer and Ohloff (1976).

Fractions of castoreum release typical scent-marking responses. The more castoreum constituents are included in the mixture, the more intense are the responses (Müller-Schwarze et al., 1986). Beaver were attracted to neutrals and phenolics, but not to carboxylic acids and amines from castoreum (Svendsen and Huntsman, 1988). The biological significance of none of the single compounds is known. Therefore we embarked on testing single castoreum constituents for their pheromonal activity in a natural setting.

METHODS AND MATERIALS

Study Areas. The castoreum components were tested between 1986 and 1988 in three beaver populations in New York State: (1) first, in 1986 and 1987, 25 castoreum constituents were screened at the Reinemann Sanctuary near Dolgeville, Fulton County, at the southern fringe of the Adirondack Park; the four most active compounds were tested further (2) at Allegany State Park (ASP) in western New York in 1987 and (3) in 1988 at the Huntington Wildlife Forest (HWF), a campus of the College of Environmental Science and Forestry (ESF), located in the center of the Adirondack Park. The beaver colonies in all three areas were located on streams and had 1–12 dams. The ponds were small and open enough to observe beaver behavior on the opposite bank. The average family size was 5.7 animals (SD = 6.8, range 1–7) at ASP and 3.8 (SD = 1.53, range 1–7) at HWF.

TABLE 1. CHEMICAL COMPONENTS OF CASTOREUM FROM CASTOR SACS OF BEAVER[a]

- Alcohols
  - Benzyl alcohol
  - Cholesterol
  - 1-Cholestanol
  - Mannitol
  - *cis*-5-Hydroxytetrahydroionol
  - 2-Hydroxycamphene
- Phenols
  - 4-Ethylphenol
  - 4-Propylphenol
  - 1,2-Dihydroxybenzene
  - 1,4-Dihydroxybenzene
  - 4-Methoxyphenol
  - *p*-allylphenol
  - 2-Hydroxy-5-ethylanisole
  - 4-Methyl-1,2-benzenediol
  - 4-Ethyl-1,2-benzenediol
  - 1-*p*-hydroxyphenylbutan-3-ol (betuligenol)
  - 2,4′-Dihydroxydiphenylmethane
  - 2′,3″-Dihydroxydibenz-2-pyrone
  - 4,4″-Dihydroxydiphenic acid dilactone
  - A phenolic ether
- Aldehydes
  - Salicylaldehyde
- Nitrogen bases (amines)
  - Castoramine
  - Isocastoramine
  - Desoxynupharidine
  - 7-*epi*-Desoxynupharidine
  - 1-*epi*-Desoxynupharidine
  - 1-*epi*,7-*epi*-Desoxynupharidine
  - 7-Desmethyl-desoxynupharidine
  - 5-(3-Furyl)-8-methyl-octahydroindolizine
  - 1,3,6,6-Tetramethyl-5,6,7,8-tetrahydroisoquinolin-8-one
  - Trimethylpyrazine
  - Tetramethylpyrazine
- Nitrogen bases (cont.)
  - 5,6,7,8-Tetrahydroquinoxaline
  - 2-Methyl-5,6,7,8-tetrahydroquinoxaline
  - 2,3-Dimethyl-5,6,7,8-tetrahydroquinoxaline
  - 1,2,3,4,6,7,8,9-Octahydrophenazine
- Ketones
  - Acetophenone
  - 3-Hydroxyacetophenone
  - 4-Methoxyacetophenone
  - Aromatic ketone
  - 2 Isomeric hydroxy ketones
  - 4-Hydroxyacetophenone
- Aromatic acids
  - Benzoic
  - 2-Phenylpropanoic
  - 4-Methoxy-salicylic
  - *o*-hydroxybenzoic (salicylic)
  - *m*-Hydroxybenzoic
  - *p*-Hydroxybenzoic
  - *p*-Methoxybenzoic
  - 2,5-dihydroxybenzoic (gentisic)
  - 5-Methoxysalicylic
- Esters
  - Cholesteryl oleate
  - Esters of ceryl alcohol
  - Esters of benzyl alcohols
  - Esters of phenols
  - Esters of gentisic acid

[a] Adapted from Lederer (1946), Valenta and Khaleque (1959), and Maurer and Ohloff (1976).

Stimulus Preparation. Of the known constituents of beaver castoreum (Lederer, 1946, 1950), 24 compounds (Tables 2 and 3) plus cholesterol (a major castoreum constituent) were obtained commercially. Samples for bioassays were prepared at concentrations representing their relative proportions in castoreum as described by Lederer (1946). The amounts dissolved in 0.25 ml ethanol as

TABLE 2. CASTOREUM CONSTITUENTS USED IN EXPERIMENTS[a]

	mg/trial		mg/trial
Alcohols:		Ketones	
Benzyl alcohol	0.2	*Acetophenone*	0.55
Cholesterol[b]	0.0	*3-hydroxyacetophenone*	0.55
Mannitol	0.99	*4-Methoxyacetophenone*	0.55
Borneol	0.99	Aromatic acids	
Glycol[1]	0.26	Benzoic	35.0
Phenols		2-Phenylpropionic	0.825
4-Ethylphenol	3.2	Cinnamic	0.825
4-Propylphenol	0.3	Salicylic	0.825
1,2-Dihydroxybenzene	0.55	*3-Hydroxybenzoic*	0.825
Hydroquinone	0.26	4-Hydroxybenzoic	0.825
4-Ethoxyphenol[c]	0.012	Anisic	0.825
1,2-dihydroxy-4-methyl-benzene	0.55	2,5-Dihydroxybenzoic (gentisic)	0.825
Aldehydes		*5-Methoxysalicylic*	0.825
Salicylaldehyde	1.0	Esters	
		Cholesteryl oleate	0.55

[a] Compounds in *italics* released at least some responses. Those also *underlined* were active immediately (during observation period) and consistently so.
[b] Used only in mixture, not as single compound.
[c] Not yet described for castoreum.

solvent and used for one trial are given in Table 2. A sample used in a particular trial represented 1/30 gland equivalent (GE), i.e., as much material as was found in 1/30 of the contents of one castor sac of average size (about 40 g, including contents and surrounding tissue). Castoreum was obtained from trappers in the western Adirondack region and kept frozen until needed.

SixMix contained the following six compounds with strong phenolic or beaverlike odors: benzoic acid, according to Lederer (1950) after cholesterol (8.0%) the second most abundant constituent of castoreum (2.6%); 4-ethylphenol, the third most abundant component (0.227%); 5-methoxysalicylic acid, 1,2-dihydroxybenzene, 3-hydroxyacetophenone, and 4-hydroxyacetophenone.

Controls (solvent only on the mudpile) and blanks (mud only) were used at ASP and HWF. Since in previous experiments (Müller-Schwarze et al., 1986) solvent controls never released any response, and the number of samples screened was already large, no controls were used at the Reinemann Sanctuary.

Stimulus Presentation. In the field, an artificial scent mound (ASM) was built by scooping mud from the pond with a trowel. The pile was about 30 cm in diameter and 10 cm high. In the center of the mud pile, a cork (4 cm diameter) was placed. The sample was applied to the cork with a disposable pipette. A tongue depressor was broken in half and one of the halves set vertically into

the scent mound next to the cork. This was to ascertain whether subtle responses had occurred during the night, such as a beaver inspecting the scent mound or moving over it without pawing and thus destroying the mound.

The ASM was placed about 50 cm from the water's edge and upwind from the main activity area, i.e., lodge and main dam, of the beaver colony. The observer sat on higher ground on the slope opposite the ASM, always downwind or crosswind from the main activity area of the beaver.

Behavior Observations. The ASM was built and scent applied at about 1700 hr, $\frac{1}{2}$ to 1 hr before the first beaver was expected to emerge from the lodge. Observation started about $\frac{1}{2}$ hr before the first beaver emerged and ended with fading daylight. Between May and August the changing times of emergence and nightfall determined the observation period. Depending on the month and the weather, the observation periods ranged from 1 to 4 hr, starting as early as 1700 hr and terminating as late as 2115 hr.

During the weeks before the experiment, as many beaver as possible of the observation colonies had been live-trapped, immobilized with Rompun and Ketaset (2 and 22 mg/kg body weight, respectively), sexed, measured, weighed, assigned to age classes (adult, 2-year-old, 1-year-old, kit), and individually marked with anodized aluminum ear tags in different color combinations for both ears. At ASP, 41 beaver were tagged (males: five adults, three 2-year-olds, and six yearlings; females: nine adults, 10 2-year-olds, six yearlings, and two kits). At HWF, 72 beaver were tagged (males: 16 adults, five 2-year-olds, and seven yearlings; females: 19 adults, five 2-year-olds, and six yearlings, one 2-year-old of unknown sex, and 13 kits).

Each individual was observed from the time it emerged until nightfall. This time period typically included several activity bouts, interspersed with visits to the lodge. The frequencies and durations of the following behaviors were recorded with a portable computer (Tandy 102) for the two seasons at ASP (1987) and HWF (1988), and with stopwatches and field notebooks for the earlier two seasons: emerging, swimming (fast, slow; near dam or in pond), feeding, tail-slapping, diving, sniffing from water, land visit, activity at ASM (sniffing, pawing, marking), and dam maintenance. If several beaver were in view simultaneously, observations focused on the animal nearest the sample. If more than one beaver went on land and responded to the sample, only the responses of the first animal were considered for analysis, because the first animal changes the stimulus situation for subsequent visitors to the ASM. The time of each behavior observation was automatically recorded by the computer, or noted in the field book, respectively. During the computer recording, a notebook and a stopwatch were held ready as backups.

If no responses to the ASM occurred during the observation period, the mound was examined the following morning for signs of beaver visits during

the night ("overnight activity"). The most unequivocal indicator of overnight beaver activity was a destroyed scent mound.

The sample sizes for 1986 and 1987 at Reinemann Sanctuary are given in the caption of Figure 1. Table 4 lists the sample sizes for the 1987 experiments at Allegany State Park, and Huntington Wildlife Forest in 1988.

Statistics. The means of the responses to the various treatments were compared with the *t* test and the Kruskal-Wallis test (Conover, 1980).

RESULTS

Responses of Beaver. The beaver responded to the ASMs with sniffing in the direction of the ASM from the water, approaching into the wind while swimming in zigzag fashion, going on land at the ASM (termed "land visit"), walking to the ASM, sniffing the ASM, pawing the ASM with the front feet, stepping forward to straddle the ASM, lowering the hindquarters and contacting

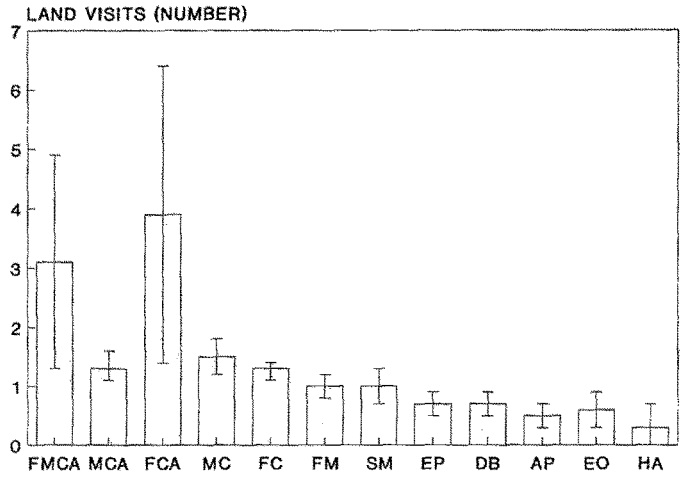


FIG. 1. The number of land visits in response to 12 treatments at Reinemann Sanctuary in 1986 and 1987. *Abscissa*: FMCA: mixture of female and male castoreum and anal gland secretion (AGS), n = 17; MCA: male castoreum and AGS, n = 21; FCA: female castoreum and AGS, n = 13; MC: male castoreum, n = 8; FC: female castoreum, n = 8; FM: full mixture of 24 compounds, n = 17; SM: six strong smelling phenolic and beaverlike compounds (SixMix), n = 8; EP: 4-ethylphenol, n = 10; DB: 1,2-dihydroxybenzene, n = 9; AP: acetophenone, n = 8; EO: 4-ethoxyphenol, n = 8; HA: 3-hydroxyacetophenone, n = 6. *Ordinate*: Mean number of land visits. Dispersion measure is standard error of mean. n refers to samples sizes.

TABLE 3. SOURCES AND PURITY OF COMPOUNDS USED IN BIOASSAYS

	Source	Purity (%)	Lot #
Alcohols			
Cholesterol	Aldrich (A)	98	TM02217HM
Mannitol			
(−)-Borneol	A	tech.	0411MK
Benzyl alcohol	Undocumented		
Mannitol	Undocumented		
Phenols			
p-Ethylphenol	Pfaltz & Barner, (PB) Waterbury, CT	97	E13750
p-Propylphenol	K & K Laboratories, Plainview, NY		44506
Catechol	A	99+	01218TL
Quinol monoethylether	PB		E03820
4-Methyl-catechol	PB	98	M14030
Quinol (Hydroquinone)	Undocumented		
Aldehydes			
Salicylaldehyde	Eastman Kodak	Practical	
Ketones			
Acetophenone			
4-Hydroxyacetophenone	A	97	6109KK
4-Methoxyacetophenone	A	98	01511PM
Aromatic acids			
Benzoic	A	99	
2-Phenylpropionic	PB		H07950
trans-Cinnamic	A	97	2427EK
Salicylic	A	99+ gold label	0404EL
3-Hydroxybenzoic	A	99	02406TJ
4-Hydroxybenzoic	A	99+ gold label	07614LL
p-Anisic	A	98	02130JJ
2,5-Dihydroxybenzoic	A	99	3201EL
5-Methoxysalicylic	A	98	4807EL
Esters			
Cholesteryl oleate	A	97	3225PH

the ASM with the cloaca, and marking the ASM with secretion from the castor sacs and/or the anal glands. The behavior at the mound itself varied from brief sniffing to elaborate obliteration of the artificial scent mound. The response was graded and did not always include the entire sequence. Beaver also hiss when encountering an ASM, but this vocalization can be heard only under optimal

conditions (short distance, no wind), so this sound was not included in the analysis. One animal may repeat the described sequence, and other colony members, except kits of the year, may have their turn at the scent mound. In the past, we have observed up to 19 visits to one experimental scent mound by various members of a colony during one evening's observations. In this study, one beaver responded by sniffing from the water in 75% of the observation nights, two individuals in 25%, and three animals on one occasion each.

Of the several behavioral measures examined, the most inclusive is the duration (in seconds) of a land visit to the ASM (Müller-Schwarze et al., 1986) (Figure 2). It includes the durations of the approach to the mound, sniffing, pawing, marking, and leaving for the water. The stronger the response at the mound, the longer the land visit lasts. On the other hand, a weak response consists in a brief sniffing at or toward the mound while the beaver is on land. Activity is operationally defined here as attracting one or more beaver of a family, inducing it to go on land, visit the scent mound and perform specific behaviors there.

Responses to Whole Castoreum Secretion. The responses to whole castoreum of males and females, with and without added anal gland secretion, were the strongest of all samples. The frequencies or durations of the various behaviors are presented in the left five bars of Figure 1–6. The number of land visits (Figure 1) did not vary significantly across the samples. The mean total durations of "time on land" (Figure 2) ranged from 27.1 sec for a mixture of male and female castoreum and AGS to 57.5 sec for male castoreum. The responses to castoreum samples from males and females, with and without AGS added,

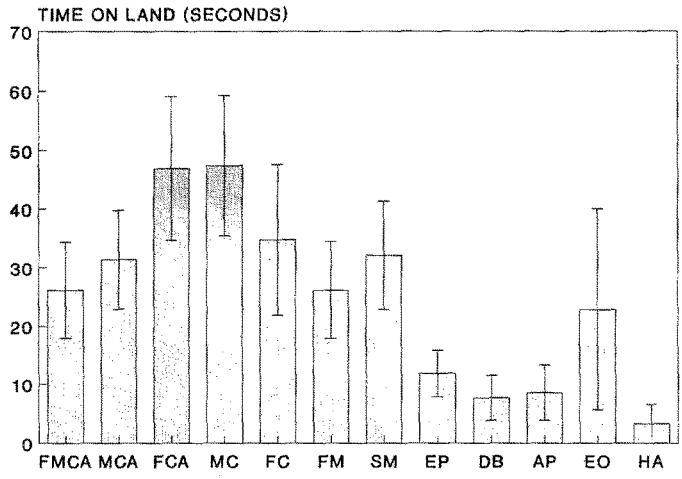


FIG. 2. Average time on land, in seconds (ordinate). Abbreviations as in Figure 1.

did not differ significantly. Sniffing the sample at close range (Figure 3) follows essentially the same pattern. The shorter durations of sniffing the most complex samples may indicate quicker recognition of a clearer signal. Pawing the scent mound was a more stereotyped behavior. With the exception of the female castoreum/AGS stimulus (FCA) the response levels (Figure 4) did not differ between the treatments as much as those for sniffing. The frequencies of straddling the scent mound, the motor pattern for scent marking (Figure 5), paralleled the sniffing durations of Figure 3. Nearly all experimental scent mounds with castoreum samples or mixtures were destroyed eventually, whether during the observation period or the subsequent night, (Figure 6).

Screening of Individual Compounds. There were five compounds among the 24 tested that were consistently active. These were the phenols 4-ethylphenol (EP), 1,2-dihydroxybenzene (DB), and 4-ethoxyphenol (EO), not yet described for castoreum, and the two ketones acetophenone (AP) and 3-hydroxyacetophenone (HA). These compounds are underlined in Table 2 and their structural formulae given in Figure 7. Five additional compounds resulted in occasional delayed destruction of the scent mound during the night, but never during the observations. These are the phenol 4-methyl-1,2-dihydroxybenzene (MD), salicylaldehyde (SA), 5-methoxysalicylic acid (MS), and 3-hydroxybenzoic acid (HB). This group of compounds is italicized in Table 2 and the structural formulae given in Figure 8. The activities of these compounds were not significantly different from zero. At this point, they should be considered merely pheromone candidates.

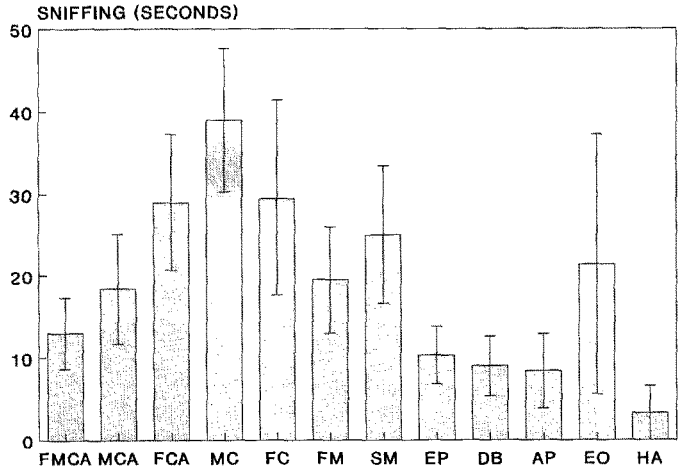


FIG. 3. Average duration of sniffing, in seconds (ordinate). Abbreviations as in Figure 1.

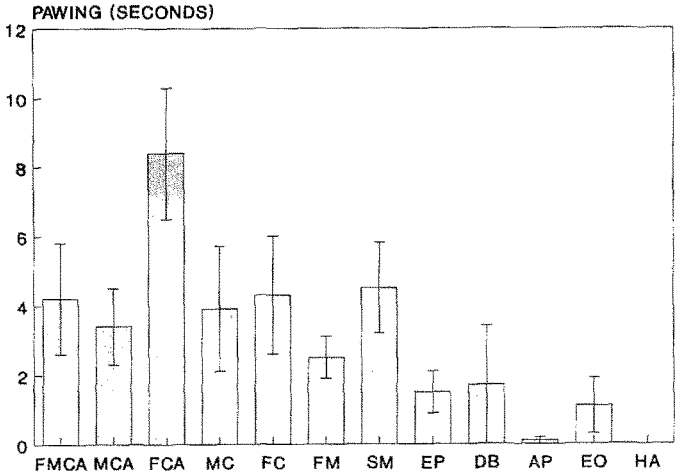


FIG. 4. Average duration of pawing the artificial scent mound, in seconds (ordinate). Abbreviations as in Figure 1.

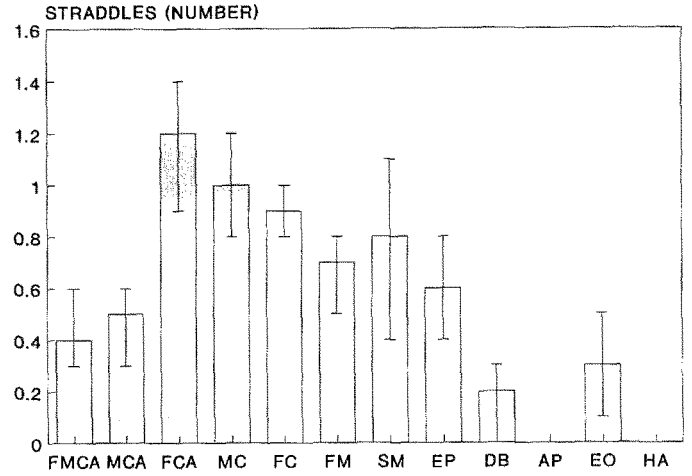


FIG. 5. Average frequencies of straddling (scent marking) the artificial scent mound. Abbreviations as in Figure 1.

The responses to the single compounds are shown in the bars on the right in Figures 1–6. Figure 2 summarizes the overall responses (time on land) for the active compounds, compared with castoreum as standard. Three single compounds were significantly less active than male castoreum (EP: $P = 0.004$; DB: $P = 0.006$; AP: $P = 0.02$). MP did not differ significantly. Only EP activity

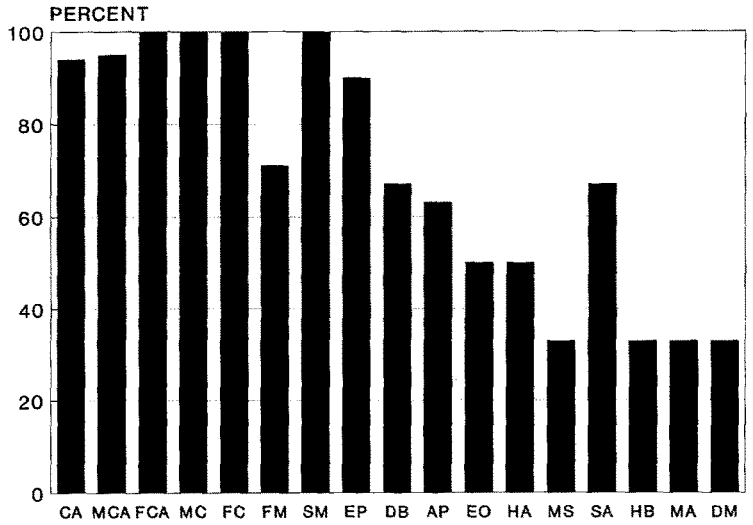


FIG. 6. Percent of artificial scent mounds obliterated. Includes incidents observed directly and scent mounds found destroyed the following morning. CA: castoreum and AGS from both sexes; MCA: same from male only; FCA: same, from female only. MC, FC: male or female castoreum, respectively. FM, SM: Mixture of 25 or six compounds, respectively. EP: 4-ethylphenol; DB: 1,2-dihydroxybenzene; AP: acetophenone. EO: 4-ethoxyphenol; HA: 3-hydroxyacetophenone; MS: 5-methoxysalicylic acid; SA: salicylaldehyde; HB: 3-hydroxybenzoic acid; MA: 4-methoxyacetophenone; DM: 4-methyl-1,2-dihydroxybenzene.

was significantly different ($P = 0.03$) from zero ($P = 0.06$ and 0.07 for DB and AP, respectively). MP did not differ significantly from zero. None of the differences between single compounds was significant.

Among the single compounds, 4-ethylphenol samples (EP) were obliterated in nine of 10 cases, 1,2-dihydroxybenzene (DB) in six of nine, and salicylaldehyde (SA) in four of six cases (Fig. 6).

Mixtures. The responses to mixtures of all 24 compounds (FullMix) or a selection of six phenol- or "beaverlike"-smelling castoreum constituents (SixMix) are shown as FM and SM in Figures 1–6. Both mixtures did not differ significantly from male or female castoreum, although FullMix was less active than SixMix and almost differed significantly ($P = 0.06$) from male castoreum. The difference between the two mixtures was not significant. FullMix ($P = 0.055$) and SixMix ($P = 0.01$) differed significantly from zero . SixMix was significantly more active than the single compounds EP ($P = 0.028$) and BD ($P = 0.03$), barely ($P = 0.068$) more active than AP, and not different from EO. There was no significant difference between FullMix and any of the single compounds.

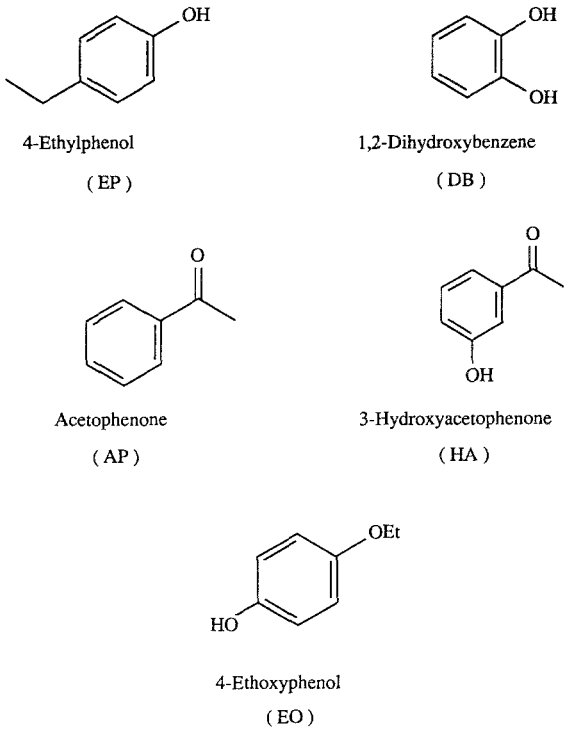


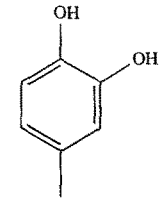
FIG. 7. Structural formulae, names, and abbreviations used in text, for the three phenols and two ketones that were most active.

FullMix was tested with and without cholesterol, the most abundant castoreum constituent (Lederer, 1946). With cholesterol, the mixture was less active. Because cholesterol is not volatile and was difficult to keep in the ethanol samples, it was not used for further tests. Therefore, all FullMix data refer to a mixture of 24 compounds, not including cholesterol.

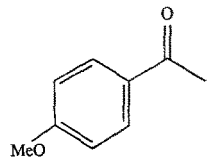
Responses by Different Populations. Further tests of the four most active compounds on two additional New York populations, in Allegany State Park (ASP) and the Huntington Wildlife Forest (HWF), confirmed the findings at the Reinemann Sanctuary.

At Allegany State Park, only one of the single compounds, 4-ethylphenol, was used. Figure 9 shows that from the water, the beaver sniffed castoreum samples the longest. Castoreum differed significantly ($P = 0.05$) from controls and blanks. Activity of EP was intermediate between blanks and controls (NS), and castoreum ($P = 0.05$). The frequencies of land visits to the samples showed a similar distribution (Figure 10).

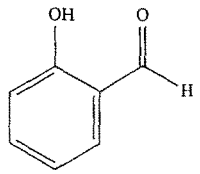
At Huntington Wildlife Forest, 4-ethylphenol, 1,2-dihydroxybenzene,



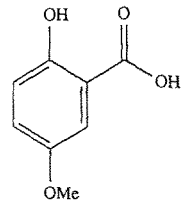
1,2-dihydroxy-4-methylbenzene
(DM)



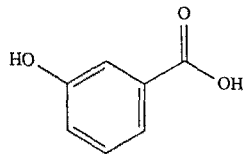
4-methoxyacetophenone
(MA)



salicylaldehyde
(SA)



5-methoxysalicylic acid
(MS)



3-hydroxybenzoic acid
(HB)

FIG. 8. Structural formulae, names, and abbreviations for five compounds with minor activity.

4-methoxyphenol, and acetophenone were tested. All four compounds elicited some sniffing from the water, but for much shorter times than castoreum (Figure 11). Table 5 lists the significant differences between pairs of samples. Land visits to the scent mounds were most frequent for castoreum, followed by 4-ethylphenol and 4-methoxyphenol, rare for 1,2-dihydroxybenzene, and absent for acetophenone. (Figure 12). More detailed information can be found in Houlihan (1989).

Beaver at Allegany State Park consistently responded more strongly to the samples than their counterparts at Huntington Wildlife Forest. This confirms earlier experiments where ASP beaver showed more intense responses to castoreum samples than Adirondack beaver at Cranberry Lake Biological Station. The behavior of beaver in the dense population at Allegany State Park will be described in detail elsewhere.

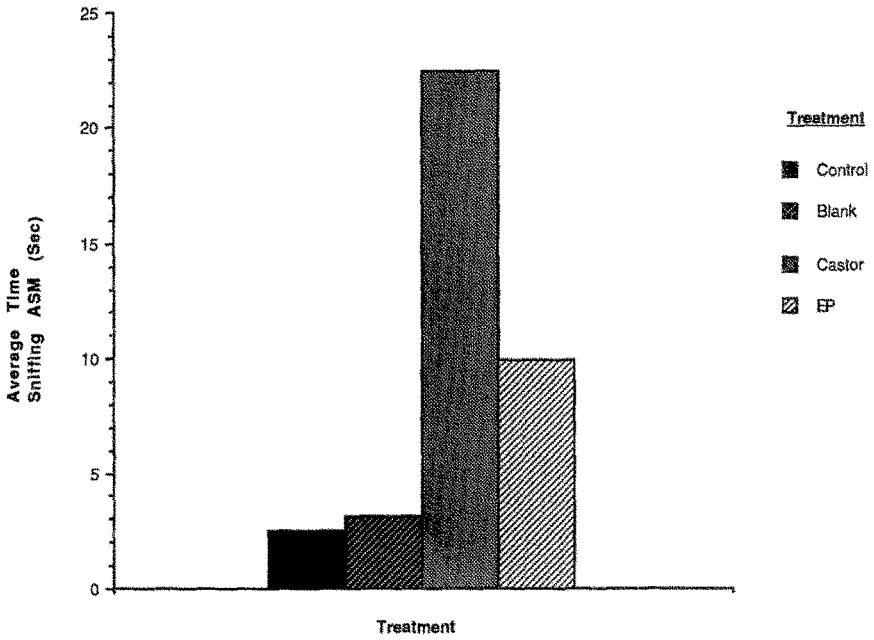


FIG. 9. Average time of sniffing from the water, in seconds (ordinate) for control (solvent = ethyl alcohol), blanks, castoreum, and 4-ethylphenol (EP). Fourteen sites received each treatment once. Allegany State Park, 1987. Differences between treatments are significant (Kruskal-Wallis, $t = 9.87$, $df = 3$, $P = 0.02$).

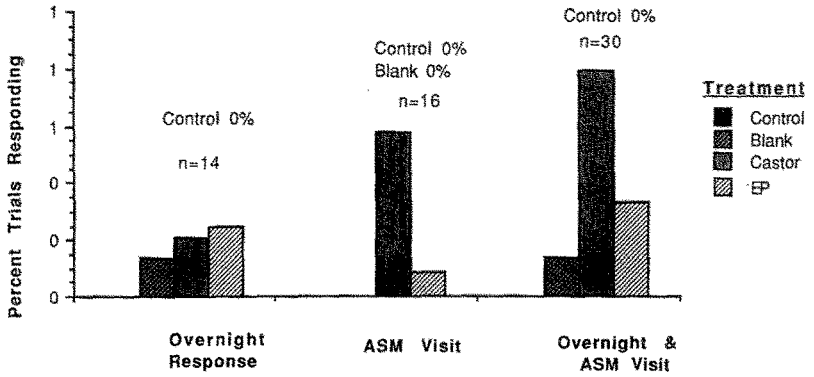


FIG. 10. Percentages of trials with overnight obliteration of the artificial scent mound (left), a visit to the mound during observations (center) and both combined (right). Allegany State Park, 1987. Treatments: control, blank, castoreum, and 4-ethylphenol (EP). Sample size n = trials with a given response.

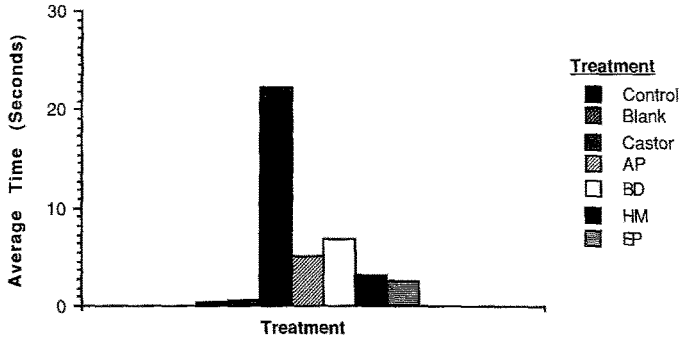


FIG. 11. Average time in seconds (ordinate) spent sniffing the artificial scent mound from the water. Samples: AP = acetophenone; DB = 1,2 dihydroxybenzene; EO = 4-ethoxyphenol; EP = 4-ethylphenol. Seven sites received each treatment once. Huntington Wildlife Forest, 1988). Differences between treatments are significant (Kruskal-Wallis, $t = 18.22$, $df = 6$, $P = 0.006$).

TABLE 4. SAMPLE SIZES FOR BIOASSAYS AT ALLEGANY STATE PARK (ASP) AND HUNTINGTON WILDLIFE FOREST (HWF)[a]

	No of Colonies	Control	Blank	Castor	EP	DB	EO	AP	Total
ASP 1987	14	27	25	27	26	0	0	0	105
HWF 1988	8	16	16	15	17	19	13	15	111

[a]EP = 4-ethylphenol; DB = 1,2-dihydroxybenzene; EO = 4-ethoxyphenol; AP = acetophenone.

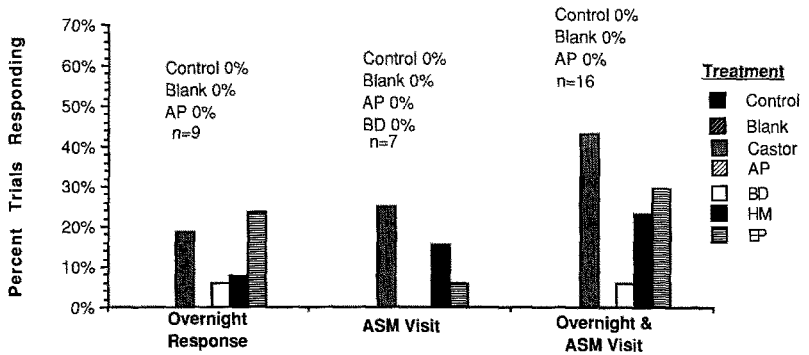


FIG. 12. Percentages of trials with overnight obliteration of the artificial scent mound (left), a visit to the mound during observations (center), and both combined (right). Samples as in Figure 11; n as in Figure 10. Huntington Wildlife Forest, 1988.

Sex Differences. At Huntington Wildlife Forest male beaver showed stronger responses than females to castoreum and 4-ethylphenol. Males sniffed the samples longer from the water (Figure 13) and also more often (44%) than other sex and age classes. In 46% of these observations, an adult of unknown sex responded, and the remaining 10% represented all other sex and age classes. Fifty percent of the land visits to the AMS were by adult males, 15% by 2-year-old males, and the remainder equally by adult females and yearling males and females. Only one animal responded in over 80% of the visits, and up to six animals responded.

DISCUSSION

This is the first time individual compounds of castoreum have been shown to be active by releasing specific behaviors in free-ranging beaver. The bioassay exploited the more tractable responses of resident beaver to strange beaver scent

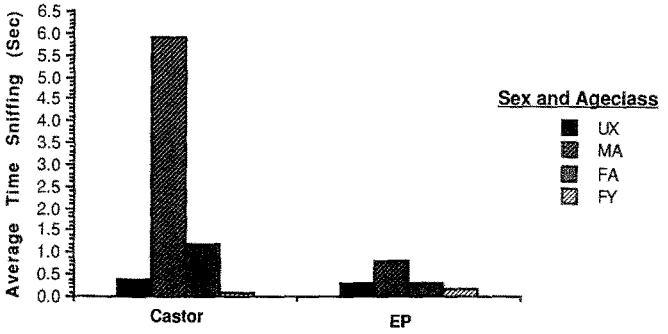


FIG. 13. Sex and age-class differences in the time (in seconds) spent sniffing the artificial scent mound from the water. UX: Unidentified sex or age; MA: adult male; FA: adult female; FY: female yearling.

TABLE 5. PAIRWISE COMPARISONS BETWEEN MEAN DURATIONS OF SNIFFING ASM FROM WATER FOR SEVEN DIFFERENT TREATMENTS, HUNTINGTON WILDLIFE FOREST[a]

	Control					
Blank	NS	Blank				
Castoreum	***	***	Castoreum			
AP	**	**	NS	AP		
DB	NS	NS	**	*	DB	
EO	+	NS	**	+	NS	EO
EP	*	*	*	NS	NS	NS

[a] AP = acetophenone; DB = 1,2-dihydroxybenzene; EO = 4-ethoxyphenol; EP = 4-ethylphenol. Conover's pairwise comparison; $df = 42$. *** $P = 0.001$; ** $P = 0.01$; * $P = 0.05$; $^{+}P = 0.1$.

marks near their lodge(s). We are well aware that castoreum most likely serves other functions, such as attracting the other sex, orientation in one's own territory, or assessing breeding condition or strength of neighboring territory owners.

Undoubtedly, different castoreum components may have different functions. The more volatile constituents may serve as long-distance signals to attract the attention of conspecifics, while other, less volatile compounds provide information at close range. Still other compounds may act as a slow-release matrix.

There was no evidence that repeated exposure of the same beaver families led to either habituation or sensitization, as response increases or decreases over time were not significant.

Sex differences of responses suggest that the adult male in the colony plays a greater role in scent marking and responding to intruders than the female. A recent study also found that male beaver show more alarm behavior and scent mark for a longer period during the summer than the female (Buech, 1989). However, in two of our three study areas, sex differences in behavior remain unknown because too few individuals were tagged.

The compounds tested so far appear to act together in additive as well as redundant fashion. The stronger effect of mixtures suggests that the activities of single compounds add up. The five most active phenols and ketones have the same effects and at the same intensities. They can be substituted for one another. This constitutes redundancy, even though their effects are far smaller than that of whole castoreum.

For some stimuli, there was a tendency for activity to decrease with increasing complexity. For example, the most complex mixture, castoreum and AGS from both sexes (left bar in Figures 1–6) was less active than the castoreum/AGS mixtures from either sex alone. Also, SixMix was more active than the mixture of 24 compounds (FullMix). At this point, it is unknown how the numerous compounds may interact to reduce activity.

The higher response level at Allegany State Park is probably related to population density. The ASP population is unexploited. All suitable and many marginal sites are occupied. The yearly turnover rate of sites is high, and the distances between colonies are short. This may result in high intercolony contact and competition, which in turn may increase the probability and intensity of a reaction to foreign scent marks in a family's home colony.

This work has demonstrated that it is possible to bioassay rigorously specified chemical stimuli of mammals in the field despite the numerous intervening variables. The disadvantages of such "noise" are more than compensated for by the natural context.

The precise functions of the various constituents remain to be elucidated. The more volatile compounds are suited to attract attention to the scent mound

over a longer distance, while mixtures of other compounds are expected to provide information on number, sex, age, and status at close range.

Acknowledgments—Most of the work was supported by the Adirondack Wildlife Program, New York State Legislature. Preparation of the manuscript was partially supported by grant GB 88-19982 from the U.S. National Science Foundation. We thank Lucinda Smarro for collecting data at the Reinemann Preserve during the 1986 and 1987 seasons. Thanks go to Dr. William Porter and Richard Sage for facilitating our work at the Huntington Wildlife Forest in Newcomb; Manager Larry Watkins for his support during our work at the Reinemann Sanctuary; and Hugh Dunne, Regional Director, and Dan Colvin for help at Allegany State Park. Undergraduate assistants and volunteers too numerous to mention helped over the years. Timothy Schwender helped to compile the data. We thank Drs. R.M. Silverstein and Francis X. Webster for advice in chemical matters and critical reading of the manuscript.

REFERENCES

ALBONE, E.S. 1984. Mammalian Semiochemistry: The Investigation of Chemical Signals Between Mammals. John Wiley & Sons, New York.

BUECH, R.R. 1989. Markov analysis of the behavior of beavers (*Castor canadensis*) living in lake habitats of a near-boreal forest region. Abstract, 3rd International Beaver Symposium, Rome, Italy, August 23, 1989.

CONOVER, W.J. 1980. Practical Nonparametric Statistics. John Wiley & Sons, New York.

EPPLE, G., BELCHER, A.M., and SMITH, A.B., III. 1986. Chemical signals in callitrichid monkeys—a comparative review, pp. 653–672 *in* D. Duvall, D. Müller-Schwarze, and R.M. Silverstein (eds.). Chemical Signals in Vertebrates, 4. Plenum Press, New York.

HOULIHAN, P.W. 1989. Scent mounding by beaver (*Castor canadensis*): Functional and semiochemical aspects. M.S. thesis. State University of New York College of Environmental Science and Forestry, Syracuse, New York. December 1989.

KATSIR, Z., and CREW, R.M. 1980. Chemical communication in *Galago crassicaudatus* : Investigation of the chest gland secretion. *South Afr. J. Zool.* 15:249–254.

LEDERER, E. 1946. Chemistry and biochemistry of the scent glands of the beaver, *Castor fiber*. *Nature* 157:231–232.

LEDERER, E. 1950. Odeurs et parfums des animaux. *Fortschr. Chem. Org. Naturst.* 6:87.

MAURER, B., and OHLOFF, G. 1976. Zur Kenntnis der stickstoffhaltigen Inhaltsstoffe von Castoreum. *Helv. Chim. Acta* 59:1169–1185.

MELROSE, D.R., REED, H.C.B., and PATTERSON, R.L.S. 1971. Androgen steroids associated with boar odour as an aid to the detection of oestrus in pig artificial insemination. *Br. Vet. J.* 127:497–501.

MÜLLER-SCHWARZE, D., MOREHOUSE, L., CORRADI, R., ZHAO, C.-H., and SILVERSTEIN, R.M. 1986. Odor images: Responses of beaver to castoreum fractions, pp. 561–570, *in* D. Duvall, D. Müller-Schwarze, and R.M. Silverstein (eds.). Chemical Signals in Vertebrates, Vol. 4. Plenum, New York.

NOVOTNY, M., HARVEY, S., JEMIOLO, B., and ALBERTS, J. 1985. Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl. Acad. Sci. U.S.A.* 82:2059–2061.

NOVOTNY, M., JEMIOLO, B., HARVEY, S., WIESLER, D., and MARCHLEWSKA-KOJ, A. 1986. Adrenal-mediated endogenous metabolites inhibit puberty in female mice. *Science* 231:722–725.

SINGER, A.G., AGOSTA, W.C., O'CONNELL, R.J., PFAFFMAN, C., BOWEN, D.V., and FIELD, F.H. 1976. Dimethyl disulfide: An attractant pheromone in hamster vaginal secretions. *Science* 191:950.

SINGER, A.G., AGOSTA, W.C., CLANCY, A.N., and MACRIDES, F. 1987. The chemistry of vomeronasally detected pheromones: Characterization of an aphrodisiac protein. *Ann. N.Y. Acad. Sci.* 519:287–298.

SVENDSEN, G.E., and HUNTSMAN, W.D. 1988. A field bioassay of beaver castoreum and some of its components. *Am. Mid. Nat.* 120:144–149.

VALENTA, Z. and KHALEQUE, A. 1959. The structure of castoramine. *Tetrahedron Lett.* 12:1–5.

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

BARK CONSUMPTION BY VOLES IN RELATION TO MINERAL CONTENTS

LENNART HANSSON

Department of Wildlife Ecology
Swedish University of Agricultural Sciences
S-750 07 Uppsala, Sweden

(Received July 31, 1990; accepted November 29, 1990)

Abstract—Recent field experiments with impregnated wooden sticks have demonstrated a pronounced use by small rodents of mineral supplies, especially sodium, and such findings seemed related to vole damage to forestry seedlings. Consumption of the bark of experimentally introduced aspen twigs and of sodium-impregnated sticks by voles (mainly or only *Microtus agrestis*) correlated significantly on clear-cuts but not on unmanipulated abandoned fields. Such a correlation appeared when abandoned fields were cut continuously in summer. At vole peak densities, bark of pine seedlings experimentally fertilized with sodium was consumed but not bark of seedlings fertilized with calcium or control seedlings. Field pine seedlings attacked by voles had significantly higher levels of calcium, sodium, and phosphorus than the nearest untouched seedling. However, sodium and phosphorus contents correlated strongly. Sodium and calcium supply to voles in laboratory feeding trials did not diminish the moderate interest in pine bark. Such conditions are, however, assumed to mimic a situation of bark sampling in low-density populations. Sodium, and possibly also calcium, requirements are concluded to be partial determinants of the destructive bark consumption by voles at the peaks of their multiannual population cycles.

Key Words—Sodium, calcium, phosphorus, vole, *Microtus agrestis*, bark consumption, field experiments.

INTRODUCTION

Voles, such as *Clethrionomys glareolus* and *Microtus agrestis*, have a diversified diet in summer (Stenseth et al., 1977, Hansson, 1985). The winter diet consists of just a few plant species but is often supplemented with bark from tree seedlings (e.g., Hansson, 1988). North Scandinavian voles fluctuate

0098-0331/91/0400-0735$06.50/0

strongly in numbers, with density peaks every three or four years (Hansson and Henttonen, 1985). Bark is consumed especially by the cyclic peak populations, e.g., on clear-cut and regenerated forest land (Hansson, 1989). Considerable damage can occur in Scandinavian forests during peak years, especially by *M. agrestis* (Myllymäki, 1977).

Voles on Swedish clear-cuts eagerly seek sodium (and sometimes also calcium), especially in strongly cyclic populations and in peak winters (Hansson, 1990). Thus, bark and sodium and/or calcium interest may be related. Bark is especially high in sodium (Lichens and Borman, 1970), and voles may seek both energy and minerals in their bark attacks during winter food shortages. Other minerals, such as phosphorus, also may be involved as they have been shown to be in generally short supply in certain models of nutrient acquisition (Barkley et al., 1980). However, field experiments failed to demonstrate any important role for phosphorus (Hansson, 1990). Laboratory experiments demonstrated that extra sodium and calcium, but not phosphorus, improved microtine reproduction (Batzli, 1986).

The hypothesis that bark consumption is due partly to a deficit in sodium or other minerals was tested by: (1) supplying both bark and sodium in various types of vole habitat—correlations in utilization would indicate some common factor behind these types of consumption; (2) fertilization of tree seedlings with preferred minerals—bark attacks on seedlings treated with a specific mineral would indicate that this is preferred or in deficit; (3) analyses of mineral contents of field seedlings debarked and untouched by voles—differences in mineral contents would indicate deficiencies; and (4) laboratory feeding of bark together with access to mineral solutions—diminished bark consumption with the supply of a certain mineral would indicate that bark consumption was stimulated by specific mineral requirements.

METHODS AND MATERIALS

Bark vs. Na Consumption. Twigs, 1 m long, taken from the top of one aspen (*Populus tremula*) clone growing at Uppsala and hence genetically identical, were put out on clear-cuts and abandoned fields in south-central and northern Sweden in 1986 and 1987. As sampling units, two twigs (ca. 1.5 cm diam.) were located 1 m from a 10 × 3 × 1-cm spruce stick impregnated with 1 M NaCl for 24 hr (cf. Weeks and Kirkpatrick, 1978; Hansson, 1990). The stick was placed under a wooden shelter. Twigs and sticks were distributed in autumn in randomly selected patches of dense shrubby vegetation on clear-cuts, interspersed with nival space in winter. The placement on more generally sheltered abandoned fields was completely random. The Na sticks could have attracted

the voles to the aspen sticks, and therefore a pilot experiment was performed with aspen sticks in direct contact with the Na stick, at 1 m distance and at 100 m distance on one large abandoned field at Uppsala. The mass of the sticks and the areas of bark removed were measured after snowmelt in spring. Studies on clear-cuts were performed both in south-central and northern Sweden, with a higher level of vole population cycling in the northern area. Abandoned fields in south and south-central Sweden had, respectively, stable and weakly cyclic populations (Hansson and Henttonen, 1985).

To see if food quality affected a possible relationship between Na and bark consumption, both items were distributed together on an abandoned field, half of which had been repeatedly cut during two summers (1986–1987) to produce continuously growing and thus, probably, more nutritious food. Cut vegetation, dry and unpalatable, was left as shelter. Vole densities did not differ substantially between the two types of vegetation (Hansson, 1990).

Another experiment was run on 10 plots fertilized with a total of 1 M NaCl/4 m^2 (Inouye et al., 1987) in May and June, 1988. The plots were supplied with both Na sticks and aspen bark in the autumn of 1988. Fifteen untreated plots acted as controls.

Fertilization. Pine (*Pinus sylvestris*) seedlings, 5–8 years old, were randomly selected in groups of three in spring 1987 for fertilization on reforested clear-cuts in south-central and northern Sweden. In May 1987, 20 seedlings in each region were provided with, respectively, 1 M NaCl or 1 M $CaCl_2$ as salt around each stem base or were left untreated. The treatments were repeated in May 1988 but seedlings already attacked by voles were replaced with new undamaged ones. The seedlings were regularly inspected for any animal damage each summer and autumn and again in the following spring. The year 1987 was a vole peak and 1988 a vole decline year on these clear-cuts.

Bark Mineral Concentrations. Pine seedlings attacked by voles (probably only *M. agrestis*) on clear-cuts in south-central Sweden during the peak vole winter 1987–1988 were examined regarding contents of Ca, Na, and P in comparison with nearby undamaged seedlings. Bark was sampled directly after snow melt in April 1988, while the ground was still frozen, on partly consumed seedlings and at the same stem height on the closest totally undamaged seedling within 2 m and thus within reach of the foraging voles. The mineral contents were determined at the Swedish Laboratory for Agricultural Chemistry, Uppsala.

Laboratory Feeding. Immature *M. agrestis* were caught in autumn and kept over winter in laboratory mouse cages on laboratory mouse pellets and water. In winter, one third of them were also given access to a 0.5% NaCl solution and one third to a 1% $CaCl_2$ solution (cf. Batzli, 1986). Every second week, starting with week 2, each animal was given two 15-cm pine stem parts,

ca. 1.5 cm diam., taken from clear-cut seedlings. Bark consumption was examined daily but comparisons between treatments were made for total weekly consumption.

RESULTS

Bark vs. Na Consumption. Consumption of aspen bark did not depend on the distance to Na sticks (Table 1), so Na and bark were consumed independently. The country-wide experiments in the vole increase and peak winters of 1986–1987 and 1987–1988 (Table 2) showed that Na and bark consumption were significantly correlated on clear-cuts but not on abandoned fields. Even on clear-cuts only a small proportion of the total variation was explained by the other variable.

Na and bark consumption on experimentally managed areas disclosed a

TABLE 1. WINTER BARK CONSUMPTION ON GENETICALLY IDENTICAL ASPEN TWIGS IN ABANDONED FIELD IN AUTUMN 1986 > 100 m FROM Na STICKS (SEPARATE), 1 m FROM Na STICKS (CLOSE) AND IN CONTACT WITH Na STICKS (ADJACENT)

		Consumption (cm^2)			
Location	*N*	Mean	SD	*F*	*P*
Separate	12	25.8	13.5		
Close	19	24.1	13.3	0.38	NS
Adjacent	18	28.6	19.0		

TABLE 2. CORRELATIONS BETWEEN WINTER CONSUMPTION OF Na-IMPREGNATED WOOD (S) AND BARK (B) IN TYPICAL *Microtus agrestis* HABITATS THROUGHOUT SWEDEN AND IN EXPERIMENTAL FIELD AT UPPSALA

Habitat	*N*	S (mean g)	B (mean cm^2)	*r*	*P*
Country-wide survey					
Clear-cuts	78	7.6	14.1	0.43	<0.001
Abandoned fields	33	4.1	22.1	0.21	NS
Experimental field					
Continuously cut vegetation	18	3.7	9.7	0.81	<0.001
Control	18	1.8	22.9	0.18	NS

highly significant correlation on repeatedly cut parts of the abandoned field but no correlation at all in areas of undisturbed vegetation (Table 2). Relatively more Na was consumed at the repeatedly cut areas. The experiment with Na-fertilized plots gave inconclusive results, as Na sticks (both on treatment and control plots) were attacked, but not the aspen bark, during the vole decline winter of 1988–1989.

Fertilization. Among the fertilized pine seedlings, only Na-treated seedlings were attacked by voles; the difference between treatments was highly significant in the vole peak year of 1987–1988 (Table 3, $G = 29.64$, $P < 0.001$). No attack was observed in a low year for vole populations, 1988–1989. However, in both summers voles and other larger animals, especially hares (*Lepus timidus*), had investigated the ground around seedlings fertilized with both Na and Ca. The soil was usually torn up and covered with vole (and hare) droppings. These effects were much more evident in the northern than in the southern study region. Likewise, significantly more Na seedlings were gnawed by voles in the northern than in the southern region ($G = 11.38$, $P < 0.001$). The voles gnawed both root and stem bark close to the root as well as pure wood both in roots and the stem. Most seedling attacks occurred in the summer of 1987. Thus, the vole attacks differed to some extent from normal bark gnawing by voles, which usually is restricted to stem bark in winter.

Bark Mineral Concentrations. Bark of pine seedlings gnawed by voles (probably only *M. agrestis*) in the peak winter of 1987–1988 (Table 4) contained significantly higher concentrations of Na (Wilcoxon matched-pairs signed-ranks test: $T = 1$, $P < 0.001$), Ca ($T = 13$, $P < 0.05$), and even P (T

TABLE 3. NUMBER OF VOLE DAMAGED STEMS (DAM.) OF *Pinus sylvestris* SEEDLINGS ON CLEAR-CUTS FERTILIZED WITH NaCl AND $CaCl_2$ AND ON UNFERTILIZED (CONTROL) SEEDLINGS IN TWO SWEDISH REGIONS[a]

Locality and year	Treatment					
	NaCl		$CaCl_2$		Control	
	N	Dam.	*N*	Dam.	*N*	Dam.
South-central Sweden						
1987–1988	20	1	20	0	20	0
1988–1989	20	0	20	0	20	0
North Sweden						
1987–1988	20	10	20	0	20	0
1988–1989	20	0	20	0	20	0

[a] Seedlings (*N*) were randomly selected for each treatment in each region, so altogether 120 seedlings were examined each year.

= 0, $P < 0.001$). The variability (SD) in Na concentrations was especially high in vole-attacked seedlings (Table 5); the voles had obviously sought the very highest Na concentrations. The mineral concentrations were highly correlated: Na–Ca with $r = 0.77$, $P < 0.001$ and Na–P with $r = 0.95$, $P < 0.001$). While Na alone explained 91% of the variation in P, a multiple correlation analysis showed that the inclusion of Ca increased this proportion to 93%.

Laboratory Feeding. The bark consumption in the laboratory varied strongly between vole individuals, as evident from the standard deviation in Table 5, and most voles did not touch the pine bark at all. The consumption increased gradually during each week and from week 2 to week 6 with access to Na. As the bark consumption was not normally distributed, comparisons between treatments were made with the Kruskal-Wallis test. No significant differences occurred in either week 2 ($H = 2.46$), week 4 ($H = 1.77$), or week 6 ($H = 1.51$) but there was a tendency for bark consumption to be higher when there was access to Ca and Na. Contingency tests with animals divided into

TABLE 4. MINERAL CONTENT (% OF BARK DRY WEIGHT) IN VOLE-GNAWED AND NEIGHBORING UNATTACKED (CONTROL) *Pinus sylvestris* SEEDLINGS ON SOUTH-CENTRAL SWEDISH CLEAR-CUTS[a]

	Vole-gnawed		Control	
Mineral	Mean	SD	Mean	SD
Ca	0.36	0.09	0.28	0.06
Na	0.027	0.031	0.005	0.002
P	0.15	0.08	0.08	0.02

[a]Twelve pairs of seedlings were randomly selected.

TABLE 5. LABORATORY CONSUMPTION (cm^2) OF *Pinus sylvestris* BARK WHEN SUPPLIED WITH LABORATORY MOUSE PELLETS AND WATER AND IN TWO TREATMENTS WITH ACCESS TO 0.5% NaCl AND 1% $CaCl_2 \cdot 2H_2O$ SOLUTIONS

	Ca			Na			Control		
Week	*N*	Mean	SD	*N*	Mean	SD	*N*	Mean	SD
2	16	0.4	0.7	14	0.4	1.1	15	0.1	0.5
4	16	2.3	4.6	14	2.4	6.7	15	0.4	1.0
6	16	2.0	4.1	14	3.7	7.8	15	0.2	0.6

attacking and nonattacking individuals did not reveal any significant differences, either. Individual voles showed consistent behavior towards bark. The correlation between consumption in weeks 2 and 4 was 0.63 ($P < 0.001$) and between weeks 2 and 6 was 0.60 ($P < 0.001$).

DISCUSSION

The main aspen experiments were performed both in southern and northern Sweden and at very contrasting densities to test for generality in the results. The differences between clear-cuts and abandoned fields may depend on a temporal difference in the craving for Na, being maximum on clear-cuts in winter and on abandoned fields in summer (Hansson, 1990). Aspen bark was only eaten in winter, when voles on abandoned fields may rely on Na-rich storage organs of grasses and forbs (Hansson, 1971, 1979, 1990) and therefore may show less interest in supplemented Na. The relatively greater use of sodium on fields with continuously growing vegetation may depend on changes in food habits, probably with higher consumption of green leaves, and possibly a higher need for Na in detoxification of plant defense substances (Pehrson, 1983). Vole diet on unmanipulated abandoned fields may include relatively more Na-rich storage organs. Na sticks were obviously preferred to bark, probably due to the much lower Na content in the bark.

The intense bark consumption in summer on Na-fertilized seedlings in northern Sweden during a population peak may relate to the very high interest in Na sticks during the peak year (Hansson, 1990). The excessive Na levels in both bark and wood of the fertilized seedlings may have induced a foraging behavior normally released only under winter food conditions at lower bark Na concentrations. However, bark is probably not attacked only as a result of its mineral content. The large amounts consumed, evident also from stomach analyses (Hansson and Larsson, 1978), imply that it is an important energy source in winter.

Sampling of bark for chemical analyses was performed while the ground was still frozen, so the mineral concentrations found should reflect true late autumn–winter conditions when the seedlings are attacked by voles. The very strong correlation between Na and P is remarkable and implies that one of the minerals should not be considered a primary target for the voles. This correlation thus will explain the significant relation between bark consumption and P content as voles otherwise seem generally to reject P (Inouye et al., 1986; Hansson, 1990). It is still unclear how important Ca is in this food selection but animals during peak populations also readily gnawed Ca-impregnated sticks (Hansson, 1990). Furthermore, bark consumption on reforested clear-cuts was more extensive in areas with high, compared to low, Ca soil levels (Larsson,

1973). Other analyses also have demonstrated higher Ca levels in attacked than rejected pine (*Pinus contorta*) seedlings (Hansson, unpublished observations). The importance of Ca in vole bark attacks needs further examination.

Laboratory experiments did not show less bark consumption when there was access to Na and Ca but rather the reverse. However, the animals examined were well-fed on a diet rich in minerals and probably behaved as animals from only slightly cyclic populations, which do not show any pronounced or density-dependent bark consumption (Hansson, 1989). Such animals seemed only to sample bark, without using it either as a main energy or nutrient source. They then may identify and remember trees with high nutrient content. Bark consumption becomes important in the diet of voles only at peak densities in highly cyclic populations (Hansson, 1988, 1989).

Certain *M. agrestis* individuals showed a much more pronounced bark consumption under laboratory conditions than others. These differences may well be related to interindividual variation of this species in the digestion of coarse food (Hansson and Jaarola, 1989). Bark-consuming individuals may be at an advantage in situations of nutrient stress.

In conclusion, Na seems to be one important factor in bark consumption of both conifer seedlings and deciduous trees and shrubs. However, substantial use may occur only under nutrient or energy stress and may be related to population density and level of resource utilization. Vole densities usually decline dramatically after widespread attacks on *P. sylvestris* bark (e.g., Hansson, 1986). The relative importance of minerals and energy in bark consumption of various tree species needs further study, as does the importance of mineral nutrients in the population dynamics of voles.

Acknowledgments—I am grateful to G.O. Batzli and Th.P. Sullivan for comments. The work was supported by the Swedish Council for Forestry and Agricultural Research.

REFERENCES

BARKLEY, S., BATZLI, G.O., and COLLIER, B. 1980. Nutritional ecology of microtine rodents: a simulation model of mineral nutrition for brown lemming. *Oikos* 34:103–114.

BATZLI, G.O. 1986. Nutritional ecology of the California vole: Effects of food quality on reproduction. *Ecology* 67:406–412.

HANSSON, L. 1971. Habitat, food and population dynamics of the field vole *Microtus agrestis* (L.) in south Sweden. *Viltrevy (Swedish Wildlife)* 8:267–378.

HANSSON, L. 1979. Food as a limiting factor for small rodent numbers: Tests of two hypotheses. *Oecologia (Berlin)* 37:297–314.

HANSSON, L. 1985. The food of bank voles, wood mice and yellow-necked mice. *Symp. Zool. Soc., London* 55:141–168.

HANSSON, L. 1988. Grazing impact by small rodents in a steep cyclicity gradient. *Oikos* 51: 31–42.

HANSSON, L. 1989. Landscape and habitat dependence in cyclic and semi-cyclic small rodents. *Holarct. Ecol.* 12:345–350.

HANSSON, L. 1990. Mineral selection in microtine populations. *Oikos* 59:213–224.

HANSSON, L., and HENTTONEN, H. 1985. Gradients in density variations of small rodents: the importance of latitude and snow cover. *Oecologia (Berlin)* 67:394–402.

HANSSON, L., and JAAROLA, M. 1989. Body size related to cyclicity in microtines: Dominance behaviour or digestive efficiency? *Oikos* 55:356–364.

HANSSON, L., and LARSSON, T.-B. 1978. Vole diet on experimentally managed reforestation areas in northern Sweden. *Holarct. Ecol.* 1:16–26.

INOUYE, R.S., HUNTLEY, N.J., and TILMAN, D. 1987. Response of *Microtus pennsylvanicus* to vegetation fertilized with various nutrients, with particular emphasis on sodium and nitrogen concentrations in plant tissues. *Holarct. Ecol.* 10:110–113.

LARSSON, T.-B. 1973. Smågnagarskador på skogskulturer i Sverige 1900–1970. *Rapp. Uppsatser, Inst. Skogszool.* 14. 26 pp.

LICHENS, G.E., and BORMAN, E.H. 1970. Chemical analyses of plant tissues from the Hubbard Brook ecosystem in New Hampshire. Yale University School of Forestry, Bulletin No. 79. 25 pp.

MYLLYMÄKI, A. 1977. Outbreaks and damage by the field vole, *Microtus agrestis* (L.), since World War II in Europe. *EPPO Bull.* 7:177–208.

PEHRSON, Å. 1983. Digestibility and retention of food components in caged mountain hares *Lepus timidus* during the winter. *Holarct. Ecol.* 6:395–403.

STENSETH, N.C., HANSSON, L., and MYLLYMÄKI, A. 1977. Food selection of the field vole *Microtus agrestis*. *Oikos* 29:511–524.

WEEKS, H.P., and KIRKPATRICK, C.M. 1978. Salt preferences and sodium drive phenology in fox squirrels and woodchucks. *J. Mammal.* 59:531–542.

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

EXTRACTABLE HYDROCARBONS AND KIN RECOGNITION IN HONEYBEE (*Apis mellifera* L.)

ROBERT E. PAGE, Jr.,[1,*] ROBERT A. METCALF,[2] ROBERT L. METCALF,[3] ERIC H. ERICKSON, Jr.,[4] and RICHARD L. LAMPMAN[3]

[1]*Department of Entomology*
University of California
Davis, California 95616

[2]*LHC-NCI, National Institutes of Health*
Building 37, Room 2C20
Bethesda, Maryland 20817

[3]*Department of Entomology*
University of Illinois
320 Morrill Hall, 505 Goodwin Avenue
Urbana, Illinois 61801

[4]*USDA-ARS Carl Hayden Bee Research Unit*
2000 E. Allen Road
Tucson, Arizona 85719

(Received July 23, 1990; accepted December 3, 1990)

Abstract—Hydrocarbons of worker honeybees of known pedigree were extracted and analyzed using gas chromatography and mass spectrometry. Variability in hydrocarbon extracts of individual workers is determined at least in part genetically. Correlations in hydrocarbon composition of extracts were highest among more closely related individuals. Individuals maintained in groups exchange hydrocarbons but still maintain enough self-produced compounds to retain genetically determined individual characteristics. These results demonstrate that extractable hydrocarbons of bees provide sufficiently reliable genetic information to function as labels for use during kin recognition.

Key Words—Honeybee, *Apis mellifera*, Hymenoptera, Apidae, hydrocarbons, kin recognition, genetic relatedness.

*To whom correspondence should be addressed.

0098-0331/91/0400-0745$06.50/0

INTRODUCTION

The question "when should animals cooperate?" has troubled biologists since Charles Darwin (1859). W.D. Hamilton formulated the rules by which adaptive cooperation or competition should be determined when he developed his theory of the evolution of altruism by kin selection (Hamilton, 1964a,b). His theory states that cooperation and competition should be decided on the basis of the genetic relationships of individuals, with those more closely related being more likely to cooperate. Although his theory does not require that individuals recognize kin, demonstration of functional kin recognition coupled with discriminating behavior on the basis of genetic relatedness provides strong evidence for the importance of kin selection in shaping social interactions of individuals.

Kin recognition systems have been demonstrated throughout the animal group that shows the most extreme forms of cooperative behavior, the Hymenoptera. Recent reviews of recognition in ants (Breed and Bennett, 1987), social bees (Page and Breed, 1987; Getz, 1991), and social wasps (Gamboa et al., 1986) attest to its widespread occurrence. The honeybee society is particularly interesting to study because it consists of a single queen mother who mates with about 17 males (see Page, 1986). This results in the colony consisting of a large number of subfamilies where individuals having the same drone father belong to the same subfamily and are super sisters while those with different drone fathers are half sisters (Page and Laidlaw, 1988). Recent research on the honeybee, *Apis mellifera* L., has demonstrated recognition abilities operating at several levels including: nestmates versus nonnestmates, super sisters versus half sisters for immature and adult workers and queens (see Getz, 1991, for a recent review of kin recognition in honeybees).

Several studies have demonstrated that the labels used by workers in recognition, under specific laboratory conditions, are endogenous in origin and genetically variable (Breed, 1981, 1983; Getz and Smith, 1983, 1986; Breed et al., 1985, 1988a). One common feature of these experiments is the confinement of teneral adults in groups of 10 for approximately five days prior to conducting behavioral assays. Breed et al. (1988a) demonstrated that cues used in recognition in laboratory studies are acquired within about the first 12 hr after emerging as adults. Unrelated individuals that are younger than about 12 hr old are indistinguishable from close relatives and are accepted or rejected without regard for genetic relatedness. Those 12 hr or older are accepted into the social group of 10 workers on the basis of genetic relatedness (cf. Getz et al., 1989).

The identities of recognition cues are unknown; however, it is likely that chemical signals used to discriminate among nestmates of different genetic relationships would function as contact pheromones within the crowded confines of the hive. Recent work on ants implicates the role of cuticular hydrocarbons in nestmate recognition. Recognition labels of individuals of *Camponotus flori-*

danus (Morel and Van der Meer, 1987), *Camponotus vagus* (Clement et al., 1987), and *Pseudomyrmex spp.* (Mintzer, 1988) are altered by rinsing their cuticle with a nonpolar solvent that removes their hydrocarbons or by applying the extracted hydrocarbons of another individual to their cuticular surface. Errard and Jallon (1987) demonstrated the relationship between the ontogeny of recognition labels, demonstrated by rejection of an unfamiliar, heterospecific individual, and the acquisition of hydrocarbons. Vander Meer et al. (1989) demonstrated that sufficient variability exists in cuticular hydrocarbon patterns between colonies to serve as nestmate recognition labels in *Solenopsis invicta.*

The olfactory system of honeybees is very sensitive to hydrocarbon compounds. Getz and Smith (1987) and Getz et al. (1988, 1989) demonstrated that worker honeybees can be conditioned to discriminate among different mixes of 10–25 carbon compounds. They also demonstrated that adult honeybee workers can discriminate between glass rods that have been rubbed on different adult workers (glass rods are assumed to be contaminated with cuticular hydrocarbons of these individuals). Breed and coworkers (unpublished data) demonstrated that contamination of the cuticle of bees with some hydrocarbon compounds, but not others, profoundly affected recognition in small social groups.

Before hydrocarbons can function as kin recognition labels for use in intracolonial interactions of individuals, they must show reliable variability that correlates with genetic relatedness (i.e., be heritable) and have the ability to be preserved on individuals without excessive blending and sharing among nestmates. In this paper, we present evidence that the hexane-extractable hydrocarbons of honeybees have these necessary properties.

METHODS AND MATERIALS

Source of Bees. Worker honeybees used in this experiment were the progeny of two different queens, designated A and B. A series of crosses was performed, using instrumental insemination (Laidlaw, 1977), that resulted in an array of genetic relationships among the progeny (Figure 1, Table 1). Both queens were homozygous for the single-gene recessive mutant, cordovan (see Tucker, 1986), and each was inseminated with the semen of two different drones: one drone carried the cordovan gene and one carried the wild type. The paternity of progeny of each queen could be distinguished by integument color. Workers belonging to the subfamily of the cordovan drone had a brown-striped integument while those of the wild-type drone had black stripes.

Handling of Bees. Combs containing pupae and emerging workers were removed from the colony of each queen and taken into the laboratory. Combs were maintained in an incubator at 33 °C. Newly emerged, teneral workers were collected from the combs and placed into 0.14-liter, paraffin-lined, cardboard

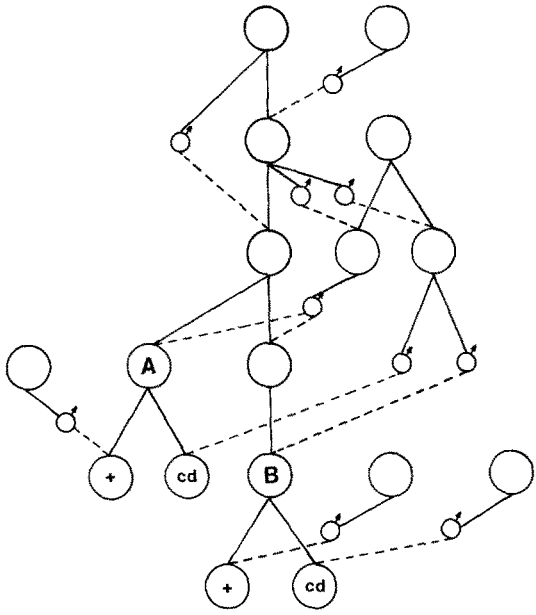


FIG. 1. Pedigree for worker progeny of queens A and B that belong to the wild-type (+) and cordovan (cd) subfamilies. Solid lines represent eggs, dashed lines sperm. The original queen and her drone mate were unrelated and selected from among commercial colonies maintained in the experimental apiary. See also the genetic relationships shown in Table 1.

TABLE 1. GENETIC RELATIONSHIPS (G; PAMILO AND CROZIER, 1982) AMONG AND BETWEEN MEMBERS OF CORDOVAN (cd) AND WILD-TYPE (+) SUBFAMILIES OF COLONIES A AND B (SEE FIGURE 1).

Colony		A		B	
		+	cd	+	cd
A	+	0.80	0.41	0.18	0.18
	cd	0.41	0.91	0.36	0.36
B	+	0.18	0.36	0.80	0.30
	cd	0.18	0.36	0.30	0.80

cups. Each cup received 10 workers. Three cups were set up for the progeny of each of the two colonies. One cup contained 10 workers of the wild-type subfamily (super sisters); one contained 10 workers of the cordovan subfamily (super sisters); and one contained five workers from the wild-type and five from the cordovan subfamilies (half sisters and super sisters). Each cup was provided with a vial of 50% sucrose solution and a vial of water. All sucrose and water came from the same homogeneous source. After five days, all surviving workers were collected and individually frozen in glass vials at −20°C until their hydrocarbons were extracted.

Hydrocarbon Analyses. Hydrocarbons were analyzed for a total of 32 individuals, four cordovan and four wild-type progeny of each queen from each of the homogeneous, super sister groups. An additional four individuals of each of the two subfamilies were analyzed from the mixed group of queen A while three cordovan and five wild type were analyzed from the mixed group of B.

For analyses, hydrocarbons were extracted from each bee for 10 min in 2 ml of hexane. Hexane extracts then were analyzed with a Hewlett Packard 5995C gas chromatograph–mass spectrometer (GC-MS) equipped with a 10-m methylsilicone capillary column. Each *n*-alkane in the series from C_{23} to C_{33} was present in the hexane extract and was individually identified. They are, in ascending order (see Figure 2) peaks 1, 2, 4, 5, 7, 9, 11, 13, 15, 17, and 19. An additional nine peaks were selected as "characters" for statistical analyses of relationships among individual samples, representing individual worker bees, for a total of 20 characters. The additional peaks probably represent unsaturated and methylated derivative compounds of the *n*-alkane series (see Blomquist et al., 1980b; Carlson and Bolton, 1984; Francis et al., 1985, 1989; Carlson, 1988; Smith, 1988).

Statistical Analyses. The total area under each peak (a quantitative measure) was considered to be an independent character for each individual. The similarity among pairs of individuals was determined for these 20 characters using a product-mean correlation coefficient (Sneath and Sokal, 1973, pp. 137–140, 190–192). The correlation coefficient yielded a measure of similarity that was independent of total quantities of each compound, it was dependent only on relative quantities within each individual sample. A correlation matrix of all pairwise comparisons of individuals was generated.

The mean correlation coefficient for all independent comparisons was calculated for each individual from the homogeneous (all super sister) group to individuals belonging to the following categories: super sisters from homogenous groups, half sisters from homogenous groups, super sisters from mixed groups, and half sisters from mixed groups (Table 2). In addition, mean correlations were calculated for each individual from a homogeneous group to all

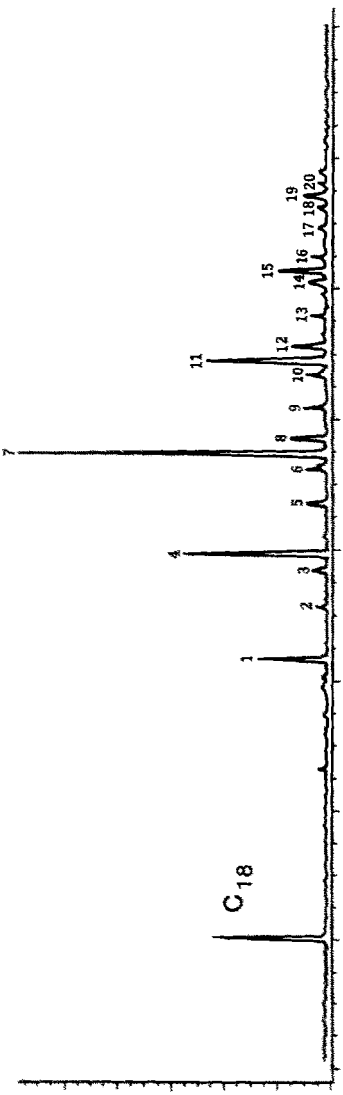


FIG. 2. Gas chromatograph of hexane-extractable compounds from a single worker. Numbered peaks were used in correlation analyses discussed in text.

TABLE 2. SAMPLE SIZES (N), MEAN CORRELATIONS (X), AND STANDARD DEVIATIONS (SD) FOR HYDROCARBON COMPOSITION OF INDIVIDUAL WORKERS[a]

Queen	Sub-family	Worker No.		Homogeneous SS	Homogeneous HS	Heterogeneous SS	Heterogeneous HS	Homogeneous nonsiblings cd	Homogeneous nonsiblings +
			N	3	4	4	4	8	8
A	cd	1	X	0.910	0.887	0.951	0.933	0.873	0.605
			SD	0.0612	0.0187	0.0152	0.0058	0.0826	0.1463
A	cd	2	X	0.925	0.841	0.953	0.920	0.874	0.622
			SD	0.0467	0.0248	0.0192	0.0158	0.0834	0.1639
A	cd	3	X	0.827	0.807	0.883	0.867	0.807	0.558
			SD	0.0694	0.0457	0.0180	0.0090	0.0775	0.1618
A	cd	4	X	0.858	0.693	0.841	0.802	0.771	0.593
			SD	0.0948	0.0164	0.0204	0.0340	0.0741	0.1268
A	+	5	X	0.952	0.808	0.942	0.939	0.933	0.735
			SD	0.0273	0.0853	0.0330	0.0126	0.0328	0.1101
A	+	6	X	0.938	0.799	0.901	0.886	0.842	0.552
			SD	0.0140	0.0872	0.0288	0.0090	0.0846	0.1153
A	+	7	X	0.951	0.791	0.902	0.892	0.874	0.622
			SD	0.0104	0.0818	0.0340	0.0054	0.0526	0.1133
A	+	8	X	0.962	0.831	0.950	0.940	0.906	0.624
			SD	0.0136	0.0889	0.0236	0.0108	0.0689	0.1417
			N	3	4	3	5	8	8
B	cd	9	X	0.931	0.757	0.920	0.858	0.880	0.899
			SD	0.0503	0.1486	0.1131	0.1021	0.0569	0.0492
B	cd	10	X	0.885	0.656	0.820	0.755	0.882	0.932
			SD	0.0650	0.1334	0.1242	0.0910	0.0650	0.0100
B	cd	11	X	0.872	0.939	0.943	0.965	0.720	0.806
			SD	0.0570	0.0387	0.0373	0.0260	0.0401	0.0655
B	cd	12	X	0.942	0.800	0.929	0.888	0.845	0.918
			SD	0.0364	0.1183	0.0895	0.0730	0.0611	0.0467
B	+	13	X	0.950	0.761	0.931	0.856	0.550	0.595
			SD	0.0616	0.1372	0.0735	0.1165	0.0288	0.0780
B	+	14	X	0.954	0.821	0.958	0.902	0.616	0.674
			SD	0.0340	0.1231	0.0524	0.0860	0.0274	0.0790
B	+	15	X	0.904	0.657	0.868	0.764	0.427	0.490
			SD	0.1109	0.1650	0.1109	0.1611	0.0337	0.0787
B	+	16	X	0.859	0.912	0.943	0.952	0.786	0.772
			SD	0.0741	0.0609	0.0539	0.0272	0.0336	0.0712

[a] Each individual worker shown (1–16) was maintained in a homogeneous subfamily group, either cordovan (cd) or wild type (+), from either queen A or B, and was compared with groups of super sisters (SS) and half sisters (HS) maintained in homogeneous and mixed-subfamily (heterogenous) groups, and with non-sibling progeny that were maintained in homogeneous groups.

nonsiblings (progeny of the other queen) of each subfamily, separately. Comparisons of the mean similarities of individuals to super sisters, half sisters in homogeneous and mixed groups, and nonsiblings in homogenous groups then were made using Wilcoxon's signed-ranks and two-sample tests (Sokal and Rohlf, 1981, pp. 449–450).

RESULTS

Individuals from homogeneous groups of both colonies were significantly more similar to their own nestmates in eight of the 12 comparisons shown (Table 3, intercolony comparisons). All four of the nonsignificant comparisons were between half sisters in homogenous groups and members of the unrelated colony. The bias was still toward a closer similarity among nestmates for three of the four nonsignificant comparisons.

The closer similarities shown between nestmates compared to nonnestmates could be either genetic or environmental in origin. Workers could obtain hydrocarbons directly from wax comb prior to emerging. However, comparisons among siblings of different subfamilies (representing different genotypic distributions) that emerged from the same combs demonstrate that the composition of hydrocarbons on individuals is genotypically determined (Table 3, intracolony comparisons). Individuals were more similar to groups of their super sisters, in both homogeneous and mixed groups, than they were to their half sisters. Similarity to their own super sisters in homogeneous groups could be explained on the basis that they shared a common "cup" environment resulting in the transfer and sharing of nongenetically variable labels through social interactions or contact with a common food source or paraffin substrate. However, comparisons of individuals from homogeneous groups with half and super sisters from "mixed" groups only can be explained on the basis of genetically acquired hydrocarbons because they did not share the same cup environment.

Hydrocarbons are transferred and shared among social groups of individuals. Workers from homogeneous groups were more similar to their half sisters maintained in the mixed groups than to their half sisters in homogeneous groups. Half sisters in heterogeneous groups were more similar to each other than were half sisters between homogeneous groups in all comparisons of Table 2. Hydrocarbons were transferred among individuals sharing a cup by either direct contact or perhaps indirectly by adsorption to the paraffin lining and then resorption onto the cuticle.

The mutant marker itself had no measurable effect on the degree of similarity among individuals. The gene for cordovan affects the deposition of melanin in the cuticle (Tucker, 1986) and could have other unknown pleiotropic effects on the hydrocarbon composition of individuals. If so, then individuals

TABLE 3. COMPARISONS OF MEAN CORRELATIONS OF CUTICULAR HYDOCARBONS[a]

Group 1[b]	Group 2	One-tailed probability
Intracolony comparisons		
A:SS/Hm	A:HS/Hm	$P < 0.01$[c]
B:SS/Hm	B:HS/Hm	$P < 0.025$
A:SS/Ht	A:HS/Ht	$P < 0.01$
B:SS/Ht	B:HS/Hm	$P < 0.025$
A:HS/Ht	A:HS/Hm	$P < 0.01$
B:HS/Ht	B:HS/Hm	$P < 0.01$
A:SS/Hm	A:SS/Ht	NS
B:SS/Hm	B:SS/Ht	NS
Intercolony comparisons		
A:SS/Hm	B:cd/Hm	$P < 0.01$
A:SS/Hm	B:+/Hm	$P < 0.01$
A:HS/Hm	B:cd/Hm	NS
A:HS/Hm	B:+/Hm	$P < 0.01$
A:HS/Ht	B:cd/Hm	$P < 0.01$
A:HS/Ht	B:+/Hm	$P < 0.01$
B:SS/Hm	A:cd/Hm	$P < 0.01$
B:SS/Hm	A:+/Hm	$P < 0.03$
B:HS/Hm	A:cd/Hm	NS
B:HS/Hm	A:+/Hm	NS
B:HS/Ht	A:cd/Hm	$P < 0.05$
B:HS/Ht	A:+/Hm	NS

[a] The mean correlations are compared for each of the eight super-sister individuals maintained in homogenous groups from each queen source (see Table 2) to the two designated groups. Wilcoxon's signed-ranks tests were conducted for statistical comparisons. SS = super sisters; HS = half sisters; Hm = from phenotypically homogeneous group (all super sisters); Ht = from phenotypically heterogeneous group (mixture of super and half sisters); cd = cordovan subfamily; + = wild-type subfamily; A = progeny of queen A; B = progeny of queen B.

[b] The mean correlation coefficient for group 1 is higher than the mean correlation for group 2 in all 20 comparisons.

[c] Using the designations in this row to refer back to Table 2, the reader will see that we are testing whether the mean correlations, X, for workers 1–8 as listed in the SS/Hm column are significantly greater than the values X in the Hs/Hm column.

that share the cordovan trait should tend to be more similar to each other than are wild-type individuals. However, an analysis of the similarity of individuals in Table 2 to their own homogeneous group of super sisters shows that this is not the case ($P > 0.05$, Wilcoxon's two-sample test). Another expectation of a "cordovan" effect would be for the same phenotypic groups of progeny of different queens to be more similar to each other. Again, an examination of Table 2 demonstrates that this did not occur ($P > 0.05$, Wilcoxon's signed-ranks test).

Differences in hydrocarbons were greater between subfamilies of colony B than colony A. This is demonstrated by larger differences in correlations between wild-type and cordovan nonnestmates for A workers compared with B workers (Table 2).

DISCUSSION

The extractable hydrocarbons of honeybee workers provide a sufficiently variable and heritable system of potential chemical signals to serve as labels in kin recognition. Honeybees contain an abundance of high-molecular-weight compounds of relatively low volatility. In laboratory studies, each individual maintains subfamily identification even though some label exchange does occur. This is essential to subgroup recognition because complete sharing and blending of compounds would eliminate any distinctive cues. Label exchange may be greater within nests of honeybees because workers are more crowded and probably have more intimate contact with each other and with the wax comb substrate.

Breed et al. (1988b) demonstrated that the wax combs of a honeybee colony provide the primary components of nestmate recognition at colony entrances. They propose that the comb may serve as a medium for adsorption and transfer of lipid-soluble compounds, such as cuticular hydrocarbons, produced by workers and queens. Cuticular hydrocarbons may be used in the absence of comb-acquired cues for nestmate recognition, such as occurs in laboratory studies. Breed et al. (1987) proposed that multiple sources of labels may be involved in recognition learning and that different learned sets of recognition cues, "templates," may be used in different behavioral contexts.

Waxes produced by bees may serve as cues for nestmate and intracolonial subgroup recognition. Nest-specific cues could reside in the wax comb while subgroup cues remain distinct on the cuticle of individuals. The wax secreted by bees for comb construction is composed of many of the same compounds found on the cuticle of bees (Blomquist et al., 1980a). Being itself a glandular secretion, it may also vary in composition among individuals as a consequence of varying genotype and introduce a genotypic component to nest recognition labels.

Unequivocal behavioral evidence is lacking for the role of hydrocarbons in nestmate and intracolonial group recognition; however, extractable hydrocarbons do fulfill the necessary requirements to function in that role. For now, at least, we have identified a candidate set of compounds, out of a vast number of possible compounds produced by bees (see Free, 1988, for a review), that we can evaluate on in our search for recognition labels.

Acknowledgments—This work was funded in part by the North-Central States, USDA-ARS, Bee Research Unit, Madison, Wisconsin, and by National Science Foundation grants BNS-8615381 and BNS-8719283 to R.E.P. We thank Dave Severson for his assistance with the genetic stocks used in this study and Wayne Getz for valuable comments.

REFERENCES

BLOMQUIST, G.J., CHU, A.J., and RAMALEY, S. 1980a. Biosynthesis of wax in the honeybee, *Apis mellifera* L. *Insect Biochem.* 10:313–321.

BLOMQUIST, G.J., HOWARD, R.W., MCDANIEL, C.A., and RAMALEY, S. 1980b. Application of methoxymercuration–demecuration followed by mass spectrometry as a convenient microanalytical technique for double-bond location in insect-derived alkenes. *J. Chem. Ecol.* 6:257–269.

BREED, M.D. 1981. Individual recognition and learning of queen odors by worker honeybees. *Proc. Natl. Acad. Sci. U.S.A.* 78:2635–2637.

BREED, M.D. 1983. Nestmate recognition in honey bees. *Anim. Behav.* 31:86–91.

BREED, M.D., and BENNETT, B. 1987. Kin recognition in highly eusocial insects, pp. 243–285, *in* D.J.C. Fletcher and C.D. Michener (eds.). Kin Recognition in Animals, John Wiley & Sons, Chichester.

BREED, M.D., BUTLER, L., and STILLER, T.M. 1985. Learning and genetic influences in the discrimination of colony members by social Hymenoptera. *Proc. Natl. Acad. Sci. U.S.A.* 82:3058–3061.

BREED, M.D., STILLER, T.M., and MOOR, M.J. 1988a. The ontogeny of kin discrimination cues in the honey bee, *Apis mellifera. Behav. Genet.* 18:439–448.

BREED, M.D., WILLIAMS, K.R., and FEWELL, J.H. 1988b. Comb wax mediates the acquisition of nest-mate recognition cues in honey bees. *Proc. Natl. Acad. Sci. U.S.A.* 85:8766–8769.

CARLSON, D.A. 1988. Africanized and European honey-bee drones and comb waxes: Analysis of hydrocarbon components for identification, pp. 264–274, *in* G.R. Needham, R.E. Page, M. Delfinado-Baker, and C.E. Bowman (eds.). Africanized Honey Bees and Bee Mites. Ellis Horwood Ltd., Chichester.

CARLSON, D.A., and BOLTON, A.B. 1984. Identification of Africanized and European honey bees using extracted hydrocarbons. *Bull. Entomol. Soc. Am.* 30:32–35.

CLEMENT, J.-L., BONAVITA-COUGOURDAN, A., and LANGE, C. 1987. Nestmate recognition and cuticular hydrocarbons in *Camponotus vagus* Scop, pp. 473–474, *in* J. Eder and H. Rembold, (eds.). Chemistry and Biology of Social Insects. Verlag J. Peperny, Munich.

DARWIN, C.R. 1859. On the Origin of Species by Means of Natural Selection, or the Preservation of Favored Races in the Struggle for Life. 1962. Collier Books Edition (6th ed.). Macmillan, New York. 512 pp.

ERRARD, C., and FALLON, J.-M. 1987. An investigation of the development of the chemical factors in ants' intra-society recognition, p. 478, *in* J. Eder and H. Rembold (eds.). Chemistry and Biology of Social Insects. Verlag J. Peperny, Munich.

FRANCIS, B.R., BLANTON, W.E., and NUNAMAKER, R.A. 1985. Extractable surface hydrocarbons of workers and drones of the genus *Apis. J. Apic. Res.* 24:13–26.

FRANCIS, B.R., BLANTON, W.E., LITTLEFIELD, J.L., and NUNAMAKER, R.A. 1989. Hydrocarbons of the cuticle and hemolymph of the adult honey bee (Hymenoptera: Apidae). *Ann. Entomol. Soc. Am.* 82:486–494.

FREE, J.B. 1987. Pheromones of Social Bees. Chapman and Hall, London.

GAMBOA, G.J., REEVE, H.K., and PFENNIG, D.W. 1986. The evolution and ontogeny of nestmate recognition in social wasps. *Annu. Rev. Entomol.* 31:431–454.

GETZ, W.M. 1991. The honey bee as a model kin recognition system, *in* P.G. Hepper (ed.). Kin Recognition. Cambridge University Press, Cambridge. In press.

GETZ, W.M., and SMITH, K.B. 1983. Genetic kin recognition: Honey bees discriminate between full and half sisters. *Nature* 302:147–148.

GETZ, W.M., and SMITH, K.B. 1986. Honey bee kin recognition: Learning self and nestmate phenotypes. *Anim. Behav.* 34:1617–1626.

GETZ, W.M., and SMITH, K.B. 1987. Olfactory sensitivity and discrimination of mixtures in the honeybee *Apis mellifera*. *J. Comp. Physiol. A* 160:239–245.

GETZ, W.M., BRÜCKNER, D., and SMITH, K.B. 1988. Variability of chemosensory stimuli within honeybee (*Apis mellifera*) colonies: Differential conditioning assay for discrimination cues. *J. Chem. Ecol.* 14:253–264.

GETZ, W.M., BRÜCKNER, D., and SMITH, K.B. 1989. Ontogeny of chemosensory cues in worker honey bees *Apis mellifera*. *Apidologie* 20:105–113.

HAMILTON, W.D. 1964a. The genetical evolution of social behavior I. *J. Theor. Biol.* 7:1–16.

HAMILTON, W.D. 1964b. The genetical evolution of social behavior II. *J. Theor. Biol.* 7:17–52.

LAIDLAW, H.H. 1977. Instrumental Insemination of Honey Bee Queens. Dadant and Sons, Hamilton, Illinois. 144 pp.

MINTZER, A. 1989. Incompatibility between colonies of acacia-ants: Genetic models and experimental results, pp. 163–182, *in* M.D. Breed and R.E. Page (eds.). The Genetics of Social Evolution. Westview Press, Boulder, Colorado.

MOREL, L., and VAN DER MEER, R.K. 1987. Nestmate recognition in *Camponotus floridanus*: Behavioral and chemical evidence for the role of age and social experience, pp. 471–472, *in* J. Eder and H. Rembold (eds.). Chemistry and Biology of Social Insects. Verlag J. Peperny, Munich.

PAGE, R.E. 1986. Sperm utilization in social insects. *Annu. Rev. Entomol.* 31:297–320.

PAGE, R.E., and BREED, M.D. 1987. Kin recognition in social bees. *Trends Ecol. Evol.* 9:272–275.

PAGE, R.E., and LAIDLAW, H.H. 1988. Full sisters and super sisters: A terminological paradigm. *Anim. Behav.* 36:944–945.

PAMILO, P., and CROZIER, R.H. 1982. Measuring genetic relatedness in natural populations: Methodology. *Theor. Popul. Biol.* 21:171–193.

SMITH, R.-K. 1988. Identification of Africanization in honey bees based on extracted hydrocarbons assay, pp. 275–280, *in* G.R. Needham, R.E. Page, M. Delfinado-Baker, and C.E. Bowman, (eds.). Africanized Honey Bees and Bee Mites, Ellis Horwood Ltd., Chichester.

SNEATH, P.H.A., and SOKAL, R.R. 1973. Numerical Taxonomy. W.H. Freeman, San Francisco. pp. 137–140, 190–192.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry. W.H. Freeman, San Francisco. pp. 449–450.

TUCKER, K.W. 1986. Visible mutants, pp. 57–87, *in* T.E. Rinderer (ed.). Bee Genetics and Breeding. Academic Press, Orlando, Florida.

VANDER MEER, ROBERT K., SALIWANCHIK, D. and LAVINE, B. 1989. Temporal changes in colony cuticular hydrocarbon patterns of *Solenopsis invicta*:: implications for nestmate recognition. *J. Chem. Ecol.* 15:2115–2125.

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

(+)-*cis*-3-PINEN-2-OL: ATTRACTANT FOR MALE CERAMBYCID BEETLE, *Monochamus alternatus* HOPE

MITSURU SAKAI and TORU YAMASAKI*

Department of Bioresource Science
Faculty of Agriculture, Kagawa University
Miki-Cho, Kagawa-Ken 761-07, Japan

(Received September 13, 1990, accepted December 3, 1990)

Abstract—A monoterpene alcohol was isolated from paraquat-induced lightwood in pines and identified as (+)-*cis*-3-pinen-2-ol [(+)-**3**]. More than a certain amount of (+)-**3** induced a laboratory flight response by the male cerambycid beetle, *Monochamus alternatus* Hope, but (+)-**3** stimulated no response by the female at any dose level tested. Compound (+)-**3** was not detected in trunks of sound pines.

Key Words—*Monochamus alternatus* Hope, Coleoptera, Cerambycidae, male, attractant, (+)-*cis*-3-pinen-2-ol, lightwood, pine.

INTRODUCTION

The herbicide paraquat drastically induces oleoresin-soaked wood (lightwood) in pines (Roberts et al., 1973). Lightwood formation, which is usually accompanied with heavy oleoresin exudation, does trigger a significant mass attack by a variety of beetles (Coleoptera: Scolytidae, Cerambycidae, Curculionidae, Rhynchophoridae, Platypodidae, and Buprestidae) (Hertel et al., 1977; Yamasaki and Suzuki, 1982). The attractive principle of lightwood has not hitherto been identified.

Recently, however, the sesquiterpene alcohol (+)-juniperol and the diterpene aldehyde (+)-pimaral were isolated from trunks of sound pines as well as lightwood and confirmed to be highly attractive to the female cerambycid beetle, *Monochamus alternatus* Hope (Sakai and Yamasaki, 1990).

The present paper describes the identification of (+)-*cis*-3-pinen-2-ol from lightwood as a male-specific attractant.

*To whom correspondence should be addressed.

0098-0331/91/0400-0757$06.50/0

METHODS AND MATERIALS

Instrumental Analysis. A mass spectrum was measured with a JEOL JMS D-300 mass spectrometer with electron impact ion at an ionizing voltage of 70 eV. A high-resolution (HR) mass spectrum also was measured with the instrument. Proton nuclear magnetic resonance ([^{1}H]NMR) and carbon-13 ([^{13}C] NMR) spectra were run at 500 MHz and 125 MHz, respectively, on a JEOL JNM-GSX 500 spectrometer. ^{13}C−^{1}H shift correlation spectroscopy (COSY), [^{1}H−^{1}H]COSY, and nuclear Overhauser effect (NOE) difference spectra were taken with the spectrometer. All NMR spectra were recorded at 27.0°C using 5% deuterochloroform solutions with tetramethylsilane as an internal standard. Optical rotation was measured with a Horiba SEPA-200 polarimeter, and specific rotation ($[\alpha]_D$) was calculated.

*Isolation of (+)-*cis*-3-Pinen-2-ol[(+)-***3***].* Eighteen-year-old *Pinus densiflora* Sieb. et Zucc. and *P. thunbergii* Parl. were treated with paraquat early in May 1988 (Roberts et al., 1973). Lightwood samples were collected in mid-July 1988. Other sound 17-year-old trees of these species also were harvested.

Essential oils were obtained by steam distillation from freshly crushed wood materials. Minor constituent fractions were separated from the essential oils, respectively, by a combination (Sakai and Yamasaki, 1990) of reduced-pressure distillation and low-temperature liquid chromatography (Yamasaki et al., 1986). Yields: less than 2% of the essential oils.

The minor constituent fractions from the lightwood samples were submitted to silica gel thin-layer chromatography (TLC) using *n*-pentane–ether (5 : 1). Fractions with R_f values of 0.61–0.85 were combined and gas chromatographed (Sakai and Yamasaki, 1990). Compound (+)-**3**, giving a relative t_R of 1.43 (longifolene = 1) was isolated from lightwood formed in *P. densiflora* and *P. thunbergii* and purified by TLC.

The compound was completely missing in the minor constituent fractions from the sound pines (Ia and IIa in Figure 1).

(+)-3 (Scheme 1). Colorless oil. $[\alpha]_D^{25}$ = + 66.7° (c = 3.0, EtOH). MS: *m/z* (%) 152(M^+, 0.2), 151(0.7), 136(2), 135(5), 134(2), 133(5), 123(7), 121(5), 119(6), 109(19), 107(23), 105(14), 103(2), 97(2), 95(15), 93(57), 91(20), 81(9), 79(10), 77(12), 71(6), 69(44), 67(5), 63(4), 59(2), 55(11), 53(10), 51(3), 43(100), 41(43), 39(22). HR-MS: Found; 152.1208, Calcd. for $C_{10}H_{16}O$; 152.1201. [^{1}H]NMR δ: 0.977 (3H, s, 6-CH_3), 1.397(3H, s, 6-CH_3), 1.447(3H, s, 2-CH_3), 1.744(1H, d, $J_{b,a}$ = 8.85Hz, 7-Hb), 2.216 (1H, q, J = 6.10 Hz, 5-H), 2.273(1H, td, $J_{1,3}$ = 2.14, $J_{1,5;1,a}$ = 6.11 Hz, 1-H), 2.415(1H, dtd, $J_{a,4}$ = 0.96, $J_{a,1;a,5}$ = 5.81, $J_{a,b}$ = 8.85 Hz, 7-Ha), 5.498(1H, ddd, $J_{3,5}$ = 0.91, $J_{3,1}$ = 2.14, $J_{3,4}$ = 8.85 Hz, 3-H), 6.269(1H, ddd, $J_{4,a}$ = 0.89, $J_{4,5}$ = 6.10, $J_{4,3}$ = 8.85 Hz, 4-H), 7.365(OH, 2-OH). [^{13}C]NMR δ: 24.14(−CH_3, 6-CH_3), 25.94(−CH_3, 6-CH_3), 27.53(−CH_3, 2-CH_3), 33.29 (>CH_2, C-7),

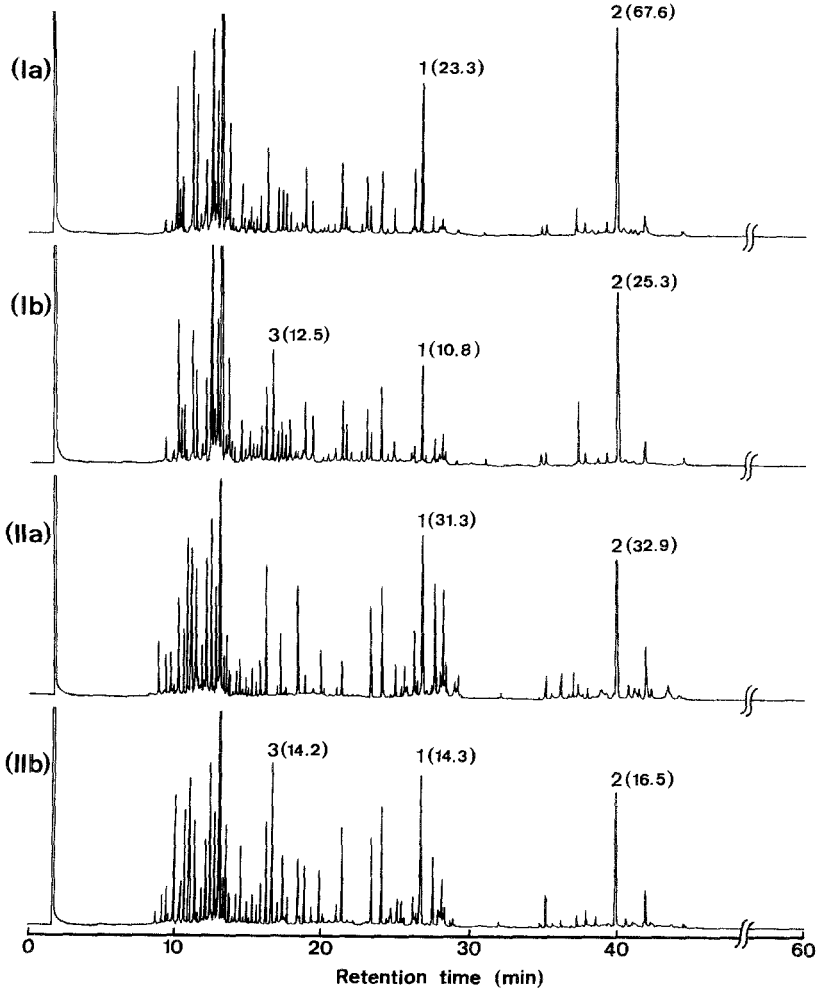
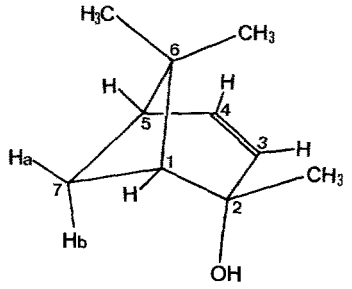


FIG. 1. Fused silica capillary gas chromatograms of minor constituent fractions prepared from essential oils. Sources: Ia, *P. densiflora*; Ib, lightwood formed in *P. densiflora*; IIa, *P. thunbergii*; IIb, lightwood in *P. thunbergii*. **1**, (+)-juniperol; **2**, (+)-pimaral; **3**, (+)-*cis*-3-pinen-2-ol. These compounds' contents (μg) of 34.8 mg each of essential oils also are shown in parenthesis. The peak at 2.0 min shows *n*-pentane as a solvent. Column: 0.25 mm ID × 30 m J & W DB-5.



SCHEME 1. (1*R*, 2*R*, 5*R*)-(+)-2,6,6-trimethyl-bicyclo[3.1.1]hept-3-en-2-ol [(+)-*cis*-3-pinen-2-ol].

42.76(>CH—, C-5), 46.92(>C<, C-6), 53.95(>CH—, C-1), 74.24(>C<, C-2), 129.98(>CH—, C-3), 138.57(>CH—, C-4). The kinds of carbons were determined by distortionless enhancement by polarization transfer (DEPT).

The Insect. Approximately 100 individuals of each sex of *M. alternatus* (being attracted to paraquat-treated pines) were collected during mid-July 1989 and kept in individual containers (Yamasaki and Suzuki, 1982). They were marked with white spot(s) to distinguish individuals and randomized before use.

Laboratory Bioassay. Hourly duplicate tests were run by a concurrent employment of two 6.8 × 2.6 × 3.8-m chamber-type olfactometers at 30-min intervals from 8:00 PM to either 1:30 or 3:00 AM according to the previous method (Sakai and Yamasaki, 1990). Twenty-five individual insects were placed in each chamber. In one case, both chambers contained either all males or all females. In the second case, one chamber contained all males, and the other chamber contained all females. Pieces of filter paper impregnated with a *n*-pentane solution (40 μl) were placed into one inlet at 15-min intervals. The other was a blank (air only) inlet. The flow rate was about 5.1 m^3/min. The rate at the 30-min intervals was about 12.3 m^3/min.

RESULTS

(+)-3 as Male-Specific Attractant. Table 1 shows flight responses of both sexes of *M. alternatus* to this compound. No male responded to doses of 1.25 × 10^{-1}μg/15 min and less. On treatment with a dose of 12.5μg, four to five males were observed arriving at this odor source. At higher dose levels, the numbers were reduced to some extent, but the control, i.e., the number of individuals arriving at the blank inlet, increased somewhat; these phenomena may be due to a decrease in the odor concentration gradient in the chambers. It therefore seems likely that the dose of 12.5 μg/15 min is close to an adequate one under the test conditions.

TABLE 1. FLIGHT RESPONSES OF *M. alternatus* TO (+)-*cis*-3-PINENE-2-OL (AUGUST 4–7, 1989)[a]

Dose (μg/15 min)	Male		Female	
	Real No.[b]	Total No.[b]	Real No.[b]	Total No.[b]
0[c]	0[0]	0[0]	0[0]	0[0]
1.25×10^{-3}	0[0]	0[0]	0[0]	0[0]
1.25×10^{-2}	0[0]	0[0]	0[0]	0[0]
1.25×10^{-1}	0[0]	0[0]	0[0]	0[0]
0.63	0.5 (0–1) [0]	0.5 (0–1) [0]	0[0]	0[0]
1.25	2 (2) [0]	2 (2) [0]	0[0]	0[0]
1.25×10	4.5 (4–5) [0.5 (0–1)]	7 (6–8) [0.5 (0–1)]	0[0]	0[0]
1.25×10^{2}	4 (3–5) [2 (2)]	4 (3–5) [2 (2)]	0[0]	0[0]
1.25×10^{3}	2.5 (2–3) [2 (1–3)]	2.5 (2–3) [2 (1–3)]	0[0]	0[0]

[a] Hourly bioassays were conducted at 30-min intervals from 8:00 PM to 3:00 AM.
[b] Number represents the mean of two replications; range in parenthesis; controls are shown in brackets.
[c] *n*-Pentane only (40 μl/15 min).

In contrast to the results obtained with males, (+)-**3** induced no flight response by females at any dose level tested. Most were at rest or walking, especially downwind. A few were flying in no specific direction.

(+)-Juniperol [(+)-**1**] and (+)-pimaral [(+)-**2**], and especially the combination of these compounds, are attractive to the female (Sakai and Yamasaki, 1990). As shown in Table 2, the attraction of (+)-**1** or (+)-**2** alone for the female and that of a blend of these two compounds in constituent composition (Figure 1) of essential oil (LW oil) from lightwood formed in *P. densiflora*, were found to be independent of adding (+)-**3**.

Attraction of (+)-3 with Other Compounds for Male M. alternatus. Table 3 shows flight responses of the male to this compound in combination with (+)-**1** and/or (+)-**2**. Compound (+)-**3** delivered in a ratio of 12.5 μg/15 min attracted four to five males, while (+)-**1** and (+)-**2** presented individually in the same ratio attracted only two and one males, respectively (August 10). Neither addition of (+)-**3** to (+)-**1** nor to (+)-**2** was effective in enhancing male attraction (August 8). Instead, a blend of these three compounds attracted seven

TABLE 2. MEAN NUMBER (RANGE IN PARENTHESES; CONTROL IN BRACKETS) OF FEMALE *M. alternatus* ATTRACTED TO (+)-JUNIPEROL (**1**), AND/OR (+)-PIMARAL (**2**) WITH (+)-*cis*-3-PINEN-2-OL (**3**) (1989)

Time period[a]	August 11			August 12		
	Sample[b] and dose (μg^c/15 min)	No.		Sample[b] and dose (μg^c/15 min)	No.	
		Real	Total		Real	Total
A	LW oil[d]	7.5 (7–8) [0]	9.5 (8–11) [0]	**1** + **3** (10.8 vs. 12.5)	3 (3) [0]	3.5 (3–4) [0]
B	**1** + **2** + **3** (10.8 vs. 25.3 vs. 12.5)	7 (7) [0]	11.5 (10–13) [0]	**2** + **3** (25.3 vs. 12.5)	2 (2) [0]	2 (2) [0]
C	**1** + **2** (10.8 vs. 25.3)	7.5 (7–8) [0.5 (0–1)]	10 (8–12) [0.5 (0–1)]	**1** (10.8)	3.5 (3–4) [0]	3.5 (3–4) [0]
D	**3** (12.5)	0[0]	0[0]	**2** (25.3)	2 (2) [0]	2.5 (2–3) [0]

[a] A, 8:00–9:00 PM; B, 9:30–10:30 PM; C, 11:00–0:00 AM; D, 0:30–1:30 AM.
[b] From essential oil (LW oil) from lightwood in *P. densiflora*.
[c] Equivalent to **1**, **2**, and **3** contents of LW oil (34.8 mg); refers to FIG. 1.
[d] The oil only (34.8 mg/15 min).

TABLE 3. MEAN NUMBER (RANGE IN PARENTHESES; CONTROL IN BRACKETS) OF MALE *M. alternatus* ATTRACTED TO (+)-JUNIPEROL (**1**), (+)-PIMARAL (**2**), AND (+)-*cis*-3-PINEN-2-OL (**3**) PRESENTED INDIVIDUALLY AND IN COMBINATION (1989)

	August 8			August 9			August 10		
Time period[a]	Sample and dose (μg[b]/15 min)	No.		Sample and dose (μg[b]/15 min)	No.		Sample and dose (μg/15 min)	No.	
		Real	Total		Real	Total		Real	Total
A	**3** (12.5)	4 (4) [0]	5.5 (5–6) [0]	**3** + **1** + **2** (12.5 vs. 10.8 vs. 25.3)	8.5 (8–9) [0]	13 (12–14) [0]	**2** (12.5)	1 (1) [0]	1 (1) [0]
B	**3** + **1** (12.5 vs. 10.8)	5 (4–6) [0.5 (0–1)]	5 (4–6) [0.5 (0–1)]	**1** + **2** (10.8 vs. 25.3)	4.5 (4–5) [0]	4.5 (4n5) [0]	**1** (12.5)	2 (2) [0]	2 (2) [0]
C	**3** + **2** (12.5 vs. 25.3)	4 (3–5) [0]	4 (3–5) [0]	LW oil[c,d]	9.5 (9–10) [0]	12 (11–13) [0]	**3** (12.5)[b]	4.5 (4–5) [0]	4.5 (4–5) [0]
D	**3** + **1** + **2** (12.5 vs. 10.8 vs. 25.3)	7.5 (7–8) [0.5 (0–1)]	12.5 (11–14) [0.5 (0–1)]	CW oil[d,e]	4 (4) [0.5 (0–1)]	4 (4) [0.5 (0–1)]	**1** + **2** (23.3 vs. 67.6)[f]	3.5 (3–4) [0.5 (0–1)]	4 (4) [0.5 (0–1)]

[a] A, 8:00–9:00 PM; B, 9:30–10:30 PM; C, 11:00–0:00 AM; D, 0:30–1:30 AM.
[b] Refers to [c] in Table 2.
[c] Refers to [b] in Table 2.
[d] The oil only (34.8 mg/15 min).
[e] Essential oil from sound *P. densiflora*.
[f] Equivalent to **1** and **2** contents of CW oil (34.8 mg); refers to FIG 1.

to nine males (August 8 and 9). The activity of the ternary blend reached almost that of 34.8 mg of LW oil (August 9).

On the other hand, two blends of (+)-**1** and (+)-**2** prepared in different ratios attracted three to five males (August 9 and 10). The other essential oil (CW oil), devoid of (+)-**3** (Figure 1), from sound *P. densiflora*, also attracted nearly the same number of males, i.e., four (August 9).

DISCUSSION

The two synthetic optical isomers of **3** are well known (Whitham, 1961; Bessière-Chrétien and Grison, 1970; Abraham et al., 1972; Jefford et al., 1973; Witkiewicz and Chabudziński, 1977; Brocki et al., 1980; Traynor et al., 1980). From data taken by means of gas chromatography–mass spectrometry, **3** has been presumed to occur in flowers of *Ophrys fusca* Link (Borg-Karlson et al., 1985), in leaves of *Eucalyptus sparsa* Boomsma (Brophy and Lassak, 1986), and in hindguts of the female *Dendroctonus frontalis* Zimm. (Renwick and Hughes, 1975). No evidence for optical isomerism has been presented in these reports. Recently, a racemate—$[\alpha]_D = 0.0°$ (c = 0.32, $CHCl_3$)—of the enantiomers of **3** was isolated from green stems, leaves, and flowers of the resinous half-shrub, *Gutierrezia sarothrae* (Epstein and Seidel, 1989). In this paper, the occurrence of the enantiomer, (+)-**3**, in paraquat-induced lightwood was clearly verified.

Neither enantiomer of **3** or racemic **3**, to our knowledge, has been known as an attractant for any insect species. Tables 1 and 2 demonstrate that (+)-**3** is an attractant specific for the male *M. alternatus*; in other words, the female is indifferent to this compound. Addition of (+)-**3** to the combination of (+)-**1** and (+)-**2** was more effective in enhancing male attraction (Table 3).

Compound (+)-**3** was not detected in the trunks of sound hosts (Figure 1). Nevertheless, we are not sure that this compound is absent in the body of *M. alternatus* or its habitats without paraquat-treated pines. Thus, we now do not know whether in this insect species (+)-**3** is related to sexual communication or has some physiological significance for the male sex.

REFERENCES

ABRAHAM, R.J., COOPER, M.A., SALMON, J.R., and WHITTAKER, D. 1972. The NMR spectra and conformations of cyclic compounds. V. Proton couplings and chemical shifts in bridged cyclobutanes. *Org. Magn. Reson.* 4:489–507.

BESSIÈRE-CHRÉTIEN, Y., and GRISON, C. 1970. Étude de diméthyl-6,6 bicyclo(3.1.1)heptanones-2 et 3. *Bull. Soc. Chim. Fr.* 00:3103–3111.

BORG-KARLSON, A.-K., BERGSTRÖM, G., and GROTH, I. 1985. Chemical basis for the relationship

between *Ophrys orchids* and their pollinators. I. Volatile compounds of *Ophrys lutea* and *O. fusca* as insect mimetic attractants/excitants. *Chem. Scr.* 25:283–294.

BROCKI, T., MOSKOVITS, M., and BOSNICH, B. 1980. Vibrational optical activity. Circular differential raman scattering from a series of chiral terpenes. *J. Am. Chem. Soc.* 102:495–500.

BROPHY, J.J., and LASSAK, E.V. 1986. The volatile leaf oils of some central Australian species of *Eucalyptus*. *J. Proc. R. Soc. N.S.W.* 119:103–108.

EPSTEIN, W.W., and SEIDEL, J.L. 1989. Monoterpenes of *Gutierrezia sarothrae*. *J. Agric. Food Chem.* 37:651–654.

HERTEL, G.D., WILLIAMS, I.L., and MERKEL, E.P. 1977. Insect attacks on and mortality of slash and longleaf pines treated with paraquat to induce lightwood formation. *U.S.D.A. For. Serv. Res. Paper* SE-169:1–13.

JEFFORD, C.W., BOSCHUNG, A.F., MORIARTY, R.T., RIMBAULT, C.G., and LAFFER, M.H. 1973. The reaction of singlet oxygen with α- and β-pinenes. *Helv. Chim. Acta* 56:2649–2659.

RENWICK, J.A.A., and HUGHES, P.R. 1975. Oxidation of unsaturated cyclic hydrocarbons by *Dendroctonus frontalis*. *Insect Biochem.* 5:459–463.

ROBERTS, D.R., JOYE, N.M., JR., PROVEAUX, A.T., PETERS, W.J., and LAWRENCE, R.V. 1973. A new and more efficient method of naval stores production. *Nav. Stores Rev.* 83:4–5.

SAKAI, M., and YAMASAKI, T. 1990. (+)-Juniperol and (+)-pimaral: Attractants for the cerambycid beetle, *Monochamus alternatus* Hope. *J. Chem. Ecol.* 16:3383–3392.

TRAYNOR, S.G., KANE, B.J., COLEMAN, J.B., and CÁRDENAS, C.G. 1980. Chemistry of the 2-pinanols. 2. Investigation of the utility of the enhanced basicities of the pinanoxide bases. *J. Org. Chem.* 45:900–906.

WHITHAM, G.H. 1961. The reaction of α-pinene with lead tetra-acetate. *J. Chem. Soc.* pp. 2232–2236.

WITKIEWICZ, K., and CHABUDZIŃSKI, Z. 1977. Oxidation of monoterpenoid olefins with manganese triacetate. II. Oxidation of (+)-α-pinene. *Rocz. Chem. Ann. Soc. Chim. Pol.* 51:475–482.

YAMASAKI, T., and SUZUKI, N. 1982. Luring of Japanese pine sawyer *Monochamus alternatus* Hope by paraquat-treated pine trees. II. The attractiveness of volatile oil from lightwood. *J. Jpn. For. Soc.* 64:340–345.

YAMASAKI, T., SAKAI, M., KANAMORI, T., and SOGO, M. 1986. Low temperature liquid chromatographic separation of oxygenated terpenes from terpene hydrocarbons. *Chromatographia* 21:478–479.

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

AVERSIVE RESPONSES OF WHITE-TAILED DEER, *Odocoileus virginianus*, TO PREDATOR URINES

ROBERT K. SWIHART,[1,*] JOSEPH J. PIGNATELLO,[2] and MARY JANE I. MATTINA[3]

[1]*Department of Plant Pathology and Ecology*
[2]*Department of Soil and Water*
[3]*Department of Analytical Chemistry*
The Connecticut Agricultural Experiment Station
Box 1106, 123 Huntington Street
New Haven, Connecticut 06504

(Received September 10, 1990; accepted December 10, 1990)

Abstract—We tested whether predator odors could reduce winter browsing of woody plants by white-tailed deer (*Odocoileus virginianus*). Urine from bobcats (*Lynx rufus*) and coyotes (*Canis latrans*) significantly reduced browsing of Japanese yews (*Taxus cuspidata*), and repellency was enhanced when urine was reapplied weekly as a topical spray. Urine of cottontail rabbits (*Sylvilagus floridanus*) and humans did not reduce damage, suggesting that deer do not respond aversively to odors of nonpredatory mammals or occasional predators with which they lack a long evolutionary association. Bobcat and coyote urine were more effective in tests conducted with eastern hemlock (*Tsuga canadensis*), which is less palatable to white-tailed deer than Japanese yew. A dichloromethane extract of bobcat urine was as effective as unextracted urine in reducing damage to hemlocks. Testing of the organic components of bobcat urine, particularly the volatile components, may enable identification of the compounds responsible for the repellency we observed.

Key Words—bobcat, browsing, *Canis latrans*, coyote, *Lynx rufus*, *Odocoileus virginianus*, predator urine, repellent, semiochemicals, white-tailed deer, wildlife damage, *Taxus cuspidata*, *Tsuga canadensis*.

INTRODUCTION

Browsing of twigs of woody plants by white-tailed deer (*Odocoileus virginianus*) in winter often damages apple trees (Katsma and Rusch, 1979) and nurs-

*To whom correspondence should be addressed.

0098-0331/91/0400-0767$06.50/0

ery stock (Anderson, 1984), leading to significant economic losses (Anderson, 1984; Phillips et al., 1987). In addition, browsing by white-tailed deer can have a negative impact on natural regeneration of important timber species (e.g., Graham, 1954; Frelich and Lorimer, 1985). In areas where other forms of control are impractical, chemical repellents often are used (Scott and Townsend, 1985; Purdy et al., 1987). However, the majority of chemical repellents are either ineffective or reduce damage only slightly (Palmer et al., 1983; Conover, 1984, Swihart and Conover, 1990), perhaps because of anthropogenic notions regarding repellency (Shumake, 1977). Thus, the identification of effective repellents with a biological basis is needed for use against white-tailed deer.

Recognition and avoidance of odors of sympatric predators by mammalian prey has been documented (Fulk, 1972; Stoddart, 1980) and may have a genetic component (Müller-Schwarze, 1972; Gorman, 1984). In an applied context, the use of predator odors has reduced damage caused by several species of herbivorous mammals, including snowshoe hares (*Lepus americanus*) (Sullivan and Crump, 1984, 1986; Sullivan et al., 1985a; Sullivan, 1986), voles (*Microtus* spp.) (Sullivan et al., 1988a), pocket gophers (*Thomomys talpoides*) (Sullivan et al., 1988b), woodchucks (*Marmota monax*) (Swihart, 1991), and mule deer (*Odocoileus hemionus*) (Sullivan et al., 1985b; Melchiors and Leslie, 1985). However, the effect of predator odors on the behavior of white-tailed deer is largely unknown. Here, we report on the ability of odors of predators to repel white-tailed deer from woody plants placed in woodlots in Connecticut, U.S.A.

The degree to which a prey species responds aversively to odors of predators probably depends on several factors, including whether or not the predator and prey occur sympatrically (Müller-Schwarze, 1972), the duration of the association between predator and prey (e.g., Gorman, 1984), and the extent to which responses to odors can be culturally transmitted among prey (Swihart, 1991). In addition, the frequency of life-threatening versus innocuous encounters with a predator species may influence selection for an aversive response to the predator or its odor. Learned aversions also are undoubtedly influenced by the frequency of these events, although on a much shorter time scale.

Bobcats (*Lynx rufus*), coyotes (*Canis latrans*), and humans occur sympatrically with white-tailed deer throughout much of the herbivore's range (Hall, 1981). Bobcats and coyotes share a long ($\approx$ 1–2 million years) association with white-tailed deer (Kurtèn and Anderson, 1980), although coyotes have expanded their range into the southern and extreme eastern portions of the United States only in recent years (Hall, 1981). Although the relative importance of bobcats and coyotes as predators of deer is uncertain, coyotes are more likely to prey upon fawns (Mech, 1984), whereas bobcats also prey upon older deer (Marston, 1942). In Connecticut, humans currently are the most significant predators of deer, whereas bobcats and coyotes occur in low numbers. However, interactions between white-tailed deer and humans have occurred in the eastern United

States only for about 10,000 years (Nash, 1974). Moreover, learned aversions to odors of humans presumably are ameliorated in Connecticut because of the high frequency of encounters with human scent in suburban areas that prove to be innocuous or beneficial (e.g., availability of food around houses). Based on these factors, we hypothesized that the order of repellency of odors of these three predators to white-tailed deer would be: bobcat > coyote > human.

METHODS AND MATERIALS

Experiments were conducted during winter, 1989–1990, near Danbury in western Connecticut. Density of deer, as estimated by pellet-group counts in 1989 and 1990, averaged 8.3/km^2, and browsing damage to nursery stock and ornamental shrubs near houses often was severe. To ensure that deer were not inhibited from visiting experimental sites for reasons other than the presence of predator odors (e.g., dogs, lights, vehicles), experiments were conducted in wooded areas. Wooded areas were separated by >1.6 km to increase the likelihood that different sites were visited by different deer (see Severinghaus and Cheatum, 1956; Larson et al., 1978). Damage was expressed as the percentage of shoots that were browsed by deer, and differences among treatment means at the end of each experiment were examined using a nonparametric Kruskal-Wallis test with one-tailed multiple pairwise comparisons (Conover, 1980, p. 230).

Trials with Japanese Yews. Trial 1 was designed to test whether the odor of small amounts of urine could reduce feeding by deer. In addition to bobcat, coyote, and human urine, we tested whether deer responded to some common component(s) of mammalian urine by examining browsing in the presence of urine from cottontail rabbits (*Sylvilagus floridanus*). Distilled water served as a control.

On November 27, 1989, 5-year-old Japanese yews (*Taxus cuspidata*) were transplanted at six woodlots. Japanese yews are popular ornamental evergreen shrubs in Connecticut, and they also are highly palatable to deer (Conover and Kania, 1988; Swihart and Conover, 1990). All yews had been grown together under partial sunlight in a lath house and subjected to the same watering and fertilization schedule before transplanting. They had not been subjected to browsing before the experiment. In each woodlot, six plots were established at the vertices of a 3×2 rectangular grid, with a 20-m spacing between adjacent vertices. Five of the plots were randomly selected, and eight yews were planted at each of the five plots using a 4×2 configuration with 1-m spacing. For each yew, counts then were made of all shoots >1.25 cm in length (≈ 600 shoots per plot). After randomly assigning a treatment to each plot, a microcentrifuge tube (1.5-ml capacity) was attached to each yew with a twist-tie. Syringes then

were used to dispense 1.2 ml of the appropriate urine into each tube. Finally, a small wooden dowel was inserted through a hole drilled in the top of each tube. The dowels protruded 1.0–1.5 cm above each tube, thereby serving as a wick to enhance dissemination of scent. Microcentrifuge tubes and dowels also were attached to control yews. Damage was monitored periodically over the next 35 days by counting the number of browsed shoots.

In trial 2 we tested whether topical application of urine at weekly intervals could enhance its repellency. The following modifications were made to the design used in trial 1. One 7-year-old Japanese yew ($\approx$350 shoots per plant) was used at each plot. At weekly intervals beginning on December 1, 1989, each yew was sprayed with a mist of 6 ml of the appropriate treatment. To enhance retention of the urine, each treatment solution (and the water control) was mixed 100:1 with Vapor Guard (Miller Chemical and Fertilizer Corporation, Hanover, Pennsylvania). Browsing damage was monitored until January 24, 1990. One of the six study sites was eliminated from subsequent analyses because of repeated visits of either a large domestic cat or a small bobcat during the trial. Upon visiting the site, the felid would bite the wooden potting stakes used to label plots and urinate on or near treated yews.

Trial with Eastern Hemlock. Trial 3 was conducted to test whether increasing the frequency of topical application increased the repellency of bobcat and coyote urine. Trials were conducted using eastern hemlock (*Tsuga canadensis*), a native species whose regeneration has been hindered in recent years by browsing of white-tailed deer (see Frelich and Lorimer, 1985). To ensure that no prior browsing of plants had occurred, we used hemlock branches collected from the sun crowns of mature trees. Mature-stage branches are more palatable to deer than juvenile-stage growth (R.K. Swihart, unpublished data); hence, the use of mature-stage growth provided a powerful test of the repellency of the predator urines. At each plot, branches were attached with wire to the bases of saplings that had been cut to a height of <15 cm, and the number of shoots was counted ($\approx$ 175 shoots per plot). Seven experimental plots were established at each of 10 wooded sites on February 20 and 21, 1990, and a separate treatment was randomly assigned to each plot. Four plots were sprayed with 6 ml of either bobcat or coyote urine, two plots at weekly intervals, and two plots twice weekly. A fifth plot sprayed with distilled water served as a control.

To determine whether aversive responses of deer were elicited by organic components of predator urine, we tested a dichloromethane extract of the bobcat urine. One liter of urine was extracted with two 50-ml portions of dichloromethane in a separatory funnel. The combined extracts were concentrated in a Kuderna-Danish apparatus to 2.4 ml and then diluted to 1 liter with methanol. The methanol solution (6 ml) then was applied at weekly intervals to a sixth plot at each site. A seventh plot sprayed with a 2.4:1000 mixture of dichloro-

methane and methanol served as a control. Final estimates of browsing damage were made on March 20, 1990.

RESULTS

Trials with Japanese Yews. Onset of browsing to Japanese yews was rapid in trial 1; after 14 days 80% of shoots of control plants had been removed (Figure 1). Moreover, the presence of urine did not prevent browsing, and browsing of treated plants increased over time. Nonetheless, plants to which tubes containing bobcat and coyote urine had been attached exhibited significantly ($P < 0.05$) lower levels of browsing than did plants treated with water, rabbit urine, or human urine at the conclusion of trial 1. Damage to yews treated with human urine, rabbit urine, or water was statistically indistinguishable. Yews treated with bobcat urine were browsed significantly ($P < 0.05$) less than those treated with coyote urine.

The repellency of bobcat and coyote urine was enhanced by repeated topical application in trial 2. Although the progression of damage to control yews was reasonably similar in trial 1 and trial 2, damage to plants treated with bobcat or coyote urine was substantially lower (Figures 1 and 2). In fact, complete suppression of browsing was achieved with bobcat urine at three sites and with coyote urine at four sites. Moreover, no progressive increase in damage

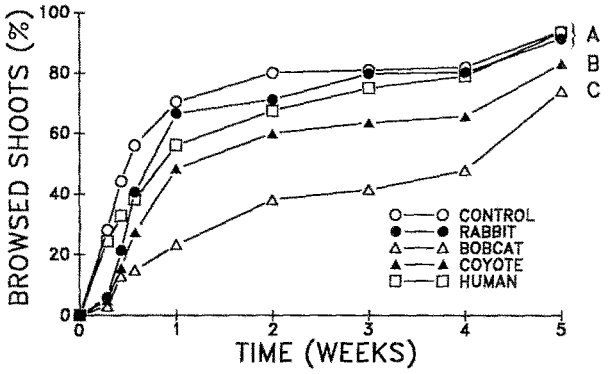


FIG. 1. Percentage of shoots of Japanese yews browsed by white-tailed deer in western Connecticut during a five-week period, winter 1989–1990. Treatments were 1.2 ml of urine placed in microcentrifuge tubes; distilled water served as a control. Significant differences occurred among treatments in the level of deer browsing at the end of the trial (Kruskal-Wallis $T = 16.3$, $P = 0.003$), although treatments sharing the same letter did not differ significantly ($\alpha = 0.05$).

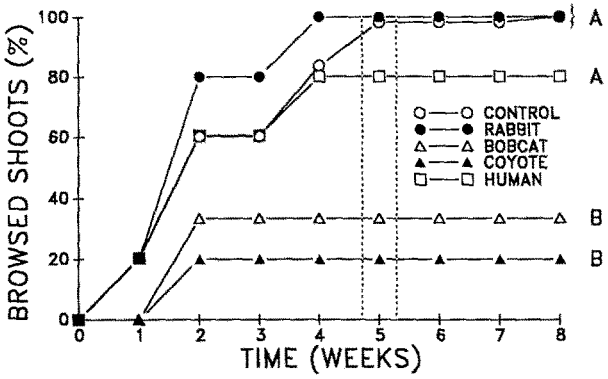


FIG. 2. Percentage of shoots of Japanese yews browsed by white-tailed deer in western Connecticut during an eight-week period, winter 1989–1990. Urine or a distilled water control (6 ml) was sprayed on each plant at weekly intervals. Significant differences occurred among treatments in the level of deer browsing at the end of the trial (Kruskal-Wallis $T = 14.8$, $P = 0.006$), although treatments sharing the same letter did not differ significantly ($\alpha = 0.05$). Dashed vertical lines enclose the levels of browsing that were present when trial 1 was terminated (Figure 1).

occurred. After eight weeks, yews treated with bobcat or coyote urine had received significantly ($P < 0.05$) less browsing by deer than had yews treated with water, rabbit urine, or human urine (Figure 2). Yews treated with human or rabbit urine did not differ from control plants. Checks of three of the study sites four weeks after the trial was terminated revealed that no yews treated with coyote urine had been browsed, whereas one yew treated with bobcat urine and the only human-treated yew that had vegetation remaining had been browsed.

Trials with Eastern Hemlock. Eastern hemlock was browsed less by deer than were the Japanese yews in the earlier trials. Indeed, no browsing occurred at three sites, necessitating their removal before analyses. For control plants, the percentage ($\bar{X} \pm$ SE) of hemlock shoots browsed after four weeks at the remaining seven sites was 30.8 ± 11.3, whereas the percentage of yew shoots browsed was 81.9 ± 10.4 in trial 1 and 83.7 ± 16.3 in trial 2.

No differences were evident in the percentage of hemlock shoots browsed that were treated with water (25.7 ± 13.4) or methanol–dichloromethane (35.9 ± 12.0). Thus, mean values of these controls were calculated at each site and used in analyses. Bobcat and coyote urine depressed levels of browsing significantly, but no additional reductions in browsing were obtained by increasing the rate at which they were applied (Figure 3). In addition, the dichloromethane extract of bobcat urine reduced browsing as effectively as the unextracted urine (Figure 3).

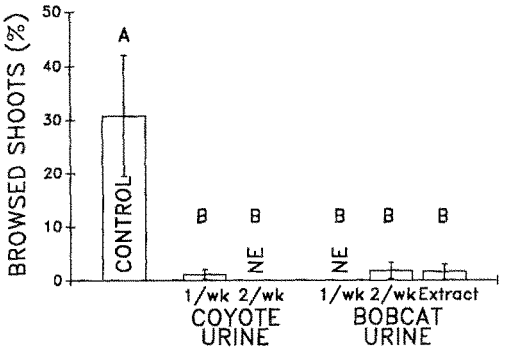


FIG. 3. Percentage of shoots of eastern hemlock browsed by white-tailed deer in western Connecticut during a four-week period, winter 1989–1990. Urine, a dichloromethane extract of bobcat urine, or a control (water or dichloromethane–methanol) was sprayed on each plant at weekly (1/wk) or twice weekly (2/wk) intervals. Significant differences occurred among treatments in the level of deer browsing at the end of the trial (Kruskal-Wallis $T = 29.9$, $P < 0.001$), although treatments sharing the same letter did not differ significantly ($\alpha = 0.05$). NE = not eaten.

DISCUSSION

In general, our prediction regarding the relative effectiveness of the three predator odors (bobcat > coyote > human) was upheld. In trial 1, bobcat urine was significantly more repellent than coyote and human urine, and coyote urine was more repellent than human urine (Figure 1). When reapplied at weekly intervals as a topical spray, both coyote and bobcat urine were more effective than human urine (Figure 2). Although yews repeatedly treated with coyote urine suffered slightly less damage than those treated with bobcat urine, the differences were not significant. In contrast, mule deer in British Columbia were repelled more by coyote than by bobcat urine (Sullivan et al., 1985b).

White-tailed deer appear more alarmed by the presence of bobcats than of coyotes, and this may be related to the contrasting styles of hunting of the two predators (Marchinton and Hirth, 1984). Coyotes, like other canids, chase deer (Marchinton and Hirth, 1984), whereas bobcats employ an ambush hunting style that enables them to kill deer that are bedded (Marston, 1942). It is possible that differences in the uncertainty of a predator's whereabouts, arising from contrasting hunting styles, could lead to differences in the aversive responses of prey exposed to fear-provoking stimuli (i.e., predator scents). In prey such as deer, which are capable of culturally transmitting aversive behaviors, conditions such as prevailing predator densities may be more influential in determining the relative magnitude of aversive responses.

At least two factors could contribute to the ineffectiveness of human urine

as a repellent. As mentioned previously, white-tailed deer and humans have existed sympatrically in the northeastern United States for a relatively brief period in evolutionary terms, thereby mitigating against the development of innate responses to human odors. In addition, the relatively high number of people inhabiting areas in the midst of deer habitat in Connecticut ensures that deer commonly encounter sensory stimuli associated with humans or their activities that do not reinforce an aversive response.

Sullivan et al. (1985b) reported that for periods of four to seven days, mule deer in British Columbia browsed untreated salal (*Gaultheria shallon*) leaves significantly more than leaves associated with bobcat or coyote urine. Based upon tests of a wide variety of predator odors, they concluded that mule deer responded aversively to odors that indicated "predator" but not to novel odors. Similarly, Gorman (1984) found that voles (*Microtus arvalis*, *M. agrestis*) responded aversively to anal gland secretions of a predator (stoat, *Mustela erminea*) but not to those of a nonpredator (guinea pig, *Cavia porcellus*). White-tailed deer reacted aversively to predator but not to nonpredator urines in our study, thus providing additional evidence that herbivorous mammals can distinguish predator odors from nonpredator odors and adjust their behavior accordingly.

The effectiveness of bobcat and coyote urine placed in microcentrifuge tubes declined over time (Figure 1), suggesting that deer either habituated to the scents or that the repellent components of the odors were lost via evaporation or degradation. Similar reductions in efficiency of predator urine placed in tubes or vials were reported by Sullivan et al. (1985a,b), Sullivan (1986), and Swihart (1991). Innate responses to fear-provoking olfactory stimuli should not habituate (Bolles, 1970, Müller-Schwarze, 1974), although habituation in response to learned avoidance responses seems possible in the absence of occasional reinforcement. Experiments with naive mule deer suggested that aversive responses to predator odors may have a genetic component (Müller-Schwarze, 1972).

The prolonged effectiveness of bobcat and coyote urine when reapplied periodically (Figures 2 and 3) supports the idea that evaporative loss of volatile components of the urine was likely the cause of the gradual decline in effectiveness observed in trial 1. Volatility appears to be an important property of semiochemicals (Wheeler, 1976), and volatile constituents of red fox (*Vulpes vulpes*) urine and mustelid anal gland secretions repel snowshoe hares (Sullivan and Crump, 1986), voles (Sullivan et al., 1988a, 1990b), and pocket gophers (Sullivan et al., 1988b, 1990a). Moreover, fermented eggs, which are repellent to deer (Bullard et al., 1978b), contain amines and volatile fatty acids that are also found in anal gland secretions of canids (Preti et al., 1976, Bullard et al., 1978a).

An alternative explanation for the increased effectiveness of the predator

urines applied as a spray in our study is that components of the urine may function as taste repellents. Baines et al. (1988) recently patented a synthetic deer repellent containing felinine, a nonvolatile amino acid found in the urine of domestic cats and bobcats (Westall, 1953; Mattina et al., 1991), as well as the acidic component of lion (presumably *Panthera leo*) feces. However, we were unable to find published documentation of the repellency of these compounds. Our results with the dichloromethane extract of bobcat urine indicate that organic constituents of the urine were responsible for the repellency we observed. It is doubtful that felinine was present above trace quantities in the extract because it is a charged compound and should not partition into the nonaqueous phase. We have identified >25 volatile constituents of bobcat urine (Mattina et al., 1991), and further trials are planned to determine the repellency and mode of action of specific components of the urine.

From a practical perspective, reduction of damage by herbivorous mammals to agricultural crops could benefit from identification and synthesis of the actively repellent compounds in excreta or glandular secretions and from development of effective slow-release devices to enhance the compounds' long-term effectiveness. Significant progress in these areas has been made for semiochemicals from red fox (Jorgenson et al., 1978, Wilson et al., 1978, Sullivan and Crump, 1986) and mustelids (Crump, 1980a,b, Brinck et al., 1983, Sullivan and Crump, 1984, Sullivan et al., 1990a,b). Because of the importance of white-tailed deer as an agricultural pest in much of the eastern United States, further clarification of the role and utility of predator urines as repellents of deer is warranted.

Acknowledgments—We thank P.M. Picone and M. Day for their diligent assistance in the field and laboratory portions of this study. M.R. Conover graciously permitted us to use his unpublished data on deer density near Danbury, Connecticut. The manuscript was improved by the comments of M.R. Conover, P.M. Picone, T.P. Sullivan, and an anonymous reviewer.

REFERENCES

ANDERSON, J.P., JR. 1984. Deer damage in Connecticut. *Conn. Conserv. Assoc. Rep.* 10:1-12.

BAINES, D.A., FAULKES, C.G., TOMLINSON, A.J., and NING, P.C.Y. 1988. European Pat. Appl. EP 280, 443.

BOLLES, R.C. 1970. Species specific defense reactions and avoidance learning. *Psychol. Rev.* 77: 32-48.

BRINCK, C., ERLINGE, S., and SANDELL, M. 1983. Anal sac secretion in mustelids. *J. Chem. Ecol.* 9:727-745.

BULLARD, R.W., LEIKER, T.J., PETERSON, J.E., and KILBURN, S.R. 1978a. Volatile components of fermented egg, an animal attractant and repellent. *J. Agric. Food Chem.* 26:155-159.

BULLARD, R.W., SHUMAKE, S.A., CAMPBELL, D.L., and TURKOWSKI, F.J. 1978b. Preparation and evaluation of a synthetic fermented egg coyote attractant and deer repellent. *J. Agric. Food Chem.* 26:160-163.

CONOVER, M.R. 1984. Effectiveness of repellents in reducing deer damage in nurseries. *Wildl. Soc. Bull.* 12:399–404.

CONOVER, M.R., and KANIA, G.S. 1988. Browsing preference of white-tailed deer for different ornamental species. *Wildl. Soc. Bull.* 16:175–179.

CONOVER, W.J. 1980. Practical Nonparametric Statistics, 2nd ed. John Wiley & Sons, New York.

CRUMP, D.R. 1980a. Thietanes and dithiolanes from the anal gland of the stoat (*Mustela erminea*). *J. Chem. Ecol.* 6:341–347.

CRUMP, D.R. 1980b. Anal gland secretion of the ferret (*Mustela putorius* forma *furo*). *J. Chem. Ecol.* 6:837–844.

FRELICH, L.E., and LORIMER, C.G. 1985. Current and predicted long-term effects of deer browsing in hemlock forests in Michigan, USA. *Biol. Conserv.* 34:99–120.

FULK, G.W. 1972. The effect of shrews on the space utilization of voles. *J. Mammal.* 53:461–478.

GRAHAM, S.A. 1954. Changes in northern Michigan forests from browsing by deer. *Trans. North Am. Wildl. Conf.* 19:526–533.

GORMAN, M.L. 1984. The response of prey to stoat (*Mustela erminea*) scent. *J. Zool. (London)* 202:419–423.

HALL, E.R. 1981. The Mammals of North America, 2nd ed. John Wiley & Sons, New York.

JORGENSON, J.W., NOVOTNY, M., CARMACK, M., COPLAND, G.B., WILSON, S.R., and WHITTEN, W.K. 1978. Chemical scent constituents in the urine of the red fox (*Vulpes vulpes* L.) during the winter season. *Science* 119:797–798.

KATSMA, D.E., and RUSCH, D.H. 1979. Evaluation of deer damage in mature apple orchards, pp. 123–142, *in* J.R. Beck (ed.). Test Methods for Vertebrate Pest Control and Management Materials. American Society for Testing and Materials Standard Technical Publication 680, Philadelphia, Pennsylvania.

KURTÈN, B., and ANDERSON, E. 1980. Pleistocene Mammals of North America. Columbia University Press, New York.

LARSON, T.J., RONGSTAD, O.J., and TERBILCOX, F.W. 1978. Movement and habitat use of white-tailed deer in southcentral Wisconsin. *J. Wildl. Manage.* 42:113–117.

MARSTON, M.A. 1942. Winter relations of bobcats to white-tailed deer in Maine. *J. Wildl. Manage.* 6:328–337.

MARCHINTON, R.L., and HIRTH, D.H. 1984. Behavior, pp. 129–168, *in* L.K. Halls (ed.). White-tailed Deer Ecology and Management. Stackpole Books, Harrisburg, Pennsylvania.

MATTINA, M.J.I., PIGNATELLO, J.J., and SWIHART, R.K. 1991. Identification of the volatile components of bobcat (*Lynx rufus*) urine. *J. Chem. Ecol.* 17:451–462.

MECH, L.D. 1984. Predators and predation, pp. 189–200, *in* L.K. Halls (ed.). White-tailed Deer Ecology and Management. Stackpole Books, Harrisburg, Pennsylvania.

MELCHIORS, M.A., and LESLIE, C.A. 1985. Effectiveness of predator fecal odors as black-tailed deer repellents. *J. Wildl. Manage.* 49:358–362.

MÜLLER-SCHWARZE, D. 1972. Responses of young black-tailed deer to predator odors. *J. Mammal.* 53:393–394.

MÜLLER-SCHWARZE, D. 1974. Olfactory recognition of species, groups, individuals, and physiological states among mammals, pp. 316–326, *in* M.C. Birch (ed.). Pheromones. North Holland, Amsterdam.

NASH, G.B. 1974. Red, White, and Black: The Peoples of Early America. Prentice-Hall, Englewood Cliffs, New Jersey.

PALMER, W.L., WINGARD, R.G., and GEORGE, J.L. 1983. Evaluation of white-tailed deer repellents. *Wildl. Soc. Bull.* 11:164–166.

PHILLIPS, M., FORSHEY, C.G., WHITE, G.B., and RICHMOND, M.E. 1987. The economic impact of wildlife damage on Hudson Valley orchards. *Proc. East. Wildl. Damage Control Conf.* 3:66–82.

PRETI, G., MUETTERTIES, E.L., FURMAN, J.M., KENNELLY, J.J., and JOHNS, B.E. 1976. Volatile constituents of dog (*Canis familiaris*) and coyote (*Canis latrans*) anal sacs. *J. Chem. Ecol.* 2:177–186.

PURDY, K.G., SIEMER, W.F., POMERANTZ, G.A., and BROWN, T.L. 1987. Deer damage control preferences and use decisions of New York orchardists. *Proc. East. Wildl. Damage Control Conf.* 3:118–127.

SCOTT, J.D., and TOWNSEND, T.W. 1985. Methods used by selected Ohio growers to control damage by deer. *Wildl. Soc. Bull.* 13:234–240.

SEVERINGHAUS, C.W., and CHEATUM, E.L. 1956. Life and times of the white-tailed deer, pp. 57–186, *in* W.P. Taylor (ed.). The Deer of North America. Stackpole Books, Harrisburg, Pennsylvania.

SHUMAKE, S.A. 1977. The search for applications of chemical signals in wildlife management, pp. 357–376, *in* D. Müller-Schwarze and M.M. Mozell, (eds.). Chemical Signals in Vertebrates. Plenum, New York.

STODDART, D.M. 1980. The Ecology of Vertebrate Olfaction. Chapman and Hall, New York.

SULLIVAN, T.P. 1986. Influence of wolverine (*Gulo gulo*) odor on feeding behavior of snowshoe hares (*Lepus americanus*). *J. Mammal.* 67:385–388.

SULLIVAN, T.P., and CRUMP, D.R. 1984. Influence of mustelid scent-gland compounds on suppression of feeding by snowshoe hares (*Lepus americanus*). *J. Chem. Ecol.* 10:1809–1821.

SULLIVAN, T.P., and CRUMP, D.R. 1986. Feeding responses of snowshoe hares (*Lepus americanus*) to volatile constituents of red fox (*Vulpes vulpes*) urine. *J. Chem. Ecol.* 12:729–739.

SULLIVAN, T.P., NORDSTROM, L.O., and SULLIVAN, D.S. 1985a. Use of predator odors as repellents to reduce feeding damage by herbivores. I. Snowshoe hares (*Lepus americanus*). *J. Chem. Ecol.* 11:903–919.

SULLIVAN, T.P., NORDSTROM, L.O., and SULLIVAN, D.S. 1985b. Use of predator odors as repellents to reduce feeding damage by herbivores. II. Black-tailed deer (*Odocoileus hemionus columbianus*). *J. Chem. Ecol.* 11:921–935.

SULLIVAN, T.P., CRUMP, D.R., and SULLIVAN, D.S. 1988a. Use of predator odors as repellents to reduce feeding damage by herbivores. III. Montane and meadow voles (*Microtus montanus* and *Microtus pennsylvanicus*). *J. Chem. Ecol.* 14:363–377.

SULLIVAN, T.P., CRUMP, D.R., and SULLIVAN, D.S. 1988b. Use of predator odors as repellents to reduce feeding damage by herbivores. IV. Northern pocket gophers (*Thomomys talpoides*). *J. Chem. Ecol.* 14:379–389.

SULLIVAN, T.P., CRUMP, D.R., WIESER, H., and DIXON, E.A. 1990a. Response of pocket gophers (*Thomomys talpoides*) to an operational application of synthetic semiochemicals of stoat (*Mustela erminea*). *J. Chem. Ecol.* 16:941–949.

SULLIVAN, T.P., CRUMP, D.R., WIESER, H., and DIXON, E.A. 1990b. Comparison of release devices for stoat (*Mustela erminea*) semiochemicals used as montane vole (*Microtus montanus*) repellents. *J. Chem. Ecol.* 16:951–957.

SWIHART, R.K. 1991. Modifying scent-marking behavior to reduce woodchuck damage to fruit trees. *Ecol. Appl.* 1:98–103.

SWIHART, R.K., and CONOVER, M.R. 1990. Reducing deer damage to yews and apple trees: Testing Big Game Repellent^{R}, Ro·pel^{R}, and soap as repellents. *Wildl. Soc. Bull.* 18:156–162.

WESTALL, R.G. 1953. The amino acids and other ampholytes of urine. *Biochem. J.* 55:244–248.

WHEELER, J.W. 1976. Properties of compounds used as chemical signals, pp. 61–70, *in* D. Müller-Schwarze and M.M. Mozell (eds.). Chemical Signals in Vertebrates. Plenum, New York.

WILSON, S.R., CARMACK, M., NOVOTNY, M., JORGENSON, J.W., and WHITTEN, W.K. 1978. Isopentenyl methyl sulfide. A new terpenoid in the scent mark in the red fox (*Vulpes vulpes*). *J. Org. Chem.* 43:4675–4676.

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

CONFORMATIONAL ANALYSES OF PERIPLANONE ANALOGS BY MOLECULAR MECHANICS CALCULATIONS

KAZUKO SHIMAZAKI,[1,*] MASATAKA MORI,[1] KENTARO OKADA, TATSUJI CHUMAN,[1,4] HITOSHI GOTŌ,[2,5] EIJI ŌSAWA,[2,5] KAZUHISA SAKAKIBARA,[3] and MINORU HIROTA[3]

[1]*Life Science Research Laboratory, Japan Tobacco Inc.*
6-2 Umegaoka, Midori-ku
Yokohama, Kanagawa 227, Japan

[2]*Department of Chemistry, Faculty of Science*
Hokkaido University
8 Kitajyujyo-nisi, Kita-ku
Sapporo, Hokkaido 060, Japan

[3]*Department of Material Engineering, Faculty of Engineering*
Yokohama National University
156 Tokiwadai, Hodogaya-ku
Yokohama, Kanagawa 240, Japan

(Received September 10, 1990; accepted December 12, 1990)

Abstract—Conformational parameters of pheromonally active analogs (**1** and **2**) of periplanones, the sex pheromones of the American cockroach, were investigated by molecular mechanics calculations. They existed in several conformers with small energy differences. These results were supported by NMR analysis. The structural features of the conformers of the analogs were compared with X-ray structures of periplanones.

Key Words—Conformational analysis, molecular mechanics, structure–activity relationships, periplanone, periplanone analogs, sex pheromone, *Periplaneta americana* L., American cockroach, Orthoptera, Blattidae.

*To whom correspondence should be addressed.
[4]Present address: Head Office, Japan Tobacco Inc., 2-2-1 Toranomon, Minato-ku, Tokyo 105, Japan.
[5]Present address: Department of Knowledge-Based Information Engineering, Toyohashi University of Technology, 1-1 Hibarigaoka, Tempakucho, Toyohashi 440, Japan.

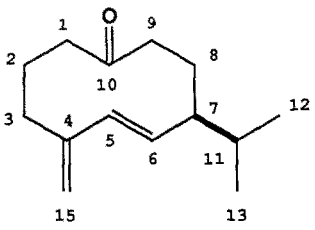
0098-0331/91/0400-0779$06.50/0

INTRODUCTION

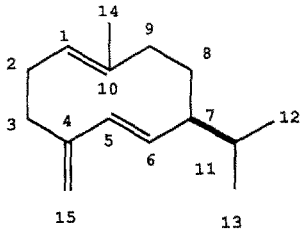
The utilization of molecular mechanics (MM) calculations on the conformational analysis of organic molecules has become an active area of research. We have already reported the application of MM analysis on the conformational property of insect pheromone serricornin (Chuman et al., 1990).

Periplanones-A (P-A) (**3**) (Hauptmann et al., 1986a) and -B (P-B) (**4**) (Figure 1) (Persoons et al., 1974) are the major sex pheromone components produced by female american cockroach (*Periplaneta americana* L.). Their activity is extremely high: threshold 10^{-5} μg for **3** and 10^{-7} μg for **4**. The X-ray crystallographic analyses of them (P-A: Kuwahara and Mori, 1989; Mori et al., 1990; P-B: Hauptmann et al., 1986b) have established that their 10-membered rings adopt similar twist-chair (TC) conformations.

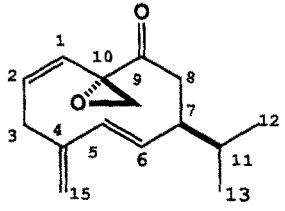
Recently, we reported the conformational analyses of periplanones and their analogs by the use of NMR and X-ray crystallographic analyses, and pointed out the conformational resemblances among the bioactive analogs (Mori et al., 1990). However, the structurally simplified analog (**1**) [(4*S*, 5*E*)-7-methylene-4-(1-methylethyl)-5-cyclodecen-1-one] and germacrene-D (**2**) (Tahara et al.,



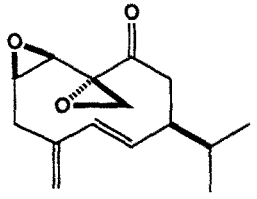
Analog (1)



Germacrene-D (2)



Periplanone-A (3)



Periplanone-B (4)

FIG. 1. Structures of analog (**1**), germacrene-D (**2**), periplanones-A (**3**) and -B (**4**). The same numbering scheme for the C atoms is employed in this paper as has been used for P-B by Persoons et al. (1974).

1975) (activity thresholds: 1 μg for **1** and 10 μg for **2**) did not yield crystalline samples suitable for X-ray analysis, and their complicated NMR spectra did not provide sufficient information (NOEs and $^3J_{HH}$'s) for conformational analyses. Thus, we attempted to investigate the conformational properties of these compounds by MM calculations to overcome the difficulty of the structural assignments by conventional methods.

METHODS AND MATERIALS

The details of the preparations and purity determinations of compounds **1–4** have been described in our previous paper. (Mori et al., 1990) Their bioassay data also have been reported. (Okada et al., 1990) The synthesis of racemic compound (**1**) was first reported by Schreiber and Santini (1984). [^{1}H]NMR (500 MHz) spectra were recorded at 27°C in C_6D_6 solution with Me_4Si as internal standard.

Molecular Mechanics Calculations. MM2 molecular mechanics program (Burkert and Allinger, 1982; version '87: Molecular Design Ltd., Farallon Drive, San Leandro, California, U.S.A.) was employed to the structural optimizations. The three-dimentional structures were visualized and superimposed using QUANTA program (Polygen Co., Waltham, Massachusetts, U.S.A.) run on an IRIS-4D/80GT computer system (Silicon Graphics Inc., Mountain View, California, U.S.A.).

The initial geometries of the ring conformations, including the rotamers, regarding isopropyl group were exhaustively generated by CONFLEX2 program (Gotō and Ōsawa, 1989; QCPE #592). They were all geometry optimized by MM2 to identify the most stable conformations. The Boltzmann population of each energy minimum at 25°C was calculated on the basis of their steric energies.

The conformers of 10-membered ring conformation on the aspect of dihedral angles were defined using a new nomenclature (in CONFLEX2, unpublished).[6]

Calculations of Vicinal Coupling Constants ($^3J_{HH}$). Averaged vicinal H/H coupling constants ($^3J_{HH}$) were calculated on MM2-geometry-optimized struc-

[6]A note on the ring nomenclature: In view of inconveniences inherent to the current ring nomenclatures, we devised the following new definition in CONFLEX2. Details will be described elsewhere. 1. Classify all the endocyclic bonds in a ring structure as g^+, g^- and anti. 2. When two contiguous gauches of the same sign exist, the midpoint is a corner. 3. When two contiguous gauches of the opposite sign exist, the midpoint is a pseudocorner, if at least one side is connected to anti. 4. Number of bonds between corners and/or pseudocorners is entered in sequence between brackets, in such a way that the smallest possible integer results. 5. When a pseudocorner appears, the number of bonds is primed. When more than two possibilities exist, corner is preferred to pseudocorner.

tures of **1** and **2** using Altona's empirical modification of Karplus equation (Haasnoot et al., 1980). These calculations were based on 93 energy minima of **1** and 47 of **2**, which were found by MM2 calculation.

RESULTS and DISCUSSION

Conformational Analysis by Molecular Mechanics Calculation

Analog 1. CONFLEX2 generated 577 initial geometries for **1**, which were structurally optimized by MM2, to identify 93 energy minima. The number of major conformers over 5% Boltzmann population at 25°C ($\Delta SE < 1.1$ kcal/mol) was five, and the number over 1% population ($\Delta SE < 2.1$ kcal/mol) was 12. The conformational parameters of the major five conformers are summarized in Table 1. The perspective drawings of these structures are reproduced in Figure 2. Regarding the ring conformation, the major five conformers can be classified into three types. For example, both **1A** and **1D** (the rotamer of **1A** with respect to the isopropyl group) possess [1′324′] type ring conformations. Similarly, both **1B** and **1E** (the rotamer of **1B**) possess [1′4′1′4′] type ring conformations. The relative ratio of ring conformations of the stable conformers of **1** at 25°C is [1′324′]:[1′4′1′4′]:[132′4′] = 56:30:14. As shown in Table 1, the dihedral angles along C6-C7-C8-C9-C10-C1 and C7–C11 atoms are practically coincident in **1A–1E** structures. Therefore, the ring conformation involving C7 isopropyl and C10 carbonyl groups are well conserved in the major conformers, in contrast to the flexible C2–C5 region of the ring.

The most stable conformer **1A** has similar conformational features as the X-ray structure of periplanones. The superimposition (Figure 3) clearly showed the overlapping of **1A** and the X-ray structure of P-B (**4**). A significant match on the location of C10 carbonyl oxygen of **1A** with that of the spiro-epoxy group in **4** was also noteworthy.

Germacrene-D (2). CONFLEX2 generated 626 initial geometries for **2**, and their structural minimization by MM2 identified 47 energy minima. There is no predominant conformer, and five conformers are found within ΔSE 0.5 kcal/mol; 11 over 1% population ($\Delta SE < 1.6$ kcal/mol). The conformational parameters of the major five conformers are summarized in Table 2. The perspective drawings of these structures are shown in Figure 4. As regards the ring conformation, the stable conformers can be classified into three types as well as those of **1**. The relative ratio of ring conformations of the major five conformers of **2** at 25°C is [1′324′]:[1′3′2′4′]:[232′3′] = 34:35:31. As can be seen from Figures 2 and 4, conformer **2A** has features analogous to **1C**. This analogy is found in the cases of **2B/1B**, **2C/1A**, **2D/1D**, and **2E/1E**, respectively. All five stable conformers have similar dihedral angles along the C6-C7-C8-C9-C10-C1-C2 regions of the rings (Table 2). Among the conformers, the

TABLE 1. CONFORMATIONAL PARAMETERS OF **1**

	1A	**1B**	**1C**	**1D**	**1E**	P-A[a] (**3**)	P-B[a] (**4**)
Ring conformation	[1′324′]	[1′4′1′4′]	[132′4′]	[1′324′]	[1′4′1′4′]	[123′1′3′]	[123′1′3′]
ΔSE (kcal/mol)	0.0	0.38	0.63	0.69	1.03		
Boltzmann dist. (%) at 25°C	34.08	18.08	11.74	10.71	5.98		
Dihedral angle (degree)							
C10–C1–C2–C3	−58.7	−80.9	−75.8	−59.9	−81.6	−3.5	−1.5
C1–C2–C3–C4	−49.5	66.1	73.8	−51.1	65.3	−93.3	−90.0
C2–C3–C4–C5	82.0	−76.3	−78.4	80.1	−75.4	72.0	80.0
C3–C4–C5–C6	24.6	140.5	−15.1	24.8	141.3	39.9	29.5
C4–C5–C6–C7	−166.7	−165.8	166.4	−165.2	−164.4	−167.0	−166.4
C5–C6–C7–C8	113.9	92.2	−68.1	119.4	92.7	114.4	110.1
C6–C7–C8–C9	−48.4	−55.7	−46.8	−52.3	−58.4	−50.0	−40.8
C7–C8–C9–C10	74.5	73.9	75.4	73.5	73.5	92.5	95.6
C8–C9–C10–C1	−133.7	−149.1	−126.8	−129.2	−147.4	140.4	−148.9
C9–C10–C1–C2	151.2	149.0	151.4	153.3	150.9	106.4	97.3
C9–C8–C7–C11	−171.2	−178.8	−173.1	−178.7	174.5	−174.6	−166.4
C8–C7–C11–H11	60.3	60.0	58.4	−53.7	−59.4	64.2	57.7

[a]The data of P-A and P-B from their X-ray crystallographic analyses.

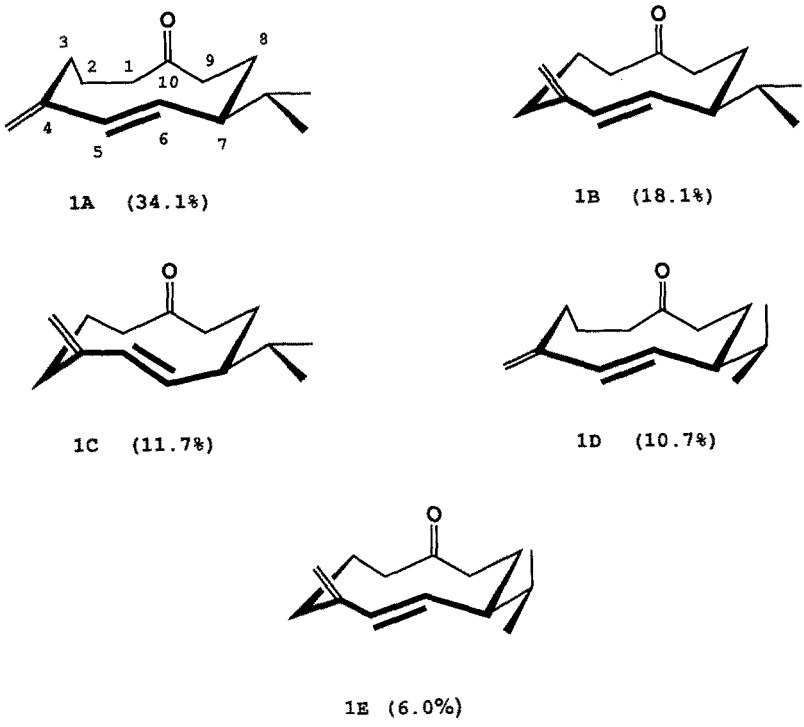


FIG. 2. Major conformers of analog **1**.

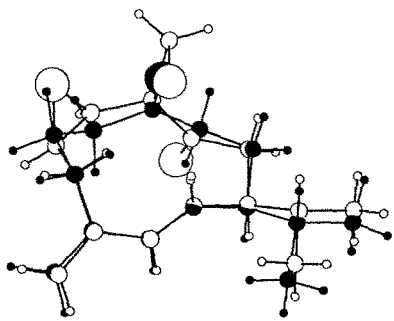


FIG. 3. Superimposition of analog **1** (conformer **1A**) (filled) and P-B.

orientations of methyl group at C10, which extends vertically above the ring plane, are well retained. The third conformer **2C** is most superimposable to P-B (**4**) (Figure 5).

Shizuri et al. (1986) proposed the most stable conformations of **2** by MM

TABLE 2. CONFORMATIONAL PARAMETERS OF **2**

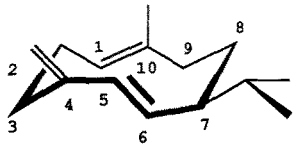
	2A	**2B**	**2C**	**2D**	**2E**
Ring conformation	[232'3']	[1'3'2'4']	[1'324']	[1'324']	[1'3'2'4']
ΔSE (kcal/mol)	0.00	0.21	0.24	0.47	0.47
Boltzmann dist. (%) at 25°C	23.76	16.60	15.83	10.82	10.79
Dihedral angle (degree)					
C10-C1-C2-C3	−99.0	−114.4	−79.3	−79.2	−115.5
C1-C2-C3-C4	65.3	56.7	−53.5	−54.7	56.7
C2-C3-C4-C5	−69.8	−69.4	69.8	68.7	−69.6
C3-C4-C5-C6	−20.3	128.7	27.1	27.6	127.7
C4-C5-C6-C7	167.5	−166.3	−167.5	−165.6	−164.3
C5-C6-C7-C8	−65.3	106.7	126.5	129.3	108.6
C6-C7-C8-C9	−48.8	−59.9	−52.5	−55.8	−62.5
C7-C8-C9-C10	71.0	67.7	65.8	65.8	67.8
C8-C9-C10-C1	−104.1	−115.1	−106.0	−104.0	−113.5
C9-C10-C1-C2	164.7	167.0	166.7	167.2	167.5
C9-C8-C7-C11	−175.2	177.5	−175.4	178.0	171.1
C8-C7-C11-H11	60.1	59.5	60.9	−52.1	−57.2

calculations and reported that **2** adopted two main conformations **2E** (CC) and **2C** (TC) in the relative ratio at 25°C of CC : TC : others = 82 : 17 : 1. The most stable conformation, **2A**, identified in our work was not found by them. Our result was more reliable in terms of the exhaustive generation of initial geometries by CONFLEX2 and the use of an improved version of MM2 program for the calculations.

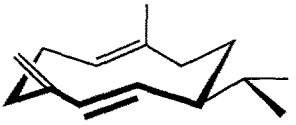
Comparisons of Calculated and Observed NMR Data

To verify the conformational properties of **1** and **2** predicted by MM calculation, their calculated NMR parameters ($^3J_{HH}$'s) were compared with those observed experimentally.

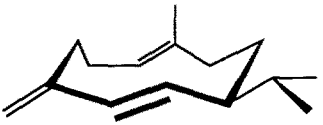
Coupling Constants ($^3J_{HH}$'s). Because of the high multiplicity and overlappings of the [^{1}H]NMR spectra of **1** and **2**, their coupling constants, except those of well-separated olefinic and methyl signals, have not been determined in our previous work. In the present study, further examination of the 1.0–2.7 ppm region of the spectrum of **1** enabled us to assign the splitting patterns of the signals of C3 and C8 geminal proton pairs. For the four vicinal couplings between the protons of H2 and H3, as well as for H8 and H9, the population-weighted coupling constants were calculated on the basis of the MM2 structures and their Boltzmann populations. As shown in Table 3, the calculated values



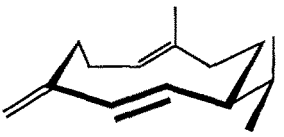
2A (23.8%)



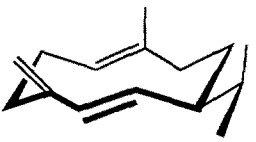
2B (16.6%)



2C (15.8%)



2D (10.8%)



2E (10.8%)

FIG. 4. Major conformers of germacrene-D (2).

essentially agreed with those observed, while the values were not accurately coincident (Masamune, et al., 1986).

CONCLUSIONS

The MM calculations on the periplanone analogs **1** and **2** revealed that they exist in several conformers with small energy differences. These results were coincident with those of NMR analysis. Regarding the ring conformation, the conformational parameters of **1** and **2** resemble each other. In the case of **2**, the substitution of the C10 carbonyl group of **1** into the methyl group and the presence of the C1–C10 *trans* double bond do not cause any drastic change on the conformation, although they must contribute to the difference of the relative ratio and the order of the conformers between **1** and **2**.

The conformers **1A** and **2C** adopt ring conformations very similar to those of natural pheromones established by X-ray analyses, and the other stable conformers can be regarded as partially altered ("flapped") ones. The similarity

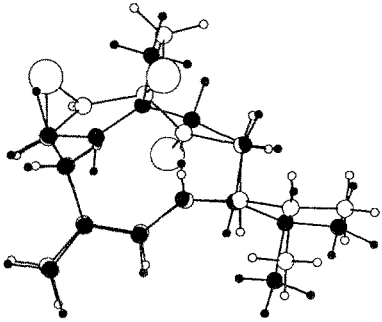
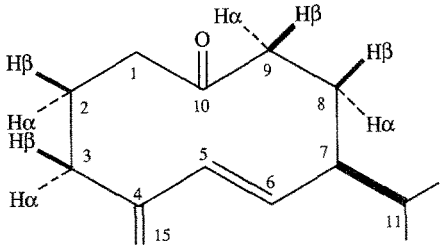


FIG. 5. Superimposition of germacrene-D (**2**) (conformer **2C**) (filled) and P-B.

TABLE 3. COMPARISON BETWEEN OBSERVED AND CALCULATED VALUES OF VICINAL NMR COUPLING CONSTANTS FOR **1**[a]

	J values (H_z) between protons							
	2α-3α	2β-3α	2α-3β	2β-3β	8α-9α	8β-9α	8α-9β	8β-9β
Calcd.	3.76	9.29	6.87	3.60	1.65	5.94	13.10	1.60
Obsd.	~1.0	12.5	5.9	1.5	2.9	4.4	11.8	2.2

[a]



of structure is not a sufficient condition for activity. For instance, there are many examples from enzyme-substrate binding studies in which the X-ray structures of enzymes do not necessarily represent the active conformers. However, we have already mentioned that the X-ray structures of the periplanones were well explained by their NMR analyses, and the close conformational resemblance between natural pheromones and the bioactive analogs was shown by X-ray and NMR analyses (Mori et al., 1990). Thus, examination of the structural resemblances of the most stable conformers of the analogs **1** and **2** with that of **4** is useful in correlating structure with activity.

The activity thresholds for the natural periplanones are 10^{-7}–10^{-5} μg, while they are 1 and 10 μg for **1** and **2**, respectively. Since the removal and

substitution of the oxygen-containing functionalities on **3** and **4** greatly reduce the activity (Mori et al., 1990), these significant decrease in activity are attributed to the lack of electrostatic effects of the functionalities, which can strongly interact with the pheromone receptor. As each of the analogs exits in a mixture of conformers, it may also contribute to the low activity.

The present work showed that the results obtained by MM analysis should be of great value for understanding structure–activity relationships for the type of compounds studied.

REFERENCES

BURKERT, U., and ALLINGER, N.L. 1982. Molecular mechanics. ACS Monograph 177. American Chemical Society. Washington, D.C.

CHUMAN, T., SHIMAZAKI, K., MORI, M., OKADA, K., GOTŌ, H., ŌSAWA, E., SAKAKIBARA, K., and HIROTA, M. 1990. Conformational analysis of serricornin application of molecular mechanics calculations to stereochemical assignment of serricornin, the sex pheromone of cigarette beetle (*Lasioderma serricorne* F.). *J. Chem. Ecol.* 16:2877–2888.

GOTŌ, H., and ŌSAWA, E. 1989. Corner flapping: A simple and fast algorithm for exhaustive generation of ring conformations. *J. Am. Chem. Soc.* 111:8950–8951.

HAASNOOT, C.A.G., DE LEEUW, F.A.A.M., and ALTONA, C. 1980. The relationship between proton–proton NMR coupling constants and substituent electronegativities-I. *Tetrahedron* 36:2783–2792.

HAUPTMANN, H., MÜHLBAUER, G., and SASS, H. 1986a. Identifizierung und Synthese von Periplanon A. *Tetrahedron Lett.* 27:6189–6192.

HAUPTMANN, H., MÜHLBAUER, G., and WALKER, N.P.C. 1986b. Synthese und Kristallstruktur von (±)-Periplanone B. *Tetrahedron Lett.* 27:1315–1318.

KUWAHARA, S., and MORI, K. 1989. Clarification of structure of Persoons's periplanone-A, an artifact derived from Hauptmann's periplanone-A. *Tetrahedron Lett.* 30: 7447–7450.

MASAMUNE, S., MA, P., MOORE, R.E., FUJIYOSHI, T., JAIME, C., and ŌSAWA, E. 1986. Computation of vicinal coupling constants in tetra- and hexa-alditol peracetates using molecular mechanics. A rational approach to conformational analysis in solution. *J. Chem. Soc., Chem. Commun.* 1986:261–263.

MORI, M., OKADA, K., SHIMAZAKI, K., CHUMAN, T., KUWAHARA, S., KITAHARA, T., and MORI, K. 1990. X-ray crystallographic and NOE studies on the conformation of periplanones and their analogues. *J. Chem. Soc. Perkin Trans. 1* 1990:1769–1777.

OKADA, K., MORI, M., KUWAHARA, S., KITAHARA, T., MORI, K., SHIMAZAKI, K., and CHUMAN, T. 1990. Behavioral and electroantennogram responses of male American cockroaches to periplanones and their analogs. *Agric. Biol. Chem.* 54:575–576.

PERSOONS, C.J., RITTER, F.J., and LICHTENDONK, W.J. 1974. Sex pheromones of american cockroach periplaneta americana. Isolation and partial identification of two excitants. *Proc. Kon. Ned. Akad. Wetensch. Amsterdam.* C77:201 (*Chem. Abstr.* 81:88 209f).

SCHREIBER, S.L., and SANTINI, C. 1984. Cyclobutene bridgehead olefin route to the american cockroach sex pheromone, periplanone-B. *J. Am. Chem. Soc.* 106:4038–4039.

SHIZURI, Y., YAMAGUCHI, S. TERADA, Y., and YAMAMURA, S. 1986. Biomimetic syntheses of oppositol, oplopanone, and aphanamol II from germacrene-D. *Tetrahedron Lett.* 27:57–60.

TAHARA, S., YOSHIDA, M., MIZUTANI, J., KITAMURA, C., and TAKAHASHI, S. 1975. A sex stimulant to the male American cockroach in the compositae plants. *Agric. Biol. Chem.* 39:1517–1518.

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

IDENTIFICATION OF MAJOR COMPONENT OF MALE-PRODUCED AGGREGATION PHEROMONE OF LARGER GRAIN BORER, *Prostephanus truncatus* (Horn) (COLEOPTERA: BOSTRICHIDAE)

A. CORK,[1,*] D.R. HALL,[1] R.J. HODGES,[1] and J.A. PICKETT[2]

[1]*Natural Resources Institute*
Central Avenue, Chatham Maritime
Chatham, Kent ME4 4TB, England

[2]*AFRC Institute of Arable Crops Research*
Rothamsted Experimental Station
Harpenden, Herts. AL5 2JQ, England

(Received October 12, 1988; accepted December 26, 1990)

Abstract—Male beetles of the larger grain borer, *Prostephanus truncatus* Horn (Coleoptera: Bostrichidae) were shown to produce material causing an electroantennographic (EAG) response from both male and female beetles. Volatiles from mixed sex cultures were collected on Porapak Q and fractionated by liquid chromatography (LC) with gradient elution. The total volatiles and the fractions were analyzed by gas chromatography (GC) linked to EAG, and two compounds eluting in the same LC fraction elicited EAG responses from male and female beetles. These were assumed to be components of the male-produced aggregation pheromone. Amounts of these compounds obtained were very low and less than 10^{-3} times the amounts of the aggregation pheromone components produced by the related species, *Rhyzopertha dominica*, under similar conditions. The most abundant of these EAG-active compounds was identified as 1-methylethyl (2*E*)-2-methyl-2-pentenoate by comparison of its GC retention times and mass spectrum with those of synthetic analogs. The synthetic compound elicited electrophysiological and behavioral responses in both male and female beetles significantly greater than those to structural analogs. This compound is attractive to *P. truncatus* beetles in the field, and has been given the trivial name of "trunc-call 1".

Key Words—Storage pests, aggregation pheromone, *Prostephanus truncatus*, larger grain borer, Coleoptera, Bostrichidae; 1-methylethyl (2*E*)-2-methyl-2-pentenoate.

*To whom correspondence should be addressed.

INTRODUCTION

The larger grain borer, *Prostephanus truncatus*, is a pest of maize and cassava and was previously considered to be a sporadic, minor pest essentially restricted to Central America, Mexico, and the southern states of the United States (Fisher, 1950; McGuire and Crandall, 1967). In 1981, its presence was confirmed in Tanzania (Dunstan and Magazini, 1981; Hodges et al., 1983a), and subsequently it has been reported in other countries in East and West Africa (Kega and Warui, 1983; Harnisch and Krall, 1984; Krall, 1984; Muhihu and Kibata, 1985). It has become established rapidly, and in Tanzania, it is now frequently the major pest in farm-stored maize, capable of causing weight losses in excess of 30% during six months (Hodges et al., 1983a; Hodges, 1986; McFarlane, 1988).

In order to survey the distribution of this pest and to determine the effectiveness of control measures against it, a simple and reliable method of detection is required. In the past, this has been undertaken by extensive sampling and visual observation. More recently, Hodges et al. (1983b) reported the use of traps for monitoring *P. truncatus* in farm stores. These were baited with dominicalures 1 and/or 2, components of the aggregation pheromone of the related lesser grain borer, *Rhyzopertha dominica*, identified by Williams et al. (1981). This paper describes the identification of one of the components of the male-produced aggregation pheromone of *P. truncatus*, undertaken with the aim of improving the specificity and effectiveness of traps for monitoring this pest. A preliminary report of this work was given by Hodges et al. (1984).

METHODS AND MATERIALS

Pheromone Collection and Purification

Prostephanus truncatus beetles from a culture of Tanzanian origin were maintained on maize kernels in 3-liter round-bottomed flasks that had been silanized with a mixture of trimethylsilyl chloride and hexamethyl-disilazane in dichloromethane. Air was drawn into the flask through a charcoal inlet filter (12 × 2 cm; 24 g; 10–18 mesh), and entrained volatiles were collected in two glass tubes packed with Porapak Q (50–80 mesh; 2.5 g; 4 × 1.5 cm). The Porapak was purified by washing with dichloromethane before use.

The cultures were maintained at 25 °C under subdued lighting, and aeration was carried out continuously at 1 liter/min. The collection tubes were extracted twice weekly by percolating through dichloromethane (25 ml). Typically, collections were made from two cultures, each containing approximately 1000 beetles of both sexes, over a three-week period. The Porapak extracts were

combined and evaporated to approximately 0.5 ml on a rotary evaporator at room temperature.

The residue was subjected to liquid chromatography (LC) on a Florisil column (100–200 mesh; 10 g; 30 × 1 cm) using gradient elution with 50-ml portions of pentane–ether mixtures containing 0, 2, 5, 10, 20, 30, 50, 75, and 100% diethyl ether. Fractions of 10 ml were collected and 2-μl aliquots checked for biological activity by electroantennography (EAG).

The effectiveness of the above procedures for collection, work-up, and chromatography of the pheromone was checked by treating a culture of *Rhyzopertha dominica* in the same manner.

Breakthrough times of model compounds on Porapak Q were studied using an empty 3-liter, round-bottomed flask with charcoal inlet filter as above. Microgram quantities of synthetic compounds were placed on a small square of aluminum foil (2 × 2 cm) in the flask, and air was drawn through the flask at 4 liters/min. Volatiles were collected on two tubes packed with purified Porapak Q (50–80 mesh; 0.1 g; 1 cm × 4 mm) connected in series. The second tube was changed at intervals to check for compounds breaking through the first tube. The Porapak was extracted with dichloromethane (1 ml), a known amount of hexyl or octyl acetate added as internal standard, and the solution analyzed directly by capillary gas chromatography (GC).

In order to determine the origin of the pheromone, five freshly emerged *P. truncatus* beetles of each sex were placed in separate screw-topped vials (8 × 2 cm) with a filter paper (7 cm diam.; Whatman No. 52) for three days. The filter paper was cut into three and the pieces inserted into separate Pasteur pipets for assay of biological activity by EAG (see below). Beetles were sexed according to the procedure of Shires and McCarthy (1976).

Gas Chromatography (GC)

Numerous GC columns and running conditions were used during the course of this work, but the definitive data presented here was obtained with the following columns: 1.8 × 2 mm ID glass columns in a Varian 2100 packed with (A) 2.5% SE 30 + 0.25% Carbowax 20 M and (B) 5% Carbowax 20 M on 100–120 mesh Chromosorb G AW DMCS, temperature programmed from 60°C at 4°C/min with nitrogen carrier gas flow of 25 ml/min; 25 m × 0.32 mm ID fused silica capillary columns in a Carlo Erba Fractovap 4160, coated with (C) nonpolar CP Sil 5 CB (chemically bonded methylsilicone; Chrompack) and (D) polar CP Wax 57 CB (chemically bonded Carbowax 20 M; Chrompack), with helium carrier gas linear velocity of 25 cm/sec, splitless injection at 220°C and the oven held at 40°C for 2 min, then raised to 50°C or 60°C at 20°C/min and temperature programmed at 1°C/min.

Electroantennography (EAG)

EAG responses from *P. truncatus* were recorded using modifications of basic techniques described previously for Lepidoptera (Moorhouse et al., 1969; Nesbitt et al., 1977) and Diptera (Hall et al., 1984). Adult *P. truncatus* were restrained by means of a metal strip in a hole drilled to fit the beetle in a Perspex disk, as described by Angst and Lanier (1979). The antennae were laid and held dorsal surface uppermost on a thin bed of plasticine. The glass recording electrode was inserted through a small hole cut in the distal segment of one antenna by means of a micro-knife (Beavor Micro-sharp, Waltham, Massachusetts, USA), and the reference electrode was inserted into a similar hole in the basal segment of the other antenna.

Crude mixtures of volatiles or fractions from LC were tested as samples (1 μl) syringed onto the inner wall of a new Pasteur pipet. The solvent was allowed to evaporate for 30 sec before positioning the pipet in such a way that the tip was 1 cm above the EAG preparation, and a 3-sec pulse of nitrogen (100 ml/min) was blown through the pipet. An interval of 3 min was allowed between tests. At least three replicates were recorded for each sample, and the results are reported as the mean $\pm$ SD. Filter papers exposed to beetles were placed in a Pasteur pipet and tested under similar conditions. Synthetic compounds were tested as samples (100 ng) in hexane (1 μl) deposited on filter paper (Whatman No. 1; 1-cm disks) in Pasteur pipets. Solvent was removed with a 3-sec pulse of nitrogen (500 ml/min), and the sample delivered to the antennal preparation in a 1-sec pulse of nitrogen (500 ml/min). Stimuli were given in random sequence at 1-min intervals, and each compound was tested three times on each of 10 insects (four males and six females). Mean responses were normalized to the mean response to 1-methylethyl (2*Z*)-2-methyl-2-pentenoate [Figure 1 (II)] for each insect. There were no obvious differences in absolute or relative magnitudes of the responses of male and female beetles, and results for each sex

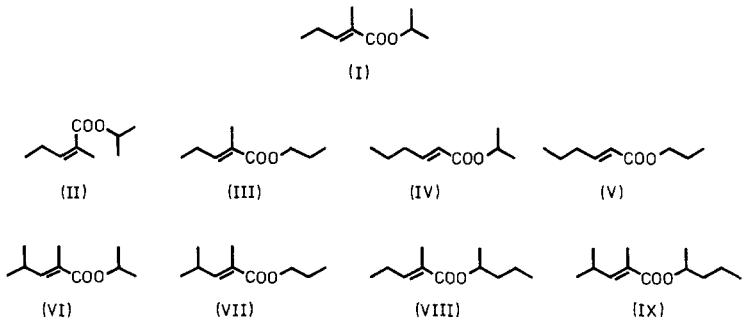


FIG. 1. Structures of synthetic compounds I–IX.

were combined. Mean responses for each compound were subjected to analysis of variance and differences between means tested for significance by Duncan's new multiple-range Test (DNMRT) (Duncan, 1955).

Linked GC-EAG analyses were carried out essentially as described by Moorhouse et al. (1969) by splitting the GC column effluent and pulsing one part at intervals over the antennal preparation. For analyses with the fused silica capillary columns, the column effluent was split 50:50 using a low-dead-volume connector (SGE) without makeup gas. One half went to the flame ionization detector, the other half to a small, three-necked reservoir (volume approx. 5 ml) through which nitrogen (100 ml/min) was pulsed for 3 sec at 17-sec intervals into a constant stream of nitrogen (100 ml/min) passing over the antennal preparation.

Mass Spectrometry

For gas chromatography coupled to mass spectrometry (GC-MS) a Pye 204 instrument was used with the column effluent led directly to the source of the mass spectrometer (VG Micromass 70-70F with Data System 2025) with electron impact ionization at 70 eV, 200°C. The GC injection system consisted of a short length of silanized glass-lined steel tube fitted into the injection port and connected directly to the column (25 m × 0.22 mm ID fused silica capillary column coated with OV-101; oven temperature held at 50°C for 10 min and then programmed at 2°/min to 100°C; helium carrier gas at 0.7 kg/cm^2).

Synthesis

For this work, (2*E*)-2-methyl-2-pentenoic acid and (2*E*)-2,4-dimethyl-2-pentenoic acid were synthesized as outlined previously by Hodges et al. (1983b) (Figure 2), although the former compound is now obtainable from Aldrich Chemical Co. (2*E*)-2-Hexenoic acid was obtained from Aldrich Chemical Co. The corresponding acid chlorides were produced by treating the acids with a slight excess of freshly distilled oxalyl chloride at room temperature, and these

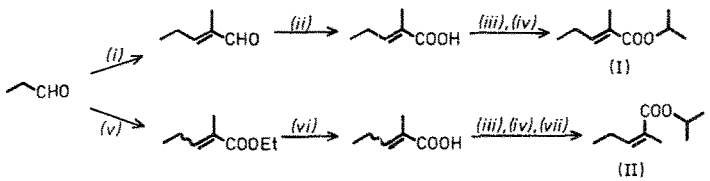


FIG. 2. Synthesis of the *E* (I) and *Z* (II) isomers of 1-methylethyl 2-methyl-2-pentenoate. Reagents: (i) aqueous NaOH; (ii) aqueous silver oxide; (iii) oxalyl chloride; (iv) 2-propanol; (v) triethylphosphonopropionate/KOBu^t/THF; (vi) aqueous KOH/EtOH; (vii) Florisil chromatography.

were converted to esters by reaction with a slight excess of 1-propanol, 2-propanol or (±)-2-pentanol in the absence of solvent. After aqueous work-up, the esters were purified by short-path distillation to give products that were greater than 95% pure by capillary GC analysis with the expected infrared (IR), [^{1}H]nuclear magnetic resonance (NMR), and mass spectra (MS). Structures of the compounds synthesized are shown in Figure 1.

1-Methylethyl (2E)-2-methyl-2-pentenoate (I) (Figure 2). (2*E*)-2-Methyl-2-pentenal was prepared on 2 M scale in 69% yield by addition of propionaldehyde to sodium hydroxide (cf. Nielsen and Houlihan, 1968). This was oxidized to (2*E*)-2-methyl-2-pentenoic acid on 0.4 M scale in 83% yield with aqueous silver oxide (cf. Pearl, 1963). Freshly distilled oxalyl chloride (32 g, 0.25 M) was added dropwise to (2*E*)-2-methyl-2-pentenoic acid (20 g, 0.2 M) in a dry, round-bottomed flask with stirring. Effervescence occurred, and, after 1 hr at room temperature, the mixture was heated gently for a few minutes. Excess oxalyl chloride then was removed on a rotary evaporator to give the crude acid chloride, IR (film) 1750 (s), 1640 (m) cm^{-1}. This was treated with 2-propanol (15 g, 0.25 M), and, after standing overnight, the IR spectrum indicated reaction was complete. The mixture was added to saturated brine, the layers separated, and the aqueous layer extracted twice with petroleum spirit (bp 40–60°C, 100 ml). The combined organic extracts were washed once with dilute sodium hydroxide solution and twice with saturated brine, dried over magnesium sulfate, and the solvent removed on a rotary evaporator. The residue was distilled to give a main fraction bp 79°C/20 mm of 1-methylethyl (2*E*)-2-methyl-2-pentenoate (23 g, 74%). Capillary GC analysis showed a major peak accounting for >97% of the volatile material. Both GC and NMR analyses showed the presence of approx. 1.5% of the *Z* isomer. IR (film): 1710 (s), 1650 (m) cm^{-1}; NMR (270 MHz, $CDCl_3$): δ 1.02 (3H, t, J = 7.7 Hz, $\underline{CH}_3-CH_2$), 1.27 (6H, d, J = 6.3 Hz, $\underline{CH}_3-CH$), 1.81 (3H, broad singlet, $\underline{CH}_3-C=C$), 2.18 (2H, broad quint, J = 7.5 Hz, $CH_3-\underline{CH}_2-C=C$), 5.05 (1H, sept, J = 6.3 Hz, $\underline{CH}-O$), 6.72 (1H, broad triplet, J = 7.7 Hz, $\underline{CH}_2-CH=C$).

1-Methylethyl (2Z)-2-methyl-2-pentenoate (II) (Figure 2). Reaction of triethylphosphonopropionate with potassium *tert*-butoxide in tetrahydrofuran at room temperature followed by addition of propionaldehyde at −78°C gave ethyl 2-methyl-2-pentenoate in 67% yield after flash chromatography on silica gel. GC analysis showed the product was a 27:73 mixture of the *Z* and *E* isomers, and this ratio was only improved to 31:69 by using trimethylphosphonopropionate as recommended by Nagaoka and Kishi (1981). The mixture of ethyl esters was hydrolyzed with 1 N potassium hydroxide in 1:1 water–ethanol at room temperature to give the corresponding acids in 86% yield with no change in isomeric ratio. The acids were converted to the 1-methylethyl esters as above in 68% yield, again with no change in isomeric ratio. Interestingly, esterification with 2-propanol using *N*, *N*′-dicyclohexylcarbodiimide and catalytic 4-pyr-

rolidinopyridine in dichloromethane (Hassner and Alexanian, 1978) caused essentially complete isomerization to the *E* isomer. The mixture of 1-methylethyl esters was separated by chromatography on Florisil eluted with 1% diethyl ether in petroleum spirit (bp 40–60°C). The *Z* isomer was eluted first and was purified by short-path distillation (bp 75°C/20 mm). GC and NMR analyses showed less than 0.1% of the *E* isomer. IR (film): 1712 (s), 1648 (m) cm^{-1}; NMR (250 MHz, $CDCl_3$): δ 1.01 (3H, t, J = 7.5 Hz, $C\underline{H}_3-CH_2$), 1.28 (6H, d, J = 6.3 Hz, $C\underline{H}_3-CH$), 1.87 (3H, overlapping double triplet, J = 1.5, 1.5 Hz, $C\underline{H}_3-C=C\underline{H}-CH_2$), 2.43 (2H, pentet of quartets, J = 7.5, 1.5 Hz, $CH_3-C\underline{H}_2-CH=C-CH_3$), 5.07 (1H, heptet, J = 6.3 Hz, $C\underline{H}-O$), 5.87 (1H, triplet of quartets, J = 7.5, 1.5 Hz, $CH_2-C\underline{H}=C-CH_3$). Spectral data for the *E* and *Z* isomers agree with those of Chan et al. (1968) for the methyl esters.

Behavioral Bioassay

Synthetic compounds were bioassayed in chambers made from plaster of Paris disks (8.5 cm diam. × 1.3 cm deep). A cartwheel pattern of channels (4 × 3 mm deep) was molded into the upper surface of each disk, consisting of a circular channel (7.5 cm outer diameter) with six equally spaced radial channels to the center. Holes (0.8 cm diam. × 1.3 cm deep) were drilled at the center and where each radial joined the outer circle. The chambers were covered with plastic lids fitted with a deep red filter (Kodak No. 70) to prevent external lighting disturbing beetles placed in them. Bioassays were performed in a darkened room at 27°C and 70% relative humidity. Male and female beetles, 7–14 days old, were removed from cultures at least four days prior to testing and conditioned on wheat flour at a density of not more than one beetle per 5 g.

Synthetic compounds (50 μg) were impregnated into the walls of polyethylene vials (1 × 0.8 cm; 1.5-mm-thick walls) by placing a solution of the compound in pentane (100 μl) in the vial, allowing the solvent to evaporate, and sealing the vial. The test vial was placed in one of the outer holes of the bioassay chamber, and untreated vials were placed in the other five outer holes and the central hole, the tops of the sources being flush with the bottom of the channels. A single beetle was placed at the center of the chamber, and observations were made by shining a light through the lid. The position of the test beetle relative to the treated vial was recorded every minute for the first 10 min of the bioassay and then every 5 min for the next 20 min. The extent to which the beetle was attracted or arrested by the test material was scored by giving one point if the beetle was observed resting within 1 cm of the treated dispenser or 2 points if it was stationary on it. After a test, the treated dispenser was discarded and the bioassay chamber baked in a ventilated oven at 60°C to remove any residual volatile materials.

Bioassays on synthetic compounds were replicated 20 times. The score for each test was expressed as a percentage of the maximum possible score (28), and these were subjected to an arcsine transformation followed by one-way analysis of variance and the Newman-Keuls multiple range test. As no significant difference could be found between the responses of male and female beetles, the results for the two tests were pooled.

RESULTS

Isolation of Biologically Active Material

Attempts to use the laboratory bioassay described above to demonstrate production of a pheromone by either sex of *P. truncatus* did not give statistically meaningful results. However, modification of the techniques used with Lepidoptera made it possible to record EAG responses from *P. truncatus*, and significant EAG responses were obtained from either sex to air blown over filter papers that had been exposed to male beetles, but not to air blown over filter papers exposed to female beetles (e.g., EAG responses from female 1.38 $\pm$ 0.15 mV and 0.15 $\pm$ 0.04 mV respectively; response to air blown over clean filter paper 0.0 mV). These results suggested that the male beetles produced an aggregation pheromone detected by both sexes.

As it is extremely time-consuming to sex large numbers of *P. truncatus* reliably and without damage to the beetles (Shires and McCarthy, 1976), volatiles were collected from large mixed-sex cultures on maize kernels. The crude mixtures of volatiles from these cultures also elicited significant EAG responses from male and female *P. truncatus*. Analyses of the crude mixtures by linked GC-EAG using packed and capillary columns defined two areas of EAG activity that occurred consistently in different collections. With both nonpolar and polar GC columns, the first of these coincided with a characteristic triplet of GC peaks. These were subsequently shown by GC-MS to be the isomeric diethylbenzenes, impurities leached from the Porapak.

On LC separation of the crude mixture of volatiles, these diethylbenzenes eluted with pentane, while EAG activity was confined to fractions eluting with 5% ether in pentane: e.g., EAG response to crude mixture of volatiles 1.18 mV; responses to five fractions eluted with 5% ether in pentane 0.09, 0.35, 1.76, 0.32, 0.18 mV; responses to solvents and other LC fractions <0.10 mV. Linked GC-EAG analyses of the active LC fractions showed the same two areas of EAG activity as were found on analysis of the crude mixture: e.g., EAG responses to active GC fractions were 1.66 mV and 1.55 mV, with all other fractions giving zero response or slight positive polarizations. The GC retention data for these EAG-active fractions 1 and 2 are shown in Table 1, and they were assumed to contain components of the male-produced aggregation pheromone of *P. truncatus*.

TABLE 1. GC RETENTION DATA FOR COMPOUNDS ON FUSED SILICA CAPILLARY COLUMNS

Compound	ECL[a]	
	CP Sil 5CB (C)	CP Wax 57CB (D)
EAG-active component 1	6.40	6.24
EAG-active component 2	9.54	9.91
I	6.40	6.24
II	5.95	5.49
III	7.03	7.15
Z isomer of III	6.54	6.30
IV	6.62	6.73
Z isomer of IV	6.18	5.88
V	7.18	7.55
Z isomer of V	6.72	6.72
VI	6.76	6.29
Z isomer of VI	6.23	5.46
VII	7.41	7.19
Z isomer of VII	6.87	6.24
VIII (dominicalure 1)	8.20	7.88
Z isomer of VIII	7.69	7.03
IX (dominicalure 2)	8.52	7.87
Z isomer of IX	7.95	6.93
Peak width at half height	0.012	0.023

[a]Equivalent chain lengths relative to the retention times of acetates of straight-chain, saturated alcohols (Harris and Habgood, 1966); retention times of hexyl, heptyl and octyl acetates: column C, 7.18, 10.35, and 13.83 min; column D, 7.57, 10.25, and 13.27 min.

The first area of activity coincided with a small GC peak, which would have represented 1–2 μg obtained from two cultures, each containing approximately 1000 beetles and entrained for three weeks. In these collections, only nanogram quantities of a component in the fraction causing the second EAG response were obtained, and it was not possible to obtain sufficient material of the requisite purity for further identification work.

It was thought possible that active material was being lost at some stage during the collection, work-up, and fractionation of the volatiles. As a check on this, a culture of *R. dominica* was treated in an identical manner. A single culture containing approximately 1000 beetles of mixed sex entrained for three weeks gave 2.81 mg of dominicalure 1, 1-methylbutyl (2*E*)-2-methyl-2-pentenoate (VIII), and 2.66 mg of dominicalure 2, 1-methylbutyl (2*E*)-2,4-dimethyl-2-pentenoate (IX) (ratio 1.06:1). LC purification gave greater than 90% recovery, the components eluting with 5% ether in pentane.

Following identification of the major pheromone component of *P. trun-*

catus, it was apparent that this is more volatile than the two dominicalures, and the effectiveness of the collection procedure was checked with synthetic pheromone. There was no breakthrough using 0.1 g Porapak and a flow rate of 4 liters/min for 24 hr, equivalent to 2.5 g Porapak at a flow rate of 0.5 liters/min for 200 days.

It was thus concluded that, under the conditions used, the major component of the aggregation pheromone of *P. truncatus* was not being lost and that it is produced at 10^{-3} the rate of each component of the pheromone of *R. dominica*.

Identification of Major EAG-Active Component

GC-MS analysis was carried out on the EAG-active LC column fractions. The mass spectrum of the first EAG-active GC peak (Figure 3a) showed similarities with that reported by Williams et al. (1981) for dominicalure 1, 1-methylbutyl (2*E*)-2-methyl-2-pentenoate (VIII), particularly dominant peaks at *m/e*

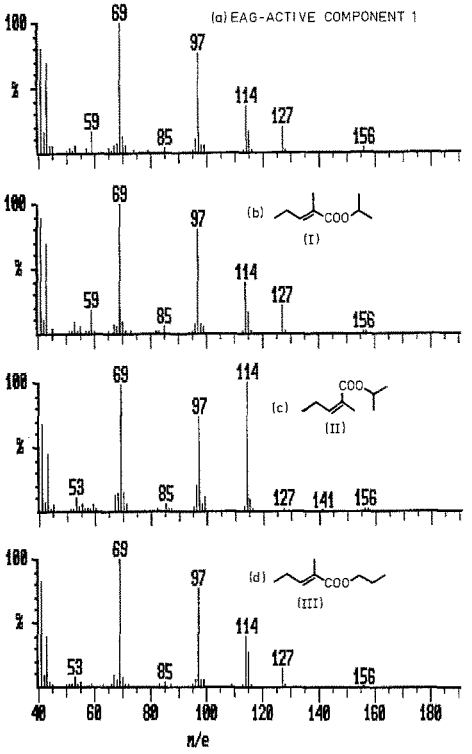


FIG. 3. Mass spectra of (a) natural and (b–h) synthetic compounds.

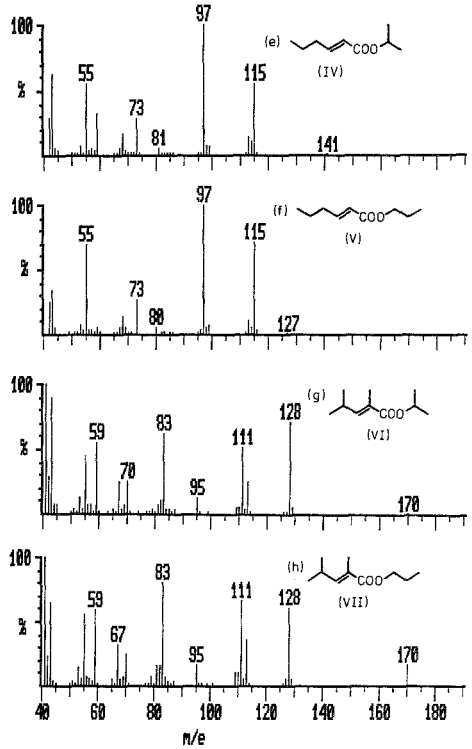


FIG. 3. Continued.

97 [$CH_3CH_2CH{=}C(CH_3)CO^+$ ·] and *m/e* 69 [$CH_3CH_2CH{=}C(CH_3)^+$ ·]. There was a possible molecular ion at *m/e* 156 corresponding to dominicalure 1 with two less carbon atoms ($C_9H_{16}O_2$). Other significant peaks occurred at *m/e* 114 [$CH_3CH_2CH{=}C(CH_3)COOH^+$ ·] (cf. *m/e* 115 in the spectrum of dominicalure 1) and *m/e* 127 ($M^+ - 29$). The mass spectrum suggested the propyl esters of 2-methyl-2-pentenoic acid as candidate structures, and these were synthesized as the *E* isomers (I) and (III). The mass spectrum of the 1-methylethyl (isopropyl) ester (I) (Figure 3b) was superimposable on that of the naturally occurring active component (Figure 3a) and that of the propyl ester (III) (Figure 3d) was similar. However, only compound I cochromatographed with the natural EAG-active compound on both nonpolar and polar capillary GC columns (Table 1). 1-Methylethyl (2*Z*)-2-methyl-2-pentenoate (II), the *Z* isomer of (I), also was prepared and shown to have different GC retention times (Table 1) and a significantly different mass spectrum (Figure 3c) from that of the natural EAG-active compound.

Other candidate structures of formula $C_9H_{16}O_2$ were the propyl esters of

2-hexenoic acid. The *E* isomers (IV and V) were synthesized, but these differed in their GC retention times (Table 1) and their mass spectra (Figure 3e and 3f) from those of the natural pheromone component. Also synthesized were the two propyl esters (VI) and (VII) of (2*E*)-2,4-dimethyl-2-pentenoic acid, the acid moiety of dominicalure 2, 1-methylbutyl (2*E*)-2,4-dimethyl-2-pentenoate (IX) (Williams et al., 1981). The GC retention times for these two compounds are shown in Table 1 and their mass spectra in Figure 3g and 3h.

Small (approx 0.5–1.5%) amounts of the corresponding *Z* isomers were detected in the synthesized *E* isomers of all the above compounds by GC-MS analysis with selective ion monitoring using the two most abundant ions in the mass spectrum of the *E* isomer. The GC retention times of these *Z* isomers are shown in Table 1.

GC analyses of biologically active fractions from LC of volatiles collected from cultures of *P. truncatus* showed that none of the compounds listed in Table 1, except compound I, was present (less than 2% of I).

Biological Activities of Synthetic Compounds

The EAG response from *P. truncatus* to compound I was compared with the responses to the other compounds synthesized. In order to demonstrate that the EAG activity used as a criterion in the isolation and identification is correlated with some behavioral activity, the synthetic compounds were also tested in a laboratory bioassay designed to show attractant and/or arrestant activity. Results are shown in Figure 4. By both EAG and bioassay, 1-methylethyl (2*E*)-2-methyl-2-pentenoate (I) was the most active compound, followed by 1-methylethyl (2*E*)-2,4-dimethyl-2-pentenoate (VI). Replacing the branched acid and/or alcohol moieties by straight-chain analogs reduced activity. The dominicalures 1 and 2 (VIII and IX) were also significantly less active than I in both assay systems, although this may have been due, at least in part, to the higher molecular weights and lower volatilities of the latter compounds. The stimulus in the EAG tests would thus be expected to contain a lower concentration of the dominicalures than of the other test compounds, and the release rate of dominicalure 2 (IX) from the polythene vial dispensers used in the bioassay is known to be lower than that of compound I (Hodges et al., 1984). The *Z* isomer of I, compound II, was not available for bioassay, but in the EAG tests it showed only low activity.

DISCUSSION

Two compounds have been detected in volatiles collected from mixed-sex cultures of *P. truncatus* that elicit EAG responses from male and female beetles. The amounts of material produced were exceptionally small, less than 10^{-3} the

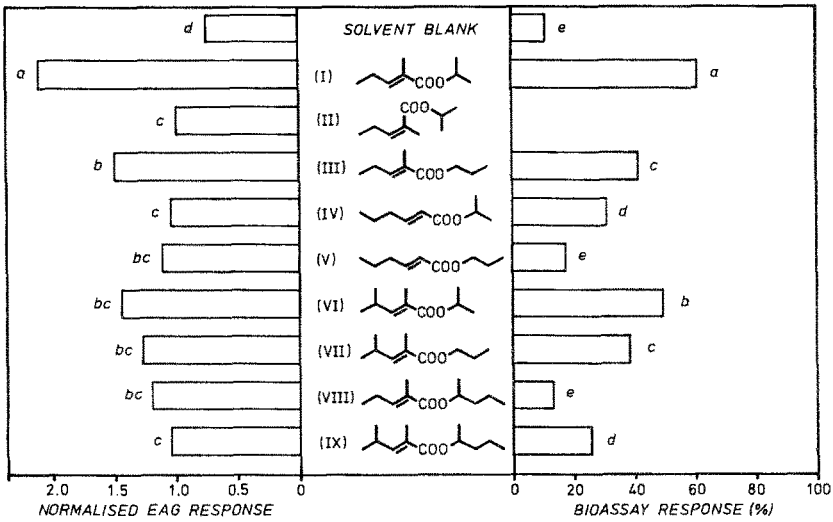


FIG. 4. EAG and bioassay responses of male and female *P. truncatus* beetles to synthetic compounds I–IX. EAG responses are means of three replicates on each of 10 insects normalized to response to compound II for each insect; responses with the same letter are not significantly different at the 5% level by DNMRT. Bioassay responses are mean percentages of maximum possible score for 20 replicates, and responses with the same letter are not significantly different at the 5% level by Newman-Keuls multiple range test after arcsine transformation of the data; compound II was not available for bioassay.

amounts of the aggregation pheromone components produced by the related species, *R. dominica*, under identical conditions. It was not possible to obtain sufficient material for identification of the minor component in this work, but the major EAG-active component was identified as 1-methylethyl (2*E*)-2-methyl-2-pentenoate (I) by comparison of its GC retention times on polar and nonpolar capillary columns and its mass spectrum with those of a range of synthetic compounds. Compound I is the isopropyl ester of the acid moiety of dominicalure 1 (VIII), one of the components of the male-produced aggregation pheromone of the related bostrichid species, *R. dominica*, identified by Williams et al. (1981).

Synthetic I was more active to *P. truncatus* than structurally related compounds in EAG tests, and this EAG activity was correlated with behavioral activity in a laboratory bioassay demonstrating attractant and/or arrestant activity. In field tests carried out in maize stores in Togo, traps made of corrugated cardboard sprayed with permethrin and baited with a polyethylene vial impregnated with 2 mg of compound I caught over twice as many *P. truncatus* beetles as similar traps baited with 2 mg of dominicalure 2 (IX) (Hodges et al., 1984).

EAG studies showed that active material is produced by the male beetles only, and 1-methylethyl (2*E*)-2-methyl-2-pentenoate (I) is proposed as the major component of the male-produced aggregation pheromone of *P. truncatus*. The trivial name "trunc-call 1" has been suggested for this compound (P. Dobie, NRI, personal communication).

During the course of writing up this work, modification of the techniques used for collection of pheromone gave sufficient of the minor EAG-active compound for it to be identified as 1-methylethyl (2*E*,4*E*)-2,4-dimethyl-2,4-heptadienoate. Lures containing mixtures of this compound with I have been shown to be significantly more attractive to *P. truncatus* beetles than lures containing I alone in both stores (Dendy et al., 1991) and the open field (Dendy et al., 1989a,b). Details of the identification of the structure of this minor pheromone component will be published separately.

Acknowledgments—Thanks are due to Mrs. Jane Smith and Mrs. Julie Dendy of NRI for assistance with the entrainment and bioassay work respectively, and to Dr. Rosemary White of NRI for synthesis of compound II.

REFERENCES

ANGST, M.E., and LANIER, G.N. 1979. Electroantennogram responses of two populations of Ips pini (Coleoptera: Scolytidae) to insect-produced and host tree compounds. *J. Chem. Ecol.* 5:131–140.

CHAN, K.C., JEWELL, R.A., NUTTING, W.H., and RAPOPORT, H. 1968. The synthesis and stereochemical assignment of *cis*- and *trans*-2-methyl-2-pentenoic acid and the corresponding esters, aldehydes, and alcohols. *J. Org. Chem.* 33:3382–3385.

DENDY, J., DOBIE, P., SAIDI, J.A., SMITH, J.L., and URONU, B. 1989a. Trapping the larger grain borer *Prostephanus truncatus* in maize fields using synthetic pheromones. *Entomol. Exp. Appl.* 50:241–244.

DENDY, J., DOBIE, P., SAIDI, J., and SHERMAN, C. 1989b. The design of traps for monitoring the presence of *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) in maize fields. *J. Stored Prod. Res.* 25:187–191.

DENDY, J., SAIDI, J.A., SMITH, J.L., and URONU, B. 1991. Trials to assess the effectiveness of new synthetic pheromone mixtures for trapping *Prostephanus truncatus*. *J. Stored Prod. Res.* 27:69–74.

DUNCAN, D.B. 1955. Multiple range and multiple *F* tests. *Biometrics* 11:3382–3385.

DUNSTAN, W.R., and MAGAZINI, I.A. 1981. Outbreaks and new records. Tanzania. The larger grain borer on stored products. *FAO Plant Prot. Bull.* 29:80–81.

FISHER, W.S. 1950. A review of the North American species of beetles belonging to the family Bostrichidae. U.S. Department of Agriculture, Miscellaneous Publication No. 698. 157 pp.

HALL, D.R., BEEVOR, P.S., CORK, A., NESBITT, B.F., and VALE, G.A. 1984. A potent olfactory stimulant and attractant for tsetse isolated from cattle odours. *Insect Sci. Appl.* 5:335–339.

HASSNER, A., and ALEXANIAN, V. 1978. Direct room temperature esterification of carboxylic acids. *Tetrahedron Lett.* 19:4475–4478.

HARNISCH, R., and KRALL, S. 1984. Further distribution of the larger grain borer in Africa. *FAO Plant Prot. Bull.* 32:113–114.

HARRIS, W.E., and HABGOOD, H.W. 1966. Programmed Temperature Gas Chromatography. John Wiley & Sons, New York.

HODGES, R.J. 1986. The biology and control of *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae)—destructive storage pest with an increasing range. *J. Stored Prod. Res.* 22:1–14.

HODGES, R.J., DUNSTAN, W.R., MAGAZINI, I., and GOLOB, P. 1983a. An outbreak of *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) in East Africa. *Prot. Ecol.* 5:183–194.

HODGES, R.J., HALL, D.R., GOLOB, P., and MEIK, J. 1983b. Responses of *Prostephanus truncatus* to components of the aggregation pheromone of *Rhyzopertha dominica* in the laboratory and the field. *Entomol. Exp. Appl.* 34:266–272.

HODGES, R.J., CORK, A., and HALL, D.R. 1984. Aggregation pheromones for monitoring the greater grain borer, *Prostephanus truncatus*. British Crop Protection Conference—Pests and Diseases, Brighton. pp. 255–259.

KEGA, V.K., and WARUI, C.W. 1983. *Prostephanus truncatus* in Coast Province, Kenya. *Trop. Stored Prod. Inf.* 46:2.

KRALL, S. 1984. A new threat to farm-level maize storage in West Africa: *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae). *Trop. Stored Prod. Inf.* 50:26–31.

MCFARLANE, J.A. 1988. Pest management strategies for *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) as a pest of stored maize grain: present status and prospects. *Trop. Pest . Manage.* 34:121–132.

MCGUIRE, J.U., and CRANDALL, B.S. 1967. Survey of insect pests and plant diseases of selected food crops of Mexico, Central America and Panama. U.S. Department of Agriculture. 157 pp.

MOORHOUSE, J.E., YEADEN, R., BEEVOR, P.S., and NESBITT, B.F. 1969. Method for use in studies of insect chemical communication. *Nature* 223:1174–1175.

MUHIHU, S.K., and KIBATA, G.N. 1985. Developing a control programme to combat an outbreak of *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) in Kenya. *Trop. Sci.* 25:239–248.

NAGAOKA, H., and KISHI, Y. 1981. Further studies on rifamycin S. *Tetrahedron* 37:3873–3888.

NESBITT, B.F., BEEVOR, P.S., HALL, D.R., LESTER, R., STERNLICHT, M., and GOLDENBERG, S. 1977. Identification and synthesis of the female sex pheromone of the citrus flower moth, *Prays citri*. *Insect Biochem.* 7:355–359.

NIELSEN, A.T., and HOULIHAN, W.J. 1968. The aldol condensation. *Org. React.* 16:1–438.

PEARL, I.A. 1963. Vanillic acid. *Org. Syn. Coll.* 4:972–973.

SHIRES, S.W., and MCCARTHY, S. 1976. A character for sexing live adults of *Prostephanus truncatus* (Horn) (Bostrichidae, Coleoptera). *J. Stored Prod. Res.* 12:273–275.

WILLIAMS, H.J., SILVERSTEIN, R.M., BURKHOLDER, W.E., and KHORRAMSHAHI, A. 1981. Dominicalure 1 and 2: Components of aggregation pheromone from male lesser grain borer *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae). *J. Chem. Ecol.* 7:759–780.

Journal of Chemical Ecology, Vol. 17, No. 4, 1991

DEFENSIVE SECRETION OF *Tenebrio molitor* (COLEOPTERA: TENEBRIONIDAE)[1]

A.B. ATTYGALLE,[2] C.L. BLANKESPOOR,[3] J. MEINWALD,[2] and T. EISNER[3,*]

[2]*Department of Chemistry*
[3]*Section of Neurobiology and Behavior*
Cornell University
Ithaca, New York 14853

(Received December 27, 1990; accepted January 2, 1991)

Abstract—The defensive secretion of *Tenebrio molitor* contains a mixture of 2-methyl-1,4-benzoquinone and *m*-cresol. The phenol had not previously been detected in the secretion, although some investigators reported presence of 2-ethyl-1,4-benzoquinone as a second component. We failed to detect the latter quinone in secretion samples from three laboratory populations of *T. molitor*.

Key Words—2-Methyl-1,4-benzoquinone, *m*-cresol, *Tenebrio molitor*, Coleoptera, Tenebrionidae, defensive glands.

INTRODUCTION

As part of a study of how parasitization encumbers an insect's ability to use its defensive glands, we recently had occasion to look into the chemistry of the defensive secretion of *Tenebrio molitor*, a beetle commonly maintained in laboratory culture and used worldwide in biological research. Unexpectedly, our findings about the composition of the beetle's secretion differed from those previously reported (Schildknecht, 1959; Tschinkel, 1975a; Kanehisa, 1978). We here present our results.

* To whom correspondence should be addressed.
[1] Paper No. 100 of the series *Defense Mechanisms of Arthropods*; No. 99 is Storey et al., *J. Chem. Ecol.*, 17, 687–693 (1991).

0098-0331/91/0400-0805$06.50/0

METHODS AND MATERIALS

Tenebrio molitor. The defensive glands of *T. molitor*, and the mechanism by which the glands are everted for defensive use, have been described (Tschinkel, 1975b,c). We used beetles from three sources: (1) our Cornell culture (replenished periodically with individuals purchased from Rainbow Mealworms, Compton, California); (2) S.C. Johnson & Sons, Inc., Racine, Wisconsin; and (3) Department of Entomology, Kansas State University, Manhattan, Kansas.

We "milked" the beetles of secretion by squeezing them gently with forceps so as to cause them to evert the glands, then wiping the glands with small pieces of filter paper, and transferring the papers to vials with hexane for extraction. Beetles were milked without regard to sex. Milkings of beetles from any one source were lumped, providing three samples [beetles milked per sample = 10 (Cornell); 4 (Wisconsin); 6 (Kansas)].

Instrumentation. Gas chromatography [Hewlett-Packard (= HP) 5890 equipped with flame ionization detector] was performed on a 25-m × 0.22-mm fused-silica capillary column coated with DB-5 (J. & W. Scientific). Temperature program: 40°C for 4 min, then to 260°C at 8°C/min.

Infrared and mass spectra were obtained with a HP 5890 gas chromatograph linked in series to a HP 5965A FTIR detector and a HP 5970 mass selective detector (MSD). Mass spectra were also obtained on a HP 5890 gas chromatograph linked to a Finnigan ion trap detector (ITD 800).

RESULTS

Capillary gas chromatography of the Cornell secretion sample (Figure 1) revealed two primary peaks, accounting for about 99.5% of the volatile components. Mass spectra (ITD and MSD) of the first component were closely similar to those given for 2-methyl-1,4-benzoquinone (McLafferty and Stauffer, 1989) and matched those of an authentic sample of this compound. The natural compound and the authentic sample also had identical gas-phase FTIR spectra.

The mass spectrum of the second component showed that it was a cresol [*m/z*(%), 108(M^+, 82), 107(100), 90(7), 79(42), 77(40), 63(6), 53(11), 51(11)], but could not unambiguously resolve the isomeric structure of the compound (McLafferty and Stauffer, 1989). However, differences that we determined between the *o*, *m*, and *p* isomers of cresol by FTIR spectrometry (Figure 2) allowed unambiguous identification of the unknown as *m*-cresol.

As further evidence for the nature of the components, peaks 1 and 2 (Figure 1) had chromatographic retention times identical to those of authentic 2-methyl-1,4-benzoquinone and *m*-cresol, respectively. Of the three cresols,

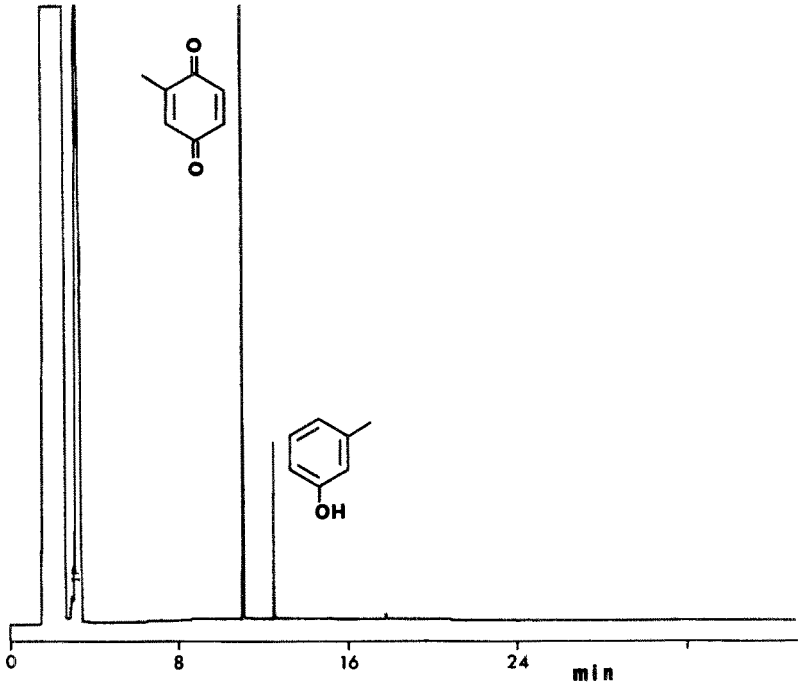


FIG. 1. Gas chromatogram (hexane) of *Tenebrio molitor* defensive secretion (Cornell sample). See text for column and temperature program.

o-cresol had the shortest retention time; *m*- and *p*-cresol eluted closely together, in that order.

The secretion samples from the Wisconsin and Kansas beetles proved to contain the same two primary components. There were some differences in the ratio of the components in the three samples (Table 1).

DISCUSSION

The finding that our three samples contained the same two primary compounds leads us to believe that 2-methyl-1,4-benzoquinone and *m*-cresol are characteristic components of the *T. molitor* secretion. Schildknecht (1959) previously had reported the presence of 2-methyl-1,4-benzoquinone in the secretion of this beetle. Tschinkel (1975a) and Kanehisa (1978) also reported presence of this quinone, but claimed the additional presence of 2-ethyl-1,4-benzoquinone. Kanehisa (1978) based his identification of the latter compound on TLC and packed column retention data only. We found no evidence for the presence of this quinone, which we would have detected at the 2-ng level.

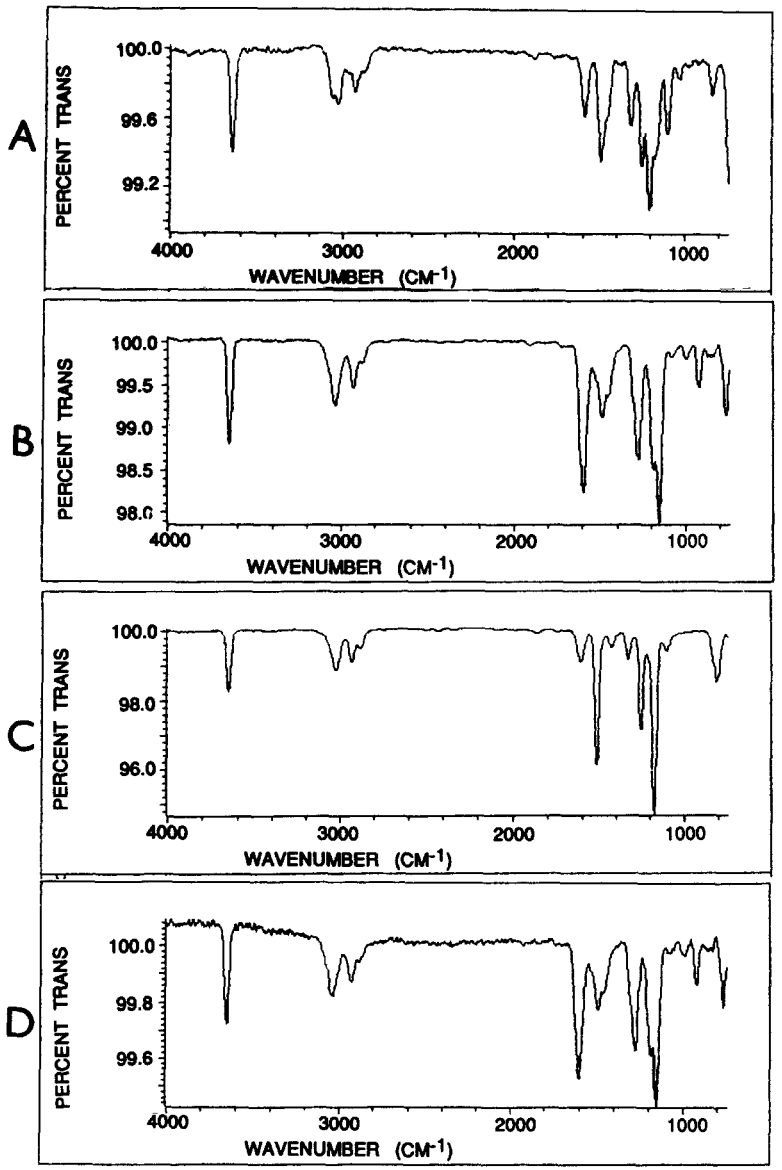


FIG. 2. Gas-phase FTIR spectra of authentic *o*-cresol (A), *m*-cresol (B), and *p*-cresol (C), and of the second-eluting component (Figure 1) of the *Tenebrio molitor* defensive secretion (D). Resolution = 8 cm^{-1}.

TABLE 1. RELATIVE PERCENT OF MAJOR COMPONENTS IN DEFENSIVE SECRETION OF *Tenebrio molitor* FROM THREE SOURCES

Source	2-Methyl-1,4-benzoquinone	*m*-Cresol
Cornell	87.5	12.5
Wisconsin	97.0	3.0
Kansas	68.0	32.0

Acknowledgments—Supported in part by grants AI-02908 and AI-12020 from NIH, and a NSF predoctoral fellowship to C.L.B. We thank Richard Hoebeke for identification of *T. molitor*, and Robert Martin from S.C. Johnson and Sons and Nahid Tavakkol from Kansas State University for shipment of beetles.

REFERENCES

KANEHISA, K. 1978. Comparative study of the abdominal defensive systems in tenebrionid beetles. *Ber. Ohara. Inst. Landwirtsch. Biol. Okayama Univ.* 17:47–56.

MCLAFFERTY, F.W., and STAUFFER, D.B. 1989. The Wiley/NBS Registry of Mass Spectral Data. John Wiley, New York.

TSCHINKEL, W.R. 1975a. A comparative study of the chemical defensive system of tenebrionid beetles: Chemistry of the secretions. *J. Insect Physiol.* 21:753–783.

TSCHINKEL, W.R. 1975b. A comparative study of the chemical defensive system of tenebrionid beetles. III. Morphology of the glands. *J. Morphol.* 145:355–370.

TSCHINKEL, W.R. 1975c. A comparative study of the chemical defensive system of tenebrionid beetles. Defensive behavior and ancillary features. *Ann. Entomol. Soc. Am.* 68:439–453.

SCHILDKNECHT, H. 1959. Über das flüchtige Sekret vom gemeinen Mehlkäfer. *Angew. Chem.* 71:524.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

ISOLATION AND IDENTIFICATION OF 4,6,8-TRIMETHYL-7,9-UNDECADIEN-5-OL, A FEMALE-SPECIFIC COMPOUND, IN TERGAL GLAND SECRETION OF *Cryptocercus punctulatus* SCUDDER (DICTYOPTERA: CRYPTOCERCIDAE)

J.L. LE QUERE,[1] R. BROSSUT,[2,*] C.A. NALEPA,[3] and O. BONNARD[2]

[1]*INRA, Laboratoire de Recherches sur les Aromes*
17 rue Sully, 21040 Dijon, France

[2]*Université de Bourgogne*
Laboratoire de Zoologie
CNRS UA 674, 21100 Dijon, France

[3]*Entomology Department*
North Carolina State University
Raleigh, North Carolina 27695-7613

(Received April 11, 1989; accepted January 7, 1991)

Abstract—The secretion of the tergal glands of *Cryptocercus punctulatus* consists of a complex mixture of 27 compounds. Of this mixture, only one compound (compound B) is specific for females. By dissecting 200 tergal glands, 50 μg of pure compound B was collected by preparative GC; it was identified as (*Z*, E-4,6,8-trimethyl-7,9-undecadien-5-ol. Its functions as well as its absolute configuration still have to be determined.

Key Words—Dictyoptera, Cryptocercidae, *Cryptocercus punctulatus*, cockroach, woodroach, 4,6,8-trimethyl-7,9-undecadien-5-ol.

INTRODUCTION

Chemical analysis of the secretions of male and female tergal glands of the woodroach, *Cryptocercus punctulatus*, shows the presence of a compound (called compound B), which is specific to females and is abundant in those

*To whom correspondence should be addressed.

0098-0331/91/0500-0811$06.50/0

females entering their oviposition period (Brossut et al., 1991). The comparative analysis of the tergal secretions from more than 200 females at different stages of their life suggests that compound B acts during sexual or parental behavior.

C. punctulatus is a primitive species of cockroach that lives in galleries in rotten wood, and its biology is still relatively unknown. Due to the lack of observations and of bioassays, currently no functional role in the behavior and/or life history of this insect can be attributed to compound B.

The composition of the tergal secretions in males and females has been reported in an earlier paper (Brossut et al., 1991). This paper is devoted to the identification of compound B isolated from female *C. punctulatus*. The structure is discussed with regard to those of other insects pheromones.

METHODS AND MATERIALS

Insects were collected from rotten hardwood logs at Mountain Lake Biological Station, Giles County, Virginia, during the spring and summer of 1984, 1985, and 1986. The tergites bearing the glands were dissected and placed individually in pure distilled pentane.

Tergal glands were dissected from 210 females (Brossut et al., 1991). After GC analysis of all the extracts from individual insects, all the samples were pooled, giving about 500 μg of secretion from which 50 μg of pure compound B was collected by preparative GC.

Gas chromatographic (GC) analyses were carried out with a Packard 429 gas chromatograph fitted with a split–splitless injector and a flame-ionization detector. The capillary column used was a DB5 fused silica column (30 m $\times$ 0.32 mm ID, J and W Scientific) with temperature programming from 60°C to 220°C at 2°C/min. Injector and detector temperatures were, respectively, 230°C and 250°C. Helium was used as the carrier gas at a flow rate of 5 ml/min. The GC traces were recorded and integrated by a CR3A Shimadzu integrator.

For preparative GC, a Varian 1440 chromatograph, fitted with a Pyrex column (3 m $\times$ 3.2 mm) packed with 10% SE 52 on 80–100 mesh Chromosorb WAW-DMCS and a flame ionization detector, was used with nitrogen as the carrier gas (flow rate 15 ml/min). Injector and detector temperatures were 230°C and 250°C, respectively, and oven temperatures were programmed from 100°C to 200°C at 2°C/min. An effluent splitter was used that allowed 10% of the effluent to flow to the detector. Fractions were collected in U-shaped glass tubes (1 mm ID) cooled to 0°C.

The sample, diluted to 100 μl with hexane, was injected in ca. 5-μl quantities into the column and the trapped component was rinsed from the tube into a Teflon-lined capped vial with distilled hexane, evaporated to dryness, and

redissolved in benzene-d_6 (99.99% D, Spectrométrie Spin et Techniques, Paris). The purity of collected fractions was monitored by capillary GC.

Gas chromatographic–mass spectrometric (GC-MS) analyses were carried out with a Nermag R 10-10-C quadrupole mass spectrometer coupled to a Girdel 31 gas chromatograph fitted with a split–splitless injector and a DB5 fused silica capillary column (60 m × 0.32 mm ID). The GC conditions described above were used. The column was directly connected to the ion source of the spectrometer through a heated transfer line maintained at 260°C.

Electron impact (EI) mass spectra were obtained at 70 eV on a 0.8-sec. cycle, the instrument scanning from 25 to 300 amu with the ion source maintained at 150°C. Chemical ionization (CI) mass spectra were generated at 90 eV using methane or ammonia at a source pressure of 0.2 and 0.3 torr, respectively, with a source temperature of 90°C, and the instrument scanning from 60 to 300 amu in 0.7 sec.

Microhydrogenation was performed on-line in the GC-MS system by means of a fused silica capillary reactor (Schomburg et al, 1982). The reactor, consisting of a 60-cm piece of a deactivated fused silica capillary column (6 m × 0.32 mm ID) statically coated with a 0.5% CH_2Cl_2 solution of palladium acetylacetonate (Fluka), was connected to the inlet of the analytical column with a zero-dead volume capillary butt-connector (Supelco). Palladium metal was precipitated at 220°C under hydrogen flow. Hydrogen then was used as the carrier gas at a flow rate of 1.5 ml/min, and the GC conditions were those previously described.

Fourier transform infrared (FT-IR) spectra were taken in the gas phase on a Bruker IFS 85 FT-IR spectrometer coupled, via a Bruker gold-coated lightpipe (36 cm × 1.5 mm ID) maintained at 240°C, to a Carlo-Erba 5160 gas chromatograph equipped with a DB5 fused silica wide-bore capillary column (30 m × 0.54 mm ID), an on-column injector, and a flame-ionization detector. The carrier gas was helium at a flow rate of 7 ml/min and temperature programming from 50 to 230°C at 5°C/min.

[^{1}H]NMR spectra were recorded on a Bruker WM400 instrument in 5 mm-ID NMR tubes using the benzene-d_6 mentioned above as solvent. The signal due to the residual protons of the deuterated solvent was used as internal reference ($S = 7.15$).

RESULTS

A typical gas–liquid chromatogram of pentane extract of female tergal glands (Figure 1) showed two main components. Compounds A was unambiguously identified as linalyl acetate by comparison of its mass spectrum, FT-IR spectrum, and retention index with those of the pure compound (Sarsynthèse).

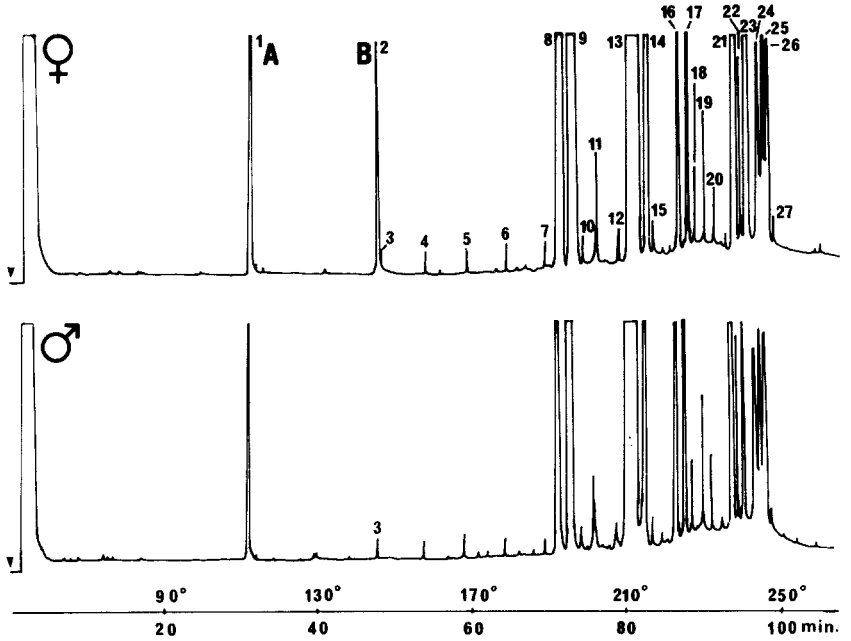


FIG. 1. Capillary gas chromatogram of the tergal gland secretion from females and males of *Cryptocercus punctulatus*. Conditions: DB5 fused silica column (30 m × 0.32 mm ID); temperature 60–220°C (2°C/min); carrier gas, helium at a flow rate of 5 ml/min. 1 (A), linalyl acetate; 2 (B), 4,6,8-trimethyl-7,9-undecadien-5-ol; 3, C-15; 4, C-16; 5, C-17; 6, C-18; 7, C-19; 8, unsaturated fatty acid; 9, hexadecanoic acid; 10, C-20; 11, unidentified; 12, C-21; 13, linoleic acid, 14, oleic acid; 15, C-22; 16, tricosene; 17, C-23; 18, unidentified; 19, tetracosene; 20, C-24; 21, pentacosene; 22, unidentified; 23, C-25; 24, 25, 26, methyl esters of diunsaturated fatty acids; 27, C-26. Compounds 8, 9, 11, 13, 14, and 18 are found everywhere on the body surface of males and females.

The mass spectrum of compound B (Figure 2) showed no molecular ion, and a possible candidate, borneol, based on a similarity of mass spectra, was promptly eliminated as pure borneol was eluted long before compound B under the GC conditions used. Peaks *m/z* 31 and *m/z* 45 are characteristic of an aliphatic alcohol (Budzikiewicz et al., 1964).

The molecular weight of B was determined by chemical ionization GC-MS with methane and ammonia as reagent gases. The highest observed peak in the methane-CI mass spectrum was *m/z* 211 accompanied with a more intense ion at *m/z* 209 and a low abundance ion at *m/z* 210. Another important ion was observed at *m/z* 193. These pseudomolecular ions characterized an alcohol of molecular weight 210, with ions at *m/z* 193, 209, 210, and 211 being respectively $[M - 17]^+$, $[M - 1]^+$, $[M]^+$, and $[M + 1]^+$ (Sarris et al., 1984).

The molecular weight and functionality of B were confirmed by the ammonia CI mass spectrum showing very intense pseudomolecular ions at *m/z* 193, $[M - 17]^+$, 211, $[M + 1]^+$, and 228 $[M + 18]^+ = [M + NH_4^+]$, again characteristic of an aliphatic alcohol of mol wt 210 (Sarris et al., 1984).

These pseudomolecular ions and their isotopic contributions suggested a possible molecular formula $C_{14}H_{26}O$ and two sites of unsaturation.

On-line microhydrogenation gave a mass spectrum (Figure 3) with the spectral pattern of a branched aliphatic saturated alcohol (spectral search in the EPA/NIH mass spectral data base) but with no molecular ion; however, an ion at *m/z* 196 attributed to $[M - 18]^+ = [M - H_2O]$ suggested a molecular weight of 214. This was confirmed by NH_3 CI-MS (*m/z* 232, M + 18) and CH_4 CI-

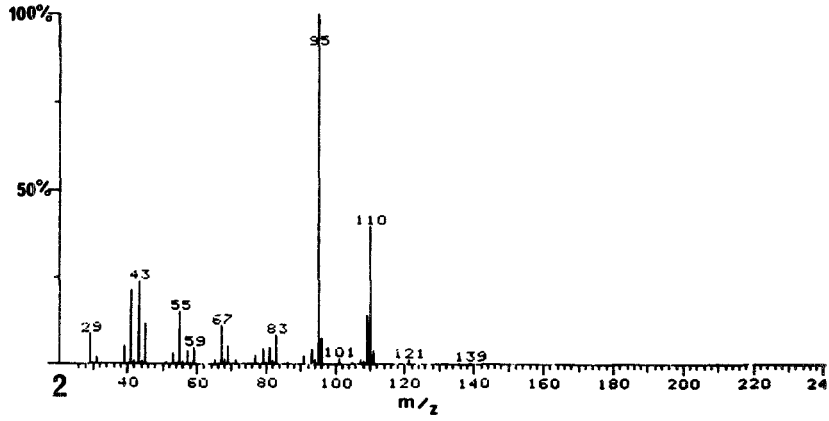


FIG. 2. Mass spectrum of compound B.

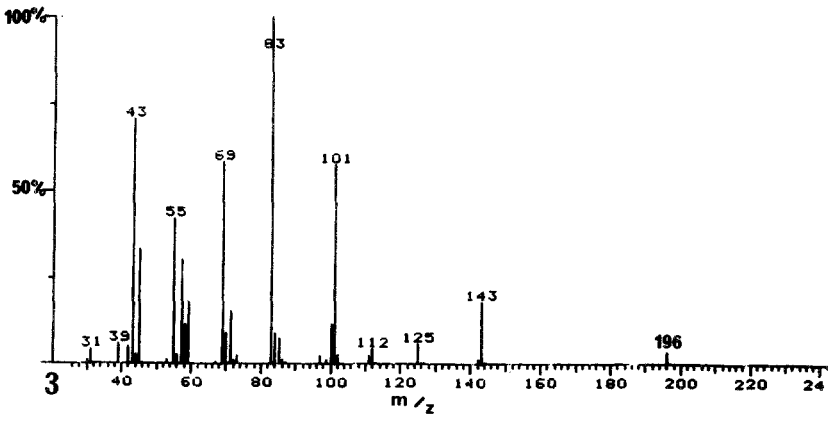


FIG. 3. Mass spectrum of compound B after hydrogenation.

MS (m/z 197, $[M - 17]^+$; 213, $[M - 1]^+$), revealing two carbon–carbon double bonds, accounting for the two sites of unsaturation.

The GC-FT-IR spectrum of B (Figure 4) confirmed the hydroxyl group, the OH stretching frequency (ν_{OH} = 3668 cm^{-1}) falling in the range of the secondary alcohols (Nyquist, 1984). Another stretching frequency (ν_{OH} = 3605 cm^{-1}) revealed an intramolecular hydrogen bonding, probably with one of the carbon–carbon double bonds, via a (5 + π) or (6 + π) system (Nyquist, 1984). An absorption at 960 cm^{-1} was tentatively attributed to a CH out-of-plane deformation (δCH) of an (*E*-disubstituted ethylenic bond —CH=CH— (Le Quere et al., 1987).

The 400-MHz [^{1}H]NMR spectrum (C_6D_6) of B isolated by preparative GC (Figure 5B) revealed signals due to three olefinic hydrogens at δ6.12, 5.47, and 5.38. Mutual couplings were solved by two dimensional NMR (chemical shifts correlation COSY, Figure 5A) and confirmed by selective irradiations. The proton at δ6.12 (dd) was coupled (J = 16 Hz) to one of the other vinyl hydrogen (δ5.47) confirming one *E* double bond, and coupled to a three-hydrogen doublet of doublet (J = 1.5 Hz) at 1.66 (methyl group). The vinyl hydrogen at δ5.47 (dg) also was coupled to the same methyl group (J = 7 Hz). Therefore, these signals were attributed to an (*E*)-propenyl group CH_3—CH=CH— bonded to a non-hydrogen-bearing carbon, since no other couplings were observed for these signals. The other olefin multiplet at δ5.38 (dq) was coupled to a one-

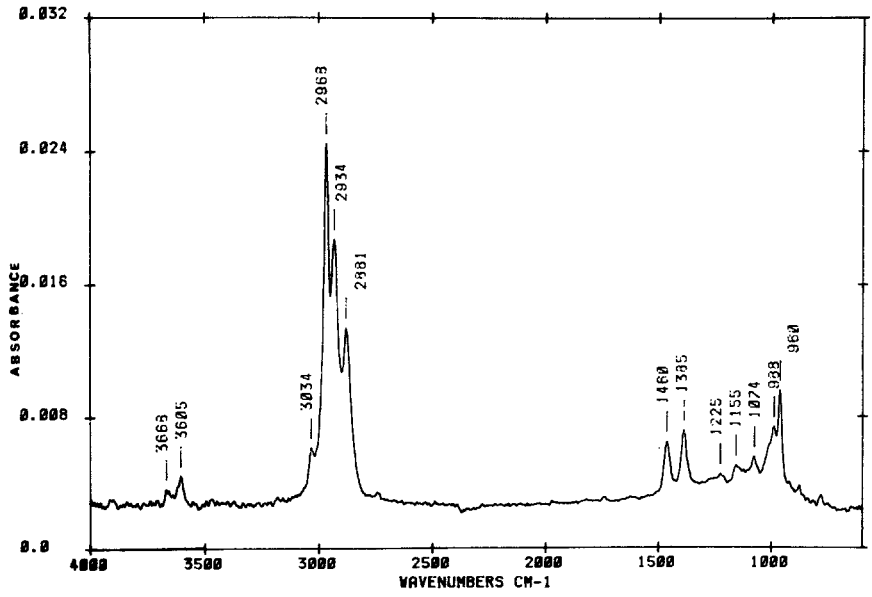


FIG. 4. Vapor-phase infrared spectrum of compound B.

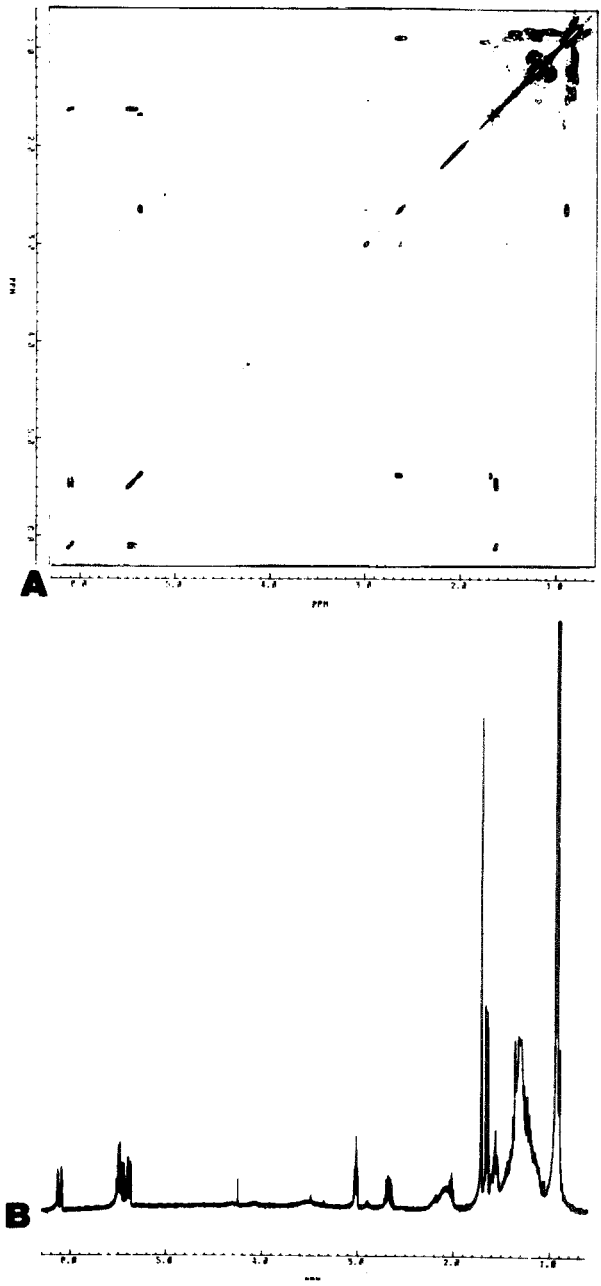


FIG. 5. [^{1}H]NMR (400.13 MHz) of B in benzene-d_6. (A) Two dimensional spectrum (COSY with a flip angle of 45° for the mixing pulse). (B) One-dimensional spectrum.

hydrogen multiplet at $\delta 2.67$ (J = 10 Hz) and to a three-hydrogen doublet at $\delta 1.71$ (J = 1.5 Hz), revealing a butenyl group $CH_3-C=CH-CH$. The geometry of this double bound could not be determined either by IR or NMR since the allylic coupling (J = 1.5 HZ) could not be considered as typically *cis* or *trans*.

The single-hydrogen multiplet at $\delta 2.67$ (ddq) also was coupled to a methyl group at $\delta 0.93$ (J = 7 Hz) and to a one-hydrogen doublet of doublet at $\delta 3.02$ (J = 6 Hz), confirming a methine hydrogen. The signal at $\delta 3.02$ which was identified by the downfield shift as a methine hydrogen geminal to the hydroxyl group, also was coupled (J = 6 Hz) to an unresolved multiplet at $\delta 1.57$. Therefore, the partial structure $CH_3-C=CH-CH(CH_3)-CHOH-CH$ could be deduced for compound B by addition of the (*E*)-propenyl group. The very upfield part of the spectrum was not completely understood, and integrals of the different overlapped multiplets at $\delta 0.85$–1.6 represented far more than the remaining 11 hydrogens necessary to complete the $C_{14}H_{26}O$ formula. These extra hydrogens possibly corresponded to water, giving rise to the broad signal at $\delta 1.1$–1.5. Traces of hexane also may contribute to the signals at $\delta 0.85$–1.0 and $\delta 1.2$–1.3.

However, as already outlined, irradiation of the methine signal at $\delta 3.02$ resulted in partial collapse of the unresolved multiplet at $\delta 1.57$; owing to the splitting pattern and coupling constants of the signal at $\delta 3.02$, the multiplet at $\delta 1.57$ was attributed to a methine hydrogen. Careful observation of the two-dimensional NMR spectrum revealed that this methine hydrogen is probably coupled to a doublet at $\delta \simeq 0.93$ overlapping the methyl group already identified, and also coupled to a multiplet at $\delta \simeq 1.22$, hidden in the broad signal at $\delta 1.1$–1.5.

More detailed assessments regarding the unknown part of the molecule could not be deduced from the NMR spectra. However, a triplet (J = 7.5 Hz) centered at $\delta 0.91$, partially overlapped and was attributed to a terminal methyl group coupled to a methylene group.

Thus, the structure of compound B was postulated to be a branched undecadienol with three methyl groups, i.e., (*E*7, *E*9)- or (*Z*7, *E*9)-4,6,8-trimethyl-7,9-undecadien-5-ol (Figure 6).

Structures B_1 and B_2 (Figure 6) are consistent with the main fragmentations displayed in the mass spectrum and also with the mass spectrum of the hydrogenated compound.

In the GC-FT-IR spectrum, the difference between the unassociated ν_{OH} and the intramolecular hydrogen bonded $\nu_{OH}:\pi$ frequencies is relatively large ($\Delta\nu_{OH}$ = 63 cm^{-1}); moreover, the $\nu_{OH}:\pi$ is located at a frequency (3605 cm^{-1}) lower than common frequencies for $\nu_{OH}:\pi$ of a (5 + π) intramolecular hydrogen bonded structure in secondary unsaturated alcohols (Nyquist, 1984).

These observations suggest an intramolecular ring structure larger than a

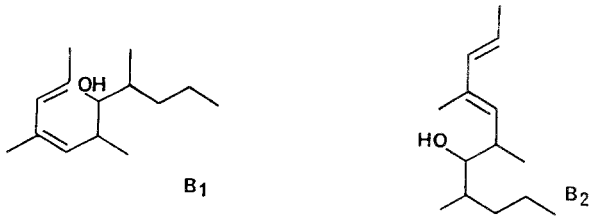


FIG. 6. The two possible structures of compound B. The GC-FT-IR spectrum favor the configuration B_1: (*Z*, *E*)4,6,8-trimethyl-7,9-undecadien-5-ol.

$(5 + \pi)$ system, therefore favoring configuration B_1, where a large $(7 + \pi)$ or $(5 + 2\pi)$ intramolecularly hydrogen-bonded ring structure is more likely to occur.

DISCUSSION

Primitive woodroaches of the genus *Cryptocercus* are the best living representatives of the ancestral blattarian–isopteran stock (Cleveland et al., 1934; McKittrick, 1964, 1965). Several aspects of their life history (Seelinger and Seelinger, 1983; Nalepa, 1984, 1988a,b) and morphology (Farine et al., 1989) strongly resemble the termites.

In the cockroaches, sex pheromones have been identified in two species: *Nauphoeta cinerea* and *Periplaneta americana*. In *N. cinerea*, the males produce a mixture of three compounds: 3-hydroxy-2-butanone, methyl 2-thiazolidine, and a derivative of the thiazoline (Brossut and Sreng, 1985). In *P. americana*, the periplanone B emitted by the females has a structure close to that of germacrene (Ritter and Persoons, 1979). Only one sex pheromone has been identified in termites: the *n*-tetradecyl propanoate produced by the females of *Reticulitermes flavipes* (Clement et al., 1989). These pheromones, including compound B from *C. punctulatus*, are very different in their structure. This chemical diversity can be explained easily by the phylogeny of these groups, where the three species of cockroaches and the termite *R. flavipes* are phylogenetically not closely related.

Compound B is basically a diunsaturated alcohol with conjugated double bonds. Such a structure is not unusual in insect pheromones, especially in beetles. For example, the enantiomers of ipsenol and of ipsdienol, although different from compound B by the length of their chain and by their configuration, are alcohols with conjugated double bonds (Silverstein, 1979).

Compound B very likely is formed from four propionate units and an acetate stopper, a general principle giving rise to numerous natural products, among which several pheromones occur (Francke, 1984). 4,6-Dimethyl-7-hydroxy-

nonan-3-one (serricornin) from the beetle *Lasioderma serricorne* (Chuman et al., 1979; Levinson et al., 1981) shows some analogies with compound B. Its reduced form would present roughly the same sequence as compound B. Another component of the sex pheromone of the cigarette beetle is closely related to compound B (Chuman et al., 1983) as it presents the same 4,6,8-methyl branching and as its cyclization would lead to the same basic heterocyclic structure. Other insect pheromones, such as invictolide from *Solenopsis invicta* and stegobinon from *Stegobium paniceum*, also possess the same trimethyl branching (Francke, 1984).

Of the 27 compounds identified from the tergal secretions of *C. punctulatus*, 20 compounds are found in both male and female; compound B is found only in the female; and six compounds are found everywhere on the body surface of both sexes (tergites, sternites, head, thorax) (Brossut et al., 1991).

Although the biological data are scarce, the morphological studies clearly indicate the existence of a secretory cycle in female tergal glands, which could be explained only by the production of a product involved in sexual or parental behavior; obviously, it must be compound B (Farine et al., 1989).

Work is in progress to determine the absolute configuration of natural 4,6,8-trimethyl-7,9-undecadien-5-ol as well as its biological function on the basis of synthetic reference samples.

Acknowledgments—We thank Dr. Wittko Francke for valuable discussions and comments on the manuscript. At the present time, the possible enantiomers are being synthesized by Dr. Kenji Mori.

REFERENCES

BROSSUT, R., and SRENG, L., 1985. L'univers chimique des Blattes. *Bull. Soc. Entomol. Fr.* 90:1266–1280.

BROSSUT, R., NALEPA, C.A., BONNARD, O., LE QUERE, J.L., and FARINE, J.P. 1991. The tergal glands of male and female *Cryptocercus punctulatus* (Dictyoptera. Cryptocercidae): Composition, sexual dimorphism and geographic variation of the secretion. *J. Chem. Ecol.* 17:823–831.

BUDZIKIEWICZ, H., DJERASSI, C. and WILLIAMS, D.H. 1964. Interpretation of Mass Spectra of Organic Compounds. Holden Day, San Francisco.

CHUMAN, T., KOHNO, M., KATO, K., and NOGUCHI, H. 1979. 4,6-Dimethyl-7-hydroxynonan-3-one, a sex pheromone of the cigarette beetle (*Lasioderma serricorne* F.). *Tetrahedron Lett.* 25:2361–2364.

CHUMAN, T., MOOCHIZUKI, K., KATO, K., OHNO, M., and OKUBO, A. 1983. Serricorone and serricorole, new sex pheromone components of cigarette beetle. *Agric. Biol. Chem.* 47:1413–1415.

CLEMENT, J.L., LLOYD, H., NAGNAN, P., and BLUM, M.S. 1989. *n*-Tetradecenyl propionate: Identification as the sex pheromone of the eastern subterranean termite (*Reticulitermes flavipes*). *Sociobiol.* 15:19–24.

CLEVELAND, L.R., HALL, S.R., SANDERS, E.P., and COLLIER, J. 1934. The woodfeeding roach

Cryptocercus. Its protozoa and the symbiosis between protozoa and roach. *Mem. Am. Acad. Arts Sci.* 17:185–342.

FARINE, J.P., BROSSUT, R., and NALEPA, C.A. 1989. Morphology of the male and female tergal glands of the woodroach *Cryptocercus punctulatus* (Insecta, Dictyoptera). *Zoomorphology* 109:153–164.

FRANCKE, W. 1984. Structural concepts in the chemistry of aliphatic pheromones. *Adv. Invert. Reprod.* 3:493–504.

LE QUERE, J.L., SÉMON, E., LATRASSE, A., and ETIEVANT, P. 1987. Gas chromatography–Fourier transform infrared spectrometry. Applications in flavour analysis. *Sci. Aliments* 7:93–109.

LEVINSON, H.Z., LEVINSON, A.R., FRANCKE, W., MACKENROTH, W., and HEEMANN, V. 1981. The pheromone activity of anhydroserricornin and serricornin from males cigarette beetles (*Lasioderma serricorne* F.). *Naturwissenschaften* 68:48.

MCKITTRICK, F.A. 1964. Evolutionary studies of cockroaches. *Mem. Cornell Univ. Agric. Exp. Stn.* 389:1–197.

MCKITTRICK, F.A. 1965. A contribution to the understanding of cockroaches-termites affinities. *Ann. Entomol. Soc. Am.* 58:18–22.

NALEPA, C.A. 1984. Colony composition, protozoan transfer and some life history characteristics of the woodroach *Cryptocercus punctulatus* (Dictyoptera, Cryptocercidae). *Behav. Ecol. Sociobiol.* 14:273–279.

NALEPA, C.A. 1988a. Reproduction in the woodroach *Cryptocercus punctulatus* (Dictyoptera, Cryptocercidae):Mating, oviposition and hatch. *Ann. Entomol. Soc. Am.* 88:637–641.

NALEPA, C.A. 1988b. Cost of parental care in *Cryptocercus punctulatus* (Dictyoptera, Cryptocercidae) and its implications for the evolution of termites eusociality. *Behav. Ecol. Sociobiol.* 23:135–140.

NYQUIST, R.A. 1984. The Interpretation of Vapor-Phase Infrared Spectra, Vol. 1. Sadler-Heyden, Philadelphia.

RITTER, F.J., and PERSOONS, C.J. 1979. Pheromones of cockroaches, pp. 225–236, *in* F.J. Ritter (ed.). Chemical Ecology:Odour Communication in Animals. Elsevier/North-Holland, Amsterdam.

SARRIS, J., ETIEVANT, P.X., LE QUERE, J.L., and ADDA, J. 1984. The chemical ionization mass spectra of alcohols, pp. 225–236, *in* J. Adda (ed.). Progress in Flavour Research. Elsevier, Amsterdam.

SCHOMBURG, G., HUBINGER, E., HUSMANA, H., and WEEKE, F. 1982. On line hydrogenation of insaturated and saturated sample components in capillary reactors coupled to either inllet or outlet of capillary columns. *Chromatographia* 16:228–232.

SEELINGER, G., and SEELINGER, U. 1983. On the social organization, alarm and fighting in the primitive cockroach *Cryptocercus punctulatus*. *Z. Tierpsychol.* 61:315–333.

SILVERSTEIN, R.M. 1979. Enantiomeric composition and bioactivity of chiral semiochemicals in insects, pp. 133–147, *in* F.J. Ritter (ed.). Chemical Ecology: Odour Communication in animals. Elsevier/North-Holland, Amsterdam.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

TERGAL GLANDS OF MALE AND FEMALE *Cryptocercus punctulatus* SCUDDER (DICTYOPTERA: CRYPTOCERCIDAE): COMPOSITION, SEXUAL DIMORPHISM, AND GEOGRAPHIC VARIATION OF SECRETION

R. BROSSUT,[1,*] C.A. NALEPA,[2] O. BONNARD,[1] J.L. LE QUERE,[3] and J.P. FARINE[1]

[1]*Université de Bourgogne*
Laboratoire de Zoologie
CNRS UA 674, 21000 Dijon, France
[2]*Entomology Department*
North Carolina State University
Raleigh, North Carolina 27695-7613
[3]*INRA, Laboratoire de Recherches sur les Aromes*
17 rue Sully, 21040 Dijon, France

(Received April 11, 1989; accepted January 7, 1991)

Abstract—Males and females of *Cryptocercus punctulatus* possess tergal glands, but they differ in position, size, morphology, and secretion chemistry. Compound A (linalyl acetate) is the most abundant of the 21 compounds found only in the secretion of these glands. Compound B, 4,6,8-trimethyl-7,9-undecadien-5-ol, is specific to the tergal secretion of females. *C. punctulatus* lives only in the United States; its distribution is disjunct. Compound A is found in samples from the eastern population but is absent in samples from the western population. The amount of compound B per gland in samples from the western population is at least twice as high as in the samples from the eastern populations.

Key Words—Glandular secretions, *Cryptocercus punctulatus*, Dictyoptera, Cryptocercidae, linalyl acetate, 4,6,8-trimethyl-7,9-undecadien-5-ol.

INTRODUCTION

Like many other species of cockroaches, adults of *Cryptocercus punctulatus* Scudder possess tergal glands (Lehrer, 1979; Seelinger and Seelinger, 1983).

*To whom correspondence should be addressed.

0098-0331/91/0500-0823$06.50/0

These glands usually produce chemical signals in adult males only and during precopulatory behavior (Roth, 1969; Brossut and Roth, 1977; Brossut et al., 1975). In most species, they are hidden by the wings but are exposed when males adopt the "wing-raised" posture in response to antennal contact with a female. The female subsequently climbs on the back of the male to feed on the secretion of these glands and is then correctly positioned for mating.

Both sexes of *C. punctulatus* possess tergal glands, but they differ in position, size, and morphology (Farine et al., 1989). In females, the tergal gland is located transversely on the intersegmental membrane at the anterior edge of the 7th tergum. The bilobed gland is narrow, 1 mm, but has a total width of 4 mm; the glandular epithelium is about 1 mm thick. The gland is normally covered by the edge of the 6th tergum but can be exposed when the female stretches her abdomen. In the male, the tergal gland is smaller and is located at the anterior edge of the 8th tergum. It is crescent shaped, with a total width of 1 mm, and is completely concealed within the anal chamber formed by the expanded 7th tergum. The glandular epithelium of these glands lacks reservoirs sensu stricto; however, several morphological modifications in the form of intracuticular reservoirs and large subcuticular spaces (Farine et al., 1989) allow extracellular, intraglandular storage of the secretion. The function of these glands is unknown, but there is some evidence to indicate that the activity of the female gland can be correlated with her reproductive status (Farine et al., 1989; Nalepa and Brossut, unpublished).

The distribution of *C. punctulatus* in the United States is disjunct. An eastern population is found in the Appalachian Mountains from Pennsylvania to Georgia, and a western population occurs in northern California, Oregon, and Washington (Cleveland et al., 1934). Here we reported the results of a chemical analysis of male and female tergal secretions of the eastern population and, additionally, an attempt to compare tergal secretions of the two populations.

METHODS AND MATERIALS

Insects were collected from rotten logs (primarily hardwood) at Mountain Lake Biological Station, Giles County, Virginia, during the spring and summer of 1984, 1985, and 1986, and from rotted coniferous logs in Douglas, Josephine, and Jackson counties, Oregon, during the summer of 1987.

Females found alone, pairs without nymphs, and pairs with nymphs of class I, II, III (Nalepa, 1984), were collected in Virginia. The physiological states of females found alone or with a male were determined by dissection. Glands from females of the same category were pooled (137 females, 10 pools, 7–35 glands per pool). Additionally, the glands of 37 females were analyzed individually. The tergal gland secretions of females from Oregon were analyzed

using two pools of five glands each. The social status of these females was unknown.

The analysis of male tergal secretions was done on a pool of 20 tergal glands and on 15 individually dissected glands from Virginia; two pools of five glands each were analyzed from Oregon.

The terga bearing the glands were dissected from the insect, immersed in pure distilled pentane, and stored at −20°C. The head, thorax, internal organs, sterna, and remaining terga also were extracted in pentane. Results of comparative extractions with pentane and methylene chloride showed that the secretion is soluble in both solvents. Only pentane was used, because methylene chloride also extracts hydrocarbons and fatty acids of high molecular weight present on the entire body surface and of no interest in this study. The extracts were filtered using glass wool and chromatographed without further purification.

A Packard 429 gas chromatograph fitted with a split–splitless injector and a flame ionization detector was used for gas chromatographic (GC) analysis. The capillary column used was a DB5 fused silica column (30 m × 0.32 mm ID, J and W Scientific), with temperature programming from 60°C to 220°C at 2°C/min. Injector and detector temperatures were, respectively, 230°C and 250°C. Helium was used as the carrier gas at a flow rate of 5 ml/min. The GC traces were recorded and integrated by a CR3A Shimatzu integrator for the quantitative analysis.

Using GC conditions just described, gas chromatographic–mass spectrometric (GC-MS) analyses were carried out with a Nermag R 10-10-C quadrupole mass spectrometer coupled to a Girdel 31 gas chromatograph fitted with a split–splitless injector and a DB5 fused silica capillary column (60 m × 0.32 mm ID). The GC conditions described above were used. The column was directly connected to the ion source of the spectrometer through a heated transfer line maintained at 260°C.

Electron impact (EI) mass spectra were obtained at 70 eV on a 0.8-sec cycle, the instrument scanning from 25 to 300 amu with the ion source maintained at 150°C.

Most of the compounds were identified by comparison of their mass spectra and retention indexes with those of pure compounds (Sigma, Sarsynthèse). The pure compounds were coinjected with the samples. The identification of compound B was carried out by pooling all samples studied here and by using additional techniques (Le Quéré et al., 1991).

RESULTS

Twenty-seven peaks observable on chromatograms were studied, and 24 were identified as indicated in the captions of Figure 1. A detailed account of the identification of compound B, 4,6,8-trimethyl-7,9-undecadien-5-ol, is given in Le Quéré et al. (1991).

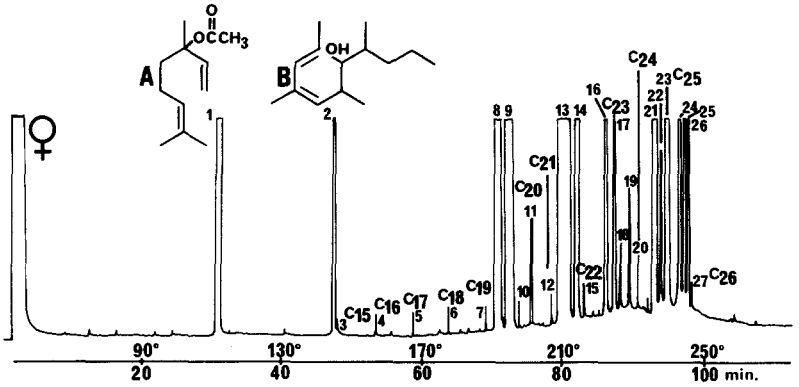


FIG. 1. Capillary gas chromatogram of the tergal gland secretion from a female *Cryptocercus punctulatus* collected in Virginia. Conditions: DB5 fused silica column (30 m × 0.32 mm ID); temperature 60–220°C (2°C/min); carrier gas, helium at a flow rate of 5 ml/min. 1 (A), linalyl acetate; 2 (B), 4,6,8-trimethyl-7,9-undecadien-5-ol; 3, C-15; 4, C-16; 5, C-17; 6, C-18; 7, C-19; 8, unsaturated fatty acid; 9, hexadecanoic acid; 10, C-20; 11, unidentified; 12, C-21; 13, linoleic acid; 14, oleic acid; 15, C-22; 16, tricosene; 17, C-23; 18, unidentified; 19, tetracosene; 20, C-24; 21, pentacosene; 22, unidentified; 23, C-25; 24, 25, 26, methyl esters of diunsaturated fatty acids; 27, C-26. Compounds 8, 9, 11, 13, 14, and 18 are found everywhere on the body surface.

Among these 27 compounds, six also were found everywhere on the body surface (8, 9, 11, 13, 14, 18). Twenty compounds (compound A, unsaturated and saturated hydrocarbons, methyl esters of diunsaturated fatty acids) were found only in the tergal secretions, and one (compound B) was specific for females.

Compound A (1), linalyl acetate, was found only in samples from Virginia (Figure 2). The amount present in male tergal glands was quite uniform, about 6 μg/gland. In the female tergal glands, however, the amount ranged from 4.6 to 29 μg/gland in the pooled samples and from 2.4 to 50.72 μg in individual samples.

Compound B, 4,6,8-trimethyl-7,9-undecadien-5-ol, occurred in female tergal secretions of both populations (Figure 2). It was absent in extracts of other parts of females and in extracts from body parts of males. The amount of compound B per gland in two pooled samples from Oregon females (235 and 265 ng) was at least twice as high as in the samples collected in Virginia (22–147 ng/gland in pooled samples; 20–199 ng in individual samples).

Except for compounds A and B, all other compounds were found in the tergal secretions from both sexes in both locations. Compounds 6, 9, 11, 13, 14, 18, four of which are fatty acids, were found adsorbed in the cuticular waxes of the several body parts analyzed (terga, sterna, head, thorax). In gland

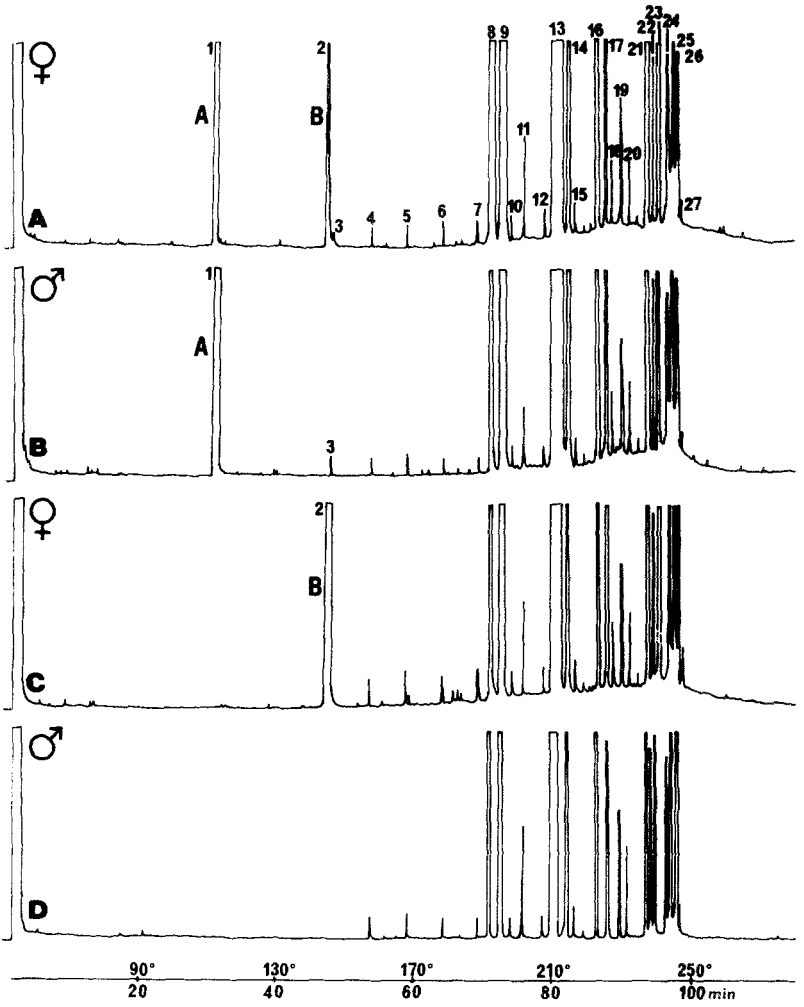


FIG. 2. Capillary gas chromatograms of tergal gland secretions from: (A) female collected in Virginia; (B) male from pair collected in Virginia; (C) pool of five females collected in Oregon; (D) pool of five males collected in Oregon. The GC conditions were the same as for Figure 1.

extracts, these six compounds must come from the part of the tergum dissected with the gland. However, the amount of linoleic acid (13) is much higher in gland extracts (about 20% in samples from Virginia, about 30% in samples from Oregon) than in other body parts (from 6% to 10%). The percentage of the other compounds varied from 0.2% (8) to 8% (9).

The three methyl esters of diunsaturated fatty acids (24–26) are found in gland extracts of both sexes and in the same percentages (about 2% for compounds 24 and 25). Compound 26 is about twice as abundant in samples from Oregon (about 8%) as in samples from Virginia (about 4%). For the most part, hydrocarbons are present in small amounts. Three exceed 1% (docosane about 1.4%, tricosene about 2%, pentacosane about 3%). Pentacosene is the only abundant hydrocarbon (12–15%).

In females, the average amount of secretion extracted from one gland was 20 μg, but varied from 6 to 30 μg. The percentages of the products reported above were usually not correlated with age or reproductive status. Preliminary evidence, however, indicates there may be a relationship between the reproductive status of females and the total amount of compound B and, to a lesser extent, of compound A present in the glands of Virginia populations. Studies designed to monitor the amount of these two compounds over the course of female life history are in progress.

DISCUSSION

Linear hydrocarbons are common in insect exocrine secretions (Blum, 1981). However, series of 10 or more hydrocarbons are found mainly in ants and bees. Often, linear hydrocarbons are found with other compounds that are responsible for the biological activity of the secretion. In the Dufour's glands of halictine bees, C-19 to C-28 hydrocarbons are found with C-16 to C-24 macrocyclic lactones (Hefetz et al., 1986). In *Andrena* bees, C-13 to C-24 hydrocarbons are found with farnesyl and geranyl esters (Tengö and Bergström, 1975). The cephalic secretions of the fire bee contain two major components with vesicatory proprieties (diketones), which occur with 13 hydrocarbons (from C-11 to C-23) and with a similar series of carboxylic acids, esters, and monoketones (Brian et al., 1984). Dufour's glands of cleptoparasitic bees contain an complex oily mixture of alkanes, alkenes, and methyl alkanes from C-23 to C-33, which, deposited on the brood cell walls and pollen balls, provides a waterproof lining for both (Hefetz et al., 1982).

The Dufour's gland secretion in *Formica* ants is a complex mixture of saturated, monounsaturated, diunsaturated, and methyl-branched alkanes from C-9 to C-19 (Lofquist and Bergström, 1980). The undecane, which is a releaser of swarming and pairing activity, always represents 50% or more of the secretion. Several species of stable flies and sciarid flies use unsaturated and saturated hydrocarbons as sex pheromones (Uebel et al., 1975; Kostlec et al., 1980). In the horn fly *Hematobia irritans*, (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene elicit male courtship behavior (Bolton et al., 1980).

In cockroaches, the aggregation pheromone produced by mandibular glands

is a mixture of undecane and tetradecane in *Blaberus craniifer* (Brossut et al., 1974). In *Eublaberus distanti*, it is a mixture of undecane, 4-heptanone, 2,6-dimethyl-4-heptanone, and octanol (Brossut, 1979). In both species, these pheromones attract conspecifics from a distance.

In *C. punctulatus*, the saturated hydrocarbons and the tetracosene are present in small amounts, from 0.1% to 0.5% of the secretion. Tricosene represents about 2%, and pentacosene, which is the only abundant hydrocarbon, from 12% to 15% of the secretion. Although we do not know the structure of the three methyl esters of diunsaturated fatty acids found in *C. punctulatus*, they are probably similar to those commonly found in the pheromonal blends of many species of Lepidoptera (see Inscoe, 1984, and references therein).

C. punctulatus from Oregon is presumed to have a biology similar to that of the eastern population, yet linalyl acetate is absent in the tergal secretions of both sexes. *C. punctulatus* lives primarily in hardwood logs in Virginia and in coniferous logs in Oregon, but it is doubtful that the presence or absence of linalyl acetate could be due to their food source; no trace of this compound was found in the internal organs.

The high amounts of compound B found in females from Oregon may be specific to the population from this area or may be related to the time of collection; the eastern population was collected later in the summer (August) than any of the western samples (June). In addition, the lack of linalyl acetate (compound A) in the tergal secretions of the individuals from Oregon suggests that tergal secretions may vary between populations. Intraspecific variations in the chemistry of exocrine secretions of insects are, for the most part, correlated with geographic separation of populations; many examples are found in defensive secretions, especially termites (Parton et al., 1981; Everaerts et al., 1988). In *C. punctulatus* variation in the secretion is not surprising since the two populations of this insect probably have been isolated geographically since the Tertiary.

In many species of termites, female tergal glands are used together with the sternal glands during the calling posture, functioning to attract males from a distance. Later, the glands initiate and control tandem behavior: the male follows the female, trying to keep his mouthparts in contact with her tergal glands (Grassé, 1982, 1984; Ampion and Quennedey, 1980). In many species of cockroaches, tergal glands are found in adult males only, where they produce aphrodisiacs. During the wing-raising posture of the male, these glands are exposed and the female feeds on them prior to copulation (Brossut and Roth, 1977). The role of tergal glands in the behavior and/or life history of *C. punctulatus* remains a matter of supposition. Because of the position of the male glands in this woodroach, it is unlikely that they produce a sexual attractant; moreover, all compounds found in the secretion of the male glands also are found in those of the female. The position of the female glands and the presence

of a female-specific compound suggests a possible role in sexual behavior, but the glands also may be involved in aggregative or parental behavior (see Farine et al., 1989).

Synthesis of reference samples of compound B is in progress. Bioassays will clarify its biological function. Tergal secretions from more than 1000 females (collected each month during one year) are being analyzed individually to monitor the amount of the different compounds over the course of their life history.

Acknowledgments—The kindness of Dr. Paul Catts and the field assistance of Dr. Richard Zack and J. Patrick Rand made the collection of the western population of *C. punctulatus* possible.

REFERENCES

AMPION, M., and QUENNEDEY, A. 1980. The abdominal epidermal glands of termites and their phylogenic significance, pp. 243–261, *in* P.E. Howse and J.L. Clement (eds.). Biosystematics of Social Insects. Academic Press, New York.

BLUM, M.S. 1981. Chemical Defenses of Arthropods. Academic Press, New York. 562 pp.

BOLTON, H.T., BUTLER, J.F., and CARLSON, D.A. 1980. A mating stimulant pheromone of the horn fly, *Haematobia irritans* (L): Demonstration of biological activity in separated cuticular components. *J. Chem. Ecol.* 6:951–964.

BRIAN, Z., FALES, H.M., BLUM, M.S., JONES, T.P., RINDERER, T.E., and HOWARD, D.F. 1984. Chemistry of cephalic secretion of fire bee *Trigona (Oxytrigona) tataira. J. Chem. Ecol.* 10:451–461.

BROSSUT, R. 1979. Gregarism in cockroaches and in *Eublaberus* in particular, pp. 237–246, *in* F.J. Ritter (ed.). Chemical Ecology: Odour Communication in Animals. Elsevier/North Holland, Amsterdam.

BROSSUT, R., and ROTH, L.M. 1977. Tergal modifications associated with abdominal glandular cells in the Blattaria. *J. Morphol.* 151:259–298.

BROSSUT, R., DUBOIS, P., and RIGAUD, J. 1974. Le grégarisme chez *Blaberus craniifer:* Isolement et identification de la phéromone. *J. Insect. Physiol.* 20:529–543.

BROSSUT, R., DUBOIS, P., RIGAUD, J., and SRENG, L. 1975. Etude biochimique de la sécrétion des glandes tergales des Blattaria. *Insect Biochem.* 5:719–732.

CLEVELAND, L.R., HALL, S.R., SANDERS, E.P., and COLLIER, J. 1934. The woodfeeding roach *Cryptocercus*, its protozoa, and the symbiosis between protozoa and roach. *Mem. Am. Acad. Arts Sci.* 17:185–342.

EVERAERTS, C., PASTEELS, J.M., ROISIN, Y., and BONNARD, O. 1988. The monoterpenoid fraction of the defensive secretion in Nasutitermitinae from Papua New Guinea. *Biochem. Syst. Ecol.* 4:437–444.

FARINE, J.P., BROSSUT, R., and NALEPA, C.A. 1989. Morphology of the male and female tergal glands of the woodroach *Cryptocercus punctulatus* (Insecta: Dictyptera). *Zoomorphology* 109:153–164.

GRASSÉ, P.P. 1982. Termitologia, Tome I. Masson, Paris.

GRASSÉ, P.P. 1984. Termitologia, Tome II. Masson, Paris.

HEFETZ, A., EICKWORT, G.C., BLUM, M.S., CANE, J., and BOHART, G.E. 1982. A comparative study of the exocrine products of cleptoparasitic bees (*Holcopasites*) and their hosts (*Calliopsis*) (Hymenoptera: Anthophoridae, Andrenidae). *J. Chem. Ecol.* 8:1389–1397.

HEFETZ, A., BERGSTRÖM, G., and TENGO, J. 1986. Species, individual and kin specific blend in Dufour's gland secretions of halictine bees. Chemical evidence. *J. Chem. Ecol.* 12:197–208.

INSCOE, M.N. 1984. Insect attractants, attractant pheromones and related compounds, pp. 201–295, *in* A.F. Kydonieus, M. Beroza, G. Zweig (eds.). Insect Suppression with Controlled Release Pheromone Systems, Vol. 2. CRC Press, Boca Raton, Florida.

KOSTLEC, J.G., GIRARD, J.E., and HENDRY, L.B. 1980. Isolation and identification of a sex attractant of a mushroom-infesting sciarid fly. *J. Chem. Ecol.* 6:1–12.

LEHRER, C.A. 1979. Intraspecific attraction in the wood roach *Cryptocercus punctulatus* Scudder. Thesis. Winston-Salem, North Carolina State University.

LE QUÉRÉ, J.L., BROSSUT, R., NALEPA, C.A., and BONNARD, O. 1991. Isolation and identification of the female specific compound in the tergal gland secretion of *Cryptocercus punctulatus* (Dictyoptera, Cryptocercidae): 4,6,8-trimethyl-7,9-undecadien-5-ol. *J. Chem. Ecol.* 17:811–821.

LOFQUIST, J., and BERGSTRÖM, G. 1980. Volatile communication substances in Dufour's gland of virgin females and old queens of the ant *Formica polyctena*. *J. Chem. Ecol.* 6:309–320.

NALEPA, C.A. 1984. Colony composition, protozoan transfer and some life history characteristics of the woodroach *Cryptocercus punctulatus* (Dictyoptera, Cryptocercidae). *Behav. Ecol. Sociobiol.* 14:273–279.

PARTON, A.H., HOWSE, P.E., BAKER, R., and CLÉMENT, J.L. 1981. Variation in the chemistry of the frontal gland secretion of European *Reticulitermes* species, pp. 183–209, *in* P.E. Howse and J.L. Clément (eds.). Biosystematics of Social Insects. Academic Press, London.

ROTH, L.M. 1969. The evolution of male tergal glands in the Blattaria. *Ann. Entomol. Soc. Am.* 62:176–208.

SEELINGER, G., and SEELINGER, U. 1983. On the social organization, alarm and fighting in the primitive cockroach *Cryptocercus punctulatus*. *Z. Tierpsychol.* 61:315–333.

TENGÖ , J., and BERGSTRÖM, G. 1975. All-*trans*-farnesyl hexanoate and geranyl octanoate in the dufour gland secretion of *Andrena* (Hymenoptera: Apidae). *J. Chem. Ecol.* 1:253–268.

UEBEL, E.C., SONNET, P.E., BIERL, B.A., and MILLER, R.W. 1975. Sex pheromone of the stable fly: Isolation and preliminary identification of compounds that induce mating strike behavior. *J. Chem. Ecol.* 1:377–385.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

BIOSYNTHESIS, PRODUCTION SITE, AND EMISSION RATES OF AGGREGATION-ATTACHMENT PHEROMONE IN MALES OF TWO *Amblyomma* TICKS

PETER A. DIEHL,* PATRICK GUERIN, MICHELE VLIMANT, and PASCAL STEULLET

Institute of Zoology, University
Chantemerle 22, CH-2007 Neuchâtel, Switzerland

(Received August 9, 1990; accepted January 7, 1991)

Abstract—The aggregation-attachment pheromone components *o*-nitrophenol (ONP) and methyl salicylate (MS) in male *Amblyomma variegatum* ticks appeared after three days of feeding on the host and reached high values after about six days. Variable quantities of 1.3–7.3 μg ONP and about 0.6 μg MS were present within ticks. ONP and MS were released at the high rates of 300–1800 ng/hr and 20–600 ng/hr per male tick, respectively. After a temporary decrease, males continued to emit at high rates after nearby attachment of females. In *A. hebraeum*, ONP showed a similar pattern, but with a delay of about a day. A male, which had fed during 14 days, contained about 2 μg and released 225–280 ng/hr. Emission in forcibly detached males of both species dropped rapidly to low levels of less than 10 ng/hr per tick. Host skin and tick feces in the vicinity of feeding males were pheromone-impregnated. The very high emission rates are consistent with the observations that the pheromone is an important component of the host-location mechanism of conspecifics. ONP and MS are produced in the dermal glands type 2 associated with the ventrolateral cuticle.

Key Words—*Amblyomma variegatum*, *Amblyomma hebraeum*, Acari, aggregation-attachment pheromone, host location, *o*-nitrophenol, methyl salicylate, pheromone glands.

INTRODUCTION

The partially fed females of at least 14 tick species from five genera of metastriate ticks attract fed males by using the volatile 2,6-dichlorophenol as sex

*To whom correspondence should be addressed.

0098-0331/91/0500-0833$06.50/0

attractant pheromone; this substance is produced by the foveal glands (Sonenshine, 1984, 1985, 1986, Sonenshine et al., 1982). After establishment of close contact, the following scenario occurs in *Dermacentor andersoni* and *D. variabilis*. A mounting sex pheromone induces the mounting and exploratory behavior that guides the male to the gonopore on the female venter (Hamilton et al., 1989). The male then probes the gonopore and inserts its chelicerae. After recognition of a genital sex pheromone, copulation is induced and a spermatophore placed into the vagina (Allan et al., 1988, 1989).

Unlike the situation in most metastriate species, the males of *Amblyomma gemma, A. hebraeum, A. maculatum, A. lepidum*, and *A. variegatum* begin to emit an aggregation–attachment pheromone after several days of feeding on the host. This pheromone attracts conspecific females and males to the feeding males, and in *A. hebraeum* even nymphs (Gladney, 1971; Gladney et al., 1974a,b; Norval and Rechav, 1979; Rechav, 1977; Rechav et al., 1976, 1977; Schöni, 1987; Schöni et al., 1984). The males clasp the attracted ticks very efficiently; this results in the formation of dense clusters at suitable feeding sites like the genitals or belly.

The pheromone is active over a considerable distance; steers infested with male *A. hebraeum* (Norval et al., 1989a), or artificial sources of pheromone for *A. hebraeum* and *A. variegatum* proved to be attractive over several meters downwind (Hess and De Castro, 1986; Norval et al., 1989b). In an extreme case, a few *A. hebraeum* males reached the steer within 13 min of first being sighted 9 m away (Norval et al., 1989a). The pheromone is thus an important component of the host-location bouquet of products, most probably together with other host volatiles such as CO_2.

The aggregation–attachment pheromone of six-day-fed male *A. variegatum* is composed of *o*-nitrophenol (ONP), methyl salicylate (MS), and nonanoic acid in a ratio of 2:1:8 μg per tick (Schöni, 1987; Schöni et al., 1984). The long-range attractant ONP induced an incomplete aggregation; MS and nonanoic acid contribute to the complete activity, but do not induce aggregation when offered separately (Schöni, 1987; Schöni et al., 1984). The closely related *A. hebraeum* emitted ONP, benzaldehyde, and 2-methylpropanoic acid, along with some nonanoic and other medium chain fatty acids (Apps et al., 1988).

The sex attractant pheromone 2,6-dichlorophenol (2,6-DCP) has been reported in females of *A. variegatum* and in both sexes of *A. maculatum* (Kellum and Berger, 1977; Wood et al., 1975). In the latter species, this pheromone has been shown to attract fed males; but no behavioral response was observed in females to explain the presence of 2,6-DCP in males.

Males of both *A. variegatum* and *A. hebraeum* become attractive only after about three or five days of feeding, respectively (Norval and Rechav, 1979; Rechav, 1977; Schöni, 1987; Schöni et al., 1984). In this paper we report on the time course of biosynthesis and emission of the aggregation–attachment

pheromone components ONP and MS in *A. variegatum* males and, to a lesser extent, of ONP in *A. hebraeum* males, from the initiation of feeding on rabbits. We also looked for the possible site of pheromone production among the different exocrine glands of *A. variegatum*.

METHODS AND MATERIALS

Ticks. Adults of *Amblyomma variegatum* Fabricius and *A. hebraeum* Koch (Acari, Ixodidae) were supplied by Ciba-Geigy Ltd. (St. Aubin, Switzerland), where they are bred on Simmenthaler calves. During molting, ticks were held at 29°C and 90% relative humidity and thereafter in constant darkness at 28°C and 80–90% relative humidity for several months. Ticks were never older than about 9 months.

For on-host experiments, the ticks were placed inside a nylon bag glued to the shaved back of mature New Zealand female rabbits (weight ca. 2 kg). Before adding the unfed *A. variegatum* males, a synthetic pheromone mixture of 100 ng of ONP, 30 ng of MS, and 250 ng of nonanoic acid dissolved in a few microliters of methanol was placed at several locations on the host skin to induce rapid attachment. Benzaldehyde and 2-methylpropanoic acid replaced MS for *A. hebraeum* (cf. Apps et al., 1988). Ticks attached generally within the first 30–45 min. Unattached ticks were removed after 1 hr to ensure good synchronization.

Chemicals. All chemicals for pheromone extractions and analysis were of analytical or HPLC (high-performance liquid chromatography) grade.

Pheromone Extractions. Five to 10 ticks were removed from the host with forceps at selected times during feeding, placed in methanol, cut into small pieces with fine scissors, and the volume made up to 1 ml/5 ticks. The extract was sonicated for about 2 min in an ultrasonic bath and then stored at −18°C. Recovery rates were estimated to be about 95% as judged from an experiment where known amounts of ONP and MS were injected into unfed males before extraction.

Hydrolysis. A 0.1-ml aliquot of the methanolic extracts from unfed males or mature males, which had fed for 14 days, corresponding to about one-half tick equivalent, was mixed with 0.4 ml H_2O and 0.5 ml KOH (5 M) and then boiled under reflux for 20 min. After cooling, an aliquot of 0.1 ml was acidified to about pH 2 with 6 M hydrochloric acid. Methanol was added to 30% before separation by HPLC.

Dissection. Dissection of ticks was performed in cell-culture medium TC199 (Seromed) under a binocular microscope with very fine scissors and forceps. The various organs were rinsed several times in fresh dissecting medium before extraction and sonication in methanol.

Histology. Pieces of dorsal and ventrolateral cuticle were fixed in 2.5% glutaraldehyde in a phosphate buffer (Sörensen, 0.1 M at pH 7.2, with 1.5% saccharose for 2 hr at 4°C) followed by postfixation in a mixture containing 1% OsO_4, 1.25% $K_2Cr_2O_7$, and 11% saccharose for 24 hr at 4°C. After three rinses in distilled water containing 11% saccharose for 30 min, the pieces were dehydrated in acetone and embedded in Spurr's medium. Semithin sections were cut with a diamond knife and stained with toluidine blue.

Some of the dehydrated cuticle pieces were critical-point-dried in CO_2 in a Balzers CPD device for scanning electron microscopy. The mounted specimens were coated with carbon and then gold sputtered in a Balzers sputtering apparatus. The dermal glands were observed in a Philips 500 PSEM scanning electron microscope.

Sampling of Volatiles. Sampling of volatile pheromones was performed by absorption to reverse-phase Sep-Pak Light cartridges (C_{18}, Millipore) coated with NaOH (Kuwata and Tanaka, 1988). To collect volatiles from ticks on the host, a simple apparatus was constructed from a polypropylene funnel, diam. 3.2 cm. A small polypropylene Y piece as an air inlet was glued into the funnel wall; the stem of this Y piece, acting as inlet, was connected by a short piece of plastic tubing (PVC) to an air-purifying system consisting of (1) an activated charcoal filter (20 cm × 2 cm), (2) a Sep-Pak cartridge, and (3) a small pipet containing Porapak (50–80 mesh, Millipore), in series. A short tubing led from the funnel outlet to another Sep-Pak cartridge followed by a Porapak-filled pipet connected to a water pump. The modified funnel was lightly pressed against the skin of the rabbit with the attached ticks, and the air sucked through the device at a rate of about 30 ml/min for 15 min.

In the case of sampling from ticks removed from the host, the funnel was replaced by a 500-ml gas-wash bottle containing the ticks.

The absorbed volatiles were eluted by gently pushing about 100 μl of methanol through the Sep-Pak cartridge with a small syringe. Generally, the first two drops were yellow, indicating the presence of ONP. We observed no breakthrough into the back-up Porapak cartridge.

HPLC. HPLC separations were performed with a Kontron liquid chromatograph consisting of two 420 pumps, a Rheodyne 7125 injector with a 1-ml sample loop, and a 250 mm × 4 mm or a 125 mm × 4 mm reverse-phase column RP-18 (Nucleosil 120-5 C_{18}, Macherey-Nagel). For separation, a gradient of methanol–aqueous buffer (KH_2PO_4, 0.02 M at pH 4) was employed at a flow rate of 1.2 ml/min at room temperature: 30 sec at 30% methanol, then step to 50% followed by a gradient to 100% methanol in 5 min and a final purge at 100% for 10 min. Before injection, sample aliquots of up to 400 μl were prepared by adjusting with buffer to contain 30% methanol and then centrifuged, if particles were present. In general, we injected aliquots of between 0.1 and 0.5 tick equivalents for whole tick extracts, and half or the entire extract in the case of the sampled volatiles.

UV-VIS detection of the eluting compounds was achieved with Hewlett-Packard HP 1040 photodiode array detector controlled by a HP ChemStation with chromatograms registered simultaneously at 210, 254, and 280 nm. For identification, the retention times and spectra of unknown compounds were compared with spectra from various reference compounds injected at the same conditions. Quantification of ONP and MS was done with the external standard method at 210 nm. The minimum quantities of ONP or MS for unambiguous identification were about 30–50 ng.

The reproducibility of retention times and area measurements was tested by repetitive injections of standards. For ONP, the retention time and the area showed coefficients of variation of 0.3 and 2.2%, respectively. In view of the good reproducibility, we injected only one aliquot of the different extracts. Between injections, both syringe and injector were carefully rinsed with methanol. Blank runs revealed no cross contamination.

GC Electrophysiology. The presence of ONP, MS, and nonanoic acid in a few extracts was tested by injection of 1-μl extract aliquots onto a capillary GC (Carlo-Erba HRGC 5160 Mega) fitted with an on-column injector and a FID and ECD detector in tandem. Separation was achieved on a G&W DB-wax fused silica capillary column (30 m, 0.32 mm ID) with H_2 (linear flow at 0.5 m/sec) using a temperature program of 80–230°C at 8°C/min. The column was simultaneously interfaced with the ONP-sensitive dII wp/sw/A sensilla of the Haller's organ on the tarsus of the first pair of legs (Schöni, 1987) via a glass Y-piece outlet splitter. The branch of the splitter leading to the biological detector passes through a heated (250°C) transfer line in the wall of the chromatograph. Upon exit to the exterior, the effluent is swept over the tick sensillum by a temperature- (22°C) and humidity- (100% relative humidity) controlled airstream in a water-jacketed glass tube, at a flow rate of 35 cm/sec. Responses of the olfactory cells were recorded with a glass electrode in contact with the severed tip of the sensillum. The reference electrode, also of glass, was placed at the base of the leg. Action and receptor potentials were recorded using an amplifier (Syntech, Hilversum, Netherlands) linked to a pulse-code modulator and video-cassette recorder (Gödde, 1985). Identification was based on the selective response of the biological detector to ONP and matching of retention times of unknowns with authentic standards.

RESULTS

Biosynthesis of Attachment–Aggregation Pheromone during Feeding. In whole extracts of *Amblyomma variegatum* males that fed on rabbits, traces of ONP were detectable by HPLC for the first time on day 4 after onset of feeding (150 ng/tick). The concentration increased during the following two days to 1.3–2 μg/tick at day 6. After this and up to day 19, variable amounts of about

1.3—7.3 μg/tick were measured (mean 3.4 ± 0.7 μg SEM er tick; $N = 9$). Methyl salicylate was detected only at day 8 (40 ng/tick), and thereafter reached about 0.6 μg/tick after day 10. Males removed at day 1, 2, or 3 after onset of feeding and kept alive at 30°C and 90% relative humidity did not contain detectable amounts of ONP or MS if assayed later at day 8. After hydrolysis of extracts of unfed males, no ONP or salicylic acid (from MS) was present. Hydrolysis of extracts from mature males also did not increase the pheromone content cited above.

Extracts of *A. hebraeum* males by day 14 of feeding contained about 1.9 μg ONP per tick; benzaldehyde was not detectable under our conditions.

Emission Rates of Attached Ticks. On one rabbit, traces of ONP emitted by *A. variegatum* males and the surrounding host skin covered by the sampling device were first detected at day 3 after onset of the blood meal (7 ng/hr per tick, see Figure 1A). Thereafter emission rose rapidly and reached a plateau at day 6 (620 ng/hr per male tick).

When four female ticks were added to these males at day 12, the ONP emission rate of 975 ng/hr per male tick just before contact, dropped after the encounter between the sexes over the next 4 hr to a level of 110 ng/hr per tick, but thereafter increased again to variable levels of 310–1650 ng/hr per male tick (Figure 1A). At day 12 we began to observe another control group of four males in parallel on the same rabbit (Figure 1A). The emission was comparable to that of the paired females–males, with variable rates between 350 and 1780 ng/hr per male tick.

Traces of volatile MS was detectable for the first time in *A. variegatum* males at day 4 of feeding (5 ng/hr per tick; Figure 1B). Increase in emission over the next days followed the pattern described for ONP. A drop occurred after pairing of the males with the females. The variable rates were lower than for ONP (20–600 ng/hour per tick). The ratio between emitted ONP and MS after 12 days of feeding varied between 2:1 and 7:1 with a mean value of about 4:1.

Host skin adjacent to 12-day feeding ticks also emitted pheromone products: ONP at 40 ng/cm^2/hr and MS at 10 ng/cm^2/hr. An area beginning about 5 cm away from the nearest tick gave off ONP at about 10 ng/cm^2/hr. No emission was detected from areas further away. In addition, feces collected near a tick contained about 140 ng ONP and 35 ng MS per milligram dry weight.

A. hebraeum males began to emit ONP one day later than *A. variegatum* males at a rate of 8 ng/hr per tick (Figure 2). The release appeared to reach a plateau after seven days of feeding with rates of 225–280 ng/hr per tick.

Emission Rates of Forcibly Removed Ticks. Mature ticks of both *Amblyomma* species, forcibly removed after 14 days of feeding, released ONP during the first few minutes at rates comparable to attached ticks (Figure 3; 950 and 250 ng/hr per male tick for *A. variegatum* and *A. hebraeum*, respectively).

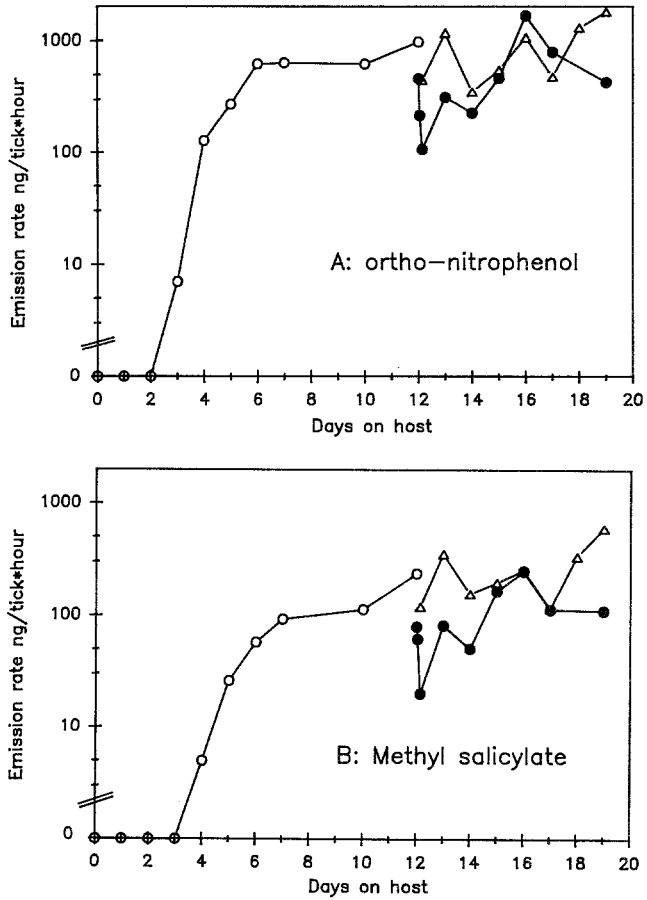


FIG. 1. Emission rate of *o*-nitrophenol (A) and methyl salicylate (B) and four males of *Amblyomma variegatum* after attachment on the rabbit (open circles). At day 12, four females were added; after an initial drop, the males continued to emit at high rates (filled circles). Open triangles: another group of four males without females on the same rabbit.

However, the emission dropped within a few hours to very low levels of less than 10 ng/hr per male tick in both species. Emission of MS in *A. variegatum* (not shown) diminished in a similar way. After one day, ticks still contained ONP in amounts comparable to attached males (one to several micrograms of ONP).

Localization of Pheromone Production. Analysis of a small piece of dorsal cuticle bearing the two foveal glands of mature *A. variegatum* or *A. hebraeum* males revealed only traces of ONP; by contrast, nearly all of the total extract-

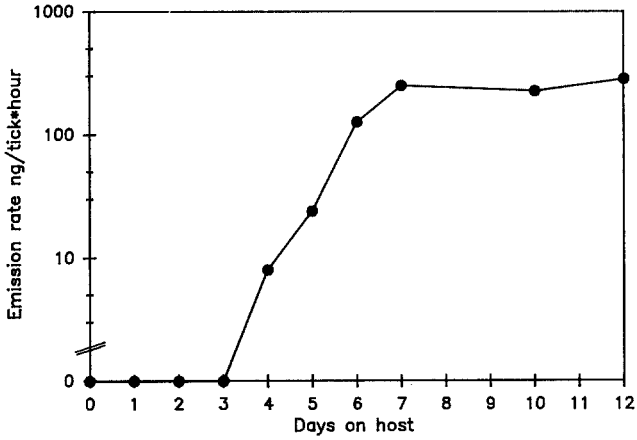


FIG. 2. Emission rate of *o*-nitrophenol from 25 males of *Amblyomma hebraeum* during the first 12 days following attachment on the rabbit.

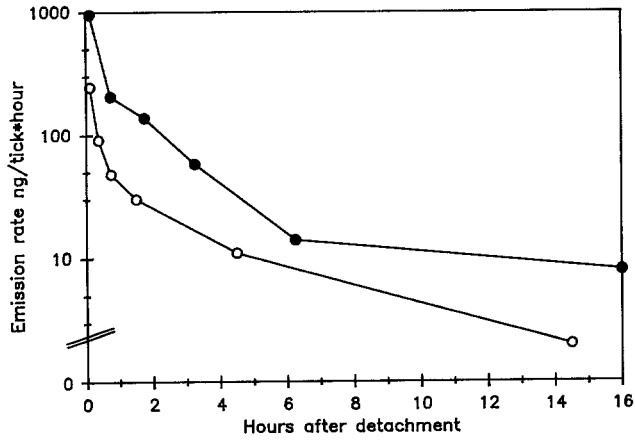


FIG. 3. Emission rates of *o*-nitrophenol in forcibly detached males of *Amblyomma variegatum* (filled circles, $N = 20$) and *Amblyomma hebraeum* (open circles, $N = 25$). Each point represents the relase rate, calculated as the mean pheromone amount collected during the sampling period per hour and per tick, at the midpoint of the sampling period.

able ONP and MS (in *A. variegatum*) were found in the rest of the tick. Systematic analysis of different organs from *A. variegatum* males showed that the pheromone was nearly absent from (1) dorsal cuticle without the lateral border, (2) legs, and (3) internal organs such as gut, salivary glands, and excretory,

nervous, and genital systems. Nearly all of the ONP and MS was associated with the ventral cuticle behind the genital opening and with the lateral border.

Dissection and histology of the cuticle revealed the presence of very large dermal glands type 2 (variable, from 200 up to 500 μm diam.) on the ventro lateral side, but not on the dorsal cuticle (Figure 4). In unfed males, these dermal glands were much smaller (60–80 μm diam.).

HPLC analysis of several extracts from carefully dissected and rinsed dermal glands type 2 allowed unambiguous detection of ONP and MS by comparison of retention times and UV spectra of the natural products with that of authentic standards (Figure 5). Presence of ONP was confirmed by combined capillary GC linked electrophysiology, using the ONP-sensitive dII wp/sw/A sensillum on the first pair of legs as a selective biological detector (Figure 6). We conclude that ONP and MS accumulate in these type 2 dermal glands and not in the dorsally located foveal glands.

DISCUSSION

Biosynthesis and Emission in A. variegatum. Our studies on the time course of biosynthesis and emission of ONP and MS in *A. variegatum* males demonstrated that unfed and feeding male ticks up to day 2 did not contain measurable amounts of ONP or MS or hydrolyzable conjugates. This contrasts with the case of the sex attractant pheromone 2,6-DCP, which is already present in unfed females of other metastriate species (Sonenshine, 1984a, 1986; Sonenshine et al., 1982). Trace of ONP emission was detected for the first time by HPLC from ticks at day 3, and within the male at the fourth day. Thus, release of ONP begins apparently as soon as synthesis starts. The mean ONP content of whole mature male ticks was 3.4 μg (range 1.3–7.3 μg). A similar amount of ONP (2 μg/tick) in males fed for six days was described (Schöni, 1987; Schöni et al., 1984). These substantial pheromone quantities represent up to about 0.25% of the weight of a fed male (average weight of 32 mg) or,in comparison, about 20 g for an average adult human. This contrasts sharply again with the sex pheromone 2,6-DCP, which occurs only in nanogram quantities, e.g., only 16 ng/tick were detected in hexane extracts of sexually active female *A. variegatum* that had been feeding for six days (Wood et al., 1975).

The timing of MS emission followed a similar pattern to that of ONP, but was only detectable one day later. Unlike ONP however, we detected MS in whole-body extracts not before day 8. This very low initial body content is very different from the reported value of about 1 μg/tick found previously in males feeding for six days on cows (Schöni, 1987; Schöni et al., 1984).

The emission from males and their surroundings on the host as measured with the Sep-Pak cartridge proved to be variable. Clearly, an impregnation of

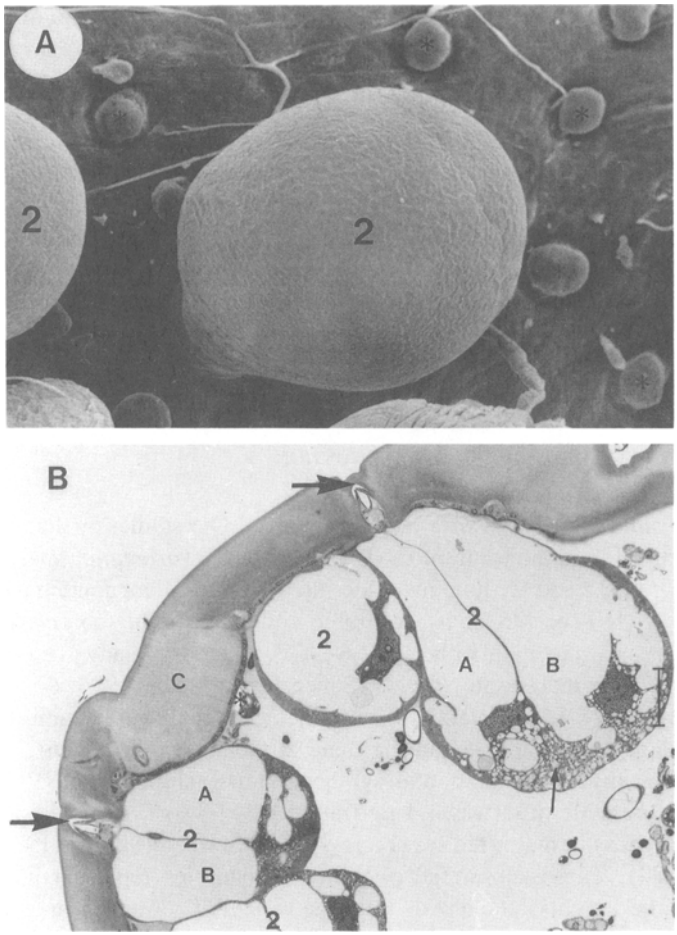


FIG. 4. Mature male of *Amblyomma variegatum* after 12 days of feeding. (A) Scanning electron micrograph of an exposed piece of ventral cuticle. Numerous small dermal glands type 1 (*) are visible; they are found all over the dorsal and ventral cuticle. Two very large dermal glands type 2 (2) are present; they are only associated with the ventrolateral cuticle. Magnification: 340× (B) Semithin section from the ventral cuticle. One small dermal gland type 1 (*) and four very large dermal glands type 2 (2) are present. The huge gland lumens (A,B) and the cytoplasm with many small secretory vesicles (⟶) are visible. (C) cuticle. ⟶: two canals with valvelike regions. Magnification: 190×.

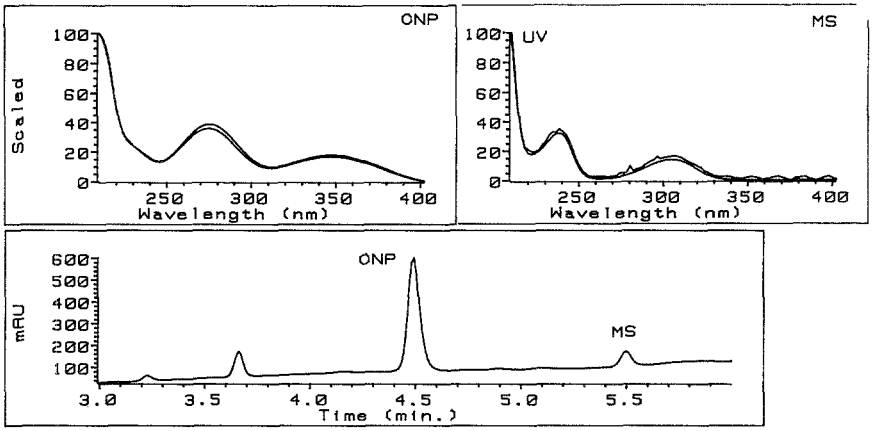


FIG. 5. Presence of *o*-nitrophenol (ONP; 890 ng) and methyl salicylate (MS; 60 ng) in a methanolic extract corresponding to about 75 dissected dermal glands type 2 of *Amblyomma variegatum* males at day 12 of feeding. Lower trace: HPLC separation on a reverse-phase column RP_{18} (125 × 4 mm) at 210 nm. The retention times and the UV-VIS spectra (upper traces) of the unknowns and of authentic standards are nearly identical.

the skin and feces occurred in the immediate vicinity, presumably through direct contact with the male, and up to a few centimeters away through adsorption from the vapor phase. We therefore collected not only from males per se, but also from the surroundings covered by the funnel. The true emission rates from attached males remains unknown; nevertheless, most of the collected pheromones should stem from the ticks because forcibly detached animals showed comparable release rates over the first few minutes in the absence of a contaminated substrate. Interestingly, release seemed to be temporarily lower during the first few hours after pairing with females. Females are apparently not disturbed by subsequent exposure to large quantities of pheromone in the immediate vicinity of the males.

Biosynthesis and Emission in A. hebraeum. Whole-body content of ONP in *A. hebraeum* was similar to *A. variegatum*, but emission rates appeared to be somewhat lower at about 250 ng/tick/hr. This value agreed with the maximum release of 400 ng/tick/hr as estimated by capillary GC-MS after tapping with the dynamic solvent effect (Apps, personal communication).

Apps et al. (1988) found that no MS, but benzaldehyde instead, was detectable in volatiles from *A. hebraeum*. Thus, the attachment–aggregation pheromone of both species contains ONP as a nonspecific main component, but differs with respect to the secondary components. *A. variegatum* probably uses MS and nonanoic acid in addition, whereas *A. hebraeum* uses benzaldehyde and 2-

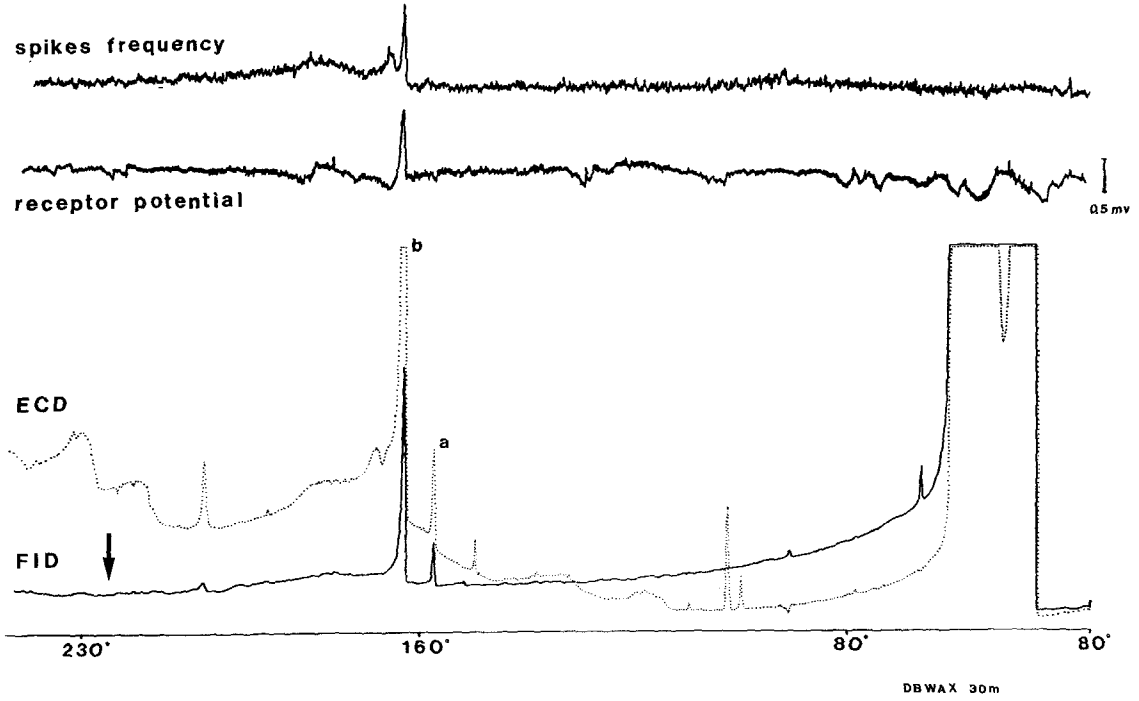


FIG. 6. Capillary gas chromatography-linked single sensillum analysis of a dermal gland type 2 methanolic extract of *Amblyomma variegatum* males at day 12 of feeding. FID, flame ionization detector; ECD, electron capture detector of the chromatograph. Recordings were made of receptor and action potentials of the *o*-nitrophenol-sensitive cell of the DII wp/sw/A sensillum on the Haller's organ of an *Amblyomma variegatum* male, placed opposite the effluent conduit of the gas chromatograph. Frequency/voltage conversion was applied to the AC trace to provide an on-line readout of the spike frequency. Note the selectivity of the olfactory unit for *o*-nitrophenol (b), with no response to methyl salicylate (a), the second major aromatic component of the extract. The elution temperature of nonanoic acid is indicated by the arrow. Detection limit for this product was about 1 ng.

methylpropanoic acid with maximum release rates of about 17 and 950 ng/tick/hr, respectively; in addition, variable amounts of medium-chain fatty acids such as nonanoic acid might be present (Apps et al., 1988).

A. hebraeum males matured at a slightly slower pace than *A. variegatum* on rabbits. This is in good agreement with earlier observations (Norval and Rechav, 1979; Rechav, 1977; Schöni, 1987; Schöni et al., 1984). In both species, the development of the clasping behavior was concomitant with the increase in pheromone release. In *A. variegatum* fed on cows, approximately 40% of the males showed clasping for the first time at day 3 (Schöni, 1987). This correlates with the time when we detected the first traces of emitted ONP in our rabbit-fed ticks. We observed that the remaining immature males developed the clasping behavior during the following two days, at a time when we noted a clear increase in ONP content and emission rates from the feeding ticks. It is tempting to speculate that the two phenomena may be controlled by the same hormones. Males prematurely detached during the first three days of feeding did not synthesize the pheromone; permanent contact with the host is a prerequisite for normal development.

Localization of Pheromone Glands. We found that ONP and MS are not associated with the foveal glands, as we initially suspected, but rather with the enormous dermal glands type 2 from the ventrolateral cuticle. Other tissues such as hypodermal cells or dermal glands type 1 from the same localization could also be implicated. However, this appears unlikely, because the dorsal cuticle, which contains these same cell types, is pheromone-free.

Dermal glands type 2 have not been reported previously to produce pheromones. Like the dermal glands type 1, in metastriate ticks, they are suspected to participate in the production of such secretions as molting fluid, the waterproofing wax layer or lipidic material on the cuticle of repleted ticks (Balashov, 1972; Lees, 1947). Our preliminary studies showed a secretory structure quite comparable to the foveal glands. Each dermal gland type 2 consists of two very large secretory cells, which contain many secretory vesicles. The glandular products are discharged into a huge lumen and gain the exterior through a cuticular duct. An innervated valvelike region in the duct might control the passage of secretion. This could represent the basis for a controlled release mechanism and explain the rapid decline in pheromone emission after forced detachment of males, and the temporary decrease in release rate after first contact with a female.

Nonanoic Acid. Schöni et al. (1984) found approximately 8 μg of nonanoic acid per tick in an ether extract of males at day 6 of feeding. Our HPLC technique was not sensitive enough to explore its biosynthesis and possible release, but neither did capillary GC did not this substrate in dermal gland extracts or in collected volatiles. Offered separately, nonanoic acid showed no influence on attraction, but together with MS in the blend it contributed to complete pheromone activity (Schöni et al., 1984; Schöni, 1987). It may function rather as a

contact chemostimulant. Male *A. hebraeum* intermittently released small amounts of nonanoic acid, among other medium-chain acids, but its behavioral role has not been elucidated (Apps et al., 1988).

Attachment–Aggregation Pheromones as Part of Host Location In both *Amblyomma* species, very high pheromone emission rates were observed. We estimate a maximum emission from a cow, infested with 152 male *A. variegatum* as reported by Hoogstraal (1956), to be in the order of about 7 mg/day! This complements various observations that indicate male infested steers or pheromone sources prove to be very attractive over a distance of several meters in the field; CO_2 further increased efficiency by rendering ticks active (Hess and De Castro, 1986; Norval et al., 1989a,b). These aggregation–attachment pheromones are thus efficient in signaling suitable hosts and must play an important part in the host-location strategy of the two *Amblyomma* species studied.

Acknowledgments—We are grateful for the generous financial support of the Swiss Office for Education and Science, the Swiss National Science Foundation (grant 3.609-0.87) and the Sandoz and Roche Foundations. We wish to thank Dr. Immler, and Misters Bouvard, Rohrer, and Cesari from Ciba-Geigy Ltd. (CRA St. Aubin) for generously supplying us with ticks. Dr. Apps (Pretoria) kindly communicated the pheromone emission rates of *A. hebraeum*.

REFERENCES

ALLAN, S.A., PHILLIPS, J.S., TAYLOR, D. and SONENSHINE, D.E. 1988. Genital sex pheromones of ixodid ticks: Evidence for the role of fatty acids from the anterior reproductive tract in mating of *Dermacentor variabilis* and *Dermacentor andersoni*. *J. Insect Physiol.* 34:315–323.

ALLAN, S.A., PHILLIPS, J.S., and SONENSHINE, D.E. 1989. Species recognition elicited by differences in composition of genital sex pheromone in *Dermacentor variabilis* and *D. andersoni* (Acari: Ixodidae). *J. Med. Entomol.* 26:539–546.

APPS, P.J., VILJOEN, H.W., and PRETORIUS, V. 1988. Aggregation pheromones of the bont tick *Amblyomma hebraeum*: Identification of candidates for bioassay. *Onderstepoort J. Vet. Res.* 55:135–137.

BALASHOV, Y.S. 1972. Bloodsucking ticks (Ixodoidea)—vectors of diseases of man and animals. *Misc. Publ. Entomol. Soc. Am.* 8:161–376 (Translation No. 500, U.S. NAMRU-3, original Russian publication by Nauka Publishers, Leningrad, 1968).

GLADNEY, W.J. 1971. Mate-seeking by female *Amblyomma maculatum* Koch (Acarina: Ixodoidea) on a bovine. *Nature* 232:401–402.

GLADNEY, W.J., ERNST, S.E., and GRABBE, R.R. 1974a. The aggregation response of the Gulf coast tick on cattle. *Ann. Entomol. Soc. Am.* 67:750–752.

GLADNEY, W.J., GRABBE, R.R., ERNST, S.E., and OEHLER, D.D. 1974b. The Gulf coast tick: Evidence of a pheromone produced by males. *J. Med. Entomol.* 11:303–306.

GÖDDE, J. 1985. Low cost storing of two electrical biosignals from DC to 20 kHz at more than 80 dB dynamic range. *Pfluegers Arch.* 403:324–327.

HAMILTON, J.G.C., SONENSHINE, D.E., and LUSBY, W.R. 1989. Cholesteryl oleate: mounting sex pheromone of the hard tick *Dermacentor variabilis* (Say) (Acari: Ixodoidea). *J. Insect Physiol.* 35:873–879.

HESS, E., and DE CASTRO, J.J. 1986. Field tests of the response of female *Amblyomma variegatum*

(Acari: Ixodoidea) to the synthetic aggregation-attachment pheromone and its components. *Exp. Appl. Acarol.* 2:249–255.

HOOGSTRAAL, H. 1956. African Ixodoidea. I. Ticks of the Sudan. U.S. Naval Department of Medical Zoology, 1–1101 pp.

KELLUM, D., and BERGER, R.S. 1977. Relationship of the occurrence and function of 2,6-dichlorophenol in two species of *Amblyomma* (Acari: Ixodoidea). *J. Med. Entomol.* 13:701–705.

KUWATA, K., and TANAKA, S. 1988. Liquid chromatographic determination of traces of phenols in air. *J. Chromatogr.* 442:407–411.

LEES, A.D. 1947. Transpiration and the structure of the epicuticle in ticks. *J. Exp. Biol.* 23:379–410.

NORVAL, R.A.I., and RECHAV, Y. 1979. An assembly pheromone and its perception in the tick *Amblyomma variegatum* (Acarina, Ixodoidea). *J. Med. Entomol.* 16:507–511.

NORVAL, R.A.I., ANDREW, H.R., and YUNKER, C.E. 1989a. Pheromone-mediation of host-selection in bont ticks (*Amblyomma hebraeum* Koch). *Science* 243:364–365.

NORVAL, R.A.I., BUTLER, J.F., and YUNKER, C.E. 1989b. Use of carbon dioxide and natural or synthetic aggregation-attachment pheromone of the bont tick, *Amblyomma hebraeum*, to attract and trap unfed adults in the field. *Exp. Appl. Acarol.* 7:171–180.

RECHAV, Y. 1977. Evidence for an assembly pheromone(s) produced by males of the bont tick *Amblyomma hebraeum* (Acarina, Ixodoidea). *J. Med. Entomol.* 14:71–78.

RECHAV, Y., WHITEHEAD, G.B., AND KNIGHT, M.M. 1976. Aggregation response of nymphs to pheromone(s) produced by the males of the tick *Amblyomma hebraeum* (Koch). *Nature* 259:563–564.

RECHAV, Y., TERRY, S., KNIGHT, M.M, and CROSS, R.M.M. 1977. Chemoreceptor organs used in detection of pheromone(s) of the tick *Amblyomma hebraeum* (Acarina, Ixodoidea). *J. Med. Entomol.* 14:395–400.

SCHÖNI, R. 1987. Das wirtsgebundene Aggregationspheromon der tropischen Buntzecke *Amblyomma variegatum* Fabricius (Acari: Ixodoidea). PhD thesis, University of Neuchâtel. 145 pp.

SCHÖNI, R., HESS, E., BLUM, W., and RAMSTEIN, K. 1984. The aggregation-attachement pheromone of the tropical bont tick *Amblyomma variegatum* Fabricius (Acari: Ixodoidea): Isolation, identification and action of its components. *J. Insect Physiol.* 30:613–618.

SONENSHINE, D.E. 1984. Pheromones of Acari and their potential use in control strategies, pp. 100–108, *in* D.A. Griffiths and C.E. Bowman (eds.). Acarology VI, Vol. 1. Ellis Horwood, Chichester.

SONENSHINE, D.E. 1985. Pheromones and other semiochemicals of the Acari. *Annu. Rev. Entomol.* 30:1–28.

SONENSHINE, D.E. 1986. Tick pheromones: An overview, pp. 342–360, *in* J.R. Sauer and J.A. Hair (eds.). Morphology, Physiology, and Behavioral Biology of Ticks. Ellis Horwood, Chicester.

SONENSHINE, D.E., SILVERSTEIN, R.M., and RECHAV, Y. 1982. Tick pheromone mechanisms, pp. 439–468, *in* F.D. Obenchain and R.L. Galun (eds.). Physiology of Ticks. Pergamon, Oxford.

WOOD, W.F., LEAHY, M.G., GALUN, R., PRESTWICH, G.D., MEINWALD, J., PRUNELL, R.E., and PAYNE, R.C. 1975. Phenols as pheromones of ixodid ticks: A general phenomenon? *J. Chem. Ecol.* 1:501–509.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

RESPONSES TO PREY CHEMICALS BY A LACERTID LIZARD, *Podarcis muralis*: PREY CHEMICAL DISCRIMINATION AND POSTSTRIKE ELEVATION IN TONGUE-FLICK RATE

WILLIAM E. COOPER, JR.

Department of Biology
Auburn University at Montgomery
Montgomery, Alabama 36117

(Received October 24, 1990; accepted January 14, 1991)

Abstract—The ability to discriminate prey chemicals from control substances and the presence of a poststrike elevation in tongue-flicking (PETF) rate are experimentally demonstrated in the lacertid lizard, *Podarcis muralis*. The tongue-flick attack score, a composite index of response strength, was significantly higher in response to integumental chemicals from cricket than to cologne or distilled water. The cricket chemicals additionally elicited a significantly greater rate of tongue-flicking and higher proportion of attacks by the lizards than did control stimuli. PETF combined with apparent searching movements strongly suggest the presence of strike-induced chemosensory searching (SICS). Experimental evidence indicates that both PETF and SICS occur in insectivorous representatives of three families of actively foraging autarchoglossan lizards, suggesting their widespread occurrence in such lizards. The adaptive roles of chemosensory behavior in the foraging behavior of *P. muralis* are discussed. It is proposed that these lizards may form chemical search images and that PETF and SICS may have been present in the lacertilian ancestors of snakes.

Key Words—Prey chemicals, tongue-flicking, feeding behavior, Reptilia, Lacertidae, *Podarcis muralis*.

INTRODUCTION

Knowledge about the importance of the chemical senses to lizards for locating and identifying prey is growing rapidly, but our understanding of chemosensory roles in foraging and other behaviors by lacertid lizards is fragmentary. *Lacerta vivipara* may be able to identify the vomodors (Cooper and Burghardt, 1990b)

0098-0331/91/0500-0849$06.50/0

of one of its predators, the snake *Vipera berus* (Van Damme et al., 1990); tongue-flicking rates in this lizard increase in cages previously occupied by the predator. Tentative experimental findings were presented by Kahmann (1939) on the use of the vomeronasal organ in feeding. Recently, an ability to discriminate integumentary chemicals of crickets from control substances was confirmed for *Podarcis hispanica* (Cooper, 1990a), but the sample size was very small ($N = 5$).

Lizards in several families of actively foraging insectivores can locate and identify prey by tongue-flicking (partially reviewed by Burghardt, 1970; Halpern, 1991; Simon, 1983; and Cooper, 1990b). Discrimination of prey chemicals has been demonstrated experimentally in representatives of several families of lizards that are insectivorous active foragers or cruising foragers (Regal, 1978), including Anguidae (Cooper, 1990c), Helodermatidae (Cooper, 1989b), Lacertidae (Cooper, 1990a), Scincidae (Loop and Scoville, 1972; Burghardt, 1973; Von Achen and Rakestraw, 1984; Nicoletto, 1985; Cooper and Vitt, 1989), Teiidae (Cooper, in press), Varanidae (Cooper, 1989b), and possibly Cordylidae (gerrhosaurines; Cooper, in press), as well as in a nocturnal gecko (Dial, 1978; Dial et al., 1989) and an herbivorous iguanid (Pedersen, 1988; Krekorian, 1989; Cooper and Alberts, 1990). Accordingly, it may be predicted that typical lacertids, the vast majority of which appear to be actively foraging insectivores, are capable of prey chemical discrimination.

Some lizards that can discriminate prey chemicals also use chemical cues in a manner analogous to strike-induced chemosensory searching (SICS) in rattlesnakes (Chiszar and Scudder, 1980). After striking and envenomating potentially dangerous prey, such as large rodents, rattlesnakes and many other venomous snakes relocate the prey after it has been incapacitated by following the prey's scent trail. Shortly after releasing the envenomated prey, a rattlesnake greatly increases its tongue-flick rate and follows the prey's trail by tongue-flicking (Chiszar and Scudder, 1980).

The elevated tongue-flicking rate and searching behavior constitute SICS; the larger behavior pattern is called the strike–release–trail strategy. Some nonvenomous snakes (Cooper et al., 1989), a varanid lizard (Cooper, 1989c), and a cordylid lizard (Cooper, in press) exhibit behavior similar to SICS despite failure to voluntarily release prey. This was discovered after removal of prey items from their mouths. Because the lizards not only increase their tongue-flicking rates, but also their locomotion, apparently searching for the removed prey (Cooper, 1989c), they show SICS.

In this paper I present experimental findings on responses to prey chemicals by a lacertid lizard, *Podarcis muralis*. The topics investigated are (1) the ability to detect and respond differentially to prey chemicals, (2) the presence of one component of SICS, the poststrike elevation in tongue-flicking (PETF),

and (3) the duration of PETF. Roles of chemical cues in lacertid foraging are discussed.

Prey odor discrimination in squamate reptiles appears to be mediated by vomerolfaction (Cooper and Burghardt, 1990c), with the tongue serving as a sampling device that delivers chemicals from the external environment to the mouth, where the chemicals are transferred via the vomeronasal ducts to the vomeronasal organs above the roof of the mouth for sensory analysis. Normal responses to food chemicals require vomeronasal participation (Halpern and Frumin, 1979; Halpern and Kubie, 1980; Graves and Halpern, 1990; Cooper and Alberts, 1991). Thus, tongue-flicking behavior has been used extensively as an observable index of chemosensory investigation (e.g., Burghardt, 1970; Cooper and Burghardt, 1990a; Halpern, 1991) and is so used here.

METHODS AND MATERIALS

Subjects and Maintenance. Sixteen adult *P. muralis* purchased from a commercial dealer were maintained in the laboratory from their arrival on December 27, 1989, for several months before the experiments. This allowed habituation to experimental conditions, especially the close proximity of an experimenter's hand. Each lizard was housed individually in a 30 × 30 × 26-cm glass terrarium containing a water bowl, a wooden shelter board, a sand substrate, and a wire screen top.

Fluorescent lighting was provided during the daylight hours. Air temperatures fluctuated from 22 to 29°C. Because these temperatures were lower than those expected to produce the highest tongue-flicking rates (Cooper and Vitt, 1986; Van Damme et al., 1990), undercage heaters were used to allow the lizards to thermoregulate and to remain fully active even when air temperatures were low. Cloacal temperatures were typically in the low thirties.

The lizards were fed crickets to satiation three times per week. Water was available ad libitum. To prepare the lizards for the experiments, the experimenter would move slowly while feeding and watering them. *Podarcis muralis* is warier than many lizard species, but a sufficient number of individuals became habituated to the experimenter's presence to allow completion of the experiments.

Prey Odor Discrimination. The ability of the lizards to discriminate prey chemicals from control substances was assessed by an experiment with a randomized blocks design in which each individual responded to three stimuli presented on cotton swabs: integumentary chemicals of domestic crickets, cologne (Mennen Skin Bracer, Spice Scent), and distilled water. Stimuli were prepared by initially dipping the cotton tip of a 15-cm wooden applicator in distilled

water; other substances were added when needed by either dipping a moistened swab in cologne or by rolling a moistened cotton over the integument of a cricket. The distilled water condition served as an odorless control to gauge responsiveness to the experimental milieu. Cologne was a pungency control to indicate degree of response to a highly odorous substance lacking trophic or social relevance. Each swab was used only once.

To begin a trial, the experimenter slowly approached a lizard's cage and carefully removed its wire cover to avoid disturbing the lizard. He then placed the cotton tip of the applicator 1–1.5 cm anterior to the lizard's snout. Tongue-flicks directed to the applicator were counted for 60 sec or until the lizard bit the applicator, whichever came first. Timing began when the first tongue-flick occurred. In addition, the presence or absence of biting and latency to biting were recorded; a latency of 60 sec was assigned if a lizard did not bite.

The experiment was conducted between 1200 and 1430 hr Central Standard Time on March 30, 1990. Nine lizards were tested with stimuli presented in random sequence, with approximately equal numbers of individuals tested with each stimulus in each of the three trials. The intertrial interval was ca. 60 min. The air temperature was only 25°C, but the sand and lizards were warmer. Although body temperature strongly affects tongue-flicking rates (Cooper and Vitt, 1986), the relative responsiveness to various chemical stimuli does not appear to be affected over a wide range (20–35°C) of temperatures (Van Damme et al., 1990).

Overall responsiveness in the three conditions was measured by the tongue-flick attack score for repeated measures experiments, TFAS(R) (Cooper and Burghardt, 1990a). If the lizard does not bite, TFAS(R) is the number of tongue-flicks it emits during the trial. If it bites, TFAS(R) is the maximum number of tongue-flicks emitted by that individual lizard in any of the three trials plus (60 − latency to bite in seconds). The stronger weight of biting than any amount of tongue-flicking in TFAS(R) reflects a conviction that a predatory attack is a stronger indication of feeding motivation than is chemosensory investigation by tongue-flicking. Numbers of tongue-flicks, latency to bite, and numbers of individuals biting in the three conditions were also compared.

Differences among stimulus conditions in TFAS(R) and number of tongue-flicks were tested for significance by analysis of variance for a single-factor experiment with a repeated measures design (Winer, 1962). Individual comparisons between pairs of means were made using Student-Newman-Keuls tests. Homogeneity of variance was tested by Hartley's tests for heterogeneity followed by logarithmic transformation where needed. Latency differences were examined by nonparametric Friedman two-way analysis of variance because the distribution of latency was nonnormal. The difference in number of lizards biting in response to cricket stimuli and the other two stimuli was assessed by a

binomial test (Siegel, 1956). Tests were two-tailed unless otherwise stated, with $\alpha = 0.05$.

PETF. In previous related studies of lizards and nonvenomous snakes (Cooper, 1989c, 1991b; Cooper et al., 1989), apparent searching movements have been noted qualitatively; only the increase in tongue-flicking rate has been treated quantitatively. Because tongue-flick rates might increase in the absence of search involving locomotion, it is important to distinguish the tongue-flicking behavior from SICS. The increase in tongue-flicking rate after the prey has been removed from the mouth of an animal is the poststrike elevation in tongue-flicking (PETF; Cooper, 1992).

The effect of biting on tongue-flicking rate was determined by counting tongue-flicks emitted in 10 consecutive minutes in four experimental conditions. In the strike condition, a cricket was tied to a white thread (ca. 25 cm) and was lowered to a position just above the sand 10 cm anterior to a lizard's snout. Immediately after the lizard bit the cricket, the experimenter removed the cricket from the lizard's mouth as gently as possible and immediately began counting tongue-flicks. The cricket was presented in the same way in the pull condition, but after the lizard approached and was poised to bite the cricket, the experimenter removed the cricket with one hand and simultaneously moved the lizard a short distance (as if separating it from bitten prey) with the other hand, then began counting tongue-flicks. In the sight condition, a cricket was held at the far side of the cage in clear view of the lizard. The cricket was removed after 10 sec or sooner if the lizard approached it. The lizard was not allowed to tongue-flick it. The string condition was similar to the sight condition except that no cricket was tied to the string. Counting of tongue-flicks was initiated upon removal of the experimental stimuli in the last two conditions.

Forced removal of prey from a lizard's mouth is a somewhat drastic experimental procedure that requires contact with the lizard. It seems likely to have behavioral effects beyond those on tongue-flicking. However, this procedure is necessary for demonstration of PETF in species that do not voluntarily release prey. The pull condition serves as a control for handling effects. In this condition, the lizard is allowed to tongue-flick the cricket. It is hoped that the only important difference from the strike condition is the absence of chemical and possibly other cues associated with biting. In a study of garter snakes, an additional control for the effects of tactile stimuli during biting was used to show that the chemical cues received during biting produced PETF (Cooper et al., 1989). The sight condition is a control for the effect of seeing the prey on tongue-flicking rates. Tongue-flicking rates expected in the experimental setting are revealed in the string condition. For further discussion of the experimental procedures and interpretation of results, see Cooper et al. (1989) and Cooper (1989c).

A repeated measures (randomized blocks) design in which each lizard responded in four conditions was used, with a partially counterbalanced sequence of presentation. Lizards were tested only once a day, two days after having been fed to satiation. Eight lizards were tested, all of which had been subjects in the study of prey odor discrimination. The experiment was conducted at 1200–1500 hr CST between April 6 and 18, 1990. Although the experiment was conducted at air temperatures of 25–27°C, the lizards were active and their bodies were warmer than the air temperature.

For each minute, the significances of differences among conditions were assessed by nonparametric Friedman two-way analysis of variance (Siegel, 1956), which was necessary because the tongue-flicking distributions were nonnormal due to frequent zero values. When the main condition effect was significant, individual comparisons were made between pairs of conditions (Hollander and Wolfe, 1973). Binomial tests were conducted to determine the significance of differences in numbers of days of greater relative magnitude for selected comparisons between conditions. Alpha was 0.05 and tests of significance were two-tailed except as noted.

RESULTS

Prey Odor Discrimination. The lizards tongue-flicked in all conditions, but emitted the most tongue-flicks in cricket trials and bit only in response to cricket stimuli, resulting in high TFAS(R) values for the cricket condition (Table 1). Variances of TFAS(R) were heterogeneous for the raw data (Hartley's $F_{max} = 15.76$; $df = 3, 8$; $P < 0.01$), but logarithmic transformation yielded homogeneity ($F_{max} = 2.49$; $df = 3, 8$; $P > 0.10$). The main condition effect for the transformed TFAS(R) data was highly significant ($F = 18.83$; $df = 2, 16$; $P < 0.002$). TFAS(R) was significantly greater in the cricket condition than in either the cologne or distilled water condition ($P < 0.01$ each), but did not differ between the two control conditions ($P > 0.10$).

The lizards tongue-flicked at a much higher mean rate in response to cricket stimuli than to the control stimuli; for the two control groups the means were similar but variance was somewhat larger in response to distilled water because two individuals emitted 85% of all tongue-flicks in that condition. Variances of tongue-flicks were heterogeneous for the raw data ($F_{max} = 11.65$; $df = 3, 8$; $P < 0.01$), but the heterogeneity was removed by logarithmic transformation ($F_{max} = 1.92$; $df = 3, 8$; $P > 0.10$). The stimulus effect was significant ($F = 9.39$; $df = 2, 16$; $P < 0.01$); with significantly more tongue-flicks emitted in response to cricket stimuli than to cologne ($P < 0.05$) or distilled water ($P < 0.01$).

TABLE 1. RESPONSES BY NINE ADULT *Podarcis muralis* TO CHEMICAL STIMULI PRESENTED ON COTTON-TIPPED APPLICATORS

	Cricket	Cologne	Distilled water
TFAS(R)			
Mean	45.56	11.67	9.33
SE	8.97	2.26	3.38
Range	19–101	3–24	1–33
Tongue-flicks			
Mean	30.56	11.67	9.33
SE	7.72	2.26	3.38
Range	7–83	3–24	1–33
Latency			
Mean	48.56	60	60
SE	6.39	0	0
Range	8–60		
Number biting	3	0	0

Three lizards bit swabs bearing cricket stimuli; no bites occurred in trials with control stimuli. Thus, no significant difference in number of lizards biting occurred between the cricket condition and either of the control conditions taken individually. However, biting is predicted to be more frequent in response to prey stimuli. If it is assumed that there is an equal chance of a lizard biting in each condition, the probability that a given lizard that bites will do so in the cricket condition is 0.333 and the combined probability of it biting in one of the other conditions is 0.667. The likelihood of all three biting in the cricket condition is thus only 0.037 (one-tailed), indicating a significantly higher frequency of biting in response to cricket stimuli than to the combined controls. Latency to bite did not differ significantly among conditions ($\chi_r^2 = 1.50$, $df = 2$, $P > 0.10$).

PETF. The highest mean tongue-flicking rates occurred in the strike condition throughout the experiment except in minutes 2 and 6, when the means were higher in the pull condition (Table 2). There was also a tie for the highest mean between the strike and pull conditions in the fourth minute. During each of the first six minutes the pull condition elicited higher total tongue-flicks rates than did the sight and string conditions and had the individual emitting the greatest number of tongue-flicks among these conditions (binomial $P = 0.016$ for each comparison, one-tailed). In addition, the sight condition elicited higher tongue-flicking rates than did the string condition and had the individual emitting the greatest number of tongue-flicks for the two conditions in each of the

TABLE 2. MEANS, STANDARD ERRORS (SE), AND RANGES OF NUMBERS OF TONGUE-FLICKS EMITTED BY *Podarcis Muralis* DURING TEN CONSECUTIVE MINUTES IN FOUR EXPERIMENTAL CONDITIONS

	Mean				SE				Range			
Minute	Strike	Pull	Sight	String	Strike	Pull	Sight	String	Strike	Pull	Sight	String
1	17.88	6.50	4.25	1.75	1.36	1.58	1.74	1.75	12–22	2–15	0–12	0–4
2	4.00	4.25	3.00	1.00	1.36	1.51	1.34	1.00	0–12	0–12	0–9	0–3
3	8.63	5.25	2.13	0.88	2.65	2.36	0.74	0.48	0–19	0–21	0–6	0–3
4	3.63	3.63	0.88	0.25	1.24	2.03	0.44	0.25	0–8	0–15	0–3	0–2
5	2.63	2.50	0.75	0.25	1.16	1.16	0.41	0.25	0–8	0–8	0–3	0–2
6	2.50	2.75	0.50	0.38	1.72	1.35	0.38	0.18	0–14	0–9	0–3	0–1
7	3.50	0.88	2.13	2.83	1.31	0.48	1.74	0.58	0–11	0–3	0–14	0–4
8	3.75	1.00	1.13	0.38	1.87	0.87	0.99	0.26	0–15	0–7	0–8	0–2
9	1.88	0.50	0.75	0.75	1.26	0.50	0.49	0.62	0–10	0–4	0–4	0–5
10	1.50	0.50	0.25	0.63	0.57	0.33	0.16	0.50	0–5	0–2	0–1	0–4

first six minutes (binomial $P = 0.016$ for each comparison, one-tailed). Overall, there were more tongue-flicks in the sight condition than in the string condition in eight of ten minutes, with one tie and one reversal [P (8 of 9) = 0.035, one-tailed].

Despite these consistent differences, numbers of tongue-flicks varied significantly among conditions only during the first three minutes. In the first minute, the main effect was significant ($\chi_r^2 = 18.56$, $df = 3$, $P < 0.001$). Significantly more tongue-flicks were emitted in the strike condition than in the pull ($P < 0.05$, one-tailed), sight ($P < 0.01$), and string ($P < 0.01$), and string ($P < 0.01$) conditions. There were no significant differences among the three control conditions. The main effect was only marginally significant during the second minute ($\chi_r^2 = 6.45$, $df = 3$, $P < 0.05$, one-tailed) and the numbers of tongue-flicks were not significantly greater in the strike condition than in the control conditions. The only significant difference was a greater response in the pull than in the string condition ($P < 0.05$, one-tailed). During the third minute, the tongue-flicking rate differed significantly among conditions ($\chi_r^2 = 7.91$, $df = 3$, $P < 0.05$), but PETF was absent because the number of tongue-flicks in the strike condition was significantly greater than that in only the string condition ($P < 0.05$). The sole other significant difference was that more tongue-flicks occurred in the pull condition than in the string condition ($P < 0.05$, one-tailed). The nonsignificant main effects for the remaining minutes all had probability values greater than 0.10 and χ_r^2 values with three degrees of freedom of 4.35, 1.88, 1.39, 1.48, 2.29, 0.56, and 2.59, consecutively).

Although movements were not quantified, individuals that tongue-flicked at high rates crawled while doing so. The locomotion, which presumably in part indicated searching movements, decreased greatly after the first few minutes, as did tongue-flicking. Most individuals that did not tongue-flick during a given minute were also still at that time. Differences among conditions, therefore, were closely related to the proportions of lizards that tongue-flicked (Figure 1). During the first few minutes, high proportions of lizards tongue-flicked in all conditions. Thereafter, the proportions fell rapidly in the three control conditions. They also decreased in the strike condition and became increasingly variable in the later minutes, but remained higher than in the control conditions throughout the experimental interval. Some individual lizards tongue-flicked in the early minutes, remained quiescent for several minutes, then tongue-flicked again later.

The proportion of individuals tongue-flicking was consistently higher in the strike condition than in the control conditions. It was higher than in the pull condition in nine of ten minutes (binomial $P = 0.02$, one-tailed), including the last seven minutes (binomial $P = 0.008$). The proportion tongue-flicking in the strike condition was higher than in the sight and string conditions in all ten minutes (binomial $P = 0.001$ for each comparison, one-tailed). Higher pro-

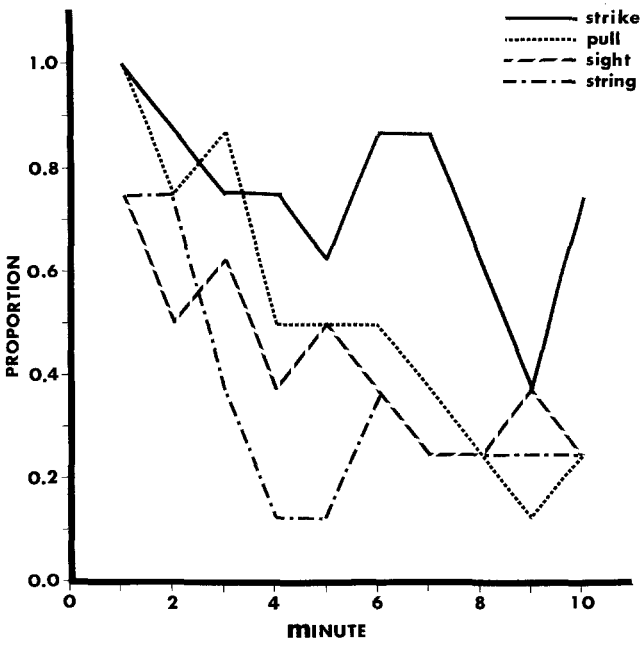


FIG. 1. Proportions of individuals of *Podarcis muralis* tongue-flicking at least once in the four experimental conditions.

portions tongue-flicked in the pull condition than in the other control conditions during the first seven days. For each comparison, the proportion was higher on six days for the pull condition, with a tie occurring on the remaining day. These differences are significant by a binomial (sign) test ($P = 0.016$, one-tailed). The proportions tongue-flicking were similar in the sight and string conditions, with ties on five days. Although greater proportions tongue-flicked in the sight condition than in the string condition in four of the remaining five days, the two conditions did not differ significantly (binomial $P = 0.19$, one-tailed).

DISCUSSION

Prey Odor Discrimination. Podarcis muralis readily detects prey chemicals and discriminates them from trophically irrelevant chemical stimuli. These results extend the previous findings for *P. hispanica* (Cooper, 1990a) to another lacertid species. In both species TFAS and numbers of tongue-flicks were significantly higher in response to prey chemicals than to control stimuli. However, *P. muralis* had TFAS and numbers of tongue-flicks over twice as great as those of *P. hispanica*. Reasons for these differences may include differences in

temperature (Cooper and Vitt, 1986), lighting, and random effects in small samples, as well as true interspecific differences.

Both biting and tongue-flicking contributed substantially to the difference between responses to prey chemicals and control chemicals. In this respect the responses of *P. muralis* are most similar to those of the varanid *Varanus exanthematicus* (Cooper, 1989b) and the anguid *Elgaria coerulea* (Cooper, 1990c), but it is now obvious that interspecific differences in the relative importance of biting and tongue-flicking exist among congeners. This is known in *Podarcis* (Cooper, 1990a, this paper) and *Elgaria* (Cooper, 1990c). Although the experimental conditions and procedures are similar in these cases, some of the apparent differences may reflect differential (defensive) responses to experimenters and uncontrolled experimental variables extraneous to chemosensory analysis and response.

Whatever the cause of the interspecific differences, it now seems likely that detection and recognition of prey chemicals are widespread in Lacertidae. The results agree with previous findings for lizard families consisting largely of active foragers (Cooper, 1989a, 1990a; Cooper and Vitt, 1989). Because information is available for only one or at most a few species per family, it will be important to determine the extent to which these responses to prey chemicals are phylogeneticaly constrained or are responsive to changes in foraging mode. Within families, most species have a similar foraging mode (Huey and Pianka, 1981; Vitt and Price, 1982); the crucial tests will require study of species that have evolved foraging modes atypical for their families.

PETF. The high level of tongue-flicking in the strike condition indicates that PETF occurs in *P. muralis*. PETF is now known to occur in all three species of actively foraging insectivorous lizards that have been studied (Cooper, 1989c, in press, this paper). Its demonstrated duration is brief in *P. muralis*, only 1 min, even shorter than the 2-min interval of PETF in a cordylid species (Cooper, in press). PETF was significant in a varanid (Cooper, 1989c) during the first 2 min but was not examined thereafter.

Although significant levels of PETF might be detected over a somewhat longer interval by using a larger sample size, it is clear that the major increase in tongue-flicking rate attributable to biting begins almost immediately and wanes rapidly. The criterion I have adopted for the existence of SICS in a particular minute is a combined significance of the condition effect and significantly higher number of tongue-flicks in the strike condition than in each of the three control conditions. This is a conservative criterion that could obscure some important findings.

The data on numbers of minutes in which more tongue-flicks were emitted and the greater proportion of lizards tongue-flicking in the strike condition than in the control conditions show that effects of biting last considerably longer than 2 min, some slight residual effects being manifest throughout the 10-min trials.

The sequelae of biting are thus a rapid increase in tongue-flicking and searching movements, followed by a rapid decrease in these behaviors, with a final, more prolonged interval of intermittent chemical monitoring at a reduced rate of tongue-flicking.

Roles of Prey Chemicals in Lacertid Foraging Behavior. As actively foraging insectivores, most lacertid species presumably search for prey that may be cryptic or hidden from view. The ability to respond selectively to prey chemicals must enhance the ability of these lizards to locate and confirm the identity of such prey. Some species of lacertids have been characterized as ambush foragers (Huey and Pianka, 1981) and would thus be expected not to use chemical cues to locate prey. These species would seemingly be good candidates for testing the intrafamilial responsiveness of reliance on prey chemical discrimination to changes in foraging. However, the movement frequencies of the putative lacertid ambush foragers are higher than those of typical phrynosomatid and polychrid (both formerly iguanid; Frost and Etheridge, 1989) ambush foragers, suggesting that prey chemical discrimination could retain importance for these lizards. Use of chemical cues intermediate to that of typical ambush and active foragers is currently unknown, but seems a distinct possibility in such cases.

The temporal pattern of responses to chemical cues in the PETF experiment is readily interpretable in terms of their probable adaptive significance in foraging. Having bitten prey that has escaped, a lizard has also sampled prey chemicals that may be used in an attempt to relocate the prey that is known to be in the vicinity. Insect prey, even if injured, are not nearly as likely to be captured by following a scent trail as are the envenomated prey of rattlesnakes. Furthermore, actively foraging lizards search for prey briefly in one area and then move to another for further search. Therefore, rapid tongue-flicking and searching movements should be beneficial only briefly, presumably explaining the brevity of these responses in *P. muralis*. This contrasts markedly with the prolonged chemosensory searching in rattlesnakes, which may last for hours (Chiszar et al., 1982, 1985) while the snake attempts to relocate envenomated prey that is unlikely to escape.

The longer-lasting intermittent tongue-flicking in *P. muralis* could be explained by periodic fluctuation of neural response to the prey chemicals to levels above threshold for tongue-flicking. Regardless of the physiological mechanism, such chemosensory investigation could be, but is not necessarily, adaptive. In a lizard that is likely to have moved to a new area to forage within a few minutes, any remaining responses to a specific prey chemical pattern might aid in the location of other individuals of the prey type likely to be in the area. This suggests the possibility that the lizards may form a chemical search image, as reported for a viperid snake by Chiszar et al. (1985).

Because PETF was accompanied by apparent searching movements, *P.*

muralis very likely shows SICS, as suggested previously in *V. exanthematicus* (Cooper et al., 1989) and *G. nigrolineatus* (Cooper, in press). The presence of PETF and very probably of SICS in three families representing the lacertoid, scincoid, and varanoid branches of Autarchoglossa (Estes et al., 1988) suggests that these behaviors are quite widespread in autarchoglossan lizards. Thus, SICS, which has been hypothesized to be a preadaptation for evolution of the strike–release–trail strategy in highly venomous viperid snakes (Cooper et al., 1989), may have been present in the lacertilian ancestors of snakes. The prediction that PETF and SICS are present in families of actively foraging lizards has now been supported for all three families studied.

Acknowledgments—This study was partially supported by a grant-in-aid from Auburn University at Montgomery.

REFERENCES

BURGHARDT, G.M. 1970. Chemical perception in reptiles, pp. 241–308, *in* J.W. Johnston, D.G. Moulton, and A. Turk (eds.). Advances in Chemoreception, Vol. I. Communication by Chemical Signals. Appleton-Century-Crofts, New York.

BURGHARDT, G.M. 1973. Chemical release of prey attack: Extension to naive newly hatched lizards, *Eumeces fasciatus*. *Copeia* 1973:178–181.

CHISZAR, D., and SCUDDER, K.M. 1980. Chemosensory searching by rattlesnakes during predatory episodes, pp. 125–139, *in* D. Muller-Schwarze and R.M. Silverstein (eds.). Chemical Signals: Vertebrates and Aquatic Invertebrates. Plenum Press, New York.

CHISZAR, D., RADCLIFFE, C.W., O'CONNELL, B., and SMITH, H.M. 1982. Analysis of the behavioral sequence emitted by rattlesnakes during predatory episodes. II. Duration of strike-induced chemosensory searching in rattlesnakes (*Crotalus viridis*, *C.* enyo). *Behav. Neural Biol.* 34:261–270.

CHISZAR, D. RADCLIFFE, C.W., OVERSTREET, R., POOLE, T., and BYERS, T. 1985. Duration of strike-induced chemosensory searching in cottonmouths *Agkistrodon piscivorus* and a test of the hypothesis that striking prey creates a specific search image. *Can J. Zool.* 63:1057–1061.

COOPER, W.E., JR. 1989a. Absence of prey odor discrimination by iguanid and agamid lizards in applicator tests. *Copeia* 1989:472–478.

COOPER, W.E., JR. 1989b. Prey odor discrimination in the varanoid lizards *Heloderma suspectum* and *Varanus exanthematicus*. *Ethology* 81:250–258.

COOPER, W.E., JR. 1989c. Strike-induced chemosensory searching occurs in lizards. *J. Chem. Ecol.* 15:1311–1320.

COOPER, W.E., JR. 1990a. Prey odor detection by teiid and lacertid lizards and the relationship of prey odor detection to foraging mode in lizard families. *Copeia* 1990:237–242.

COOPER, W.E., JR. 1990b. Prey odour discrimination by lizards and snakes, pp. 533–538, *in* D. W. Macdonald, D. Müller-Schwarze, and S. E. Natynczuk (eds.). Chemical Signals in Vertebrates 5. Oxford University Press, Oxford. In press.

COOPER, W.E., JR. 1990c. Prey odor discrimination by anguid lizards. *Herpetologica* 46:183–190.

COOPER, W.E., JR. Prey odor discrimination and poststrike elevation in tongue-flicking by a cordylid lizard, *Gerrhosaurus nigrolineatus*. In press.

COOPER, W.E., JR., and ALBERTS, A.C. 1990. Responses to chemical food stimuli by an herbivorous actively foraging lizard, *Dipsosaurus dorsalis*. *Herpetologica* 46:259–266.

COOPER, W.E., JR., and ALBERTS, A.C. 1991. Tongue-flicking and biting in response to chemical food stimuli by an iguanid lizard (*Dipsosaurus dorsalis*) having sealed vomeronasal ducts: Vomerolfaction may mediate these behavioral responses. *J. Chem. Ecol.* 17:135–146.

COOPER, W.E., JR., and BURGHARDT, G.M. 1990a. A comparative analysis of scoring methods for chemical discrimination of prey by squamate reptiles. *J. Chem. Ecol.* 16:45–65.

COOPER, W.E., JR., and BURGHARDT, G.M. 1990b. Vomerolfaction and vomodor. *J. Chem. Ecol.* 16:103–105.

COOPER, W.E., JR., and VITT, L.J. 1986. Thermal dependence of tongue-flicking and comments on use of tongue-flicking as an index of squamate behavior. *Ethology* 71:177–186.

COOPER, W.E., JR., and VITT, L.J. 1989. Prey odor discrimination by the broad-headed skink (*Eumeces laticeps*). *J. Exp. Zool.* 249:11–16.

COOPER, W.E., JR., MCDOWELL, S.G., and RUFFER, J. 1989. Strike-induced chemosensory searching in the colubrid snakes *Elaphe g. guttata* and *Thamnophis sirtalis*. *Ethology* 81:19–28.

DIAL, B.E. 1978. Aspects of the behavioral ecology of two Chihuahuan desert geckos (Reptilia, Lacertilia, Gekkonidae). *J. Herpetol.* 12:209–216.

DIAL, B.E., WELDON, P.J., and CURTIS, B. 1989. Predator-prey signals: Chemosensory identification of snake predators by gekkonid lizards and its ecological consequences. *J. Herpetol.* 23:224–229.

ESTES, R., QUEIROZ, K. DE, and GAUTHIER, J. 1988. Phylogenetic relationships within Squamata, pp. 119–281, *in* R. Estes and G. Pregill (eds.). Phylogenetic Relationships of the Lizard Families, Essays Commemorating Charles L. Camp. Stanford University Press, Stanford, California.

FROST, D.R., and ETHERIDGE, R. 1989. A phylogenetic analysis and taxonomy of iguanian lizards (Reptilia: Squamata). *Misc. Publ. Univ. Kans. Mus. Nat. Hist.* 81:1–65.

GRAVES, B.M., and HALPERN, M. 1990. Vomeronasal organ chemoreception in tongue-flicking, exploratory and feeding behaviour of the lizard, *Chalcides ocellatus*. *Anim. Behav.* 39:692–698.

HALPERN, M. 1991. Nasal chemical senses in reptiles: Structure and function, pp. xxx–xxx, *in* D. Crews and C. Gans (eds.). Biology of the Reptilia, Vol. 18, Physiology and Behavior. University of Chicago Press, Chicago. In press.

HALPERN, M., and FRUMIN, N. 1979. Roles of the vomeronasal and olfactory systems in prey attack and feeding in adult garter snakes. *Physiol. Behav.* 22:1183–1189.

HALPERN, M., and KUBIE, J.L. 1980. Chemical access to the vomeronasal organs of garter snakes. *Physiol. Behav.* 24:367–371.

HOLLANDER, A., and WOLFE, D.A. 1973. Nonparametric Statistical Methods. John Wiley & Sons, New York.

HUEY, R.B., and PIANKA, E.R. 1981. Ecological consequences of foraging mode. *Ecology* 62:991–999.

KAHMANN, H. 1939. Uber das Jakobsonische Organ der Echsen. *Z. Vergl. Physiol.* 26:669–695.

KREKORIAN, C.O. 1989. Field and laboratory observations on chemoreception in the desert iguana. *J. Herpetol.* 23:267–273.

LOOP, M.S., and SCOVILLE, S.A. 1972. Response of newborn *Eumeces inexpectatus* to prey-object extracts. *Herpetologica* 28:254–256.

NICOLETTO, P.F. 1985. The roles of vision and the chemical senses in predatory behavior of the skink, *Scincella lateralis*. *J. Herpetol.* 19:487–491.

PEDERSEN, J.M. 1988. Laboratory observations on the function of tongue extrusion in the desert iguana (*Dipsosaurus dorsalis*). *J. Comp. Psychol.* 102:193–196.

REGAL, P.J. 1978. Behavioral differences between reptiles and mammals: An analysis of activity and mental capabilities, pp. 183–202, *in* N. Greenberg and P.D. MacLean (eds.). Behavior

and Neurobiology of Lizards. United States Department of Health, Education, and Welfare, Washington, D.C.

SIEGEL, S. 1956. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York.

SIMON, C.A. 1983. A Review of Lizard Chemoreception, pp. 119–133 and 443–447, *in* R.B. Huey, E.R. Pianka, and T.W. Schoener (eds.). Lizard Ecology: Studies of a Model Organism. Harvard University Press, Cambridge, Massachusetts.

SIMON, C.A., GRAVELLE, K., BISSINGER, B.E., EISS, I., and RUIBAL, R. 1981. The role of chemoreception in the iguanid lizard *Sceloporus jarrovi*. *Anim. Behav.* 29:46–54.

VAN DAMME, R., BAUWENS, D., VANDERSTIGHELEN, D., and VERHEYEN, R.F. 1990. Responses of the lizard *Lacerta vivipara* to predator chemical cues: The effects of temperature. *Anim. Behav.* 40:298–305.

VITT, L.J., and PRICE, H.J. 1982. Ecological and evolutionary determinants of relative clutch mass in lizards. *Herpetologica* 38:237–255.

VON ACHEN, P.L., and RAKESTRAW, J.L. 1984. The Role of Chemoreception in the Prey Selection of Neonate Reptiles, pp. 163–172, *in* R.A. Seigel, L.E. Hunt, J.L. Knight, L. Malaret, and N.L. Zuschlag (eds.). Vertebrate Ecology and Systematics—A Tribute to Henry S. Fitch. Special Publication, University of Kansas Museum of Natural History, Lawrence, Kansas.

WINER, B.J. 1962. Statistical Principles in Experimental Design. McGraw-Hill, New York.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

EFFECTS OF EXOGENOUSLY APPLIED FERULIC ACID, A POTENTIAL ALLELOPATHIC COMPOUND, ON LEAF GROWTH, WATER UTILIZATION, AND ENDOGENOUS ABSCISIC ACID LEVELS OF TOMATO, CUCUMBER, AND BEAN[1]

LYNN D. HOLAPPA and UDO BLUM*

Department of Botany
North Carolina State University
Raleigh, North Carolina 27695-7612

(Received October 31, 1990; accepted January 14, 1991)

Abstract—To determine the relative sensitivities of tomato, cucumber, and bean to exogenously applied concentrations of ferulic acid (FA) and to determine whether FA-induced stress responses increase endogenous levels of abscisic acid (ABA), wild-type and *flacca* (ABA-deficient mutant) tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig), cucumber, (*Cucumis sativus* L. cv. Early Green Cluster), and bean (*Phaseolus vulgaris* L. cv. Oregon 91) were treated with FA (0.0, 0.2, 0.4, 0.8 mM) in nutrient solution every other day for a total of two or three treatments. FA inhibited leaf growth and water utilization of wild-type tomato, *flacca* tomato, and cucumber, but not of bean. Acclimation to FA was observed following the first FA treatment and increased endogenous ABA levels were found in wild-type tomato, *flacca* tomato, and cucumber following multiple FA treatments. Induction of ABA biosynthesis occurred in wild-type tomato within 8 hr of FA treatment and maximum ABA levels were observed 24 hr after treatment. At that time, ABA levels of tomato treated with 0.4 and 0.8 mM FA were 13.7 times and 2.6 times higher than control levels, respectively. A second FA (0.4 or 0.8 mM) treatment, 48 hr after the first, did not appear to affect ABA levels. Ninety-six hours after the first treatment, ABA levels of tomato treated with 0.4 mM FA approached control levels; ABA levels of plants treated with 0.8 mM FA were 1.9 times higher than control levels. Control ABA levels increased gradually with time. The data showed that plant sensitivity and

*To whom correspondence should be addressed.

[1] The use of trade names in this publication does not imply endorsement by North Carolina Agricultural Research Service of the products named, nor criticism of similar ones not mentioned.

0098-0331/91/0500-0865$06.50/0

ability of subsequent acclimation to phenolic acids, such as FA, were taxa dependent.

Key Words—Tomato, *Lycopersicon esculentum*, wild type and *flacca* mutant, cucumber, *Cucumis sativus*, bean, *Phaseolus vulgaris*, ferulic acid, leaf expansion, water utilization, abscisic acid, allelopathy.

INTRODUCTION

During episodes of water deficit, it has been well established that abscisic acid (ABA) levels increase dramatically in leaf and root tissue of mesophytic plants (Walton, 1980; Neill et al., 1984). Since endogenous ABA levels also can be increased by a variety of stresses, it has been suggested that the increase in ABA may be a general stress response that is associated with the process of acclimation (Zeevaart and Creelman, 1988). ABA accelerated the acclimation of cultured tobacco cells to high salt concentrations (LaRosa et al., 1987) and increased freezing and cold tolerance in potato and wheat (Chen et al., 1983; Lalk and Dorffling, 1985). Tomato plants exposed to stressful day/night temperatures ranging from 10/5 to 45/35°C accumulated ABA without alteration of plant water potential (Daie and Campbell, 1981), and ABA levels of tomato increased as a result of salt stress (Walker and Dumbroff, 1981). Waterlogged pea plants also exhibited increased ABA levels in an apparent absence of foliar water deficit (Jackson and Hall, 1987). In many of these cases, however, plant water status is believed to be modified, thereby enhancing ABA biosynthesis (Davies et al., 1986).

Recently, the effects of ferulic acid (FA) on the growth and metabolism of cucumber seedlings grown in nutrient solution and in soil systems have been extensively investigated. FA was taken up by the roots, translocated throughout the plant, and used as a precursor in lignin biosynthesis (Shann and Blum, 1987a,b). Physiologically, FA reduced leaf area expansion and shoot dry weight, suppressed germination, inhibited radicle growth, and inhibited seedling root growth in cucumber (Blum et al., 1984; 1985a,b; Blum and Rebbeck, 1989). FA also influenced photosynthetic rates, uptake of various minerals and ions, and certain enzyme (soluble cell wall peroxidase) activities (Balke, 1985; Shann and Blum, 1987b; Blum and Rebbeck, 1989).

Although all of these responses were clearly FA-induced, the initial impact of FA on plants appeared to occur at the root plasmalemma (Glass and Dunlop, 1974; Balke, 1985). The actual mechanism of perturbance at the plasmalemma has not been determined definitively. Circumstantial evidence, including ion uptake studies (Balke, 1985), has suggested that hydraulic conductivity of the roots decreases and that unabated water loss, via transpiration, in conjunction

with the FA-induced decrease in hydraulic conductivity results in visible wilting. Wilting occurred within a few hours upon addition of FA (above 0.5 mM) to nutrient solutions in which cucumber seedlings were grown (Blum and Dalton, 1985). There was no change in water potential (as detected by freezing-point depression) of nutrient solution to which FA had been added (Blum and Dalton, 1985). Stomatal closure occurred sometime during the latter stages of wilting. Leaf turgor returned 12–24 hr later. Stomata remained closed for several days even when roots were removed from the FA treatment (Blum et al., 1985a,b). Stomatal closure following FA treatment also was reported in soybean and grain sorghum (Patterson, 1981; Einhellig et al., 1985). Treatments of soybean and grain sorghum with FA also resulted in lowered leaf water potential (i.e., water potential became more negative) and in lowered stomatal diffusive conductance (Patterson, 1981; Einhellig et al., 1985). Since exogenously applied FA noticeably alters the water balance of various plant species, it appears that one of the subsequent physiological responses to FA treatment should be an increase in endogenous ABA levels.

ABA has been implicated in the regulation of water balance within plants (Walton, 1980; Davies et al., 1986; Zeevaart and Creelman, 1988). Endogenous ABA levels increased dramatically in water-stressed leaves (Harrison and Walton, 1975; Neill et al., 1984; Cornish and Zeevaart, 1988). Increased ABA levels were associated with reduced leaf water potential and with stomatal closure (Beardsell and Cohen, 1975; Raschke and Zeevaart, 1976). Mild water stress also increased endogenous root ABA levels in bean and sunflower (Walton, et al., 1976; Robertson et al., 1985).

The tomato mutant, *flacca*, has been reported to contain 17–26% of the endogenous ABA level of the wild type of the isogenic parent line, *Lycopersicon esculentum* Mill. cv. Ailsa Craig (Tal and Nevo, 1973; Neill and Horgan, 1985). These mutants have a "droopy, wilty" phenotype compared to wild type. They were identified as ABA-deficient primarily because complete reversion to the wild-type phenotype occurred when *flacca* plants were sprayed with ABA (Karssen et al., 1987). *Flacca* tomato plants wilt even under conditions of mild water stress (Darby et al., 1978). Both the failure to synthesize ABA in response to water stress and the low ABA levels in nonstressed leaves have corresponded very well with stomatal behavior (Karssen et al., 1987). Stomatal resistance to closure and higher root resistances are reported phenotypic characteristics (Tal and Nevo, 1973; Tal and Imber, 1971).

The present investigation was conducted to determine whether such FA-induced stress modified endogenous ABA levels. Three species were utilized. Two tomato taxa, the wild type and ABA-deficient mutant, *flacca*, of the isogenic parent line *Lycopersicon esculentum* cv. Ailsa Craig were chosen for their known differences in endogenous ABA levels. Tomato was chosen also for its reported sensitivity to phenolic compounds (gallotannic, gallic, and chlorogenic

acids) and its ability to accumulate ABA in stressed leaves (Floyd and Rice, 1967; Walker and Dumbroff, 1981; Neill and Horgan, 1985). Cucumber and bean species were chosen for their rapid growth rates and their predictable sensitivities and behaviors to FA (Blum et al., 1985a,b; Waters and Blum, 1987). ABA levels in bean leaves increased in response to water stress (Pierce and Raschke, 1981). The objectives of this research were: (1) to determine the relative sensitivities of four plant taxa (wild-type tomato, *flacca* tomato, cucumber and bean) to exogenously applied FA by monitoring leaf growth and water utilization and (2) to determine the effects of exogenously applied FA on the endogenous levels of ABA within these taxa.

METHODS AND MATERIALS

General Aspects. In the first experiment, seed coats of wild-type and *flacca* tomato of the isogenic parent line, *Lycopersicon esculentum* Mill. cv. Ailsa Craig (Institute of Horticultural Research, Worthing Road, Littlehampton, West Sussex, England) were scarified for 30 min with 10 N sulfuric acid. After a deionized-water rinse, seeds were placed in autoclaved glass Petri dishes (9 cm diameter) containing Whatman No. 1 filter paper and 10 ml deionized water. Twenty seeds were placed on the filter paper of each dish. The dishes were placed in the dark at 30°C until radicle-hypocotyls were approximately 1–2 cm in length. These 4-day-old seedlings then were transferred to styrofoam cups (with drainage holes) containing vermiculite moistened with modified Hoagland's nutrient solution, pH 5.5 (Hoagland and Arnon, 1950). KNO_3 and KH_2PO_4 were increased by 25% and 50%, respectively, and 0.01 mg Sequestrene 330 Fe per liter was substituted for EDTA-Fe solution. The seedlings were grown for six days in a lighted chamber with a photosynthetic photon flux density (PPFD) of 140 μEinsteins/m^2/sec (Blum and Dalton, 1985).

Tomato seedlings then were moved to a standard B-chamber of the Southeastern Plant Environmental Laboratory (NCSU Phytotron; Downs and Thomas, 1983) with a 26/22°C day/night temperature, 60–80% relative humidity, a 9-hr photoperiod, PPFD of 240–250 μEinsteins/m^2/sec provided by cool white fluorescent bulbs. Preliminary experiments revealed that these conditions were optimal for *flacca* tomato. On day 14, each seedling was transferred to a foil-covered jar containing 350 ml nutrient solution. Each seedling was suspended at the hypocotyl with a foam collar. Beginning on day 22, wild-type ($N = 24$) and *flacca* ($N = 24$) seedlings were treated with 0.0, 0.2, 0.4, or 0.8 mM ferulic acid (FA; Sigma Chemical Co., St. Louis, Missouri) in fresh nutrient solution every other day for the total of three treatments.

In a second experiment, cucumber seeds, *Cucumis sativus* L. cv. Early Green Cluster (Wyatt Quarles Seed Company, Raleigh, North Carolina), and

bean seeds, *Phaseolus vulgaris* L. cv. Oregon 91 (Rogers Brothers Seed Company, Boise, Idaho), were germinated in vermiculite moistened with nutrient solution. Cucumber and bean seedlings were transferred to 350 ml foil-covered jars when 3 or 5 days old, respectively, and moved to a standard B-chamber of the NCSU Phytotron with the same environmental conditions as experiment one. Twelve-day-old cucumber seedlings ($N = 24$) and 7-day-old bean seedlings ($N = 24$) were treated with 0.0, 0.2, 0.4, or 0.8 mM FA in fresh nutrient solution every other day for a total of three treatments.

In a third experiment, 22-day-old wild-type tomato seedlings ($N = 75$) were treated with 0.0, 0.4, or 0.8 mM FA in fresh nutrient solution every other day for a total of two treatments. Sets of 15 plants were harvested at 8, 24, and 48 hr after the first treatment and 24 and 48 hr after the second treatment. Each treatment had five replicates.

In each experiment, beginning with the phytotron-transfer day and prior to the first treatment, nutrient solutions were replaced every other day. Water was added daily to compensate for transpirational water loss. All solutions were continually aerated. All seedlings were arranged in a completely randomized block design in the B chamber.

Leaf Measurements. The length (L), width (W), and area of bean and cucumber leaves were used to develop leaf area models. The cucumber and bean leaf area models were: Area $= -0.58548 + [0.87881 \times (L \times W)]$; $r^2 = 0.99$; $N = 395$, where length and width are in centimeters; Area $= -1.457 + [0.00769 \times (L \times W)]$; $r^2 = 0.98$; $N = 121$, where length and width are in millimeters (Blum and Dalton, 1985), respectively. Leaf length and width measurements for the bean model included only primary leaves. Length and width were recorded just prior to treatments every other day starting on days 12 and 7 for cucumber and bean plants, respectively.

Due to the low r^2 values (wild type, $r^2 = 0.85$; *flacca*, $r^2 = 0.84$) models for tomato leaf areas could not be used. Leaf lengths (mm) were recorded just prior to treatments every other day, starting on day 22.

Water Utilization. Transpirational water loss from the foil-covered jars was measured between 12 and 1 PM daily starting on days 22, 12, and 7 for tomato, cucumber, and bean plants, respectively.

Leaf Measurement and Water Utilization Data Analysis. Mean absolute rate of leaf elongation (ARLE) and mean absolute rate of leaf area expansion (ARLAE) were determined as follows: ARL(A)E = Σ[seedling leaf (lengths or areas) on day_{x+2}] $-$ Σ [seedling leaf (lengths or areas) on day_x], where ARLE is mm/2 days and ARLAE is cm^2/2 days. Mean relative rates of leaf elongation (RRLE) and mean relative rates of leaf area expansion (RRLAE) were determined as follows: RRL(A)E = ln [Σ seedling leaf (lengths or areas) on day_{x+2}] $-$ ln [Σ seedling leaf (lengths or areas) on day_x], where RRLE is mm/mm/2 days and RRLAE is cm^2/cm^2/2 days. See Radford (1967) for details

that describe the derivation of these equations. Water utilization was calculated per unit leaf length for each tomato taxon and per unit leaf area for cucumber and bean. Analysis of variance and regression analysis (Steele and Torrie, 1980) were used to determine significant responses. Data were analyzed using Statistical Analysis System programs (SAS Institute Inc., 1988).

Tissue Processing for ABA Analysis. In the first experiment, when tomato seedlings were 28 days old, leaflets of the oldest fully expanded leaves (i.e., leaves 1 and 2) and leaflets of the remaining shoot were separated into samples designated "old" or "young" leaves, respectively. In the second experiment, when cucumber seedlings were 18 days old, four leaf disks (2 cm diameter/disk) were excised from the oldest fully expanded true leaf and from the third oldest leaf, and designated "old" and "young," respectively. Four leaf disks also were excised from each primary leaf of 15-day-old bean seedlings. In the third experiment, all wild-type tomato leaflets per seedling were harvested 8, 24, 48, 72, and 96 hr after the first treatment on day 22. Major veins were avoided when excising leaf disks. Each sample was immediately frozen in liquid nitrogen, lyophilized, and stored at −20°C until ABA analysis.

Lyophilized and powdered samples were suspended in extracting methanol (methanol, containing 0.1 g/liter butylated hydroxytoluene and 0.5 g/liter citric acid monohydrate at a ratio of 0.01 g dry tissue to 1.0 ml extracting methanol) and stirred overnight at 4°C in the dark. A C-18 Sep-Pak (Waters Co., Milford, Massachusetts) was utilized to clean up each leaf sample. Five milligrams of plant tissue in 70% extracting methanol was applied to each Sep-Pak. Each sample was eluted from the column with 70% extracting methanol. For tomato samples, 500 μl of each 70% methanol eluant was dried in a Speed Vac Concentrator (Savant, Farmingdale, New York). Dried tomato samples were resuspended in 1 ml Tris buffered saline (TBS; 6.05 g Tris/liter, 0.20 g $MgCl_2 \cdot 6H_2O$/liter, 8.8 g NaCl/liter, pH 8.8) solution. For cucumber and bean samples, 1500 μl of each 70% methanol eluant was dried in a Speed Vac Concentrator and then resuspended in 500 μl TBS solution.

Five samples of each taxon were spiked with 5.5 nCi/sample (±)-cis, trans-[^{3}H]-ABA, specific activity = 115 Ci/mmol (Amersham, Arlington Heights, Illinois) at the beginning of the extraction procedure to estimate recovery, which averaged >90% ± 0.09 SE. Similar recovery estimates were reported by Walker-Simmons (1987), Morris et al. (1988), and Norman et al. (1988). When unlabeled (+)-*cis, trans*-ABA, in the absence of plant sample, was carried through this extraction protocol, recovery ranged from 65% to 70%. ABA values were not corrected for percent recovery.

ABA Quantification. ABA was measured utilizing an immunoassay. Monoclonal antibody (Mab) to free *cis, trans*-(+)-ABA was purchased from Idetek Inc. (1057 Sneath Lane, San Bruno, California). The assay was an indirect ELISA (enzyme-linked immunosorbant assay) as described by Walker-Sim-

mons (1987). For this assay, abscisic acid-4′-bovine serum albumin (ABA-4′-BSA) conjugate was prepared according to Weiler (1979) and stored at −20°C.

The standard curve for the ELISA was linear ($r^2 = 0.99$) when plotted as a log by logit function. A linear regression was established for ABA levels from 5 to 150 pg/200 μl. When C-18 Sep-Pak-purified plant samples were added to standard amounts of ABA, no interference in the linearity of the immunoassay was detected. When 70% extracting methanol, in the absence of plant tissue, was carried through the C-18 Sep-Pak and ELISA protocols, no false positives were detected.

ABA quantification using this Mab has been evaluated and validated by numerous laboratories using GC-MS, GC-FID, and HPLC-UV (Raikhel et al., 1987; Walker-Simmons, 1987; Norman et al., 1988). The Mab used in this study has been demonstrated to possess little (< 1000-fold) or no cross-reactivity with ABA-related compounds (Weiler, 1980; Mertens et al., 1983).

ABA Data Analysis. Analysis of variance and regression analysis were conducted on the four taxa separately. In the third experiment, regression analyses were run for ferulic acid concentrations within each time interval.

RESULTS

Growth: Experiment 1. Regression analyses using data summarized in Table 1 showed that FA inhibited leaf elongation rates of both wild-type and *flacca* tomato. As FA concentrations were increased, concurrent decreases were observed in both ARLE and RRLE (Figure 1). ARLE of wild-type tomato exhibited linear trends for all three growth periods, while RRLEs were linear for the first two growth periods and convex curvilinear for the third. ARLE of *flacca* exhibited a concave curvilinear trend after initial FA treatment (growth period 22–24), a linear trend after the second treatment (growth period 24–26), and a convex curvilinear trend after the third treatment (growth period 26–28). RRLE of *flacca* exhibited a concave curvilinear trend during the first growth period and convex curvilinear trends for subsequent growth periods.

The effect of the initial treatment (day 22) of FA at 0.2, 0.4, and 0.8 mM on wild type was not as severe as it was on *flacca*. ARLE of treated plants, when compared to control plants, decreased 29%, 49%, and 90% for the wild type compared to 32%, 85%, and 100% for *flacca*, respectively (Table 1). Similarly, RRLEs decreased 15%, 44%, and 89% for wild type and 26%, 82%, and 100% for *flacca*, respectively. Thus, percent reductions were lower for wild-type than for mutant tomato. *Flacca* at 0.8 mM became necrotic after the initial treatment; visually, wild-type tomato appeared normal.

When comparing treated to control plants, it appeared from the shift in percent change over time that the wild type was less affected by subsequent

TABLE 1. MEAN ABSOLUTE AND RELATIVE RATES OF LEAF ELONGATION FOR WILD-TYPE AND *Flacca* SEEDLINGS GIVEN MULTIPLE FERULIC ACID TREATMENTS[a]

		Ferulic acid (mM)	Growth period (days)		
			22–24	24–26	26–28
Wild type	ARLE[b] (mm/2 days)	0.0	161.83	194.17	215.33
			(4.38)	(7.44)	(7.50)
		0.2	114.50	157.83	205.17
			(5.95)	(8.27)	(9.34)
		0.4	81.33	130.00	180.83
			(9.49)	(7.76)	(14.27)
		0.8	16.00	61.33	95.83
			(5.98)	(5.04)	(6.67)
	RRLE[c] (mm/mm/2 days)	0.0	0.331	0.289	0.247
			(0.021)	(0.008)	(0.015)
		0.2	0.280	0.290	0.284
			(0.009)	(0.007)	(0.010)
		0.4	0.185	0.239	0.259
			(0.018)	(0.012)	(0.019)
		0.8	0.036	0.136	0.178
			(0.012)	(0.014)	(0.010)
Flacca	ARLE (mm/2 days)	0.0	115.83	132.50	128.50
			(6.49)	(10.90)	(22.39)
		0.2	78.17	117.00	98.83
			(4.89)	(8.22)	(6.99)
		0.4	17.00	79.50	117.33
			(10.95)	(27.41)	(7.14)
		0.8	00.00	00.00	00.00
			(00.00)	(00.00)	(00.00)
	RRLE (mm/mm/2 days)	0.0	0.269	0.237	0.182
			(0.014)	(0.011)	(0.023)
		0.2	0.198	0.237	0.168
			(0.006)	(0.008)	(0.016)
		0.4	0.049	0.168	0.226
			(0.031)	(0.050)	(0.017)
		0.8	00.00	00.00	00.00
			(00.00)	(00.00)	(00.00)

[a] On treatment days, leaves were measured between 9 and 11 AM. Seedlings were treated at 2 PM. $N = 6$. Values in parentheses are standard errors.
[b] ARLE = mean absolute rates of leaf elongation.
[c] RRLE = mean relative rates of leaf elongation.

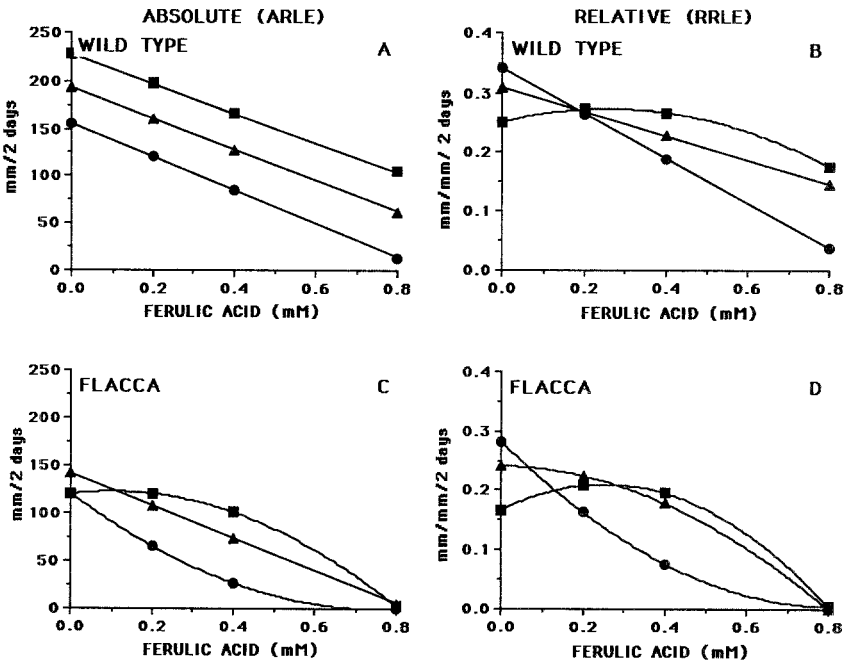


FIG. 1. Effects of ferulic acid concentrations (0.0, 0.2, 0.4, 0.8 mM) supplied in nutrient solution (pH 5.5) on mean absolute rates of leaf elongation (ARLE: A, C) and on mean relative rates of leaf elongation (RRLE: B, D) for wild-type tomato plants (A, B) and *flacca* plants (C, D). Each line was generated from a model for a specific two-day growth period: (●) 22–24, (▲) 24–26, and (■) 26–28 days from seed. The three points above each ferulic acid concentration represent either the predicted mean of the absolute rate of leaf elongation (mm/2 days) or the predicted mean of the relative rate of leaf elongation (mm/mm/2 days) of same six plants treated at the three different growth periods. Seedlings were treated on days 22, 24, and 26. For wild-type tomato seedlings, r^2 values ranged from 77% to 92% and from 60% to 90% for ARLE and RRLE, respectively. For *flacca* tomato seedlings, r^2 values ranged from 71% to 87% and from 73% to 85% for ARLE and RRLE, respectively. For all regression equations, $P \leq 0.0001$.

treatments. Reduction of ARLE for wild type in the third growth period (days 26–28) were six, three, and two times lower than ARLE of the first growth period (days 22–24) for 0.2, 0.4, and 0.8 mM, respectively (Table 1). These changes in response are even more apparent in RRLE, since in several instances, stimulation or no growth inhibition occurred for the second and third growth periods at 0.2 and 0.4 mM concentrations of FA. For *flacca*, 100% inhibition of ARLE and RRLE occurred at 0.8 mM for all growth periods. The magnitude of inhibition of ARLE and RRLE for *flacca* plants treated with 0.2 and 0.4 mM

FA was less for growth periods 2 and 3 than for the first growth period. In fact, either stimulation or no growth inhibition was observed in two instances.

Shifts in regression equations (Figure 1) for growth also suggested a reduction in the effect of FA after the first treatment. For example, RRLE equations of wild-type tomato shifted from linear to concave curvilinear from the first to third growth period, while *flacca* RRLE equations shifted from convex curvilinear for growth period 1 to concave curvilinear for growth periods 2 and 3.

Growth: Experiment 2. Regression analyses using data summarized in Table 2 showed that FA inhibited leaf area expansion rates of cucumber. FA had no apparent effect on bean growth or water utilization throughout the entire experiment (data not presented). During the first growth period (days 12–14), as FA concentrations were increased, concurrent decreases in ARLAE and RRLAE were observed in cucumber (Figure 2). In the initial growth period, ARLAE decreased in a concave curvilinear manner; RRLAE decreased linearly. ARLAE for subsequent growth periods decreased linearly. There were no effects of FA on RRLAE during the second and third growth periods.

The impact of FA on cucumber ARLAE for the first treatment (day 12) was more severe than subsequent treatments (Table 2). Reductions of ARLAE in the third growth period (days 16–18) were three, two, and 1.5 times lower than the reductions of the first growth period (days 12–14), for 0.2, 0.4, and 0.8 mM respectively. RRLAE of the first period was reduced 16%, 30%, and 56% from that of the controls. RRLAE of cucumber was not affected by subsequent treatments. The decline in response of FA was also evident in the shifts in regression equations. Cucumber ARLAE shifted from a convex curvilinear to linear trend for growth periods 2 and 3, respectively. Cucumber RRLAE was significant only for the first growth period.

Water Utilization: Experiment 1. Regression analyses using data summarized in Table 3 indicated that FA inhibited tomato mean relative rates of water utilization (RRWU) at all concentrations. As FA concentrations increased, concurrent decreases (linear or quadratic) in RRWU were observed 24 and 48 hr after each treatment (Figure 3). Following the first and second treatments, tomato responses were linear; 48 hr after the third treatment, the response was curvilinear. RRWU of *flacca* 24 hr after the first and second treatments decreased in a concave curvilinear manner, and linearly after the third treatment. RRWU of *flacca* decreased linearly 48 hr after the first and third treatments, and curvilinearly 48 hr after the second treatment.

The initial effect of FA was greater on *flacca* than wild-type tomato at 0.2 and 0.4 mM. RRWU were reduced 24% and 43% for wild type and 52% and 87% for *flacca* at 0.2 and 0.4 mM FA, respectively (Table 3). On subsequent days, relationships of RRWU for wild type and *flacca* were inconsistent. RRWU for both tomato taxa was essentially eliminated at 0.8 mM, except on day 28 when wild-type RRWU was reduced 64%.

TABLE 2. MEAN ABSOLUTE AND RELATIVE RATES OF LEAF AREA EXPANSION AND MEAN RELATIVE RATES OF WATER UTILIZATION FOR CUCUMBER SEEDLINGS GIVEN MULTIPLE FERULIC ACID TREATMENTS[a]

	Ferulic acid (mM)	Growth period (days)		
		12–14	14–16	16–18
ARLAE[b] (cm^2/2 days)	0.0	61.32	88.46	136.16
		(1.50)	(2.82)	(9.55)
	0.2	44.85	73.18	121.89
		(1.17)	(6.86)	(8.15)
	0.4	33.65	70.90	106.13
		(1.03)	(2.32)	(4.27)
	0.8	20.96	54.79	76.87
		(4.24)	(6.78)	(14.43)
RRLAE[c] (cm^2/cm^2/2 days)	0.0	0.814	0.589	0.521
		(0.010)	(0.010)	(0.027)
	0.2	0.680	0.587	0.554
		(0.011)	(0.043)	(0.010)
	0.4	0.572	0.651	0.539
		(0.019)	(0.019)	(0.015)
	0.8	0.356	0.578	0.463
		(0.055)	(0.025)	(0.049)

	Ferulic acid (mM)	Days from seed					
		13	14	15	16	17	18
RRWU[d] (ml/cm^2/day)	0.0	0.291	0.228	0.202	0.234	0.152	0.245
		(0.024)	(0.019)	(0.027)	(0.022)	(0.020)	(0.017)
	0.2	0.153	0.156	0.085	0.259	0.084	0.214
		(0.053)	(0.044)	(0.042)	(0.027)	(0.023)	(0.016)
	0.4	0.126	0.196	0.118	0.269	0.080	0.196
		(0.045)	(0.043)	(0.027)	(0.043)	(0.023)	(0.019)
	0.8	0.000	0.066	0.029	0.160	0.000	0.146
		(0.000)	(0.034)	(0.020)	(0.032)	(0.000)	(0.025)

[a] Leaves were measured at 11–12 AM. Water measurements were recorded between 12 and 1 PM daily. Cucumber seedlings were treated between 1 and 2 PM on 12, 14, and 16 days from seed. $N = 6$. Values in parentheses are standard errors.
[b] ARLAE = mean absolute rates of leaf area expansion.
[c] RRLAE = mean relative rates of leaf area expansion.
[d] RRWU = mean relative rates of water utilization.

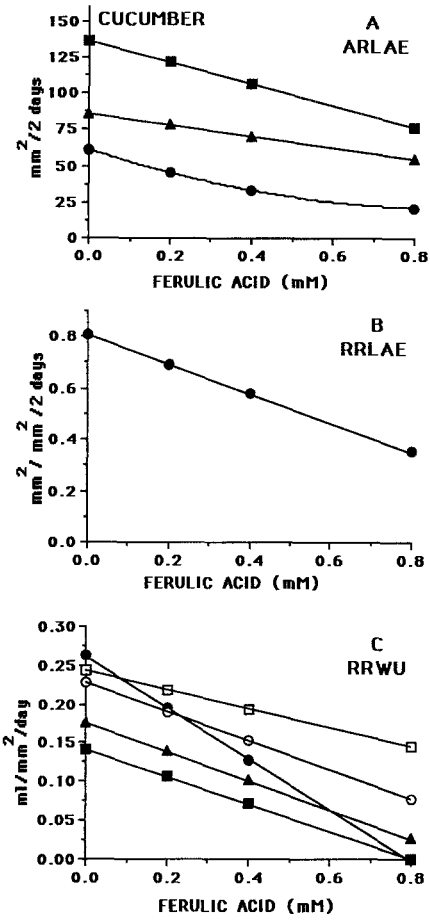


FIG. 2. Effects of ferulic acid concentrations (0.0, 0.2, 0.4, and 0.8 mM) supplied in nutrient solution (pH 5.5) on mean absolute rates of leaf area expansion (ARLAE: A), mean relative rates of leaf area expansion (RRLAE: B), and mean relative rates of water utilization (RRWU: C) of cucumber seedlings. Each ARLAE or RRLAE line was generated from a model for a specific two-day growth period: (●) 12–14, (▲) 14–16, and (■) 16–18. Each RRWU line was generated from a model for a specific day following treatment. The closed symbols represent measurements taken 24 hr after treatment; the open symbols represent measurements taken 48 hr after treatment. Water utilization 48 hr after treatment 2 was not significant and, thus, not shown. The three points above each ferulic acid concentration represent the predicted mean rate of absolute leaf area expansion (ARLAE: A), the predicted mean rates of relative leaf area expansion (RRLAE: B), or the mean relative rate of water utilization (RRWU: C) of the same six plants at the three different growth periods. Seedlings were treated on days 12, 14, and 16. For RRWU symbols are (●) day 13, (○) day 14, (▲) day 15, (■) day 17, (□) day 18. Cucumber r^2 values ranged from 48% to 86% for ARLAE, was 83% for RRLAE of the first growth period, and ranged from 32% to 64% for RRWU, respectively. For all regression equations, $P \leq 0.004$, most $P \leq 0.0009$.

TABLE 3. MEAN VALUES OF WATER UTILIZATION FOR WILD-TYPE AND *Flacca* TOMATO SEEDLINGS TREATED WITH FERULIC ACID SUPPLIED IN NUTRIENT SOLUTION[a]

		Ferulic acid (mM)	Days from seed					
			23	24	25	26	27	28
Wild type	RRWU[b]	0.0	0.042	0.036	0.031	0.044	0.048	0.042
			(0.003)	(0.004)	(0.004)	(0.006)	(0.004)	(0.005)
		0.2	0.032	0.022	0.028	0.024	0.029	0.021
			(0.005)	(0.003)	(0.003)	(0.005)	(0.004)	(0.005)
		0.4	0.024	0.012	0.006	0.016	0.019	0.020
			(0.008)	(0.005)	(0.005)	(0.006)	(0.003)	(0.003)
		0.8	0.000	0.001	0.000	0.001	0.000	0.015
			(0.000)	(0.001)	(0.000)	(0.001)	(0.000)	(0.005)
Flacca	RRWU	0.0	0.071	0.038	0.032	0.055	0.056	0.047
			(0.011)	(0.005)	(0.006)	(0.007)	(0.010)	(0.006)
		0.2	0.034	0.030	0.027	0.018	0.030	0.025
			(0.006)	(0.007)	(0.004)	(0.003)	(0.007)	(0.005)
		0.4	0.009	0.012	0.018	0.014	0.014	0.032
			(0.003)	(0.007)	(0.010)	(0.003)	(0.008)	(0.007)
		0.8	0.000	0.000	0.000	0.000	0.000	0.000
			(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)

[a] Water measurements were recorded between 12 and 2 PM daily. Tomato seedlings were treated at 2 PM at 22, 24, and 26 days from seed. $N = 6$. Values in parentheses are standard errors.
[b] RRWU = mean relative rates of water utilization (ml/mm/day).

Water Utilization: Experiment 2. Regression analyses using data summarized in Table 2 indicated that FA inhibited cucumber RRWU at all concentrations of FA for all growth periods, except growth period 15–16. A steeper slope was observed for RRWU 24 hr after the first treatment (days 12–13) than for subsequent growth periods (Figure 2). No significant inhibition in RRWU was detected in bean.

Levels of ABA: Experiments 1 and 2. Results from regression analyses using data summarized in Table 4 for ABA levels in tomato and cucumber are presented in Figure 4. For wild-type tomato, leaf tissue ABA levels increased linearly with increasing concentrations of FA (Figure 4). No significant difference was detected between old and young leaves. ABA levels of *flacca* increased curvilinearly in young leaf tissue and linearly in old leaf tissue. No values at 0.8 mM FA were shown for *flacca* since leaf tissue was necrotic.

ABA levels in wild-type tomato leaves, compared to those of control leaves, increased 0–24%, 26–42%, and 75–101% for 0.2, 0.4, and 0.8 mM FA, respectively. ABA levels of young *flacca* leaves were increased 28% and

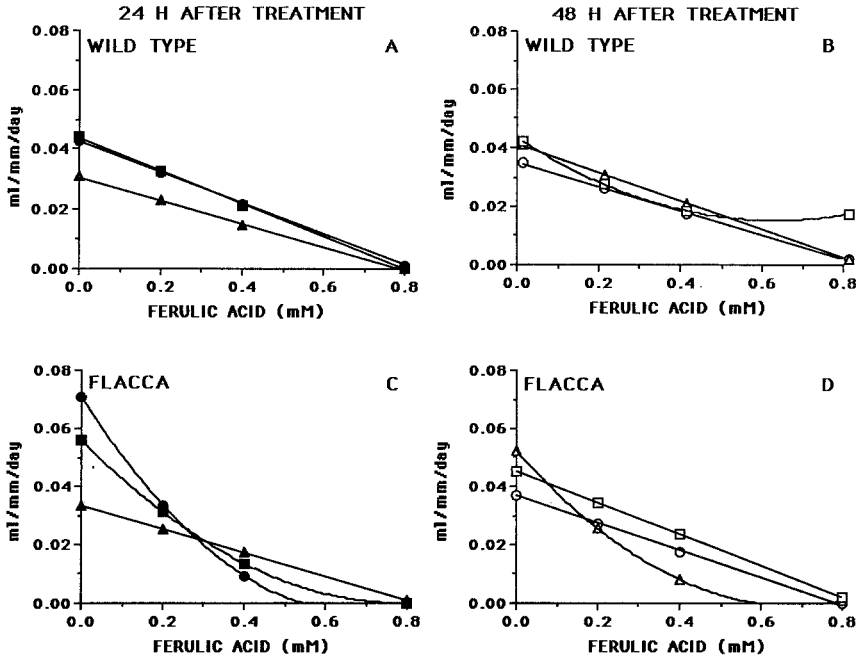


FIG. 3. Effects of increasing ferulic acid concentrations (0.0, 0.2, 0.4, 0.8 mM) supplied in nutrient solution (pH 5.5) on mean relative rates of water utilization (RRWU) of wild-type tomato plants (A, B) and *flacca* tomato plants (C, D). Each line was generated from a model for a specific day following treatment. The three points above each ferulic acid concentration represent the predicted means of relative rate of water utilization (ml/mm/day) of the same six plants treated at three different growth periods. Seedlings were treated on days 22, 24, and 26. For RRWU symbols are (●) day 23, (○) day 24, (▲) day 25, (△) day 26, (■) day 27, and (□) day 28. RRWU r^2 values ranged from 45% to 83% and from 44% to 83% for wild type and *flacca*, respectively. For all regression equations, $P \leq 0.0004$.

162%, while old leaves increased 37% and 93% for 0.2 and 0.4, respectively. *Flacca* contained 31–65% of the ABA levels of wild type.

In cucumber, ABA levels increased linearly in young leaf tissue, but curvilinearly in old leaf tissue. Compared to control leaves, ABA levels of young leaves increased 9%, 7%, and 139%, while old leaves increased 18.5%, 27%, and 383% for 0.2, 0.4, and 0.8 mM, respectively.

Levels of ABA Over Time: Experiment 3. Growth and water-utilization data for wild-type plants in the third experiment were consistent with that of the first experiment and thus are not presented.

ABA levels of wild-type tomato were modified by FA. Within each time

TABLE 4. MEAN ABSCISIC ACID LEVELS OF OLD AND YOUNG LEAVES FOR WILD-TYPE AND *Flacca* TOMATO SEEDLINGS AND CUCUMBER SEEDLINGS TREATED WITH FERULIC ACID SUPPLIED IN NUTRIENT SOLUTION.[a]

Ferulic acid (mM)	Wild type (ng)		*Flacca* (ng)		Cucumber (pg)	
	Old	Young	Old	Young	Old	Young
0.0	1.60	2.06	0.54	0.65	24.05	22.26
	(0.16)	(0.23)	(0.04)	(0.08)	(3.67)	(5.57)
0.2	2.11	2.05	0.74	0.83	28.51	24.35
	(0.20)	(0.22)	(0.03)	(0.10)	(4.37)	(6.95)
0.4	2.27	2.60	1.04	1.70	30.23	23.75
	(0.44)	(0.40)	(0.14)	(0.14)	(3.43)	(4.19)
0.8	3.22	3.60	[b]	[b]	116.10	53.29
	(0.26)	(0.28)	[b]	[b]	(25.94)	(6.50)

[a] $N = 5$. Values in parentheses are standard errors. Tomato leaflets were excised from 28-day old seedlings. Cucumber leaf disks were excised from 18-day-old seedlings. For additional details describing leaf tissue processing, see Methods and Materials.
[b] No values were determined.

interval, significant linear or convex curvilinear (at 24 hr) relationships were observed between FA concentrations and endogenous ABA levels (Figure 5). As FA concentrations were increased, concurrent increases in ABA levels occurred. ABA levels in control plants increased throughout the experiment. Compared to control levels in the respective time periods, ABA levels in plants treated with 0.4 mM FA were 1.5-, 2.6-, 2.7-, 1.6-, and 1.0-fold higher; those treated with 0.8 mM FA were 2.2-, 13.7-, 8.4-, 3.1-, and 1.9-fold higher at 8, 24, 48, 72, and 96 hr after the initial treatment, respectively.

ABA in plants treated with FA reached maximum levels 24 hr after the initial treatment, although ABA levels in plants treated with 0.8 mM FA were five times higher than those treated with 0.4 mM FA. ABA levels did not appear to be affected by the second FA treatment.

DISCUSSION

Leaf Growth and Water Utilization. Increasing concentrations of FA inhibited leaf growth and water utilization rates of three of the four taxa examined. Both leaf elongation and water utilization rates of two tomato taxa were severely affected by FA. The observed inhibition by FA of leaf area expansion and water utilization of cucumber were consistent with earlier studies (Blum and Dalton, 1985; Blum et al., 1985a,b). Bean leaf area expansion rates and water utilization rates were not inhibited by FA.

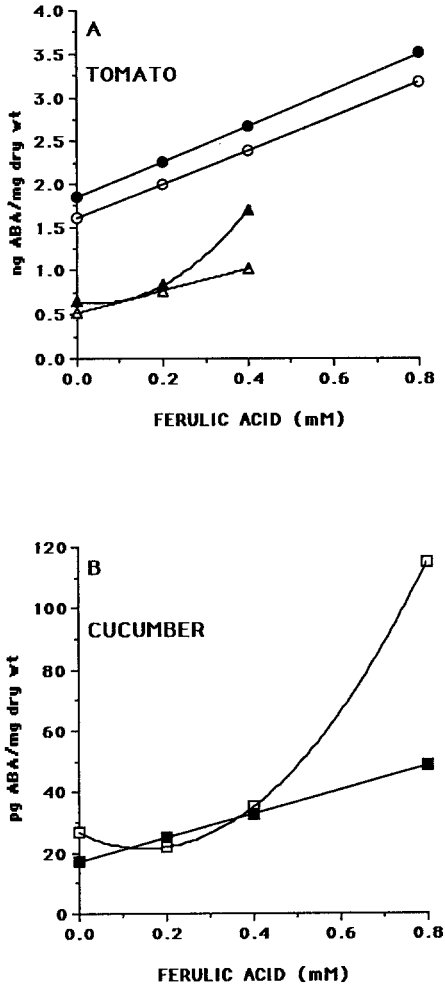


FIG. 4. Effects of ferulic acid concentrations (0.0, 0.2, 0.4, and 0.8 mM) supplied in nutrient solution (pH 5.5) on the mean level of endogenous abscisic acid in leaf tissue of wild-type tomato plants [A; (○) old, (●) young], *flacca* tomato plants [A; (△) old, (▲) young], and cucumber plants [B; (□) old, (■) young]. Regression analyses were based on ABA/mg dry weight leaf tissue. r^2 values ranged from 43% to 49% for wild type, 61% to 78% for *flacca*, and from 44% to 66% for cucumber. $P \leq 0.0001$, except for young cucumber were $P \leq 0.0015$.

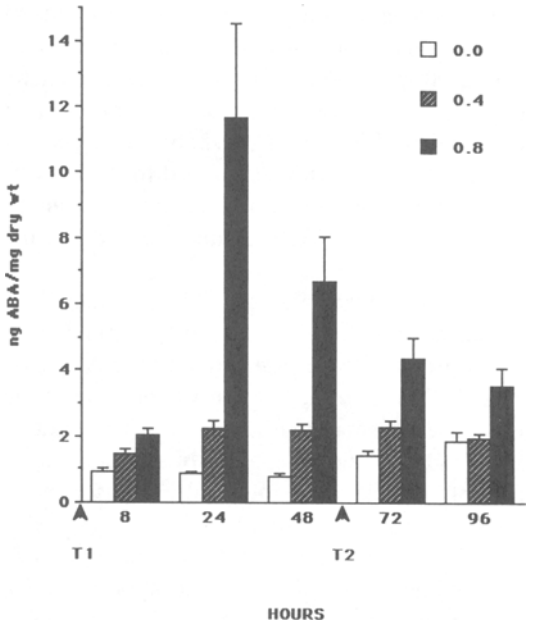


FIG. 5. Effects of ferulic acid concentrations (0.0, 0.4, and 0.8 mM) supplied in nutrient solution (pH 5.5) on the actual mean level of endogenous abscisic acid in leaf tissue on wild-type tomato plants at time intervals after the first treatment. Arrows indicate when treatments were given (T1 = treatment 1; T2 = treatment 2). No distinction between old and young leaves were made. Each column represents the actual mean with standard error of ABA levels for five plants. Regression analyses, based on ng ABA/mg dry weight leaf tissue, revealed significant linear, except for 24 hr where it was concave curvilinear, trends for ferulic acid treatments within time intervals. r^2 values ranged from 64% to 68% for all hours after treatment 1, except 96 hr when $r^2 = 42\%$. $P \leq 0.0003$ for 8, 48, and 72 hr. $P \leq 0.0012$ for 24 hr. $P \leq 0.0089$ for 96 hr.

Differential sensitivities of the taxa to FA were evident. For example, following the first FA treatment (growth period 1), at 0.4 mM FA, leaf growth of wild-type tomato was inhibited 44–49%, *flacca* 82–85%, and cucumber 30–45%; bean was unchanged. The magnitude of the FA-induced water stress was greatest in *flacca*. The mutants wilted at all concentrations of FA following each treatment. In addition, *flacca* treated with 0.8 mM FA became necrotic after the initial exposure. Wild-type tomato wilted only at 0.8 mM, following each of the first two treatments; cucumber wilted only following the first treatment. No water stress-related responses were detected in bean seedlings; they were considered FA-tolerant.

The responses of leaf growth rates of tomato and cucumber to FA changed

over time. Based on the changes in percent inhibitions and shifts in regression equations over time, tomato and cucumber were less affected by subsequent treatments. The reduced inhibitions of growth rates of tomato in this study by FA treatments after the first FA treatment was similar to what has been observed previously with cucumber (Blum et al., 1985b; Klein and Blum, 1990). This suggested that cucumber and tomato acclimated to the presence of FA. Acclimation was not apparent, however, when cucumber in an earlier study were pretreated with 0.1 and 0.2 mM FA, and later exposed to 1 mM FA (Blum and Dalton, 1985).

When considering acclimation as a possible explanation for the suppression of the FA-induced inhibition, one should be reminded that measurements of dry weight or leaf growth are only sensitive for actively growing portions of the plant (Waters and Blum, 1987). Leaf area expansion of the entire seedling, as used in this investigation, may partially or completely obscure responses or recoveries. Even though significant changes may not be detected using these types of measurements, specific physiological processes, not directly involved in leaf expansion, may be significantly altered.

Levels of ABA. Wild-type tomato contained the highest ABA levels of the four taxa. *Flacca* contained 31–65% of the wild-type ABA levels. *Flacca* have been reported to contain between 17% and 26% of the wild-type levels (Tal and Nevo, 1973; Neill and Horgan, 1985). These mutants are still capable of limited biosynthesis (Neill and Horgan, 1985). The level present depends on the conditions under which the tomatoes are grown (Taylor, 1987). Tal and Imber (1970) identified an ABA-like compound that increased with age in both *flacca* and wild-type genotypes of the Rheinland Rhum tomato cultivar. Levels of ABA from control cucumber and bean were 69- to 88-fold less than those from wild-type tomato controls.

FA-induced stress increased ABA levels in three of the four taxa. Leaf ABA levels were increased roughly 0–24%, 26–42%, and 75–101% in wild-type tomato and 9–18.5%, 7–27%, and 139–383% in cucumber, for 0.2, 0.4, and 0.8 mM FA, respectively. Leaf ABA levels were increased roughly 28–37% and 93–162% in *flacca* for 0.2 and 0.4 mM FA, respectively. The lack of change observed in bean leaf ABA levels was expected, since stress-related responses were not detected at any time during the experiment. The elevated ABA levels observed in the other three taxa may have resulted from an FA-induced water stress. The unexpected rise in endogenous ABA levels in *flacca*, although consistently below those of wild type, suggested that mutants were capable of responding to FA-induced stress.

The objective of the third experiment was to establish more definitively when ABA levels rise after FA treatment and whether these levels fluctuate or are maintained over time. In wild-type tomato utilized for this experiment,

induction of ABA biosynthesis occurred within 8 hr after treatment. Maximum levels of ABA were observed 24 hr after the initial treatment. At that time, ABA levels in plants treated with 0.4 or 0.8 mM FA were 2.6 and 13.7 times higher, respectively, than control levels. Tomato appeared to have adjusted physiologically (i.e., acclimated) to the FA in their root environment because ABA levels were apparently not affected by a second FA treatment. At 96 hr after the initial treatment, ABA levels of plants treated twice with 0.4 mM FA approach control levels; ABA levels of those treated twice with 0.8 mM FA were at 1.9 times higher than those of control levels.

Acclimation. A possible explanation for the reduced effect of subsequent FA treatments may be stomatal closure associated with increased ABA levels resulting from the initial FA treatment. In addition to induction of ABA biosynthesis, plants may adjust physiologically in other ways. Shann and Blum (1987b) observed an increase in soluble cell wall peroxidases and in protein level of bound enzyme in cucumber tissue harvested 24 hr after FA treatment at 0.5 and 1.0 mM. Radiotracer also was found in the residues of lignin isolated from seedling tissue treated with [U-ring-^{14}C] FA (Shann and Blum, 1987b). The initial FA treatment may have stimulated the detoxification of FA by its incorporation into bound forms, which are not active (Balke et al., 1987). Upon subsequent treatment, such FA detoxification mechanisms, once induced, could quickly bind or incorporate FA, and thus reduce the effects of subsequent FA treatments. Root uptake of FA also may decline as additional FA treatments are given (Shann and Blum, 1987b). All or various combinations of these mechanisms may be operating to produce the decline in response to multiple FA treatments. Some of these mechanisms also may help to explain the differential sensitivities of the four taxa to FA.

Final Comments. Under field conditions, plants are exposed continually to allelopathic compounds. The low estimates (<0.1 mM) of individual phenolic acids in soil solutions (Whitehead et al., 1982) are not representative of what may interact with roots. Turnover rates of phenolic acid pools in soils can be substantial. Thus, plant roots very likely come in contact with higher concentrations. At present, no information is available on actual uptake of phenolic acids by plants from natural soil environments. Consequently, unequivocal statements of how FA may affect plant growth and ABA levels in field environments cannot be made (Williamson and Weidenhamer, 1990). What should be evident from this study, however, is that (1) plant sensitivity and capacity for subsequent acclimation to phenolic acids, such as FA, are taxon dependent; (2) FA-sensitive taxa under mild water stress and growing in the presence of phenolic acids such as FA, may be more severely impacted than FA-tolerant taxa; and (3) severe drought stress or any other stress that closes stomata and reduces water loss may be antagonistic to FA-induced water stress.

Acknowledgments—We would like to thank Drs. R.S. Boston, E.L. Fiscus, and M.K. Walker-Simmons for their critical reviews of the manuscript. We would also like to thank K. Klein and Dr. S.W. Lyu for their help in data collection, Dr. S.W. Chilton for use of his Speed Vac concentrator, and Dr. J.F. Roberts for use of his monoclonal antibody facilities.

REFERENCES

BALKE, N.E. 1985. Effects of allelochemicals on mineral uptake and associated physiological processes, pp. 161–178, *in* A.C. Thompson (ed.). The Chemistry of Allelopathy. American Chemical Society Monograph Series 268, American Chemical Society, Washington, D.C.

BALKE, N.E., DAVIS, M.P., and LEE, C.C. 1987. Conjugation of allelochemicals by plants. Enzymatic glucosylation of salicylic acid by *Avena sativa*, pp. 214–227, *in* G.R. Waller (ed.). Allelochemicals Role in Agriculture and Forestry. American Chemical Society Monograph Series 330, American Chemical Society, Washington, D.C.

BEARDSELL, M.F., and COHEN, D. 1975. Relationships between leaf water status, abscisic acid levels, and stomatal resistance in maize and sorghum. *Plant Physiol.* 56:207–212.

BLUM, U., and DALTON, B.R. 1985. Effects of ferulic acid, an allelopathic compound, on leaf expansion of cucumber seedlings grown in nutrient culture. *J. Chem. Ecol.* 11:279–301.

BLUM, U., and REBBECK, J. 1989. Inhibition and recovery of cucumber roots given multiple treatments of ferulic acid in nutrient solution. *J. Chem. Ecol.* 15:917–928.

BLUM, U., DALTON, B.R., and RAWLINGS, J.O. 1984. Effects of ferulic acid and some of its microbial products on the radicle growth of cucumber. *J. Chem. Ecol.* 10:1169–1191.

BLUM, U., DALTON, B.R., and SHANN, J.R. 1985a. Effects of various mixtures of ferulic acid and some of its microbial metabolic products on cucumber leaf expansion and dry matter in nutrient solution. *J. Chem. Ecol.* 11:619–641.

BLUM, U., DALTON, B.R., and SHANN, J.R. 1985b. Effects of ferulic and p-coumaric acids in nutrient culture on cucumber leaf expansion as influenced by pH. *J. Chem. Ecol.* 11:1567–1582.

CHEN, H.H., LI, P.H., and BRENNER, M.L. 1983. Involvement of abscisic acid in potato cold acclimation. *Plant Physiol.* 71:362–365.

CORNISH, K., and ZEEVAART, J.A.D. 1988. Phenotypic expression of wild-type tomato and three wilty mutants in relation to abscisic acid accumulation in roots and leaflets of reciprocal grafts. *Plant Physiol.* 87:190–194.

DAIE, J., and CAMPBELL, W.F. 1981. Response of tomato plants to stressful temperatures: Increase in abscisic acid concentrations. *Plant Physiol.* 67:26–29.

DARBY, L.A., RITCHIE, D.B., and TAYLOR, I.B. 1978. Isogenic line in the tomato variety "Ailsa Craig." Rep. Glass House Crops Res. Inst. pp. 168–184, Little Hampton, West Sussex, England.

DAVIES, W.J., METCALFE, J., LODGE, T.A., and DA COSTA, A.R. 1986. Plant growth substances and the regulation of growth under drought. *Aust. J. Plant Physiol.* 13:105–125.

DOWNS, R.J., and THOMAS, J.F. 1983. Phytotron Procedural Manual for Controlled Environmental Laboratories. North Carolina Agricultural Experimental Station Technical Bulletin No. 244 (Revised).

EINHELLIG, F.A., STILLE MUTH, M., and SCHON, M.K. 1985. Effects of allelochemicals on plant-water relationships, pp. 170–195, *in* A.C. Thompson (ed.). The Chemistry of Allelopathy. American Chemical Society Monograph Series 268, American Chemical Society, Washington, D.C.

FLOYD, G.L., and RICE, E.L. 1967. Inhibition of higher plants by three bacterial growth inhibitors. *Bull. Torrey Bot. Club* 94:125–129.

GLASS, A.M., and DUNLOP, J. 1974. Influence of phenolics on ion uptake IV. Depolarization of membrane potentials. *Plant Physiol.* 54:855–858.

HARRISON, M.A., and WALTON, D.C. 1975. Abscisic acid metabolism in water-stressed bean leaves. *Plant Physiol.* 56:250–254.

HOAGLAND, D.R., and ARNON, D.I. 1950. The water culture method for growing plants without soil. *Calif. Agric. Exp. Stn. Circ.* 347.

JACKSON, M.B., and HALL, K.C. 1987. Early stomatal closure in waterlogged pea plants is mediated by abscisic acid in the absence of foliar water deficits. *Plant Cell Environ.* 10:121–130.

KARSSEN, C.M., GROOT, S.P.C., and KOORNEEF, M. 1987. Hormone mutants and seed dormancy in Arabidopsis and tomato, pp. 119–133, *in* H. Thomas and D. Grierson (eds.). Developmental Mutants in Higher Plants. Cambridge University Press, Cambridge, U.K.

KLEIN, K., and BLUM, U. 1990. Inhibition of cucumber leaf expansion by ferulic acid in split root experiments. *J. Chem. Ecol.* 16:455–463.

LALK, I., and DORFFLING, K. 1985. Hardening, abscisic acid, proline, and freezing resistance in two winter wheat varieties. *Physiol. Plant.* 63:287–292.

LAROSA, P.C., HASEGAWA, P.M., RHODES, D., CLITHERO, J.M., WATAD, A.-E.E., and BRESSAN, R.A. 1987. Abscisic acid stimulated osmotic adjustment and its involvement in adaptation of tobacco cells to NaCl. *Plant Physiol.* 85:174–181.

MERTENS, R., DEUS-NEUMANN, B., and WEILER, E.W. 1983. Monoclonal antibodies for the detection and quantification of the endogenous plant growth regulator, abscisic acid. *FEBS Lett.* 160:269–272.

MORRIS, P.C., WEILER, E.W., MADDOCK, S.E., JONES, M.G.K., LENTON, J.R., and BOWLES, D.J. 1988. Determination of endogenous abscisic acid levels in immature cereal embryos during in vitro culture. *Planta* 173:110–116.

NEILL, S.J., and HORGAN, R. 1985. Abscisic acid production and water relations in wilty tomato mutants subjected to water deficiency. *J. Exp. Bot.* 36:1222–1231.

NEILL, S.J., HORGAN, R., and WALTON, D.C. 1984. Biosynthesis of abscisic acid, pp. 43–70, *in* A. Crozier and J.R. Hillman (eds.). The Biosynthesis and Metabolism of Plant Hormones. Society for Experimental Biology, Seminar Series 23. Cambridge University Press, Cambridge, U.K.

NORMAN, S.M., POLING, S.M., and MAIER, V.P. 1988. An indirect enzyme-linked immunosorbent assay (+)-abscisic acid in *Citrus*, *Ricinus*, and *Xanthium* leaves. *J. Agric. Food Chem.* 36:225–231.

PATTERSON, D.T. 1981. Effects of allelopathic chemicals on growth and physiological responses of soybean (*Glycine max*). *Weed Sci.* 29:53–59.

PIERCE, M., and RASCHKE, K. 1981. Synthesis and metabolism of abscisic acid in detached leaves of *Phaseolus vulgaris* L. after water loss and recovery of turgor. *Planta* 153:156–165.

RADFORD, P.J. 1967. Growth analysis formulae—their use and abuse. *Crop Sci.* 7:171–175.

RAIKHEL, N.V., HUGHES, D.W., and GALAU, G.A. 1987. An enzyme-immunosorbant assay for quantitative analysis of abscisic acid in wheat, pp. 197–207, *in* J.E. Fox and M. Jacobs (eds.). Molecular Biology of Plant Growth Control, Alan R. Liss, New York.

RASCHKE, K., and ZEEVAART, J.A.D. 1976. Abscisic acid content, transpiration, and stomatal conductance as related to leaf age in plants of *Xanthium strumarium* L. *Plant Physiol.* 58:169–174.

ROBERTSON, J.M., PHARIS, R.P., HUANG, Y.Y., REID, D.M., and YEUNG, E.L. 1985. Drought-induced increases in abscisic acid levels in root apex of sunflower. *Plant Physiol.* 79:1086–1089.

SAS INSTITUTE INC. 1988. SAS/SAT User's Guide, Release 6.03 edition. SAS Institute Inc., Cary, North Carolina.

SHANN, J.R., and BLUM, U. 1987a. The uptake of ferulic acid and *p*-hydroxybenzoic acids by *Cucumis sativus*. *Phytochemistry* 26:2959–2964.

SHANN, J.R., and BLUM, U. 1987b. The utilization of exogenously supplied ferulic acid in lignin biosynthesis. *Phytochemistry* 26:2977–2982.

STEELE, R.G.D., and TORRIE, J.H. 1980. Principles and Procedures of Statistics, 2nd edition. McGraw-Hill, New York.

TAL, M., and IMBER, D. 1970. Abnormal stomatal behavior and hormonal imbalance in *flacca*, a wilty mutant of tomato. II. Auxin- and abscisic acid-like activity. *Plant Physiol.* 46:373–376.

TAL, M., and IMBER, D. 1971. Abnormal stomatal behavior and hormonal imbalance in *flacca*, a wilty mutant of tomato. III. Hormonal effects on the water status in the plant. *Plant Physiol.* 47:849–850.

TAL, M., and NEVO, Y. 1973. Abnormal stomatal behavior and root resistance, and hormonal imbalance in three wilty mutants of tomato. *Biochem. Gen.* 8:291–300.

TAYLOR, I.B. 1987. ABA-deficient tomato mutants. pp. 197–217. *in* H. Thomas and D. Grierson (eds.). Developmental Mutants in Higher Plants. Cambridge University Press, Cambridge, U.K.

WALKER, M.A., and DUMBROFF, E.B. 1981. Effects of salt stress on abscisic acid and cytokinin levels in tomato. *Z. Pflanzenphysiol. Bd.* 101:461–470.

WALKER-SIMMONS, M. 1987. ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. *Plant Physiol.* 84:61–66.

WALTON, D.C. 1980. Biochemistry and physiology of abscisic acid. *Annu. Rev. Plant Physiol.* 31:453–489.

WALTON, D.C., HARRISON, M.A., and COTE, P. 1976. The effects of water stress on abscisic acid levels and metabolism in roots of *Phaseolus vulgaris* L. and other plants. *Planta* 131:141–144.

WATERS, E.R., and BLUM, U. 1987. Effects of single and multiple exposures of ferulic acid on the vegetative and reproductive growth of *Phaseolus vulgaris* BBL-290 *Am. J. Bot.* 74:1635–1645.

WEILER, E.W. 1980. Radioimmunoassays for the differential and direct analysis of free and conjugated abscisic acid in plant extracts. *Planta* 144:262–272.

WHITEHEAD, D.C., DIBB, H., and HARTLEY, R.D. 1982. Phenolic compounds in soil as influenced by the growth of different plant species. *J. Appl. Ecol.* 19:579–588.

WILLIAMSON, G.B., and WEIDENHAMER, J.D., 1990. Bacterial degradation of juglone—evidence against allelopathy? *J. Chem. Ecol.* 16:1739–1742.

ZEEVAART, J.A.D., and CREELMAN, R.A. 1988. Metabolism and physiology of abscisic acid. *Annu. Rev. Physiol. Plant Mol. Biol.* 39:439–473.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

USE OF ANTIOXIDANTS IN EXTRACTION OF TANNINS FROM WALNUT PLANTS

SHUYUN PENG* and CHRISTAN JAY-ALLEMAND

Station d'Amélioration des Arbres Forestiers
Institut National de la Recherche Agronomique
Ardon, F-45160 Olivet, France

(Received November 12, 1990; accepted January 17, 1991)

Abstract—The radial diffusion assay was applied to estimate the amount of total tannins in extracts from walnut plant materials. We found that the protein-precipitating activity of the tannin extracts were increased by 30–75% when antioxidants (ascorbic acid or sodium metabisulfite, 5.5 mM) were added in extraction solvents (50% aqueous methanol). The extracts obtained with $Na_2S_2O_5$ were much less brown than control. Moreover, two-dimensional thin-layer chromatography revealed some additional polyphenolic compounds in the extracts made with $Na_2S_2O_5$. These results suggest that the amount of tannins and/or their protein-precipitating property may be strongly affected by oxidation events during extraction procedure. The addition of antioxidants in the extraction solvents is useful to limit this problem.

Key Words—Tannins, antioxidants, extraction, protein precipitation, walnut.

INTRODUCTION

Tannins are complex polyphenolic compounds found widely in higher plants. There are two types of tannins chemically different as hydrolyzable and condensed tannins (Swain, 1965; Haslam, 1979). Hydrolyzable tannins are mainly polyesters of gallic or hexahydroxydiphenic acid with glucose or other polyols. Condensed tannins (proanthocyanindins) are flavonoid polymers, with carbon–carbon bonds joining the individual flavonoid monomers. The most outstanding common characteristic of tannins is their ability to bind and/or precipitate proteins (McManus et al., 1985). At present, the characterization of new tannin

*To whom correspondence should be addressed.

0098-0331/91/0500-0887$06.50/0

molecules and the physiological and ecological significance of these secondary metabolites are widely studied (Beart et al., 1985; Okuda et al., 1989). For example, a high content of tannins decreases the nutrient value of food and feed products (Deshpande et al., 1986). Some evidence indicates that tannins may be important in plant defense (Robbins et al., 1987) and responsible for the great durability of some woods (Hart and Hillis, 1972).

Tannins can be estimated by different chemical assays (Scalbert et al., 1989). Alternatively, some methods based on the protein-precipitating property of tannins have been developed (for review, see Hagerman and Butler, 1989). The radial diffusion assay (Hagerman, 1987) is highly reproducible and suitable for testing a great number of samples. According to this method, tannin extracts are injected in wells in an agarose gel containing bovine serum albumin (BSA). Tannins diffuse in the gel and interact with BSA, marked by a visible ring of tannin–BSA precipitation. However, there are few examples of its application in tannin studies (Hagerman, 1988).

The walnut tree is a woody plant producing wood of high quality and edible fruit. Biochemical analyses showed an abundance of polyphenols in the tissues (Gupta et al., 1972; Muller and Leistner, 1976; Feucht and Schmid, 1983; Jay-Allemand et al., 1988). For example, numerous naphthoquinoids such as juglone and its derivatives and flavonoids such as sakuranetine and myricetine have been identified in walnut pericarp, fruit, and bark (Binder et al., 1989). Furthermore, two ellagitannins (corilagin and its isomer, juglanin) have been isolated from walnut pellicle (Jurd, 1958). By coloration tests, Scalbert et al. (1989) found ellagitannins and condensed tannins in walnut wood. The present paper is devoted primarily to the effect of antioxidants added in the extraction solvents on the BSA-precipitating activity of the tannin extracts in order to improve this quantitative method and adapt it to walnut tissues.

METHODS AND MATERIALS

Plant Material. Seedlings, stump sprouts, annual shoots from adult trees and in vitro shoots in micropropagation (Cornu and Jay-Allemand, 1989) of different species of *Juglans* were collected during growth period. After defoliation, the samples were put in liquid nitrogen and lyophilized before extraction. Leaves freshly sampled from annual shoots and from shoots grown in vitro were either immediately extracted or extracted after lyophilization.

Extraction. Plant material (50 mg dry wt) was extracted by 50% aqueous methanol (1.5 ml × 1 hr × 2 times). In order to study the effect of antioxidants on tannin extraction, ascorbic acid (5.5 mM, Merck) or diethyldithiocarbamic acid (DIECA, 5.5 mM, Sigma), or sodium metabisulfite ($Na_2S_2O_5$, Merck), all at different concentrations, were added to the solvents. In some experiments,

juglone (Aldrich) also was added at different concentrations to the solvents. Extractions were performed in test tubes at 4°C with ultrasound. The homogenates were centrifuged (5000 *g*) at 0°C for 15 min. The supernatants obtained were immediately analyzed.

Radial Diffusion Assay of Total Tannins. The radial diffusion assay was performed according to Hagerman (1987). Agarose gels were prepared with an acetic acid buffer (50 mM, pH 5) containing no antioxidant, or 60 μM antioxidant (ascorbic acid, DIECA, or sodium metabisulfite). The data were expressed as square centimeters of BSA precipitation (the area of the visible ring subtracted by the area of the well) per gram of tissues (dry wt). The BSA-precipitation activity of a commercial tannin mixture (tannic acid, from Extrasynthèse) also was tested. Each value is the mean of nine determinations (three extracts, triplicate for each extract).

TLC Analysis. Two-dimensional thin-layer chromatography (TLC) analysis was performed with Merck DC-Alufolien cellulose plates (20 × 20 × 0.1 mm); first dimension: upper phase of the mixture methyl isobutylketone–formic acid–H_2O (3:1:2; v/v/v); second dimension: formic acid–H_2O (6:94, v/v). Polyphenolic compounds separated on the plates were visualized under UV light in the presence of liquid nitrogen and after fuming with ammonia.

RESULTS

Effect of Antioxidants Added in Agarose Gel. Agarose gels were prepared with or without different antioxidants for the determination of the BSA-precipitation area of the same tannin extracts. Defoliated shoots from an adult walnut tree were extracted by 50% aqueous methanol. As compared to control (gel without antioxidant), the addition of ascorbic acid or $Na_2S_2O_5$ had no effect (Figure 1A). However, the gel containing DIECA showed a precipitation area 20% larger than the control.

Effect of Antioxidants Added in Extraction Solvents. Shoots from an adult walnut tree were extracted by 50% aqueous methanol, 50% aqueous methanol with ascorbic acid, $Na_2S_2O_5$, or DIECA at 5.5 mM. The radial diffusion assay was conducted in the DIECA-containing gel (Figure 1B). The presence of DIECA in 50% methanol did not affect the BSA-precipitating activity of the extracts. On the other hand, ascorbic acid and $Na_2S_2O_5$ addition increased the BSA-precipitation activity by 30% and 75%, respectively. Similar results were obtained when the radial diffusion assay was performed in the gels containing no antioxidant (data not shown).

In the experiments illustrated by Figure 2, $Na_2S_2O_5$ was added to 50% aqueous methanol at different concentrations (1.4–55.0 mM). The BSA-precip-

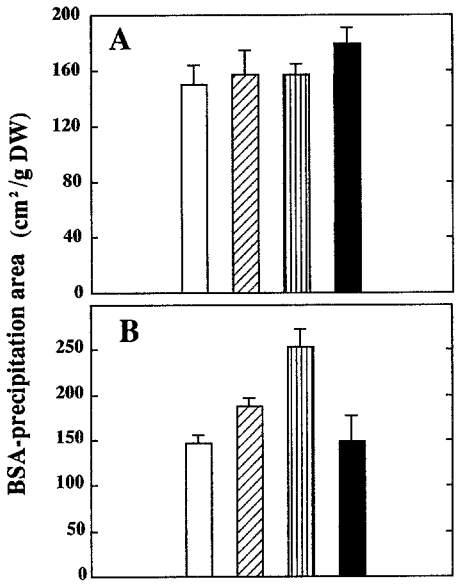


FIG. 1. Effect of antioxidants on BSA precipitation of walnut tannin extracts. Defoliated shoots from walnut trees (*J. nigra* × *J. regia*) were extracted by 50% aqueous methanol with and without antioxidants (5.5 mM). Aliquots of 36-μl extracts were injected in wells in agarose gels with or without antioxidants (60 μM). No addition (control, □), ascorbic acid (▨), sodium metabisulfite (▥), DIECA (■). Each value is the mean (± SD) of nine determinations. (A) Antioxidants in agarose gels for BSA-precipitation assay of the extracts made without antioxidant; (B) antioxidants in extraction solvents, BSA-precipitation assay conducted in gel containing no antioxidant.

itation area of the extracts increased as the concentration of sodium metabisulfite in extraction solvent increased from 0 to 5.5 mM, which is the best condition. No positive effect was observed when the concentration was higher than 27.5 mM. In addition, we noticed that the extracts made without $Na_2S_2O_5$ were dark brown. The extracts became less and less colored with the increasing concentration of sodium metabisulfite in the extraction solvent. Furthermore, our experiments also showed that NaCl (11.0 mM) could not replace $Na_2S_2O_5$ in the extraction solvents (data not shown).

A series of different walnut plant materials were extracted with or without $Na_2S_2O_5$ in 50% aqueous methanol (Table 1). In all the cases, the presence of $Na_2S_2O_5$ in the extraction solvents resulted in a higher BSA-precipiting activity, although the amplitude of the increase varied from 16% to 146% according to plant materials (genotype, plant age, and storage method). The most important effect was found with leaves extracted when fresh.

In order to get information about qualitative changes, walnut leaf extracts

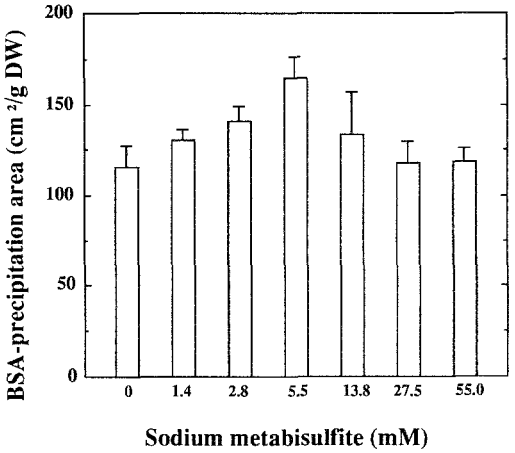


FIG. 2. Effect of $Na_2S_2O_5$ concentrations on tannin extraction. Defoliated shoots from walnut trees (*J. nigra* × *J. regia*) were extracted by 50% aqueous methanol containing $Na_2S_2O_5$ at different concentrations. The BSA-precipitation area (cm^2) was determined in an agarose gel containing 60 μM DIECA. Each value is the mean (± SD) of six determinations.

TABLE 1. EFFECT OF SODIUM METABISULFITE ON TANNIN EXTRACTION[a]

	Juglans species	Tannins (cm^2/g dry wt)		Increase in tannin amount (%)
		Control	Sodium metabisulfite	
Stems				
Seedlings	*J. nigra*	95 ± 1	179 ± 3	88
	J. nigra × *J. rigia*	113 ± 1	134 ± 12	19
Stump sprouts	*J. nigra* × *J. regia*	207 ± 7	354 ± 51	71
Annual shoots	*J. nigra*	248 ± 3	296 ± 12	19
	J. nigra × *J. regia*	150 ± 6	300 ± 6	100
In vitro shoots	*J. major* × *J. regia*	135 ± 3	201 ± 20	49
Leaves				
Annual leaves	*J. major* × *J. regia*	50 ± 5	77 ± 5	54
In vitro leaves	*J. major* × *J. regia* (A)	180 ± 8	240 ± 8	33
	J. major × *J. regia* (B)	151 ± 6	371 ± 22	146

[a] Freshly sampled walnut leaves (A) and lyophilized leaves (B) and stems were extracted by 50% aqueous methanol without (control) or with 5.5 mM sodium metabisulfite. The amount of tannins was determined by the radial diffusion assay in an agarose gel containing 60 mM DIECA. Each value is the mean ±SD of nine determinations.

made in the presence or absence of sodium metabisulfite were compared by two-dimensional TLC analysis. The extracts made with antioxidant showed four marked spots that were not detectable in the control (Figure 3). The compound, corresponding to spot d, has been identified as hydrojuglone glucoside (Jay-allemand et al., 1990).

Effect of Juglone on BSA-Precipitation Activity of Tannins. Stems from stump sprouts of an adult tree were used in these studies. Preliminary experiments showed that the addition of juglone to plant extracts did not change their BSA-precipitation activity (data not shown). Alternatively, juglone was added at two concentrations (0.1 and 1 mM) in 50% aqueous methanol before extracting plant material. The BSA-precipitation activity of the extracts was not affected by the coextraction with juglone (data not shown). However, the positive effect of $Na_2S_2O_5$ added in the extraction solvents was observed in all cases.

Effect of Antioxidants on Commercial Tannins. A commercial tannin mixture (tannic acids, Extrasynthèse) was dissolved in 50% aqueous methanol. Its BSA-precipitation activity in the agarose gel was proportional to the injected tannin amount at the range of 0–0.5 μg/well. No effect of antioxidant addition in 50% aqueous methanol or in agarose gel was observed with commercial tannins (data not shown).

DISCUSSION

Tannins are commonly extracted from plant materials by different solvents such as boiling aqueous methanol, aqueous acetone, or acidic methanol (Deshpande et al., 1986; Hagerman, 1988). Little work has been done to combine the extraction solvents with antioxidants. However, some phenols tend to oxi-

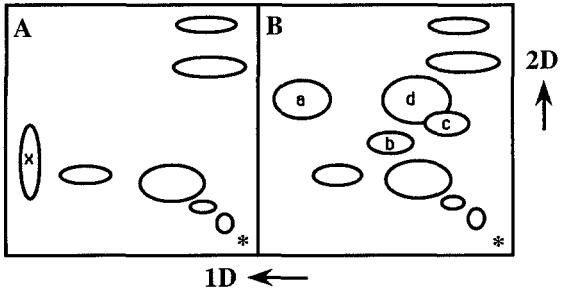


FIG. 3. Two-dimensional TLC analysis of walnut leaf extracts. Leaves from in vitro grown walnut shoots (*J. major* × *J. regia*) were extracted when fresh by 50% aqueous methanol without (A) or with 5.5 mM $Na_2S_2O_5$ (B). Only the most marked spots observed under UV light in the presence of liquid N_2 are drawn. The letter x indicates a spot noticed only for extract A; the letters a, b, c, and d indicate those for extract B.

dize easily during sample preparation and extraction (Deshpande et al., 1986). By the radial diffusion assay, we found that the protein-precipitation activity of walnut tannin extracts is strongly increased when antioxidants are included in the extraction solvents (Table 1).

In enzyme and metabolite extraction, and in cell and tissue cultures, browning often occurs and brings about some detrimental effects. One of the main causes for browning may be polyphenol oxidation (Loomis and Bataille, 1966; Christiansen and Fonnesbech, 1975; Rouet-Mayer et al., 1990). In some cases, the detrimental effects can be limited by addition of antioxidants, such as bisulfite, ascorbic acid (Golan-Goldhirsh and Whitaker, 1984), and cysteine (Dudley and Hotchkiss, 1989), or polyvinylpyrrolidone, which binds polyphenols by hydrogen bonding and prevents their interaction with proteins (Christiansen and Fonnesbech, 1975). According to Hagerman (1987), ascorbic acid is incorporated in the agarose gel for radial diffusion assay of tannins, but in our experiments, the presence of ascorbic acid and bisulfite in the gel did not affect BSA precipitation although the gel containing DIECA showed a slightly larger area of BSA precipitation than the control. On the contrary, when ascorbic acid or bisulfite (5.5 mM) was added to the extraction solvents, the BSA-precipitation activity of tannin extracts was strongly increased. Walnut extracts obtained with 50% aqueous methanol are dark brown. The color is much weaker when $Na_2S_2O_5$ is added to the extraction solvent, suggesting a limited polyphenol oxidation. Alternatively, bisulfite may react directly, like sulfhydryl compounds, with quinones to form colorless adducts (Sanada et al., 1972). These adducts may or may not have the capacity to precipitate proteins. However, ascorbic acid also shows a positive effect on tannin extraction. So the effect of bisulfite on tannin extraction may be due mainly to antioxidation, although we do not know the reasons for the ineffectiveness of DIECA addition in extraction solvents and the reduction of bisulfite effect when its concentration is higher than 5.5 mM. The mechanism involved in the oxidation of tannins and other polyphenols in 50% methanol has not been well understood. The oxidation of polyphenols is catalyzed in higher plants by polyphenoloxidase, which is responsible for the oxidation of *o*-diphenols to *o*-quinones (Vaughn and Duke, 1984; Rouet-Mayer et al., 1990) and which may be partially active in the extraction solvents. Spontaneous polyphenol oxidation may also take place in the solvents.

To form soluble or insoluble complexes under mild conditions, the interaction between tannins and proteins is based on hydrogen bonds, for example, the hydrogen bonds between the phenolic hydroxyl groups of tannins and carbonyl groups of the protein peptide bonds. This interaction may increase with the number of phenolic groups (Calderon et al., 1968; Haslam, 1979; McManus et al., 1985). We suppose that the effect of antioxidants on the BSA-precipitation activity of tannins extracts may be attributed mainly to the prevention of

oxidation of phenolic function. In addition, two-dimensional TLC analysis shows that the walnut extracts obtained with $Na_2S_2O_5$ contain some polyphenolic compounds such as hydrojuglone glucoside, which may have been destroyed during extraction without antioxidant addition (Figure 3). The possibility that similar changes occurred for tannins could not be dismissed. Further studies also are required to determine if some of the spots observed in the presence of bisulfite correspond to the colorless adducts formed between bisulfite and quinones (Sanada et al., 1972).

Hagerman (1987) reported that the presence of nontannin phenolics such as flavonoids, benzoic acids, or hydroxycinnamic acids do not interfere with the radial diffusion assay of tannins. This conclusion has been confirmed further with juglone addition to walnut tannin extracts. In addition, plant materials were extracted with added juglone in order to know if eventual reduction or oxidation of simple phenolic compounds in the presence or absence of sodium metabisulfite brings about interference with the assay. There is no observable interference caused by juglone addition for the BSA-precipitation activity.

The results presented here suggest that the amount of tannins and/or their BSA-precipitating activity may be affected strongly by oxidation events during extraction procedure. When we compare the tannin amounts extracted with or without antioxidant, the difference varies considerably, from 19% to 146%, according to plant material (genotype, plant age, tissue, and storage method). The addition of antioxidants in extraction solvents may allow more accurate determination and comparison of the tanning amounts in different plant materials and will be useful in experiments evaluating the relationship between the tannin amount and a physiological or ecological parameter. It also may allow isolation and characterization of labile tannins and other phenolic compounds (Jay-Allemand et al., 1990), which may be destroyed or modified by oxidation in commonly used extraction solvents containing no antioxidants.

Acknowledgments—The authors wish to express their sincere gratitude to Dr. M.-D. Deng for helpful advices, to Dr. A. Scalbert for critical reading of the manuscript, and to Miss P. Capelli for in vitro culture.

REFERENCES

BEART, J.E., LILLEY, T.H., and HASLAM, E. 1985. Plant polyphenols—secondary metabolism and chemical defence: Some observations. *Phytochemistry* 24:33–38.

BINDER, R.G., BENSON, M.E., and FLATH, R.A. 1989. Eight 1,4-naphthoquinones from *Juglans*. *Phytochemistry* 28:2799–2801.

CALDERON, P., VAN BUREN, J., and ROBINSON, W.B. 1968. Factors influencing the formation of precipitates and hazes by gelatin and condensed and hydrolyzable tannins. *J. Agric. Food Chem.* 16:479–482.

CORNU, D., and JAY-ALLEMAND, C. 1989. Micropropagation of hybrid walnut trees (*Juglans nigra* × *Juglans regia*) through culture and multiplication of embryos. *Ann. Sci. For.* 46S:113–116.

CHRISTIANSEN, J., and FONNESBECH, M. 1975. Prevention by polyvinylpyrrolidone of growth inhibition of *Hamamelis* shoot tips grown in in vitro and of browning of the agar medium. *Acta Hortic.* 54:101–104.

DESHPANDE, S.S., CHERYAN, M., and SALUNKHE, D.K. 1986. Tannin analysis of food products. *CRC Crit. Rev. Food Sci. Nutr.* 24:401–449.

DUDLEY, E.D., and HOTCHKISS, J.H. 1989. Cysteine as an inhibitor of polyphenol oxidase. *J. Food Biochem.* 13:65–75.

FEUCHT, W., and SCHMID, P.P.S. 1983. Selective histochemical staining of flavanols (catechins) with *p*-dimethylaminocinnamaldehyde in shoots from some fruits crops. *Gartenbauwissenschaft* 48:119–124.

GOLAN-GOLDHIRSH, A., and WHITAKER, J.R. 1984. Effect of ascorbic acid, sodium bisulfite, and thiol compounds on mushroom polyphenol oxidase. *J. Agric. Food Chem.* 32:1003–1009.

GUPTA, S.A., RAVINDRANATH, B., and SESHADRI, T.R. 1972. Polyphenols of *Juglans nigra*. *Phytochemistry* 11:2634–2636.

HAGERMAN, A.E. 1987. Radial diffusion method for determining tannin in plant extracts. *J. Chem. Ecol.* 13:437–449.

HAGERMAN, A.E. 1988. Extraction of tannin from fresh and preserved leaves. *J. Chem. Ecol.* 14:453–461.

HAGERMAN, A.E., and BUTLER, L.G. 1989. Choosing appropriate methods and standards for assaying tannin. *J. Chem. Ecol.* 15:1795–1810.

HART, J.H. and HILLIS, W.E. 1972. Inhibition of wood-rooting fungi by ellagitannins in the heartwood of *Quercus alba*. *Phytopathology* 62:620–626.

HASLAM, E. 1979. Vegetable tannins. *Recent Adv. Phytochem.* 12:475–523.

JAY-ALLEMAND, C., CORNU, D., and MACHEIX, J.J. 1988. Biochemical attributes associated with rejuvenation of walnut tree. *Plant Physiol. Biochem.* 26:139–144.

JAY-ALLEMAND, C., KERAVIS, G., LANCELIN, J.M., CORNU, D., and MACHEIX, J.J. 1990. Naphthoquinones and flavonols associated with walnut rejuvenation and involved in different physiological processes. *Plant Physiol.* 93S:147.

JURD, L. 1958. Plant polyphenols. II. The isolation of a new ellagitannin from the pellicle of the walnut. *J. Am. Chem. Soc.* 80:2249–2252.

LOOMIS, W.D., and BATAILLE, J. 1966. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* 5:423–438.

MCMANUS, J.P., DAVIS, K.G., BEART, J.E., GAFFNEY, S.H., LILLEY, T.H., and HASLAM, E. 1985. Polyphenol interactions. Part 1. Introduction; Some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *J. Chem. Soc. Perkin Trans.* 2:1429–1438.

MULLER, W.U., and LEISTNER, E. 1976. 1,4-Naphthoquinone, an intermediate in juglone (5-hydroxy-1,4-naphthoquinone) biosynthesis. *Phytochemistry* 15:407–410.

OKUDA, T., YOSHIDA, T., and HATANO, T. 1989. New methods of analyzing tannins. *J. Nat. Prod.* 52:1–31.

ROBBINS, C.T., HANLEY, T.A., HAGERMAN, A.E., HJELJORD, O., BAKER, D.L., SCHWARTZ, C.C., and MAUTZ, W.W. 1987. Role of tannins in defending plants against ruminants: reduction in protein availability. *Ecology* 68:98–107.

ROUET-MAYER, M.A., RALAMBOSOA, J., and PHILIPPON, J. 1990. Roles of *o*-quinones and their polymers in the enzymic browning of apples. *Phytochemistry* 29:435–440.

SANADA, H., SUZUE, R., NAKASHIMA, Y., and KAWADA, S. 1972. Effect of thiol compounds on melanin formation by tryosinase. *Biochim. Biophys. Acta* 261:258–266.

SCALBERT, A., MONTIES, B., and JANIN, G. 1989. Tannins in wood: Comparaison of different estimation methods. *J. Agric. Food Chem.* 37:1324–1329.

SWAIN, T. 1965. The tannins, pp. 552–580, *in* J. Bonner and J.E. Varner (eds.). Plant Biochemistry. Academic Press, New York.

VAUGHN, K.C., and DUKE, S.O. 1984. Function of polyphenol oxidase in higher plants. *Physiol. Plant.* 60:106–112.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

IDENTIFICATION OF SEX PHEROMONE OF BROWNTAIL MOTH, *Euproctis chrysorrhoea* (L.) (LEPIDOPTERA: LYMANTRIIDAE)

B.A. LEONHARDT,[1,*] V.C. MASTRO,[2] M. SCHWARZ,[1] J.D. TANG,[3,4] R.E. CHARLTON,[3,4] A. PELLEGRINI-TOOLE,[2] J.D. WARTHEN, JR.,[1] C.P. SCHWALBE,[2] and R.T. CARDÉ[3]

[1]*USDA, ARS, Insect Chemical Ecology Laboratory, BARC-West Beltsville, Maryland 20705*

[2]*USDA, APHIS, Otis Methods Development Laboratory Bldg. 1398, Otis ANGB, Massachusetts 02542*

[3]*Department of Entomology University of Massachusetts Amherst, Massachusetts 01003*

(Received August 29, 1990; accepted January 22, 1991)

Abstract—A unique sex attractant pheromone was isolated and identified from extracts of ovipositor tips from the female browntail moth (Lepidoptera: Lymantriidae). The pheromone compound, (*Z,Z,Z,Z*)-7,13,16,19-docosatetraen-1-ol isobutyrate, $CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_4CH{=}CH(CH_2)_6O_2CCH(CH_3)_2$, was identified by a combination of gas chromatography, mass spectrometry, and microreactions and was confirmed by synthesis. Traps baited with 5–50 μg of the synthetic pheromone, dispensed from rubber septa treated with an antioxidant and a UV stabilizer, gave male moth captures that were comparable to traps baited with three virgin females. Higher (250 μg) and lower (0.04–2.5 μg) quantities of the synthetic pheromone on septa captured somewhat fewer males. Captures were the same for similar quantities of the natural and synthetic pheromone. Minor, inactive components in the tip extract were identified as a mixture of 7- and 8-pentacosanone.

Key Words—Browntail moth, *Euproctis chrysorrhoea* (L.), Lepidoptera,

*To whom correspondence should be addressed.
[4]Present address: Department of Entomology, NYS-Agricultural Experiment Station, Cornell University, Geneva, New York 14456.

0098-0331/91/0500-0897$06.50/0

Lymantriidae, pheromone, (Z,Z,Z,Z)-7,13,16,19-docosatetraen-1-ol isobutyrate, sex attractant, dose dependence.

INTRODUCTION

The browntail moth, *Euproctis chrysorrhoea* (L.), is native to Eurasia, where periodic outbreaks result in defoliation of forest, shade, and ornamental trees. Accidently introduced into the United States in the late 1890s, the insect had spread into Long Island, New York, most of New England, and southeastern Canada by the early 1900s. Although the infested area in North America expanded rapidly, populations began to decline after 1915 (Burgess and Baker, 1938). At present, known U.S. infestations are limited to coastal islands and neighboring coast in Casco Bay, Maine and coastal dunes on Cape Cod, Massachusetts (Schaefer, 1974; Leonard, 1988) where the larvae feed primarily on beach plum (*Prunus maritima*) and black cherry (*Prunus serotina*). In addition to the damage caused by defoliation, human contact with larval setae can result in severe dermatitis.

The presence of a female-produced attractant was first demonstrated by Komarek and Pfeffer (1939) and traps baited with females have been used for monitoring moth populations (Jacobson, 1972; Schaefer, 1974). The synthetic sex attractant pheromone would facilitate moth detection. This paper describes the isolation and identification of the browntail moth female sex attractant pheromone, the bioassay of the natural and synthetic compounds, and the design of an effective dispenser for use in traps to monitor populations.

METHODS AND MATERIALS

Insects. In late June 1986, last-instar larvae were collected from beach plum at the Cape Cod National Seashore, North Truro, Massachusetts. Larvae were transferred to freshly cut apple foliage to complete their development under conditions of 16 hr:8 hr light–dark photoperiod at 21 ± 1°C. In 1987–1989, field-collected pupae, extricated from their webs, were sexed, held in separate containers, and checked daily for eclosion.

Isolation. Ovipositor tips, excised from 2-day-old females 2–5 hr into scotophase, were extracted with hexane for 10 min (5 tips/100 μl hexane). Whole female bodies were similarly extracted but with 250 μl hexane per insect. All solvents were HPLC grade (Baker Analyzed).

The tip extracts (10–50 female equivalents) were added to 1.2 g Florisil (60–200 mesh, precleaned with hexane in a Soxhlet extractor for 6 hr and dried at 80°C) held in a Pasteur pipet with a small plug of glass wool at the bottom and topped with a thin layer of dry sodium sulfate. Components were eluted

successively with 1 ml hexane, 1 ml 5% ether in hexane, and 4 ml 10% ether in hexane. Fractions of about 0.2 ml were collected and used for electroantennographic (EAG) measurement, flight-tunnel bioassay, and gas chromatographic (GC) analysis and peak collection.

Bioassay. EAG measurements were conducted using excised antennae from 2-day-old males (Roelofs, 1984). Aliquots (about one female equivalent) of the whole tip extract and the Florisil fractions were evaporated in Pasteur pipets. A 4-cc pulse of air through the pipets was used to introduce the stimulus into the continuous airstream, 4 cm upstream from the antenna. Two successive responses (in mV) were recorded for each sample or blank (5 μl hexane). The process was repeated using a new aliquot of each treatment, giving a total of four readings. The EAG activities of the GC-collected fractions also were measured.

Flight-tunnel (1.4 × 0.8 × 2.8 m, w × h × l) bioassays (Cardé and Hagaman, 1979) were used to verify that EAG-active fractions elicited upwind-oriented flight by males. An inactive (fraction 6) and two active (fractions 8 and 9) fractions were selected as being representative of the Florisil fractions for wind-tunnel bioassay. Aliquots (2 μl containing 0.3 female equivalents) were evaporated on filter paper disks (8 mm diam.) which were mounted at the upwind end of the tunnel. Air flow and temperature in the tunnel were 0.5 m/min and 21 $\pm$ 2°C, respectively. Individual males were placed under 30-ml clear plastic cups on small screens in the tunnel to acclimate for at least 1 hr before testing. The behaviors recorded were: activation (wing fanning, flight initiation), zigzag upwind flight, and contact with source.

Field tests were conducted at the Cape Cod National Seashore in mid-July using sticky, wing-type traps (Pherocon 1C). Traps were suspended 1.4 m above the ground from steel stakes. The tests were carried out in a randomized complete block with the traps checked and rerandomized daily. Reference traps were baited with three live, virgin females held in small wire cages (32 mesh, 4 cm high × 7 cm diam.). Aliquots of tip extracts or synthetic compounds were applied to filter paper disks (22 mm diam.) or slices of cotton dental roll (6 mm × 13 mm diam.) for the 1987 and 1988 field tests. Gray synthetic elastomer septa (No. 1888 West Co.) were used for the 1989 and 1990 tests and were extracted for 2 hr with 1 : 1 hexane–acetone and dried at room temperature prior to use. The septa were modified by removing all but 1 mm of the cup end. Each septum was treated with 20 μl of 5 μg/μl hexane solutions (i.e., 100 μg) each of butylated hydroxytoluene (BHT), an antioxidant, and Tinuvin 328 (Ciba-Geigy), a UV stabilizer, prior to sample addition. The quantities of synthetic compound per dispenser ranged from 0.04 to 250 μg. Male capture data were analyzed using analysis of variance (ANOVA) and means were separated using Duncan's (1955) multiple-range test.

Identification. Extracts of female ovipositor tips and fractions from Florisil

chromatography were initially analyzed by gas chromatography (GC) on a Hewlett-Packard model 5890 chromatograph fitted with an SP-2340 (75% cyanopropyl) fused silica capillary column (0.32 mm ID × 30 m; Supelco, Inc.). The column temperature was set at 80°C for 4 min and then programmed at 10°C/min to 210°C, where it was held for 10 min. The injection port and detector temperatures were 180° and 220°C, respectively. The same column (in a Varian 3700 chromatograph) was used to collect fractions in glass capillary tubes connected to the extinguished flame jet with temperature-resistant Teflon tubing. Collection times were 30 sec. The collection tubes were rinsed with 25 μl of hexane, which was concentrated to ca. 10 μl for GC and EAG analysis. Gas chromatographic–mass spectrometric (GC-MS) analyses were conducted on a Finnigan 4500 spectrometer fitted with a DB-1 (methyl silicone) fused silica capillary column (0.25 mm ID × 60 m; J & W Scientific). The column temperature was set at 120°C for 0.6 min and then programmed at 25.5°C/min to 280°C, where it was held. The injection port was set at 200°C. Electron-impact (EI) spectra were recorded at 2-sec intervals at 70 eV. Chemical ionization (CI) spectra were obtained using isobutane reagent gas. Retention indices were measured on an SP-2340 column (0.25 mm ID × 15 m; Supelco) set at 120°C for 6 min and then programmed at 2°/min to 225°C, where it was held.

Tip extracts (about 10 female equivalents) were subjected to microchemical reactions for structural identification. The extract was hydrogenated in hexane in the presence of Adams catalyst at room temperature for 1 hr. Other portions were ozonized for 20 sec (Beroza and Bierl, 1967) in carbon disulfide and in *n*-hexadecane. These treated extracts were subjected to GC-MS analyses with the DB-1 column. The column conditions were modified for the extracts ozonized in carbon disulfide (90°C for 2 min, then programmed at 25.5°/min to 280°C where it was held) and in *n*-hexadecane (70°C for 4 min, then 10°/min to 280°C).

Synthesis. The identified pheromone, (*Z*,*Z*,*Z*,*Z*)-7,13,16,19-docosatetraen-1-ol isobutyrate [(*Z*,*Z*,*Z*,*Z*)-7,13,16,19-22:OiBu] was synthesized (Schwarz et al., in preparation). The synthetic compound was first purified by liquid chromatography on a silica gel (activated at 120°C) column (1.5 cm ID × 60 cm) using hexane, 5% and 15% ether in hexane, and ether as eluents. The fraction containing the compound of interest was further purified by high-pressure liquid chromatography (HPLC) on a Millipore Waters Assoc. Delta Prep 3000 instrument using two passes through a 5-μm Hypersil (YNC Co.) column (2.0 cm ID × 25 cm). A Millipore Waters Lambda-max model 481 LC spectrophotometer was used for UV absorbance detection at 214 nm and an ISCO model 2150 peak separator plus an ISCO Foxy fraction collector were used to collect fractions. The mobile phase was 0.5% diethyl ether in hexane (UV grade, American Burdick & Jackson). The target compound eluted between 28 and 31 min. The final preparation for field examination was >90% pure as

determined by GC on a CP Wax 52 (polyethylene glycol) fused silica capillary column (0.25 mm ID × 10 m; Chrompack) set at 100°C for 2 min and then programmed at 10°C/min to 200°C, where it was held. The retention time of the (Z,Z,Z,Z)-7,13,16,19-22 : OiBu was 29.38 min.

Other synthetic compounds were prepared by conventional methods or procedures to be reported later. These included: (*E*,Z,Z,Z)-7,13,16,19-22 : OiBu, (Z,Z,Z,Z)-6,13,16,19-22 : OiBu, (Z,Z,Z,Z)-8,13,16,19-22 : OiBu, *n*-docosan-1-ol isobutyrate (22 : OiBu), (Z)-7-dodecen-1-ol isobutyrate, (Z,Z,Z,Z)-7,13,16,19-docosatetraen-1-ol [(Z,Z,Z,Z)-7,13,16,19-22:ol], 7-pentacosanone (7-25 : one) and 8-pentacosanone (8-25 : one).

RESULTS AND DISCUSSION

Identification. The chromatographic separation of tip extracts on Florisil resulted in eight fractions (5-10% ether in hexane) that showed an EAG response of 0.3 mV or greater (Figure 1). All other fractions gave a response of <0.3 mV. The flight tunnel bioassay showed that fractions that gave high EAG signals also caused activation, upwind zigzag flight, and source contact by most or all of the male moths (Table 1).

GC analyses (SP-2340) of the Florisil fractions showed the presence of six peaks that were present in all fractions showing high EAG response. The areas of only two of these peaks gave positive correlation ($P = 0.05$) with EAG responses: the peak at 20.25 min, which later was identified as the pheromone, and a minor peak at 18.91 min, which was not isolated for bioassay. The 20.25 min peak, when collected from the GC, gave a similar EAG response (1.20 ±

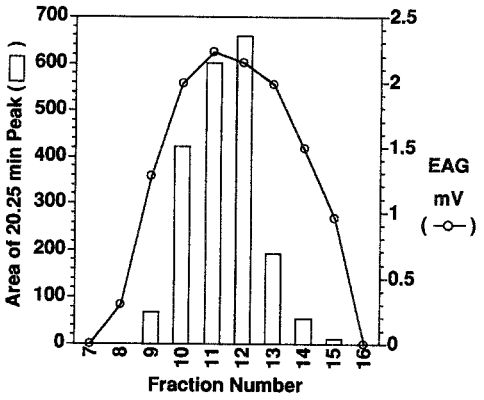


FIG. 1. Tests of fractions from Florisil chromatography of an extract of 10 female tips. Electroantennographic (EAG) response as a function of pheromone peak abundance.

TABLE 1. BEHAVIORAL RESPONSES OF MALE MOTHS IN FLIGHT TUNNEL[a] TO EAG[b] ACTIVE AND INACTIVE FLORISIL FRACTIONS

Fraction number	EAG response (mV ± SD)	No. males in tunnel	No. males exhibiting behavior		
			Activation[c]	Upwind flight[d]	Touch source
6	0.03 ± 0.03	2	0	0	0
8	2.21 ± 0.12	7	7	6	5
9	2.31 ± 0.14	5	5	5	4

[a] 0.3 female equivalents; flight tunnel: 1.4 × 0.8 × 2.8 m (w × h × l).
[b] 0.8 female equivalents.
[c] Within 3 min.
[d] Greater than 80 cm.

0.20 mV) as did the crude Florisil fraction 10 (1.63 ± 0.28 mV). The quantity of the 20.25 min peak in the tip extracts was estimated to be about 30–60 ng/female and that of the minor peak (18.91 min) about 3–10 ng/female. Kovats (1964) retention indices of the pheromone peak on SP-2340 and DB-1 capillary GC columns were 3007 and 2695, respectively. On the same columns, the minor peak had indices of 2900 and 2680. These differences in retention indices for polar vs. nonpolar columns were consistent with a single, oxygen-containing functional group in each component.

The mass spectrum of the unidentified major component (Figure 2A) showed that the molecular weight (M^+) was *m/z* 388 (2.8%). This was confirmed by CI-MS with the ion abundances as: $M^+ + 1$ (*m/z* 389) 100%, $M^+ + 2$ (*m/z* 390) 25%, and $M^+ - 1$ (*m/z* 387) 4%. Assuming one oxygenated functional group, the pheromone was thus either $C_{26}H_{44}O_2$ or $C_{27}H_{48}O$ with three to five or three to four double bonds and/or rings, respectively.

GC-MS analysis showed that the minor peak was a mixture of two compounds, each with M^+ of *m/z* 366. Although these compounds did not separate on polar or nonpolar columns, the DB-1 column provided sufficient resolution for the mass spectra, taken on the leading (19.73 min) and tailing (19.76) edges of the peak, to show that the two components were 8–25 : one and 7–25 : one, respectively (Figure 3A–D). A 1 : 1 mixture of the synthesized ketones gave the same retention time and approximately the same relative abundances of the ions that are characteristic of each ketone.

Hydrogenation of the tip extract eliminated the major peak and produced a later-eluting compound with a GC retention index of 2760 on the DB-1 column. The difference in retention index (2760 − 2695 = 65) is consistent with saturation of methylene-interrupted and/or isolated double bonds. The mass

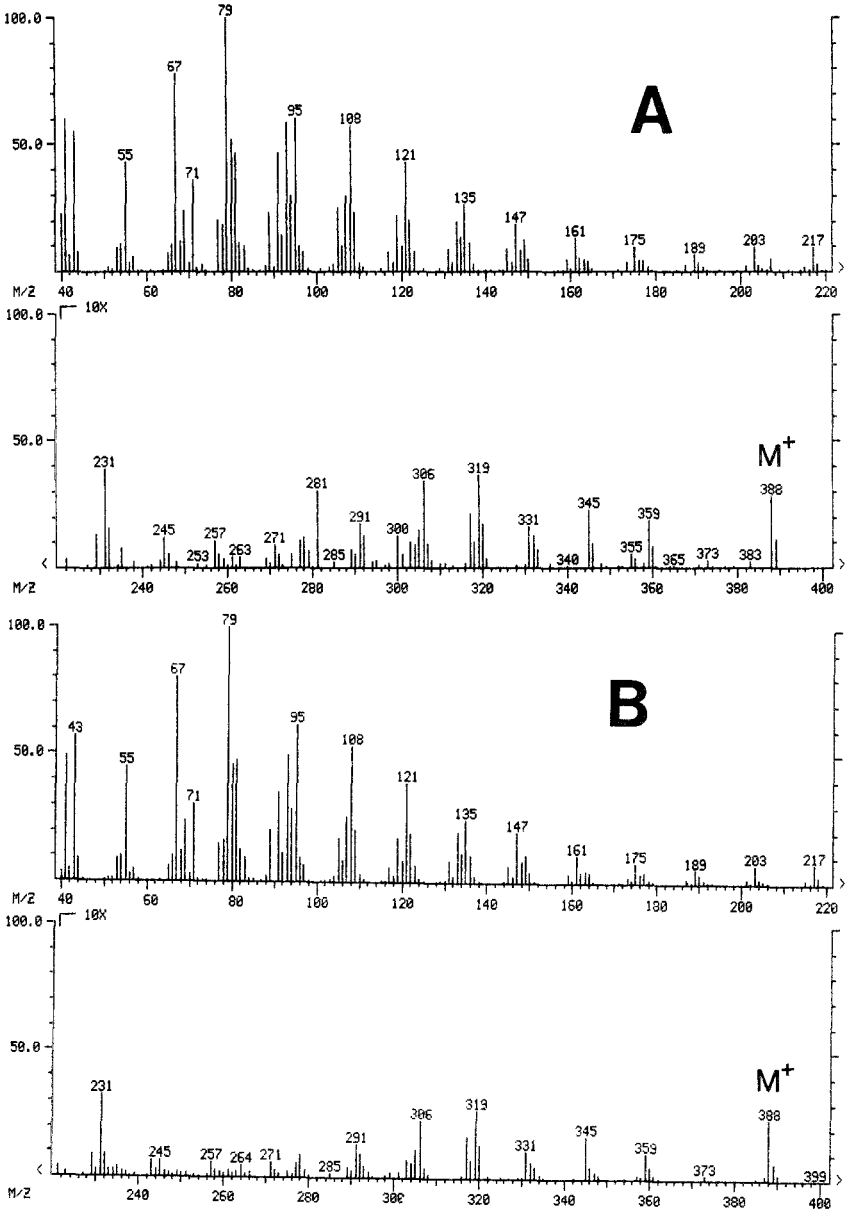


FIG. 2. Mass spectra (EI; 70 eV) of the natural (A) and synthetic (B) pheromone; abundances of ions higher than m/z 220 shown magnified 10×.

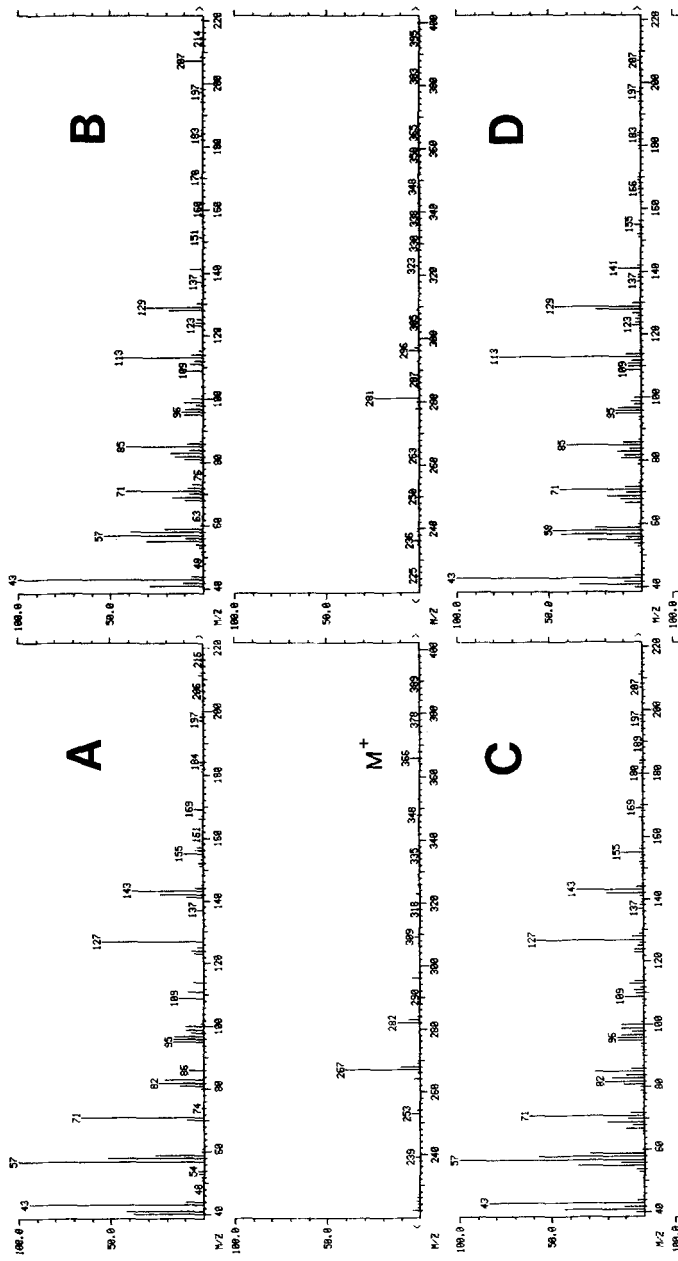


FIG. 3. Mass spectra (EI; 70 eV) of the leading (A) and trailing (B) edges of the minor peak in the female extract and of synthetic 8–25 : one (C) and 7–25 : one (D).

spectrum of the hydrogenation product showed M^+ at *m/z* 396 (31%) and fragment ions at *m/z* 353 (67%), 308 (10%), and 89 (100%), which correspond to $M^+-C_3H_7$, $M^+-C_3H_7CO_2H$, and $C_3H_7CO_2H_2^+$, respectively. The increase in molecular weight established that the pheromone had four double bonds and the *m/z* 89 ion confirmed that the pheromone was an ester of a $C_3H_7CO_2H$. Indeed, the mass spectrum and retention times of the hydrogenation product on polar and nonpolar columns matched those of synthetic 22:OiBu. Coinjection of the hydrogenated extract and the authentic 22:OiBu resulted in a single peak at 21.0 min (DB-1 column). Under the same conditions, *n*-docosan-1-ol *n*-butyrate has a retention time of 22.0 min. Thus, the major peak was an *n*-docosatetraen-1-ol isobutyrate ($C_{26}H_{44}O_2$). The minor peak was present in the hydrogenated extract, although it is not known whether a portion of it had been reduced.

The positions of the four double bonds in the alcohol portion of the major compound were identified by the *m/z* 108 ion in its mass spectrum and by ozonolysis. The *m/z* 108 ion (58%) has been shown to be characteristic of $[CH_3CH_2(CH{=}CH)_3H]^+$ produced by a 3,6,9-triene, counting from the methyl end of the chain (Connor et al., 1980; Underhill et al., 1983). In a docosatetraene isobutyrate, this corresponds to double bonds in the 13,16,19-positions. Ozonolysis of the major compound in carbon disulfide produced a peak at 9.26 min, which showed prominent ions at *m/z* 172 (1%), 157 (13%), 95 (15%), 94 (14%), 89 (53%), 84 (11%), 79 (7%), 71 (64%), 55 (22%), and 43 (100%). Ozonolysis of authentic (Z)-7-dodecen-1-ol isobutyrate produced the same product, i.e., $(CH_3)_2CHCO_2(CH_2)_6CHO$. Ozonolysis of the extract in *n*-hexadecane produced a peak whose retention time (3.2 min) and mass spectrum matched that of *n*-propanal, confirming the placement of the last double bond in the 19 position. These results indicated that the pheromone was 7,13,16,19-docosatetraen-1-ol isobutyrate, $CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_4CH{=}CH(CH_2)_6O_2CCH(CH_3)_2$. The minor peak remained unchanged on ozonolysis.

Coinjection of synthetic (Z,Z,Z,Z)-7,13,16,19–22:OiBu and the tip extract gave a single peak by GC analysis on polar and nonpolar columns. The mass spectrum of synthetic (Z,Z,Z,Z)-7,13,16,19–22:OiBu (Figure 2B) was the same as that of the natural compound. On the DB-1 column, the retention time of (E,Z,Z,Z)-7,13,16,19–22:OiBu was 6 sec longer (20.15 min) than that of the all-*cis* compound (20.05 min), and coinjection produced two peaks. Therefore, the unsaturation in the isolated compound was identified as all *cis*, i.e., (Z,Z,Z,Z)-7,13,16,19–22:OiBu. Coinjection of (Z,Z,Z,Z)-7,13,16,19–22:OiBu with (Z,Z,Z,Z)-8,13,16,19–22:OiBu also produced two peaks; the compound with the 7,13,16,19 double bonds eluted 4 sec later than the structure with the 8,13,16,19 double bonds. Coinjection of (Z,Z,Z,Z)-7,13,16,19–22:OiBu and (Z,Z,Z,Z)-6,13,16,19–22:OiBu produced a single but broad peak.

The mass spectra of the three synthetic isobutyrates with the first double bond in the 6, 7, and 8 position are very similar. However, differences in ion abundances were observed for the two C_nH_{2n-7} fragments which were characteristic of the first double-bond position of each compound. The pattern of ion abundances for the series of C_nH_{2n-7} fragments (Figure 4) showed elevated abundances for the two ions that include one and both, respectively, of the two carbons carrying the inner double bond. Both the natural and synthetic (Z,Z,Z,Z)-7,13,16,19-22:OiBu showed increases in intensity at *m/z* 203 ($C_{15}H_{23}^+$) and *m/z* 217 ($C_{16}H_{25}^+$) corresponding to $[CH_3(CH_2CH{=}CH)_3C_3H_8]^+$ and $[CH_3(CH_2CH{=}CH)_3C_4H_{10}]^+$, respectively. The spectrum of (Z,Z,Z,Z)-6,13,16,19-22:OiBu showed enhanced intensity at *m/z* 217 ($C_{16}H_{25}^+$) and *m/z* 231 ($C_{17}H_{27}^+$) and the spectrum of (Z,Z,Z,Z)-8,13,16,19-22:OiBu showed high abundances at *m/z* 189 ($C_{14}H_{21}^+$) and *m/z* 203 ($C_{15}H_{23}^+$) (Figure 4). Thus, these spectral data provide additional support the assignment of the isolated double bond at the 7 position.

Field Studies. Initial trapping tests in 1987–1988 with tip extracts and the synthetic compound gave poor and inconsistent results. Traps baited with the synthetic compound or extracts deposited on filter paper disks or cotton wicks captured some males during the first night. This number increased if the baits were prepared and the traps deployed just before sunset. However, trap captures

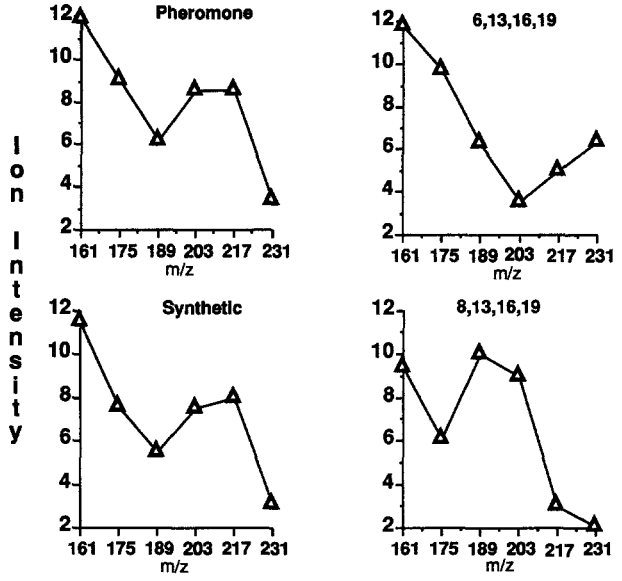


FIG. 4. Ion abundances of $C_nH_{2n-7}^+$ fragments in the mass spectra of three docosatetraen-1-ol isobutyrates and the natural pheromone.

TABLE 2. CAPTURES OF MALE *E. chrysorrhoea* IN TRAPS BAITED WITH SYNTHETIC (Z,Z,Z,Z)-7,13,16,19-DOCOSATETRAEN-1-OL ISOBUTYRATE AND FEMALE EXTRACTS IN FIELD TESTS, CAPE COD NATIONAL SEASHORE, JULY 1989

Source[b]	Total (μg)	Mean No. of males captured[a] July 13–17 test[c]	July 17–27 test[c]	July 18–21 test[c]
(Z,Z,Z,Z)-7,13,16,19-22:OiBu	250		5.5bc	
	50	30.3a		
	25	18.3abc	8.3ab	18.2a
	5	18.3abc		12.1b
	2.5		4.1c	
	1.0			3.8c
	0.5	2.3d		
	0.2			0.8d
	0.04			0.3d
Female tip extract	1.0[d]			3.4c
	0.5[d]	5.0cd		
Female whole body extract	0.4[d]			0.8d
Live virgin females		25.5ab	13.9a	14.9ab
Blank		1.0d	0.2c	0.3d

[a]Means within each column followed by the same letter are not significantly different at $P = 0.5$ according to Duncan's multiple range test after $(n + 0.5)$ transformation of data.
[b]Tinuvin 328 (100 μg) and BHT (100 μg) were added to each treatment. All samples were dispensed from gray septa.
[c]Mean number of male moths captured per trap per reading; two replicates and two readings were used in the July 13–17 test, four replicates and six readings in the July 17–27 test, and four replicates and three readings in the July 18–21 test.
[d]μg of the pheromone compound.

in subsequent nights were very low in comparison to those in female-baited traps.

Laboratory tests revealed that the reason for these low trap captures was decomposition of pheromone on filter disks or cotton wicks. When the synthetic pheromone (15 μg) was applied to filter paper disks or cotton wicks, very little of the compound was recovered by extraction following 24 hr of exposure at room temperature. However, similar recovery experiments with 22:OiBu showed that most of this compound remained on the filter paper or cotton wicks. Therefore, evaporation of the pheromone was not occurring and decomposition must have caused the disappearance of the pheromone. However, similar quantities of (Z,Z,Z,Z)-7,13,16,19-22:OiBu and the 22:OiBu, when applied to septa, were recovered by extraction after 24 hr of exposure, suggesting that the septa protected the pheromone from decomposition. BHT and Tinuvin 328 UV

stabilizer were added to all subsequently used septa to help further stabilize the pheromone.

Outdoor exposure of septa treated with 50 μg of synthetic (Z,Z,Z,Z)-7,13,16,19-22 : OiBu or 22 : OiBu resulted in similar recoveries of the two compounds after one, two, and five days of exposure. A longer study measured the residual quantity of (Z,Z,Z,Z)-7,13,16,19-22 : OiBu in septa exposed outdoors in Maryland during late July and early August 1989. The septa were protected from rain but not sun or wind. GC analyses showed that about 60% of the isobutyrate remained after 21 days of exposure.

Dose–response field tests showed (Table 2) that male moth captures in traps baited with 5–50 μg of synthetic (Z,Z,Z,Z)-7,13,16,19-22 : OiBu per septum were equivalent to those baited with three caged virgin females. Higher (250 μg) and lower (0.04–2.5 μg) quantities of the isobutyrate resulted in somewhat diminished moth captures.

No significant differences in male moth captures were detected in comparisons of similar (0.4–1.0 μg) amounts of the synthetic (Z,Z,Z,Z)-7,13,16,19-22 : OiBu and of the natural compound as present in extracts of female ovipositor tips or whole bodies (Table 2). In a 1990 test, male moth captures showed that 7-25 : one and 8-25 : one, alone or in 1 : 1 combination, were not attractive. Addition (0.5, 2.5, and 12.5 μg) of a 1 : 1 blend of these ketones with 25 μg of the synthetic pheromone had no significant effect on attraction (Table 3).

(Z,Z,Z,Z)-7,13,16,19-22 : OiBu is a novel structure and has not been reported previously as an insect pheromone. It has the highest molecular weight (*m/z* 388) thus far recorded for a sex attractant pheromone. However, aliphatic

TABLE 3. CAPTURES OF MALE *E. chrysorrhoea* IN TRAPS BAITED WITH SYNTHETIC (Z,Z,Z,Z)-7,13,16,19-DOCOSATETRAEN-1-OL ISOBUTYRATE AND 7- AND 8-PENTACOSANONE IN FIELD TESTS, CAPE COD NATIONAL SEASHORE, JULY 13–20, 1990

(Z,Z,Z,Z)-7,13,16,19-22 : OiBu (μg)	7–25 : one (μg)	8–25 : one (μg)	Mean No. of males captured[a]
25	0	0	12.8a
0	25	0	0.5c
0	0	25	0.5c
25	6.25	6.25	7.5b
25	1.25	1.25	9.3ab
25	0.25	0.25	9.3ab
0	6.25	6.25	1.0c

[a] Mean number of male moths captured per trap in each of four replicates. Means followed by the same letter are not significantly different at $P = 0.5$ according to Duncan's multiple range test after $(n + 0.5)$ transformation of data.

unsaturated hydrocarbons with similar 3,6,9-unsaturation and their monoepoxides have been reported as attractants for a large number of Lepidoptera, including noctuids, geometrids, lymantriids, and arctiids (Arn et al., 1986). The browntail moth pheromone is exceptional in that it has an additional isolated double bond further towards the ester end of the chain and is terminated by an alcohol functionality esterified by isobutyric acid, heretofore not encountered in pheromone chemistry. The only other sex pheromone identified in the genus *Euproctis* is that of *E. similis xanthocampa*, which is a four-component mixture of valerate and isovalerate esters of four octadecen-1-ols (Tan et al., 1984). However, the biological activity of these compounds has not been described.

In summary, the sex attractant pheromone was isolated from the female browntail moth and identified as (*Z*,*Z*,*Z*,*Z*)-7,13,16,19-docosatetraen-1-ol isobutyrate. This structure has not been reported previously and is dissimilar to other lepidopteran sex attractants because of its high molecular weight and its degree of unsaturation. In the field, the synthetic compound, when properly formulated with an antioxidant and a UV stabilizer, is as attractive as live females to conspecific males.

REFERENCES

ARN, H., TOTH, M., and PRIESNER, E. 1986. List of Sex Pheromones of Lepidoptera and Related Attractants. Swiss Federal Research Station, Wädenswil, Switzerland. 123 pp.

BEROZA, M., and BIERL, B.A. 1967. Rapid determination of olefin position in organic compounds in microgram range by ozonolysis and gas chromatography. *Anal. Chem.* 39:1131–1135.

BURGESS, A.F., and BAKER, W.L. 1938. The gypsy and brown-tail moths and their control. Bulletin No. 464. U.S. Department of Agriculture, Washington D.C.

CARDÉ, R.T., and HAGAMAN, T.E. 1979. Behavioral responses of the gypsy moth in a wind tunnel to air-borne enantiomers of disparlure. *Environ. Entomol.* 8:475–484.

CONNOR, W.E., EISNER, T., VANDER MEER, R.L., GUERRERO, A., GHIRINGELLI, D., and MEINWALD, J. 1980. Sex attractant of an arctiid moth (*Utethesia ornatrix*): A pulsed chemical signal. *Behav. Ecol. Sociobiol.* 7:55–63.

DUNCAN, D.B. 1955. Multiple range and multiple F tests. *Biometrics* 11:1–42.

JACOBSON, M. 1972. Insect sex pheromones. Academic Press, New York. 382 p.

KOMAREK, J., and PFEFFER, —. 1939. A new biological method of recording forest pests, pp. 2005–2010, *in* Forstentomologie. Verh. 7. International Congress on Entomology, Berlin, 1938.

KOVATS, E. 1964. The Kovats retention index system. *Anal. Chem.* 36:31A–35A.

LEONARD, D.E. 1988. Browntail moth *Euproctis chrysorrhoea* (Lepidoptera: Lymantriidae) on Cape Cod, Massachusetts, pp. 14–23, *in* Proc. Conference on Science in the National Parks, 1986, Vol. 5. Colorado State University, Fort Collins, Colorado.

ROELOFS, W.L. 1984. Electroantennogram assays: Rapid and convenient screening procedures for pheromones, pp. 131–159, *in* H.E. Hummel and T.H. Miller (eds). Techniques in Pheromone Research. Springer-Verlag, New York.

SCHAEFER, P.W. 1974. Population ecology of the browntail moth (*Euproctis chrysorrhoea* L.) (Lepidoptera: Lymantriidae) in North America. Thesis. University of Maine, Orono, Maine. 248 pp.

TAN, Z., WU, Y., LIN, G., WU, B., LIU, H., XU, X., ZHOU, W., PU, G. and ZHANG, M. 1984. Study on identification and synthesis of insect pheromone. XVII. The sex pheromone of *Euproctis similis xanthocampa*. Acta Chim. Sin. 42:1178–1181.

UNDERHILL, E.W., PALANISWAMY, P., ABRAMS, S.R., BAILEY, B.K., STECK, W.F., and CHISHOLM, M.D. 1983. Triunsaturated hydrocarbons, sex pheromone components of *Caenurgina erechtea*. *J. Chem. Ecol.* 9:1413–1423.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

SYNTHESIS AND FIELD SCREENING OF CHIRAL MONOUNSATURATED EPOXIDES AS LEPIDOPTERAN SEX ATTRACTANTS AND SEX PHEROMONE COMPONENTS[1]

J.G. MILLAR,* M. GIBLIN,[2] D. BARTON,[2] and E.W. UNDERHILL[2]

Department of Entomology
University of California
Riverside California 92521

(Received November 16, 1990, accepted January 23, 1991)

Abstract—Enantiomerically enriched forms of (*Z*)-6-*cis*-9,10-epoxymonoenes and (*Z*)-9-*cis*-6,7-epoxymonoenes of chain lengths C_{17-21} were synthesized by Sharpless asymmetric epoxidation of allylic alcohol intermediates, followed by tosylation or halogenation and chain extension. The resulting monounsaturated epoxides were field tested as sex attractants for lepidopteran species. *Euchlaena madusaria* Walker males were attracted to blends of the enantiomers of (*Z*)-6-*cis*-9,10-epoxynonadecene {6*Z*-*cis*-9,10-epoxy-19:H; IUPAC name [2α,3α(*Z*)]-2-pentyl-3-(2-dodecenyl)oxirane} in combination with 6*Z*,9*Z*-19:H. The response was antagonized by 9*Z*-*cis*-6,7-epoxy-19:H. 6*Z*,9*Z*-19:H was tentatively identified in pheromone gland extracts. *Xanthotype sospeta* Drury male moths were attracted to lures containing 6*Z*-9*S*,10*R*-epoxy-19:H; the response was antagonized by the opposite enantiomer. *Palthis angulalis* Hübner males were attracted to 9*Z*-6*S*,7*R*-epoxy-19:H; the opposite enantiomer was antagonistic. 6*Z*,9*Z*-19:H and 9*Z*-*cis*-6,7-epoxy-19:H and 9*Z*-*cis*-6,7-epoxy-19:H were tentatively identified in pheromone gland extracts from *Anacamptodes humaria* Guenée females. In field trails, 9*Z*-6*R*,7*S*-epoxy-19:H proved to be the attractive enantiomer, and the response was potentiated by 6*Z*,9*Z*-19:H. Mechanisms by which unique chemical communication channels are maintained by each species are discussed.

Key Words—Sex attractant, pheromone, enantiomer, Lepidoptera, Noctuidae, Geometridae, *Euchlaena madusaria, Xanthotype sospeta, Palthis angu-*

*To whom correspondence should be addressed.
[1] Issued as NRCC #32455.
[2] Present address: Plant Biotechnology Institute, National Research Council, 110 Gymnasium Road, Saskatoon, Saskatchewan S7N OW9 Canada.

0098-0331/91/0500-0911$06.50/0

lalis, *Anacamptodes humaria*, (Z,Z)-6,9-nonadecadiene, (Z)-6-*cis*-9,10-epoxynonadecene, (Z)-9-*cis*-6,7-epoxynonadecene.

INTRODUCTION

There have been a number of reports of (Z,Z,Z)-3,6,9-trienes and the related *cis*-3,4-,6,7-, or 9,10-monoepoxydienes as lepidopteran sex attractants and sex pheromone components (compilation to 1986, Arn et al., 1986; Millar et al., 1990a–d, and references therein). There also have been several reports of (Z,Z)-6,9-diunsaturated hydrocarbons as sex attractants and pheromones. For example, (Z,Z)-6,9-nonadecadiene (6Z,9Z-19:H; subsequent abbreviations will follow the same pattern) has been reported as a sex attractant for the geometrid species *Bupalus piniaria* L. (Bestmann and Vostrowsky, 1982), *Boarmia repandata* L. (Bogenschütz et al., 1985), and *Alsophila quadripunctata* Esp. (Szöcs et al., 1984), and as a sex pheromone component for *Sabulodes caberata* Gn. (McDonough et al., 1986). 6Z,9Z-21:H has been identified in extracts of the pheromone glands of the arctiid moths *Utetheisa ornatrix* L. (Jain et al., 1983) and *Phragmatobia fuliginosa* L. (Descoins and Frérot, 1984), and the noctuid *Mocis latipes* Gn. (Descoins et al., 1986). To our knowledge, however, there has been only a single report of a monoepoxide analog of these dienes as a sex attractant or pheromone component; Descoins and Frérot (1984) reported 6*Z*-9*S*,10*R*-epoxy-21:H as a component of the sex pheromone of the ruby tiger moth, *Phragmatobia fuliginosa* L. (Lepidoptera: Arctiidae).

As an extension of our field screening of (Z,Z,Z)-3,6,9-trienes and the related monoepoxydienes, we have synthesized and field screened racemic and enantiomerically enriched forms of (Z)-6-*cis*-9,10- and (Z)-9-*cis*-6,7-epoxymonoenes with carbon chain lengths C_{17}–C_{21}. We report here: (1) the identification and optimization of sex attractants containing chiral or racemic monoene monoepoxides for four species of noctuid and geometrid moths; (2) the tentative identification by coupled gas chromatography–mass spectrometry with selected ion monitoring of 6Z,9Z-19:H and *cis*-monoepoxynonadecenes from insect pheromone gland extracts; and (3) the identification of behavioral antagonists which suppress attraction to otherwise attractive lures.

METHODS AND MATERIALS

Insects and Electroantennography

Female moths were field collected with sweep nets or in black-light traps. Male moths were collected as described above or in sticky wing traps (Pherotech Inc., Vancouver, British Columbia) containing attractant lures. Field tests

were carried out in a mixed forest area approx. 100 km north of Saskatoon, Saskatchewan. Sticky wing traps, similar in design to Pherocon 1CP traps (Scentry Inc., Buckeye, Arizona) were used throughout. Traps were hung at heights of approx. 1.5 m, in randomized block design, with traps spaced at least 10 m apart. Survey traps were replicated twice, while specific experiments for a given insect were replicated three or four times.

Trap lures consisted of red rubber septa (#1780J07, Thomas Scientific, Philadelphia, Pennsylvania) loaded with hexane solutions of test compounds, followed by a few drops of a 10% acetone solution of the antioxidant butylated hydroxytoluene. Lure dosages are listed with the appropriate tables.

Summed trap captures were transformed $[(X + 1/2)^{1/2}]$ and subjected to analysis of variance. Treatments with zero trap captures overall were not included in the ANOVA, as the lack of variance within the treatment captures would violate an assumption of the ANOVA. The variances of the transformed data were tested for homogeneity with Bartlett's test (Sokal and Rohlf, 1981). Significantly different means were separated by Duncan's (1955) multiple range test.

Extracts of sex pheromone glands were made by immersion of excised female ovipositor tips in methylene chloride for 20 min. The resulting extracts were concentrated under a stream of N_2, and internal standards (heptadecane and tetracosane) were added. The extracts then were analyzed by coupled gas chromatography–electroantennogram detection (GC-EAD) as previously described (Millar et al., 1987) using a DB-1701 capillary column (30 m × 0.32 mm ID, J&W Scientific, Folsom California; temperature program: initial temperature 40°C, heat ballistically to 90°C, then 4°C/min to 240°C, He carrier gas) or gas chromatography–mass spectrometry. GC-MS analyses were carried out with a Finnigan 4000-E instrument interfaced to an Incos 2300 data system, in electron impact (70 eV) or chemical ionization (isobutane) modes. An Ultra-2 capillary column (50 m × 0.32 mm ID, Hewlett-Packard, Avondale, Pennsylvania) was used with helium carrier gas, programming from 40 to 150°C ballistically, then 4°C/min to 275°C, in on-column injection mode.

Synthetic Chemicals

The syntheses of the (*Z*,*Z*)-6,9-dienes (Underhill et al., 1983) and the geometric isomers of 6*Z*,9*Z*–19:H (Miller et al., 1990d) have been described previously. Samples of 3*Z*,9*Z*–19:H and 3*Z*,6*Z*–19:H were prepared by partial hydroboration of 3*Z*,6*Z*,9*Z*–19:H with 9-borabicyclononane (9-BBN) in refluxing THF, followed by protonolysis with propionic acid in diglyme at 160°C for 3 hr (Brown, 1975). The resulting mixture was separated cleanly into fractions containing monoenes, dienes, and unreacted triene by flash chromatography on 15% $AgNO_3$ on silica gel, eluting with 10% ether in hexane.

Glassware was oven-dried at 120°C and cooled under N_2. All reactions were run under a positive pressure of N_2. THF was distilled from sodium benzophenone ketyl, methylene chloride was distilled from CaH_2, and hexamethylphosphoric triamide (HMPA) was distilled from BaO under reduced pressure. Flash chromatography was performed with 230–400 mesh silica gel (Terochem Laboratories, Edmonton, Alberta). Proton NMR spectra were obtained with a Bruker WM-360 wide-bore instrument, operating at 360 MHz. Infrared spectra were recorded with a Perkin-Elmer 237-B instrument, using neat films on NaCl plates. Mass spectra (EI, 70 eV) were obtained as described above and are reported as *m/z* (relative intensity). Optical rotations were measured with a Perkin-Elmer 141 polarimeter, in a 1-d, 1 ml cell.

Synthesis of (Z)-6-cis-9,10-Monoene Epoxides

2,5-Undecadiyn-1-ol (3). To a solution of 2-octyn-1-ol **1** (6.3 g, 50 mmol) and tosyl chloride (10.1 g, 53 mmol) in dry ether (150 ml) at −10°C was added in portions over 15 min powdered KOH (250 mmol) (Brandsma, 1971). The resulting slurry was stirred 30 min at 0°C, then poured into ice-water (200 ml). The layers were separated, and the aqueous layer was extracted with ether (2 × 50 ml). The combined ether layers were washed with brine, dried (Na_2SO_4), concentrated, and pumped under vacuum (0.1 torr) to remove traces of solvent. The crude tosylate **2** (13.2 g, 94%) was used without further purification.

Ethylmagnesium bromide (approx. 300 mmol) was prepared from Mg turnings (7.3 g, 300 mmol) and ethyl bromide (34 g, 312 mmol) in THF (250 ml). 2-Propyn-1-ol (8.4 g, 150 mmol) was added dropwise over 1 hr, maintaining the temperature <30°C. The resulting slurry was stirred at 20°C for 2.5 hr, then cooled to 0°C, and CuI (380 mg, 2 mmol) was added. Tosylate **2** (13.2 g, 47 mmol) in THF (20 ml) was added dropwise, and the mixture was allowed to warm to 20°C, then heated at 60°C for 24 hr. The mixture was cooled and poured into 10% aqueous NH_4Cl (300 ml), and extracted with ether (1 × 200 ml, 2 × 100 ml). The combined ether layers were washed with brine, dried (Na_2SO_4), concentrated, and distilled, giving diynol **3** (6.53 g, 84%), bp 96–101°C (0.16 torr), lit, bp 100°C (0.09 torr) (Eiter et al., 1978), as a colorless oil, which yellowed on contact with air. Spectral data matched those previously reported (Eiter et al., 1978).

(Z,Z)-2,5-Undecadien-1-ol (4). A solution of P-2 nickel catalyst (Brown and Ahuja, 1973) was prepared by addition of 7.2 ml of a 1 M solution of $NaBH_4$ in EtOH to a solution of $Ni(OAc)_2 \cdot 4H_2O$ (1.79 g, 7.2 mmol) in 95% EtOH (100 ml), while flushing the reaction flask with N_2. When the effervescence subsided, the flash was flushed with H_2, and ethylene diamine (0.96 ml,

14.4 mmol) was added. The mixture was stirred 10 min, then diynol **3** (5.93 g, 36 mmol) was added, and the flask was maintained under a slight positive pressure of H_2 until the reduction was complete ($\simeq$6 hr.) The mixture then was filtered through a 1-cm pad of activated charcoal, rinsing the charcoal well with ethanol, and the filtrate was concentrated. The concentrate was taken up in ether (100 ml), washed with 1 M HCl (50 ml) and brine, dried (Na_2SO_4), and concentrated again. The crude dienol was flash chromatographed on silica, eluting with 20% EtOAc in hexane, giving the pure dienol **4** (4.5 g, 74%). The dienol has been previously described only in communications (Gleason et al., 1980; Mills and North, 1983) with no spectral details reported. [^{1}H]NMR ($CDCl_3$): δ 5.60 (dtt, 1H, J = 10.8, 6.6,1.5 Hz, H-2), 5.51 (dtt, 1H, J = 10.8, 7.2, 1.3 Hz, H-3), 5.40 (dtt, 1H, J = 10.7, 7.2, 1.5 Hz, H-5), 5.30 (dtt, 1H, J = 10.7, 7.2, 1.5 Hz, H-6), 4.21 (br. dd, 2H, J = 5, 5 Hz, H-1), 2.81 (m, 2H, H-4), 2.03 (m, 2H, H-7), 1.4–1.2 (m, 6H, H-8 to H-10), 0.87 (t, 3H, J = 6.8 Hz, H-11). IR (neat) λ_{max}: 3600–3100 (br. s), 3020 (m), 2965 (s), 2940 (s), 2865 (s), 1020 (br. s) cm^{-1}. MS: 168 (M^+, trace), 150 (9.8), 121 (2.2), 107 (4.1), 95 (9.9), 93 (28.6), 91 (16.4), 83 (17.5), 81 (24.3), 80 (83.6), 79 (100), 77 (26.2), 70 (46.4), 67 (55.9), 57 (28.7), 55 (71.8), 54 (35.3), 43 (31.9).

(Z)-cis-*2,3-Epoxy-undec-5-en-l-ols (5).* To 30 ml of CH_2Cl_2 (dried over CaH_2) at $-$ 25°C was added sequentially at 20-min intervals with vigorous stirring titanium isopropoxide (3.0 ml, 10 mmol), (−)-diisopropyl tartrate (2.6 ml, 12 mmol), dienol **4** (1.73 g, 10.3 mmol), and *t*-butyl hydroperoxide (4.16 M solution in CH_2Cl_2; 5 ml, 20.8 mmol). The solution was sealed and stirred at −25°C for one week. The mixture then was worked up by addition of 10% aqueous tartaric acid (25 ml) dropwise over 2 hr, maintaining the temperature at −23°C in a CCl_4/Dry Ice slush bath. The mixture was warmed to 20°C, and stirred until the aqueous layer was clear. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 20 ml). The combined CH_2Cl_2 layers were cooled to 0°C, 10% aqueous Na_2SO_3 (25 ml) was added dropwise, and the mixture was warmed to 20°C and stirred overnight, to decompose excess *t*-butyl hydroperoxide. The layers were separated, the aqueous phase was extracted again with CH_2Cl_2 (2 × 20 ml), and the combined organic extracts were concentrated. The concentrate was taken up in ether (100 ml), cooled to 0°C, 2 N NaOH (150 ml) was added, and the mixture was stirred until all the diisopropyl tartrate had been hydrolyzed ($\simeq$1 hr). The layers were separated, the aqueous phase was extracted with ether (2 × 25 ml), and the combined ethereal extracts were washed with brine, dried (Na_2SO_4), concentrated, and flash chromatographed on silica (4 cm ID × 20 cm), eluting with 30% EtOAc in hexane. (2*R*,3*S*)-Epoxy alcohol **5** was obtained as a clear oil (1.71 g, 90%), $[\alpha]_D^{24}$ = +10.6° (c = 6.32, CH_2Cl_2), lit. $[\alpha]_D^{20}$ = +11° (c = 2.60, CCl_4)

(Rollin and Pougny, 1986). Spectral data were entirely analogous to those previously reported (Rollin and Pougny, 1986).

(2*S*,2*R*)-**5** was obtained in 76% yield by the same procedure, except that (+)-diisopropyl tartrate was used in the formation of the chiral catalyst; $[\alpha]_D^{24} = -10.9°$ (c = 9.72, CH_2Cl_2), lit. $[\alpha]_D^{25} = -10.2°$ (c 1.5, $CHCl_3$) (Mills and North, 1983).

The enantiomeric excesses of the two enantiomers of **5** were checked by formation of diastereomeric derivatives (Slessor et al., 1985). Thus, (*S*)-2-acetoxypropionyl chloride (20 μl) was added to a solution of epoxy alcohol **5** (10 μl) and pyridine (50 μl) in ether (1 ml), and the mixture was stirred 1 hr. The supernatant was pipetted off, washed with water, and dried (Na_2SO_4). The resulting derivatives were gas chromatographed isothermally on a DB-210 capillary column (30 m × 0.25 mm) at 180°C. Baseline separation of the diastereomers was obtained. (2*R*,3*S*)-**5**: ee = 85.7%; (2*S*,3*R*)-**5**: ee = 88.1%.

(Z)-1-Iodo-2,3-epoxy-undec-5-enes (6). Iodine (2.16 g, 8.5 mmol) was added to a ice-bath-cooled solution of triphenylphosphine (2.23 g, 8.5 mmol) and imidazole (0.58 g, 8.5 mmol) in ether–CH_3CN, 3 : 1 (30 ml), and the resulting slurry was stirred briskly until it was a uniform bright yellow color. (2*R*,3*S*)-epoxy alcohol **5** (1.53 g, 8.3 mmol) was added in one portion, and the mixture was stirred 1 hr, allowing the mixture to warm to 20°C. Pentane (125 ml) then was added, and the mixture was stirred a further 10 min. The top layer was decanted off, and the gummy bottom layer was triturated with pentane (2 × 25 ml). The combined pentane extracts were washed with 1 M $Na_2S_2O_3$, dried (Na_2SO_4), concentrated, and flash chromatographed on silica (4 cm ID × 20 cm), eluting with 6% ether in pentane. The (2*S*,3*S*) iodide **6** (2.43 g, 83%) was obtained as a pale yellow oil, $[\alpha]_D^{22} = -32.9°$ (c = 15.35, CH_2Cl_2). [^{1}H]NMR ($CDCl_3$): δ 5.54 (dtt, 1H, J = 10.7, 7.2, 1.4 Hz, H-6), 5.44 (dtt, 1H, J = 10.7, 7.2, 1.4 Hz, H-5), 3.32 (m, 2H, H-1), 3.05 (m, 2H, H-2 and H-3), 2.29 (m, 2H, H-4), 2.04 (dt, 2H, J = 7.2, 7.0 Hz, H-7), 1.5–1.2 (m, 6H, H-8 to H-10), 0.88 (t, 3H, J = 6.8 Hz, H-11). IR (neat): λ_{max} 3020 (m), 2965 (s), 2935 (s), 2865, (s), 1450 (s), 1265 (m), 1160 (m) cm^{-1}. MS (CI, isobutane): m/z 295 (M + 1). MS (EI): 223 (M-71, 0.6), 183 (4.5), 155 (5.4), 127 (2.3), 123 (3.7), 111 (9.7), 99 (7.6), 95 (8.2), 93 (7.2), 83 (15.7), 81 (35.4), 79 (16.0), 69 (47.1), 67 (62.5), 57 (55.2), 55 (100), 43 (32.2).

(2*R*,3*R*)-**6** was obtained in 80% yield by the same procedure, $[\alpha]_D^{22} = +31.9°$ (c = 4.45, CH_2Cl_2), from 2*S*,3*R*-**5**.

(Z)-cis-9,10-Epoxyalk-6-enes (7) {IUPAC Names, [2α(Z),3α]-2-(2-Octenyl)-3-alkyloxiranes}. The following procedure describes the synthesis of (9*S*,10*R*)-**7a** from (2*S*,3*S*)-**6**. The syntheses of (9*S*,10*R*)-**7b–e** were performed under identical conditions, using the appropriate dialkyl lithium cuprate reagents. The syntheses of the (9*R*,10*S*)-**7a–e** series were carried out in similar fashion, using (2*R*,3*R*)-**6**.

A solution of hexylmagnesium bromide ($\simeq$ 1.5 mmol), prepared from hexyl

bromide (248 mg, 1.5 mmol) and Mg turnings (49 mg, 2 mmol) in THF (2.5 ml), was added dropwise by syringe to a mixture of (2*S*,3*S*)-**6** (200 mg, 0.68 mmol) and CuI (20 mg, 0.11 mmol) in THF–HMPA (3:1, 2.4 ml) maintained at −23°C in a CCl_4–Dry Ice slush bath. The reaction was stirred 20 min, then quenched by addition of sat. aq. NH_4Cl (1 ml). The mixture was poured into water (10 ml) and extracted with ether (3 × 5 ml). The combined ether extracts were backwashed with water and brine, dried (Na_2SO_4), concentrated, and flash chromatographed on silica gel impregnated with $AgNO_3$ (7.5%; 1 cm ID × 20 cm), eluting with 3% ether in pentane. Final purification was achieved by Kugelrohr distillation (0.1 torr, oven temp. ≃ 150°C) to remove traces of solvent and chromatographic packing material, yielding (9*S*,10*R*)-**7a** as a clear oil (96 mg, 56%), $[\alpha]_D^{21} = +3.2°$ (c = 2.29, CH_2Cl_2).

The [^{1}H]NMR spectrum of (9*S*,10*R*)-**7a** was entirely comparable to those of the previously reported analogs, (6*Z*,9*S*,10*R*)-epoxyheneicosene and (6*Z*,9*S*,10*R*)-epoxypentadecene (Rollin and Pougny, 1986). The IR spectrum also matched those reported for the analogs; in addition to the previously reported IR absorbtions, we also noted a sharp, medium intensity band at 3020 cm^{-1} (olefinic C-H stretch). MS: 252 (M+, trace), 243 (0.1), 223 (0.1), 209 (0.3), 195 (0.6), 181 (1.9), 167 (1.3), 153 (2.10, 141 (2.0), 139 (1.8), 127 (3.6), 124 (5.0), 113 (9.2), 110 (14.5), 97 (17.6), 96 (13.9), 95 (27.0), 83 (39.8), 82 (33.8), 81 (57.0), 69 (83.0), 68 (62.0), 67 (76.5), 57 (75.30, 55 (100), 54 (88.1), 43 (62.2).

The mass spectra of the higher homologs **7b–e** were characterized by very low intensity molecular ions, a weak M-18 ion, and a series of ions of *m/z* M-29, M-43, ... , M-99. The mass spectra in the mass range *m/z* 43–130 were virtually identical to that of **7a**. There were diagnostic fragments at *m/z* 110 $(C_4H_9CH{=}CH{-}CH{=}CH_2)^+$ and 124 $(C_5H_{11}CH{=}CH{-}CH{=}CH_2)^+$ from cleavage and rearrangement of the epoxide, and at *m/z* 153, from simple cleavage α to the epoxide.

Optical rotations of (9*S*,10*R*) series: **7b** $[\alpha]_D^{21} = +2.7°$ (c = 1.97, CH_2Cl_2); **7c** $[\alpha]_D^{21} = +2.6°$ (c = 0.86, CH_2Cl_2); **7d**, $[\alpha]_D^{21} = +2.6°$ (c = 2.22, CH_2Cl_2); **7e** $[\alpha]_D^{21} = +2.5°$ (c = 1.32, CH_2Cl_2).

(9*R*,10*S*) series: **7a**, $[\alpha]_D^{21} = -1.1°$ (c = 0.44, CH_2Cl_2); **7b**, $[\alpha]_D^{21} = -2.7°$ (c = 2.08, CH_2Cl_2); **7c**, $[\alpha]_D^{21} = -2.7°$ (c = 2.52, CH_2Cl_2); **7d**, $[\alpha]_D^{21} = -2.2°$ (c = 2.35, CH_2Cl_2); **7e**, $[\alpha]_D^{21} = -2.3°$ (c = 2.15, CH_2Cl_2).

Synthesis of (Z)*-cis-6,7-mon-9-ene epoxides (14)* {*IUPAC Names, [2α,3α(Z)]-2-pentyl-3-(2-alkenyl)oxiranes*}

(Z)*-2-octen-1-ol (8).* 2-Octyn-1-ol **2** was reduced with 2.5 mmol of P-2 nickel catalyst, prepared as described above in the synthesis of **4**. The crude product was distilled, giving (Z)-2-octen-1-ol **8** (5.02 g, 78%), bp 89–91°C (12 torr); lit bp 98–102°C (10–15 torr) (Osbond, 1961).

*cis-2,3-Epoxy-octan-1-ols (**9**).* The recently reported modification of the Sharpless asymmetric epoxidation method,[1] using molecular sieves, was attempted. Thus powdered 4 Å molecular sieves (5 g, freshly activated) were added to CH_2Cl_2 (40 ml) at −23°C (CCl_4–Dry Ice slush bath). To this mixture was added sequentially at 20-min intervals titanium isopropoxide (0.6 ml, 2.0 mmol), (+)-diisopropyl tartrate (0.54 ml, 2.5 mmol), alcohol **8** (2.45 g, 19.4 mmol), and *t*-butyl hydroperoxide (4.75 M in CH_2Cl_2; 8.5 ml, 40 mmol). The mixture was stirred at −25°C for 40 hr. The reaction was worked up by addition of aq. tartaric acid (10%, 10 ml) over 1 hr, while stirring vigorously and maintaining the temperature at −23°C. Stirring at this temperature was continued for 1 hr, then the mixture was allowed to warm to 20°C, and stirred another 2 hr. The mixture then was filtered with suction through a bed of Celite (4 cm). The layers were separated and the aqueous phase was reextracted with CH_2Cl_2 (2 × 10 ml). The combined CH_2Cl_2 extracts were cooled to 0°C, and aq. Na_2SO_3 (1.3 g in 10 ml H_2O) was added dropwise over 30 min. The resulting mixture was warmed to 20°C and stirred overnight. The layers were separated, and the aq. phase was extracted again with CH_2Cl_2 (2 × 10 ml). The combined organic extracts were washed with brine, dried (Na_2SO_4), concentrated, and flash chromatographed on silica gel (4 cm ID × 20 cm), eluting with 30% EtOAc in hexane, yielding (2*S*,3*R*)-**9** (1.73 g, 62%), $[\alpha]_D^{21} = -5.42°$ (c = 9.08, CH_2Cl_2). [^{1}H]NMR ($CDCl_3$): δ 3.83 (ddd, 1H, *J* = 12.0, 7.3, 4.1 Hz, H-1), 3.66 (ddd, 1H, *J* = 12.0, 6.8, 4.8 Hz, H-1′), 3.13 (dt, 1H, *J* = 6.8, 4.2 Hz, H-3), 3.01 (m, 1H, H-20, 1.67 (dd, 1H, *J* = 7.3, 4.8 Hz, OH), 1.67–1.25 (m, 8H, H-4 to H-7), 0.88 (br. t, 3H, *J* ≃ 7 Hz, H-8). IR (neat): 3600–3100 (br. s), 2965 (s), 2940 (s), 2965 (s), 1460 (m), 1380 (w), 1040 (s) cm^{-1}. MS: 113 (0.2, M-31), 95 (0.8), 84 (2.5), 83 (37.7), 71 (3.6), 69 (5.2), 67 (3.1), 61 (4.2), 57 (28.2), 56 (18.0), 55 (100), 45 (17.2), 43 (73.8).

The enantiomeric excess was determined by formation of diastereomeric derivatives of the alcohol with (*S*)-2-acetoxypropionyl chloride (Slessor et al., 1985), as described above for compounds **6**. Baseline separation of the derivatives was obtained on a DB-210 capillary column (30 m × 0.25 mm) operated isothermally at 150°C. Ee of (2*S*,3*R*)-**9**: 74.6%.

The (2*R*,3*S*) enantiomer was prepared concurrently in similar fashion in 75% yield, using (−)-diisopropyl tartrate in the asymmetric epoxidation step; $[\alpha]_D^{21} = +5.61°$ (c = 9.65, CH_2Cl_2), ee 76.1%. Mass, NMR, and IR spectra were satisfactory. The (2*R*,3*S*) enantiomer has been previously reported in a communication (Nicolau and Webber, 1985).

*1-Iodo-cis-2,3-epoxy-octanes **10**.* The iodides **10** were prepared exactly as described above for the iodides **6**. The purified products were also vacuum

[1] Referred to in an Aldrich Chemical Co. advertisement on the back cover of *J. Org. Chem. 50*(25), 1985; R.M. Hanson, S.Y. Ko, J.M. Chang, and K.B. Sharpless, unpublished results.

distilled, Kugelrohr over temperature 75°C (0.1 torr), yielding, for example (2*R*,3*R*)-**10** (2.38 g, 88%), $[\alpha]_D^{21}$ = +35.3° (c = 9.41, CH_2Cl_2). [^{1}H]NMR($CDCl_3$): δ 3.29 (m, 2H, H-1), 3.05 (m, 1H, H-3), 2.98 (m, 1H, H-2) 1.6–1.4 (m, 4H, H-4 and H-5), 1.38-1.27 (m, 4H, H-6 and H-7), 0.89 (t, 3H, *J* = 7.0 Hz, H-8). IR (neat): 2965 cm (s), 2935 (s), 2870(s), 1460 (s), 1380 (m), 1265 (m), 1165 (s) cm^{-1}. MS: 254 (0.2), 183 (1.0), 171 (1.5), 155 (4.3), 141 (2.4), 127 (56.7), 83 (73.3), 57 (100), 55 (96.5), 43 (75.2). Isobutane CI-MS: *m/z* 255 (M + 1).

The (2*S*,3*S*) enantiomer was prepared in identical fashion in 92% yield, $[\alpha]_D^{21}$ = −36.5 (c = 6.26, CH_2Cl_2). Mass, NMR, and IR spectra were satisfactory.

(Z)-cis-*6,7-epoxyalk-9-enes 14.* The alkenyl bromides **12a–e** were prepared from 1-alkynes **11**, exactly as previously described (Millar and Underhill, 1986).

The following procedure describes the synthesis of (6*R*,7*S*)-**14a** from (2*R*,3*R*)-epoxyiodide **10**. The syntheses of (6*R*,7*S*)-**14b–e** were performed under identical conditions, using the appropriate alkenyl Grignard reagents **13b–e**. The syntheses of the (6*S*,7*R*)-**14a–e** series were carried out in similar fashion, using (2*S*,3*S*)-**10**.

Grignard reagent **13a** was prepared by slow addition of (*Z*)-1-bromonon-1-ene **12a** (620 mg, 3.0 mmol) to Mg turnings (120 mg, 5 mmol) in THF (6 ml) at 20°C, and stirring for 2 hr. The Grignard solution was added dropwise over 10 min to a stirred solution of (2*S*,3*S*)-**10** (381 mg, 1.5 mmol), CuI (29 mg, 0.15 mmol), and HMPA (1.4 ml, 6 mmol) in THF (2 ml) at −23°C (CCl_4–Dry Ice slush bath). The resulting mixture was stirred for 30 min at −23°C, then poured into 10% aq. NH_4Cl (25 ml), and extracted with hexane (1 × 25, 2 × 10 ml). The combined hexane extracts were washed with water and brine, dried (Na_2SO_4), and filtered with suction through a plug of silica gel (4 cm), rising the silica well with ether. The filtrate was concentrated and flash chromatographed on $AgNO_3$-impregnated silica gel (7.5%; 2 cm ID × 20 cm), eluting with 2.5% ether in hexane. The purified compound was then freed from traces of column packing by vacuum distillation, Kugelrohr oven temperature 140°C (0.05 Torr), yielding (6*R*,7*S*)-**14a** (228 mg, 60%), $[\alpha]_D^{24}$ = +2.86° (c = 1.99, CH_2Cl_2). [^{1}H]NMR($CDCl_3$): δ 5.51 (dtt, 1H, *J* = 10.8, 7.8, 1.2 Hz, H-10), 5.40 (dtt, 1H, *J* = 10.8, 7,2, 1.2 Hz, H-9), 2.91 (m, 2H, H-6 and H-7), 2.45 (m, 1H, H-8), 2.36 (m, 1H, H-8′), 2.02 (br. dt, 2H, *J* = 6.8, 6.8 Hz, H-11), 1.5–1.2 (m, 10H, H-12 to H-16), 0.88 (m, 6H, H-1 and H-17). IR (neat): 3920 (m), 2970 (s), 2935 (s), 2865 (m), 1460 (m), 1380 (m) cm^{-1}. MS: 252 (0.2, M^+), 234 (0.4), 209 (0.4), 196 (0.3), 195 (0.2), 181 (1.4), 167 (1.3), 153 (2.1), 141 (2.9), 138 (3.2), 123 (2.4), 113 (6.50, 110 (8.0), 99 (15.40, 97 (14.6), 96 (15.10, 95 (16.10, 83 (32.6), 81 (33.1), 71 (19.60, 69 (38.7), 68 (36.8), 67 (52.2), 57 (40.50, 55 (92.2), 54 (50.9), 43 (100).

The mass spectra of **14b–e** were characterized by small but distinct ions at M^+, M-18, M-43, M-56, M-111, M-142, and *m/z* 113. The mass spectra were otherwise very similar to that of **14a**. NMR and IR spectra were all satisfactory.

Optical rotations of 6*R*,7*S* series. **14b**, $[\alpha]_D^{24} = +2.51°$ (c = 1.95, CH_2Cl_2); **14c** $[\alpha]_D^{24} = +2.34°$ (c = 1.97, CH_2Cl_2); **14d**, $[\alpha]_D^{24} = +2.32°$ (c = 1.99, CH_2Cl_2); **14e**, $[\alpha]_D^{24} = +1.78°$ (c = 2.08, CH_2Cl_2). (6*S*,7*R*) series: **14a**, $[\alpha]_D^{24} = -2.83°$ (c = 0.92, CH_2Cl_2); **14b**, $[\alpha]_D^{24} = +2.45°$ (c = 1.68, CH_2Cl_2); **14c**, $[\alpha]_D^{24} = +2.35°$ (c = 2.04, CH_2Cl_2); **14d**, $[\alpha]_D^{24} = +1.92°$ (c = 1.98, CH_2Cl_2); **14e** $[\alpha]_D^{24} = +2.12°$ (c = 2.03, CH_2Cl_2).

RESULTS

Euchlaena madusaria Walker. Male *E. madusaria* moths were first caught in 1985. Traps baited with 6*Z-cis*-9,10-epoxy-19:H (replicated three times, 500 μg dosage) caught 89 specimens, while the corresponding traps baited with 9*Z-cis*-6,7-epoxy-19:H, the combined monoepoxide mixture from nonspecific monoepoxidation of 6*Z*,9*Z*–19:H or 6*Z*, 9*Z*-19:H caught no moths. This experiment conclusively demonstrated that 6*Z-cis*-9,10-epoxy-19:H was an attractant for this species, and that the 9*Z-cis*-6,7 regioisomer was antagonistic, as the mixture of monoepoxides caught no moths.

Pheromone gland extracts (one, two, and three female equivalents) from field-collected female moths were analyzed by coupled gas chromatography–electroantennogram detection (GC-EAD) using a DB-1701 column. Male antennae consistently responded to a compound at the retention time of 6*Z-cis*-9,10-epoxy-19:H with a weaker response to a compound at the retention time of 6*Z*,9*Z*–19:H. On this column, 6*Z-cis*-9,10-epoxy-19:H was separated cleanly from the 9*Z-cis*-6,7-epoxy-19:H regioisomer, and a standard sample composed of 3*Z*,6*Z*–19:H, 3*Z*,9*Z*–19:H, and 6*Z*,9*Z*–19:H gave three clearly resolved peaks (relative retention times versus heptadecane: 6*Z*,9*Z*–19:H, 1.226; other two isomers, 1.236 and 1.251) providing additional proof of the position of the double bond. It had been demonstrated previously that all the geometric isomers of 6,9-nonadecadiene also were resolved cleanly under the standard GC-EAD conditions (Millar et al., 1990d).

A pheromone gland extract from two females was analyzed by coupled gas chromatography–mass spectrometry (GC-MS) using an Ultra-2 capillary column, in selected ion mode, with isobutane chemical ionization (CI). A perfect retention time match, and a good ion ratios fit with three ions was obtained for 6*Z*,9*Z*–19:H vs. a 400 pg standard [standard, *m/z* (rel. abundance): 264 (100 M^+), 263 (87, M-1), 155 (11); unknown from insect extract: *m/z* 264 (100), 263 (96), 155 (13)]. The corresponding monoepoxide was not detected.

A field test in 1986 (replicated three times) indicated that *E. madusaria* males were not attracted to either enantiomer of 6*Z*-*cis*-9,10-epoxy-19:H (one moth caught in traps with 9*R*,10*S* enantiomer; 0 moths with 9*S*,10*R* enantiomer), while the racemate was highly attractive (136 moths caught).

Further field tests demonstrated that *E. madusaria* males were optimally attracted to mixtures of the enantiomers of 6*Z*-*cis*-9,10-epoxy-19:H (Table 1), with the most attractive lures containing a 1:1 or 4:1 blend of the (9*S*,10*R*) and (9*R*,10*S*) enantiomers, respectively.

Finally, in an experiment run late in the flight season of this moth, traps (replicated three times) baited with a 4:1 blend of 6*Z*-*cis*-9,10-epoxy-19:H–6*Z*,9*Z*-19:H caught significantly more moths (23 specimens) than traps baited with 6*Z*-*cis*-9,10-epoxy-19:H alone (five specimens), suggesting that 6*Z*,9*Z*-19:H may potentiate the response to the epoxide.

Xanthotype sospeta Drury. This species is sympatric with and has approximately the same flight season as *E. madusaria*. A preliminary field test indicated that the *X. sospeta* males were selectively attracted by 6*Z*-9*S*,10*R*-epoxy-19:H, as traps (replicated three times) containing this compound caught 17 specimens, while the corresponding traps containing the opposite enantiomer or the racemate caught none. This selectivity was confirmed in further field tests (Table 1), wherein only traps containing a high proportion of the 9*S*,10*R* enantiomer caught moths. Thus, the data suggest that 6*Z*-9*S*,10*R*-epoxy-19:H is a powerful attractant for this species, and that the (9*R*,10*S*) enantiomer antagonizes the attractive response.

Palthis angulalis Hübner. Males of this species were first captured in 1984

TABLE 1. CAPTURES OF *Euchlaena madusaria* AND *Xanthotype sospeta* IN TRAPS BAITED WITH BLENDS OF ENANTIOMERS[a] OF (*Z*)-*cis*-9,10-EPOXYNONADEC-6-ENE

Lure blend 6*Z*-9*R*,10*S*-epoxy-19:H (μg)	6*Z*-9*S*,10*R*-epoxy-19:H (μg)	Males captured (X + SE)[b]	
		E. madusaria	*X. sospeta*
500	0	0	0
475	25	2.0 ± 0.6c	0
400	100	15.3 ± 5.8bc	0
250	250	52.7 ± 10.7a	0
100	400	47.7 ± 11.9a	0
25	475	18.3 ± 0.9b	3.7 ± 1.2a
0	500	0	19.0 ± 7.8a

[a] Enantiomeric excess of 6*Z*-9*R*,10*S*-epoxy-19:H, 88.1%, 6*Z*-9*S*,10*R*-epoxy-19:H, ee 85.7%.
[b] Traps replicated three times. Traps were set out from June 26 to July 16, 1986. Trap captures followed by the same letter are not significantly different ($P > 0.05$).

in several traps containing the combined monoepoxides mixture from nonspecific oxidation of 6*Z*,9*Z*–19 : H as the major component (up to 22 moths caught/lure). A 1985 field experiment (replicated three times) indicated that the 9*Z*-*cis*-6,7-regioisomer was the attractive component, as traps containing this compound (250 μg) caught 16 specimens, while traps containing the 6*Z*-*cis*-9,10-regioisomer (250 μg) caught none. In addition, traps containing the combined monoepoxides mixture (500 μg) caught 16 specimens, indicating that the 9*Z*-*cis*-6,7-19 : H regioisomer was not antagonistic.

An experiment in 1986 demonstrated that 9*Z*-6*S*,7*R*-epoxy-19 : H was the more attractive enantiomer, as traps (N = 3) containing this compound (250 μg) caught 39 specimens, while the corresponding traps containing 9*Z*-6*Z*,7*S*-epoxy-19 : H (250 μg) caught one specimen.

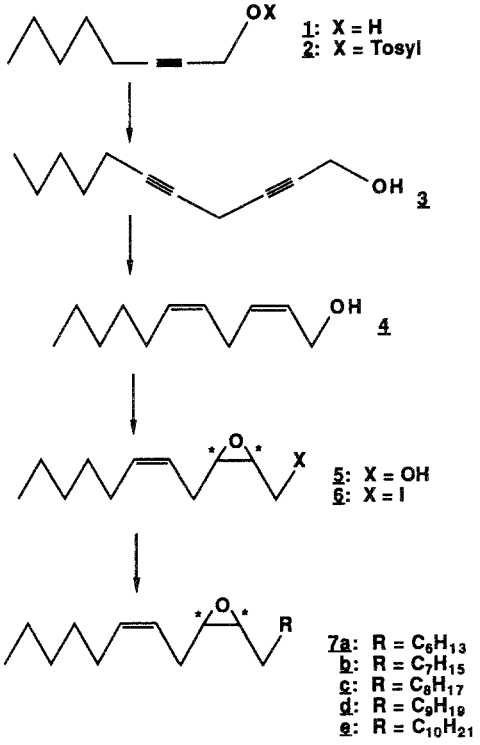
Anacamptodes humaria Guenée. A number of specimens of *A. humaria* (37) were caught in 1986 field survey traps (replicated twice) containing a 1 : 1 blend of 9*Z*-6*R*,7*S*-epoxy-19 : H (250 μg) with 6*Z*,9*Z*–19 : H (250 μg). The corresponding traps containing the opposite enantiomer (6*S*,7*R*) in combination with 6*Z*,9*Z*–19 : H caught no moths. When present as a single component, 9*Z*-6*R*,7*S*-epoxy-19 : H caught 10 specimens, while the corresponding traps containing either 9*Z*-6*S*,7*R*-epoxy-19 : H, 6*Z*,9*Z*–19 : H, or 9*Z*-*cis*-6,7-epoxy-19 : H as single components caught no moths.

6*Z*,9*Z*–19 : H and 9*Z*-*cis*-6,7-epoxy-19 : H were tentatively identified by selected ion monitoring (SIM) GC-MS analysis of the pheromone gland extract from three field-collected female moths on an Ultra-2-capillary column. Perfect retention time and good ion ratios matches were made for 6*Z*,9*Z*–19 : H [400 pg standard, *m*/*z* 264 (89, M^+), 263 (100, M-1), 155 (14); unknown from insect extract: 264 (100), 263 (95), 155 (14)] and 9*Z*-*cis*-6,7-epoxy-19 : H [standard, *m*/*z* 281 (100, M + 1), 279 (4, M-1), 263 (22, M + 1-18), 155 (1); unknown from insect extract: 281 (100), 279 (4), 263 (22), 155 (0)]. The lack of a sizable ion at *m*/*z* 155 was significant, as the 6*Z*-*cis*-9,10-epoxy-19 : H regioisomer, which has a retention time very similar to that of 9*Z*-*cis*-6,7-epoxy-19 : H, has a strong diagnostic ion at *m*/*z* 155 (40% of the *m*/*z* 281 base peak). Thus, the field data and the analyses of pheromone gland extracts both implicate 9*Z*-6*R*,7*S*-epoxy-19 : H and 6*Z*,9*Z*–19 : H as sex pheromone components of this species.

Synthesis. The (*Z*)-*cis*-9,10-epoxymono-6-enes were prepared by the route outlined in Scheme 1. This route was similar to those which had been used to synthesize the enantiomerically enriched forms of the analogous (*Z*,*Z*)-3-6-*cis*-9,10-epoxydienes (Mori and Ebata, 1981, 1986; Wong et al., 1985). The stoichiometric version of the Sharpless asymmetric epoxidation was used (Katsuki and Sharpless, 1980), resulting in enantiomeric excesses of approximately 88%. The enantiomeric excesses of the resulting epoxy alcohols **5** were determined by esterification of the alcohols with (2*S*)-2-acetoxypropionyl chloride (Slessor et al., 1985), and separation of the resulting diastereomeric derivatives by cap-

illary GC on a DB-210 column. The epoxyalcohols **5** were converted to iodides **6** (Garegg and Samuelsson, 1980; Corey et al., 1983), and the syntheses were completed by the dropwise addition of a THF solution of the appropriate alkylmagnesium bromide (2 equiv) to a cooled (−23°C) slurry of iodide (1 equiv), CuI (0.1 equiv), and HMPA (4 equiv) (Millar and Underhill, 1986; Nicolaou et al., 1984). The epoxides **7** were obtained in approx. 25% overall yield from 2-octyn-1-ol **1**. The reaction was sensitive to conditions (Nicolaou et al., 1984), as attempted alkylation of the iodides in ether by addition of dialkyllithium cuprates, as had been reported for 1-tosyloxy-2,3-epoxyalkenes (Mori and Ebata, 1981, 1986; Wong et al., 1985) gave almost exclusively the rearrangement product (*Z*)-1,5-undecadien-3-ol.

The synthetic route to the enantiomerically enriched forms of the (*Z*)-*cis*-6,7-epoxymono-9-enes was similar to that which we had used in the synthesis of the analogous (*Z*,*Z*)-3,9-*cis*-6.7-epoxydienes (Millar and Underhill, 1986), as shown in Scheme 2. However, the catalytic modification of the key Sharpless asymmetric epoxidation step using powdered molecular sieve (Hanson and



SCHEME 1

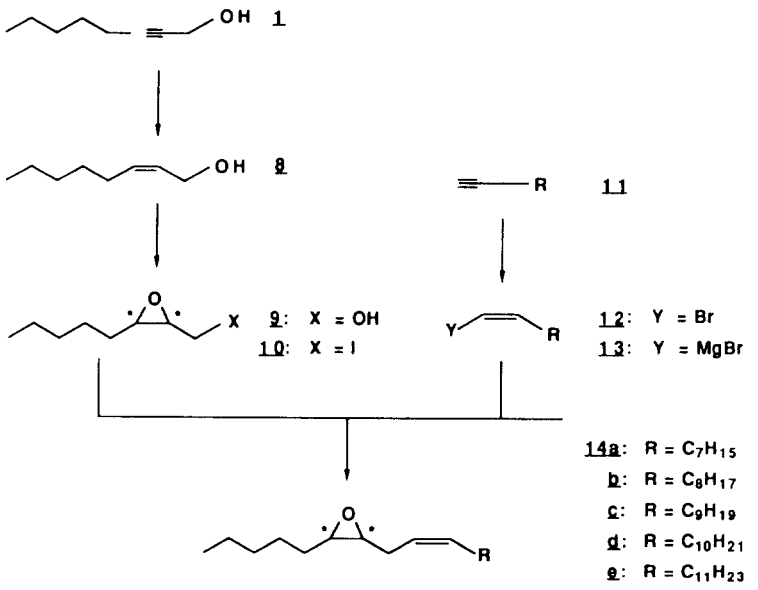
Sharpless, 1986), the details of which were at the time unpublished (see footnote 1, p. 917) was tried, resulting in a much faster reaction but with a somewhat lower enantiomeric excess (approx. 75% ee).

The epoxy alcohols were subsequently converted to the (Z)-*cis*-6,7-epoxymono-9-enes according to the previously reported methods used in synthesizing the epoxydiene analogs (Millar and Underhill, 1986). Overall yields were approximately 25%.

DISCUSSION

In parallel with the increasing number of identifications of pheromones and sex attractants that are derived from epoxidation of (Z,Z,Z)-3,6,9-trienes, it was reasonable to expect that there may be analogous sets of monounsaturated monoepoxides with sex attractant activity. The work reported here confirms this expectation.

The identification of 9Z-6*S*,7*R*-epoxy-19:H as a sex attractant for the noctuid species *P. angulalis* is the first report of a (Z)-*cis*-6,7-epoxymono-9-ene as an insect sex attractant. It was to be expected that compounds such as these would be identified eventually as sex attractants and pheromones, as the cor-



SCHEME 2

responding (*Z*,*Z*)-6,9-dienes and analogous diene monoepoxides had been reported from a number of lepidopteran species (references in Introduction).

To our knowledge, this is the second report of (*Z*)-*cis*-9,10-epoxymono-6-enes as sex attractants and possible sex pheromone components for geometrid moth species; 6*Z*-*cis*-9,10-epoxy-21:H has been reported as an attractant for the geometrid *Caustoloma flavicaria* L. (Kovalev and Nikolaeva, 1986). The same compound has been identified as a sex pheromone component for the arctiid moth *Phragmatobia fuliginosa* L. (Descoins and Frérot, 1984), and the chirality of the epoxide was presumed to be 9*S*,10*R* based on electroantennographic responses to the analogous 3*Z*,6*Z*-9*S*,10*R*-epoxy-21:H (Rollin and Pougny, 1986).

The biologically active compounds described here were discovered by field screening of potential attractants and optimized using information from GC-EAD analyses of standard mixtures and of pheromone gland extracts. There are several clear indications of the intricate balance of attraction and behavioral antagonism, with the chirality of the epoxide components playing a crucial role, as was found with the diene monoepoxide analogs (Millar et al., 1990a–d, 1991a). The results are summarized in Table 2. Distinct communication channels may be maintained by a combination of synergism between components, blend ratio, and antagonism in the cases of blends sharing common components.

For example, *E. madusaria* males were attracted only by blends of the enantiomers of 6*Z*-*cis*-9,10-epoxy-19:H. This represents one of several known examples of enantiomeric synergism in the lepidoptera (Wong et al., 1985; Millar et al., 1991a).

TABLE 2. BIOLOGICAL ACTIVITIES OF COMPOUNDS TESTED AGAINST FOUR LEPIDOPTERAN SPECIES

	Compounds[a]				
Species	6*Z*,9*Z*–19:H	6*Z*-9*R*,10*S*-epoxy-19:H	6*Z*-9*S*,10*R*-epoxy-19:H	9*Z*-6*R*,7*S*-epoxy-19:H	9*Z*-6*S*,7*R*-epoxy-19:H
E. madusaria	P	A	A	I	I
X. sospeta		I	A		
P. angulalis				I(?)	A
A. humaria	P			P	

[a]P = component tentatively identified from pheromone gland extracts; A = sex attractant; I = compound inhibiting attraction.

The sympatric species, *X. sospeta*, was attracted to only one of the enantiomers, with the opposite enantiomer being a strong antagonist (Table 2). Thus, these two species were not attracted to the same lures.

In similar fashion, *P. angulalis* was attracted to lures containing the single component 9*Z*-6*S*,7*R*-epoxy-19:H, while *A. humaria* was attracted by blends of the opposite enantiomer in combination with 6*Z*,9*Z*–19:H (Table 2).

There is considerable but not conclusive evidence to support the tentative identification of 6*Z*,9*Z*–19:H and 6*Z*-*cis*-9,10-epoxy-19:H as sex pheromone components for *E. madusaria*. For 6*Z*,9*Z*–19:H, this evidence consists of exact retention time matches on two capillary GC columns versus a synthetic standard, a good SIM ion ratios match versus a standard, and biological activity in terms of electroantennographic responses and attractant activity in the field. Furthermore, it has been shown that the other geometric isomers of this compound have retention times different from that of 6*Z*,9*Z*–19:H under the GC conditions used (Millar et al., 1990d), and we have shown here that the 3*Z*,6*Z*–19:H and 3*Z*,9*Z*–19:H positional isomers, which might also be potential pheromone candidates, also have retention times different from 6*Z*,9*Z*–19:H under the GC-EAD conditions used. In addition 6*Z*,9*Z*–19:H is a logical biosynthetic precursor to the attractive 6*Z*-*cis*-9,10-epoxy-19:H. However, on the basis of the available data, we cannot absolutely rule out the slim possibility that the insect-produced compound is actually a positional isomer of 6*Z*,9*Z*–19:H.

The evidence for 6*Z*-*cis*-9,10-epoxy-19:H is more tenuous. A compound in the female pheromone gland extract that gave strong electroantennogram activity exactly matched the retention time of a synthetic standard. The biological activity in the field, coupled with the demonstrated behavioral antagonism of the 9*Z*-*cis*-6,7-epoxy-19:H regioisomer, also provided circumstantial evidence for this compound being a pheromone component for *E. madusaria*.

There was reasonably conclusive evidence that 6*Z*,9*Z*–19:H and 9*Z*-6*S*,7*R*-epoxy-19:H are female-produced pheromone components of the species *Anacamptodes humaria*. Both compounds were identified in pheromone gland extracts by a combination of GC retention time matches, and by matches of SIM chemical ionization mass spectra with those of standards. In light of the retention time match with a standard, the possibility of the diene insect-produced compound being an isomer other than 6*Z*,9*Z*–19:H is small, as described above for *E. madusaria*.

Synthesis

Three syntheses of a component of the sex pheromone blend of the ruby tiger moth, 6*Z*-*cis*-9,10-epoxy-21:H, or its enantiomers have been reported. In the first, Rollin and Pougny (1986) synthesized the 9*S*,10*R* enantiomer in 10

steps from D-xylose, providing material of high chiral purity. However, the last step delivered a 9:1 mixture of the 6*Z*:6*E* geometric isomers. In addition, the synthesis was only amenable to the production of the 9*S*,10*R* enantiomer or series of homologous enantiomers.

Ebata and Mori (1989) described a synthesis of both of the enantiomers of 6*Z*-*cis*-9,10-epoxy-21:H, using Sharpless asymmetric epoxidation of the achiral intermediate (*Z*)-tetradecen-2-ol as a key step. Repeated recrystallization of crystalline derivatives of the intermediate epoxy alcohols was used to increase the chiral purities to high levels. However, this synthetic route could only be used to produce the C_{11} compounds, that is, it could not be used to efficiently and convergently synthesize homologs.

It also must be mentioned that the optical rotations reported for the tiger moth pheromone component 6*Z*-9*S*,10*R*-epoxy-21:H by both Rollin and Pougny (1986) ($[\alpha]_D^{20} = +5.5°$) and Ebata and Mori (1989) ($[\alpha]_D^{23} = +9.4°$) are considerably different from each other, and from our result ($[\alpha]_D^{21} = +2.5°$). Rollin and Pougny indicated that their product was contaminated with 10% of the 6*E* isomer, and this or other impurities may have contributed to a possibly erroneous optical rotation. Possible impurities in the pheromone prepared by Ebata and Mori (1989) may have had a similar effect.

Bell and Ciaccio (1988) published a short synthesis of racemic 6*Z*-*cis*-9,10-epoxy-21:H based on an ingenious alkylative rearrangement of an epoxytosylate intermediate. With several minor modifications, this synthesis could be adapted to the production of either enantiomer of this compound or to the production of homologs.

Our synthetic strategies were designed to satisfy two criteria. First, we wanted facile access to both enantiomers of each monoene epoxide. This was expeditiously accomplished by use of the Sharpless asymmetric epoxidation sequence on appropriate readily available allylic alcohol precursors. Although this procedure gives enantiomeric excesses of approximately 90% at best (i.e., a 19:1 ratio of enantiomers) for 1,2-disubstituted allylic alcohol subtrates (Katsuki and Sharpless, 1980), our previous experiences with the analogous diene monoepoxides had demonstrated that compounds of this enantiomeric purity were in many cases sufficient to demonstrate that biological activity, either as an attractant or a behavioral antagonist, could be attributed to one of the two enantiomers.

Our second requirement was for a synthetic route that would be amenable to the efficient production of homologous series of compounds, that is, for syntheses that used a common intermediate to which a chain of variable length could be attached in the penultimate step. The chiral epoxy alcohols obtained by the Sharpless epoxidations, when converted to epoxy iodides, fulfilled this requirement.

REFERENCES

ARN, H., TÓTH, M., and PRIESNER, E. 1986. List of sex pheromones of Lepidoptera and related attractants. OILB-SROP/IOBC-WPRS, Secrétariat General, 149 Rue de Bercy, F-75595 Paris cedex 12; ISBN 92-9067-002-9.

BELL, T.W., and CIACCIO, J.A. 1988. Alkylative epoxide rearrangement. Application to stereoselective synthesis of chiral pheromone epoxides. *Tetrahedron Lett.* 29:865–868.

BESTMANN, H.J. and VOSTROWSKY, O. 1982. Struktur und Vorkommen-Isolierung und Strukturaufklärung-Synthese-Biologische Aktivität und Verhaltensauslösung—Anwendung im Pflanzenschutz. *Naturwissenschaften* 69:457–471.

BOGENSCHÜTZ, H., KNAUF, W., TRÖGER, E.J., BESTMANN, H.J., and VOSTROWSKY, O. 1985. Pheromone 49: Freilandfänge von Geometriden-Männchen mit C_{19}-Polyenen in Kiefernbeständen. *Z. Angew. Entomol.* 100:349–354.

BRANDSMA, L. 1971. Preparative Acetylenic Chemistry, 1st ed. Elsevier, New York. 207 pp.

BROWN, C.A., and AHUJA, V.K. 1973. "P-2 nickel" catalyst with ethylenediamine, a novel system for highly stereospecific reduction of alkynes to *cis*-olefins. *J. Chem. Soc. Chem. Commun.* 1973:553–554.

BROWN, H.C. 1975. Organic Synthesis via Boranes. John Wiley & Sons, New York. pp. 105–107.

COREY, E.J., PYNE, S.G., and SU, W.-G. 1983. Total synthesis of leukotriene B_5. *Tetrahedron Lett.* 24:4883–4886.

DESCOINS, C. and FRÉROT, B. 1984. XVIIth International Symposium of Entomology, Hamburg, August 20–26, 1984.

DESCOINS, C., LALANNE-CASSOU, B., MALOSSE, C., and MILAT, M.-L. 1986. Analyse de la phéromone sexuelle émisé par la femelle vierge de *mocis latipes* (Guenée) (Noctuidae, Catocalinae de Guadeloupe, Antilles Francaises). *C.R. Acad. Sci. Paris* 302(Serie III, 13):509–512.

DUNCAN, D.B. 1955. Multiple range and multiple *F* tests. *Biometrics* 11:1–41.

EBATA, T., and MORI, K. 1989. Synthesis of both the enantiomers of (*Z*)-*cis*-9,10-epoxy-6-heneicosene. *Agric. Biol. Chem.* 53:801–804.

EITER, K., LIEB, F., DISSELNKÖTTER, H., and OEDIGER, H. 1978. Synthese hochungesättigter Fettsäuren und einiger Derivative; neue C-C-Verknüpfungsmethode zum Aufgbau von Vorstufen der biologischen Prostaglandin-Synthese. *Justus Liebigs Ann. Chem.* 00:658–674.

GAREGG, P.J., and SAMUELSSON, B. 1980. Novel reagent system for converting a hydroxy group into an iodo group in carbonhydrates with inversion of configuration. Part 2. *J. Chem. Soc. Perkin I* 1980:2866–2869.

GLEASON, J.G., BRYAN, D.B., and KINZIG, C.M. 1980. Convergent synthesis of leukotriene A methyl ester. *Tetrahedron Lett.* 21:1129–1132.

HANSON, R.M., and SHARPLESS, K.B. 1986. Procedure for the catalytic asymmetric epoxidation of allylic alcohols in the presence of molecular sieves. *J. Org. Chem.* 51:1922–1925.

JAIN, S.C., DUSSOURD, D.E., CONNOR, W.E., EISNER, T., GUERRERO, A., and MEINWALD, J. 1983. Polyene pheromone components from an arctiid moth (*Utethesa ornatrix*): Characterization and synthesis. *J. Org. Chem.* 48:2266–2270.

KATSUKI, T., and SHARPLESS, K.B. 1980. The first practical method for asymmetric epoxidation. *J. Am. Chem. Soc.* 102:5974–5976.

KOVALEV, B.G., and NIKOLAEVA, L.A. 1986. Attractiveness of some epoxy compounds for males of a tiger moth, *Phragmatodia fuliginosa* (Arctiidae), and of a geometrid moth, *Caustoloma* (*Therapis*) *flavicaria* (Geometridae). *Zool. Zh.* 65:802–804.

MCDONOUGH, L.M., BAILEY, J.B., HOFFMAN, M.P., LEONHARDT, B.A., BROWN, D.F., SMITHHISLER, C.L., and OLSEN, K. 1986. *Sabulodes caberata* Guenée (Lepidoptera: Geometridae). Components of its sex pheromone gland. *J. Chem. Ecol.* 12:2107–2116.

MILLAR, J.G., and UNDERHILL, E.W. 1986. Synthesis of chiral bishomoallylic epoxides, a new class of lepidopteran sex attractants. *J. Org. Chem.* 51:4726–4728.

MILLAR, J.G., UNDERHILL, E.W., GIBLIN, M., and BARTON, D. 1987. Sex pheromone components of three species of *Semiothisa* (Geometridae), (*Z,Z,Z*)-3-6,9-heptadecatriene and two monoepoxydiene analogs. *J. Chem. Ecol.* 13:1371–1383.

MILLAR, J.G., GIBLIN, M., BARTON, D., and UNDERHILL, E.W. 1990a. 3*Z*,6*Z*,9*Z*-nonadecatriene and enantiomers of 3*Z*,9*Z*-*cis*-6,7-epoxy-nonadecadiene as sex attractants of two geometrid and one noctuid moth species. *J. Chem. Ecol.* 16:2153–2166.

MILLAR, J.G., GIBLIN, M., BARTON, D., and UNDERHILL, E.W. 1990b. 3*Z*,6*Z*,9*Z*-Trienes and unsaturated epoxides as sex attractants for geometrid moths. *J. Chem. Ecol.* 16:2307–2316.

MILLAR, J.G., GIBLIN, M., BARTON, D., MORRISON, A., and UNDERHILL E.W. 1990c. Synthesis and field testing of enantiomers of 6*Z*,9*Z*-*cis*-3,4-epoxydienes as sex attractants for geometrid moths. Interactions of enantiomers and regioisomers. *J. Chem. Ecol.* 16:2317–2339.

MILLAR, J.G., GIBLIN, M., BARTON, D., REYNARD, D.A., NEILL, G.B., and UNDERHILL, E.W. 1990d. Identification and field testing of female-produced sex pheromone components of the spring cankerworm, *Paleacrita vernata* Peck (Lepidoptera: Geometridae). *J. Chem. Ecol.* 16:3393–3409.

MILLAR, J.G., GIBLIN, M., BARTON, D., and UNDERHILL, E.W. 1991a. Chiral lepidopteran sex attractants: Blends of optically active C_{20} and C_{21} diene epoxides as sex attractants for geometrid and noctuid moths. *Environ. Entomol.* In press.

MILLS, L.S., and NORTH, P.C. 1983. A short syhnthesis of a leukotriene B_4 synthon. *Tetrahedron Lett.* 24:409–410.

MORI, K., and EBATA, T. 1981. Synthesis of optically active pheromones with an epoxy ring. (+)-disparlure and the saltmarsh caterpillar moth pheromone, [(*Z,Z*)-3,6-*cis*-9,10-epoxyheneicosadiene]. *Tetrahedron Lett.* 22:4281–4282.

MORI, K., and EBATA, T. 1986. Synthesis of optically active pheromones with an epoxy ring, (+)-disparlure and both the enantiomers of (3*Z*,6*Z*)-*cis*-9,10-epoxy-3,6-heneicosadiene. *Tetrahedron* 42;3471–3478.

NICOLAOU, K.C., and WEBBER, S.E. 1985. A general strategy for the synthesis of the presumed lipoxin structures. *J. Chem. Soc. Chem. Commun.* 1985:297–298.

NICOLAOU, K.C., DUGGAN, M.E., and LADDUWAHETTY, T. 1984. Reactions of 2,3-epoxyhalides. Synthesis of optically active allylic alcohols and homoallylic epoxides. *Tetrahedron Lett.* 25:2069–2072.

OSBOND, J.M. 1961. Essential fatty acids. Part II. Synthesis of (±)-vernolic, linoleic, and γ-linolenic acid. *J. Chem. Soc.* 1961:5270–5275.

ROLLIN, P., and POUGNY, J.-R. 1986. Synthesis of 6*Z*-*cis*-9*S*,10*R*-epoxyheneicosene, a component of the ruby tiger moth pheromone. *Tetrahedron* 42:3479–3490.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry, 2nd ed. W.H. Freeman, New York.

SZÖCS, G., TÓTH, M, BESTMANN, H.J., and VOSTROWSKY, O. 1984. A two-component sex attractant for males of the geometrid moth *Alsophilia quadripunctata*. *Entomol. Exp. Appl.* 36:287–291.

SLESSOR, K.N., KING, G.G.S., MILLER, D.R., WINSTON, M.L., and CUTFORTH, T.L. 1985. Determination of chirality of alcohol or latent alcohol semiochemicals in individual insects. *J. Chem. Ecol.* 11:1659–1667.

UNDERHILL, E.W., PALANISWAMY, P., ABRAMS, S.R., BAILEY, B.K., STECK, W.F., and CHISHOLM, M.D. 1983. Triunsaturated hydrocarbons, sex pheromone components of *Caenurgina erechtea*. *J. Chem. Ecol.* 9:1413–1423.

WONG, J.W., UNDERHILL, E.W., MACKENZIE, S.L., and CHISHOLM, M.D. 1985. Field trapping and electroantennographic responses to triene hydrocarbons and monoepoxydiene derivatives. *J. Chem. Ecol.* 11:727–756.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

ATTRACTION OF NORTHERN FALSE CHINCH BUG *Nysius niger* (HETEROPTERA: LYGAEIDAE) TO MUSTARD OILS[1]

KENNETH A. PIVNICK,[2,*] DARWIN W. REED,[3] JOCELYN G. MILLAR,[3,4] and EDWARD W. UNDERHILL[3]

[2]*Agriculture Canada Research Station*
107 Science Crescent
Saskatoon, Saskatchewan S7N 0X2 Canada

[3]*Plant Biotechnology Institute*
National Research Council
Saskatoon, Saskatchewan S7N 0W9 Canada

(Received September 24, 1990; accepted January 24, 1991)

Abstract—Selected isothiocyanates (mustard oils) were tested as attractants for adult *Nysius niger* Baker, a pest of mustard crops in the Canadian prairies. Individuals of both sexes, but predominantly females, were caught in yellow boll-weevil traps baited with certain mustard oils. Initial testing was done with compounds loaded on rubber septa, a procedure that resulted in a great disparity in release rates between compounds due to differences in volatility. In subsequent experiments, glass tubes of varying dimensions were used so that release rates of each compound could be controlled and maintained at a constant rate. Of mustard oils tested, ethyl 4-isothiocyanatobutyrate, the corresponding methyl ester of which is found in seeds in the cruciferous genus *Erysimum*, was the most attractive. However, the methyl ester itself was either less attractive or not attractive at all. Allyl and *n*-propyl isothiocyanates were less attractive than ethyl 4-isothiocyanatobutyrate, and 2-phenylethyl isothiocyanate was not attractive. Insects were caught in traps almost exclusively when traps were in proximity to canola and mustard fields in bloom.

Key Words—Isothiocyanate, attractant, Cruciferae, host-plant location, Brassicaceae, ethyl 4-isothiocyanatobutyrate, *Nysius niger*, Heteroptera, Lygaeidae.

*To whom correspondence may be addressed.
[1] Agriculture Canada Research Station Manuscript No. 1031.
[4] Present address: Department of Entomology, University of California, Riverside, California 92521.

INTRODUCTION

The northern false chinch bug, *Nysius niger* Baker, formerly known as the false chinch bug, *N. ericae* (Schilling) (Ashlock, 1977), is a minor pest of a wide variety of crops throughout Canada with a preference for crucifers (Beirne, 1972; L. Burgess, personal communication). This pattern of host-plant preference is similar to that of the congeneric U.S. pest, *N. raphanus* Howard (Barnes, 1970). *N. niger* is a vector of the yeast, *Nematospora sinecauda* Holley, formerly known as *N. coryli* Peglion (Holley et al., 1984), which infects mustard crops, *Brassica juncea* (L.) Czern. and *Sinapis alba* L., in the Canadian prairie provinces (Burgess et al., 1983). In Saskatchewan, *N. niger* has two to three generations per year and overwinters in the egg stage (L. Burgess, personal communication). In a July 1985 insect trapping survey using isothiocyanates loaded on rubber septa as baits, appreciable numbers of *N. niger* were collected with two of the baits tested: a mixture (1 : 1) of ethyl and methyl 4-isothiocyanatobutyrate (ethyl–methyl ICB) and, to a lesser extent, 3-methylthiopropyl isothiocyanate (IC). Many insects also were found feeding on the vegetation near the trap baited with ethyl–methyl ICB. All other mustard oils tested, including allyl IC, *n*-butyl IC, ethyl IC, *p*-methoxybenzyl IC, 2-phenylethyl IC and phenyl IC, caught one or no insects (unpublished). Isothiocyanates, or mustard oils, are among the major volatile hydrolysis products of glucosinolates, a group of nonvolatile anions that are found predominantly in five dicotyledonous families including all cruciferous species (family Brassicaceae) Fenwick et al., 1983). Isothiocyanates are released when plant tissue is damaged (Underhill, 1980), and are known to be attractive to a number of crucifer-feeding insect species (Feeny et al., 1970; Matsumoto, 1970; Eckenrode and Arn, 1972; Free and Williams, 1978; Hawkes and Coaker, 1979; Finch and Skinner, 1982). In light of the potential usefulness of an attractant for *N. niger*, we decided to investigate this chemical attraction further.

METHODS AND MATERIALS

Trapping. In 1987, a trapping experiment was carried out using yellow boll-weevil traps (Pest Management Specialists, Inc., Starkville, Mississippi) as described by Burgess and Wiens (1980). The experiment was designed to distinguish between the response of *N. niger* to ethyl and methyl ICB and was arranged in a randomized complete block design with three replicates. Traps were placed in a line, 10 m apart alongside blooming mustard, *B. juncea*, or canola, *B. napus* L. and *B. campestris* L., plots, as this is where all insects were trapped in 1985. Traps were in place from June 16 to July 16 near Saskatoon and all insects caught were sexed. The treatments were baits containing 20 mg of: (1) ethyl ICB; (2) ethyl–methyl ICB mixture; (3) methyl ICB; and

(4) a solvent blank. Each compound was loaded on a rubber septum (No. 1780J07, Arthur H. Thomas Scientific, Philadelphia, Pennsylvania) and washed into the rubber with two drops of solvent (dichloromethane) and one drop of antioxidant (10% butylated hydroxytoluene in acetone). Two of the trap replicates were moved to new flowering *Brassica* plots on June 22 because the plots used initially had finished blooming.

To determine if differences in rates of release of compounds from rubber septa might have been the cause of the large differences in trap captures in response to different mustard oils in 1985, the following experiment was carried out. Release rates of allyl IC and ethyl ICB, loaded on rubber septa, were compared in the laboratory. Twenty milligrams of each compound were loaded on separate rubber septa ($n = 4$ replicates) and these were weighed daily for a week, then twice weekly for a month. Weight losses of less than 10 μg per week could not be detected, and, therefore, once release rates fell below this level, loss of compounds was measured by gas chromatography: septa contents dissolved in dichloromethane were compared to known quantities of standard. Release rates of the compounds were determined from the log-log linear regression. Simple decay curves were not obtained, probably because release rates were functions of both evaporation of the compounds and the physical interaction between the compounds and the rubber septa. All release rate measurements were calculated in terms of weight loss (micrograms per day).

In 1989, an additional trapping experiment was carried out using yellow boll-weevil traps to examine the specificity of response of *N. niger* to mustard oils. Traps were placed in a canola field near Saskatoon in a randomized complete block design, replicated four times. All insects caught were sexed. Traps were in place from July 10 to August 8 and were emptied weekly; baits were changed after two weeks. The treatments were: allyl IC, *n*-propyl IC, ethyl ICB, and 2-phenylethyl IC, at release rates of 400 μg/day and 4000 μg/day, and, for allyl IC only, 40,000 μg/day. Ethyl ICB was chosen for its demonstrated attractiveness, allyl and 2-phenylethyl IC for their widespread occurrence in crucifers, and *n*-propyl IC for its structural similarity to allyl IC. To produce the 40,000 μg/day release of allyl IC, the release device was a serum bottle with a pipe cleaner wick and a rubber septum top as described by Burgess and Wiens (1980). For other release rates, glass tubes, sealed at one end, were used. The tubes were filled with the desired compound so that a specified length of air column extended above the compound. The release rate for any given compound varied directly with the diameter of the tube and inversely with the air column length. The appropriate dimensions for each compound were arrived at by application of the mathematical model of Weatherston et al. (1985) and subsequent empirical testing. Over the course of the experiment, the release rates in the glass tubes did not change appreciably. The inside diameter and air column lengths for each bait releasing at 4000 μg/day were, respectively, 3.7

and 40 mm for allyl IC, 5.0 and 18 for ethyl ICB, and 6.5 and 6 for 2-phenylethyl IC. At 400 μg/day, they were 1.3 and 60 mm, 2.5 and 45, and 2.5 and 9, respectively. Glass tube dimensions for *n*-propyl IC were the same as for allyl IC, as their boiling points are almost identical: 153°C for *n*-propyl IC vs. 152°C for allyl IC. The length of the column of air above the compound in each tube was measured before and after the experiment to determine the actual rate of release. The capillary tubes were taped to stand upright inside the bases of the traps. Square-root transformed trap capture data were analyzed with a two-way ANOVA followed by a protected LSD test.

Compounds. 3-Methylthiopropyl IC and a 1 : 1 mixture of methyl ICB and ethyl ICB were previously synthesized in this laboratory (NRC) according to published methods (Kjaer et al., 1955; Kjaer and Gmelin, 1957). The mixture was intended to be a synthesis of methyl ICB, but GC-MS analysis revealed the presence of ethyl ICB. The individual syntheses of methyl ICB and ethyl ICB are described below. All other compounds were obtained from commercial sources.

Methyl ICB and ethyl ICB were individually synthesized by a modification of the methods of Kjaer and Gmelin (1957) and Kim and Yi (1986). [^{1}H]NMR spectra were taken on a Brucker Am-360-WB instrument at 360 MHz, in $CDCl_3$. Infrared spectra were recorded on a Perkin-Elmer 237B instrument, using films of neat material on NaCl plates. Mass spectra (EI, 70 eV) were recorded with a Finnigan 4000E GC-MS instrument, with an INCOS 2300 data system.

Ethyl 4-aminobutyrate hydrochloride was obtained from Aldrich Chemical Co. Methyl 4-aminobutyrate was synthesized as follows. HCl gas was bubbled into a water-bath cooled slurry of 4-aminobutyric acid (2.0 g, 19.4 mmol) in MeOH (25 ml). When all the solid had dissolved, the solution was cooled in an ice bath, and saturated with HCl gas. The resulting solution was warmed to 20°C, and stirred overnight. The solvent was evaporated, followed by sequential addition and evaporation of aliquots of MeOH and ether (25 ml) to remove traces of HCl. The residue was dissolved in warm MeOH (5 ml), and the product was precipitated as white crystals by slow addition of ether (25 ml). The crystals were filtered off, rinsed with ether, and air dried, giving a quantitative yield of methyl 4-aminobutyrate (3.0 g as the hydrochloride salt).

To prepare ethyl 4-isothiocyanatobutyrate, 1,1′-thiocarbonyldiimidazole (1.98 g, 90% pure, 20 mmol; Aldrich Chemical Co.) was added to a solution of ethyl 4-aminobutyrate hydrochloride (1.68 g, 20 mmol) in 20 ml CH_3CN at 0°C. The mixture was warmed to room temperature while stirring, giving a yellow solution, which subsequently precipitated imidazole by-products. The mixture was stirred at room temperature for 3 hr, water was added (5 ml), and stirring was continued for 30 min. The mixture then was concentrated on a rotary evaporator, and the residue partitioned between water and ether. The ether layer was washed with water and brine, dried (anhydrous Na_2SO_4), and

Kugelrohr distilled, bp ~80°C (0.05 mm Hg), giving 1.61 g (93%) of the isothiocyanate as an oil, 98% pure by capillary GC (DB-5, 20 m × .32 mm ID). NMR: δ 4.14 (quartet, 2H, J = 7.1 Hz; $CH_3C\underline{H_2}O$), 3.60 (t, 2H, J = 6.5 Hz; CH_2-N) 2.43 (t, 2H, J = 7.1 Hz; CH_2COOR), 1.99 (quintet, 2H, J ~ 6.8 Hz; $CH_2C\underline{H_2}CH_2$), 1.25 (t, 3H, J = 7.1 Hz; CH_3). IR: 2990 (m), 2945 (m), 2190 (s), 2105 (s), 1730 (s), 1180 (s), 1030 (m) cm^{-1}. MS m/z (relative abundance): 175 (2; M + 2), 173 (37: M^+), 129 (64), 128 (61), 127 (54), 100 (51), 88 (93), 85 (33), 70 (46), 61 (64), 60 (100), 45 (46).

The analogous methyl 4-isothiocyanatobutyrate was synthesized in similar fashion from methyl 4-aminobutyrate. The NMR and IR spectra were satisfactory. MS m/z: 161 (2; M + 2), 159 (43; M^+), 128 (23), 127 (35), 100 (25), 74 (100), 72 (65), 59 (68), 43 (85).

RESULTS

The results from the trapping experiment in 1987 indicated that the major attractive compound in the ethyl–methyl ICB mixture was ethyl ICB (Table 1).

Release rates of the two mustard oils, allyl IC and ethyl ICB, from rubber septa were very different (Table 2), as a result of differences in their volatility (see Table 3) and differential absorption by the rubber septa. The majority of allyl IC actually evaporated in the 24 hr between loading and the start of the test. On the other hand, 24 hr was required for 20 mg ethyl ICB to be absorbed into the rubber, during which time very little evaporated. At 24 hr after loading, only 5% of allyl IC remained on the septa, compared to 98.5% of the ethyl ICB. Following absorption into the rubber septa, ethyl ICB began to evaporate rapidly, presumably because of the much greater surface area available. How-

TABLE 1. MEAN NUMBER OF *Nysius niger* ADULTS CAUGHT IN YELLOW BOLL-WEEVIL TRAPS BAITED WITH MUSTARD OILS IN CANOLA PLOTS NEAR SASKATOON, JUNE 16–JULY 16, 1987

Compound (20 mg/rubber septum)[a]	Number of insects caught ($\bar{X}$ ± SE; N = 3)[b]	Females (%)
Ethyl ICB	64.3 ± 49.9a	86
Ethyl/methyl ICB	21.0 ± 14.8ab	79
Methyl ICB	2.3 ± 0.7b	100
Control	3.0 ± 2.0b	89

[a]ICB = 4-isothiocyanatobutyrate. More than 90% of insects were caught when release rates of both ethyl and methyl ICB were estimated at between 80 μg/day (day 15) and 15 μg/day (day 30).

[b]Means in columns followed by different letters are significantly different at P = 0.05.

TABLE 2. CALCULATED RELEASE RATES OF ISOTHIOCYANATES FROM RUBBER SEPTA

Day[a]	Release rates (μg/day)	
	Allyl isothiocyanate	Ethyl 4-isothiocyanatobutyrate
1	1250	7,500
8	2.0	250
15	.20	80
22	.05	30
29	.02	15

[a] Day 1 is considered the day after loading septa as it took 24 hr for ethyl ICB to soak into septa. During this initial 24 hr, only 1.5% of ethyl 4-isothiocyanatobutyrate evaporated. During the same period, 95% of allyl isothiocyanate evaporated.

ever, allyl IC still evaporated proportionately more rapidly for the next few days. The results were counterintuitive in that less (in absolute terms) of the more volatile compound (allyl IC) was released over the entire test (which did not include the 24 hr after loading). After one week, only about 9 μg of allyl IC remained.

The 1989 trapping experiment showed that ethyl ICB was the most attractive mustard oil tested based on trap catch (sexes pooled) (Table 3). In addition, this experiment demonstrated that at least some other mustard oils are also attractive at appropriate release rates. When allyl IC was released at 40,000 μg/day, its attractiveness was similar to that of ethyl ICB at between one tenth and one hundredth the rate. *N*-Propyl IC, which is similar in structure to allyl IC except for the lack of a double bond, was similar to allyl IC in attractiveness. 2-Phenylethyl IC was not attractive.

The trap catch increased initially in 1989 and remained at a plateau for the last two weeks of the trapping period while the proportion of females increased (Table 4).

DISCUSSION

N. niger is attracted to mustard oils with specific side-chain structures. Of the mustard oils tested, it is attracted to some strongly, to some weakly, and to others not at all. This was unexpected because *N. niger* is not a crucifer specialist, although a number of crucifer species are preferred food sources (Beirne, 1972; L. Burgess, personal communication). This study is the first to demonstrate mustard oil side-chain specificity in an insect species, although many insects are known to be attracted to mustard oils. Only three previous studies have compared attraction of insects to different mustard oils (Matsumoto, 1970;

TABLE 3. MEAN NUMBER OF *Nysius niger* ADULTS[a] CAPTURED IN YELLOW BOLL-WEEVIL TRAPS BAITED WITH MUSTARD OILS IN CANOLA FIELD NEAR SASKATOON, JULY 10–AUGUST 8, 1989

Compound[b]	Boiling point (°C at 1 atm.)	Release rates (μg/day) Estimated	Measured ($\bar{X} \pm$ SE; sample size in parentheses)	Number of insects caught per trap ($\bar{X} \pm$ SE; $N = 4$)[c]
Ethyl ICB	186[d,e]	4000	2700 ± 1500(4)	336 ± 92a
		400	130 ± 50(7)	220 ± 61abc
n-Propyl IC	153[f]	4000	3600 ± 400(7)	184 ± 52bcd
Allyl IC	152[f]	4000	5900 ± 200(4)	109 ± 23cde
		400	590 ± 30(8)	97 ± 28de
n-Propyl IC		400	350 ± 60(8)	44 ± 18ef
Control				16 ± 6f
2-Phenylethyl IC	236[e,g]	400	140 ± 60(8)	13 ± 5f
		4000	3600 ± 1200(3)	11 ± 5f
Allyl IC		40,000	68,000 ± 9,000(8)	248 ± 15ab

[a] Thirteen late-instar nymphs were also captured in the final week. These were not included in analyses.
[b] ICB = 4-isothiocyanatobutyrate; IC = isothiocyanate.
[c] Means followed by different letters are significantly different at $P = 0.05$
[d] Boiling point is for methyl 4-isothiocyanatobutyrate as reported in Kjaer and Gmelin (1957).
[e] Boiling point at 1 atm. calculated from boiling point at reduced pressure according to the method in Weast (1971).
[f] Reported in Weast (1971).
[g] Reported in Aldrich catalogue 1988–89, Aldrich Chemical Co., Milwaukee, Wisconsin.

TABLE 4. TOTAL NUMBER AND SEX RATIO OF *Nysius niger* ADULTS CAUGHT WEEKLY IN YELLOW BOLL-WEEVIL TRAPS BAITED WITH MUSTARD OILS IN CANOLA FIELD NEAR SASKATOON, JULY 10–AUGUST 8, 1989

Period	Total number caught	Females (%)
July 10–17	25	76
July 17–24	439	86
July 24–31	2129	96.5
July 31–August 8	1955	97.1

Finch and Skinner, 1982; Tuttle et al., 1988): the first in the vegetable weevil *Listroderes obliquus* (Klug), and the latter two in cabbage root flies, *Delia* spp. In these studies no clear preference patterns were demonstrated, and in none of them was the release rate well controlled, possibly resulting in problems similar to those encountered in our 1985 experiment (described in introduction).

The lack of attraction to 2-phenylethyl IC may be related to the feeding habits of *N. niger* as it feeds primarily on flower buds, flowers, seeds and seedlings. We infer these feeding preferences based on the prevalence of *N. niger* on flixweed *Descurainia sophia* (L.) in June in Saskatchewan when this plant is in flower and producing seed (L. Burgess, personal communication), the diet upon which they thrive in captivity (Burgess and Weegar, 1986), and by the fact that we could trap *N. niger* in mustard and canola plots and fields only when these had begun to bloom. In crucifers, these plant parts rarely produce much 2-phenylethyl IC, which is mainly found in roots and leaves (Kjaer, 1960; Sang et al., 1984).

The strong response to ethyl ICB and lack of response to the corresponding methyl ester is intriguing. Methyl ICB is present in seeds of three species of the cruciferous genus *Erysimum* (Kjaer and Gmelin, 1957), but ethyl ICB is not known to occur naturally. Comparisons of release rates of the two mustard oils from glass tubing (unpublished) do not indicate differences in evaporation rates that could explain the differences in capture of *N. niger* using the two mustard oils released from rubber septa. Ethyl ICB or a related compound may be found in a host plant or produced by *N. niger* itself, and this may explain its specific response. Alternatively, the compound may have no biological significance but has, by chance, a high affinity for certain *N. niger* chemoreceptors. This would be analogous to male lures for tephritid fruit flies (Cunningham et al., 1990). Our results do not indicate that methyl ICB is not attractive to *N. niger*; the insect may simply require higher release rates. The estimated rates of release during this experiment were low enough (see footnote in Table 1) that no mustard oil tested, other than ethyl ICB, would likely have elicited a response. The intermediate attraction to other mustard oils including allyl IC, *n*-propyl IC, and 3-methylthiopropyl IC is possibly an indication of the general preferences of *N. niger* for plants in the family Brassicaceae where these or closely related compounds are found (Fenwick et al., 1983).

N. niger was not caught in mustard-oil baited traps at all times. For example, in a trapping experiment in July 1985, five traps baited with five doses of ethyl–methyl ICB were placed beside plots of canola, wheat, and weeds. A total of 145 *N. niger* were caught in traps beside the canola plot while none were caught beside the other plots (unpublished). From June 22 to July 6, 1989, four traps, which released 40,000 μg/day of allyl IC, were placed alongside an alfalfa field that contained high densities of both flixweed and *N. niger* adults, yet none were caught. Two weeks later, in a nearby canola field, large numbers

of adults were attracted to the same kind of traps as this field came into bloom. Large numbers of adults continued to be caught in these traps for the next three weeks (Table 4). Other trapping experiments were also successful only when nearby canola or mustard was in bloom. The most likely explanation is that *N. niger* responded to the mustard oil baits only when they were actively host- or mate-seeking and in the vicinity of the traps. *N. niger* probably does not respond to mustard oils from very far away. The crucifer flea beetle, *Phyllotreta cruciferae* (Goeze), responds to 40,000 μg/day of allyl IC from less than 2 m (Pivnick, unpublished). *N. niger* probably move into canola fields once flowering begins, perhaps responding to the bright yellow flower color, the pungent floral odor, or both. Lygus bugs, *Lygus* spp., are also known to move into canola fields at this time (Butts, 1989) as they, like *N. niger* feed primarily on flowers, fruits, and seeds. This movement is likely also induced by the development of a new generation of *N. niger* adults in July (Pivnick, personal observation) at the time that the early season host plant, flixweed, is maturing and is no longer desirable. Dingle (1972) has summarized a wealth of data indicating that insects tend to increase flight activity early in adult life. Other lygaeid species are known to fly more under unfavorable local conditions particularly under food or mate deprivation (Dingle, 1966, 1968; Solbreck and Pehrson, 1979). Females but not males of the congeneric, *Nysius vinitor* Bergroth, have an increased tendency for sustained flight when starved as adults, particularly for the first few days after emergence (Kehat and Wyndham, 1974).

N. niger does form dense aggregations. We observed large numbers feeding on plants around the ethyl–methyl ICB trap in 1985. This aggregative behavior has been observed before in *N. niger* (Burgess et al., 1983) and in *N. raphanus* (Byers, 1973). We propose that the response to mustard oils is one method by which *N. niger* adults find new food sources, as high levels of mustard oils are released when plant tissue is damaged (Benn, 1977), which occurs when insects are feeding. Moreover, the relatively high specificity of response to mustard oils may allow *N. niger* to find preferentially the plant species and the phenological stages it prefers. Mustard oil response also may be important in producing the aggregations seen in this species, a behavior that likely contributes to its status as a disease vector. Whether the response to mustard oils is related to mating activity is not known, although clearly females were the primary respondents in our tests. The low incidence of males in trap catches may indicate a shorter adult life for males than females under field conditions. It also may reflect a mate location strategy, higher female vagility, or greater use of plants by females. The last possibility is supported by the finding that adult *N. groenlandicus* (Zett.) sampled on host plants are predominantly females even when the sex ratio of individuals found on the ground nearby is approximately equal (Böcher, 1972). In any case, our results indicate that *N. niger*, even though it is polyphagous, does make use of mustard oils in a discriminat-

ing manner that may help it to exploit specific food plants in the family Brassicaceae. Its response to mustard oils also makes possible the investigation of this insect by field trapping.

Acknowledgments—We thank the late Peter Ashlock, formerly of the Department of Entomology, University of Kansas, Lawrence, Kansas, for insect identification, and B.J. Jarvis, H. H. Weegar, and B. Pittendrigh for assistance with trapping. C.F. Hinks and J.J. Soroka reviewed an earlier version of this manuscript.

REFERENCES

ASHLOCK, P.D. 1977. New records and name changes of North American Lygaeidae (Hemiptera: Heteroptera: Lygaeidae.) *Proc. Entomol. Soc. Wash.* 79:575–582.

BARNES, M.M. 1970. Genesis of a pest: *Nysius raphanus* and *Sisymbrium irio* in vineyards. *J. Econ. Entomol.* 63:1462–1463.

BEIRNE, B.P. 1972. Pest insects of annual crop plants in Canada. IV. Hemiptera-Homoptera. *Mem. Entomol. Soc. Can.* 85:1–37.

BENN M. 1977. Glucosinolates. *Pure Appl. Chem.* 49:197–210.

BÖCHER, J. 1972. Feeding biology of *Nysius groenlandicus* (Zett.) (Heteroptera: Lygaeidae) in Greenland. *Medd. Gronl.* 191:3–41.

BURGESS, L., DUECK, J., and MCKENZIE, D.L. 1983. Insect vectors of the yeast *Nematospora coryli* in mustard, *Brassica juncea*, crops in southern Saskatchewan. *Can. Entomol.* 115:25–30.

BURGESS, L., and WEEGAR, H.H. 1986. A method for rearing *Nysius ericae* (Hemiptera: Lygaeidae), the false chinch bug. *Can. Entomol.* 118:1059–1061.

BURGESS, L., and WIENS, J.E. 1980. Dispensing allyl isothiocyanate as an attractant for trapping crucifer-feeding flea beetles. *Can. Entomol.* 112:93–97.

BUTTS, R.A. 1989. Biology and damage assessments of *Lygus* spp. infesting oilseed *Brassica* grown in Alberta. PhD thesis. University of Manitoba, Winnipeg, Canada.

BYERS, G.W. 1973. A mating aggregation of *Nysius raphanus*. *J. Kans. Entomol. Soc.* 46:281–282.

CUNNINGHAM, R.T., KOBAYASHI, R.M., and MIYASHITA, D.H. 1990. The male lures of tephritid fruit flies, pp. 255–267, *in* R.L. Ridgway, R.M. Silverstein, and M.N. Inscoe (eds.). Behavior-Modifying Chemicals for Insect Management, Marcel Decker, New York.

DINGLE, H.H. 1966. Some factors affecting flight activity in individual milkweed bugs (*Oncopeltus*). *J. Exp. Biol.* 44:335–344.

DINGLE, H.H. 1968. The influence of environment and heredity on flight activity in the milkweed bug (*Oncopeltus*). *J. Exp. Biol* 48:175–184.

DINGLE, H.H. 1972. Migration strategies of insects. *Science* 175:1327–1335.

ECKENRODE, C.J., and ARN, H. 1972. Trapping cabbage maggots with plant bait and allyl isothiocyanate. *J. Econ. Entomol.* 65:1343–1345.

FEENY, P., PAAUWE, K.L., and DEMONG, N.J. 1970. Flea beetles and mustard oils: host plant specificity of *Phyllotreta cruciferae* and *P. striolata* adults (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 63:832–841.

FENWICK, G.R., HEANEY, R.K., and MULLIN, W.J. 1983. Glucosinolates and their breakdown products in food and food plants. *CRC Crit. Rev. Food Chem. Nutr.* 18:123–201.

FINCH, S., and SKINNER, G. 1982. Trapping cabbage root flies in traps baited with plant extracts and with natural and synthetic isothiocyanates. *Entomol. Exp. Appl.* 31:133–139.

FREE, J.B., and WILLIAMS, I.H. 1978. The responses of the pollen beetle, *Meligethes aeneus*, and the seed weevil, *Ceuthorhynchus assimilis* to oil-seed rape, *Brassica napus*, and other plants. *J. Appl. Ecol.* 15:761–774.

HAWKES, C., and COAKER, T.H. 1979. Factors affecting the behavioural responses of the adult cabbage root fly, *Delia brassicae*, to host plant odour. *Entomol. Exp. Appl.* 25:45–58.

HOLLEY, R.A., ALLAN-WOJTAS, P., and PHIPPS-TODD, B.E. 1984. *Nematospora sinecauda* sp. nov., a yeast pathogen of mustard seeds. *Antonie van Leeuwenhoek* 50:305–320.

KEHAT, M., and WYNDHAM, M. 1974. Differences in flight behavior of male and female *Nysius vinitor* Bergroth (Hemiptera: Lygaeidae). *J. Austr. Entomol. Soc.* 13:27–29.

KIM, S., and YI, K.Y. 1986. 1,1′-Thiocarbonyldi-2,2′-pyridone. A new useful reagent for functional group conversions under essentially neutral conditions. *J. Org. Chem.* 51:2613–2615.

KJAER, A. 1960. Naturally derived isothiocyanates (mustard oils) and their parent glucosides, pp. 122–176, *in* L. Zechmeister (ed.). Progress in the Chemistry of Organic Natural Products, Springer-Verlag, Vienna.

KJAER, A., and GMELIN, R. 1957. Isothiocyanates. XXV. Methyl 4-isothiocyanatobutyrate, a new mustard oil present as a glucoside (glucoerypestrin) in *Erysimum* species. *Acta Chem. Scand.* 11:577–578.

KJAR, A., GMELIN, R., and LARSEN, I. 1955. Isothiocyanates. XII. 3-Methylthiopropyl isothiocyanate (ibervin), a new naturally occurring mustard oil. *Acta Chem. Scand.* 9:1143–1147.

MATSUMOTO, Y. 1970. Volatile organic sulfur compounds as insect attractants with special reference to host selection, pp. 133–160, *in* D.L. Wood, R. Silverstein, and M. Nakajima (eds.) Control of Insect Behavior by Natural Products. Academic Press, New York.

SANG, J.P., MINCHINTON, I.R., JOHNSTONE, P.K., and TRUSCOTT, R.J.W. 1984. Glucosinolate profiles in the seed, root and leaf tissue of cabbage, mustard, rapeseed, radish and swede. *Can. J. Plant Sci.* 64:77–93.

SOLBRECK, C., and PEHRSON, I. 1979. Relations between environment, migration and reproduction in a seed bug, *Neacoryphus bicrucis* (Say) (Heteroptera: Lygaeidae). *Oecologia* 43;51–62.

TUTTLE, A.F., FERRO, D.N., and IDOINE, K. 1988. Role of visual and olfactory stimuli in host finding of adult cabbage root flies, *Delia radicum*. *Entomol. Exp. Appl.* 47:37–44.

UNDERHILL, E.W. 1980. Glucosinolates, pp. 493–511, *in* E.A. Bell, and B.V. Charlwood (eds.). Secondary Plant Products, Encyclopedia of Plant Physiology, New Series, Vol. 8, Springer-Verlag, Berlin.

WEAST, R.C. (ed.). 1971. Handbook of Chemistry and Physics, 52nd ed. The Chemical Rubber Co., Cleveland, Ohio.

WEATHERSTON, I., MILLER, D., and DOHSE, L. 1985. Capillaries as controlled release devices for insect pheromone and other volatile substances—a re-evaluation. Part I. Kinetics and development of predictive model for glass capillaries. *J. Chem. Ecol.* 11:953–965.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

ISOLATION OF SUBSTANCE FROM SWEET POTATO (*Ipomoea batatas*) PERIDERM TISSUE THAT INHIBITS SEED GERMINATION

JOSEPH K. PETERSON* and HOWARD F. HARRISON, JR.

U.S.D.A., Agricultural Research Service
U.S. Vegetable Laboratory
2875 Savannah Highway
Charleston, South Carolina, 29414

(Received November 21, 1990; accepted January 28, 1991)

Abstract—Chromatographic procedures were used to isolate inhibitors of seed germination from sweet potato root periderm tissue. The inhibitory activity of all fractions was monitored using a proso millet seed germination bioassay. A single HPLC peak, representing approximately 1.2% of the periderm dry weight, accounted for most of the inhibitory activity. The active fraction was labile in methanolic solution. Further fractionation of this peak by HPLC methods was not successful. In vitro seed germination dose–response relationships were established for the peak. The various seed species exhibited an extremely wide range of sensitivity. The I_{50} values were 0.16, 0.013 and 0.011 mg/ml for redroot pigweed, velvetleaf, and proso millet, respectively. Tall morning glory was not inhibited by any concentration tested.

Key Words—Allelopathy, seed germination, inhibition, sweet potato, *Ipomoea batatas*, proso millet, *Panicum milliaceum*, velvetleaf, *Abutilon theophrasti*, tall morning glory, *Ipomoea purpurea*, redroot pigweed, *Amaranthus retroflexis*.

INTRODUCTION

Interest in allelopathy has kept pace with increasing public pressure against the use of environmentally damaging agrochemicals. In particular, potential weed suppression by crop plants deserves attention. A multitude of studies indicate that some degree of weed suppression can be expected from various crops or

*To whom correspondence should be addressed.

0098-0331/91/0500-0943$06.50/0

their residues. Among the cereals are corn (*Zea mays* L.; Guenzi and McCalla, 1966), oats (*Avena sativa* L.; Fay and Duke, 1977), rye (*Secale cereale* L.; Barnes and Putnam; 1983, 1986), and sorghum [*Sorghum bicolor* (L.) Moench; Alsaadawi et. al., 1986]. Dicotyledonous crops with allelopathic properties include cucumbers (*Cucumis sativus* L.; Putnam and Duke, 1974; Lockerman and Putnam, 1979, 1981), sunflower (*Helianthus annuus* L.; Leather, 1983; Hall et al., 1983) and soybean [*Glycine max* (L.) Merr.; Maun, 1977; Huber and Abney, 1986].

Sweet potatoes are known to contain allelopathic compounds. Taylorson (1967) reported the occurrence of a compound that inhibited the growth of cucumber hypocotyls. Growth of yellow nutsedge (*Cyperus esculentus* L.) and alfalfa (*Medicago sativa* L.) was significantly reduced when plants were grown in field soil that had supported sweet potatoes previously (Harrison and Peterson, 1986). Decaying sweet potato plant residues incorporated into the soil caused significant inhibition of growth of sweet potato vine cuttings and cowpea plants, and nodulation in cowpea was negligible (Walker and Jenkins, 1986). The uptake of Ca, Mg, and S was impaired under these conditions (Walker et al., 1989).

The related species, *Ipomoea aquatica* and *I. tricolor* have been shown to have allelopathic properties. *I. aquatica* contains terpenoid compounds that were inhibitory to pearl millet (*Pennisetum typhoideum* Rich.) seedling growth (Singhvi and Sharma, 1984). Anaya et al. (1990) isolated a jalapinolic acid (11-hydroxyhexadecanoic acid) glycosidic complex that is the most inhibitory chemical component of *I. tricolor* in seed germination and seedling growth bioassays.

Crude polar extracts of sweet potato periderm tissue, tested on nine weed species, inhibited germination differentially. I_{50} values (estimated concentrations at which 50% inhibition occurs) ranged from 0.1 mg periderm eq/ml for velvetleaf to 8.0 mg periderm eq/ml for pigweed (Peterson and Harrison, 1991). These observations led to attempts to isolate inhibitory substances from sweet potato periderm tissue.

METHODS AND MATERIALS

Sample Tissue. Sweet potatoes, cv. Regal, were harvested in the fall of 1989. Within a few days the roots were gently scrubbed under flowing water and the periderm tissue was carefully scraped off with a scalpel. This tissue easily separates from the rest of the storage root (Schalk et al., 1986). Only healthy, nondamaged roots were used to avoid interference by phytoalexins (Hyodo et al., 1969). The periderm tissue was dried at 50°C and subsequently frozen at −20°C until use.

Extractions. Sequential extractions were carried out on a shaker in the dark at ambient laboratory temperature. After the solvents were added (15 ml solvent per gram of dry tissue), they were degassed and blanketed with nitrogen. Each individual extraction lasted 24 hr. The extraction sequence was as follows: hexane (dry, four repetitions), methanol (dry, redistilled, four repetitions), 50% aqueous methanol (2 repetitions), and finally water (two repetitions). Extracts with the same solvents were combined and condensed under vacuum at 40°C.

Low-Pressure Column Chromatography. The methanol (100%) extract was applied to three borosilicate microfibrous filters at a rate of 1.0 g equivalent (extract obtained from 1.0 g periderm tissue). After drying, three filters were placed on top of a Sephadex LH-20 column[1] (4.5 × 14 cm in ethyl acetate) and 1 cm of the stationary phase was placed on top of the sample. A step-gradient elution was performed with mixtures of methanol–ethylacetate, 1:20 (625 ml, 25 ml/min, peak 1), 1:20 (450 ml, 25 ml/min, peak 2), 1:2 (475 ml, 20 ml/min, peak 3), 1:2 (750 ml, 20 ml/min, peak 4), 100% methanol (1500 ml, 20 ml/min, peak 5), and distilled water (1000 ml, 20 ml/min, peak 6). Elution was monitored at 280 nm. The six collected fractions were condensed under vacuum at 40°C and bioassayed (see Figure 2 below).

High-Pressure Liquid Chromatography. Fraction 3 from Sephadex LH-20 chromatography was condensed to dryness, redissolved in methanol, centrifuged, and resolved by HPLC. Column: Waters μ Bondapak 25 mm × 8 cm, C_{18}, 125 Å, 10 μm. Injection: 500 mg eq in 150 μl methanol. Detection: absorption at 230 nm. Solvents: water (solvent A) and methanol–acetonitrile, 50:50 (solvent B). Elution program: (1) 100% A → 56% A, 4 ml/min → 6 ml/min, 8 min. (2) isocratic, 12 min. (3) 56% A → 14% A, 6 ml/min, 4 min. (4) 14% A → 10% A, 6 ml/min, 6 min. (5) 10% A → 100% B, 6 ml/min → 8 ml/min, 8 min. (6) isocratic, 8 ml/min. Solvent and flow gradients were linear in all cases.

UV Absorption. Freshly isolated peak 3 from the HPLC was condensed to dryness, redissolved in redistilled methanol and its UV absorption spectrum determined against methanol with a Shimadzu UV-160 spectrometer.

Seed Sources. Proso millet, tall morning glory, and velvetleaf seeds were obtained from Valley Seed Service (Fresno, California) and redroot pigweed seeds were collected locally.

Bioassays. The crude extracts (hexane, methanol, 50% methanol and water, six fractions from the Sephadex LH-20 column, and three fractions from the HPLC) were tested for inhibition of proso millet germination. Fraction 3 from the HPLC was tested for dose–response based on its actual weight, using

[1] Sigma Chemical Co., St Louis, Missouri 63178. Mention of a propriety product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

proso millet, redroot pigweed, tall morning glory, and velvetleaf seed germination bioassays.

Seeds were germinated in Petri dishes with two Whatman No. 1 filter papers on the bottom. The filter papers were washed with methanol to remove inhibitory residues. Dilute extracts or chromatography fractions in the original solvents were added to Petri dishes, then the solvents were evaporated. All solvents used were pretested for residue effects; the methanol was redistilled. After adding water and seeds, Petri dishes were sealed in plastic and incubated. Controls were incubated in distilled, deionized water. A seed was considered to have germinated when the protruding radicle had achieved the length of the longest dimension of the seed. Seeds that required scarification were submerged in concentrated sulfuric acid. Subsequently the seeds were submerged in 2% $NaHCO_3$, then rinsed in tap water for 15 min and quickly blotted dry. The germination protocol is listed in Table 1.

Incubation periods were chosen so that nontreated seeds were approximately 50% germinated. All seeds were germinated at 35°C in the dark except proso-millet (30°C). These data were used to calculate the relative percent germination of all treatments. This approach allows detection of stimulatory effects as evidenced in the crude water extract. In order to determine the quality of the seeds, maximum germination (germinability) was also determined (Table 1).

Statistical Analyses. The bioassays were conducted with five replicates for each treatment, including the controls, and were arranged in a completely random design within the incubator. Each bioassay was repeated using fractions from separate extractions or chromatography fractions, and data from two repetitions of the experiments were combined for statistical analyses. Data from the various bioassays were converted to percent control germination, and treatment means and standard errors of the means were calculated. The extract concentration required to cause 50% inhibition of seed germination (I_{50}) was estimated using log-probit analyses after the method of Finney (1971).

TABLE 1. SEED GERMINATION PROTOCOLS[a] AND GERMINABILITY[b]

Species	Petri dish diameter (cm)	Water (ml)	Seeds per dish	Scarification (min)	Incub. period (hr)	Germinability (%)
Proso millet	10	5.0	100		28.5	89
Redroot pigweed	5	1.5	100		24.0	71
Tall morning glory	10	5.0	50	5	22.0	82
Velvetleaf	10	5.0	100	15	32.0	76

[a] Dishes were rotated throughout the incubator twice daily to ensure equal temperature exposure.
[b] Maximum germination obtained in water.

RESULTS AND DISCUSSION

Two notations are used to express concentrations, i.e., milligram equivalent per milliliter (mg eq/ml) and milligram per milliliter (mg/ml). The former indicates the amount of periderm extracted, the solute of which is dissolved in 1 ml solvent, and the latter represents the actual weight of extractable material per milliliter of solvent.

Of the four sequentially obtained extracts, the methanol extract (I_{50} = 0.3 mg eq/ml) and the aqueous methanol extract (I_{50} = 4.1 mg eq/ml) showed significant inhibition of germination (Figure 1). Since this study was designed to isolate and purify the major inhibitors of sweet potato, only the methanol fraction was further explored. Preliminary research (unpublished data) showed a large number of UV-absorbing compounds on TLC plates; however, most of the activity was lost during this process. A typical separation profile of the methanol fraction on a Sephadex LH-20 column using gradient elution with organic solvents is shown in Figure 2. Six fractions were collected and bioassayed. At their highest concentration (16 mg eq/ml), fractions 1 and 6 caused 42% and 20% inhibition, respectively, but their I_{50}s could not be calculated due to lack of dose-response. Fractions 2, 3, 4, and 5 produced I_{50} values of 32, 0.4, 9.5, and 14.7 mg eq/ml, respectively. The I_{50} for all fractions recombined

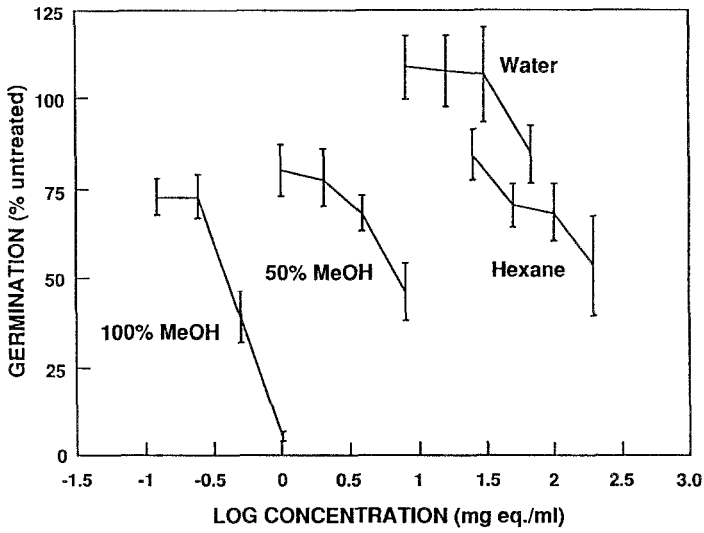


FIG. 1. Proso millet germination bioassays of crude extracts of sweet potato periderm tissue. The I_{50} values are 254, 0.3, and 4.1 mg eq/ml for the hexane, 100% methanol, and 50% methanol extracts, respectively. The I_{50} for the water extract could not be calculated due to the lack of dose-response.

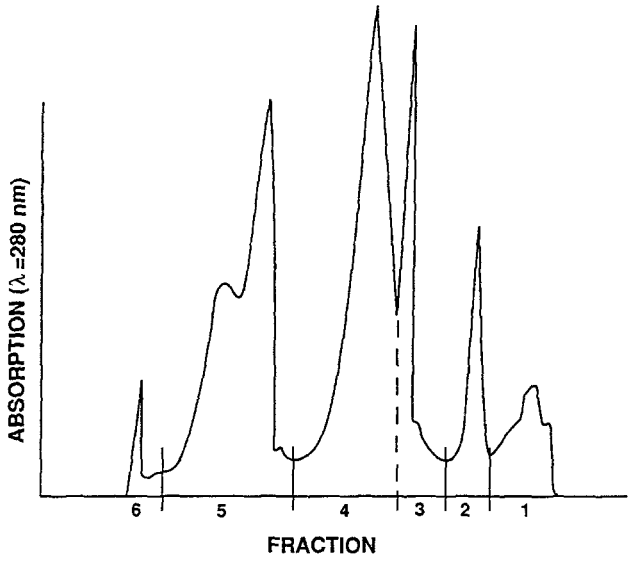


FIG. 2. Low-pressure column chromatography (Sephadex LH-20) of a crude 100% methanol extract.

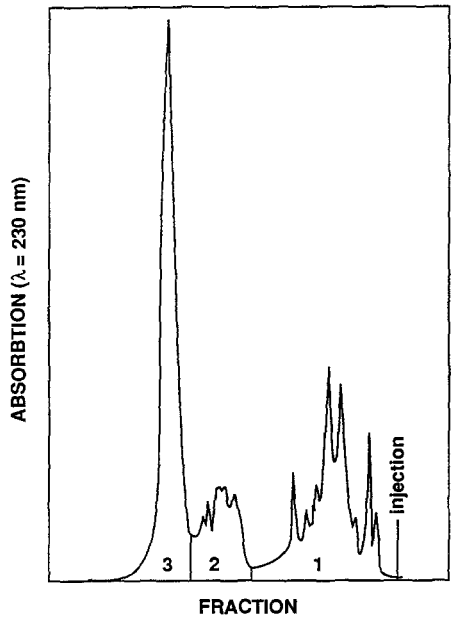


FIG. 3. HPLC record of gradient elution of Sephadex LH-20 column fraction 3, indicating the three collected fractions. Column: Waters μBondapak 25 mm × 10 cm, C_{18}, 125 Å, 10 μm. Injections: 500 mg eq in 150 μl methanol. Detection: absorption at 230 nm. Gradient: linear, H_2O-(MeOH–ACN, 50:50), 100% H_2O → 100% MeOH–ACN.

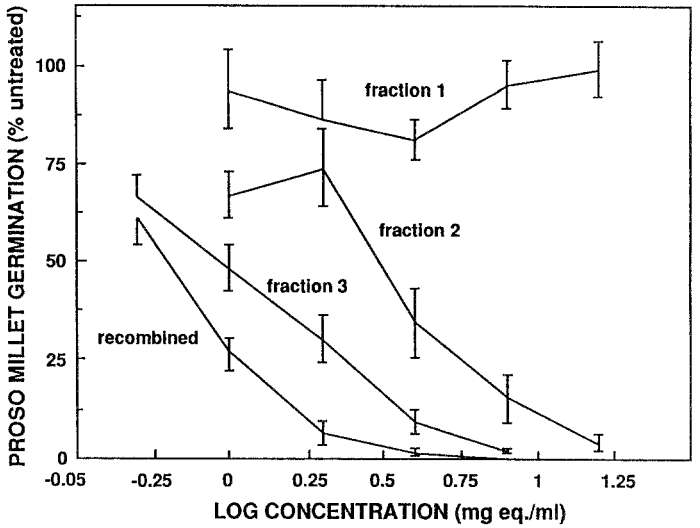


FIG. 4. Proso millet germination bioassays of the HPLC fractions and recombined fractions. The I_{50} values are 2.5, 0.9, and 0.6 mg eq/ml for fractions 2 and 3 and the recombined fractions, respectively. The I_{50} value for fraction 1 could not be calculated due to lack of dose–response.

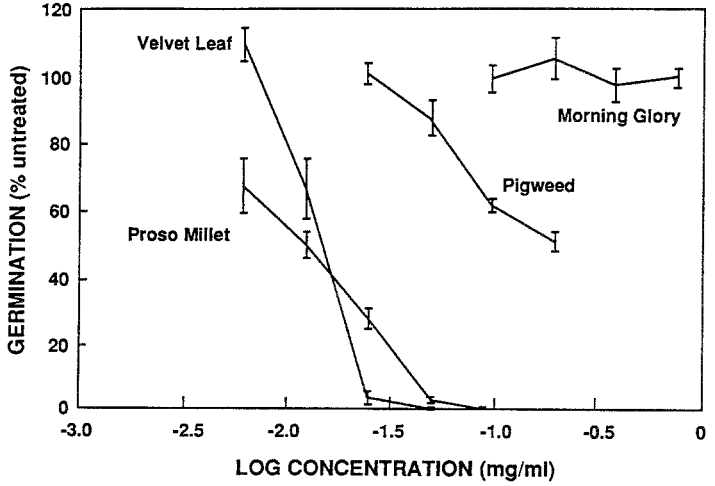


FIG. 5. Seed germination bioassays. The concentrations represent actual weights of HPLC fraction 3. The I_{50} values are 0.16, 0.013, and 0.011 mg/ml for redroot pigweed, velvetleaf, and proso millet, respectively. The I_{50} value for tall morning glory could not be calculated due to lack to dose–response.

was 0.3 mg eq/ml, similar to the 100% methanol extract, indicating that no significant losses had occurred.

Since fraction 3 from the Sephadex chromatography accounted for almost all of the inhibitory activity it was further separated into three fractions by HPLC (Figure 3). Most of the inhibitory activity was found in fraction 3 (Figure 4), which eluted as a single peak (Figure 3). Further attempts to resolve fraction 3 by HPLC or TLC were unsuccessful. The peak was monitored at two wavelengths, the absorption ratios (230/280) were almost constant across the peak. This indicates that the peak may be a pure compound or a mixture of isomers containing the same chromophores. The UV absorption spectrum showed a single maximum at 216 nm. HPLC peak 3, due to its high activity, was tested for dose–response relationships using seeds of four weed species (Figure 5). In this case, the actual weight of material present was used. The total recovery in this peak represented 1.2% of the periderm dry weight. The results of these bioassays showed large differences in sensitivity to the inhibitor(s) between species. Such large differences have been demonstrated previously with crude methanolic extracts (Peterson and Harrison, 1991). The I_{50} were 0.011, 0.013, and 0.16 mg/ml for proso millet, velvetleaf, and redroot pigweed, respectively; tall morning glory showed no response to the fraction. The strong weed-suppressing properties of the sweet potato clone, Regal, as previously observed in field and greenhouse (Harrison and Peterson, 1986, 1991) may be explained by the presence in the periderm of a single compound or a mixture of closely related isomers. The active principle was highly selective with respect to germination inhibition of various seeds and may have potential as a natural herbicide.

Acknowledgments—The authors wish to thank Ms. Naomi Kelly for her excellent technical assistance.

REFERENCES

ALSAADAWI, I.S., AL-UQAILI, J.K., ALRUBEEA, A.J.,and AL-HADITHY, S.M. 1986. Allelopathic suppression of weed and nitrification by selected cultivars of *Sorghum bicolor* (L.) Moench. *J. Chem. Ecol.* 12:209-219.

ANAYA, A.L., CALERA, M.R., MATA, R., and PEREDA-MIRANDA, R. 1990. Allelopathic potential of compounds isolated from *Ipomoea tricolor* Cav. (Convolvulaceae). *J. Chem. Ecol.* 16:2145-2152.

BARNES, J.P., and PUTMAN, A.R. 1983. Rye residues contribute to weed suppression in no-tillage cropping systems. *J. Chem. Ecol.* 9:1045-1057.

BARNES, J.P., and PUTMAN, A.R. 1986. Evidence for allelopathy by residues and aqueous extracts of rye (*Secale cereale*). *Weed Sci.* 34:384-390.

FAY, P.K., and DUKE, W.B. 1977. An assessment of allelopathic potential in *Avena* germplasm. *Weed Sci.* 25:244-228.

FINNEY, D.J. 1971. Probit Analysis. Cambridge Press, London, pp. 10-49.

GUENZI, W.D., and MCCALLA, T.M. 1966. Phenolic acids in oats, wheat, sorghum and corn residues and their phytotoxicity. *Agron. J.* 58:303-304.

HALL, A.B., BLUM, U., and FITES, R.C. 1983. Stress modification of allelopathy of *Helianthus annuus* L. debris on seedling biomass production of *Amaranthus retroflexus* L. *J. Chem. Ecol.* 9:1213–1222.

HARRISON, H.F, JR. and PETERSON, J.K. 1986. Allelopathic effects of sweetpotatoes (*Ipomoea batatas*) on yellow nutsedge (*Cyperus esculentus*) and alfalfa (*Medicago sativa*) *Weed Sci.* 34:623–627.

HARRISON, H.F., JR. and PETERSON, J.K. 1991. Evidence that sweetpotatoes (*Ipomoea batatas*) are allelopathic to yellow nutsedge (*Cyperus esculentus*). *Weed Sci.* (In press).

HUBER, D.M., and ABNEY, T.S. 1986. Soybean allelopathy and subsequent cropping. *J. Agron. Crop Sci.* 157:73–78.

HYODO, H., URITANI, I., and AKAI, S. 1969. Production of furanoterpenoids and other compounds in sweet potato root tissue in response to infection by various isolates of *Ceratocystis fimbriata. Phytopathol. Z.* 65:332–340.

LEATHER, G.R. 1983. Sunflowers (*Helianthus annuus*) are allelopathic to weeds. *Weed Sci.* 31:37–42.

LOCKERMAN, R.H., and PUTMAN, A.R. 1979. Evaluation of allelopathic cucumbers (*Cucumis sativus*) as an aid to weed control. *Weed Sci.* 27:54–57.

LOCKERMAN, R.H., and PUTMAN, A.R. 1981. Growth inhibitors in cucumber plants and seeds. *J. Am. Soc. Hortic. Sci.* 106:418–422.

MAUN, M.A. 1977. Suppressing effect of soybeans on barnyard grass. *Can. J. Plant Sci.* 57:485–490.

PETERSON, J.K., and HARRISON, H.F., JR. 1991. Differential inhibition of seed germination by extracts of sweetpotato (*Ipomoea batatas*) root periderm. *Weed Sci.* (In press).

PUTNAM, A.R., and DUKE, W.B. 1974. Biological suppression of weeds. Evidence for allelopathy in accessions of cucumber. *Science* 185:370–372.

SCHALK, J.M., PETERSON, J.K., JONES, P.D., and WALTER, W.M. 1986. The anatomy of sweetpotato periderm and its relationship to wireworm, *Diabrotica*, *Systena* resistance. *J. Agric. Entomol.* 3:350–356.

SINGHVI, N.R., and SHARMA, K.D. 1984. Allelopathic effects of *Ludwigia adscendens* Linn. and *I. aquatica* Forsk on seedling growth of pearl millet (*Pennisetum typhoideum Rich.*). *Transactions of the Indian Society of Desert Technology*, University Center for Desert Studies, Jodhpur, India, 9:95–100.

TAYLORSON, R.B. 1967. Some properties of a growth inhibitor in *Ipomoea. Proc. S. Weed. Sci. Soc.* 19:370.

WALKER, D.W., and JENKINS, D.D. 1986. Influence of sweetpotato plant residues on growth of sweetpotato vine cuttings and cowpea plants. *HortScience* 21:426–428.

WALKER, D.W., HUBBELL, T.J., and SEDBERRY, J.E. 1989. Influence of decaying sweet potato crop residues on nutrient uptake of sweetpotato plants. *Agric. Ecosyst. Environ.* 26:45–52.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

Heliothis virescens: RESPONSE CHARACTERISTICS OF RECEPTOR NEURONS IN SENSILLA TRICHODEA TYPE 1 AND TYPE 2

TOR J. ALMAAS and HANNA MUSTAPARTA*

Department of Zoology
University of Trondheim
AVH, 7055 Dragvoll, Norway

(Received August 17, 1990, accepted January 28, 1991)

Abstract—Partial electroantennograms (EAGs) and single cell recordings from *Heliothis virescens* males have demonstrated the presence of pheromones receptor neurons in sensilla trichodea type 2 as well as in type 1. This is supported by cobalt tracing experiments, showing that primary axons from the distal flagellum, containing only s. trichodea type 2, project into the macroglomerulus complex in the male antennal lobes. Four types of finely tuned pheromone receptor neurons were found in males, whereas in females the corresponding neurons responded mainly to host odors. In males the majority (75 and 18%, respectively) were tuned to the major *Heliothis virescens* pheromone components (Z)-11-hexadecenal (Z11-16:Al) and (Z)-9-tetradecenal (Z9-14:Al). The others (5 and 2%, respectively) responded specifically to (Z)-11-hexadecen-1-ol (Z11-16:OH) and (Z)-11-hexadecen-1-ol acetate (Z11-16:Ac). No neurons responding selectively to the minor pheromone components were found. The Z11-16:Al neurons of both sensilla types possessed similar specificity. However, the sensitivity decreased toward the medial and distal part of the flagellum, where s. trichodea type 2 are located. This suggests that the pheromone concentrations can be detected peripherally by a spatial as well as a temporal mechanism. Differences in temporal response patterns (pronounced phasic vs. tonic component) were found within the same type of neurons, suggesting different ability to encode intermittency of the pheromone plume as well as to mediate maintenance of flight.

Key Words—Electroantennogram, single-cell recordings, olfactory receptor

*To whom correspondence should be addressed.

0098-0331/91/0500-0953$06.50/0

cells, cobalt tracing, sex pheromones, (Z)-11-hexadecenal, (Z)-9-tetradecenal, host odors, *Heliothis virescens*, Lepidoptera, Noctuidae.

INTRODUCTION

Pheromone reception in the tobacco budworm moth *Heliothis virescens* (Lepidoptera: Noctuidae) has been studied previously by electrophysiological recordings from single cells of the male specific sensilla trichodea type 1 (Almaas and Mustaparta, 1990). It was found that the majority of receptor neurons of these sensilla are finely tuned to either one of the two major pheromone components: (Z)-11-hexadecenal (Z11–16:Al) (58%) and (Z)-9-tetradecenal (Z9–14:Al) (27%). In the natural ratio 16:1, these compounds are necessary and sufficient to elicit behavioral responses in males (Roelofs et al., 1974; Tumlinson et al., 1975). In addition to the receptor neurons tuned to the two major components, another type of receptor neuron was found that was tuned to the *Heliothis*-produced compound (Z)-11-hexadecen-1-ol (Z11–16:OH). Whether this alcohol has an intra- or interspecific function remains controversial, since it is present in the *H. virescens* female gland, but is not emitted (Teal et al., 1986). Furthermore, in wind-tunnel experiments it does not influence close-range precopulatory male behavior (Teal et al., 1986), but increases trap catches when added to a three-component blend of pheromone aldehydes (Ramaswamy et al., 1985). However, it is possible that Z11–16:OH disrupts orientation of *H. virescens* males, as in other species of *Heliothis* (cf. Heath et al., 1990).

Receptor neurons tuned to the minor components (Z)-9-hexadecenal (Z9–16:Al), (Z)-7-hexadecenal (Z7–16:Al), hexadecenal (16:Al), and tetradecenal (14:Al) (shown by Teal et al., 1986, to influence the close-range male behavior) were not identified in the male-specific s. trichodea type 1 (Almaas and Mustaparta, 1990). Furthermore, tests with mixtures did not reveal any influence by the minor components on the responses of receptor neurons tuned to the major compounds. However, the fact that minor components elicited some electroantennogram (EAG) response suggests that there might exist some receptor neurons responsive to minor components, perhaps in other sensilla. Therefore, the present study was undertaken to investigate the specificity of receptor neurons in s. trichodea type 2, which are located along the whole antenna of both males and females.

The aims of the present study were primarily (1) to find out whether the receptor neurons of the s. trichodea type 2 are involved in pheromone reception in males and, if they differ from those of females, (2) to determine the response characteristics of the neurons with respect to specificity, sensitivity, and temporal response patterns, and (3) to compare these characteristics with those of receptor neurons in s. trichodea type 1.

METHODS AND MATERIALS

Animals. Pupae of the tobacco budworm moth *Heliothis virescens* from a laboratory culture were kindly provided by Dr. Max Angst, Ciba-Geigy, Basel, Switzerland. The pupae were sex determined, and the sexes were kept separate in closed containers. After eclosion at room temperature, the moths were placed in containers marked with date of eclosion and fed a sucrose solution ad libitum. Most animals were used within four days posteclosion while some were not used until 6 days of age.

Morphology. A standard procedure for in vivo diffusion of cobalt chloride via cut axons was used (Altman and Tyrer, 1980; Obermayer and Strausfeld, 1980). A 5% cobalt chloride (Sigma) solution (aq.) was applied at the top of the cut end of the flagellum, allowing cobalt to migrate at room temperature (22°C) inside the cut axons along the antennal nerve to the glomeruli in the antennal lobe (Figure 1A). For comparison, the flagellum in three specimens was cut proximally (segment 35–45) and in four distally (segment 65–75). After two and three days, respectively, the brain was removed and transferred to a saline solution saturated with hydrogen disulfide for precipitation of cobalt sulfide. After 10 min, the tissue was rinsed in saline (10 min) and fixed (2 hr) in alcoholic Bouin fixative. The silver intensification method of Bacon and Altman (1977) for cobalt-filled neurons in whole-mount preparations was employed. After dehydration in ethanol, the brains were embedded in Durcupan (Fluka) and sectioned (20 μm) for light microscopy of cobalt sulfide precipitation in the antennal lobe. For control, the same procedure was applied to intact antennae.

Electrophysiological Recordings. The experimental procedure and the recording technique were previously described (Almaas and Mustaparta, 1990). The insect body and antenna were fixed in position using a Plexiglas holder and pieces of tape and wax. Tungsten wire hooks were used to prevent any movement of the antennae that could distort the electrodes positioned on the flagellum.

A standard technique was employed for recording the summated receptor potential of the electroantennogram (EAG) (Schneider, 1957; Kaissling, 1971). In males, partial EAG responses also were recorded from the proximal part, containing both s. trichodea type 1 and type 2 (flagellar segments 1–45), and from the distal part, containing s. trichodea type 2 but no type 1 (flagellar segments 46–81). This was carried out by covering the distal and proximal parts, respectively, with a domed sheet of wax, in a manner not touching the sensilla. The edge of the wax cover was gently pressed toward the wax platform, making a seal to prevent odors from reaching the covered sensilla. After recording responses from the proximal part, the wax was removed distally and repositioned proximally. In all experiments the partial EAG recordings were preceded by EAG recordings from the whole antenna. The EAG responses were mea-

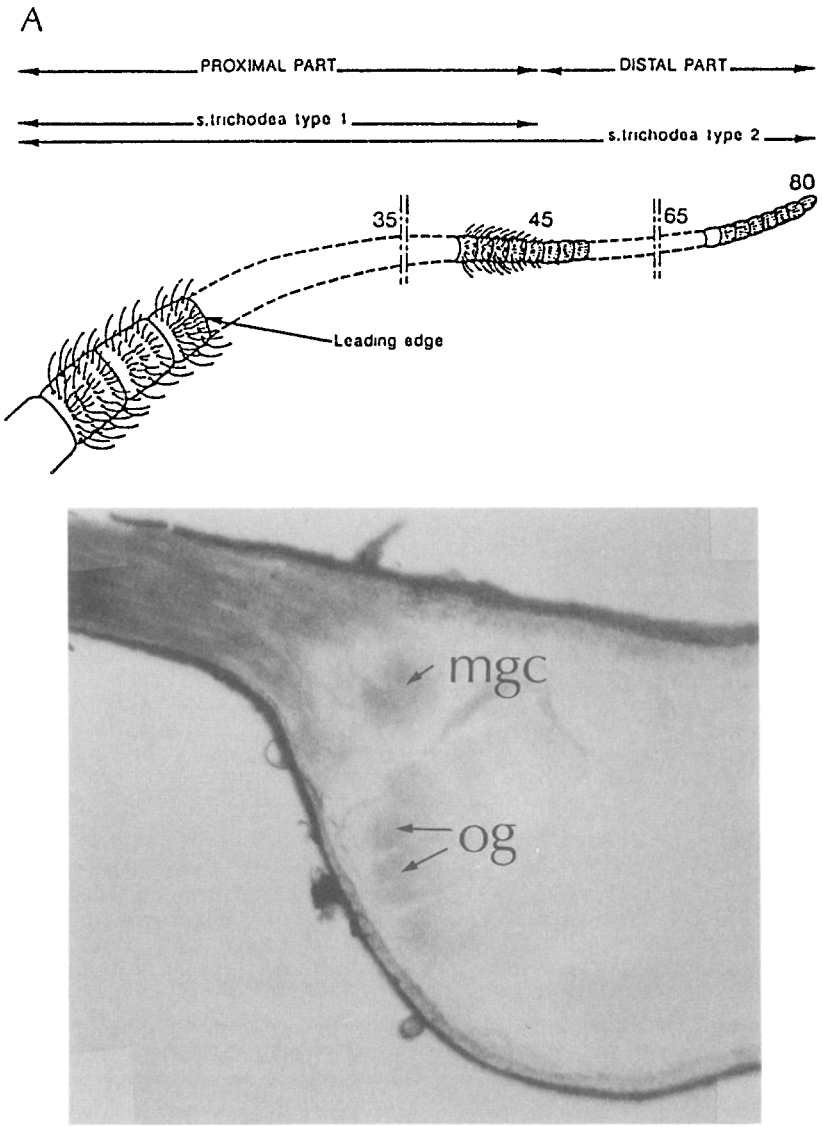


FIG. 1. (A) Schematic drawing of the flagellum in a *H. virescens* male, indicating the position of the s. trichodea type 1 on the proximal part and the s. trichodea type 2 on the distal part. The antenna was cut proximally (segment 35–45) and distally (segments 65–75), and the cut end was placed inside a vaseline cup allowing the cobalt to migrate inside the cut axons towards the antennal lobe. (B) Light microscopy micrograph showing silver-stained cobalt sulfide deposits in the antennal lobe of a *H. virescens* male. The flagellum was cut distally (segment 65). The deposits are restricted to the antennal nerve, the macroglomerulus complex (mgc), and a few ordinary glomeruli (og) close to the entrance of the antennal nerve. Magnification 170×.

sured as peak amplitude (mV) to each odor stimulus from which the response (ca. 1 mV) to the control stimulus was subtracted. All responses (both in males and females) are presented as percentage of the average response (3.2 mV) of male antennae to 100 μg of Z9–14 : Al.

For single cell recordings, tungsten microelectrodes were used (Boeckh, 1962; Mustaparta et al., 1979). Recordings were primarily made from s. trichodea type 2 on the medial side (the leading edge) of the proximal part, and from the medial and lateral sides of the distal part. Some recordings also were made from the longest sexually dimorphic s. trichodea type 1 in order to compare the responses from the two sensilla types when using the same stimulation cartridges. In the females, most recordings originated from the medial side.

The spontaneous activity was 0–10 impulses/0.5 sec and the spike amplitude varied from 30 to 200 μV. The spontaneous activity was not subtracted from the response values. The recordings often displayed impulse activity from more than one neuron. Only cells that were unambiguously identifiable from their spike characteristics were used for further analysis.

Stimuli. The synthetic pheromone compounds Z11–16:Al, Z9–14:Al, Z9–16 : Al, Z7–16 : Al, 16 : Al, and Z11–16 : OH were kindly provided by Dr. Peter Beevor, Natural Research Institute, Kent, England. The purity (in excess of 99%) was rechecked before use by gas–liquid chromatography (GLC) (cf., Almaas and Mustaparta, 1990). Other compounds used were the acetate analogs (Z)-11-hexadecen-1-ol acetate (Z11–16 : Ac), (Z)-9-hexadecen-1-ol acetate (Z9–16 : Ac), and (Z)-7-hexadecen-1-ol acetate (Z7–16 : Ac) (Shin-Etsu Chemical Co., Japan), which are pheromone components in *H. subflexa*. Several synthetic compounds known to be common in plants were also tested: (Z)-3-hexen-1-ol, (Z)-3-hexenyl acetate (Sigma), (E)-2-hexen-1-ol, (E)-2-hexenal, 1-hexanol (Aldrich Chem. Co.), and benzaldehyde (Merck). Other "green odor" stimuli employed were hexane extracts and fresh materials of various host plants (leaves from cotton, tobacco, and corn) and of various fruits of nonhost plants (orange, apple, and banana), as well as fresh material from pot plants. As "green odor" stimuli in the EAG recordings, only fresh materials of nonhost plants were used. Furthermore, these recordings also included two mixtures, where the minor pheromone components were added to Z11–16 : Al and Z9–14 : Al, respectively. For details regarding preparation of stimuli, see Almaas and Mustaparta (1990). The compounds (50 mg) were solved in hexane (1 ml) and diluted in decade steps. The concentrations were checked by GLC, and 200 μl of each dilution was applied on pieces of filter paper, each placed inside a glass cartridge. Four or two series of each compound were made independently in order to check recording conditions and stimulus concentration. In addition to these test series, separate cartridges were used initially for screening the sensitivity of the receptor neurons to the various compounds. The cartridges were stored at $-20°C$ and used within three months. To prevent adaptation, a low concen-

tration (0.1–1.0 μg) was employed for screening. Host odors were used only for qualitative tests.

Stimulation. The stimuli were applied with a "syringe-olfactometer" (Kafka, 1970; Mustaparta et al., 1980). For details of the stimulus apparatus and stimulation procedure, see Almaas and Mustaparta (1990). The cartridges were fastened to the outlet of a clean 20-ml syringe that served to deliver the airflow (7 ml/0.5 sec) through the cartridge. The interstimulation intervals were 1 min for low stimulation intensities (below 1 μg amount in the cartridge) and 1–4 min at higher intensities. Application of air from control cartridges (hexane on filter paper) was regularly carried out during the experiments.

RESULTS

Morphology. In preparations where the flagellum was cut proximally, massive precipitation of cobalt sulfide was observed both in the antennal lobe macroglomerulus complex (MGC) (male-specific glomerulus particularly transmitting pheromone information; Christensen and Hildebrand, 1987; Christensen et al., 1989) and in the ordinary glomeruli (probably dealing with plant odor information; Masson and Mustaparta, 1990). Sections of the antennal lobe in a preparation for which the flagellum were cut distally at segment 65 showed definite cobalt sulfide precipitation in the MGC (Figure 1B), but less pronounced than in the preparations with a proximally cut flagellum. Furthermore, here the precipitate was much more restricted, i.e., confined to the MGC and a few ordinary glomeruli close to the entrance of the antennal nerve. No cobalt was found to migrate into the antennal lobe when using the same procedure with an intact antenna.

Electroantennograms. The summated receptor responses (EAGs) from the whole antenna, its proximal and distal parts respectively, were recorded in eight males of *H. virescens*. Responses to the five pheromone aldehydes and the alcohol analog were compared. Z11–16:Al and Z9–14:Al (major components) elicited considerably higher responses than Z9–16:Al (minor component) and Z11–16:OH (minor component or interspecific inhibitor), whereas Z7–16:Al and 16:Al (minor components) had the lowest effects. This range of effects, measured for the whole antenna, was the same also in the proximal and distal parts. The responses from the proximal part were in general higher than those from the distal part (Figure 2A–C).

The dose–response relationships for Z11–16:Al, Z9–14:Al, and Z11–16:OH are plotted in Figure 2A–C. The diagrams show augmented responses with increased concentrations for both the proximal and distal parts of the antenna. In general, the sum of responses from the two parts matched the responses recorded from the whole antenna. For Z11–16:Al (Figure 2A), the

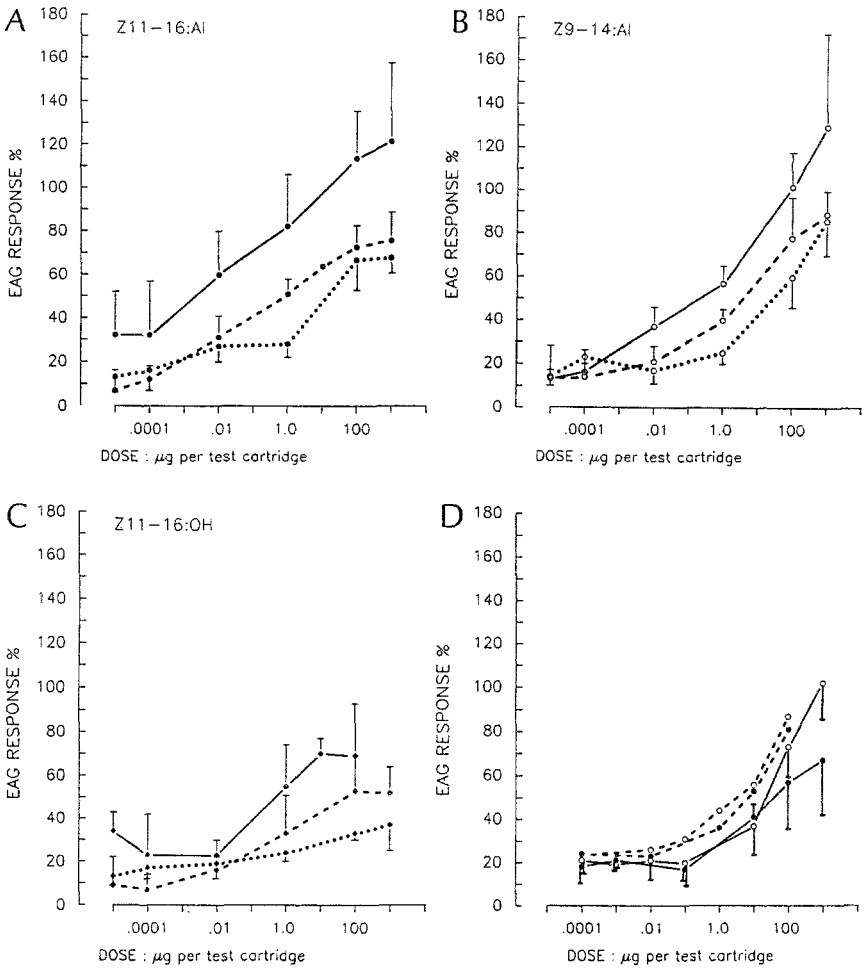


FIG. 2. Electroantennogram dose–response curves from six males (A–C) and seven females (D) of *H. virescens*. In males, the curves are shown for Z11–16:Al (A), Z9–14:Al (B), and Z11–16:OH (C), and recorded from the whole antenna (solid line), proximal part (broken line), and distal part (dotted line). In females (D) the curves are shown for Z11–16:Al (•), Z9–14:Al (○)and the mixtures with minor components (broken lines), recorded from the whole antenna. The data are presented as the mean values calculated as percentage of the average response of male antennae to 100 μg of Z9–14:Al. Standard deviation is indicated by vertical bars.

dose–response curve for the proximal part parallelled roughly (with a shift of 2–3 log units to the right) that of the whole antenna, whereas the curve of the distal part showed a steeper slope starting at a higher concentration (1.0–10 μg). For Z9–14 : Al (Figure 2B), the dose–response curves for the proximal and distal parts were shifted 1–2 and 2–3 log units, respectively. Similar dose–response relationships of the different parts were obtained for Z9–16 : Al and Z11–16 : OH. For the alcohol (Figure 2C), as for Z9–16 : Al, all responses were lower and the dose–response curves less steep.

EAG responses to the pheromones and fresh plant materials were recorded from seven female antennae. Here, responses only from the whole antenna were compared, since s. trichodea type 1 is not present in females. As shown in Figure 2D, the dose–response curves for the major pheromone Z11–16 : Al and Z9–14 : Al were shifted about 4 and 2 log units, respectively, to the right compared to those of the male antennae. When adding minor components, the responses showed a slight increase.

Among the odors from plant material tested qualitatively only, that of orange peel elicited the strongest responses both in males and females; these were even higher than the maximum male response recorded for pheromones. In males, the orange peel response was higher in the distal part than in the proximal part. The mean values for orange peel were 150% in the whole male antenna and 130% in the female antenna (calculated as percentage of the male response to 100 μg Z9–14 : Al). The proximal and distal flagellar responses in males were 77% and 102%, respectively.

Single-Cell Recordings. A total of 96 neurons from *H. virescens* males were investigated, of which 77 were characterized as olfactory receptor neurons. The other 19 neurons did not respond to any of the test odors or to air puffs. The olfactory neurons were divided into five groups, each specifically responsive to one of the following pheromone compounds: Z11-16:Al, Z9-14:Al, Z11–16 : OH, Z11–16 : Ac, or to "green odors." The number and distribution of these receptor neurons are shown in Table 1. The responses to 1.0 μg of *Heliothis* pheromone components from neurons tuned to Z11–16 : Al, Z9–14 : Al, Z11–16 : OH on the medial side of the proximal flagellum are demonstrated in Figure 3A–C, respectively. No receptor neurons selectively activated by the minor aldehydes of the conspecific pheromone blend were found in s. trichodea type 2.

In female *H. virescens* a total of 31 cells were studied, of which 17 could be classified as olfactory receptor neurons. These recordings originated mainly from the medial side of the proximal and distal flagellum. Thirteen of the neurons were classified as host odor cells whereas four responded better to a pheromone compound than to "green odor" stimuli.

Receptor Neurons Tuned to Z11–16 : Al. Twenty-seven male receptor neurons of s. trichodea type 2 were finely tuned to Z11–16 : Al (Table 1). In accord-

TABLE 1. CLASSIFICATION AND DISTRIBUTION OF RECEPTOR NEURONS IDENTIFIED ON THE FLAGELLUM OF *H. virescens* MALES[a]

		Proximal		Distal	
Compound	N	Lateral s.t. 1	Medial s.t. 2	Lateral s.t. 2	Medial s.t. 2
Z11-16:Al	41	14	16	5	6
Z9-14:Al	10	3	7	0	0
Z11-16:OH	3	0	1	2	0
Z11-16:Ac	1	0	0	1	0
Green odours	22	0	11	8	3
No modality	19	1	7	3	8

[a] s.t. = *sensilla trichodea*.

ance with type 1 receptor neurons recorded in the present and a previous study (Almaas and Mustaparta, 1990), responses to other pheromone components appeared only at higher concentrations (Figure 4A). Some of them (both type 1 and 2) responded to the extract of orange peel, eliciting a response corresponding to 0.1–1.0 μg Z11–16:Al. As in type 1, the type 2 receptor neurons responded second best to Z9–14:Al, which was only 1/100 as effective as Z11–16:Al (Figure 4B–D). However, different sensitivities between type 1 and 2 receptor neurons were observed. Both in the proximal and distal parts, the cells of s. trichodea type 2 showed lower sensitivities. Furthermore, their sensitivity decreased from proximal to more distal annuli as well as from the lateral to the medial side of the distal part (Figure 4 and 5). On the proximal part, it was only recorded from medially located s. trichodea type 2. The dose–response relationships of single receptor neurons are shown in Figure 4, where the dose–response curves of receptor neurons from s. trichodea type 2 (Figure 4B–D) are compared with that of one cell from s. trichodea type 1 (Figure 4A). The dose–response curves show a shift to the right from the lateral (Figure 4A) to the medial (Figure 4B) side of the proximal part, from the lateral (Figure 4C) to the medial side (Figure 4D) of the distal part, as well as from proximal to distal annuli. The average dose–response relationships of receptor neurons in these areas are shown in Figure 5, demonstrating the different sensitivities.

Variations of temporal response characteristics were found for receptor neurons of both s. trichodea type 1 and 2. Some neuron responses showed a pronounced phasic component with a fast decay, whereas others showed a more tonic component with slow decay and responses far outlasting the stimulation period. This is exemplified in Figure 6, showing temporal response characteristics for neurons of each response type from s. trichodea type 2 located at the

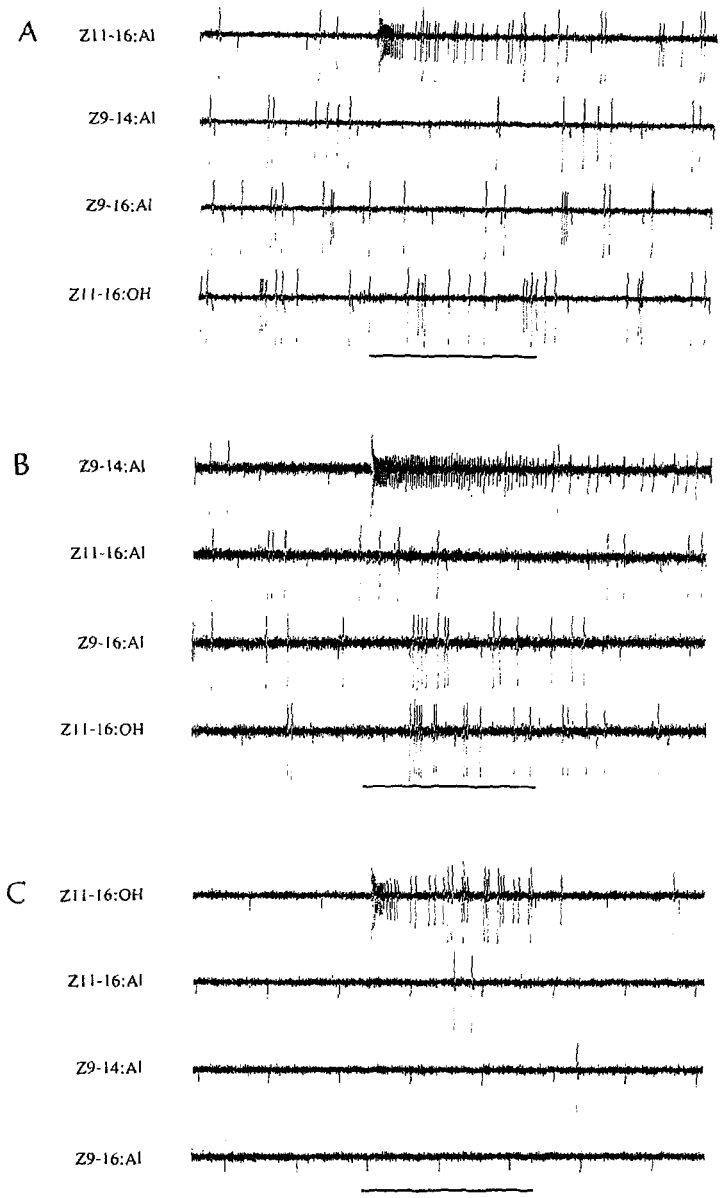


FIG. 3. Electrophysiological recordings of spike activity from single olfactory receptor cells tuned to Z11-16:Al (A), Z9-14:Al (B) and Z11-16:OH (C) in *H. virescens* males. All stimulations were carried out at the same concentration (1.0 μg). The stimulation period of 500 msec is indicated by horizontal bar below. Regular deflections in recordings are time markers.

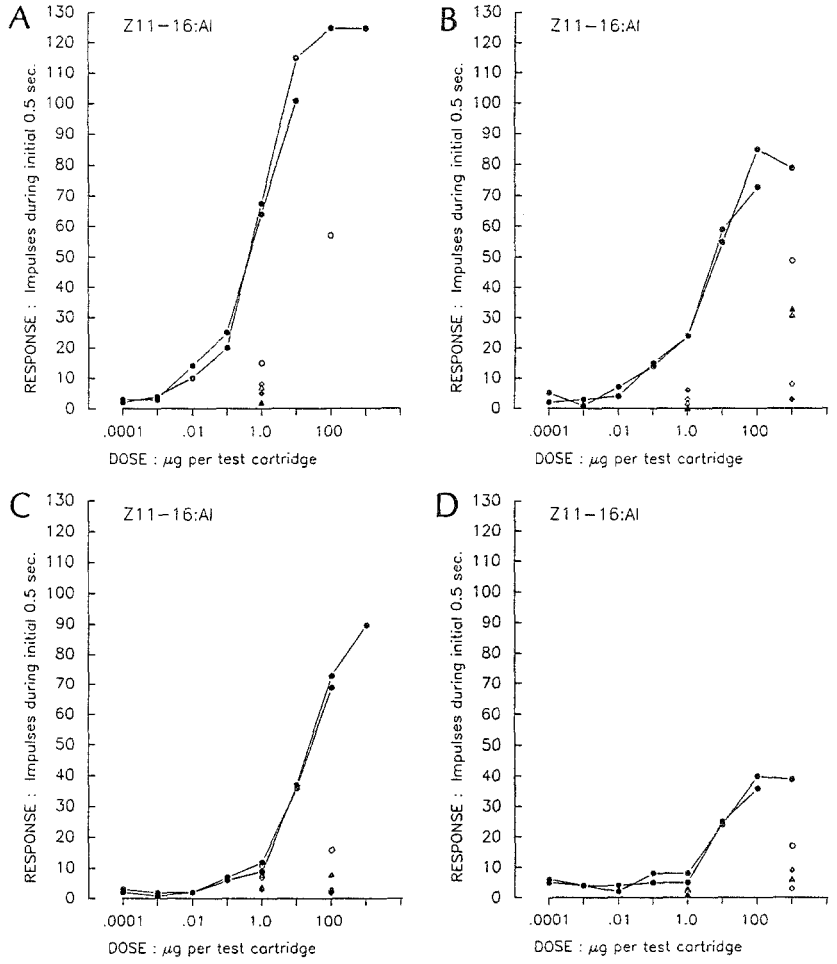


FIG. 4. Dose–response curves obtained for four receptor cells in male *H. virescens*, all tuned to the major pheromone compound Z11-16:Al. The duplicated curves represent independently performed test series of the key compound. The receptor neurons were positioned on the lateral and medial sides of the proximal part (A, B) and distal part (C, D) of the flagellum, respectively. Responses to the other test compounds are indicated by: ○ Z9-14:Al, ▲ Z9.16:Al, △ Z7-16:Al, ◇ 16:Al, ◆ Z11-16:OH. (B is the same neuron as demonstrated in Figure 3A).

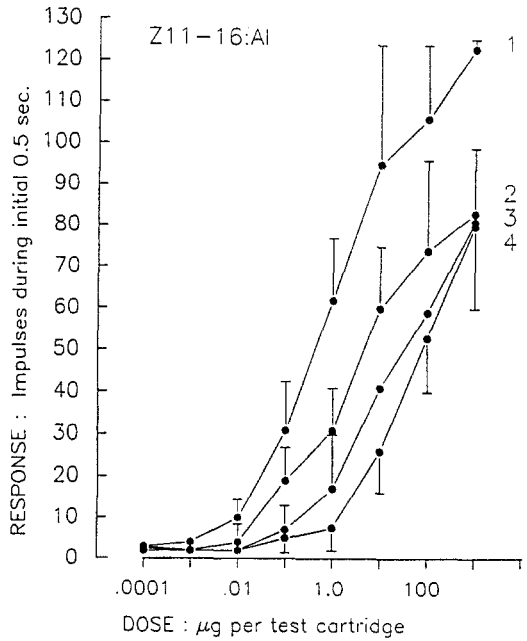


FIG. 5. Average dose-response curves for olfactory receptor neurons in *H. virescens* males, tuned to the major pheromone component Z11-16: Al. The curves represent neurons located laterally (line 1, $N = 7$) and medially (line 2, $N = 14$) on the proximal part; the laterally (line 3, $N = 5$) and medially (line 4, $N = 5$), on the distal part of the flagellum. Standard deviation is indicated by vertical bars, but omitted at the lowest concentrations for clarity.

medial side of the proximal part (A: phasic, B: tonic) and from s. trichodea type 1 located at the lateral side of the proximal part (C: phasic, D: tonic). The type of response patterns (phasic vs. tonic) was consistent at both low and high concentrations, as demonstrated in Figure 6A vs. B. Some neurons showed intermediate types of temporal response patterns.

The variability of temporal response characteristics did not correlate with sensitivity of the receptor neurons, i.e., neurons showing phasic responses did not necessarily show highest sensitivity. However, on the proximal part, more neurons of the phasic type were obtained from the lateral trichodea type 1 (10 of 14) than from the medial s. trichodea type 2 (six of 16) (Table 1). On the distal part, four of five neurons laterally and three of five medially (one not determined) were phasic.

Receptor Neurons Tuned to Z9-14:Al. Ten of the receptor neurons were finely tuned to Z9-14: Al (Table 1). Of these, three were located on the lateral (s. trichodea type 1) and seven on the medial side (s. trichodea type 2) of the

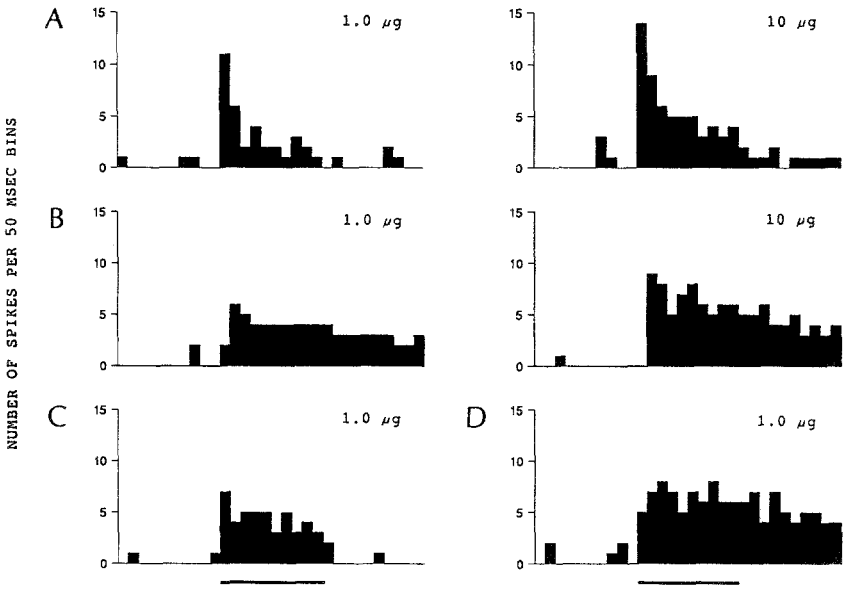


FIG. 6. Temporal response patterns of olfactory receptor neurons tuned to Z11–16:Al on the proximal part of the flagellum in *H. virescens* males. The figures demonstrate response patterns of a more pronounced phasic vs. tonic type, from receptor neurons on the medial side (A vs. B; s. trichodea type 2) and on the lateral side (C vs. D; s. trichodea type 1) . Stimulation period of 500 msec is indicated by horizontal bar below. Note the prolonged tail of discharge at the end of stimulus in the tonic type (B, D). Neurons in A and C are the same as in Figure 4B and A, respectively.

proximal part. Like the type 1 Z9–14:Al neurons previously studied (Almaas and Mustaparta, 1990), these cells had a low response to the other pheromone components at high concentrations (Figure 7) and to the extract of orange peel. In all neurons activated by this "green odor," the responses were approximately the same as for 0.1–1.0 μg of Z9–14:Al. Z9–16:Al was the second best stimulant, being 1/1000 to 1/10000 as effective as Z9–14:Al (Figure 3 and 7), which might either be an analog effect or due to impurities of Z9–14:Al.

The average dose–response relationships for four medially located Z9–14:Al receptor neurons (for which the full dose–response curve was recorded) are shown in Figure 7. The sensitivity of these neurons was not significantly different from that of the laterally located s. trichodea type 1 receptor neurons previously studied (Almaas and Mustaparta, 1990), as indicated by the dotted line in Figure 7. In the present study two of the laterally recorded Z9–14:Al neurons showed a markedly lower sensitivity. No receptor cells selective for Z9–14:Al were detected in the distal part.

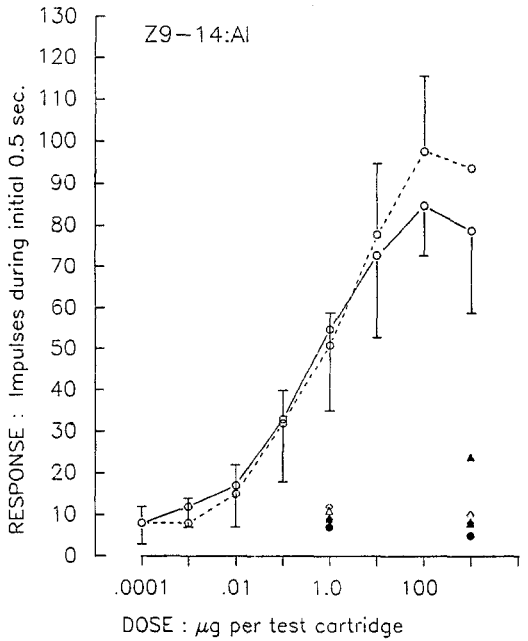


FIG. 7. Average dose–response curves from olfactory receptor neurons in *H. virescens* males, tuned to the major pheromone compound Z9–14 : Al. The recordings were obtained from s. trichodea type 2 on the medial side ($N = 4$, solid line) and from s. trichodea type 1 on the lateral side ($N = 4$, broken line) of the proximal part of the flagellum. Standard deviation is indicated by vertical bars. The average responses to other test compounds are indicated: • Z11-16:Al, ▲ Z9-16:Al, △ Z7-16:Al, ◇ 16:Al, ◆ Z11-16:OH.

The receptor neurons both of s. trichodea type 2 and 1 showed variations regarding phasic and tonic characteristics. This is demonstrated in Figure 8, showing the temporal characteristics of each type in medially and laterally located receptor neurons. The phasic component with a rapid decay and a fast resumption of spontaneous activity was observed for neurons of both sensilla types (Figure 8A and C). More pronounced tonic characteristics with slow decay and responses that far outlasted the stimulation period are demonstrated in Figure 8B and D. Figure 8A and B show that the type of response pattern (phasic or tonic) is consistent at both low and high concentrations.

Due to the relatively low number of Z9–14 : Al receptor neurons, no correlation could be made between temporal response pattern, sensitivity, and location.

Other Pheromone Components. Of the 77 olfactory receptor neurons identified in the present study, only four responded specifically to other pheromone

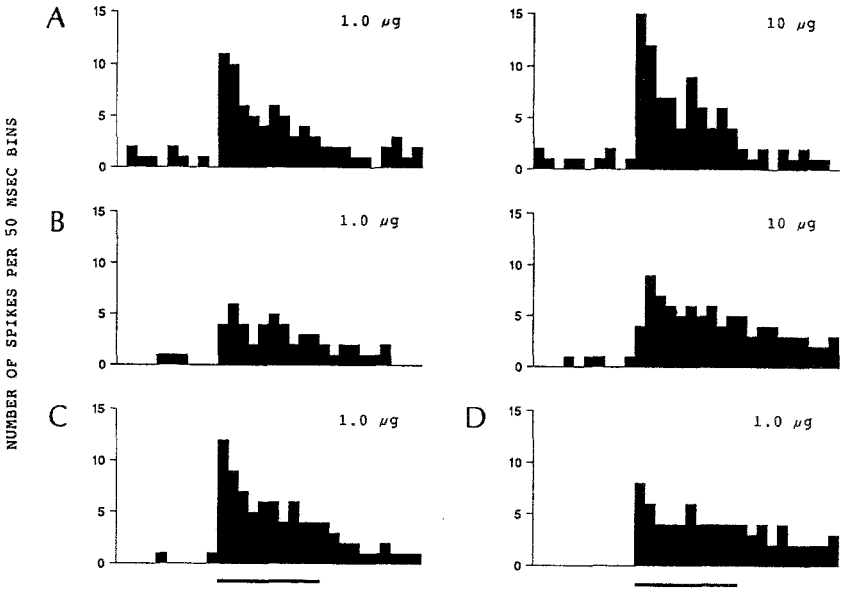


FIG. 8. Temporal response patterns of olfactory receptor neurons from the proximal part of the flagellum tuned to Z9-14:Al in *H. virescens* males. The figure demonstrates response patterns of a more pronounced phasic vs. tonic type, from receptor neurons on the medial side (A vs. B; s. trichodea type 2) and on the lateral side (C vs. D; s. trichodea type 1). Stimulation period of 500 msec is indicated by horizontal bar below. Note the prolonged tail of discharge at end of stimulation in the tonic type (B, D).

components (three to Z11-16:OH and one to Z11-16:Ac) of the genus *Heliothis* (Table 1). The Z11-16:OH neurons had a similar type of specificity and sensitivity. The dose-response relationships of an alcohol and an acetate receptor neuron are shown in Figure 9A and B. The sensitivity of these neurons was similar to the Z11-16:Al receptor neurons located in corresponding areas; i.e., medially on the proximal part (Figure 9A; Figure 5, line 2) and laterally on the distal part (Figure 9B; Figure 5, line 3). The acetate receptor neuron exhibited a pronounced tonic response with a very slow decay.

Host Odors. Twenty-two of the olfactory receptor neurons in males (Table 1) could be classified as "host odor detectors," i.e., they showed strongest response to various plant odors and no response to *Heliothis* pheromones.

In general, these units fired below 60 impulses per 0.5 sec when exposed to several of the "green odor" stimuli. Certain response groups could be distinguished: (1) Three neurons reacted to fresh materials of cotton, and two of these also responded to the cotton extract. (2) Five neurons were activated by the odors of tobacco and/or corn, but also responded to other "green odor"

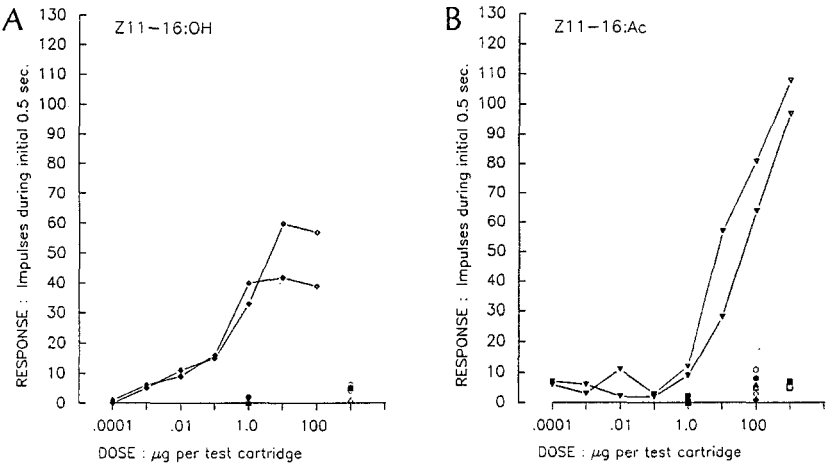


FIG. 9. Dose-response curves of two olfactory receptor neurons in male *H. virescens*, tuned to Z11-16:OH (A, same neuron as in Figure 3C) and Z11-16:Ac (B). The duplicated curves represent independently performed test series with the key compound. Responses to the other test compounds are indicated: • Z11-16:Al, ○ Z9-14:Al, ▲Z9-16:Al, △ Z7-16:Al, ◇16:Al, ◆ Z11-16:OH, ■ Z9-16:Ac, □ Z7-16:Ac.

stimuli. (3) One neuron was activated selectively by the synthetic alcohols. (4) Thirteen units responded best to extracts of fruit (primarily orange peel extract). Four of them also reacted to the synthetic plant volatiles, either the alcohols or the acetate. The synthetic compounds consistently elicited phasic responses, whereas the bouquet of natural plant odors elicited firing patterns that varied with the response strength and predominantly were tonic at high responses.

Receptor Neurons in H. virescens *Females.* In females, 13 of the 17 olfactory neurons studied responded to the "green odor" stimuli with minimal or no reaction to the pheromone components tested. The other four neurons, however, responded better to high concentrations (100 μg) of pheromone components than to any of the "green odor" stimuli. Compared to male pheromone receptor neurons in corresponding areas, their sensitivity was much lower. Furthermore, the specificity differed from that of the units in males by being less selective for one particular compound. Thus, two neurons responded to Z9-14:Al (50 and 52 impulses per 0.5 sec), Z11-16:Al (38 and 42 impulses), Z9-16:Al (30 and 36 impulses) and Z7-16:Al (21 and 19 impulses). The other two were activated by the acetates, one of them somewhat better to Z7-16:Ac (62 impulses, dose-response curve shifted 1 log unit to the left) than to Z11-16:Ac (30 impulses) and Z9-16:Ac (26 impulses). The 13 "green odor" units were divided between the same response groups as found in males (see above), and the response strength of these neurons was similar to that recorded in males.

One neuron was specific for fresh cotton leaves and cotton leaf extracts. Also in the female, the synthetic compounds elicited a phasic response pattern, whereas the responses to the bouquet of "green odors" predominantly were of the tonic type.

DISCUSSION

The observed specific responses of receptor neurons in s. trichodea type 2 indicate that these neurons are involved in pheromone reception. This is supported by the results of cobalt tracing, showing that primary axons from the distal flagellum project into the MGC of the male antennal lobes (Figure 1B). Furthermore, specificities of these receptor neurons were different in males and females. While finely tuned to one pheromone compound in males, they generally did not respond to *Heliothis* pheromone components in females. However, the finding of four units in *H. virescens* females responding to these compounds suggests that females might possess a few receptor neurons detecting *Heliothis* pheromones.

The similar specificity of the pheromone receptor neurons in male s. trichodea types 1 and 2 (Figure 4) suggests that these neurons posses functionally similar membrane receptor types. It is interesting to note that the type 2 receptor neurons tuned to Z11–16:Al exhibited a lower sensitivity than those of s. trichodea type 1. A further decrease of sensitivity occurred medially and distally (Figures 4 and 5). The variation in sensitivities between neurons may be due to several reasons, e.g., differences in filtering capacities of the sensilla, numbers of membrane receptors, or amplification in the transduction (features discussed by Kaissling, 1986). The specificity and sensitivity of the Z11–16:Al receptor neurons are reflected by the EAG responses of the proximal and the distal parts, since no other olfactory receptor neurons contribute by significant analog responses to Z11–16:Al. The stronger EAG response by the proximal part, which parallels the responses of the whole antenna (Figure 2A), can be explained by both a larger number and a higher sensitivity of the receptor neurons in this part of the antenna. The lower sensitivity of the Z11–16:Al receptor neurons on the distal part (Figures 4 and 5) is in accordance with the EAG dose–response curve (Figure 2A). Lower sensitivities of receptor neurons in shorter than in longer s. trichodea also have been observed in two *Antheraea* species (Meng et al., 1989). In the noctuid species *Trichoplusia ni*, however, a higher sensitivity was found for neurons in the shortest s. trichodea, which are located laterally and possess a higher number of pores (O Connell et al., 1983).

The large proportion of receptor neurons (Table 1) tuned to Z11–16:Al indicates that this compound might be detected at lower concentrations and thus

have a long-distance effect, whereas Z9–14 : Al, detected by fewer neurons, mediates the species-specific signal at a closer range. Furthermore, the presence of receptor neurons tuned to the same compound (Z11–16 : Al), but with a different sensitivity, suggests that the concentration (and thus the distance from the odor source) can be determined peripherally by a temporal as well as a spatial mechanism, i.e., by increased firing rates of equally sensitive neurons and by activation of additional neurons contributing to the antennal lobe information when the male approaches the pheromone source.

The present results do not reveal any different sensitivities for the Z9–14 : Al receptor neurons located laterally and medially on the proximal part of the flagellum. Only two lateral (present study) and three medial neurons (Almaas and Mustaparta, 1990) exhibited a lower sensitivity. It seems, therefore, that these neurons contribute less to the spatial mechanism of concentration detection. In the present and a previous study (Almaas and Mustaparta, 1990) a total of 24 Z9–14 : Al neurons and 71 Z11–16 : Al neurons were identified in *H. virescens* males (i.e., ratio of 1 : 3). This is consistent with the observed lower average EAG response to Z9–14 : Al than to Z11–16 : Al (Figure 2A and B). However, in EAG responses, the analog effect of Z9–14 : Al on Z11–16 : Al receptor neurons should not be neglected, since it would increase the EAG response to Z9–14 : Al.

In addition to the Z11–16 : Al and Z9–14 : Al receptor neurons, 10 Z11–16 : OH neurons (including seven units from Almaas and Mustaparta, 1990) and one Z11–16 : Ac neuron were found. It is possible that the two latter types mediate important interspecific messages. It is surprising that, of 106 receptor neurons identified, no neuron tuned to any minor pheromone compound was found. Furthermore, no host odor receptor neuron responded to either major or minor components. Since the minor components, added to the major pheromones did not influence any responses of the Z11–16 : Al and Z9–14 : Al receptor neurons (Almaas and Mustaparta, 1990), it is difficult to explain how the minor components are detected. Still they might exist in a very low number or in sensilla types not examined. It might be that the minor components are important only for some individuals of the population or that there are differences between lab-reared and wild *H. virescens* (Raina et al., 1989).

The temporal response patterns observed in *H. virescens* male receptor neurons are generally in accordance with previous results obtained in *H. virescens* males (Almaas et al., 1990). Both as regards the Z11–16 : Al and Z9–14 : Al neurons, phasic as well as tonic temporal response patterns were observed. As for *H. zea*, these differences probably can be correlated with the ability to follow pulsed stimuli; i.e., a phasic neuron follows the pulsed stimuli better than a tonic neuron and may, therefore, more easily encode the intermittency of the pheromone plume. While phasic and tonic responses for neurons tuned to the same compound were found in *H. virescens* and *H. zea* (Almaas

et al., 1990), these characteristics could be correlated to the specificity of the receptor neurons in *Antheraea* (Meng et al., 1989; Rumbo and Kaissling, 1989).

Responses to "green odors" were revealed in males and females both as results of the single cell and EAG recordings. Because of the markedly higher number of olfactory sensilla in females than in males (Almaas and Mustaparta, 1990) a higher female EAG response to "green odors" would be expected. However, an explanation for the similar responses seen here in the two sexes is that "green odors," e.g., orange peel (perhaps containing pheromone analog), also activate the numerous pheromone receptor neurons in males. The higher distal EAG response to the orange peel in males indicates that the largest numbers of host odor neurons are distally located in this sex.

In conclusion, the peripheral olfactory system in *H. virescens* males possesses receptor neurons that distinguish pheromone blends of conspecifics and other species by finely tuned receptor neurons located in two morphological types of s. trichodea. Concentrations of the blend can apparently be detected by a temporal as well as by a spatial mechanism. Furthermore, the *H. virescens* males have receptor neurons with a high ability to encode intermittency of the pheromone plume as well as neurons that might be of importance for long-term maintenance of flight.

Acknowledgments—The study was financially supported by the Norwegian Research Council for Sciences and Humanities (NAVF) (project No. 452.89/006). We are also grateful to Dr. Max Angst (Ciba-Geigy, Basel) for continuously supplying insect materials and to Dr. Peter Beevor (Natural Resource Institute, Kent) for providing pheromone components of high purity. For valuable help with the graphics, we thank Sturla Graabrœk and Jan Fredrik Børseth.

REFERENCES

ALMAAS, T.J., and MUSTAPARTA, H. 1990. Pheromone reception in tobacco budworm moth, *Heliothis virescens*. *J. Chem. Ecol.* 16:1331–1347.

ALMAAS, T.J., CHRISTENSEN, T.A., and MUSTAPARTA, H. 1990. Encoding of different features of an olfactory stimulus by the sex pheromone receptors in *Heliothis zea* (Lepidoptera: Noctuidae). *J. Comp. Physiol. A.* (Accepted)

ALTMAN, J.S., and TYRER, N.M. 1980. Filling selected neurons with cobalt through cut axons, pp. 377–405, *in* N.J. Strausfeld, and T.A. Miller (eds.). Neuroanatomical Techniques. Springer, Berlin. Springer Series in Experimental Entomology.

BACON, J.P., and ALTMAN, J.S. 1977. A silver intensification method for cobalt-filled neurons in wholemount preparations. *Brain Res.* 138:359–363.

BOECKH, J. 1962. Electrophysiologische Untersuchungen an einzelnen Geruchsrezeptoren auf den Antennen des Totengrabers (*Necrophorus*, Coleoptera). *Z. Vergl. Physiol.* 46:212–248.

CHRISTENSEN, T.A., and HILDEBRAND, J.G. 1987. Male-specific, sex-pheromone selective projection neurons in the antennal lobes of the moth *Manduca sexta*. *J. Comp. Physiol. A* 160:553–569.

CHRISTENSEN, T.A., MUSTAPARTA, H., and HILDEBRAND, J.G. 1989. Discrimination of sex pheromone blends in the olfactory system of the moth. *Chem. Senses* 14:463–477.

HEATH, R.R., MITCHELL, E.R., and TOVAR, J.C. 1990. Effect of release rate and ratio of (Z)-11-hexadecen-1-ol from synthetic pheromone blends on trap capture of *Heliothis subflexa* (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 16:1259–1268.

KAFKA, W.A. 1970. Molekulare Wechselwirkung bei der Erregung einzelner Riechzellen. *Z. Vergl. Physiol.* 70:105–143.

KAISSLING, K.-E. 1971. Insect olfaction, pp. 351–431, *in* L.M. Beidler (ed.). Chemical Senses Part 1. Olfaction. Handbook of Sensory Physiology, Vol. IV. Springer, Berlin.

KAISSLING, K.-E. 1986. Chemo-electrical transduction in insect olfactory receptors. *Annu. Rev. Neurosci.* 9:121–145.

MASSON, C., and MUSTAPARTA, H. 1990. Chemical information processing in the olfactory system of insects. *Physiol. Rev.* 70:199–245.

MENG, L.Z., WU, C.H., WICKLEIN, M., KAISSLING, K.-E., and BESTMANN, H.J. 1989. Number and sensitivity of three types of pheromone receptor cells in *Antheraea pernyi* and *A. polyphemus*. *J. Comp. Physiol. A* 165:139–146.

MUSTAPARTA, H., ANGST, M., and LANIER, G.N. 1979. Specialization of olfactory cells to insect- and host-produced volatiles in the bark beetle *Ips pini* (Say). *J. Chem. Ecol.* 5:109–123.

MUSTAPARTA, H., ANGST, M., and LANIER, G.N. 1980. Receptor discrimination of enantiomers of the aggregation pheromone ispdienol, in two species of *Ips*. *J. Chem. Ecol.* 6:689–701.

OBERMAYER, M., and STRAUSFELD, N.J. 1980. Silver-staining cobalt sulfide deposits within neurons of intact ganglia, pp. 406–430, *in* N.J. Strausfeld, and T.A. Miller, (eds.). Neuroanatomical Techniques. Springer Series in Experimental Entomology.

O'CONNELL, R.J., GRANT, A.J., MAYER, M.S., and MANKIN, R.W. 1983. Morphological correlates of differences in pheromone sensitivity in insect sensilla. *Science* 220:1408–1410.

RAINA, A.K., STADELBACHER, E.A., and RIDGWAY, R.L. 1989. Comparison of sex pheromone composition and pheromone-mediated male behavior of laboratory-reared and wild *Heliothis zea* (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 15:1259–1265.

RAMASWAMY, S.B., RANDLE, S.A., and MA WAI KEUNG. 1985. Field evaluation of the sex pheromone components of *Heliothis virescens* (Lepidoptera: Noctuidae) in cone traps. *Environ. Entomol.* 14:293–296.

ROELOFS, W.L., HILL, A.S., CARDÉ, R.T., and BAKER, T.C. 1974. Two sex pheromone components of the tobacco budworm moth, *Heliothis virescens*. *Life Sci.* 14:1555–1562.

RUMBO, E.R., and KAISSLING, K.-E. 1989. Temporal resolution of odour pulses of three types of pheromone receptor cells in *Antheraea polyphemus*. *J. Comp. Physiol. A* 165:281–291.

SCHNEIDER, D. 1957. Elektrophysiologische Untersuchungen von Chemound Mechanorezeptoren der Antenne des Seidenspinners *Bombyx mori* L. *Z. Vergl. Physiol.* 40:8–41.

TEAL, P.E.A., TUMLINSON, J.H., and HEATH, R.R. 1986. Chemical and behavioral analysis of volatile pheromone components released by calling *Heliothis virescens* (F) females (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 12:107–126.

TUMLINSON, J.H., HENDRICKS, P.E., MITCHELL, E.R., DOOLITLE, R.E., and BRENNAN, M.M. 1975. Isolation, identification and synthesis of the sex pheromone of the tobacco budworm. *J. Chem. Ecol.* 1:203–214.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

IMPACT OF UV RADIATION ON ACTIVITY OF LINEAR FURANOCOUMARINS AND *Bacillus thuringiensis* var. *kurstaki* AGAINST *Spodoptera exigua*:[1] IMPLICATIONS FOR TRITROPHIC INTERACTIONS

J.T. TRUMBLE,* W.J. MOAR,[2] M.J. BREWER and W.G. CARSON

Department of Entomology
University of California
Riverside, California 92521

(Received October 25, 1990; accepted January 28, 1991)

Abstract—Acidic fogs with a pH of 2.0 and duration of 2 hr did not reduce the efficacy of *Bacillus thuringiensis* var. *kurstaki* (Berliner). Therefore, the impact of UV radiation was investigated on the interactions between (1) levels of the antibacterial linear furanocoumarins psoralen, bergapten, and xanthotoxin in *Apium graveolens* (L.) occurring following a 2.0 pH acidic fog episode, (2) the noctuid *Spodoptera exigua* (Hübner), and (3) a sublethal dosage of the microbial pathogen *B. thuringiensis* var. *kurstaki*. Mean time to pupation in the absence of UV radiation (survival was too low to conduct this analysis for insects exposed to UV) was significantly extended by the addition of either psoralens or *B. thuringiensis*. Larvae developing on diets containing *B. thuringiensis* plus psoralens required nearly 40% longer to pupate than controls, but their effects were additive as the interaction was not significant. Although the mean times to adult emergence were significantly different, time spent in the pupal stage did not vary significantly between treatments, indicating that increases in larval developmental time were responsible for the observed decrease in developmental rate. Mean time to mortality, a weighted average time of death, was not significantly affected by any of the treatments. In a $2 \times 2 \times 2$ factorial analysis, all main effects (linear furanocoumarins. *B. thuringiensis*, UV radiation) reduced survival significantly, as did the three-way interaction. Thus, antagonistic interactions with psoralens that would reduce the effectiveness of *B. thuringiensis* in the field were not observed. When pairs of main effects were nested within the two levels (presence and absence) of the third factor, several two-way inter-

*To whom correspondence should be addressed.
[1] Lepidoptera: Noctuidae.
[2] Present address: Department of Entomology, Auburn University, Auburn, Alabama 36849.

0098-0331/91/0500-0973$06.50/0

actions were found. Interestingly, the activity of *B. thuringiensis* and the psoralens, individually or in combination, was enhanced by exposure to UV radiation. Implications of this research are discussed for both natural and agricultural ecosystems.

Key Words—Linear furanocoumarins, *Spodoptera exigua*, Lepidoptera, Noctuidae, UV light, tritrophic interactions, *Bacillus thuringiensis*.

INTRODUCTION

Few reports are available on the in vivo impact of plant dietary constituents on the susceptibility of insects to pathogens (Felton and Dahlman, 1984; Krischik et al., 1988). Some plants, such as *Apium graveolens* (L.) (celery), are known to contain the phototoxic linear furanocoumarins psoralen, bergapten, and xanthotoxin (hereafter referred to collectively as psoralens) (Trumble et al., 1990). These compounds generally exhibit increased toxicity in the presence of UV radiation (Igali et al., 1970; Berenbaum, 1978). In addition, the insecticidal activity of *B. thuringiensis* is rapidly degraded in the presence of UV radiation (Dunkle and Shasha, 1988), presumably due to trytophan destruction (Pozgay et al., 1987). To our knowledge, no reports document investigation of such tritrophic interactions in the presence of UV radiation.

Antibacterial substances from plants have been implicated in the reduced insecticidal activity of *B. thuringiensis* (Kushner and Harvey, 1962; Maksymiuk, 1970; Smirnoff, 1972). A primary mode of action of the psoralens is to alkylate DNA (Scott et al., 1976), producing profound mutagenic, carcinogenic, and sometimes lethal effects on bacterial cells (Fowlks et al., 1958; Ashwood-Smith et al., 1986). A second reported activity is as an insect antifeedant (Yajima and Munakata, 1979; Muckenstrum et al., 1981). Thus, the psoralens could theoretically degrade or inhibit (through feeding reduction) the insecticidal activity of microbial pathogens such as *Bacillus thuringiensis* (Berliner), which are under consideration for control of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) in commercial celery production (Trumble, 1990).

Increased production of psoralens in *A. graveolens* can be induced by a variety of environmental stresses (Berenbaum, 1981). Beier and Oertli (1983) demonstrated that his phytoalexin response could be initiated by general elicitors including copper sulfate, UV light, and cold temperatures. Mechanical damage occurring during harvesting and storage also have been shown to increase concentrations from about 2 μg/g to 95 μg/g (Chaudhary et al., 1985). In addition, Berenbaum (1981) and Zangerl and Berenbaum (1987) demonstrated that distribution of psoralens in wild parsnip was significantly correlated with increasing nitrogen content. Not surprisingly, the high-nitrogen acidic fogs of the type and duration occurring in the Los Angeles Basin are known to gen-

erate exceptionally high levels of linear furanocoumarins in celery (Dercks et al., 1989).

Therefore, a multifactor experiment testing several hypotheses was designed to elucidate the various potential interactions. Initially, we wanted to determine if acidic fogs would directly affect the activity of *B. thuringiensis* against *S. exigua*. A second objective was to test the hypothesis that the enhanced levels of psoralens resulting from acidic fog-induced stress on *A. graveolens* would have an impact on the susceptibility of *S. exigua* to *B. thuringiensis*. A third objective was to investigate these interactions in the presence and absence of UV radiation. In addition, the effects of UV radiation on larval developmental rates and survival were documented.

METHODS AND MATERIALS

Simulated Acidic Fogs. Simulated acidic fogs were prepared by adjusting distilled water to pH 2.0 with reagent grade nitric and sulfuric acid mixed at a 2.5:1 (v/v) ratio. This acid ratio is typical of fogs in California (Waldman et al., 1982). The pH levels also are consistent with fogs occurring coastally near Los Angeles (Hoffman, 1984) in areas where the annual value of field-grown celery exceeds $5 million dollars (U.S.) (Ivey and Johnson, 1986). Additional ionic components known to occur in such fogs were also added (R.T. Musselman, U.C. Statewide Airpollution Resource Center, U.C. Riverside, Riverside, California 92521; personal communication). Control fogs consisted of distilled water and background ions adjusted to pH 6.2–6.3. Fogs were created within a 1-m^3 chamber using a fogging apparatus designed by Musselman et al. (1985), which operated at 7.03 kg/cm^2 and produced droplets averaging 20 μm in diameter. Temperature during treatment averaged 22–26°C. Shade cloth was used to reproduce incident radiation levels consistent with coastal fogs of no more than 300 $\mu E/m^2/sec^{-1}$.

Experiment I—Effects of Acidic Fogs on B. thuringiensis *Activity.* Commercially available *B. thuringiensis* (Dipel 2X, Abbott Labs, Chicago, Illinois, containing the HD-1 isolate of *B. thuringiensis* susbsp. *kurstaki*) was spread evenly at 1-mm thickness in standard, uncovered Petri dishes and exposed to acidic or control fogs for 3 hr. Following treatment, the *B. thuringiensis* was dried by pulling air over the Petri dishes with a vacuum for approximately 12 hr. The resulting material then was incorporated into artificial diets using procedures reported previously (Moar and Trumble, 1987).

Larvae used in all tests were obtained from a laboratory colony established in 1982 from insects collected in Orange County, California, and maintained on artificial diet (Patana, 1969) at 27 ± 1°C with a light–dark period of 16:8 hr. New genetic material was added annually.

Bioassays consisted of eight concentrations of *B. thuringiensis* plus a control for each fog treatment. Concentrations tested were as follows: 25, 50, 100, 200, 300, 400, and 800 μg/ml diet. Thirty neonate larvae were tested at each concentration, and the entire test was repeated twice. Larvae were held on diets in an environmental chamber at 27 ± 1°C and mortality was assessed at day 7.

Statistical Analyses for Experiment I. Bioassay data were analyzed using the Proc Probit procedure (SAS Institute, 1985) after correction for control mortality with Abbott's (1925) formula and then judged for suitability using the overlapping fiducial limits criteria described by Vandekar and Dulmage (1982). Control mortality was less than 10%.

Experiment II—Interactions of UV Radiation, B. thuringiensis, *and Psoralens on Development and Survival of* S. exigua. For diets containing phototoxic furanocoumarins (Aldrich Chemical Company, Milwaukee, Wisconsin), psoralen (14.94 μg/ml diet), bergapten (5-methoxypsoralen; 37.75 μg/ml), and xanthotoxin (8-methoxypsoralen; 82.4 μg/ml) were incorporated at the levels found in celery leaves following a single acidic fog incident (pH 2.0, Dercks et al., 1989). These materials were dissolved in ethanol and adsorbed onto alphacel following removal of the ethanol by vacuum as described by Chan et al. (1978). The amount of alphacel constituted 5% of the entire diet media. The ethanol and alphacel procedure was used for all treatments including the control. Diet media then was added to the alphacel and blended for 5 min. *B. thuringiensis* (Dipel 2X, Abbott Labs) was added last, and blended for 5 min prior to dispensing into 30 ml clear plastic cups. The *B. thuringiensis* was incorporated at a level (25 μg/ml diet) expected to kill 25% of the test population as estimated in Experiment I. *B. thuringiensis* and the psoralens were incorporated individually and in combination in the diets.

Diet cups were covered with Teflon FEP Fluorocarbon film (E.I. DuPont de Nemours & Co., Wilmington, Delaware). This film is much more transparent to ultraviolet light than glass. For treatments exposed to UV radiation, cups with larvae were placed beneath UV-producing fluorescent lamps (40-W Sylvania 350 Blacklight, Inland Lighting Supplies, Riverside, California). UV lamps with a peak intensity at 350 nm were chosen because wavelengths between 300 and 400 nm are believed to be critical for activation of the psoralens (Musajo and Rodighiero, 1962). The lamps were adjusted in height such that the intensity of UV light beneath a layer of the Teflon film was 1.023 mW/cm^2, a value less than the 1.3–1.6 mW/cm^2 range observed in coastal southern California at midday in September (Trumble, personal observation) and more typical of the early morning. All UV measurements were made with a System 371 Optical Power Meter equipped with a model 268 detector head (United Detector Technology, Hawthorne, California).

A 2-hr exposure was chosen on the basis of a study by Griswold and Trum-

ble (1985), which showed the first through third instars to be positively photoactic and the fifth and sixth instars to be negatively phototactic. Thus, due to either thermoregulation or light aversion, larvae are directly exposed to UV radiation at the canopy surface only for approximately 2 hr. Our results may be somewhat conservative for early instars in that test larvae were not exposed to additional low levels of UV that would be encountered beneath foliage. Exposure times for larger larvae should be accurate, as later instars often hide beneath the soil and would not be exposed to UV radiation during the peak intensity periods. Insects not exposed to UV radiation were kept in the same room and protected from UV radiation by wooden barriers. An incandescent bulb maintained photoperiod during the 2-hr exposure. When not being exposed, all insects were held at 27 $\pm$ 1°C in a photoperiod of 16:8 hr light–dark.

Thirty neonate larvae were placed on each treatment (all combinations of psoralens, *B. thuringiensis*, and exposure to UV radiation = eight treatment combinations). This test was replicated three times. Larvae were examined daily for mortality, development to the pupal stage, and emergence of the adults. Examinations were stopped when all insects were dead or had emerged as adults. Mean time to pupation was calculated for each treatment by averaging the number of days required to pupate for those larvae reaching the pupal stage. The mean time to emergence was calculated similarly for those insects successfully emerging as adults.

The mean time of mortality (after Moar and Trumble, 1987) was calculated for each treatment by dividing the number of larvae dying on a given day by the total mortality at the end of the study. This value then was multiplied by the respective day. Values for all days then were summed to produce a weighted average time of death.

Statistical Analyses for Experiment II. An analysis of variance of the 2 $\times$ 2 $\times$ 2 factorial was used to compare the survival variables among the eight treatment combinations following an arcsine square root transformation. Each of the factors (psoralens, *B. thuringiensis*, UV radiation) was treated as a fixed effect, each with two levels (presence or absence). A blocking factor was included as a random effect in the model because the experiment was replicated three times. The error mean square of the 2 $\times$ 2 $\times$ 2 factorial was used to construct the F tests. If a three-way interaction was present, dependency of the two-way combined effects on a third factor was removed; the two-way interaction sums of squares were calculated within each level of the third factor (Steel and Torrie, 1980, Chapter 15).

All four treatment combinations with UV radiation exposure were absent when analyzing mean time to emergence and mean time spent as pupae (all insects died before or during the pupal stage). Three treatment combinations with UV radiation exposure were missing when analyzing the mean time to pupation variable (only the insects from the "no psoralen $\times$ no *B. thuringiensis*

× UV radiation'' treatment survived to pupation). The effect of the psoralens and *B. thuringiensis* treatments, in the absence of UV radiation, therefore, was considered with a two-way ANOVA comparing four treatment combinations within a replicated experiment. This analysis allowed comparisons of psoralen and *B. thuringiensis* treatments and their interactions in the absence of UV exposure.

RESULTS AND DISCUSSION

Experiment I—Effects of Acidic Fogs on B. thuringiensis *Activity.* Exposure of *B. thuringiensis* to acidic fogs with a pH of 2.0 produced no significant effects on *S. exigua* as compared to the control treatment by the criteria of Vandekar and Dulmage (1982). In the first repetition, the LC_{50} (95% fiducial limits) values were 185 (144–234) μg formulated material/ml diet and 166 (103–249) μg/ml for the acidic fog-treated *B. thuringiensis* and the control fog-treated *B. thuringiensis*, respectively. Slopes (± SEM) of log dose probit lines were 1.86 (0.211) and 1.86 (0.287), respectively. In the second repetition, conducted several months later, LC_{50} values were 340 (253–461) μg formulated material/ml diet and 384 (306–477) μg/ml for the treated *B. thuringiensis* and the untreated *B. thuringiensis*, respectively. Slopes (± SEM) of log dose probit lines were 1.63 (0.238) and 2.60 (0.390), respectively.

The variability in LC_{50} values between repetitions probably is due to changes occurring in susceptibility over time in colonies due to founder effects and the introduction of new genetic material. Such variability is not uncommon among populations of other noctuid moths (Vandekar and Dulmage, 1982). The LC_{50} values found in this study are similar to those reported previously (Moar et al., 1986, LC_{50} = 196; Moar and Trumble, 1987, LC_{50} = 299). Thus, even in populations with variable susceptibility to *B. thuringiensis*, no differences in treatment effects were detected.

Experiment II—Interactions of UV Radiation, B. thuringiensis *and Psoralens on Development and Survival of* S. exigua. Mean time to pupation in the absence of UV radiation (survival was too low to conduct the analysis for insects exposed to UV) was significantly extended by the addition of either psoralens ($F = 13.11$; $df = 1,6$; $P < 0.01$) or *B. thuringiensis* ($F = 73.55$; $df = 1,6$; $P < 0.01$) (Figure 1). Developmental rate reductions on diets containing *B. thuringiensis* were not unexpected as *Bacillus* species-induced feeding inhibition has been reported previously (Hegazy and Antonious 1987; Salama and Sharaby 1988a). Larvae developing on diets containing *B. thuringiensis* plus psoralens required nearly 40% longer to pupate than controls, but the effects were additive as the interaction was not significant ($F = 0.02$; $df = 1,6$; $P = 0.883$).

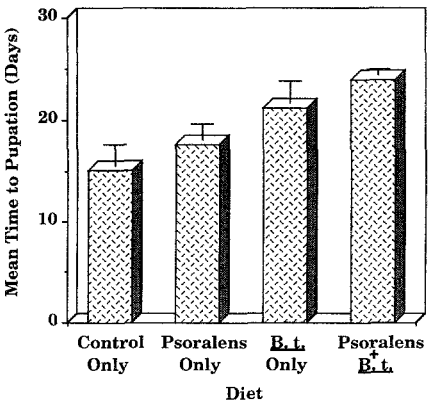


FIG. 1. Mean time to pupation of *S. exigua* in the absence of UV radiation on diets containing psoralens, *B. thuringiensis*, or combinations of both. Thirty neonate larvae were placed on each treatment (all combinations of psoralens, *B. thuringiensis*, and exposure to UV radiation = 8 treatment combinations). This test was replicated three times. Extensions above bars denote standard errors.

This increase in developmental time during the larval stage could have substantial implications for the population dynamics of *S. exigua* in the celery system. Because the majority of the mortality observed in the field occurs in the larval stage (Hogg and Gutierrez, 1980), increased time in this susceptible stage, as opposed to the more protected egg and underground pupal stages (Oatman and Platner, 1972; Tingle et al., 1978), may allow additional opportunity for parasites and predators to reduce populations. Such increases (or decreases) in longevity of larvae previously have been implicated as critical factors in population regulation for other insects (Cardona and Oatman, 1971; Trumble et al., 1987).

The time to emergence of the adult stage in the absence of UV radiation was significantly prolonged by the addition of either psoralens (control mean = 23.64 days; psoralens mean = 26:33; $F = 60.93$; $df = 1,6$; $P < 0.01$) or *B. thuringiensis* (mean = 30.59 days; $F = 431.65$; $df = 1,6$; $P < 0.01$). However, because the mean time spent in the pupal stage (range = 8.5 to 9.3 days) did not vary significantly for either psoralens ($F = 0.03$; $df = 1,6$; $P = 0.88$) or *B. thuringiensis* ($F = 1,63$; $df = 1,6$; $P = 0.25$) as compared to the controls, the increases in developmental time during the larval stage therefore were responsible for the observed decrease in developmental rate. As in the mean time to pupation analysis, there were no significant interactions between psoralens and *B. thuringiensis* for mean time to emergence ($F = 0.16$; $df = 1,6$; $P = 0.70$).

Mean time to mortality, a weighted average time of death, was not signif-

icantly different between treatments ($F = 1.721$; $df = 4,9$; $P = 0.229$). Substantial differences would have suggested either a variable mode or site of action, or the presence of an additional toxic component or interaction. Just such a change in mortality rate was an important factor leading to the discovery of toxic impurities in another insecticidal compound (Umetsu et al., 1981). Because no differences were detected in mean time of mortality for larvae feeding on diets containing psoralens or *B. thuringiensis* does not necessarily suggest that the modes or sites of action may be similar; we simply conclude that this analysis does not clearly separate modes or sites of action.

Larval survival to the pupal stage was variable. In the absence of UV radiation, larval survival on diets containing psoralens > diets with *B. thuringiensis* alone > diets with both psoralens and *B. thuringiensis* (Figure 2A). In

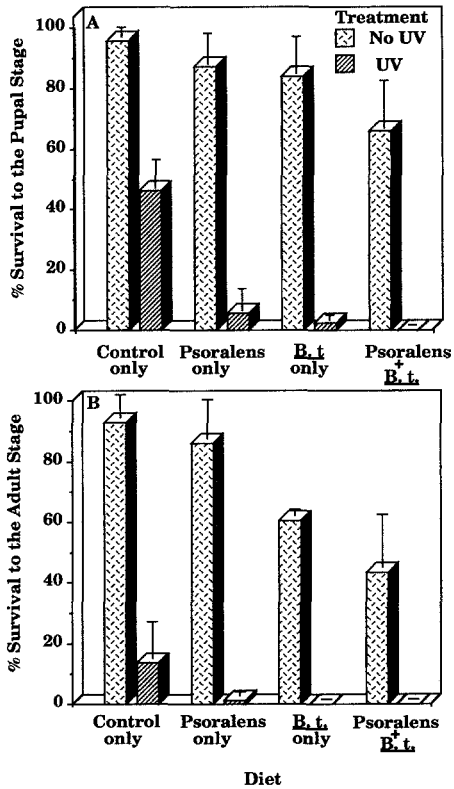


FIG. 2. Percent survival of *S. exigua* to the pupal (A) or adult (B) stages in the presence or absence of UV radiation on diets containing psoralens, *B. thuringiensis*, or combinations of both. See Figure 1 or Methods section for sample sizes. Extensions above bars delineate standard errors.

the presence of UV radiation survival was always reduced, but the same trend was evident.

All main effects (psoralens, *B. thuringiensis*, UV radiation) were significant ($P < 0.01$), with UV radiation being the predominant mortality factor ($F = 239$; $df = 1,14$; $P < 0.01$). Mean square error was 0.0208. Nearly all larvae (95.5%) on control diets that were not exposed to UV light survived to pupate (Figure 2A). Because first to third instars of *S. exigua* prefer to feed on foliage rather than petioles in celery, and the larvae are positively phototactic in the first three stadia (Griswold and Trumble, 1985), the low survival observed in the field (Hogg and Gutierrez, 1980) may be explained in part by the toxic effects triggered by UV light. However, in the aforementioned study all of the phototaxis experiments with early instar larvae were conducted at a single temperature, and larval movements associated with potential thermoregulating activities were not investigated.

The survival of larvae to the pupal stage was reduced significantly by the three-way interaction of exposure to psoralens and *B. thuringiensis* in the presence of UV light ($F = 5.01$; $df = 1,14$; $P < 0.042$) (Figure 2A). Thus, since UV radiation is ubiquitous in field situations, concerns that antagonistic interactions with psoralens would reduce the effectiveness of *B. thuringiensis* are unwarranted.

No two-way interactions were detected ($P > 0.10$) in the $2 \times 2 \times 2$ factorial analysis. However, three-way interactions may mask the two-way combined effects (Steel and Torrie, 1980). Therefore, pairs of main effects were nested within the two levels (presence and absence) of the other main effect to determine if combined two-way effects were important.

Nesting Psoralen and B. thuringiensis *Treatments within UV Radiation Treatment.* The psoralen × *B. thuringiensis* interaction was not significant ($F = 0.14$; $df = 1, 14$; $P = 0.72$) when the diet was not exposed to UV radiation (Figure 3A). When the additional stress of UV exposure was added, the psoralen × *B. thuringiensis* interaction produced a significant decrease in survival rate ($F = 7.79$; $df = 1,14$; $P = 0.015$; Figure 3B). However, this interaction should be interpreted with caution because adding the mortality caused by psoralens and *B. thuringiensis* in combination cannot equal less than 0% survival. Therefore, because the lower bound (0%, Figure 3A) of the mortality measure was reached, the psoralen × *B. thuringiensis* interaction may be constrained by the statistical approach and may not have biological meaning.

Results from similar studies presented in the literature provide no consistent antagonistic trend between potentially defensive plant chemicals and bacteria. Krischik et al. (1988) demonstrated that plant allelochemicals could interfere with microbials such that specialist herbivores feeding on plants or plant parts with high levels of toxins could gain protection from *B. thuringiensis* var. *kurstaki*. Although *S. exigua* is a generalist, the levels of psoralens in our study

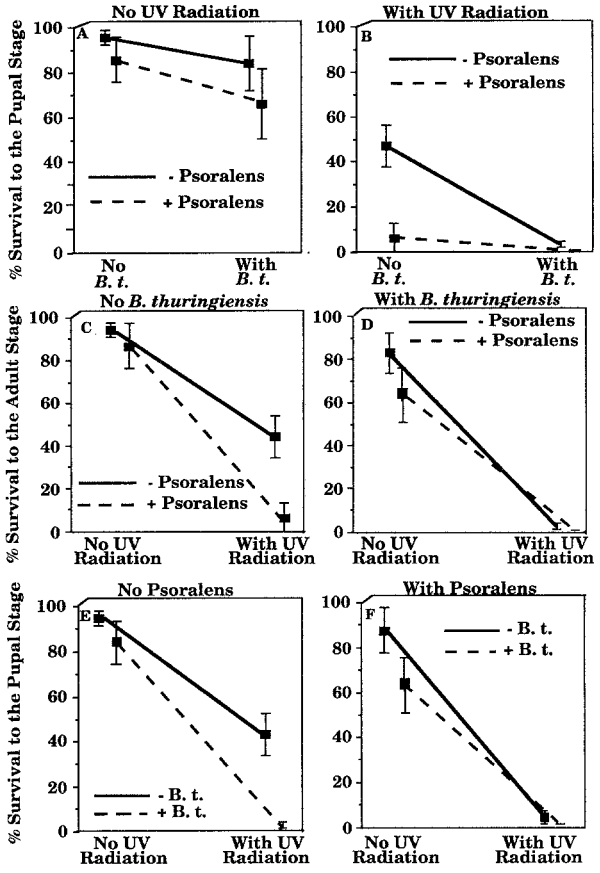


FIG. 3. Nested interaction plots for a 2 × 2 × 2 factorial with the main factors psoralens, *B. thuringiensis* (*B.t.*), and UV radiation. See Figure 1 or Methods section for sample sizes. Two-way interactions were calculated within each level (presence or absence) of the third factor (see text for details). Extensions above bars delineate standard errors. Data points were slightly offset to allow discrimination of standard error bars.

were high, and the percent survival resulting from a combination of *B. thuringiensis* and psoralens was less than observed for either chemical alone (Figure 2A,B). Thus, antagonistic interactions between psoralens and *B. thuringiensis* that would cause the combination of chemicals to become markedly less effective were not indicated. In contrast, our data were not in agreement with the findings of Salama and Sharaby (1988b), where an increase in potency of *B. thuringiensis* was observed in combination with powdered plants known to contain allelochemicals active against another *Spodoptera* species. Similar in vivo

studies with *Manduca sexta* (L.) indicated an enhanced effect of *B. thuringiensis* in the presence of cavanine (Felton and Dahlman, 1984). Unfortunately, direct comparisons are difficult because these studies were conducted in environmental chambers, and the UV radiation exposure levels were not specified.

Nesting Psoralen and UV Radiation Treatments within B. thuringiensis *Treatment.* The psoralen × UV interaction was significant ($F = 5.71$; $df = 1,14$; $P = 0.032$) when larvae were not exposed to *B. thuringiensis* (Figure 3C). Similar results have been summarized by Murray et al. (1982) for a variety of species. In the presence of *B. thuringiensis*, the psoralen × UV interaction was not significant ($F = 0.60$; $df = 1,14$; $P = 0.45$; Figure 3D). Even if photoactivation of the psoralens was occurring in the presence of *B. thuringiensis*, the mortality level could not exceed 100% (the level reached). Under these conditions the interaction would not appear statistically significant.

Nesting B. thuringiensis *and UV Radiation Treatments within Psoralen Treatment.* The *B. thuringiensis* × UV radiation interaction significantly increased mortality beyond a simple additive effect ($F = 7.23$; $df = 1,14$; $P = 0.018$) when the psoralens were not incorporated in the diet (Figure 3E). One potential explanation for this effect is that a partial paralysis resulting from feeding on could serve to enhance the effect of UV light by preventing larvae from moving away from exposed foraging positions. Although this interaction may be enhanced in our study by incorporating the *B. thuringiensis* into the diet (thereby providing some UV radiation protection), the effect would be similar to what can occur if *B. thuringiensis* protein production is incorporated in plants or if commercially available *B. thuringiensis* is applied in the evening.

The *B. thuringiensis* × UV radiation interaction was not significant ($F = 0.23$; $df = 1,14$; $P = 0.64$) when the additional stress of psoralen was added in the diet (Figure 3F). As noted for the previous analysis, survival reaches the lower extreme of the measure (0%), and any potential interaction may be masked by the additional mortality factor of the psoralens.

Survival to Adult Stage. No interactions were detected when measuring survival to the adult stage. UV radiation again triggered the single most important mortality factor, resulting in significant (80–100%) decreases in percent survival as compared to treatments in which larvae were protected from UV exposure ($F = 149$; $df = 1,14$; $P < 0.01$) (Figure 2B). In addition, approximately 65% more mortality occurred during the pupal stage on control diets for those insects exposed to UV light as larvae. Examination of these "pupae" indicated that many had not entirely completed metamorphosis. While this response is typical for nutritionally deprived larvae, no attempt was made to determine the mechanism responsible for the incomplete metamorphosis in our study.

The main effect of *B. thuringiensis* exposure was also significant ($F =$

17.8; $df = 1,14$; $P < 0.01$). This was not unexpected as survival-threatening physical defects and low weights in pupae developing from larvae that have ingested *B. thuringiensis* have been reported previously (Salama and Sharaby, 1988a).

Several conclusions can be drawn from these results. First, acute incidences of acidic fogs as low as pH 2.0 will not reduce the efficacy of *B. thuringiensis* if the bacteria are not physically washed from the leaves. In agricultural systems, spray adjuvants can provide "rain fastness," but the impact of fogs during naturally occurring epizootics has not been quantified. Second, a three-way interaction between psoralens, *B. thuringiensis*, and UV radiation significantly increases mortality, indicating that, in the field, antagonistic interactions with psoralens would not reduce the effectiveness of *B. thuringiensis*. Third, *S. exigua* larvae feeding on celery with high content of psoralens following acidic fog episodes will suffer substantial additional mortality (survival reduced from 50% to 8%, Figure 2A), with the resulting reduction in survival likely to produce substantial economic consequences (Trumble, 1990). Because the psoralens are strong mutagens, and adult fitness (oviposition capacity, longevity, etc.) was not examined, the potential impact on subsequent generations may be greater from psoralens than this study suggests. However, effects on adult populations will be mitigated by migratory movements in the Los Angeles Basin from (1) outside agroecosystems, (2) other geographic locations, or (3) crops that do not contain psoralens providing a consistent supply of fit adults. (Trumble and Baker, 1984). One caveat of this study is that a single concentration of psoralens was used; no effort was made to determine the variability of larval responses with dose.

In addition, the activity of *B. thuringiensis* and the psoralens, individually or in combination, was enhanced by the levels of UV radiation used in this study. While this response was not surprising for the psoralens (Berenbaum, 1978, 1981), which are reportedly activated by UV radiation (Scott et al., 1976), the results for *B. thuringiensis* were unexpected given previous reports that UV radiation rapidly degrades insecticidal activity (Pozgay et al., 1987; Dunkle and Shasha, 1988). We suspect that the enhanced effect of *B. thuringiensis* may have been due to UV radiation causing a general weakening of the larvae, which interferes with their normal immune response.

Acknowledgments—The assistance of J. McFall, E. Younce, and A. Jones in the laboratory is appreciated. Support for this project was provided, in part, by the Academic Senate of the University of California and the California Celery Research Advisory Board. The critical reviews of R. Redak, T. Paine, and T. Meade improved this manuscript and are appreciated.

REFERENCES

ABBOTT, W.S. 1925. A method for computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18:265–267.

ASHWOOD-SMITH, M.J., CESKA, O., CHAUDHARY, S.K., WARRINGTON, P.J., and WOODCOCK, P. 1986. Detection of furanocoumarins in plants and plant products with an ultrasensitive biological photoassay employing a DNA-repair-deficient bacterium. *J. Chem. Ecol.* 12:915–932.

BERENBAUM, M. 1978. Toxicity of a furanocoumarin to armyworms: A case of biosynthetic escape from insect herbivores. *Science* 201:532–534.

BERENBAUM, M. 1981. Patterns of furanocoumarin distribution and insect herbivory in the umbelliferae: Plant chemistry and community structure. *Ecology* 62:1254–1266.

BEIER, R.C. and OERTLI, E.H. 1983. Psoralen and other linear furocoumarins as phytoalexins in celery. *Phytochemistry* 22:2595–2597.

CARDONA, C., and OATMAN, E.R. 1971. Biology of *Apanteles dignus* (Hymenoptera: Brachonidae) a primary parasite of the tomato pinworm. *Ann. Entomol. Soc. A m.* 64:996–1007.

CHAN, B.C., WAISS, A.C. JR., STANLEY, W.L., and GOODBAN, A.E. 1978. A rapid diet preparation method for antibiotic phytochemical bioassay. *J. Econ. Entomol.* 71:366–368.

CHAUDHARY, S.K., CESKA, O., WARRINGTON, P.J., and ASHWOOD-SMITH, M.J. 1985. Increased furanocoumarin content of celery during storage. *J. Agric. Food Chem.* 33:1153–1157.

DERCKS, W., TRUMBLE, J.T., and WINTER, C. 1989. Impact of atmospheric pollution on linear furanocoumarin contentin celery. *J. Chem. Ecol.* 16:443–454.

DUNKLE, R.L., and SHASHA, B.S. 1988. Starch-encapsulated *Bacillus Thuringiensis*: A potential new method for increasing environmental stability of entomopathogens. *Environ. Entomol.* 17:120–126.

FELTON, G.W., and DAHLMAN, D.L. 1984. Alleolochemical induced stress: Effects of L-cavanine on the pathogenicity of *Bacillus thuringiensis* in *Manduca sexta. J. Invert. Pathol.* 44:187–191.

FOWLKS, W.L., GRIFFITH, D.G., and OGINSKY, E.L. 1958. Photosensitization of bacteria by furocoumarins and related compounds. *Nature* 181:571–572.

GRISWOLD, M.J., and TRUMBLE, J.T. 1985. Responses of *Spodoptera exigua* (Lepidoptera: Noctuidae) larvae to light. *Environ. Entomol.* 14:650–653.

HEGAZY, E., and ANTONIOUS, A.G. 1987. Feeding deterrent efficacy of certain microbial insecticides against the cotton leafworm, *Spodoptera littoralis* (Boisd.). *Ann. Agric. Sci.* 32:1765–1778.

HOFFMAN, M.R. 1984. Comment on acid fog. *Environ. Sci. Technol.* 18:61–64.

HOGG, D.B., and GUTIERREZ, A.P. 1980. A model of the flight phenology of the beet armyworm (Lepidoptera: Noctuidae) in central California. *Hilgardia* 48:1–35.

IGALI, S., BRIDGES, B.A., ASHWOOD-SMITH, M.J., and SCOTT, B.R. 1970. IV. Photosensitization to near ultraviolet light by 8-methoxypsoralen. *Mutat. Res.* 9:21–30.

IVEY, L., and JOHNSON, H. 1986. Page 3, *in* Vegetable Crops: Acreage and Value at a Glance. U.C. Riverside, Cooperative Extension Publications.

KRISCHIK, V.A., BARBOSA, P., and REICHLDORFER, C.F. 1988. Three trophic level interactions: Allelochemicals, *manduca sexta* (L.), and *Bacillus thuringiensis* var *kurstaki* Berliner. *Environ. Entomol.* 17:476–482.

KUSHNER, D.J., and HARVEY, G.T. 1962. Antibacterial substances in leaves: Their possible role in insect resistance to disease. *J. Insect Pathol.* 4:155–184.

MAKSYMIUK, B. 1970. Occurrence and nature of antibacterial substances in plants affecting *Bacillus thuringiensis* and other entomogenous bacteria. *J. Invert. Pathol.* 15:356–371.

MOAR, W.J., and TRUMBLE, J.T. 1987. Toxicity, joint action, and mean time of mortality of Dipel 2X, avermectin b1, neem, and thuringiensin against beet armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 80:588–592.

MOAR, W.J., OSBRINK, W.L.A., and TRUMBLE, J.T. 1986. Potentiation of *Bacillus thuringiensis* var. *kurstaki* by thuringiensin against *Spodoptera exigua* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 79:1443–1446.

MUCKENSTRUM, B., DUPLAY, D., ROBERT, P.C., SIMONIS, M.T., and KIENLEN, J. 1981. Substances antiappetantes pour insectes phytophages presentes dans *Angelica silvestris* et *Heracleum sphondylium. Biochem. Syst. Ecol.* 9:289–292.

MURRAY, R.D.H., MENDEZ, J., and BROWN, S.A. 1982. The Natural Coumarins. J. Wiley & Sons, Chichester, U.K.

MUSAJO, L., and RODIGHIERO, G. 1962. The skin-photosensitizing furanocoumarins. *Experientia* 18:153–200.

MUSSELMAN, R.C., STERRETT, J.L., and GRANETT, A.L. 1985. A portable fogging apparatus for field or greenhouse use. *HortScience* 20:1127–1129.

OATMAN, E.R., and PLATNER, G.R. 1972. An ecological study of lepidopterous pests affecting lettuce in coastal southern California. *Environ. Entomol.* 1:202–204.

PATANA, R. 1969. Rearing cotton insects in the laboratory. *U.S. Dept. Agric. Prod. Res. Rep.* 108.

POZGAY, M., FAST, P., KAPLAN, H., and CARY, P.R. 1987. The effect of sunlight on the protein crystals from *Bacillus thuringiensis* var *kurstaki* HD1 and NRD12: A raman spectroscopic study. *J. Invert. Pathol.* 50:246–253.

SALAMA, H.S., and SHARABY, A. 1988a. Effects of exposure to sublethal doses of *Bacillus thuringiensis* (Berl.) on the development of the greasy cutworm, *Agrotis ypsilon* (Hufn.). *J. Appl. Entomol.* 106:396–401.

SALAMA, H.S., and SHARABY, A., 1988b. Feeding deterrence induced by some plants in *Spodoptera littoralis* and their potentiating effect on *Bacillus thuringiensis* Berliner. *Insect Sci. Appl.* 9:573–577.

SAS INSTITUTE. 1985. SAS User's Guide: Statistics, Version 5. SAS Institute, Cary, North Carolina.

SCOTT, B.R., PATHAK, M.A., and MOHN, G.R. 1976. Molecular and genetic basis of furocoumarin reactions. *Mutat. Res.* 39:29–74.

SMIRNOFF, W.A. 1972. Effects of volatile substances released by foliage of *Abies balsamera. J. Invert. Pathol.* 19:32–35.

STEEL, R.G.D., and TORRIE, J.H. 1980. Principles and Procedures of Statistics, A Biometrical Approach, 2-ed. McGraw-Hill, New York.

TINGLE, F.C., ASHLEY, T.R., and MITCHELL, E.R. 1978. Parasites of *Spodoptera exigua, S. eridania* (Lep: Noctuidae) and *Herpetogramma bipunctalis* (Lep.: Pyralidae) collected from *Amaranthus hybridus* in field corn. *Entomophaga* 23:343–347.

TRUMBLE, J.T. 1990. Vegetable insect control with minimal use of insecticides. *HortScience* 25:159–164.

TRUMBLE, J.T., and BAKER, T.C. 1984. Flight phenology and pheromone trapping of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) in southern coastal California. *Environ. Entomol.* 13:1278–1282.

TRUMBLE, J.T., HARE, J.D., MUSSELMAN, R.C., and MCCOOL, P.M. 1987. Ozone-induced changes in host plant suitability: Interactions of *Keiferia lycopersicella* and *Lycopersicon esculentum. J. Chem. Ecol.* 13:203–218.

TRUMBLE, J.T., DERCKS, W., QUIROS, C.F., and BEIER, R.C. 1990. Host plant resistance and linear furanocoumarin content of *Apium* accession. *J. Econ. Entomol.* 83:519–525.

UMETSU, N., MALLIPUDI, M., TOIA, R.F., MARCH, R.B., and FUKUTO, T.R. 1981. Toxicological properties of phosphorothioate and related esters present as impurities in technical organophosphorus insecticides. *J. Toxicol. Environ. Health* 7:481–497.

VANDEKAR, M., and DULMAGE, H.T. 1982. Guideline for production of *Bacillus thuringiensis* H-14, pp. 39–45, *in* Proceedings, Special Programme for Research and Training in Tropical Diseases, Geneva.

WALDMAN, J.M., MUNGER, J.W., JACOB, D.J., FLAGEN, R.C., MORGAN, J.J., and HOFFMAN, M.R. 1982. Chemical composition of acid fog. *Science* 218:677–680.

YAJIMA, T., and MUNAKATA, K. 1979. Phloroglucinol-type furocoumarins, a group of potent naturally-occurring insect antifeedants. *Agric. Biol. Chem.* 24:1701–1706.

ZANGERL, A.R., and BERENBAUM, M.R. 1987. Furanocoumarins in wild parsnip: Effects of photosynthetically active radiation, ultraviolet light, and nutrients. *Ecology* 68:516–520.

Journal of Chemical Ecology, Vol. 17, No. 5, 1991

ROLE OF STEROIDAL GLYCOALKALOID α-TOMATINE IN HOST-PLANT RESISTANCE OF TOMATO TO COLORADO POTATO BEETLE

J.D. BARBOUR and G.G. KENNEDY*

Department of Entomology
North Carolina State University
Raleigh, North Carolina 27695-7630

(Received July 30, 1990; accepted January 28, 1991)

Abstract—The role of the steroidal glycoalkaloid α-tomatine in the host-plant resistance of tomato to the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) was examined in short- (24 hr; using first- and fourth-instar larvae) and long-term (first-instar larvae reared through the prepupal stage) feeding experiments. Consumption rate, growth rate, efficiency of conversion of ingested food to body mass, and survival were compared for *L. decemlineata* provided foliage from susceptible (*Lycopersicon esculetum* Mill. cv. Walter), resistant (*L. hirsutum* f. *glabratum* C.H. Mull accession PI 134417), and F_1 hybrid plants. Values obtained for dietetic indices were regressed against corresponding values for α-tomatine content of foliage provided to larvae. Differences in dietetic indices could not be attributed to variation in foliar α-tomatine content despite a long-standing literature showing the *ex planta* α-tomatine inhibits feeding and growth by *L. decemlineata*.

Key Words—Host-plant resistance, host-plant selection, glycoalkaloids, *Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae, *Lycopersicon*, α-tomatine.

INTRODUCTION

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) is an oligophagous insect (*sensu* Otte and Joern, 1977) that feeds, except under extraordinary conditions, exclusively on several species of plants belonging to the Solanaceae (Hsiao and Fraenkel, 1968; Hsiao, 1981).

*To whom correspondence should be addressed.

0098-0331/91/0500-0989$06.50/0

Although most commonly known as an egregious pest of potato (*Solanum tuberosum* L.), *L. decemlineata* is also a pest of several other solanaceous crop plants including tomato, *Lycopersicon esculentum* Mill. (Hofmaster, 1977; Lange and Bronson, 1981). Feeding and economic damage to tomato by *L. decemlineata* can be substantial, particularly in the mid- and northeastern Atlantic states (Schalk and Stoner, 1979; Kennedy et al., 1983). Although resistance in tomato to *L. decemlineata* has been known for some 30 years (Buhr, 1956; Schalk and Stoner, 1976), to date, there are no commercial tomato cultivars resistant to this insect.

The wild tomato species *L. hirsutum* f. *glabratum* C.H. Mull accession PI 134417 has been shown to express a high degree of resistance to many insect pests of tomato including *L. decemlineata* (Kennedy et al., 1985). The type VI glandular trichomes (sensu Luckwill, 1943), which abound on the foliage of PI 134417, play an important role in the resistance of this plant to *L. decemlineata*. The methyl-ketone 2-tridecanone, a major constituent of the glandular tips of these trichomes, is toxic to *L. decemlineata* at levels occurring in PI 134417 foliage (Kennedy and Dimock, 1983) and resistance that results from its presence is manifest as extensive mortality of young larvae (Kennedy and Sorenson, 1985).

Although 2-tridecanone plays an important role in the trichome-based resistance of PI 134417 to *L. decemlineata*, other, nontrichome factors are also involved. Type VI trichome glands, and hence 2-tridecanone, can be removed by washing PI 134417 foliage. *L. decemlineata* fed such washed foliage suffer extensive mortality during the late larval and pupal stages, whereas those fed PI 134417 foliage with glandular trichomes intact suffer extensive mortality during the first larval instar. In both instances mortality is greater than that of larvae fed susceptible, *L. esculetum* cv. Walter foliage or hybrid (Walter × PI 134417) tomato foliage containing or divested of type VI trichome glands (Kennedy and Sorenson, 1985).

Several alkaloids occurring in tomato, most notably the leptines and α-tomatine, have been shown to inhibit feeding and growth in *L. decemlineata* when provided to adults and larvae *ex planta* (Kuhn and Löw, 1955; Buhr et al, 1958; Schreiber, 1968; Sturköw and Löw, 1961; Sinden et al, 1980, 1986). Largely on the basis of these and similar studies, reduced feeding by *L. decemlineata* on resistant hosts has been attributed to the feeding-deterrent properties of these compounds when present in planta (Hsiao, 1969; Hsiao and Fraenkel, 1968; Schreiber, 1957, 1968; Sinden et al., 1978, 1980, 1986).

Sinden et al. (1978) suggested that α-tomatine is involved in the resistance of PI 134417 to *L. decemlineata*. In experiments using adult beetles, they demonstrated a positive relationship between inhibition of feeding and the level of α-tomatine infiltrated into *L. esculetum* cv. C28 leaflet disks. They also found that feeding on foliage from cv. C28 and PI 134417 plants of different ages or

grown under different photoperiod regimes was negatively correlated with α-tomatine content. Although their data are suggestive, they are difficult to interpret since the experiments were confounded by both the presence of glandular trichomes, hence 2-tridecanone, on PI 134417 foliage (Kennedy et al., 1985) and by differences in photosynthate levels between plants grown under long and short day regimes (Kennedy et al., 1981).

In other studies, inhibition of feeding by *L. decemlineata* has been observed to vary directly with the amount of α-tomatine used to treat tomato and potato foliage (Sturköw and Löw, 1961; Sinden et al., 1978) or artificial diets (Hsiao and Fraenkel, 1968; Hare, 1983, 1987). However these studies fail to distinguish clearly between preingestive behavioral effects (i.e., deterrency) and postingestive physiological effects (i.e., toxicity) of α-tomatine on *L. decemlineata* (Mitchell and Harrison, 1985; Harrison and Mitchell, 1988). Neither is there any work, of which we are aware, demonstrating that the effects of α-tomatine on *L. decemlineata* observed *ex planta* are operative as resistance factors *in planta*.

α-Tomatine has been found in all *Lycopersicon* species surveyed and has been detected in all plant tissues examined, although epigeal plant tissues, especially fruits and flowers, tend to have higher α-tomatine levels (Roddick, 1974). Since α-tomatine has been found, at least in the commercial *L. esculentum* cultivars examined thus far, to be associated with the leaf lamella and not with glandular trichome exudate (Duffey and Isman, 1981), it is possible that the resistance of PI 134417 to *L. decemlineata* results from the combined effects of trichome-based 2-tridecanone resistance and lamellar-based α-tomatine resistance. The study reported herein was undertaken to determine what role, if any, α-tomatine plays in the resistance of PI 134417 to *L. decemlineata*.

METHODS AND MATERIALS

Tomato Plants. Susceptible (*L. esculetum* cv. Walter), resistant (*L. hirsutum* f. *glabratum* accession PI 134417), and F_1 hybrid (cv. Walter × PI 134417) plants were grown in a greenhouse from seed germinated directly in soil (Terra Lite Metro Mix 220®), in 9.1-cm clay pots (greenhouse-grown plants) or plastic transplanting trays (field-grown plants). Plants were fertilized weekly (Peters Professional® 20:20:20), and natural light was augmented with radiation from metal halide lamps to provide a 16-hr photophase. At four weeks, plants to be used for greenhouse experiments were transplanted into 20-cm pots. Plants to be used for field experiments were placed outdoors to condition for two weeks before transplanting into methyl bromide-fumigated soil at the Agricultural Research Station at Clayton, Johnston County, North Carolina, in early May (first planting) and early June (second planting). Fertilizer (12:6:24 N-

P-K at 896 kg/ha) was incorporated at planting, and weeds were controlled with biodegradable plastic mulching.

The field plants were arranged in a randomized complete block design with six rows (= blocks) and five single-plant plots of each plant line per row. Plants within a row were separated by 1.5 m with 3.0 m separating rows. Maneb®, applied at 2.24 kg/ha, was used for control of foliar disease. In the greenhouse, each plant line was maintained on individual, adjacent benches in the same greenhouse.

Insects. L. decemlineata were obtained from colonies maintained in a greenhouse on potato (*Solanum tuberosum* cv. Pungo) foliage. Colonies were initiated from, and infused annually with, beetles collected from potato at Plymouth, Washington County, North Carolina. Photoperiod was maintained as described above for plant rearing.

Short-Term Feeding Experiments: Fourth Instar. Insects molting to fourth instar within a 24-hr period were collected and placed in individual 5.5-cm Petri dishes containing filter paper disks moistened with four to five drops of water. The Petri dishes were sealed with Parafilm® and the larvae held 24 hr at 27°C to allow voiding of gut contents. The starved insects then were weighed to provide an initial wet weight (IWWL) for each larva. Twenty-four starved larvae were frozen, oven dried (40°C) to a constant weight, and individually reweighed to provide a proportion dry weight (PDWL) estimate for larvae used in experiments.

Larvae, maintained individually in Petri dishes, were provided excess foliage, containing or divested of glandular trichome exudate, as whole leaflets, preweighed to provide an initial wet weight (IWWF) for foliage provided to each insect. A proportion dry weight (PDWF) estimate of foliage provided to larvae was obtained, analogously to PDWL, for each foliage treatment, using leaflets on the opposite side of the rachis from those provided to larvae. Insects were held in sealed Petri dishes for 24 hr at 27°C before tests were halted by freezing (−20°C).

Frozen larvae and uneaten foliage were rinsed with clean water to remove feces. Final dry weight of larvae (FDWL) and foliage (FDWF) was determined directly by weighing oven dried material. Initial dry weight of larvae (IDWL) and foliage (IDWF) was calculated as the product of their initial wet weight values and their respective proportion dry weight (e.g., IDWL = IWWL × PDWL). The quantity of foliage consumed (FC) was calculated, for each larva, as IDWF − FDWF, and consumption rate (RCR_i) as FC/IDWL. The weight gained by larvae during the feeding period (WGL) was calculated as FDWL − IDWL. Growth rate (RGR_i), was calculated analogously to consumption rate, as WGL/IDWL. Efficiency of conversion of ingested food to body weight (ECI) was calculated, for each larva, as the weight gained during the test period divided by the amount of foliage consumed during that same time interval (WGL/FC).

Since *L. decemlineata* produces a semiliquid feces, the amount of food digested (AD) and the efficiency of conversion of digested food (ECD) were not calculated.

Short Term Feeding Experiments: First Instar. The protocol for first-instar experiments was similar to that used in fourth-instar experiments, although some changes were required to accommodate first-instar biology and to reduce error in estimated parameters. After they had dispersed from egg masses, first instars were starved 6 hr instead of the 24 hr used in fourth-instar experiments. This was necessary since the duration of this stadium is ca. 1.5 days under experimental conditions. Individual observations in first-instar experiments were based on the results of five larvae per Petri dish rather than the single larva used in fourth-instar experiments. Whole leaflets provided to fourth-instar insects were replaced with leaflet disks in first-instar experiments. Leaflet disks were excised from the distal portion of leaflets (divested of or containing a full complement of type VI trichome glands) with a No. 10 cork borer (1.91 cm^2), one disk per leaflet. Leaflet disks excised from leaflets on the opposite side of the rachis from those provided to larvae were used to obtain a percent dry weight estimate for each foliage treatment. These changes were required since preliminary experiments indicated that consumption and weight gain by first-instar insects were small compared to the dry weight of foliage when provided as whole leaflets.

It is important to note that the explicit (RCR_i, RGR_i) or implicit (ECI) divisor used in construction of dietetic indices was the initial (after Farrar et al., 1989; denoted by the subscript "i"), rather than the mean (see Waldbauer, 1968) dry weight of larvae used in tests. This was done, since we were interested primarily in the behavioral response of insects to their food source, to avoid confounding preingestive and postingestive effects of food source on the insects (see Farrar et al., 1989).

Foliage used in short-term experiments consisted of the first fully expanded leaflets below the shoot apex of 10- to 12- (greenhouse-grown) or 10- to 14- (field-grown) week-old plants. Trichome- and lamellar-based effects of foliage were partitioned by providing larvae with foliage either divested of, or containing a full complement of, intact type VI trichome glands, providing a 3 × 2 factorial arrangement of treatments (3 plant lines × 2 trichome conditions). Removal of trichome glands was accomplished by first gently wiping foliage with a dry cotton ball, then with a cotton ball saturated with a 0.1% solution of Triton X-155®, a nonphytotoxic emulsifying agent. Foliage was then rinsed, first in 0.1% Triton X-155®, then in clean water. Excess moisture was removed from washed foliage by gently pressing between several layers of clean tissue paper.

All short-term experiments were conducted using foliage from both greenhouse- and field-grown plants. Experiments consisted of 10 observations per

treatment and were repeated five and six times for greenhouse- and field-grown plants, respectively. Data from plants grown at the different locations were analyzed separately by factorial ANOVA using the GLM Procedure (SAS Institute, Cary, North Carolina).

Long-Term Feeding Experiments. Neonate *L. decemlineata* were weighed and placed into individual 7 × 10-cm (h × diam.) plastic cups containing a filter paper disk moistened with water. Larvae were provided preweighed foliage, which was replaced after 48 hr and every 24 hr thereafter with similarly prepared foliage. Water was added as required to maintain adequate humidity. Uneaten foliage was removed, washed to remove feces, and oven dried to determine the final dry weight of foliage remaining after each feeding period. After larvae molted to the fourth instar, ca. 3 cm of soil was added to cups to provide a substrate for pupation. When they died or became prepupae (indicated by burrowing behavior), larvae were frozen, oven dried, and final dry weight determined.

Amount of foliage consumed (FC) and percent survival were determined for each feeding period, and values were accumulated over the entire larval period, which for experimental purposes was considered to end when larvae either died or became prepupae. Foliage consumed was calculated similarly to RCR_i but contained no correction for initial insect weight. ECI was calculated as WGL (determined at the time of death or pupation), divided by the total amount of foliage consumed by the larvae. ECI in the long-term experiments thus represents an overall ECI of larvae from neonate to death or pupation. Except that only greenhouse-grown plants were used, foliage used in long-term experiments was similar to that used in short-term experiments. Experiments consisted of 20 observations per treatment, and the entire experiment was repeated four times. Data were analyzed by factorial ANOVA.

Analysis of Foliage for α-Tomatine Content. Procedures for extraction and quantification of α-tomatine were modified from Juvick and Stevens (1982). Uneaten foliage from insect experiments was pooled according to plant line, oven dried, and ground to a fine powder. Aliquots (ca. 100 mg) were Soxhlet extracted in 100 ml of 95% MeOH for 12 hr and the resulting extract concentrated to 2 ml on a rotary evaporator. The samples were acidified with 2 ml of 1.0 N HCl and washed with 4 ml of benzene. After phase separation (12 hr), the organic phase was discarded, the aqueous phase adjusted to pH 7.9–8.1 with 1.0 N NaOH, and the final volume adjusted to 10.0 ml with 95% MeOH.

Quantification of α-tomatine was by red blood cell hemolysis (Arneson and Durbin, 1968). Sheep red blood cells in Alsever's solution (Cleveland Scientific Co.) were centrifuged (3500*g* for 15 min), and 2.5 ml of packed cells diluted to 1.0 liter with normal saline. Samples (50–200 μl) were added to 10.0-ml portions of the sheep red blood cell reagent, the contents vortexed, centrifuged (1500*g* for 15 min), and the optical density (540 nm) of the supernatent

determined as the average of three replicate readings per sample. α-Tomatine content of samples was determined by reference to the mean optical density of triplicate readings of similarly prepared standards [0.05–0.30 mg/ml α-tomatine (Sigma Chemical Co.) in MeOH]. Percent recovery was determined for each plant line by comparing the α-tomatine content of plant material containing no added α-tomatine with that of similar plant material containing a known amount of α-tomatine in standard solution. Analysis was performed separately for foliage from short- and long-term bioassays.

To confirm that α-tomatine was responsible for the observed hemolysis, samples for each plant line, a standard α-tomatine solution, and an MeOH blank were divided into equal (2.0-ml) portions. One of each pair received 2.0 mg cholesterol (Sigma Chemical Co.). Both portions were vortexed, incubated (15 min at room temp.), centrifuged (5000*g*, 20 min), and analyzed for α-tomatine as described above. Cholesterol forms a 1 : 1 molecular complex with α-tomatine in methanolic solution (Roddick, 1979), and its addition to samples containing α-tomatine should inhibit hemolysis.

To test assumptions concerning the role of α-tomatine in the lamellar-based resistance of PI 134417 to *L. decemlineata*, performance, as measured by the previously defined dietetic indices, was regressed against corresponding values for foliar α-tomatine content. In order to avoid confounding of trichome- and lamellar-based resistance effects on *L. decemlineata* (see Kennedy et al., 1985), only observations using foliage from which glandular type VI trichome glands had been removed were used in this analysis.

RESULTS

Short-Term Experiments: Fourth Instar. When type VI glandular trichomes were intact, RCR_i, RGR_i, and ECI were significantly lower for larvae provided PI 134417 foliage, than for larvae provided cv. Walter or F_1 foliage (Table 1). When the type VI trichome glands were removed by washing, plant line had no significant effect on either RCR_i, RGR_i, or ECI of *L. decemlineata* provided foliage from either field- or greenhouse-grown plants. Removal of type VI trichome glands consistently and significantly improved the RCR_i, RGR_i and ECI of larvae provided PI 134417 foliage. The removal of type VI trichome glands on cv. Walter foliage enhanced the RCR_i but not the RGR_i and ECI of *L decemlineata*; this effect was not seen with greenhouse grown plants. Removal of trichome glands did not affect insect performance on F_1 foliage.

Short-Term Experiments: First Instar. Results of first-instar experiments were similar in all essential details to the results of fourth-instar experiments. In the presence of type VI trichome glands, dietetic indices, except for the ECI of larvae fed field-grown foliage, were lower for larvae provided PI 134417

foliage. In the noted exception, the ECI of larvae fed F_1 foliage was higher than the ECI of larvae fed cv. Walter or PI 134417 foliage (Table 2). When type VI trichome glands were removed, the dietetic indices of larvae provided PI 134417 foliage were always as high or higher than the corresponding indices of larvae provided cv. Walter or F_1 foliage. While removal of type VI trichome glands did not, in general affect the performance of larvae fed cv. Walter or F_1 foliage, ECI was higher and RCR_i lower for first-instar larvae provided field-grown

TABLE 1. MEAN DIETETIC INDICES OF FOURTH-INSTAR *L. decemlineata* IN 24 HR FEEDING EXPERIMENTS, USING FOLIAGE FROM FIELD-GROWN AND GREENHOUSE-GROWN PLANTS

		RCR_i		RGR_i		ECI	
Planting location	Plant line	Trichomes present	Trichomes absent	Trichomes present	Trichomes absent	Trichomes present	Trichomes absent
Field	Walter	1.777 An[a]	2.228 Am	0.682 Am	0.779 Am	0.329 Am	0.343 Am
	F_1	1.706 Am	1.971 Am	0.610 Am	0.781 Am	0.239 Am	0.324 Am
	PI 134417	0.294 Bn	2.200 Am	−0.115 Bn	0.818 Am	−0.336 Bn	0.338 Am
Greenhouse	Walter	1.958 Am	1.782 Am	0.806 Am	0.641 Am	0.392 Am	0.329 Am
	F_1	1.478 Bm	1.788 Am	0.450 Bm	0.615 Am	0.239 Bm	0.320 Am
	PI 134417	0.269 Cn	1.895 Am	−0.231 Cn	0.588 Am	−0.561 Cn	0.258 Am

[a] Vertical means (capital letters) separated, within planting location, by protected LSD (Snedecor and Cochran, 1980) ($P > 0.05$). Horizontal means (lowercase letters) separated by F test ($P > 0.05$) within planting location.

TABLE 2. MEAN DIETETIC INDICES OF FIRST-INSTAR *L. decemlineata* IN 24 HR FEEDING EXPERIMENTS USING FOLIAGE FROM FIELD-GROWN AND GREENHOUSE-GROWN PLANTS

		RCR_i		RGR_i		ECI	
Planting location	Plant line	Trichomes present	Trichomes absent	Trichomes present	Trichomes absent	Trichomes present	Trichomes absent
Field	Walter	3.290 Am[a]	2.372 Bn	0.816 Am	0.827 Bm	0.268 Bn	0.376 ABm
	F_1	2.666 Bm	2.156 Bn	0.890 Am	0.845 Bm	0.350 An	0.424 Am
	PI 134417	1.427 Cn	2.765 Am	−0.395 Bn	0.949 Am	0.260 Bn	0.353 Bm
Greenhouse	Walter	2.214 Am	2.166 Am	0.721 Am	0.719 Am	0.382 Am	0.337 Am
	F_1	2.084 Am	2.054 Am	0.491 Bn	0.655 Am	0.229 Bn	0.307 Am
	PI 134417	0.566 Bn	2.231 Am	−0.267 Cn	0.685 Am	−0.575 Cn	0.326 Am

[a] Vertical means (capital letters) separated, within planting location, by protected LSD (Snedecor and Cochran, 1980) ($P > 0.05$). Horizontal means (lowercase letters) separated by F test ($P > 0.05$) within planting location.

foliage lacking trichome glands As in the fourth-instar experiments, removal of type VI trichome glands always increased the RCR_i, RGR_i and ECI of larvae provided PI 134417 foliage compared to the same indices for larvae provided cv. Walter or F_1 foliage.

Long-Term Experiments. Total amount of foliage (mg dry weight) consumed by *L. decemlineata*, reared from neonate to prepupae, was significantly affected by plant line in both the presence and absence of type VI trichome glands (Table 3). The presence of type VI trichomes significantly reduced the total amount of foliage consumed by larvae provided F_1 and PI 134417 foliage but did not affect the amount of foliage consumed by larvae provided cv. Walter foliage. The ECI of *L. decemlineata*, over the entire larval period, also was affected by plant line in the presence and absence of type VI trichome glands. When these glands were present, ECI was significantly lower for larvae provided PI 134417 foliage than for larvae provided either F_1 or cv. Walter foliage (Table 3). When type VI trichome glands were absent, the ECI of larvae provided PI 134417 foliage was lower than that of larvae provided cv. Walter foliage, but not lower than the ECI of larvae provided F_1 foliage. The presence of glandular type VI trichomes significantly lowered the ECI of larvae provided F_1 and PI 134417 foliage, but not that of larvae provided cv. Walter foliage. Survival of larvae to prepupal stage in the presence of type VI trichome glands was lower for larvae provided F_1 and PI 134417 foliage than for larvae provided cv. Walter foliage (Table 3). When these glands were removed, survival of larvae provided PI 134417 foliage was lower than survival of larvae provided cv. Walter foliage but not lower than the survival of larvae provided F_1 foliage.

TABLE 3. MEAN FOLIAGE CONSUMED (FC[a]), ECI, AND MEAN PERCENT SURVIVAL OF *L. decemlineata* REARED FROM NEONATE TO PREPUPAE ON FOLIAGE FROM INDICATED PLANT LINE

	FC		ECI		Percent survival to prepupal stage	
Plant line	Trichomes present	Trichomes absent	Trichomes present	Trichomes absent	Trichomes present	Trichomes absent
Walter	42.36 Am[b]	39.93 Am	0.031 Am	0.042 Am	27.5 Am	30.0 Am
F_1	17.03 Bn	28.83 Bm	−0.095 Bn	−0.016 ABm	11.3 Bm	15.0 ABm
PI 134417[c]	0.57 Cn	16.76 Cm	−0.331 Cn	−0.042 Bm	0 Bm	10.0 Bm

[a] Measured as mg dry weight foliage consumed over the entire test period.
[b] Vertical means (capital letters) separated by protected LSD (Snedecor and Cochran, 1980) ($P > 0.05$). Horizontal means (lowercase letters) separated by F test ($P > 0.05$).
[c] Although all larvae died before becoming prepupae, calculation of FC and ECI was possible since most larvae consumed some foliage before dying.

For a given plant line, the presence of type VI trichome glands did not significantly affect the number of larvae surviving to the prepupal stage.

α-Tomatine Analysis. When grown under field conditions α-tomatine content differed significantly among the plant lines test. α-Tomatine was higher in foliage from F_1 plants than in foliage from cv. Walter or PI 134417 plants and lowest in foliage from PI 134417 plants. There was no significant difference, among plant lines, in the α-tomatine content of foliage from greenhouse-grown plant lines. Analysis of foliage from greenhouse-grown plants used in long-term experiments yielded patterns of α-tomatine content resembling those of similarly grown plants from short-term experiments (Table 4). The α-tomatine content of F_1 foliage in these tests was significantly higher than that of either PI 134417 or cv. Walter foliage. Levels of α-tomatine found in the foliage of all greenhouse-grown plant lines and in foliage from F_1 plants grown in the field were consistent with α-tomatine levels found, by other researchers, to inhibit feeding and growth of *L. decemlineata* (Sturcköw and Löw, 1961; Sinden et al., 1978; Hare, 1987).

Hemolysis of sheep red blood cells (as indicated by optical density) by α-tomatine in standard solution was linear over the range of concentrations tested ($y = 0.0323 + 0.0044x$, $R^2 = 0.96$, $P > F = 0.05$; short-term tests: $y = 0.0215 - 0.0018x$, $R^2 = 0.95$, $P > F = 0.05$; long-term tests). Recovery of α-tomatine was high but variable (98.34 ± 13.78% in short term experiments; 94.35 ± 20.9% in long-term experiments). There was no significant difference in the percent recovery of α-tomatine among plant lines tested. Results of the cholesterol inhibition test (Table 5) demonstrate that the addition of cholesterol to standards and/or samples containing α-tomatine quenches the production of color in this assay. The results of this test provide confirmation that α-tomatine is the principal agent of hemolytic activity in the samples examined (Juvick and Stevens, 1982).

TABLE 4. MEAN α-TOMATINE CONTENT (mg/g DRY WEIGHT) OF FOLIAGE USED IN SHORT AND LONG-TERM EXPERIMENTS

	Planting location		
	Short-term experiments		Long-term experiments
Plant line	Field	Greenhouse	Greenhouse
Walter	10.94 B[a]	27.76 A	22.91 B
F_1	14.42 A	27.25 A	26.26 A
PI 134417	5.14 C	22.98 A	19.73 B

[a] Vertical means separated by protected LSD (Snedecor and Cochran, 1980)($P > 0.05$).

TABLE 5. RESULTS OF CHOLESTEROL INHIBITION TEST

Plant line/ treatment[a]	OD540[b] Without added cholesterol	OD540[b] With added cholesterol[c]
Walter	0.190	0.016
F_1	0.205	0.017
PI 134417	0.143	0.018
Standard[d]	0.209	0.022
Blank[e]	0.018	0.022

[a] Test solutions prepared as described in text.
[b] Optical density at 540 nm.
[c] 1 mg/ml of test solution.
[d] 0.20 mg α-tomatine/ml MeOH.
[e] MeOH, no α-tomatine or plant material.

Regression Analysis. Regression analysis demonstrated no significant relationship between *L. decemlineata* performance, as measured by RCR_i, RGR_i, and ECI, in short-term feeding experiments (Table 6) or, as measured by FC and ECI, in long-term feeding experiments (Table 7) and α-tomatine content of foliage provided to larvae.

DISCUSSION

Results of the short-term experiments indicate that poorer performance of *L. decemlineata* fed PI 134417 foliage from field- and greenhouse-grown plants can be explained largely by the presence of type VI trichome glands. When foliage is divested of these glands, dietetic indices of *L. decemlineata* fed PI 134417 foliage are as high or higher than dietetic indices of larvae fed F_1 or cv. Walter foliage (Tables 1 and 2). There is no evidence, in these short-term experiments, that PI 134417 foliage, divested of glandular trichomes, is deterrent or repellent to *L. decemlineata* since, in the absence of type VI trichome glands, larvae consume as much or more PI 134417 foliage as they do F_1 or cv. Walter foliage. Neither do these short-term tests provide evidence that PI 134417 foliage, in the absence of type VI trichome glands, is acutely toxic to *L. decemlineata* larvae. Growth rate and ECI of larvae provided PI 134417 foliage divested of trichome glands are at least as great as the growth rate and ECI of larvae provided similarly prepared F_1 and cv. Walter foliage (Tables 1 and 2).

Results of the long-term feeding experiments clearly demonstrate that in

TABLE 6. STATISTICS FOR REGRESSION OF *L. decemlineata* PERFORMANCE AGAINST FOLIAR α-TOMATINE CONTENT IN SHORT-TERM (24 HR) EXPERIMENTS

Experiment	Dietetic index	Regression statistic	Planting location	
			Field	Greenhouse
Fourth instar	RCR_i	R^2	0.001	0.148
		Slope	0.003	0.042
		$P > F$	0.8888	0.5230
	RGR_i	R^2	0.047	0.322
		Slope	0.007	0.027
		$P > F$	0.3863	0.3177
	ECI	R^2	0.023	0.002
		Slope	0.002	−0.001
		$P > F$	0.5458	0.9398
First instar	RCR_i	R^2	0.062	0.004
		Slope	−0.026	−0.006
		$P > F$	0.3355	0.8210
	RGR_i	R^2	0.001	0.080
		Slope	−0.001	0.008
		$P > F$	0.8963	0.3270
	ECI	R^2	0.066	0.233
		Slope	−0.005	0.005
		$P > F$	0.3187	0.0802

TABLE 7. STATISTICS FOR REGRESSION OF *L. decemlineata* PERFORMANCE AGAINST FOLIAR α-TOMATINE CONTENT IN LONG-TERM (THROUGHOUT LARVAL PERIOD) EXPERIMENTS

Dietetic index	Regression statistic	Planting location, Greenhouse[a]
FC	R^2	0.003
	Slope	−0.022
	$P > F$	0.8456
ECI	R^2	0.012
	Slope	0.010
	$P > F$	0.6693

[a] Foliage from field-grown plants was not used in long-term experiments.

both the presence and the absence of type VI glandular trichomes, PI 134417 is resistant to *L. decemlineata* as compared to *L. esculentum*: consumption, ECI, and survival to prepupa are all lower for *L. decemlineata* provided PI 134417 foliage divested of glandular trichome exudate compared to similarly prepared cv. Walter foliage.

However, regression analysis demonstrated that differences in *L. decemlineata* performance observed in feeding experiments were not associated with variation in foliar α-tomatine content (Tables 6 and 7). Examination of mean foliar α-tomatine content (Table 4) and measures of performance for larvae in short- (Table 2) and long- (Table 3) term feeding experiments provide further evidence. In short-term feeding experiments, foliar α-tomatine content varies significantly among plant lines (ca. 2.5-fold). Despite these differences in α-tomatine content, no differences in performance of *L. decemlineata* larvae were observed. Conversely, in long-term experiments, significant differences in the performance of *L. decemlineata* provided cv. Walter and PI 134417 foliage were demonstrated, even though α-tomatine content of foliage from these plant lines did not differ.

Mitchell and Harrison (1985) examined the role of the sensory system in the feeding deterrence of α-tomatine and other steroidal glycoalkaloids to *L. decemlineata*. They demonstrated that although steroidal glycoalkaloids, including α-tomatine clearly damage chemosensilla, there was no evidence for the existence of cells, in the galeal or tarsal sensilla of *L. decemlineata* capable of providing differential sensitivity to the alkaloids tested. Although deterrent-specific receptors are not essential for disruption of feeding behavior (Schoonhoven, 1982, Mitchell and Sutcliffe, 1984), Mitchell (1987) and Harrison and Mitchell (1988) found no evidence that α-tomatine acted in any capacity to reduce feeding by *L. decemlineata*. Harrison and Mitchell (1988) suggested that rather than acting to modify feeding behaviorally, α-tomatine may disrupt feeding by exerting toxic effects. Our results demonstrate that the lamellar-based resistance of PI 134417 to a tomato-adapted *L. decemlineata* population does not result from deterrent or acute antibiotic properties of α-tomatine. Further, they provide no evidence that α-tomatine exerts a chronic, antibiotic effect that could be associated with this resistance.

Previous work linking α-tomatine to feeding deterrence against *L. decemlineata* has been performed using relatively artificial systems. α-Tomatine has been found to deter feeding by *L. decemlineata* when offered to the insects in artificial diets (Hare, 1987; Hsiao and Fraenkel, 1968) or when using potato (Sturköw and Löw, 1961; Barbour, 1987) and tomato (Sinden et al., 1978) foliage coated or infiltrated with α-tomatine. To our knowledge, in the only previous experiments in which in planta α-tomatine levels were determined for foliage provided to *L. decemlineata* (Sinden et al., 1978), measurements of consumption were confounded by differences in photosynthate levels in plants

tested and by the presence of type VI trichome glands, and hence 2-tridecanone, on PI 134417 foliage. It is possible that the effects of α-tomatine observed *ex planta* are simply not manifest *in planta.*

α-Tomatine is known to form a stable 1 : 1 molecular complex with steroids having a free 3-β-hydroxyl group (Schultz and Sanders, 1957; Roddick, 1979). Alleviation of α-tomatine-induced toxicity by addition of steroids to artificial diets has been shown for both herbivorous insects (see reviews by Dimock and Tingey, 1985; Duffey and Bloem, 1986) and insect parasitoids (Campbell and Duffey, 1981; Duffey and Bloem, 1986). Whether the feeding deterrent properties of α-tomatine are likewise affected is not known. It also has been demonstrated that addition of protein to diets containing α-tomatine increases the growth rates of *L. decemlineata* larvae compared to the growth rates of larvae fed a similar diet containing α-tomatine but without additional protein (Hare, 1987). Both the trichome-and lamellar-based resistance of PI 134417 can be reduced dramatically by increasing the rate and/or frequency of fertilizer applications (Barbour et al., 1990; see also Hare and Dodds, 1987 and Janssen and Smilowitz, 1986a,b for similar work in tomato and potato, respectively).

Compared to potato, tomato is a poor host for *L. decemlineata* (Latheef and Harcourt, 1972; de Wilde and Hsiao, 1981). α-Tomatine has been found in all *Lycopersicon* accessions assayed but has not been found in *S. tuberosum* accessions (Roddick, 1979). Our work was conducted with tomato-adapted *L. decemlineata* and included no plant line completely lacking α-tomatine. Therefore, the role of α-tomatine, if any, in the relative quality of *Lycopersicon* and *S. tuberosum* as hosts for *L. decemlineata* is not addressed by our experiments. However, in view of the lack of a deterrent or antibiotic response to α-tomatine of the tomato-adapted *L. decemlineata* used in this study, and the absence of a deterrent response to α-tomatine of beetles from several *L. decemlineata* populations (including both potato and tomato adapted populations) tested by Mitchell and Harrison (1988), raises questions concerning the role of α-tomatine in the relative suitability of *L. esculentum* and *S. tuberosum* as hosts for *L. decemlineata* merit further study.

In this study, no difference in *L. decemlineata* performance could be attributed to variation in the foliar α-tomatine content of tomato plant lines expressing different levels of the alkaloid. These results appear to be at odds with much of the previous work demonstrating that α-tomatine inhibits feeding and growth in *L. decemlineata* (but see Mitchell and Harrison, 1985; Mitchell, 1987; Harrison and Mitchell, 1988). However, the expression of α-tomatine-based resistance in tomato may depend on concurrent levels of phytosteroids, proteins, and/or other as yet undetermined factors. Although demonstrated to have a genetic basis (Juvick and Stevens, 1982), foliar α-tomatine content is also sensitive to environmental factors, as demonstrated in the present study (see also Sinden et al., 1978). It is difficult to imagine how a compound, whose expres-

sion and effects are so readily influenced by its immediate environment, could be reliably useful in the protection of cultivated crops from insect pests.

Acknowledgments—We wish to thank D. Bumgarner, V. Covington, and E. Eckenrode for their technical assistance; S. Chilton and R. M. Roe for providing material, equipment, and advice towards the completion of study; and S. Chilton, R. M. Roe, and J. Walgenbach for their comments on earlier drafts of this document. This research was funded in part by grant 85-CRCR-1-1615 from the USDA Competitive Research Grants Program and the North Carolina Agricultural Research Service, Raleigh.

REFERENCES

ARNESON, P.A., and DURBIN, R.D. 1968. Studies on the mode of action of tomatine as a fungitoxic agent. *Plant Physiol.* 13:683–686.

BARBOUR, J.D. 1987. Investigation of the role of α-tomatine in the lamellar-based resistance of *Lycopersicon hirsutum* f. *glabratum* C.H. Mull to the Colorado potato beetle. Masters thesis. North Carolina State University. Raleigh, North Carolina 132 pp.

BARBOUR, J.D., FARRAR, R.R., and KENNEDY, G.G. 1990. Interaction of fertilizer regime with host-plant resistance in tomato. *Entomol. Exp. Appl.* In press.

BUHR H. 1956. Problems of breeding for resistance to the potato beetle. *Pflanzenschutzkong, Berlin.* pp. 105–130.

BUHR, H., TOBALL, T., and SCHREIBER, K. 1958. The effect of several plant-derived compounds, particularly alkaloids, on the development of larvae of the Colorado potato beetle (*Leptinotarsa decemlineata* Say). *Entomol. Exp. Appl.* 1:209–224 (in German).

CAMPBELL, B.C., and DUFFEY, S.S. 1981. Alleviation of α-tomatine-induced toxicity to the parasitoid, *Hyposoter exiguae*, by phytosterols in the diet of the host, *Heliothis zea*. *J. Chem. Ecol.* 7:927–946.

DE WILDE J., and HSIAO, T. 1981. Geographic diversity of the Colorado potato beetle and its infestation in Eurasia. pp. 47–68, *in* J.H. Lashomb and R.A. Casagrande (eds.). Advances in potato pest management Hutchinson & Ross, Stroudsburg, Pennsylvania.

DIMOCK, M.B., and TINGEY, W.M. 1985. Resistance in *Solanum* spp. to the Colorado potato beetle: Mechanisms, genetic resources and potential, pp. 79–106, *in* D.N. Ferro and R.H. Voss (eds.). Proceedings of the Symposium on the Colorado Potato Beetle, XVII International Congress of Entomology. Mass Agric. Exp. St. Res. Bull. 704.

DUFFEY, S.S., and BLOEM, K.A. 1986. Plant defense-herbivore-parasite interactions and biological control, pp. 135–183, *in* M. Kogan (ed.). Ecological Theory and Integrated Pest Management Practice. John Wiley & Sons, New York.

DUFFEY, S.S., and ISMAN, M.B. 1981. Inhibition of insect larval growth by phenolics in glandular trichomes of tomato leaves. *Experientia* 37:574–576.

FARRAR, R.R. JR., BARBOUR, J.D., and KENNEDY, G.G. 1989. Quantifying food consumption and growth in insects. *Ann. Entomol. Soc. Am.* 82(5):593–598.

HARE, J.D. 1983. Seasonal variation in plant-insect associations: Utilization of *Solanum dulcamara* by *Leptinotarsa decemlineata*. *Ecology* 64(2):345–361.

HARE, J.D. 1987. Growth of *Leptinotarsa decemlineata* in response to simultaneous variation in protein and glycoalkaloid concentration. *J. Chem. Ecol.* 13(1):39–46.

HARE, J.D. and DODDS, J.A. 1987. Survival of the Colorado potato beetle on virus-infected tomato in relation to plant nitrogen and alkaloid content. *Entomol. Exp. Appl.* 44(3):31–36.

HOFMASTER, R.N. 1977. Insect pests of tomatoes. *Veg. Growers News* 31:1–3.

HARRISON, G.D., and MITCHELL, B.K. 1988. Host-plant acceptance by geographic populations of

the Colorado potato beetle, *Leptinotarsa decemlineata*: Role of Solanaceous alkaloids as sensory deterrents. *J. Chem. Ecol.* 14(3):777–788.

HSIAO, T.S. 1969. Chemical basis of host selection and plant resistance in oligophagous insects. *Entomol. Exp. Appl.* 12:777–788.

HSIAO, T.S. 1981. Ecophysiological adaptations among geographic populations of Colorado potato beetle in North America, pp. 69–85, *in* J.H. Lashomb and R.A. Casagrande (eds.). Advances in Potato Pest management. Hutchinson & Ross, Stroudsburg, Pennsylvania.

HSIAO, T.H., and FRAENKEL, G. 1968. The role of secondary plant substances in the food specificity of the Colorado potato beetle. *Ann. Entomol. Soc. Am.* 6(2):485–493.

JANSSON, R.K., and SMILOWITZ, z. 1986a. Influence of potato persistence, foliar biomass and foliar nitrogen on abundance of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). *Environ. Entomol.* 15:726–732.

JANSSON R.K., and SMILOWITZ, Z. 1986b. Influence of nitrogen on population parameters of potato insects: Abundance, development and damage of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). *Environ. Entomol.* 14:500–506.

JUVICK, J.A. and STEVENS, M.A. 1982. Inheritance of foliar α-tomatine content in tomatoes. *J. Am. Soc. Hortic. Sci.* 107(6):1061–1065.

KENNEDY, G.G., and DIMOCK, M.B. 1983. 2-Tridecanone: A natural toxicant in a wild tomato responsible for insect resistance, pp. 123–128, *in* J. Miyamoto and P.C. Kearney (eds.). Pesticide Chemistry: Human Welfare and the Environment, Vol. 2. Pergamon, Tokyo.

KENNEDY, G.G., and SORENSON, C.E. 1985. The role of glandular trichomes in the resistance of *Lycopersicon hirsutum* f. *glabratum*. *J. Econ. Entomol.* 78:547–551.

KENNEDY, G.G., YAMAMOTO, R.T., DIMOCK, M.B., WILLIAMS, W.G., and BORDNER, J. 1981. Effect of daylength and light intensity on 2-tridecanone levels and resistance in *Lycopersicon hirsutum* f. *glabratum* to *Manduca sexta*. *J. Chem. Ecol.* 7:707–716.

KENNEDY, G.G., ROMANOW, L.R., JENKINS, S.F., and SANDERS, D.C. 1983. Insect and diseases damaging tomato fruits in the Coastal Plains if North Carolina. *J. Econ. Entomol.* 76:168–173.

KENNEDY, G.G., SORENSON, C.E., FERY, R.L. 1985. Mechanisms of resistance to Colorado potato beetle in tomato, pp. 107–116, *in* D.N. Ferro and R.H.Voss (eds.). Proceedings of the Symposium on the Colorado Potato Beetle, XVII International Congress of Entomology Mass. Agric. Exp. St. Bull. No. 704. 144 pp.

KUHN, R., and LÖW, I. 1955. Resistance factors against *Leptinotarsa decemlineata* Say, isolated from the leaves of wild Solanum species, pp. 122–132, *in* M.G. Sevage, R.D. Reid, and D.E. Reynolds (eds.). Origins of resistance to Toxic Agents. Academic Press, New York.

LANGE, W.H. and BRONSON, L. 1981. Insect pests of tomatoes *Annu. Rev. Entomol.* 26:345–371.

LATHEEF, M.A. and HARCOURT, D.G. 1972. A quantitative study of food consumption assimilation and growth in *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) on two host plants. *Can. Entomol.* 104:1271–1276.

LUCKWILL, L.C. 1943. The Genus *Lycopersicon*, An Historical, Biological and Taxonomic Survey of the Wild and Cultivated Tomatoes. Aberdeen University Press Aberdeen, Scotland. 44 pp.

MITCHELL, B.K. 1987. Interactions of alkaloids with galeal chemosensory cells of Colorado potato beetle. *J. Chem. Ecol.* 13(10):2009–2022.

MITCHELL, B.K. and HARRISON, G.D. 1985. Effects of *Solanum* glycoalkaloids on chemosensilla in the Colorado potato beetle: A mechanism of feeding deterrence? *J. Chem. Ecol.* 113(1):73–83.

MITCHELL, B.K. and SUTCLIFFE, J.F. 1984. Sensory inhibition as a mechanism of feeding deterrence by three alkaloids on leaf beetle feeding *Physiol. Entomol.* 9:57–64.

OTTE, D. and JOERN, A. 1977. On feeding patterns in desert grasshoppers and the evolution of specialized diets. *Proc. Acad. Nat. Sci. Philadelphia* 128:89–126.

RODDICK, J.G. 1974. The steriodal glycoalkaloid α-tomatine. *Phytochemistry* 13:9–25.

RODDICK, J.G. 1979. Complex formation between solanaceous steroidal glycoalkaloids and free sterols in vitro. *Phytochemistry* 18:1467–1470.

SCHALK, J.M. and STONER, A.K. 1976. A bioassay differentiates resistance to the Colorado potato beetle on tomato. *J. Am. Soc. Hortic. Sci.* 101:74–76.

SCHALK, J.M., and STONER, A.K. 1979. Tomato production in Maryland: Effect of different densities of larvae and adults of the Colorado potato beetle. *J. Econ. Entomol.* 72:826–829.

SCHOONHOVEN, L.M. 1982. Biological aspects of antifeedants. *Entomol. Exp. Appl.* 31:57–69.

SCHREIBER, K. 1957. Natural plant resistance compounds against the Colorado potato beetle and their possible mode of action. *Züchter* 27:289–299 (in German).

SCHREIBER, K. 1968. Steroid alkaloids: The *Solanum* group, pp. 1–192, *in* R.H.F. Manske (ed.). The Alkaloids. Academic Press, New York.

SCHULTZ, G., and SANDERS, H. 1957. Über Cholesterin-Tomatid eine neue Molekulverbindung zur Analyse und präparativen Gewinnung von Steroiden. *Z. Physiol. Chem.* 308:122–126.

SINDEN, S.L., SCHALK, J.M., and STONER, A.K., 1978. Effects of daylength and maturity of tomato plants on α-tomatine content and resistance to the Colorado potato beetle. *J. Am. Soc. Hortic. Sci.* 103:596–600.

SINDEN, S.L., SANFORD, L.L., and OSMAN, S.F. 1980. Glycoalkaloids and resistance to the Colorado potato beetle in *Solanum chacoense* Bitter. *Am. Potato J.* 57:331–343.

SINDEN, S.L., SANDFORD, L.L., CANTELO, W.W., and DEAHL, K.L. 1986. Leptine glycoalkaloids and resistance to the Colorado potato beetle (Coleoptera: Chrysomelidae) in *Solanum chacoense. Environ. Entomol.* 15:1057–1062.

SNEDECOR, G.W. and COCHRAN, W.M. 1980. Statistical Methods. Iowa State University Press, Ames. 507 pp.

STURKÖW, B., and LÖW I. 1961. Die Wirkung einige *Solanum*-Alkaloidglykoside auf den Kartoffelkafer, *Leptinotarsa decemlineata* (Say). *Entomol. Exp. Appl.* 4:133–142.

WALDBAUER, G.P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol.* 5:229–288.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

BEHAVIORAL AND NEUROSENSORY RESPONSES OF THE BOLL WEEVIL, *Anthonomus grandis* BOH. (COLEOPTERA: CURCULIONIDAE), TO FLUORINATED ANALOGS OF ALDEHYDE COMPONENTS OF ITS PHEROMONE

JOSEPH C. DICKENS[1,*], GLENN D. PRESTWICH,[2] and WEI-CHUAN SUN[2]

[1]*USDA, ARS*
Boll Weevil Research Unit
Mississippi State, Mississippi 39762

[2]*Department of Chemistry*
State University of New York
Stony Brook, New York 11794-3400

(Received October 24, 1990; accepted January 30, 1991)

Abstract—Competitive field tests with α-fluorinated analogs of compounds III and IV (III-α-F and IV-α-F, respectively) of the boll weevil, *Anthonomus grandis* Boh., aggregation pheromone showed these compounds, when combined with the other pheromone components [(±)-I and II], to be as attractive as grandlure [(±)-I, II, and III + IV]. Dose–response curves constructed from electroantennograms of male boll weevils to serial stimulus loads of III, IV, III-α-F, IV-α-F, and the corresponding acyl fluorinated analogs (III-acyl-F and IV-acyl-F) showed the α-fluorinated analogs to be as active as the pheromone components (threshold = 0.1 μg), while the acyl fluorinated analogs had a 10–100 × higher threshold (= 1–10 μg). Single-neuron recordings showed that IV neurons and II neurons (Dickens, 1990) responded to IV-α-F and III-α-F, respectively, while IV-acyl-F and III-acyl-F were inactive. Since a previous study showed compounds I, II, and IV to be essential for behavioral responses in the field, it seems likely that the activity of the α-fluorinated analogs observed here is due to the stimulation of IV neurons by IV-α-F as indicated in single neuron recordings.

Key Words—Pheromone, analog, parapheromone, behavior, neurobiology, olfaction, single neuron, electroantennogram, boll weevil, *Anthonomus grandis*, Coleoptera, Curculionidae.

*To whom correspondence should be addressed.

0098-0331/91/0600-1007$06.50/0

INTRODUCTION

Chemical signals emitted by insects may communicate the location of mates as for sex attractants, or host plants and mates as for aggregation pheromones (Dickens and Payne, 1985). The use of pheromones for detection of emergent insect populations has been valuable for the timing and application of pest control strategies (Mitchell, 1981). Pheromones also have been used to disrupt chemical signaling in indigenous insect populations, e.g., for lepidopterous species (Sanders, 1981) and coleopterous pests (Vité et al., 1976; Richerson et al., 1980), including the boll weevil (Villavaso, 1982). Alternatively, analogs of pheromone molecules have been designed and synthesized and found to inhibit response to the pheromone (Mitchell et al., 1975, 1976; Camps et al., 1990), enhance response to the pheromone (Camps et al., 1988; Bengtsson et al., 1990), or mimic the pheromone component (Camps et al., 1988; Briggs et al., 1986; Eidmann et al., 1986). The acyl fluoride analogs of *Heliothis* pheromone components acted as hyperagonists, eliciting aphrodisia and disorientation in male moths (Prestwich et al., 1986).

Pheromone analogs have potential not only for use in alternative pest control strategies, but also as probes of olfactory mechanisms. For example, functional, geometric, and fluorinated analogs of compound II of the boll weevil aggregation pheromone have been used to elucidate the nature and specificity of receptor sites on identified olfactory neurons (Dickens et al., 1990), while fluorinated analogs of moth pheromones have been used to probe local hydrophobicity requirements of receptor sites on pheromone-sensitive neurons (Sun and Prestwich, 1990; Prestwich et al., 1990).

Fluorinated analogs of bioactive molecules offer the advantage of isosteric substitution of a fluorine atom for a hydrogen atom while modifying the charge distribution of the molecule by the electrophilic nature of the fluorine atom (Pavlath, 1986; Prestwich, 1986). Fluorinated analogs of various molecules have been synthesized, including potential pesticides (Pavlath, 1986) and naturally occurring compounds such as hormones and pheromones (Briggs et al., 1986; Prestwich, 1986, 1987). Acyl and α-fluorinated analogs of the aldehyde components of the boll weevil pheromone were synthesized and evaluated for activity in female boll weevils using electroantennograms (EAGs) (Prestwich et al., 1988). While the α-fluoro analogs were as active as the pheromone components in these tests, the acyl fluorides showed little activity.

Here we sought to evaluate the behavioral activity of the α-fluorinated analogs of the aldehyde components of the boll weevil pheromone and to further elucidate the mechanisms by which these compounds are perceived by olfactory receptors on the boll weevil antenna.

METHODS AND MATERIALS

Insects

Adult *A. grandis* used in field releases and electrophysiological experiments were obtained from a small laboratory colony annually infused with feral insects. Upon emergence, insects were sexed and fed either cotton squares or an artificial diet (Lindig, 1979). Insects were not used until substantial olfactory receptor maturation at four days postemergence (Dickens and Moorman, 1990). For electrophysiological studies, groups of five insects of the same sex were maintained on moist filter paper (Whatman No. 1) in Petri dishes until use at 4–6 days of age. For field experiments, groups of 50 insects of the same sex were held in paper cartons (ca. 0.5 liter) with screen tops. Insects used in field releases were 6–13 days postemergence and were fed cotton (STV-213) at least two days prior to use. All insects were held in incubators at 26°C under a photoregime of 16 hr of light (ca. 700 lux) and 8 hr of darkness.

Chemicals

The boll weevil aggregation pheromone, grandlure, is composed of compounds I [racemic grandisol, (±)-*cis* -2-isopropenyl-1-methylcyclobutaneethanol]; II (*cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol); and III + IV (a 50:50 *cis–trans* mixture of 3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde) (Tumlinson et al., 1969) (Figure 1). Each component was >95% chemically pure as obtained from Albany International, Controlled Release Division, Buckeye, Arizona. Isomeric pure III and IV were obtained by separation of a commercially available mixture of III and IV as previously described (Dickens and Prestwich, 1989; Dickens et al., 1990).

Selectively fluorinated analogs of components of compounds III and IV of the boll weevil pheromone have been described previously (Prestwich et al., 1988). These compounds were: (*E*)- and (*Z*)-3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-α-fluoroacetaldehyde [(*E*vt)- = III-α-F]; [(*Z*)- = IV-α-F]; and (*E*)- and (*Z*)-3,3-dimethylcyclohexane-$\Delta^{1,\alpha}$-acetyl fluoride [(*E*)- = III-acyl-F; (*Z*)- = IV-acyl-F] (Figure 1). While quantities of the individual geometric isomers of the fluorinated compounds were adequate for our electrophysiological experiments, it was necessary to use a mixture of geometric isomers of the α-fluorinated compounds, i.e., III-α-F + IV-α-F, in field tests. Serial dilutions of experimental odorants were prepared in hexane. 1-Hexanol used as a standard in EAG studies was diluted in pentane as in previously reported experiments on boll weevil olfaction (Dickens, 1984; Dickens and Mori, 1989; Dickens and Prestwich, 1989).

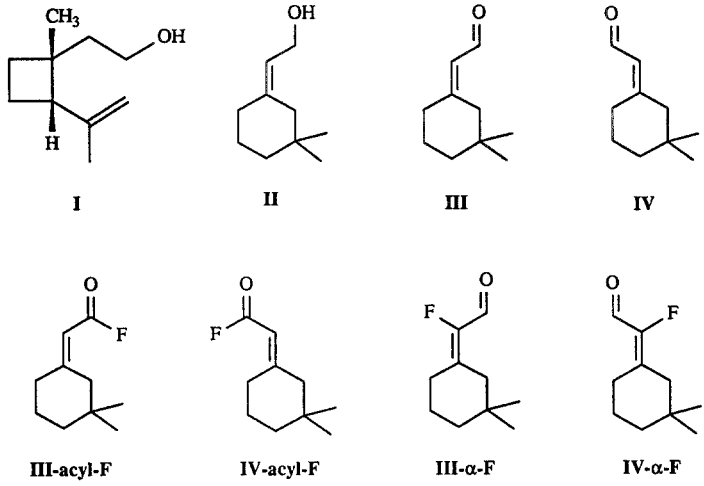


FIG. 1. Structure of boll weevil pheromone components I–IV and fluorinated analogs.

Behavior

Traps used for all field experiments were those currently used by the Boll Weevil Eradication Program (BWEP) and were a modification of a previously described trap (Mitchell et al., 1976).

Competitive Field Tests with Released Weevils. A competitive field test involving released boll weevils in which (±)-I (0.6 mg):II (0.8 mg):III + IV (0.6 mg) (= 2 mg total) in a ratio of 3:4:3 (grandlure) was placed in competition with (±)-I (0.6 mg):II (0.8 mg):III-α-F + IV-α-F (0.6 mg) (= 2 mg total) in the identical ratio. This experiment involved an array of four traps. One trap was placed at each corner of a 20-m square. The two treatments being tested simultaneously in this competitive arrangement were placed alternately in the traps; i.e., the two traps baited with the same treatment were located diagonally across from each other. In order to avoid competition with cotton or pheromone-producing boll weevils, both this initial test and the second series of experiments described below were conducted in open fields located more than 1 mile from cotton fields. Cigarette filters were used as release substrates for the experimental odorants, which had been diluted in 0.5 ml of the following mixture by weight: polyethylene glycol 1000, 20%; methanol, 42.5%; glycerol, 25%; and distilled water, 12.5% (McKibben et al., 1980). The ratio of the pheromone components used in the cigarette filters was that currently used in the BWEP [I(3):II(4):III + IV(3)]. This ratio was chosen for use in the BWEP based on economic considerations even though the ratio of components in the natural pheromone was more attractive in competitive tests (Hardee, 1974). One hundred insects (50 males and 50 females) were released along a

20-m line parallel to each side of a square at a distance of 20 m (400 insects total) at ca. 0900 hr, prior to baiting of the traps at 1200 hr. On the first day, traps were checked and insects removed at 1400 and 1600 hr. On the second day, traps were checked at 0900 hr and their positions rotated at 1630 hr. Following this rotation of the traps, collections were made at one- to four-day intervals for the next 11 days. The same filters remained in the traps for the entire test period.

A second series of experiments also involved an array of four traps. In this experiment, (±)-I, II, III + IV, and III-α-F + IV-α-F diluted in hexane were released from 2.2-ml vials as 10 μg/μl dilutions using glass capillaries (Dickens and Mori, 1989). One vial placed in each trap contained (±)-I (0.4 mg); the second vial in each trap contained either II (0.3 mg) and III + IV (0.1 mg), or II (0.3 mg) and III-α-F + IV-α-F (0.1 mg). The ratio of pheromone components used in this experiment [I(4) : II(3) : III + IV(1)] approximated that of the natural pheromone as identified from the frass of male boll weevils (Tumlinson et al., 1969). One hundred insects (50 males, 50 females) were released along a 20-m line parallel to each side of the square at a distance of 20 m (400 insects total) at 1400 hr. Traps were then immediately baited. In each test, traps were checked and insects removed at 1530 and 1700 hr on the day of release and before 0900 hr the next day, at which time the test was terminated. This test was replicated five times.

Competitive Test in Cotton Field with Indigenous Boll Weevil Population. A third experiment was also a competitive test and involved an indigenous population of boll weevils in a cotton field. This experiment was conducted in October 1989 in a field of mature cotton located at Mississippi State, Mississippi. The experimental design consisted of placing one trap at each corner of a 20-m square within a cotton field. Four additional traps were placed ca. 2 m outside the cotton field at a distance of ca. 40–60 m from the traps in the field and located near imaginary diagonal lines extending through the corners of the square within the field. The result resembled an "X" array of traps placed across the field with each arm of the "X" consisting of four traps, two within and two outside of the field. Cigarette filters prepared as in the first experiment released either (±)-I (0.3 mg), II (0.4 mg), and III + IV (0.3 mg)(1 mg total), or (±)-I (0.3 mg), II (0.4 mg), and III-α-F + IV-α-F (0.3 mg)(1 mg total). Traps within one arm contained (±)-I, II, III + IV; those within the other arm were baited with (±)-I, II, III-α-F + IV-α-F. Insects were collected daily from the traps for six days, at which time the test was terminated.

Electrophysiology

Electroantennograms. EAG techniques were described in detail elsewhere (Dickens, 1979, 1984). Briefly, two glass microcapillaries filled with physiological saline (Pantin, 1948) were inserted into the antenna of an intact animal:

the ground electrode was placed into the distal end of the scape and the recording electrode into the distal end of the club. Electrical contact was provided by a Ag–AgCl wire placed into the shank of each capillary. Electrical activity was amplified by a Grass P-16 DC preamplifier. EAGs were recorded using a stripchart recorder.

Serial dilutions of the pheromone components and their fluorinated analogs were delivered from the lowest to the highest concentration. Odorous stimuli were delivered as 1-μl aliquots placed on filter paper (8 mm $\times$ 18 mm) inserted into glass cartridges (80 mm $\times$ 5 mm ID) oriented toward the preparation from a distance of 1 cm. Molecules evaporating from the filter paper were carried over the preparation by hydrocarbon-free air, which also had been filtered and dried. Stimulus duration was 1 sec. Three minutes were allowed between stimuli at lower doses; 5–7 min were allowed at doses greater than 1 μg. These times were adequate for complete recovery of the EAG (Dickens, 1984).

Three replicates were recorded for serial dilutions of each odorant for both male and female *A. grandis*. 1-Hexanol (100 μg; 10 μl of 10 μg/μl dissolved in pentane) served as a standard to: (1) normalize responses; (2) control for viability and constancy of the preparation; and (3) allow relative comparisons with previously recorded species (Dickens, 1984; Dickens and Boldt, 1985). Stimulation with the standard both preceded and followed each serial dilution level. Although preparations were stimulated with a solvent control, seldom was a response noted. EAGs to test chemicals were expressed as a percent of the mean of the two nearest responses to the standard (Dickens, 1981, 1984). The threshold of response was considered to be the stimulus load at which a statistically significant response was noted.

Single-Neuron Recordings. Single-neuron recording techniques are described in detail elsewhere (Dickens, 1979, 1990; Dickens and Mori, 1989). In brief, microelectrodes were constructed from 50.8-μm-diameter tungsten wire electrolytically sharpened to a tip of 1–2 μm. The recording electrode was positioned near the proximal edge of one of the three sensory bands encircling the club. The ground electrode was inserted in the distal end of the scape. Action potentials were amplified by a Grass P-15 preamplifier and stored on cassette tapes using a Teac R51-D data recorder. Action potentials were analyzed using a microcomputer equipped with software designed for the acquisition and analysis of insect chemoreceptor data (SAPID; Department of Entomology, University of Alberta, Edmonton, Alberta, Canada).

Upon obtaining a single neuron preparation, the first two stimuli tested were volatiles emanating from 1 μg of grandlure, followed by volatiles emanating from a crushed cotton square. These two stimuli served to determine whether the neuron was activated by the aggregation pheromone of the boll weevil and/or volatiles emanating from its host and food source. If the neuron were activated by grandlure, stimulation with 1-μg doses of the individual pher-

omone components followed. Once a neuron was found to be responsive to II, III, or IV, stimulation with the various analogs followed. Serial dilutions were delivered from the lowest to the highest concentration.

Apparatus for stimulus delivery and airflow were the same as described for EAGs. Stimulus duration was 0.5 sec with an airflow of ca. 1 liter/min. Two or three minutes were allowed between each stimulation. The number of impulses given by a neuron in response to a stimulus was tabulated over the 0.5-sec stimulation period.

Statistical Analyses

EAGs were evaluated by analysis of variance (Ostle, 1963). Where differences were noted among compounds, comparisons were made using Duncan's new multiple-range test (Duncan, 1955).

Numbers of released weevils captured using the four-trap array in the second field experiment were analyzed for significant differences by a paired t test (Ostle, 1963). Competitive tests in cotton fields with natural boll weevil populations also were evaluated by a paired t test. Daily trap captures of boll weevils were compared over the six-day test period.

RESULTS

Behavior

Competitive Field Tests with Released Weevils. Results of our initial field test with released weevils indicated that III-α-F and IV-α-F could be substituted for compounds III and IV of the boll weevil aggregation pheromone in a slow-release formulation without loss of activity over the 13-day test period (Table 1A). Not only were numbers of insects captured by each treatment nearly identical, but the sex ratios were also similar.

A second field test with released weevils, which utilized a different method of pheromone delivery and a shortened test period, showed no significant difference in either the number of weevils captured in traps or the sex ratio of responding weevils ($P < 0.05$) (Table 1B).

Competitive Field Tests with Indigenous Population of Weevils. Our results showed that the combination of ($\pm$)-I, II, and III-α-F + IV-α-F captured as many insects as ($\pm$)-I, II, and III + IV in cotton fields (Table 1C). In fact, the numbers of weevils captured each day of the six-day test period were nearly identical except that the combination with the fluorinated compounds was still active on the final day while the combination with pheromone components was not (Figure 2).

TABLE 1. COMPETITIVE FIELD TESTS WITH RELEASED BOLL WEEVILS USING SLOW-RELEASE FORMULATION OVER AN EXTENDED PERIOD, 13 DAYS, (A), AND SECOND METHOD OF RELEASE REPLICATED SEVERAL TIMES OVER A SHORTER PERIOD (B), AND COMPETITIVE FIELD TEST USING SLOW-RELEASE FORMULATION IN COTTON FIELD WITH INDIGENOUS POPULATION OF BOLL WEEVILS (C)

A. Total number of boll weevils captured in a competitive field test of (±)-I, II, III + IV vs. (±)-I, II, III-α-F + IV-α-F in four-trap array. Test was conducted over 13-day test period (May 14–26, 1988) and utilized slow release formulation.

Treatment	Total trap capture	Sex ratio (M/F)
(±)-I, II, III + IV (2 mg)	101	0.61:1
vs		
(±)-I, II, III-α-F + IV-α-F (2 mg)	102	0.65:1

B. Mean number of boll weevils captured in five replicates of competitive field test in four-trap array with (±)-I, II, III + IV vs. (±)-I, II, III-α-F + IV-α-F. Tests were conducted from May 16–September 23, 1988.

Treatment pair	Mean ± SE weevils/replicate	Sex ratio (M/F)
(±)-I, II, III + IV (0.8 mg)	35.6 ± 10.0	0.80:1
vs		
(±)-I, II, III-α-F + IV-α-F (0.8 mg)	25.4 ± 7.6	1.44:1

C. Mean (±SE) daily trap capture of boll weevils in competitive field tests in cotton field with traps baited with (±)-I, II, III + IV or (±)-I, II, III-α-F + IV-α-F. Test was conducted over six days (October 27–November 1, 1989) and utilized slow release formulation.

Treatment	Mean ± SE daily weevil capture	Sex ratio (M/F)
(±)-I, II, III + IV (1 mg)	5.7 ± 2.03	0.48:1
vs		
(±)-I, II, III-α-F + IV-α-F (1 mg)	5.7 + 1.25	1.00:1

Electrophysiology

Electroantennograms. Dose–response curves constructed from EAGs of male boll weevils to III and IV and their fluorinated analogs show significant differences in activity of the various compounds (Figure 3). IV and IV-α-F were the most active compounds tested with responses to either compound not differing significantly except at the highest stimulus load tested. EAGs in response to III-α-F did not differ from those to IV-α-F at any stimulus load. III, III-acyl-

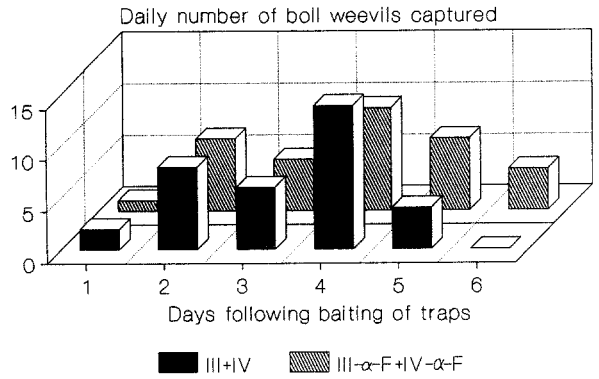


FIG. 2. Daily number of boll weevils captured in traps in cotton fields in traps baited with the slow-release formulation of (+)-I (0.3 mg), II (0.4 mg), and III + IV (0.3 mg) (III + IV in figure) versus (+)-I (0.3 mg), II (0.4 mg), III-α-F + IV-α-F (0.3 mg) (III-α-F + IV-α-F in figure).

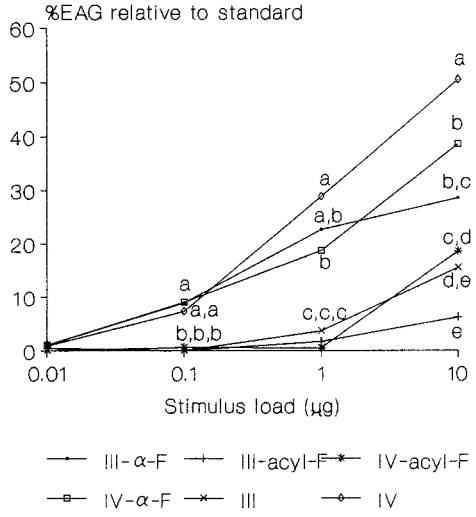


FIG. 3. Dose–response curves constructed from EAGs of male boll weevils to serial stimulus loads of III-α-F, IV-α-F, III-acyl-F, IV-acyl-F, III, and IV. Means followed by different letters are significantly different at that stimulus load.

F, and IV-acyl-F had a threshold stimulus load of 1–10 μg and were thus considerably less active than the other compounds except for IV-acyl-F, which was as active as III-α-F at the highest dose tested ($P < 0.05$). EAGs of female boll weevils to III, IV, and their α-and acyl fluorinated analogs have been reported previously (Prestwich et al., 1988).

Single-Neuron Recordings. While neurons responsive to IV are infrequently encountered in single-neuron recordings from boll weevil antennae (ca. 2% of neurons recorded are responsive to IV) (Dickens, 1990), responses of one IV neuron associated with a type I sensillum within the sensory band region of an *A. grandis* female was recorded to serial dilutions of IV, IV-α-F, and IV-acyl-F (Figure 4A). Responses of this single neuron substantiated the EAG results by clearly showing that IV-α-F could stimulate the IV neuron while IV-acyl-F was inactive.

Since previously reported single-neuron recordings from boll weevil antennae revealed no specialized neurons for III (Dickens, 1990), II neurons that were shown to respond to a lesser degree to III were stimulated with the fluorinated analogs of III and IV (III is the aldehydic analog of II). Results from neurons in a male and a female showed III-α-F to be nearly as active as III, while III-acyl-F and IV showed little activity (Figure 4B).

DISCUSSION

Behavior

The ability of III-α-F + IV-α-F to substitute for III + IV in the boll weevil pheromone blend is clearly demonstrated in our field experiments involving both released and indigenous boll weevil populations (Table 1). This is the first report of a compound capable of substituting for a boll weevil pheromone component and, to our knowledge, the only report of a fluorinated analog as active as the targeted pheromone component in Coleoptera. A previous report of boll weevil pheromone analogs had shown that certain derivatives may inhibit boll weevil pheromone responses in laboratory behavioral bioassays (Hedin et al., 1985). Although a 2-fluorinated analog (4-methyl-2-fluoro-3-heptanol) of the pheromone component, 4-methyl-3-heptanol, in the European elm bark beetle, *Scolytus multistriatus*, was active in EAG and laboratory bioassays, the analog showed little activity in field tests (Pignatello and Grant, 1982).

Electrophysiology

The activity and similarity in dose–response curves for IV and IV-α-F, as well as the lack of activity of the acyl fluorinated analogs, in EAG tests is not surprising (Figure 3). Similar results were found for females in a previous study (Prestwich et al., 1988). The activities of these analogs is further elucidated in our current study by results of our single-neuron recordings (Figure 4). The IV neuron recorded responded with increasing doses of IV and IV-α-F, while little response was elicited by IV-acyl-F even at the highest dose tested. Similarly, III and III-α-F elicited significant numbers of impulses from II neurons, while

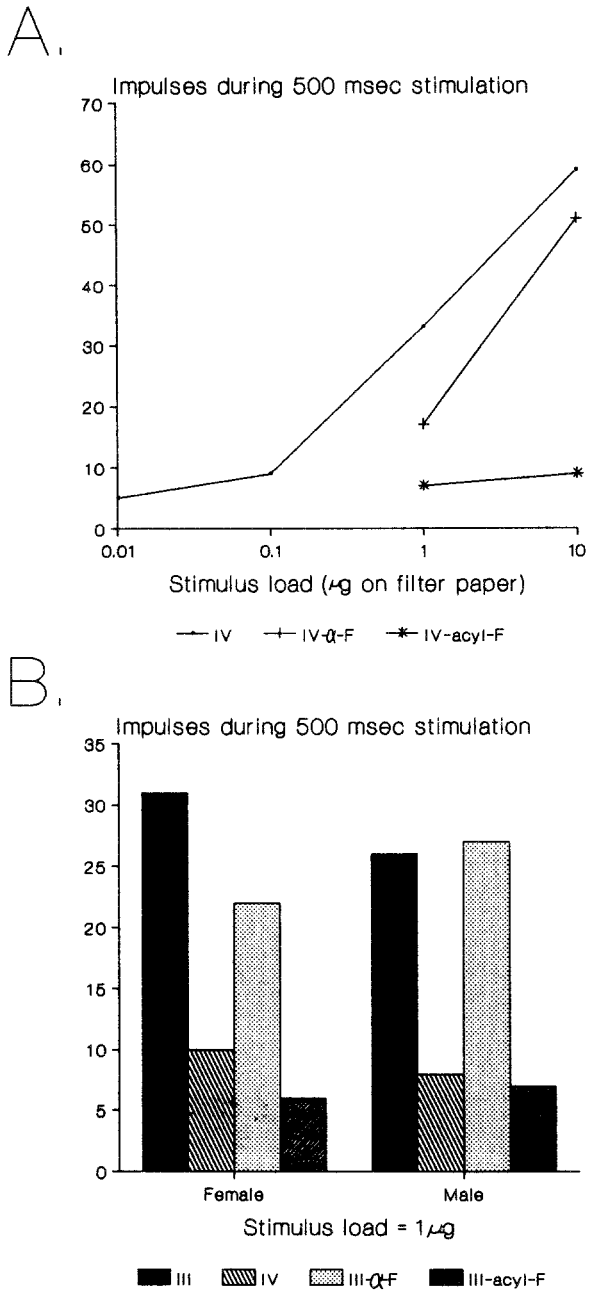


FIG. 4. (A) Dose–response curve of a IV neuron associated with a type I sensillum in a male boll weevil to serial stimulus loads of IV, IV-α-F, and IV-acyl-F. (B) Number of impulses elicited from II neurons associated with a type I sensilla in male and female boll weevils by 1 μg stimulus loads of III, IV, III-α-F, and III-acyl-F.

III-acyl-F was inactive. We recognize that stimulus loading and not the number of molecules available for interaction with responsive receptors is being compared in our study. The number of molecules of a compound reaching the receptors would be a result of its volatility and release rate from the filter paper. A measure of volatility, Kovats indices (Kovats, 1958), calculated for each of the compounds (III = 1253; III-α-F = 1211; III-acyl-F = 1167; IV = 1261; IV-α-F = 1217; IV-acyl-F = 1170) shows them to differ by less than one methylene group. Thus, it seems that the large differences observed in activity of the compounds is largely due to differences in their interaction with the receptors.

The lack of activity of the acyl fluorides is in contrast with the activity of the acyl fluoride analogs of the aldehyde components of the pheromone of *Heliothis virescens* (Prestwich et al., 1986). Despite the ease of hydrolysis of saturated acyl fluorides to the corresponding acids, conjugated unsaturated acyl fluorides are quite stable. For example, a 3-year-old sample of the mixed III-acyl-F and IV-acyl-F still contained 25% acyl fluoride and 75% acid. Nonetheless, we cannot rule out the unlikely possibility that the low activity of the acyl fluoride of the boll weevil pheromone is due in part to selective hydrolysis in the receptor lymph.

In conclusion, the α-fluorinated analogs of the boll weevil pheromone components, III and IV, are active in stimulating identified neurons sensitive to the pheromone components. Furthermore, a mixture of these geometric isomers combined with the other pheromone components, ($\pm$)-I and II, is as active as the combination with III + IV in behavioral tests in the field. Since boll weevils require only compounds (+)-I, II, and IV for attraction to pheromone traps in the field (Dickens and Prestwich, 1989), it seems likely that the mechanism for this attraction is the stimulation of IV neurons (Dickens, 1990) by IV-α-F as indicated in single neuron recordings.

As a final note, the α-fluorinated analogs are substantially more stable than III + IV, as indicated by our experience in chemical oxidation of the α-fluorinated compounds, which require more vigorous conditions than chemical oxidation of the parent compounds, III + IV (Prestwich et al., 1988). Thus the α-fluorinated analogs of III + IV may be useful as substitutes for these pheromone components in baits for survey traps used in the Boll Weevil Eradication Program and in an attracticide bait that is currently being developed.

Acknowledgments—The authors acknowledge critical reviews of the manuscript provided by: Dr. A.R. Alford, Department of Entomology, University of Maine, Orono, ME; Dr. W.L. Parrott, USDA, ARS, Crop Science Research Laboratory, and Dr. W.L. McGovern, USDA, ARS, Boll Weevil Research Unit, Mississippi State, MS. JCD thanks E. Mattix and D. Wu for technical assistance. GDP and WCS thank the Herman Frasch Foundation for support of the chemical synthesis.

REFERENCES

BENGSSTON, M., RAUSCHER, S., and ARN, H. 1990. Fluorine-substituted pheromone components affect the behavior of the grape berry moth. *Experientia*. In press.

BRIGGS, G.G., CAYLEY, G.R., DAWSON, G.W., GRIFFITHS, D.C., MACAULAY, E.D.M., PICKETT, J.A., PILE, M.M., WADHAMS, L.J., and WOODCOCK, C.M. 1986. Some fluorine-containing pheromone analogs. *Pestic. Sci.* 17:441–448.

CAMPS, F., FABRIAS, G., GASOL, V., GUERRERO, A., HERNANDEZ, R., and MONTOYA, R. 1988. Analogs of sex pheromone of processionary moth, *Thaumetopoea pityocampa:* Synthesis and biological activity. *J. Chem. Ecol.* 14:1331–1346.

CAMPS, F., GASOL, V., and GUERRERO, A. 1990. Inhibitory pheromonal activity promoted by sulfur analogs of the sex pheromone of the female processionary moth *Thaumetopoea pityocampa* (Denis and Schiff). *J. Chem. Ecol.* 16:1155–1172.

DICKENS, J.C. 1979. Electrophysiological investigations of olfaction in bark beetles. *Mitt. Schweiz. Entomol. Ges.* 52:203–216.

DICKENS, J.C. 1981. Behavioural and electrophysiological responses of the bark beetle, *Ips typographus*, to potential pheromone components. *Physiol. Entomol.* 6:251–261.

DICKENS, J.C. 1984. Olfaction in the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae): Electroantennogram studies. *J. Chem. Ecol.* 10:1759–1785.

DICKENS, J.C. 1990. Specialized receptor neurons for pheromones and host plant odors in the boll weevil, *Anthonomus grandis* Boh., (Coleoptera: Curculionidae). *Chem. Senses* 15:311–331.

DICKENS, J.C., and BOLDT, P.E. 1985. Electroantennogram responses of *Trirhabda bacharides* (Weber) (Coleoptera: Chrysomelidae) to plant volatiles. *J. Chem. Ecol.* 11:767–779.

DICKENS, J.C., and MOORMAN, E.E. 1990. Maturation and maintenance of electroantennogram responses to pheromone and host odors in boll weevils fed their host plant or an artificial diet. *Z. Angew. Entomol.* 109:470–480.

DICKENS, J.C., and MORI, K. 1989. Receptor chirality and behavioral specificity of the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae) for its pheromone, (+)-grandisol. *J. Chem. Ecol.* 15:517–528.

DICKENS, J.C., and PAYNE, T.L. 1985. Chemical messengers and insect behavior, pp. 201–230, *in* N.B. Mandava (ed.). Handbook of Natural Pesticides: Methods, Vol. 1: Theory, Practice and Detection. CRC Press, Boca Raton, Florida.

DICKENS, J.C., and PRESTWICH, G.D. 1989. Differential recognition of geometric isomers by the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae): Evidence for only three essential components in the aggregation pheromone. *J. Chem. Ecol.* 15:529–540.

DICKENS, J.C., PRESTWICH, G.D., SUN, W.-C., and MORI, K. 1990. Receptor site analysis using neurosensory responses of the boll weevil to analogs of the cyclohexylideneethanol of its aggregation pheromone. *Chem. Senses* Submitted.

DUNCAN, D.B. 1955. Multiple range and multiple *F* tests. *Biometrics* 11:1–42.

EIDMANN, H.H., WESLIN, J., HARDING, S., BAECKSTROM, P., NORIN, T., and VRKOC, J. 1986. A compound replacing a natural pheromone component of the spruce bark beetle. *Naturwissenschaften* 73:629.

HARDEE, D.D., MCKIBBEN, G.H., RUMMEL, D.R., HUDDLESTON, P.M., and COPPEDGE, J.R. 1974. Response of boll weevils to component ratios and doses of the pheromone, grandlure. *Environ. Entomol.* 3:135–138.

HEDIN, P.A., BURKS, M.L., and THOMPSON, A.C. 1985. Synthetic intermediates and byproducts as inhibitors of boll weevil attractancy. *J. Agric. Food Chem.* 33:1011–1017.

KOVATS, E. 1958. Gas-chromatographische Charakterisierung organisher Verbindungen. *Helv. Chim. Acta* 41:1915–1932 .

LINDIG, O.H. 1979. A replacement for cottonseed meal and meats in boll weevil diets. *J. Econ. Entomol.* 72:291–292.

MCKIBBEN, G.H., JOHNSON, W.L., EDWARDS, R., KOTTER, E., KEARNY, J.F., DAVICH, T.B., LLOYD, E.P., and GANYARD, M.C. 1980. A polyester-wrapped cigarette filter for dispensing grandlure. *J. Econ. Entomol.* 73:250–251.

MITCHELL, E.B., HARDEE, D.D., and DAVICH, T.B. 1976. In-field boll weevil trap. U.S. Patent No. 3,949,515.

MITCHELL, E.R. 1981. Management of Insect Pests with Semiochemicals. Plenum Press, New York. xiv + 514 pp.

MITCHELL, E.R., JACOBSON, M., and BAUMHOVER, A.H. 1975. *Heliothis* spp.: Disruption of pheromonal communication with (Z)-9-tetradecen-1-ol formate. *Environ. Entomol.* 4:577–579.

MITCHELL, E.R., BAUMHOVER, A.H., and JACOBSON, M. 1976. Reduction of mating potential of male *Heliothis* spp. and *Spodoptera frugiperda* in field plots treated with disruptants. *Environ. Entomol.* 5:484–486.

OSTLE , B. 1963. Statistics in Research. Iowa State University Press, Ames, Iowa. xv + 585 pp.

PANTIN, C.F.A. 1948. Notes on Microscopical Techniques for Zoologists. Cambridge University Press, Cambridge, UK.

PAVLATH, A.E. 1986. Theoretical considerations for the application of aromatic and heterocyclic fluorine compounds as pesticides. *Pestic. Sci.* 7:412–417.

PIGNATELLO, J.J., and GRANT, A.J. 1983. Structure–activity correlations among analogs of 4-methyl-3-heptanol, a pheromone component of the European elm bark beetle (*Scolytus multistriatus*) *J. Chem. Ecol.* 9:615–643.

PRESTWICH, G.D. 1986. Fluorinated sterols, hormones and pheromones: Enzyme-targeted disruptants in insects. *Pestic. Sci.* 37:430–440.

PRESTWICH, G.D. 1987. Chemistry of pheromone and hormone metabolism in insects. *Science* 237:999–1006.

PRESTWICH, G.D., CARVALHO, J.F., DING, Y.S., and HENDRICKS, D.E. 1986. Acyl fluorides as reactive mimics of aldehyde pheromones: Hyperactivation and aphrodisia in *Heliothis virescens. Experientia* 42:964–966.

PRESTWICH, G.D., SUN, W.-C., and DICKENS, J.C. 1988. Fluorinated analogs of aldehyde components of boll weevil pheromone: Synthesis and biological activity. *J. Chem. Ecol.* 14:1427–1439.

PRESTWICH, G.D., SUN, W.-C., MAYER, M.S., and DICKENS, J.C. 1990. Perfluorinated moth pheromones: Synthesis and electrophysiological activity. *J. Chem. Ecol.* 16:1761–1778.

RICHERSON, J.V., MCCARTY, F.A., and PAYNE, T.L. 1980. Disruption of southern pine beetle infestations with frontalure. *Environ. Entomol.* 9:90–93.

SANDERS, C.J. 1981. Spruce budworm: Effects of different blends of sex pheromone components on disruption of male attraction. *Experientia* 37:1176–1177.

SUN, W.-C., and PRESTWICH, G.D. 1990. Partially fluorinated analogs of (Z)-9-dodecenyl acetate: Probes for pheromone hydrophobicity requirements. *Tetrahedron Lett.* 31:801–804.

TUMLINSON, J.H., HARDEE, D.D., GUELDNER, R.C., THOMPSON, A.C., HEDIN, P.A., and MINYARD, J.P. 1969. Sex pheromones produced by male boll weevils: Isolation, identification, and synthesis. *Science* 166:1010–1012.

VILLAVASO, E.J. 1982. Boll weevil: Isolated field plot studies of disruption of pheromonal communication. *J. G. Entomol. Soc.* 17:347–350.

VITÉ, J.P., HUGHES, P.R., and RENWICK, J.A.A. 1976. Southern pine beetle: Effect of aerial pheromone saturation on orientation. *Naturwissenschaften* 63:44.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

EVIDENCE FOR A TWO-COMPONENT EXTERNAL MARKING PHEROMONE SYSTEM IN AN APHID HYPERPARASITOID

C. HÖLLER,[1,*] H.J. WILLIAMS,[2] and S.B. VINSON[2]

[1]*Institut für Phytopathologie*
Universität Kiel
2300 Kiel, Germany

[2]*Department of Entomology*
Texas A&M University
College Station, Texas 77843

(Received October 24, 1990; accepted January 31, 1991)

Abstract—The megaspilid *Dendrocerus carpenteri* (Curtis) is a solitary ecto-hyperparasitoid of aphid primary parasitoids. It avoids superparasitism, but the avoidance rate depends on the amount of time elapsed after the first oviposition. In addition, females discriminate in a time-dependent manner between hosts parasitized by themselves and hosts parasitized by conspecifics. They recognize parasitized hosts by means of two externally perceivable chemical markers that are deposited on the mummy shell. One of the markers is highly active, short-lived, not passable through gas chromatography (GC), and freezing sensitive; the other is moderately active, persistent, GC-passable and freezing tolerant. Using two markers is believed to be advantageous for the hyperparasitoid female, as their ratio provides information on mark age and on the producer of the mark (the female herself or a conspecific female).

Key Words—*Dendrocerus carpenteri*, Hymenoptera, Megaspilidae, hyperparasitoid, primary parasitoid, marking pheromone, host discrimination, self-recognition, superparasitism.

INTRODUCTION

Aphid hyperparasitoids develop on or in the body of aphid primary parasitoids and can be important secondary pests. Even though their detrimental effect on

*To whom correspondence should be addressed.

0098-0331/91/0600-1021$06.50/0

primary parasitoids has still to be clearly demonstrated, many field data suggest that at least the more polyphagous hyperparasitoids are significant promoters of pest outbreaks (Bennett, 1981; Van den Bosch, 1981; Sullivan, 1988). The species dealt with here, *Dendrocerus carpenteri* (Curtis) (Hymenoptera, Megaspilidae), is a polyphagous hyperparasitoid. It parasitizes a wide range of primary parasitoid hosts associated with many aphids and plants (Dessart, 1972; Takada, 1973; Stary, 1977).

The efficiency of aphid primary parasitoids cannot usually be enhanced by inundative releases, because this would lead to the augmentation of hyperparasitoids in opposition to the desired effect (Höller, 1988). Instead, an indirect enhancement through the manipulation of hyperparasitoids is much more promising. The use of marking pheromones could perhaps achieve this goal by dispersing hyperparasitoids from areas where pest populations increase and thus freeing primary parasitoids from their attack (Vinson, 1977). Marking pheromones have been shown to be produced for host discrimination by a number of primary parasitoids (see for reviews: van Lenteren, 1981; Vinson, 1984; van Alphen and Visser, 1990), but they have not been documented for hyperparasitoids. Marking pheromones produced by parasitoids are either patch marks, i.e., pheromones used to label the substrate, or host marks, which are applied on or injected into the host. In this paper, we deal with marking pheromones as host marks exclusively. We investigated the biological activity of the marking pheromones of *D. carpenteri*, and we attempted partial chemical characterization of the compounds.

D. carpenteri is a solitary ectohyperparasitoid of aphidiids and aphelinids. Primary parasitoid hosts are attacked after death of the aphid host during the postparasitic phase of development in the mummy (= the cocoon surrounded by the remaining aphid cuticle wherein the primary parasitoid pupates). The female drills a hole through the mummy shell, applies venom, and lays her egg on the surface of the host (Bocchino and Sullivan, 1981). Fecundity is rather low in *D. carpenteri*, since, on average, only 75 progeny are produced per female (Walker and Cameron, 1981).

METHODS AND MATERIALS

Insect Cultures

Two different plant–aphid–primary parasitoid systems were used to investigate the biological activity of the marking pheromone (system 1) and to attempt chemical characterization (system 2). System 1: oat (cv. Bojar)–*Sitobion avenae* F.–*Aphidius rhopalosiphi* De Stefani-Perez maintained at 20°C in a 16:8 hr light–dark cycle. Experiments using system 1 were conducted in Kiel, Germany. System 2: wheat (cv. McGregor)–*Schizaphis graminum* (Rondani)–

Aphidius matricariae Hal. maintained at 26°C in a 16:8 hr light–dark cycle. Experiments using system 2 were conducted in College Station, Texas. *A. rhopalosiphi* ex *S. avenae* and *A. matricariae* ex *S. graminum* were similarly suitable hosts for the hyperparasitoid *D. carpenteri*. A choice experiment in which both hosts were offered simultaneously could not be conducted due to quarantine laws.

Bioassay

Six hosts were offered to one female *D. carpenteri* for 1 hr in a 3.5-mm-diameter Petri dish. The hosts were mummies containing a primary parasitoid larva, prepupa, or young pupa. Mummies containing a diapausing larva, an older pupa, or an adult, as evidenced by their dark appearance, were not used. The six hosts were glued on their ventral side in a matrix of equal distances to a 15 × 30-mm cardboard rectangle, which was placed in the Petri dish. To investigate the biological activity of the marking pheromone, an experienced and mated hyperparasitoid female taken directly from the rearing culture was allowed to oviposit in three of the six hosts and then was removed (females that did not parasitize three hosts within 20 min were discarded). After a given time interval elapsed, the same female or another female with the same amount of experience was again confined with these hosts, half of which had been attacked previously. Any completed drillings were noted during an observation period of 1 hr. The hosts attacked were later dissected in order to verify oviposition. Eggs laid on hosts attacked twice were classified as having been laid during the first or the second attack, as evidenced by visible development of the first-laid egg. The minimum amount of time necessary for accurate classification of eggs was 6 hr. For every combination, 20 replicates were performed.

For experiments with time intervals of 6 hr or longer, storage of hosts at 15°C prevented rapid development of unparasitized primary parasitoids. The longest time interval investigated was 48 hr, as longer intervals result in hatching of unparasitized primary parasitoids. Hyperparasitoid females were stored at 15°C between experiments and were fed during this time with 15% sucrose solution.

The experimental procedure had to be modified when time intervals of 5 min or 1 hr were investigated: only two hosts were offered, the female was allowed to oviposit only once, the same female was used, the recording time was 20 min, and 60 replicates were performed. This modification was necessary because of the time required for oviposition (about 1 min 14 sec) in *D. carpenteri* (Le Ralec, 1988).

The same bioassay was used during efforts to characterize the chemical nature of the marking pheromone. However, this time the hosts were arranged in the matrix

1 5 3
4 2 6

where 1–3 are controls and 4–6 are treatments, with a single female being observed for 1 hr as described above. The matrix was used to investigate the response of *D. carpenteri* females towards: (1) artificial drilling hole (one hole ca. 0.3 mm diameter drilled in 4–6 each; none in 1–3); (2) a piece of mummy shell from a host parasitized previously (one mummy shell piece ca. 0.7 mm^2 surrounding the drilling hole of a host parasitized ca. 24 hr ago detached and glued on 4–6 each; similar piece of unparasitized host mummy shell on 1–3); and (3) solvent extracts (1 μl solvent extract applied dorsally on 4–6 each; 1 μl solvent on 1–3).

Solvent Extraction

Preliminary studies revealed that methanol was a better extraction solvent than acetone, diethylether, hexane, or water. Three different extracts were prepared:

Extract A (Female Body Rinses). Live *D. carpenteri* females (25 at a time) were placed in 100 μl methanol in a 1-ml beaker. After 2 min, the methanol was decanted into a glass reactivial. The 25 females were again extracted with 100 μl of solvent, which was added to the first extract. In total, 800 rinsed females yielded 6.5 ml extract. The extract was concentrated under nitrogen to 1.5 ml, which corresponds to a concentration of 0.53 female equivalents (FE) per microliter and was stored at $-60°C$.

Extract B (Host and Egg Extracts). Parasitized hosts were dissected within 24 hr after oviposition. The mummified aphid cuticle, the primary parasitoid, and the egg of the hyperparasitoid (numbers were identical between fractions but differed between samples) were placed together and separately into glass reactivials. The meconium was discarded. A given amount of methanol was added to obtain a concentration of 0.6 HE (host equivalents)/μl. After 10 min, the extract was carefully withdrawn by means of a syringe. Higher concentrations of mummified aphid cuticles (24 HE/μl MeOH) also were prepared. Mummified aphid cuticle and primary parasitoid extracts (0.6 and 24 HE/μl MeOH) from unparasitized mummies were used as controls. All extracts B were stored at $-60°C$.

Extract C (Female Reproductive System Extracts). The reproductive system is the source of the marking pheromones in *D. carpenteri* (Höller and Vinson, in preparation). Females of the hyperparasitoid were dissected in Pringle's saline solution (Pringle, 1938) by gently pulling out the ovipositor and the adjacent reproductive system. The latter was disconnected by exerting pressure with a minute needle on the caudal part of the common oviduct until detachment. Subsequently, the reproductive system was immediately transferred in a reac-

tivial containing methanol. The extract was concentrated under nitrogen to 0.4 FE/μl MeOH. Extract C was divided in two parts of equal sizes, one being stored at 4°C and one at −60°C for 24 hr before being bioassayed.

Gas Chromatography

Analytical GC was performed using a 0.22-mm-ID × 25-m vitreous silica capillary column coated with a 0.5-μm film of BP1 (SGE) methyl silicone stationary phase using a Varian 3700 instrument equipped with an on-column injector and a flame ionization detector (FID). Injection sizes were 1 or 2 μl. For preparative purposes, a Tracor 550 chromatograph equipped with a 4-mm-ID × 1.8-m glass column packed with 3% OV-101 on chromosorb 750 (100–120 mesh) was used. A 100-0:1 variable glass splitter divided the effluent between a Brownlee-Silverstein collector (Brownlee and Silverstein, 1968) and an FID detector. Sample sizes between 15 and 50 μl were injected for preparative separation, using a temperature of 60°C at injection (held for 1 min) to 300°C at 20°C/min.

RESULTS

Biological Activity of Female-Deposited Marking Pheromone

These experiments were conducted using the plant–aphid–primary parasitoid–hyperparasitoid system (1). Females were strongly repelled by their own mark when this mark was 5 min old, but showed a significantly (chi-squared goodness-of-fit test, $P < 0.070$) weaker response towards marks having been applied by themselves 1 hr or more previously (Figure 1). Responses towards self-produced marks of 1, 6, 12, 24, and 48 hr old were similar ($P > 0.376$). Females encountering a host marked by a conspecific female 6 or 12 hr before were repelled to the same degree as to their own marks of the same age. In contrast, conspecific marks 24 or 48 hr old led to a significantly ($P < 0.099$) higher acceptance of hosts than the corresponding self-produced marks (Figure 1).

Egg-laying was the last step in a series of behaviors following the encounter of a *D. carpenteri* female with a suitable host: (1) the host was thoroughly examined with the antennae, (2) a hole was drilled through the mummified aphid cuticle and the underlying cocoon of the primary parasitoid, (3) the contents of the mummy were palpated by means of the ovipositor, and (4) the motionless ovipositor remained in contact for some time with the mummy contents. Rejection of a marked host occurred either after step 1, i.e., hole drilling was suppressed as a result of exterior antennal examination of the host, or at steps 3–4, i.e., the host was attacked but an egg was not laid. Seventy-four to

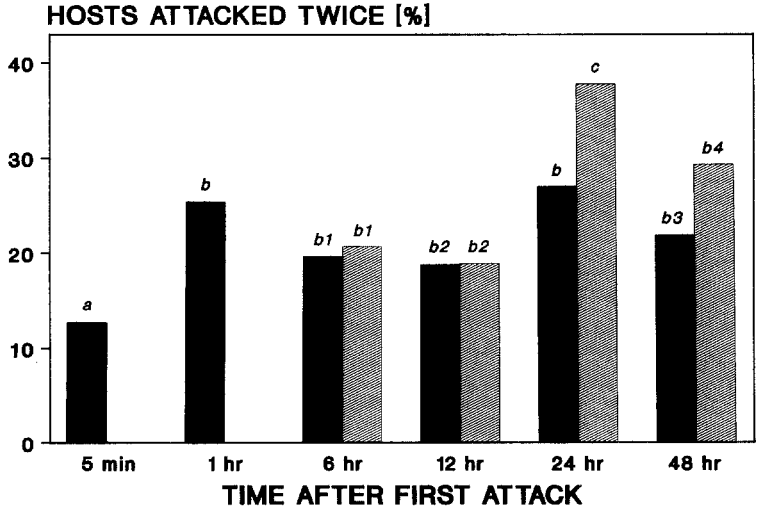


FIG. 1. Percentages of hosts attacked twice as a time-dependent phenomenon in *Dendrocerus carpenteri*. Solid bars: hosts previously attacked by the same female; shaded bars: hosts previously attacked by another female. Different letters and numbers indicate significant differences between bars at all time intervals or at one time interval, respectively (chi-squared goodness-of-fit test, $P < 0.070$ and $P < 0.099$, respectively); all values were significantly different from 50% ($P < 0.055$).

90% of the females oviposited on a primary parasitoid that had not been attacked previously, but only 35–67% did so when the host had been attacked before, and thus in most cases already contained an egg (Table 1). Hence, *D. carpenteri* was able to recognize parasitized hosts externally with the antennae and internally with the ovipositor. The data presented in Table 1 give no evidence for internal discrimination between self-laid eggs and eggs laid by a conspecific female.

In order to prove that host discrimination in *D. carpenteri* is not a result of visual, tactile, or acoustical stimuli, hosts with artificial drilling holes were offered. No differences were recorded between treated and untreated hosts (Table 2). In another experiment, a piece of mummy shell surrounding the drilling hole of a parasitized host was detached and glued on a healthy host. The treated hosts were offered to females that had not initially made the hole. This time, significantly less treated hosts were attacked in comparison to the control (Table 2). Thus, host discrimination in *D. carpenteri* was based on chemical markers.

Activity of Extract A (Female Body Rinses)

These and all following experiments were conducted using the plant–aphid–primary parasitoid–hyperparasitoid system (2). The complete collection of extract A after GC separation was not as active as the crude extract (data not

TABLE 1. OVIPOSITION BY *Dendrocerus carpenteri* IN HOSTS ATTACKED FOR FIRST TIME AND IN HOSTS ATTACKED TWICE

	Hosts attacked for the first time		Hosts attacked twice		
Time after first attack (hr)	No. hosts attacked	Attacks with oviposition (%)	Female	No. hosts attacked	Attacks with oviposition (%)[a]
6	49	81.6	Same	15	40.0
6	42	88.1	Other	12	66.6
12	43	90.1	Same	14	35.7
12	34	82.4	Other	8	37.5
24	46	78.3	Same	18	44.4
24	38	73.7	Other	23	56.5
48	43	79.1	Same	14	50.0
48	46	89.1	Other	20	35.0

[a] Second attacks only.

TABLE 2. ACTIVITY LEVELS OF ARTIFICIAL DRILLING HOLES (TREATMENT I) AND PIECES OF PARASITIZED HOST MUMMY SHELLS (TREATMENT II)

Treatment	No. hosts attacked[a]	Attacks on treated hosts (%)	Chi^2 value[b]	*P*
I	84	52.4	0.19	0.663
II	72	36.1	5.56	0.019

[a] Totals of 20 replicates.
[b] Chi-squared goodness-of-fit test; expected: No. attacks on treated hosts is not different from half of total attacks.

shown). Analysis of various GC fractions revealed activity only from a fraction eluting from 18.30 to 27.00 min (fraction A3; Table 3). Doubling the injected concentration from 1.06 to 2.12 FE/μl MeOH (fractions A3 and A6, respectively; Table 3) did not increase the response of the hyperparasitoids. A10 was the narrowest fraction yielding activity. A comparison with hydrocarbon standards showed that fraction A10 eluted between the C-31 and C-33 hydrocarbon fractions. However, it was impossible to visualize any peaks in the chromatograms of the active fractions, which indicates that the active compound(s) were present only at very low concentrations. Rates of oviposition did not differ between attacked extract-treated and methanol-treated control hosts.

Activity of Extracts B (Host and Egg Extracts)

Hyperparasitoid eggs, freshly parasitized primary parasitoids, and the corresponding mummy shells were extracted together and separately in methanol. As shown in Table 4, joint extracts, primary parasitoid extracts, and mummy shell extracts (0.6 HE/μl) alone provoked a significant (chi-squared goodness-of-fit test, P values given in Table 4) response in terms of activity. In contrast,

TABLE 3. ACTIVITY LEVELS OF EXTRACT A (FEMALE BODY RINSES) AND ITS ACTIVE FRACTIONS SEPARATED BY GAS CHROMATOGRAPHY

Treatment	No. hosts attacked[a]	Attacks on treated hosts (%)	Chi^2 value[b]	P
Extract A	60	33.3	5.59	0.019
Fraction A3[c]	76	39.5	3.37	0.067
Fraction A6[d]	48	33.3	5.33	0.021
Fraction A10[e]	70	38.6	3.66	0.056

[a] Totals of 20 replicates.
[b] Chi-squared goodness-of-fit test; expected: No. attacks on treated hosts is not different from half of total attacks.
[c] Concentration 1.06 FE/μl MeOH; retention time 18.30–27.00 min.
[d] Concentration 2.12 FE/μl MeOH; retention time 18.30–27.00 min.
[e] Concentration 2.12 FE/μl MeOH; retention time 19.30–22.00 min.

TABLE 4. ACTIVITY LEVELS OF EXTRACTS B (HOST AND EGG EXTRACTS)

Treatment	No. hosts attacked[a]	Attacks on treated hosts (%)	Chi^2 value[b]	P
Joint extract[c]	34	29.4	5.76	0.017
Primary parasitoid extract[c]	63	38.1	3.57	0.059
Hyperparasitoid egg extract[c]	64	45.3	0.56	0.454
Mummy shell extract[c]	55	29.1	9.62	0.002
Mummy shell extract[d]	56	14.3	28.57	0.001
Mummy shell extract[e]	84	42.9	1.71	0.191
Mummy shell extract[e] fraction B3	60	40.0	2.40	0.122

[a] Totals of 20 replicates.
[b] Chi-squared goodness-of-fit test; expected: No. attacks on treated hosts is not different from half of total attacks.
[c] Concentration 0.6 HE/μl MeOH.
[d] Concentration 24 HE/μl MeOH.
[e] Concentration 24 HE/μl MeOH, collected from preparative GC.

hyperparasitoid egg extracts were not or at most slightly active. Rates of oviposition did not differ between attacked extract-treated and methanol-treated control hosts.

Highly concentrated mummy shell extracts (24 HE/μl MeOH) were prepared to enable a GC comparison between healthy and parasitized hosts. The chromatograms differed in two peaks (Figure 2), but the peak appearing at 18.05 min was an artifact of contamination as it was lacking in an active additional replicate. The second peak (retention time 20.36 min), however, was present in both replicates. Therefore, and because this peak fell within the retention time of the active fraction A10 prepared from extract A, peak 20.36 was subsequently purified by means of preparative GC. A dotriacontane (hydrocarbon C-32) standard injection yielded a peak of very similar retention time, i.e., 20.18 min (Figure 2). By comparing peak 20.36 to the peak obtained from dotriacontane, an amount of 0.3 ng/ml of pheromone was estimated to be deposited per *D. carpenteri* female during host marking. Dotriacontane, applied in a concentration of ca. 100 ng/ml MeOH (maximum solubility at room temperature), was not active when bioassayed (data not shown). Apparently, the marking pheromone was not dotriacontane, despite its retention time being similar to the retention time of the active compound.

Treating hosts with a crude highly concentrated mummy shell extract (24 HE/μl MeOH) resulted in increased activity (approximately two times greater than the standard concentration 0.6 HE/μl MeOH; see Table 4). The biological

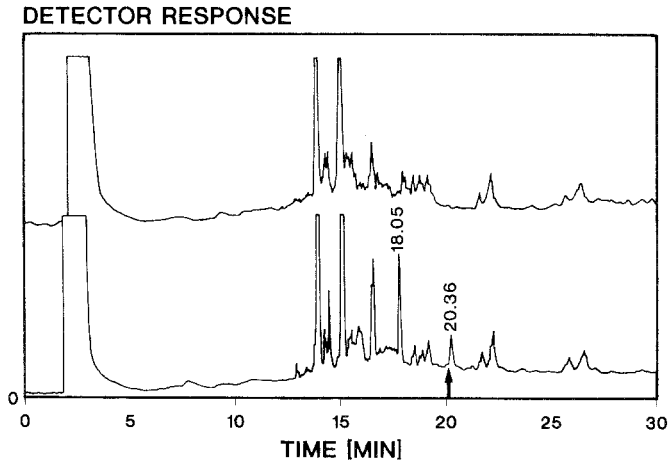


FIG. 2. Gas chromatograms of highly concentrated methanol mummy shell extracts (24 HE/μl MeOH). Above: unparasitized hosts; below: parasitized hosts; arrows: peaks appearing in chromatogram of parasitized hosts, exclusively; retention times 18.05 and 20.36 min (average values of two replicates, calculated from hundredth sec).

activity of the mummy shell extract, although present, was significantly reduced when totally collected following preparative GC. In contrast, similar activities were obtained from the totally collected GC-passed mummy shell sample and its one purified peak at 20.36 min (fraction B3; Table 4).

Activity of Extract C (Female Reproductive System Extracts)

Extract C was highly active when stored overnight at 4°C but lost much of its activity when frozen in the same time interval at −60°C (Table 5). Thus, freezing significantly decreased activity of the female reproductive system extract. Rates of oviposition did not differ between attacked extract-treated and methanol-treated control hosts.

DISCUSSION

The data of the present study clearly demonstrate that the hyperparasitoid *D. carpenteri* uses marking pheromones for host discrimination. Many parasitoids do so, but most of the cases reported so far concern endoparasitoids. Host discrimination of ectoparasitoids, and especially solitary ones, has been studied only occasionally (Lloyd, 1956; van Lenteren and De Bach, 1981) and is reported here for the first time in a hyperparasitoid.

The following results provide evidence that the marking pheromone of *D. carpenteri* consists of at least two different components:

1. Persistence. The avoidance rate of already parasitized hosts was high when a time interval of 5 min elapsed between the first attack and subsequent encounter. After 1 hr, the avoidance level had significantly decreased. However, it remained stable between 1 and 48 hr. Apparently, one component caus-

TABLE 5. ACTIVITY LEVELS OF EXTRACT C (FEMALE REPRODUCTIVE SYSTEM EXTRACTS)

Treatment	No. hosts attacked[a]	Attacks on treated hosts (%)	Chi^2 value[b]	P
Extract C, previously frozen[c]	80	42.5	1.80	0.180
Extract C, not previously frozen[d]	52	17.3	22.23	0.001

[a] Totals of 20 replicates.
[b] Chi-squared goodness-of-fit test; expected: No. attacks on treated hosts is not different from half of total attacks.
[c] Concentration 0.4 FE/μl MeOH; frozen overnight at −60°C.
[d] Concentration 0.4 FE/μl MeOH; stored overnight at 4°C.

ing strong responses became inactive quickly, whereas another component was not so repellent, but much more long-lasting.

2. Transferability. Gluing the piece of mummy shell surrounding the drilling hole of a host parasitized ca. 24 hr to an unparasitized host resulted in a decreased attack rate. The rate of avoidance was comparable to that of hosts having been attacked 24 or 48 hr earlier by a different female. Thus, a stable component was transferable and was deposited on the mummy shell.

3. GC stability. Methanol extracts from mummy shells resulted in a concentration-dependent response by searching hyperparasitoid females. However, after passing the extract through a preparative GC and collecting all fractions combined, the extract lost much of its activity. The remaining activity probably was due to a stable component, a more active component failing to be collected following GC fractionation.

4. Freezing stability. Extracts of the female reproductive system stored overnight at 4°C yielded high activity the next day. In contrast, the same extracts caused significantly weaker responses when stored for the same time at −60°C. Thus, one component was sensitive to freezing, in opposition to a stable component that was still active after freezing.

Taken together, points 1–4 allow the conclusion that a two-component marking pheromone system is used for external host discrimination in *D. carpenteri*. One component is highly active, but unstable (sensitive to GC conditions and freezing), whereas a second component is less active but persistent (GC stable and freezing tolerant). The possible advantages of using a two-component mixture as an externally perceivable marking pheromone are:

1. Information on mark age. Simultaneously applying two components with different levels of persistence would enable a female to deduce the mark's age from the ratio of components. In fact, it has been shown repeatedly that parasitoid females recognize the age of marks (e.g., Rogers, 1972; Chow and Mackauer, 1986; Hofsvang; 1988), but the question of how a parasitoid tells the time interval between successive ovipositions was never answered (see Hubbard et al., 1987).

2. Self-recognition. There is now considerable evidence that at least some parasitoid species differentiate between self-produced marks and marks produced by conspecifics (Hubbard et al., 1987; Völkl and Mackauer, 1990; see also results of this study). Again, self-recognition might be enabled through specifities in the ratios of components.

3. Dispersal after oviposition. Parasitoids like *D. carpenteri* are not easily disturbed once they are in contact with a suitable host. Contact kairomones originating from the mummy shell elicit thorough examination of the area (Höller, unpublished data). Hence, after oviposition there must be some stimulus strong enough to cause the female to leave before beginning to search for a new host. We believe that the unstable, highly active component has this function

in *D. carpenteri*, being a dispersal pheromone (Vinson, 1984). Once the female has left, the dominance of the this component attenuates and its less active, but the much more stable counterpart prevails, which allows a careful examination of the host when encountered again. The alternative explanation for the function of short-lived marking pheromones given by Roitberg and Mangel (1988) does not apply here, considering the persistence of the stable compound.

The two-component theory is also helpful in explaining other data. For instance, Hubbard et al. (1987) recorded striking differences in self- and conspecific superparasitism of *Nemeritis canescens* (Grav.) 0–24 hr after the first oviposition, but not thereafter (2–8 days). The combined action of an unstable and highly active (towards the same female) component and a persistent component might have been occurring here too. The theory is further substantiated by the fact that many parasitoids discriminate not only externally through information perceived at receptors located on the antennae, but also internally after ovipositor insertion in the host. This behavior was first observed in *Trichogramma evanescens* Westw. (Salt, 1937). Since then, numerous examples have been published (including this study). It seems not unlikely that the secretion deposited in the host's body (or, in *D. carpenteri*, on the host's cuticle inside the mummy) remains externally detectable for a short time after oviposition, before being metabolized, moved, or diffused. Thus, the highly active, nonpersistent component and the secretion are possibly identical.

In spite of this sophisticated marking system, levels of superparasitism were quite high throughout the study. Self-superparasitism can be advantageous, i.e., when two or more eggs in or on a host increase the probability of gaining an offspring from that host (van Alphen and Visser, 1990). This would be the case when there is a high risk of a later attack by a conspecific female or when defense mechanisms from the host reduce the survival rate of the offspring. Obviously, a mother benefits from conspecific superparasitism when hosts are scarce and when the probability of the second egg to win is reasonably high. If the ovipositions are sufficiently close together, then the probability of the second egg winning may be great enough to warrant the risk (Hubbard et al., 1987). However, just the contrary happened in the present study, since only 24 and 48 hr after the first oviposition were higher levels of conspecific superparasitism recorded. We believe the reason for this is the following: females laid more eggs in hosts bearing older eggs or early larvae of conspecifics because the survival rate of their offspring would be enhanced if they act as tertiary parasitoids (or, in this case, autoparasitoids). Haviland (1920) showed very early that tertiary and even higher levels of parasitism occur in *D. carpenteri*, with conspecifics or other hyperparasitoids as hosts. We hypothesize that the venom that is applied prior to oviposition toxifies both the primary parasitoid host and a possibly concurrent hyperparasitoid, as long as the latter has hatched from its egg. Should this hypothesis prove true, conspecific superparasitism on hosts

parasitized 24 or 48 hr before (time required from egg deposition until hatching of the first instar larva; Le Ralec, 1988) must be regarded as being advantageous to the second female.

The source of the marking pheromone is often one of the accessory glands, either the Dufour gland (Guillot and Vinson, 1972; Vinson and Guillot, 1972; Harrison et al., 1985; Hubbard et al., 1987) or the poison gland (Yamaguchi, 1986). Guillot et al. (1984) identified hydrocarbons, sterol esters, and free fatty acids as active fractions from the poison gland of *Cardiochiles nigriceps* Viereck. Even though each of the neutral lipid fractions proved to be active at a given concentration, much higher activities were recorded from the composite sample, indicating synergism among the active fractions. These findings support the two-component theory. Mudd et al. (1982) identified volatile hydrocarbons from the Dufour gland of *N. canescens* and suggested that heneicosane (C21) acts as an external marker pheromone in this parasitoid. In the present study, the stable component had a GC retention time near that of dotriacontane (C32), which itself was not active. The pheromones used for host marking in *D. carpenteri* are not yet identified.

Acknowledgments—We thank Dr. Franco Pennacchio and Tom Syvertsen for their suggestions and ideas, Dr. Jacques van Alphen and an anonymous referee for reviewing the manuscript, and Dr. Eline Hågvar and Dr. Trond Hofsvang for commenting on an earlier draft of the manuscript. Leslie McKinney provided the initial parasitoid and aphid material in Texas, which is gratefully acknowledged. This research was supported in part by a German Research Council (DFG) grant Wy 9/11-1 and a DFG traveling grant Ho 1182/1-1.

REFERENCES

BENNETT, F.D. 1981. Hyperparasitism in the practice of biological control, pp. 43–49, *in* D. Rosen (ed.). The Role of Hyperparasitism in Biological Control: A Symposium. Division of Agricultural Sciences, University of California, Berkeley.

BOCCHINO, F.J., and SULLIVAN, D.J. 1981. Effects of venoms from two aphid hyperparasitoids, *Asaphes lucens* and *Dendrocerus carpenteri* (Hymenoptera: Pteromalidae and Megaspilidae), on larvae of *Aphidius smithi* (Hymenoptera: Aphidiidae). *Can. Entomol.* 113:887–889.

BROWNLEE, R.G., and SILVERSTEIN, R.M. 1968. A micro-preparative gas chromatograph and a modified carbon skeleton determinator. *Anal. Chem.* 40:2077–2079.

CHOW, F.J., and MACKAUER, M. 1986. Host discrimination and larval competition in the aphid parasite *Ephedrus californicus*. *Entomol. Exp. Appl.* 41:243–254.

DESSART, P. 1972. Révision des espèces européennes du genre *Dendrocerus* Ratzeburg, 1852 (Hymenoptera Ceraphronoidea). *Mem. Soc. R. Belge Entomol.* 32:1–310.

GUILLOT, F.S., and VINSON, S.B. 1972. Sources of substances which elicit a behavioural response from the insect parasitoid, *Campoletis perdistinctus*. *Nature* 235:169–170.

GUILLOT, F.S., JOINER, R.L., and VINSON, S.B. 1974. Host discrimination: Isolation of hydrocarbons from Dufour's gland of a braconid parasitoid. *Ann. Entomol. Soc. Am.* 67:720–721.

HARRISON, E.G., FISHER, R.C., and ROSS, K.M. 1985. The temporal effects of Dufour's gland secretion in host discrimination by *Nemeritis canescens*. *Entomol. Exp. Appl.* 38:215–220.

HAVILAND, M.D. 1920. On the bionomics and development of *Lygocerus testaceimanus*, Kieffer, and *Lygocerus cameroni*, Kieffer (Proctotrypoidea—Ceraphronidae), parasites of *Aphidius* (Braconidae). *Q. J. Microsc. Sci.* 65:101–127.

HÖLLER, C. 1988. Effizienzanalyse der Parasitoiden an Getreideblattläusen. PhD thesis. University of Kiel.

HOFSVANG, T. 1988. Mechanisms of host discrimination and intraspecific competition in the aphid parasitoid *Ephedrus cerasicola*. *Entomol. Exp. Appl.* 48:233–240.

HUBBARD, S.F., MARRIS, G., REYNOLDS, A., and ROWE, G.W. 1987. Adaptive patterns in the avoidance of superparasitism by solitary parasitic wasps. *J. Anim. Ecol.* 56:387–401.

LE RALEC, A. 1988. Study on the model *Sitobion avenae–Aphidius uzbekistanicus–Dendrocerus carpenteri:* The hyperparasitoid ovipositor, sting, egg and first instars of development, pp. 125–126, *in* B. Bouletreau and G. Bonnot (eds.). Parasitoid Insects. Les Colloques de l'INRA No. 48, INRA, Paris.

LLOYD, D.C. 1956. Studies on parasite oviposition behaviour. I *Mastrus carpocapsae* Cushman. (Hymenoptera: Ichneumonidae). *Can. Entomol.* 88:80–89.

MUDD, A., FISHER, R.C., and SMITH, M.C. 1982. Volatile hydrocarbons of the Dufour's gland of the parasite *Nemeretis canescens* (Grav.) (Hymenoptera; Ichneumonidae). *J. Chem. Ecol.* 8:1035–1042.

PRINGLE, J.W.S. 1938. Proprioception in insects. *J. Exp. Biol.* 15:101–113.

ROGERS, D.J. 1972. The ichneumon wasp *Venturia canescens:* Oviposition and the avoidance of superparasitism. *Entomol. Exp. Appl.* 15:190–194.

ROITBERG, B.D., and MANGEL, M. 1988. On the evolutionary ecology of marking pheromones. *Evol. Ecol.* 2:289–315.

SALT, G. 1937. Experimental studies in insect parasitism. V. The sense used by *Trichogramma* to distinguish between parasitized and unparasitized hosts. *Proc. R. Soc. London, Ser. B.* 122:57–75.

STARY, P. 1977. *Dendrocerus* hyperparasites of aphids in Czechoslovakia (Hymenoptera, Ceraphronoidea). *Acta Entomol. Bohemoslov.* 74:1–9.

SULLIVAN, D.J. 1988. Hyperparasites, pp. 189–204, *in* A.K. Minks and P. Harrewijn (eds.). Aphids. Their Biology, Natural Enemies and Control. Vol. B. Elsevier, Amsterdam.

TAKADA, H. 1973. Studies on aphid hyperparasites of Japan. I. Aphid hyperparasites of the genus *Dendrocerus* Ratzeburg (Hymenoptera: Ceraphronidae). *Insecta Matsum.* 2:1–37.

VAN ALPHEN, J.J.M., and VISSER, M.E. 1990. Superparasitism as an adaptive strategy for insect parasitoids. *Annu. Rev. Entomol.* 35:000–000.

VAN DEN BOSCH, R. 1981. Specifity of hyperparasites, pp. 27–33, *in* D. Rosen (ed.). The Role of Hyperparasitism in Biological Control: A Symposium. Division of Agricultural Sciences, University of California, Berkeley.

VAN LENTEREN, J.C. 1981. Host discrimination by parasitoids, pp. 153–179, *in* D.A. Nordlund, R.L. Jones, and W.J. Lewis (eds.). Semiochemicals, Their Role in Pest Control. John Wiley, New York.

VAN LENTEREN, J.C., and DE BACH, P. 1981. Host discrimination in three ectoparasites (*Aphytis coheni, A. lingnanensis* and *A. melinus*) of the oleander scale (*Aspidiotus nerii*). *Neth. J. Zool.* 31:504–532.

VINSON, S.B. 1977. Behavioral chemicals in the augmentation of natural enemies, pp. 237–279, *in* R.L. Ridgway and S.B. Vinson (eds.). Biological Control by Augmentation of Natural Enemies. Plenum Press, New York.

VINSON, S.B. 1984. Parasitoid-host relationship, pp. 205–233, *in* W.J. Bell and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

VINSON, S.B., and GUILLOT, F.S. 1972. Host marking: Source of a substance that results in host discrimination in insect parasitoids. *Entomophaga* 17:241–245.

VÖLKL, W., and MACKAUER, M. 1990. Age-specific pattern of host discrimination by the parasitoid *Ephedrus californicus* Baker (Hymenoptera: Aphidiidae). *Can. Entomol.* 122:349–361.

WALKER, G.P., and CAMERON, P.J. 1981. The biology of *Dendrocerus carpenteri* (Hymenoptera: Ceraphronidae), a parasite of *Aphidius* species, and field observations of *Dendrocerus* species as hyperparasites of *Acyrthosiphon. N.Z. J. Zool.* 8:531–538.

YAMAGUCHI, H. 1987. [The role of venom in host discrimination of Ascogaster reticulatus Watnabe.] In Japanese. *Jpn. J. Appl. Entomol. Zool.* 31:80–82.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

DIFFERENCE IN HYDROXAMIC ACID CONTENT IN ROOTS AND ROOT EXUDATES OF WHEAT (*Triticum aestivum* L.) AND RYE (*Secale cereale* L.): POSSIBLE ROLE IN ALLELOPATHY

FRANCISCO J. PÉREZ* and JUAN ORMEÑO-NUÑEZ

Departamento de Química
Facultad de Ciencias
Universidad de Chile
Casilla 653, Santiago, Chile
Estación Experimental La Platina (INIA)
Casilla 493-3, Santiago, Chile

(Received October 31, 1990; accepted January 31, 1991)

Abstract—Hydroxamic acids (Hx) produced by some cereal crops have been associated with allelopathy. However, the release of Hx to the soil by the producing plant—an essential condition for a compound to be involved in allelopathy—has not been shown. GC and HPLC analysis of roots and root exudates of wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.) cultivars, with high Hx levels in their leaves, demonstrated the presence of these compounds in the roots of all cultivars analyzed and in root exudates of rye. Moreover, bioassays employing root exudates collected from wheat and rye seedlings demonstrated that only rye exudates inhibited root growth of wild oats, *Avena fatua* L., a weed whose root growth is inhibited by Hx. These results suggest that rye could potentially interfere with the growth of *Avena fatua* in nature and that this interference could be due to the release of Hx to the soil by way of roots.

Key Words—Hydroxamic acids, root exudates, allelopathy, *Triticum aestivum* L., *Secale cereale* L., wild oats, *Avena fatua*.

INTRODUCTION

Hydroxamic acids (Hx) isolated from wheat and other cereals, have been proposed to impart plant resistance to pests and diseases (Niemeyer, 1988). Hx

*To whom correspondence should be addressed.

0098-0331/91/0600-1037$06.50/0

isolated from rye, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), and its decomposition product, benzoxazolin-2-one (BOA), have been implicated as potential allelopathic agents of residues of this crop (Barnes and Putnam, 1987; Barnes et al., 1987). Recently, it was reported that these compounds are decomposed by microorganisms in the soil to 2,2′-oxo-1,1′-azobenzene (AZOB) or its methoxy derivative (MAZOB) and that these transformed compounds have stronger herbicidal activity than the original molecules (Nair et al., 1990). Previous results obtained by us indicated that 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), the main Hx isolated from wheat, and its decomposition product, 6-methoxybenzoxazolin-2-one (MBOA), inhibit root growth of wild oats (Pérez, 1990), a weed that normally infests wheat fields (Bell and Nalewaja, 1968; Cudvey et al., 1989).

One of the essential features of allelopathic interactions between plants is that phytotoxins or substrate for phytotoxins must be released from a plant to the soil and absorbed by a second plant (Rice, 1984). In this paper we present data on Hx levels in roots and root exudates of three different wheat cultivars and two rye cultivars. The presence or absence of toxicity of root exudates collected from wheat and rye plants was assessed with wild oat bioassays.

METHODS AND MATERIALS

Reference Compounds. DIMBOA was isolated from ether extracts of *Zea mays* L. cv. T129 s, as described by Queirolo et al. (1983). DIBOA was synthesized as described by Jernow and Rosen (1975).

Seeds. Seeds of spring wheat cultivars Andifén, Alifén, and Likay-INIA and of wild oats (*A. fatua*) were obtained from the Agricultural Experimental Station, La Platina (INIA). Seeds of rye cultivar Forrajero Baer and Tetra Baer were obtained from E. von Baer Seeds Co., Temuco, Chile.

Collection of Root Exudates. Seeds of wheat and rye cultivars were germinated in Petri dishes containing Whatman No. 1 filter paper. After radicle emergence, seeds were transferred to a continuous root exudate trapping system using vermiculite as an inert medium (Tang and Young, 1982). The system was autoclaved for 40 min at 15 psi before use and covered with aluminum foil. Thirty seedlings of each cultivar were planted in vermiculite and grown in a light bank at 20 ± 5°C and a 12 light–12 dark photoregime. Each pot was irrigated continuously by recycling distilled H_2O at a rate of 15 ml/min until the seedlings reached the first leaf stage. At this point, the Amberlite column was removed from the system and washed with 10 ml of distilled H_2O. Adsorbed organic materials were eluted from the column with 100 ml MeOH. The MeOH solution was evaporated to dryness under reduced pressure at a temperature of 35°C, and the residue was redissolved in 1 ml MeOH. This latter solution was

employed for the bioassays, HPLC, and GC analysis. Control solutions were obtained by the same procedures from systems without seedlings.

Root Extracts. Once the seedlings reached the first leaf stage, roots were removed from the vermiculite in the root exudate system and washed with distilled H_2O. Root tissue (100–150 mg) was macerated successively with 3 × 0.33 ml of distilled H_2O. The extract was adjusted to pH 3 with 0.1 N H_3PO_4 to avoid Hx decomposition and then centrifuged at 6000 *g*. The resulting supernatant was analyzed by HPLC (Niemeyer et al., 1989).

HPLC Analysis. Analysis was performed in a Knauer HPLC system equipped with two pumps (type 364), mixing chamber, variable UV-Vis detector and HPLC software package interfaced to a personal computer. Isolations of the compounds of interest were obtained by using a reverse-phase Zorbax ODS C-18 (4.6 × 25) column with a 5-μm particle size. A linear gradient of H_2O (pH 3)–MeOH, 80:20 to 50:50 for the first 10 min with an additional 20 min at 50:50 was used. The flow was 1.0 ml/min, and the eluate was analyzed at 254 nm. Samples were filtered through a 0.45-μm filter prior to injection. The volume injected was 20 μl. Concentrations of DIBOA and DIMBOA in the samples were obtained by extrapolation from the corresponding calibration curves obtained with pure compounds.

GC Analysis. Aqueous root extracts (1 ml) were evaporated to dryness and redissolved in 100 μl MeOH. Fifty microliters of this solution were derivatized with 50 μl of *N*, *O*-bis(trimethylsilyl) acetamide for 20 min at 50°C. Methanolic solutions of root exudates and standard samples were derivatized in the same way. GC analysis was performed in a Shimadzu GC-PA system equipped with a capillary column and a FID detector. The N_2 was used as carrier gas and the injector and detector temperatures were 250°C. Samples were run with a temperature gradient between 180 and 206°C at a rate of 8°C/min.

Bioassays. Bioassays were carried out in Petri dishes containing Whatman No. 1 filter paper. Different volumes of root exudates dissolved in MeOH ranging from 100 to 250 μl were added to the filter paper. After solvent evaporation, 3 ml of distilled H_2O were added and 10 seeds of wild oats were placed on each disk. Root lengths were measured after 72 hr of incubation in the dark at 20 ± 3°C.

RESULTS

HPLC and GC Analysis of Hx in Roots and Root Exudates of Wheat and Rye Plants. Figure 1 depicts typical HPLC chromatograms of root extracts and root exudates from wheat and rye plants. The peak at 8:06 ± 0.05 min was identified as DIBOA and the peak at 10:15 ± 0.15 min was identified as DIMBOA. Identification was based on retention times and UV spectra of authentic

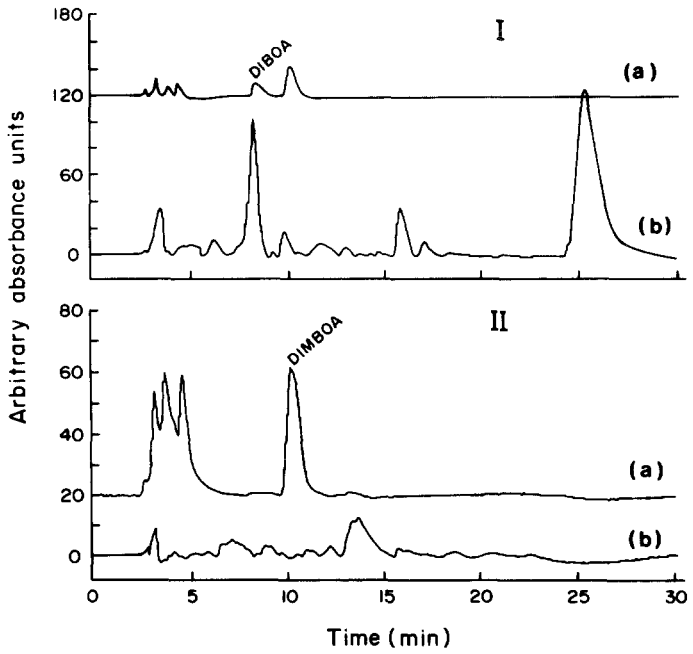


FIG. 1. HPLC chromatograms of root extracts (a) (100–150 mg tissue in 1 ml H_2O) and root exudates (b) (collected from 30 plants and dissolved in 1 ml MeOH), of rye cultivar Forrajero Baer (I) and wheat cultivar Andifén (II).

standards. The same samples were also analyzed by capillary GC and its retention times compared with those of DIBOA 3.1 ± 0.01 min and DIMBOA 5.0 ± 0.01 min. In the HPLC chromatogram of root extracts from rye, a peak appeared with a similar retention time as DIMBOA, but the UV spectrum of this peak did not correspond with the standard nor was DIMBOA observed in the GC chromatogram.

In root extracts, DIBOA was detected by HPLC and GC in wheat and rye cultivars, while DIMBOA was detected only in wheat cultivars. In root exudates, DIBOA was the only Hx detected, and it was found only in rye (Table 1).

Bioassays. Root exudates collected from the wheat cultivar Andifén and from the rye cultivar Forrajero Baer between emergence and the first leaf stage were tested for toxicity on seeds of *A. fatua*, a weed whose root growth is inhibited by Hx (Pérez, 1990). The results (Figure 2) demonstrate that only root exudates collected from rye inhibited root growth of wild oats and that the level of inhibition was determined by the volume of exudate tested.

TABLE 1. QUANTITATION OF HYDROXAMIC ACIDS IN ROOT EXTRACTS AND ROOT EXUDATES COLLECTED BETWEEN EMERGENCE AND FIRST LEAF STAGE FROM WHEAT AND RYE CULTIVARS

	Root extracts (mmol/kg fr wt)[a]		Root exudates (μmol/kg fr wt)	
Cultivar	DIBOA	DIMBOA	DIBOA	DIMBOA
Rye				
Tetra Baer	0.8	nd[b]	0.07	nd
Forrajero Baer	1.3	nd	25.0	nd
Wheat				
Alifén	0.9	2.3	nd	nd
Andifén	nd	0.8	nd	nd
Likay-INIA	0.01	1.6	nd	nd

[a] Root fresh weight of plants at the first leaf stage.
[b] nd = not detected.

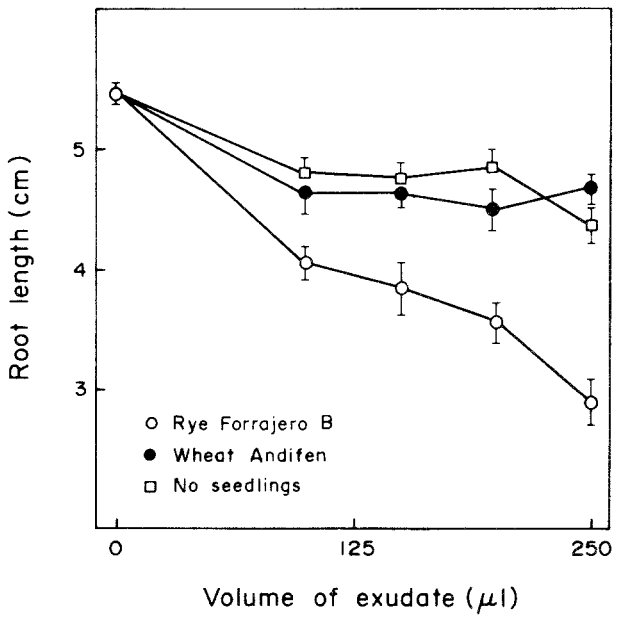


FIG. 2. Radicle lengths of wild oats (*A. fatua* L.), treated with different amounts of root exudates collected from wheat cultivar Andifén (●), rye cultivar Forrajero Baer (○) and without seedlings (□). Data are mean ± SE.

DISCUSSION

Root Exudates. It has been known for many years that organic compounds are exuded from healthy undamaged plant roots (Rovira, 1969). "Normal exudates" are thought to be composed of soluble, low-molecular-weight compounds that are mixed in the rhizosphere with root mucilage, root cap cells, dead root hairs, and epidermal cells (Nye and Tinker, 1977). Thus the distinction between "normal exudates" and other organic materials in the rhizosphere is unlikely even in hydroponic cultures (Barnes et al., 1986) and washings of rooting medium such as sand (Abdul-Wahab and Rice, 1967) include materials that were initially part of the plant structure. However, the results in Table 1 show that hydroxamic acids retained by the XAD-4 column are not a product of cell rupture; i.e., they are "normal exudates," since the levels found in the rhizosphere do not correlate with levels in the roots. Thus, it would seem plausible that exudation of hydroxamic acids is an active process.

Specifity of Root Exudates. DIBOA was the only Hx detected in root exudates and was found only in rye. The amount of DIBOA exuded by rye plants is not proportional to its content in the roots. Additionally, the wheat variety Alifén contained both Hxs in its roots and none of them was exuded. These results suggest that root exudation of Hx depends on a specific regulating factor that is not related with either concentration or type of Hx present in the root tissue.

These observations suggest that simply identifying roots with high contents of Hx is not adequate for the selection of varieties with allelopathic potential. Root exudate analysis will also be required. It has been reported that stress and other factors such as plant age, plant nutrition, light, and moisture can greatly increase root exudation (Nye and Tinker, 1977). The assessment of these factors, together with the search for rye germplasm with a high capacity to exude Hx by its roots, are of interest relative to the potential of Hxs as weed inhibitors through root exudation.

Toxicity of Root Exudates. In a previous paper we demonstrated that Hx can inhibit root growth of wild oats (Pérez, 1990), a weed normally associated with wheat. On the other hand, it has been suggested that rye interferes with the growth of wild oats among other plants (Barnes et al., 1986). The findings that Hx is not lost from roots and is not leached from leaves (Argandoña et al., 1987) of wheat suggest that Hx is not an important allelopathic agent associated with living wheat plants. The detection of DIBOA in root exudates of rye plants and that these root exudates inhibited the growth of wild oats suggest that Hx or its transformation products (Nair et al., 1990) may be important in inhibiting the growth of wild oats in nature.

Acknowledgments—The technical assistance of Ms. Patricia Muñoz and the financial support from Departamento Tecnico de Investigaciones Universidad de Chile, International Foundation for Science, International Program in Chemical Science, and the Agency for International Development are gratefully acknowledged. Supply of rye seeds by E. von Baer Seeds Co. is also acknowledged.

REFERENCES

ABDUL-WAHAB, A.S., and RICE, E.L. 1967. Plant inhibition by Johnson grass and its possible significance in old-field succession. *Bull. Torrey Bot. Club* 94:486–493.

ARGANDOÑA, V.H., ZUÑIGA, G.E., and CORCUERA, L.J. 1987. Distribution of gramine and hydroxamic acids in barley and wheat leaves. *Phytochemistry* 26:1917–1918.

BARNES, J.P., and PUTNAM, A.R. 1987. Role of benzoxazinones in allelopathy by rye (*Secale cereale* L.). *J. Chem. Ecol.* 13:889–905.

BARNES, J.P., PUTNAM, A.R., and BURKE, A.B. 1986. Allelopathic activity of rye (*Secale cereale* L.), pp. 276–286, *in* The Science of Allelopathy. A.R. Putnam and C.S. Tang (eds.). John Wiley & Sons, New York.

BARNES, J.P., PUTNAM, A.R., BURKE, A.B., and AASEN, J.A. 1987. Isolation and characterization of allelochemicals in rye herbage. *Phytochemistry* 26:1385–1390.

BELL, A.R., and NALEWAJA, J.D. 1968. Competition of wild oat in wheat and barley. *Weed Sci.* 16:505–508.

CUDVEY, D.W., JORDAN, L.S., HOLT, J.S., and REINTS, J.S. 1989. Competitive interactions of wheat (*Triticum aestivum*) and wild oats (*Avena fatua*) grown at different densities. *Weed Sci.* 37:538–543.

JERNOW, J.L., and ROSEN, P. 1975. 2H-1,4 Benzoxazin-3(4H)-ones. U.S. Patent 3,862,180.

NAIR, E.M., WHITENACK, C.J., and PUTNAM, A.R. 1990. 2,2′-Oxo-1,1′-azobenzene a microbially transformed allelochemical from 2,3-benzoxazolinone. *J. Chem. Ecol.* 16:353–364.

NIEMEYER, H.M. 1988. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defense chemicals in the Gramineae. *Phytochemistry* 27:3349–3358.

NIEMEYER, H.M., PESEL, E., FRANKE, S., and FRANCKE, W. 1989. Ingestion of the benzoxazinone DIMBOA from wheat plants by aphids. *Phytochemistry* 28:2307–2310.

NYE, P.H., and TINKER, P.B. 1977. Solute movement on the soil root system. Studies in Ecology, Vol. 4. Blackwell, Oxford. pp. 342–355.

PÉREZ, F.J. 1990. Allelopathic effect of hydroxamic acids from cereals on *A. sativa* and *A. fatua*. *Phytochemistry* 29:773–776.

QUEIROLO, C.B., ANDREO, C.S., NIEMEYER, H.M., and CORCUERA, L.J. 1983. Inhibition of ATPase from chloroplast by hydroxamic acid from the Gramineae. *Phytochemistry* 22:2455–2458.

ROVIRA, A.D. 1969. Plant root exudates. *Bot. Rev.* 35:35–59.

RICE, E.L. 1984. Allelopathy, 2nd ed. Academic Press, Orlando.

TANG, C.S., and YOUNG, C.C. 1982. Collection and identification of allelopathic compounds from the undisturbed root system of Bigakta Limpograss (*Hemarthria altissima*). *Plant Physiol.* 69:155–160.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

PHENOLIC ACID CONTENT OF SOILS FROM WHEAT–NO TILL, WHEAT–CONVENTIONAL TILL, AND FALLOW–CONVENTIONAL TILL SOYBEAN CROPPING SYSTEMS[1]

U. BLUM,[2,*] T.R. WENTWORTH,[2] K. KLEIN,[2] A.D. WORSHAM,[3] L.D. KING,[4] T.M. GERIG,[5] and S.-W. LYU[2]

Departments of Botany,[2] Crop Science,[3] Soil Science,[4] and Statistics[5]
North Carolina State University
Raleigh, North Carolina 27695

(Received October 31, 1990; accepted February 1, 1991)

Abstract—Soil core (0–2.5 and/or 0–10 cm) samples were taken from wheat–no till, wheat–conventional till, and fallow–conventional till soybean cropping systems from July to October of 1989 and extracted with water in an autoclave. The soil extracts were analyzed for seven common phenolic acids (*p*-coumaric, vanillic, *p*-hydroxybenzoic, syringic, caffeic, ferulic, and sinapic; in order of importance) by high-performance liquid chromatography. The highest concentration observed was 4 μg/g soil for *p*-coumaric acid. Folin & Ciocalteu's phenol reagent was used to determine total phenolic acid content. Total phenolic acid content of 0- to 2.5-cm core samples was approximately 34% higher than that of the 0- to 10-cm core samples. Phenolic acid content of 0- to 2.5-cm core samples from wheat–no till systems was significantly higher than those from all other cropping systems. Individual phenolic acids and total phenolic acid content of soils were highly correlated. The last two observations were confirmed by principal component analysis. The concentrations were confirmed by principal component analysis. tions of individual phenolic acids extracted from soil samples were related to soil pH, water content of soil samples, total soil carbon, and total soil nitrogen. Indirect evidence suggested that phenolic acids recovered by the water-autoclave procedure used came primarily from bound forms in the soil samples.

Key Words—Wheat, *Triticum aestivum*, soybean, *Glycine max*, no till, con-

*To whom correspondence should be addressed.

[1] The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of products named, nor criticism of similar ones not mentioned.

ventional till, soil extracts, allelopathy, phenolic acids, Folin & Ciocalteu's phenol reagent, HPLC.

INTRODUCTION

Straw residues of wheat, barley, oats, rye, grain sorghum, and sudangrass have effectively suppressed weeds, primarily annual broadleaf weeds (Barnes and Putnam, 1983; Putnam and De Frank, 1983; Putnam et al., 1983; Shilling et al., 1985, 1986b; Liebl and Worsham, 1983). In North Carolina, for example, Worsham (1989) noted that straw management and tilling in no-till-planted crops affected the level of early-season weeds. He found that: (1) removing wheat or rye straw plus tilling resulted in a 9–30% suppression; (2) removing straw without tilling resulted in a 43–50% suppression; (3) removing straw, tilling, and then replacing the straw resulted in a 60% suppression; and (4) leaving straw without tilling resulted in a 76–81% suppression of broadleaf weeds such as redroot pigweed, common lambsquarters, common ragweed, morning-glory, prickly sida, and sicklepod, when compared to no-cover-crop–tilled plots.

Studies have suggested that inhibition of germination and seedling growth by small grain mulches may be due, in part, to allelopathic interactions (Chou and Patrick, 1976; Liebl and Worsham, 1983; Shilling et al., 1985, 1986a,b; Barnes et al., 1986). To actually establish the role of allelopathic interactions in such systems will require: (1) identification of inhibitors involved; (2) determination of the rates of uptake of inhibitors by seeds or roots; and (3) establishing that uptake was of a sufficient magnitude to bring about the observed levels of inhibition.

Concentrations of inhibitors available to interact with seeds and roots of seedlings in soils are determined by the rate of release of potential allelopathic compounds from plant debris, the action of microorganisms, the fixation by the soil, and the rates of soil leaching (Skujins, 1967; Haider and Martin, 1975; Huang et al., 1977; Hartley and Whitehead, 1985, Blum et al., 1987; Dalton et al., 1987; Blum and Shafer, 1988). The level of inhibition observed for a given species will depend on the sensitivity of the species, on an adequate "source" and rate of release of inhibitors, and on the activity and strengths of the various "sinks" (i.e., clays, organic matter, microbes, seeds, and roots) within the soil. Seeds and roots are essentially in "competition" for inhibitors with other sinks in the soil. The "available" fraction in the soil (i.e., that which has not been irreversibly bound, leached, taken up by seeds and roots, or metabolized by microorganisms) at any time represents a residual but dynamic pool through which inhibitors move.

A variety of potentially allelopathic compounds have been identified from small grain mulches, including phenolic acids (Chou and Patrick, 1976; Liebl and Worsham, 1983; Shilling et al., 1985; Barnes et al., 1986). The presence

of simple phenolic acids, usually in bound form, such as caffeic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, sinapic, syringic, and vanillic, is almost universal in plant tissue (Bates-Smith, 1956; Harborne, 1980). All of the above listed phenolic acids have been isolated from soils (Whitehead et al., 1982; Hartley and Whitehead, 1985; Kuiters and Denneman, 1987) and have been identified as potential allelopathic agents (Rice, 1984).

The objectives of this research were to characterize the phenolic acid pools in soils of no-till and conventional-till wheat/soybean cropping systems and to establish correlations between easily obtained soil characteristics and phenolic acid pools. Good correlations between soil characteristics and phenolic acid pools could help in the rapid identification of systems with potential allelopathic interactions.

METHODS AND MATERIALS

General Background Information. A long-term rotation study was initiated in the fall of 1985 to study various low-input cropping systems on a 6-hectare site located 5 km south of the North Carolina State University campus. Forty treatments (only five of which were used for this study) were arranged in a randomized complete block design with four replicates (only three replicates were used for this study). Blocking was based on landscape position and soil texture. Plots of 30 × 8 m with eight rows were used throughout the study. Tillage, planting, spraying, cultivating, and harvesting were done with two-row farm equipment. A Case International no-till planter was used to plant corn (*Zea mays* L. Dekalb 798 or 689), and soybeans (*Glycine max* L. Merill Deltapine 417), and a KMC no-till drill was used to plant wheat (*Triticum aestivum* L. Coker 916) and clover (*Trifolium incarnatum* L. Tibbee or *Trifolium pratense* L. Kenland). Beginning in 1988, a Hiniker cultivator was used to cultivate for weed control. A partial history of the treatments sampled and their codes is outlined in Table 1. Wheat was harvested on June 14, and soybeans were planted on June 19 or 20, 1989.

Sampling. Treatment plots were divided into four sections (15 × 4 m) for sampling. On July 3 and 31, August 31, and October 12, 1989, two soil cores (5.5 cm in diameter; 0–10 cm) per section were taken and combined. For some treatments, additional cores were taken to a depth of 2.5 cm adjacent to the previous sampling locations. The 0- to 2.5-cm core samples were taken for wheat–no till (WNT) and wheat–conventional till in four-year rotation (WT4R) for all sampling dates and for all treatments at the final sampling date. Soil samples were sieved (3 mm sieve), placed in plastic bags, and frozen (−20°C). Maximum storage in the freezer before soils were extracted was three months. Most soil samples were extracted within a month.

TABLE 1. HISTORY OF TREATMENTS SAMPLED AND IDENTIFICATION CODES[a]

1985–86	1986–87	1987–88	1988–89	Weed control summer of 1989	Code for 1988–1989 season
fallow/corn	wheat/soybean	fallow/corn	wheat/soybean	herbicides	WNT
C clv/corn	wheat/soybean	C clv/corn	wheat/soybean	cultivated	WT2R
C clv/corn	R clv/R clv	R clv/corn	wheat/soybean	cultivated	WT4R
fallow/soybean	fallow/corn	fallow/corn	fallow/soybean	herbicides	FTH
fallow/soybean	fallow/corn	fallow/corn	fallow/soybean	cultivated	FTC

[a] WNT = wheat-no till; WT2R and WT4R = wheat-conventional till in two and four-year rotation, respectively; FTH and FTC = fallow-conventional till with weed control by herbicides and cultivation, respectively; C clv = crimson clover; R clv = red clover; herbicides on soybeans, 1989: 3.4 kg glyphosate, 3.4 kg alachlor + 0.14 kg imazaquin per hectare were applied at planting on June 19; 0.3 kg sethoxydim was applied per hectare on August 7; cultivated: tilled with field cultivator (maximum depth 15 cm) on June 14, disked (maximum depth 8 cm) on June 15 or 16, cultivated for weed control (maximum depth 5 cm) on July 3 and 27.

Aboveground soybean and weed biomass were determined on June 13, July 3 and 31, and August 31 by clipping 0.5-m^2 quadrats centered around the soil cores in each section. Standing and surface debris were collected from the quadrats in the no-till plots on the same dates plus October 12. The soybean, weed, and debris samples in each section were combined by type, oven dried (70°C), and weighed.

Aboveground wheat biomass for litter bags was collected on June 13. Litter bags ($N = 54$; 19 × 19 cm; constructed of plastic screen with 1-mm mesh) containing 10 g of dried (40°C for five days) material were half buried between soybean rows on June 26 in the winter wheat plots. Two litter bags per plot were recovered on July 31, August 31, and October 12. Material in the litter bags was freeze-dried. Organic remains were separated from the soil material and weighed.

Site Description. Treatment plots were chosen among available plots of a low-input sustainable agriculture (LISA) cropping system rotation study. Soybean plots selected (see Table 1) included plots with wheat stubble (WNT), plots in which the wheat stubble had been tilled under (WT2R, WT4R), and plots without wheat (fallow plots; FTH, FTC). The soils were Cecil and Appling gravelly sandy loams (Typic Kanhaludults) with 2–6% slope and moderate erosion. Soils were composed of 67 ± 2% (mean ± SE, $N = 15$) sand, 18 ± 0.5% silt, and 16 ± 2% clay with 22 ± 2% gravel. Due to timing of herbicide applications, cultivation, and sampling, weed biomass and surface debris were highly variable (Table 2).

June to November mean maximum and minimum temperatures were 27.8

TABLE 2. SOYBEAN AND WEED BIOMASS, SOIL SURFACE DEBRIS, AND LITTER BAG CONTENT FOR 1989[a]

Sampling date	Code	Soybean (g/m of row)	Weeds (g/m^2)		Surface debris (g/m^2)	Wheat litter in bags (g)
6/13	WNT		32.88 (8.35)	12	110.07 (6.91)	Starting
	WT2R		40.54 (6.93)	12	53.25 (6.85)	weight
	WT4R		64.92 (13.37)	12	105.16 (10.73)	6/26
	FTH		6.63 (2.62)	12		10.05 (0.05)[b]
	FTC		7.49 (2.57)	12		
7/3	WNT	2.88 (0.23)	6.96 (1.82)	9	200.16 (19.77)	
	WT2R	3.04 (0.13)	1.38 (0.41)	11		
	WT4R	3.23 (0.13)	5.29 (0.99)	12		
	FTH	3.42 (0.19)	0.16 (0.05)	3		
	FTC	4.12 (0.25)	1.09 (0.29)	9		
7/31	WNT	51.59 (3.84)	13.69 (3.95)	9	188.45 (12.09)	7.49 (0.24)[c]
	WT2R	60.71 (3.80)	25.27 (6.81)	12		7.78 (0.14)
	WT4R	78.31 (6.14)	44.33 (8.28)	12		7.46 (0.13)
	FTH	77.38 (6.62)				
	FTC	101.22 (10.91)	21.60 (4.41)	11		
8/31	WNT	349.70 (13.08)			268.04 (25.50)	6.42 (0.18)
	WT2R	397.34 (30.58)	108.00	1		7.16 (0.20)
	WT4R	355.70 (31.34)	94.43 (19.25)	7		6.52 (0.15)
	FTH	348.97 (30.42)				
	FTC	433.16 (26.27)	43.25 (32.25)	2		
10/12	WNT				197.83 (28.69)	6.02 (0.13)
	WT2R					6.01 (0.26)
	WT4R					4.88 (0.31)
	FTH					
	FTC					
		N = 12	N value after ()		N = 12	N = 6

[a]WNT = wheat–no till; WT2R and WT4R = wheat–conventional till in two- and four-year rotation, respectively; FTH and FTC = fallow-conventional till with weed control by herbicides and cultivation, respectively; () = standard errors.
[b]Oven ried at 40°C for five days.
[c]Freeze-dried.

and 17.2°C, respectively. There were 54 rain events, which provided a total of 76 cm (maximum event = 9.52 cm). Weather data were collected 1.6 km from the research site. The max/min temperatures and precipitation were 0.65°C lower/0.94°C higher, and 21 cm higher than normal for Raleigh, North Carolina, respectively.

Soybean plant biomass was not significantly affected by any of the treatments selected during July and August of 1989 (Table 2). Soybean biomass/m row increased 100-fold from July 3 to August 31. This rapid increase was partially due to excellent weather conditions. There were four rain events over 2.54 cm during this period.

The decline of wheat straw in the half-buried litter bags was not significantly different for the WNT, WT2R, and WT4R treatments. A 35% reduction (after adjusting for water content) in biomass was observed in the litter bags after 109 days. No significant differences in surface debris, however, were observed for the WNT plots over the same interval. This is consistent with the observations of Summerell and Burgess (1989) that the rate of decomposition of partially incorporated and buried wheat residue is much faster than that of surface debris.

Soil and Litter Analyses. Soil samples were defrosted overnight in a refrigerator (10°C). Three soil subsamples were taken to determine soil pH, water content, and phenolic acid content. The pH was determined by mixing 25 g soil with 100 ml deionized water and reading the pH 60 min later. Water content was determined gravimetrically on 10–15 g of soil before and after oven drying (100°C). Nitrogen and carbon content of soil samples were determined with a Perkin-Elmer 2400 C, H, N analyzer (Perkin-Elmer, Norwalk, Connecticut).

To determine phenolic acid content, 50 g of soil was placed in a 500-ml Erlenmeyer flask with 100 ml deionized water. Loosely capped flasks were autoclaved for 45 min at 1.2 kg/cm^2 and 121°C, removed from the autoclave, and then allowed to come to room temperature (20–30 min). The slurry in the flask was centrifuged for 10 min at 27,200 *g*. The resulting supernatant was filtered (Whatman No. 42). The filtered supernatant (extract 1) was adjusted to pH 2 with HCl, centrifuged for 10 min at 27,200 *g*, and the resulting supernatant was adjusted to pH 7 with NaOH (extract 2).

To determine the phenolic acid content of wheat litter samples, freeze-dried litter samples were ground in a Wiley mill (20 mesh), and 400-mg samples were extracted by the same procedures used for the soil samples.

HPLC Analysis. Protocatechuic acid (200 μl; 0.25 mM; pH 7) was mixed with 1.8 ml of extract 2 from soil and litter samples. The resulting solution was filtered through a 0.2 -μm Supor-200 filter (Gelman Sciences Inc., Ann Arbor, Michigan). Phenolic acids were determined with a Waters (Milford, Massachu-

setts) HPLC at 245 nm, using a model 440 absorption detector and a Baseline 810 Chromatographic Workstation (Dynamic Solutions, Ventura, California). A Waters reverse-phase 5-μm Nova-Pak C_{18} column was used to isolate and quantify eight different phenolic acids [i.e., caffeic (CAF), ferulic (FER), *p*-coumaric (PCO), *p*-hydroxybenzoic (POH), protocatechuic (PRO), sinapic (SIN), syringic (SYR), and vanillic (VAN)]. Standard phenolic acids were obtained from Sigma Chemical Company (St. Louis, Missouri). Extracts were analyzed using two mobile phases: (A) 2% methanol, 0.25% ethyl acetate, and 0.5% acetic acid, and (B) 80% methanol, 1% ethyl acetate, and 2% acetic acid. Linear gradients starting with 92% A and ending with 66% A were used over the first 40 min of a 60-min run. The flow rate of the mobile phase was 0.5 ml/min. Protocatechuic acid was used as a marker in each sample. Identification and quantification were confirmed by comparing retention times and areas with those of the appropriate standards (prepared in deionized water) and by spiking unknown samples with standards. Additional confirmations of the identity of the extracted phenolic acids were obtained with paper chromatography and by UV spectral comparisons in ethanol and ethanol plus sodium hydroxide (Harborne, 1984).

FC Method. Extracts 1 or 2 (0.5 ml) of soils plus 4.5 ml deionized water or 2.5 ml of standard phenolic acid solution plus 2.5 ml deionized water (pH 7) were mixed with 0.75 ml of 1.9 M Na_2CO_3, and 0.25 ml Folin & Ciocalteu's phenol reagent (Sigma Chemical Company, St. Louis, Missouri). This mixture was allowed to stand in darkness at room temperature (20–25°C) for at least 1 hr before its absorption was read at 750 nm (Box, 1983). Ferulic acid was chosen as the standard because it has been identified as an allelopathic agent in wheat mulch (Liebl and Worsham, 1983), and it was somewhat intermediate in color development when compared to the other phenolic acids (Figure 1). Units of total phenolic acid in soils determined by the FC method are thus in micrograms ferulic acid equivalents per gram of soil.

Data Analyses. Data were analyzed, using statistical analysis system programs for analysis of variance, regressions, correlations, and principal component analysis (SAS Institute Inc., 1988). Principal component analysis was run on mean plot values. Phenolic acids in soils were analyzed either as a split-plot design with whole plot subsampling (0- to 10-cm core samples for all sampling dates), a bivariate split-plot design with whole plot subsampling (0- to 2.5- and 0- to 10-cm soil core samples for treatments WNT and WT4R at all sampling dates), or a bivariate randomized block design with whole plot subsampling (0- to 2.5- and 0- to 10-cm soil core samples for all treatments at final sampling date). The bivariate was core type, the whole plot was treatment, and the subplot was sampling date. Means comparisons were based on Bonferroni *t* tests.

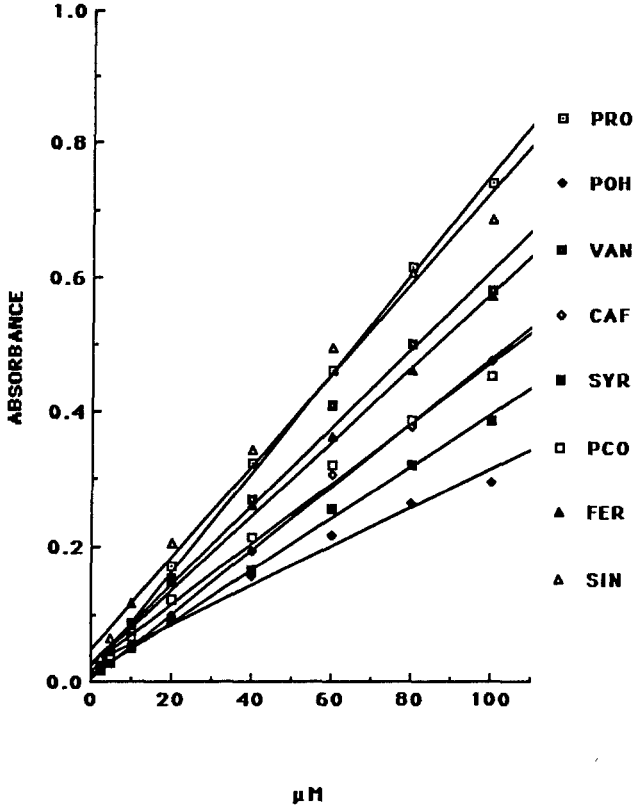


FIG. 1. Standard curves for protocatechuic (PRO), *p*-hydroxybenzoic (POH), vanillic (VAN), caffeic (CAF), syringic (SYR), *p*-coumaric (PCO), ferulic (FER), and sinapic (SIN) acids determined by the FC method. Order based on HPLC retention times.

RESULTS

Soil Extraction Procedures. To determine how the extraction procedures might affect the stability of phenolic acids, we autoclaved, filtered, etc. (see Methods and Materials), a mixture of caffeic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, sinapic, syringic, and vanillic acids (0.25 mM each, 100 ml, pH 5.5). Caffeic acid and sinapic acid were reduced 20 $\pm$ 0.004% (mean $\pm$ SE) and 61 $\pm$ 0.03% in extract 1 and 28 $\pm$ 0.002% and 69 $\pm$ 0.02% in extract 2, respectively. No other major losses or gains were observed. Values subsequently presented have not been adjusted for these losses, because changes noted in these solutions may not be representative of changes in soil extracts. This information is presented only to alert the reader to limitations of this extraction procedure.

To estimate the proportion of "available" to "bound" phenolic acids in the extract, soil samples (75 g) from all the treatments ($N = 60$) were placed into Petri dishes with 25 ml water or 25 ml Hoagland's nutrient solution and incubated in the dark at 30°C. Soils were extracted and analyzed by HPLC at the start and after one and two weeks. Total phenolic acid content for the 0- to 2.5-cm soil samples decreased by 8% and 13% in the water treatment and increased by 3% and 18% in the nutrient solution treatment at the ends of week 1 and 2, respectively. When water was added to 0- to 10-cm soil core samples, total phenolic acid content of the samples was not changed. When nutrient solution was added, the total phenolic acid content was reduced by 10% by the end of week 1, but no additional reduction occurred during week 2. This suggested that phenolic acids in these soils were primarily in a bound form and that decomposition in these soil samples was nutrient-limited. Since, however, we do not have data on actual flows in and out of the phenolic acid pools, these conclusions are lacking in certainty. It is also not certain what proportion of the "bound" phenolic acids extracted by the autoclave procedure may be released into the soil solution by the action of microorganisms.

Soil Phenolic Acids. Treatment effects ($P \leq 0.05$) were noted only for *p*-coumaric acid (PCO). PCO of WNT (2.24 μg/g) and FTH (0.97 μg/g) were significantly different (Table 3). Significant effects of sampling date were noted for all but total phenolic acid (sum of seven phenolic acids determined by HPLC). Highest concentrations (μg/g) were observed for *p*-hydroxybenzoic (POH, 0.95), caffeic (CAF, 0.68), syringic (SYR, 0.80), and PCO (1.80) on August 31 and for vanillic (VAN, 1.43), sinapic (SIN, 0.18), and ferulic (FER, 0.53) on October 12. Lowest concentrations (μg/g) were observed for POH (0.82), CAF (0.48), VAN (0.94), and SYR (0.57) on July 31; for SIN (0.05) and FER (0.42) on August 31; and for PCO (1.30) on October 12. The coefficients of variation for all but PCO, FER, and SIN were less than 45% over all sampling dates for each treatment (Table 3).

Sampling dates (for all but PCO and SIN), core depth, and core depth by sampling date interactions (for all but PCO and SIN) were significantly different ($P \leq 0.05$) when individual and total phenolic acid values were compared for WNT and WT4R. For these treatments both 0- to 2.5- and 0- to 10-cm cores were taken at all sampling dates. The 0- to 2.5-cm soil core samples had on average 34% higher concentrations than the 0- to 10-cm soil core samples (Table 3). For example, the micrograms per gram of total phenolic acids for the 0- to 2.5-cm cores were 10.22, 8.14, 9.26, and 11.15 for sampling dates 1, 2, 3, and 4, respectively (July 3 and 31, August 31 and October 12, 1989, respectively). The values for the 0- to 10-cm cores were 6.14, 5.85, 7.27, and 6.41 μg/g, respectively. The differences between cores were 4.08, 2.29, 1.99, and 4.74 μg/g for sampling dates (SD) 1, 2, 3, and 4, respectively. Thus, values of 1.79, 2.09, 0.66, 0.30, 2.45, and 2.75 were observed for SD1–SD2, SD1–

TABLE 3. PHENOLIC ACID CONTENT, pH, AND PERCENTAGE WATER OF SOIL SAMPLES AVERAGED OVER SAMPLE DATES[a]

Variable	Treatment	0– to 2.5-cm cores		0– to 10-cm cores	
		Mean ± SE (μg/g)	CV	Mean ± SE (μg/g)	CV
POH	WNT	1.51 ± 0.06	28	0.94 ± 0.04	31
	WT2R	1.33 ± 0.15	38	1.00 ± 0.05	35
	WT4R	1.18 ± 0.05	32	1.03 ± 0.05	35
	FTH	0.96 ± 0.07	24	0.68 ± 0.03	29
	FTC	1.00 ± 0.05	19	0.78 ± 0.04	30
VAN	WNT	2.06 ± 0.11	38	1.34 ± 0.07	35
	WT2R	1.52 ± 0.06	15	1.12 ± 0.04	24
	WT4R	1.35 ± 0.05	25	1.25 ± 0.05	28
	FTH	1.13 ± 0.07	22	0.91 ± 0.03	24
	FTC	1.34 ± 0.07	17	1.10 ± 0.04	26
CAF	WNT	1.30 ± 0.06	34	0.74 ± 0.04	39
	WT2R	0.76 ± 0.03	15	0.57 ± 0.02	21
	WT4R	0.76 ± 0.02	23	0.59 ± 0.02	28
	FTH	0.50 ± 0.04	25	0.42 ± 0.02	29
	FTC	0.57 ± 0.03	19	0.51 ± 0.02	38
SYR	WNT	1.53 ± 0.07	33	0.88 ± 0.05	39
	WT2R	0.90 ± 0.04	15	0.67 ± 0.02	21
	WT4R	0.90 ± 0.03	23	0.70 ± 0.03	28
	FTH	0.60 ± 0.04	25	0.49 ± 0.02	29
	FTC	0.67 ± 0.04	19	0.60 ± 0.03	30
PCO	WNT	4.08 ± 0.22	37	2.24 ± 0.17	53
	WT2R	1.59 ± 0.11	23	1.41 ± 0.08	38
	WT4R	2.10 ± 0.08	41	1.74 ± 0.10	40
	FTH	0.93 ± 0.12	43	0.97 ± 0.06	42
	FTC	1.52 ± 0.11	25	1.55 ± 0.10	42
FER	WNT	1.21 ± 0.08	44	0.59 ± 0.03	40
	WT2R	0.76 ± 0.05	22	0.50 ± 0.03	43
	WT4R	0.65 ± 0.04	41	0.50 ± 0.03	44
	FTH	0.41 ± 0.06	46	0.32 ± 0.03	65
	FTC	0.55 ± 0.03	22	0.44 ± 0.03	51
SIN	WNT	0.60 ± 0.07	86	0.16 ± 0.02	90
	WT2R	0.32 ± 0.02	27	0.13 ± 0.02	132
	WT4R	0.18 ± 0.03	103	0.14 ± 0.02	104
	FTH	0.12 ± 0.02	69	0.05 ± 0.01	159
	FTC	0.23 ± 0.02	36	0.12 ± 0.03	154
Total	WNT	12.30 ± 0.58	32	6.89 ± 0.38	38
	WT2R	7.18 ± 0.32	15	5.83 ± 0.14	18
	WT4R	7.11 ± 0.24	23	5.94 ± 0.23	27
	FTH	4.64 ± 0.34	26	3.83 ± 0.13	23
	FTC	5.88 ± 0.27	16	5.10 ± 0.23	32

TABLE 3. Continued

Variable	Treatment	0 to 2.5-cm cores Mean ± SE	CV	0 to 10-cm cores Mean ± SE	CV
pH	WNT	5.5 ± 0.05	6	5.4 ± 0.05	6
	WT2R	5.3 ± 0.08	5	5.2 ± 0.05	7
	WT4R	5.5 ± 0.06	8	5.3 ± 0.06	8
	FTH	5.3 ± 0.09	6	5.2 ± 0.06	7
	FTC	5.7 ± 0.18	11	5.5 ± 0.06	8
% Water	WNT	16 ± 0.49	21	15 ± 0.50	23
	WT2R	13 ± 0.64	17	13 ± 0.37	19
	WT4R	13 ± 0.38	20	14 ± 0.31	15
	FTH	8 ± 0.36	15	9 ± 0.29	22
	FTC	11 ± 0.58	18	13 ± 0.28	8

[a] POH = *p*-hydroxybenzoic acid CAF = caffeic acid; VAN = vanillic acid; SYR = syringic acid; PCO = *p*-coumaric acid; FER = ferulic acid; SIN = sinapic acid; WNT = wheat–no till; WT2R and WT4R = wheat–conventional till in two- and four-year rotation, respectively; FTH and FTC = fallow–conventional till with weed control by herbicides and cultivation, respectively. Number for 0 to 2.5-cm cores: WNT $N = 47$; WT4R $N = 48$; WT2R, FTH, and FTC $N = 12$ (last sample date only). Number for 0 to 10-cm cores: $N = 48$.

SD3, SD2–SD3, SD4–SD3, SD4–SD2, and SD4–SD3 comparisons, respectively (LSD = 1.26). In each case the first sampling date cited had the greater difference between the cores. Thus, the differences in concentration between the core depths for the sampling dates were significantly different for all but SD2–SD3 and SD4–SD3. For a discussion of a bivariate split-plot design with whole plot subsampling, see chapter 15 for factorial experiments and chapter 16 for split-plot designs in Steel and Torrie (1980).

For the final sampling date, a sampling date where both core depths were taken for all treatments, treatment, core depth, and the treatment by core depth interactions were significant ($P \leq 0.05$) for all phenolic acids and for the total phenolic acid content of soil sampled. Only concentrations of WNT and FTH were significantly different. For example, total phenolic acid concentrations for WNT and FTH were 10.18 and 4.42 μg/g, respectively. The 0- to 2.5-cm soil core samples were 32% higher in phenolic acid concentration than the 0- to 10-cm core soil samples. In all instances, the difference in phenolic acid concentrations between the core depths for WNT was greater than that of all other treatments. All other comparisons were not significantly different. For example, the micrograms per gram of total phenolic acids for the 0- to 2.5-cm core samples were 14.09, 7.18, 8.13, 4.94, and 5.88 for WNT, WT2R, WT4R, FTH, and FTC, respectively. The values for the 0- to 10-cm cores were 6.28, 5.66,

6.54, 3.90, and 4.88, respectively. The differences between cores were 7.81, 1.52, 1.59, 1.04, and 1.00 μg/g for WNT, WT2R, WT4R, FTH, and FTC, respectively (LSD = 5.18). For a discussion of a bivariate randomized block design with whole plot subsampling, see chapters 15 and 16 in Steel and Torrie (1980).

Principal component analysis (PCA) has been successfully used in interpreting patterns of objects with several attributed variables. Objects in this case were treatments and the attributed variables were the seven different phenolic acids. PCA takes data that initially have a high dimensionality and creates new variables, called components, which are linear combinations of the original variables. Typically, the first two or three components (referred to as axes in this paper) account for much of the variation in the data. This results in an efficient summarization, which lends itself well to visual presentation and interpretation (Gauch, 1982).

PCA on the 0- to 10-cm core data did not result in clear groupings of treatments or in significant correlations between phenolic acids and any axes; thus only PCA on 0- to 2.5-cm core data are presented. The results of the PCA on phenolic acid content for WNT and WT4R are given by sampling date in Figure 2. Treatments are grouped with the exception of replicate plot 3 (block 3) of WNT, which appears to be more closely related to WT4R than WNT. The soil in this WNT plot was very sandy (78% sand, 17% silt, and 6% clay) compared to the other WNT plots (mean = 60% sand, 19% silt, and 21% clay). The first PCA axis accounted for 88% of the variation (Table 4). All phenolic acids, as well as total phenolic acid, were positively correlated with the first axis. Individual phenolic acids were also highly correlated with total phenolic acid.

The results of the PCA on phenolic acid content of soils sampled (0–2.5 cm) for all treatments at the final sampling date are given in Figure 3. Treatments are generally grouped with some overlapping of treatments. WNT is the most distinct in its distribution. The first PCA axis accounted for 95% of the variation (Table 4). All phenolic acids, as well as total phenolic acid, were positively correlated with the first axis. Individual phenolic acids were also highly correlated with total phenolic acid. Not only were individual phenolic acids correlated with total phenolic acid, but individual phenolic acids were also correlated with each other (Table 5). This latter correlation was based on an independent phenolic acid analysis of randomly chosen soil samples.

Phenolic Acid Concentrations in Litter Bag Samples. Concentrations of individual phenolic acids in extract 2 from litter bag samples for the WNT, WT2R, and WT4R treatments and the three litter sampling dates (July 31, August 31, and October 12) were not significantly different. The mean values and standard errors (N = 18) for POH, VAN, CAF, SYR, PCO, FER, SIN, and total were 12.49 $\pm$ 1.17, 33.35 $\pm$ 2.90, 61.52 $\pm$ 3.77, 40.03 $\pm$ 6.63,

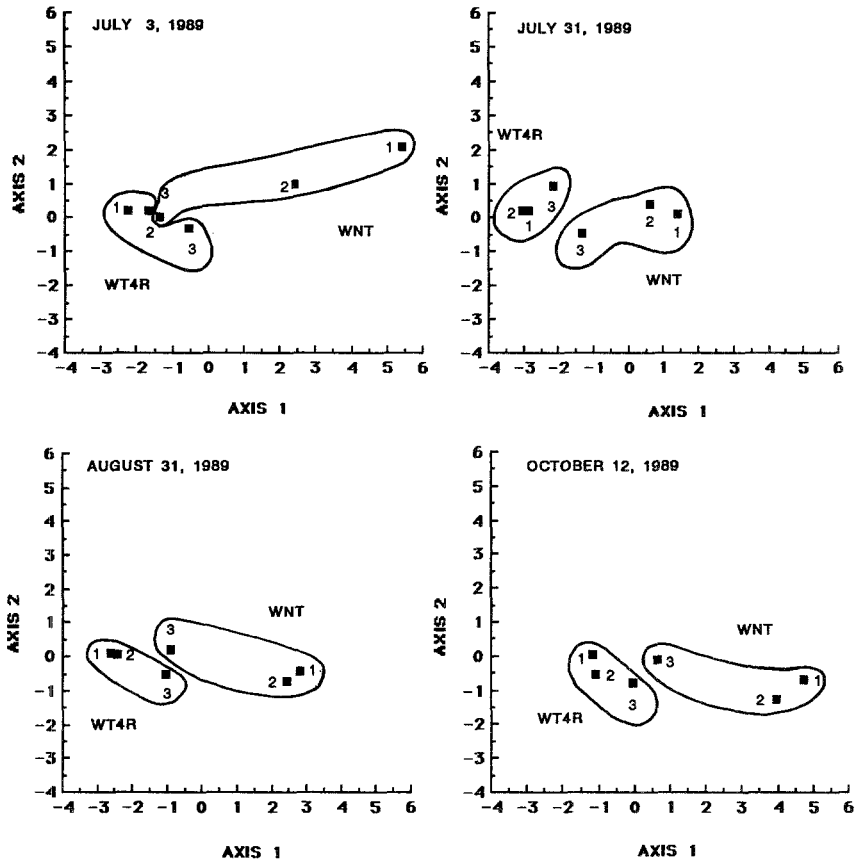


FIG. 2. Principal component analysis for phenolic acid content of soils (0- to 2.5-cm cores) from wheat–conventional till (WT4R) and wheat–no till (WNT) plots. Numbers associated with points are blocks. PCA analysis was based on all four sample dates. Sample dates presented individually only for ease of interpretation.

260.96 ± 26.19, 43.58 ± 3.45, 97.22 ± 22.96, and 549.15 ± 32.41 μg/g, respectively. Concentrations of the individual phenolic acids extracted from wheat straw placed into the litter bags (starting material) were similar to those extracted from the partially decomposed wheat straw, with the exception of FER, SIN, and SYR. FER content was 23% higher and SIN and SYR contents were 49% and 60% lower, respectively.

A 35% reduction in biomass was observed in the partially buried litter bags after 109 days (Table 2). No significant differences in surface debris, however, were observed for the WNT plots over the same interval.

Estimates of Total Phenolic Acids in Soils by FC Method. Phenolic acid

TABLE 4. CORRELATIONS (R) OF INDIVIDUAL PHENOLIC ACIDS FROM 0– TO 2.5-cm SOIL SAMPLES WITH PCA AXIS 1, AND PERCENTAGE OF TOTAL VARIATION ACCOUNTED FOR BY AXIS 1[a]

	Figure 2		Figure 3	
	Axis 1	Total phenolic acid	Axis 1	Total phenolic acid
POH[b]	0.92	0.90	0.92	0.91
VAN	0.95	0.93	0.98	0.98
CAF	0.97	0.97	0.99	0.99
SYR	0.97	0.97	0.99	0.99
PCO	0.94	0.96	0.98	0.98
FER	0.95	0.94	0.99	0.99
SIN	0.84	0.84	0.96	0.96
Total Phenolic Acid	1.00	—	1.00	—
Variation (%)	0.88		0.95	

[a] Correlations of individual phenolic acids with total phenolic acid (HPLC analysis) are also presented.
[b] POH = *p*-hydroxybenzoic; VAN = vanillic; CAF = caffeic; SYR = syringic; PCO = *p*-coumaric; FER = ferulic; SIN = synapic.

content of soil was determined on both extract 1 and extract 2 (injected into the HPLC). Extract 1 (crude extract) was adjusted to pH 2, centrifuged, and then adjusted to pH 7 to produce extract 2. Centrifugation of the acidified extract produced a reddish brown pellet, probably humic acid. The supernatant was straw-yellow in color, probably due to fulvic acid (Stevenson, 1982). Total phenolic acid of extract 2 (μg ferulic acid equivalents/g soil) estimated with the Folin & Ciocalteu's reagent was highly correlated, except for SIN, with the individual phenolic acids and the total phenolic acid (sum of seven phenolic acids) estimated by HPLC (Table 5; Figure 4). Thus, total phenolic acid determined by the FC method could be used for these soil samples to estimate total phenolic acid based on HPLC, as well as individual phenolic acid content of extract 2 (Table 6).

Finally, total phenolic acid of extract 1 based on the FC method was highly correlated with total phenolic acid of extract 2 based on the FC method (Figure 4). This suggested that the amount of reddish brown precipitate removed by acidification and centrifugation of extract 1 was proportional to total concentration, since the relationship of total phenolic acid estimated for extract 1 and extract 2 was linear.

Soil Characteristics and Total Phenolic Acid Content of Extract 1. Total phenolic acid of soil samples estimated by the FC method for extract 1 was

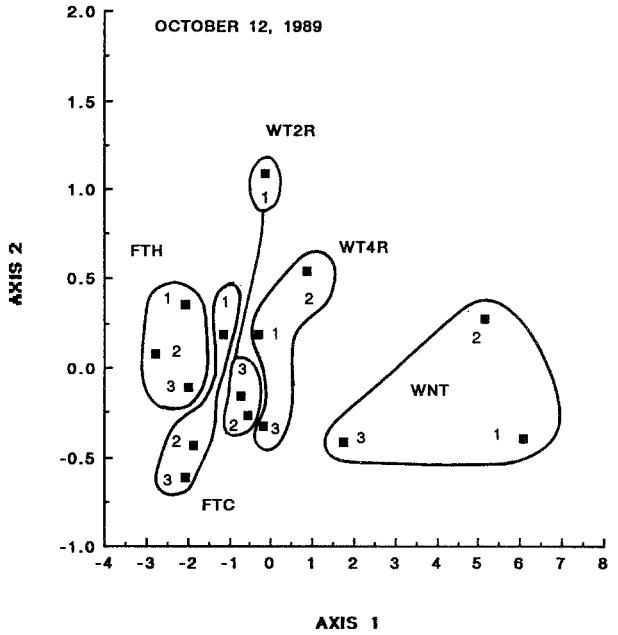


FIG. 3. Principal component analysis of phenolic acids in soils (0- to 2.5-cm cores) from fallow–conventional till (FTH, FTC), wheat–conventional till (WT2R, WT4R) and wheat–no till (WNT) plots for the last sampling date. Numbers associated with points are blocks.

correlated with carbon (C), nitrogen (N), soil pH, and soil water content. R values ranged from 0.51 to 0.83. Stepwise regression with maximum r^2 improvement was used, subsequently, to generate the best one, two, etc., variable models for total phenolic acid. Only nontransformed and squared values (i.e., no interaction terms) were included in the stepwise regressions. The best single-variable model was obtained with N ($r^2 = 0.69$) and the best two-variable model was obtained with N and pH ($r^2 = 0.76$). No improvement in r^2 values occurred with the addition of other variables. Regressions for total phenolic acid content in extract 1 with C, N, soil water content, and pH are presented in Figure 5.

Since C and N were linearly correlated ($r = 0.92$; $\%N = -0.0076 + 0.0527\ \%C$, $r^2 = 0.843$, $P \leq 0.0001$, $N = 48$), N was removed from the data set and the stepwise procedure was repeated. The best single-variable model was now obtained with C ($r^2 = 0.54$), the best two-variable model was obtained with C and the squared soil water content ($r^2 = 0.66$), and the best three-variable model was obtained with C, pH, and the squared soil water content (r^2

TABLE 5. CORRELATION COEFFICIENTS FOR INDIVIDUAL PHENOLIC ACIDS AND TOTAL PHENOLIC ACID DETERMINED BY HPLC (SUM OF INDIVIDUAL PHENOLIC ACID) OR FC METHOD[a]

	POH	VAN	CAF	SYR	PCO	FER	SIN	Total HPLC	Total FC
POH		0.79	0.66	0.79	0.63	0.67	NS	0.79	0.78
VAN			0.81	0.93	0.70	0.89	NS	0.89	0.91
CAF				0.89	0.77	0.83	NS	0.87	0.85
SYR					0.78	0.94	0.28	0.94	0.95
PCO						0.80	0.30	0.91	0.82
FER							0.26	0.92	0.95
SIN								0.45	NS
Total HPLC									0.93

[a] Soil samples were chosen at random from all samples collected during 1989 ($N = 64$). POH = *p*-hydroxybenzoic acid; VAN = vanillic acid; CAF = caffeic acid; SYR = syringic acid; PCO = *p*-coumaric acid; FER = ferulic acid; SIN = sinapic acid; NS = not significantly different at 0.05. Extract 2 data.

= 0.66). No improvement in r^2 values occurred with the addition of other variables.

DISCUSSION

Soil Extraction Procedures. An unequivocal quantification of the "available" fraction of phenolic acids in soil pools has not been made for any soil. The amounts of phenolic acids recovered from a given soil depend on a variety of factors, including the extractant and the extraction procedures used (Hartley and Whitehead, 1985; Dalton et al., 1987). In general, however, most researchers would agree that extractants such as water, low concentrations of calcium hydroxide, sodium acetate, or mild chelating agents provide the most biologically meaningful estimates of the "available" phenolic acid pools. The "available" fraction in the soil is essentially the "free" and/or "readily freed" phenolic acids present at any time.

Phenolic acids, such as ferulic, *p*-coumaric, vanillic, etc., generally are slow to dissolve in water at ambient temperatures. The speed with which phenolic acids solubilize and the amount that can stay in solution can be increased by raising the pH and/or the temperature of the solution. Increasing the temperature and/or the pH of an extraction solution may at times be more desirable than using long-term extractions, because of the presence of microbes. Blum and Shafer (1988), for example, noted that $<1\%$ of exogenously applied phe-

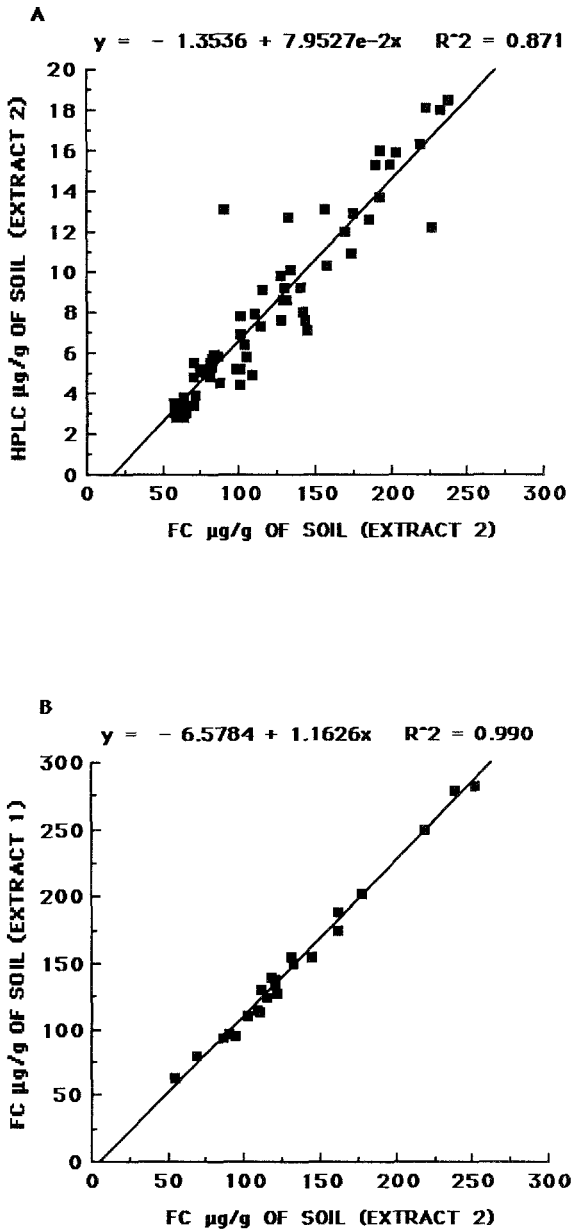


FIG. 4. Relationship of total phenolic acid content of soil determined by HPLC and by the FC method for extract 2 (A). Relationship of total phenolic acid content of soil determined by the FC method for extracts 1 and 2 (B). FC values are in ferulic acid equivalents.

TABLE 6. PARTIAL REGRESSION COEFFICIENTS AND r^2 VALUES FOR INDIVIDUAL PHENOLIC ACIDS AND SUM OF PHENOLIC ACIDS (TOTAL HPLC) REGRESSED AGAINST TOTAL PHENOLIC ACID DETERMINED BY FC METHOD[a]

Phenolic acid	Line intercept	Linear	r^2
POH	0.3444	0.0074	0.61
VAN	−0.0682	0.0158	0.82
CAF	−0.1220	0.0057	0.72
SYR	−0.2254	0.0095	0.90
PCO	−0.8463	0.0278	0.67
FER	−0.3357	0.0095	0.90
SIN	NS		
Total HPLC	−1.3395	0.0794	0.87
Total HPLC-SIN	−1.2532	0.0758	0.91

[a] Soil samples were chosen at random from all soil samples collected during 1989 ($N = 64$). Soil samples in Tables 5 and 6 are identical. POH = *p*-hydroxybenzoic acid; VAN = vanillic acid; CAF = caffeic acid; SYR = syringic acid; PCO = *p*-coumaric acid; FER = ferulic acid; SIN = synaptic acid; $P \leq 0.0001$ in all cases; NS = not significant at 0.05; dependent and independent variables in μg/g of soil. Extract 2 data.

nolic acids could be extracted with water 24 hr after addition to nonsterilized Portsmouth soil, but >90% could be recovered from sterilized Portsmouth soil. Thus, shaking field soil samples for long periods to extract "available" phenolic acids could lead to substantial underestimations of these pools. On the other hand, extractants with artificially high pH or extractions at high temperatures will lead to the extraction of both "available" and some "bound" phenolic acids.

We chose water as the extractant and an extraction procedure that was rapid [i.e., high temperature (121°C) and pressure (1.2 kg/cm^2)], that had only a minimal impact on soil pH, and that eliminated the concern about microbial activity. With the exception of caffeic and sinapic acid, which were reduced in concentration, this procedure appeared to be satisfactory for extracting some of the common phenolic acids found in soil and litter samples. The proportion of "available" and "bound" phenolic acids extracted cannot be stated with certainty, but indirect evidence would suggest that the phenolic acids extracted came primarily from "bound" forms.

Individual Phenolic Acids. With the exception of sinapic acid, the individual phenolic acids were not only correlated with total phenolic acid determined by the FC and HPLC methods, but also to each other. Soil samples analyzed to establish these relationships included samples from all treatments, i.e., WNT, WT2R, WT4R, FTH, and FTC. This indicated that differences in soil phenolic acid content between the crop management systems were primarily quantitative

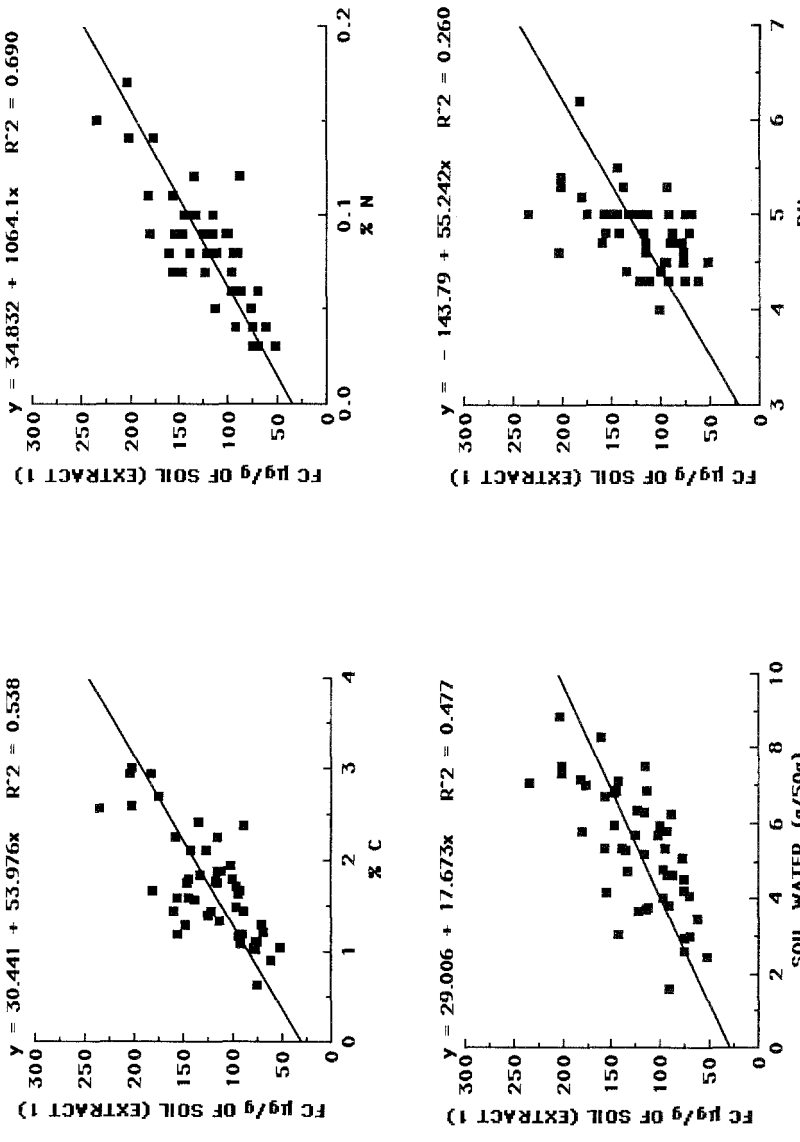


FIG. 5. Relationship of total phenolic acid content of soil and %C, %N, soil water content, and pH. Total phenolic acid values were based on extract 1 and are in ferulic acid equivalents.

and not qualitative. One arrives at the same conclusion by the use of principal component analysis (PCA). For both PCAs (Figures 2 and 3), the strong positive correlations of all individual phenolic acids with the first axis, and the large amount of variance accounted for by the first axis, were indicative of the high level of multicollinearity among the phenolic acids. There is evidently little residual variation that may be attributed to independent behavior of any of the phenolic acids. The first axis, then, is a surrogate for joint variation of all the phenolic acids, as evidenced by its strong positive correlation with total phenolic acid. Positioning of the treatments along the first axis is a reflection of the substantially higher concentrations of all phenolic acids in the WNT treatment and of somewhat greater concentrations in the WT2R and WT4R treatments, relative to the FTH and FTC treatments. This is not to say that concentrations of the individual phenolic acids recovered from soil did not differ from each other. With minor exceptions, PCO > VAN > POH > SYR > CAF > FER > SIN. The benzoic acid derivatives (i.e., POH, SYR, and VAN) were higher in concentration than the cinnamic acid derivatives (i.e., CAF, FER, and SIN), with the exception of PCO.

The types and amounts of plant residue present in the soil and the action of various soil processes (i.e., microbes, soil fixation, leaching, etc.) would obviously be important in determining the absolute and relative amounts of individual phenolic acids present. Such factors may account for the higher concentrations of phenolic acids in the 0- to 2.5-cm than in the 0- to 10-cm core samples (the former were approximately 34% higher than the latter), and the quantitative differences between individual phenolic acids extracted from soil and litter bag samples (i.e., soil samples: PCO > VAN > POH > SYR > CAF > FER > SIN; litter bag samples: PCO > SIN < CAF > FER > SYR > VAN > POH). That the importance of individual phenolic acids in soils is influenced by plant species growing in the soil has been demonstrated by Whitehead et al. (1981, 1982) and Kuiters and Denneman (1987), among others. Whitehead et al. (1981) suggested that substantial proportions of the phenolic acids extracted from soil were either derived from organic residues more than 4 years old or were the result of microbial synthesis. This last observation may suggest one possible reason for the lack of clear differences in the importance of individual phenolic acids in the wheat–no-till (WNT), wheat–conventional-till (WT2R, WT4R), or the fallow–conventional-till (FTH, FTC) system soils.

Phenolic Acids and Sampling Dates. No dramatic changes or clear trends for soil phenolic acid concentrations were observed over the first 109 days of the growing season. Maximum changes for the total phenolic acid content based on HPLC analysis were on the order of 20–30%. Slow changes of phenolic acid concentrations in these nutrient limited soils would be expected if the major sources of these phenolic acids were a result of microbial action on plant litter and/or organic residues (Turner and Rice, 1975; Lodhi, 1978; Whitehead et

al., 1979). We suspect that the fluctuations observed were, in part, a result of the proximity of rain events to the soil sampling dates. This observation is based on the correlations between water content of the soil samples and total phenolic acid content. Additional fluctuations resulted from the variation in litter distribution. As stated before, we do not have data on flows in and out of the phenolic acids pools and, thus, cannot state these conclusions with certainty.

Total Phenolic Acid Content of Soil Extracts. The sum of the individual phenolic acids in extract 2 determined by HPLC analysis (μg/g of soil) was highly correlated with total phenolic acid estimated by the FC method (μg ferulic acid equivalents/g soil) for extract 2. Values for extracts 1 and 2 based on the FC method were also highly correlated with each other. Total phenolic acid content of soil extracts estimated by the FC method was 1.11 times greater for extract 1 than for extract 2. The difference between extracts 1 and 2 was most likely the result of the removal of humic acid by acid precipitation. The small difference observed between extracts 1 and 2 suggested that little humic acid was extracted by the procedure. This was not unexpected, since humic acid is only sparingly soluble in water (Hartley and Whitehead, 1985). The total phenolic acid estimated for extracts 1 and 2 by the FC method were 16.75 and 15 times higher, respectively, than that determined by the HPLC method. Extracts 1 and 2 would include phenolic acids not identified, humic and/or fulvic acid, and other compounds that reduce the Folin & Ciocalteu's phenol reagent. Box (1983) provides a list of organic substances with or without a phenolic hydroxyl group and inorganic substances that reduce the FC reagent. Others have used the FC method to estimate total phenolic acid content of soils (for example, Kuiters and Denneman, 1987), but this is the first time that correlations between the FC and HPLC methods have been made. Whether such correlations will occur for data of other soil systems is not certain. Similar correlations between the FC and HPLC methods, however, have been found for Durham sandy loam soils underneath crimson clover, hairy vetch, subterranean clover, and rye cover crops (unpublished data). Once such correlations are defined for a given soil and management system, the FC method would appear to be useful in surveys to identify potential systems in which allelopathic interactions due to phenolic acids may be important. We would, however, not recommend the use of the FC method to estimate absolute total phenolic acid concentrations in soil extracts, since the Folin & Ciocalteu's phenol reagent reacts differently with individual phenolic acids (Figure 1) and also reacts with other organic and inorganic substances (Box, 1983).

Total phenolic acid content of soil as determined by the FC method was also correlated with soil carbon (C), nitrogen (N), pH, and water content. Since the total amount of phenolic acids dissolved is determined by the volume of water and the pH of the solution, this was not entirely unexpected. The percent C for the soil samples was 2% $\pm$ 0.17 for WNT, 1.7% $\pm$ 0.12 for WT2R,

1.9% ± 0.11 for WT4R, 1.3% ± 0.09 for FTH, and 1.5% ± 0.16 for FTC. The C/N ratios of the soils were 20 ± 0.77 for WNT, 21 ± 1.98 for WT2R, 20 ± 0.60 for WT4R, 26 ± 2.17 for FTH, and 24 ± 1.79 for FTC. C/N ratios above about 14 are a strong indication that the soil contains much partially decomposed plant material (Wild, 1988). Both Iritani and Arnold (1960) and Harmsen and Van Schreven (1955) suggested that a C/N ratio of 20 was the approximate dividing line between positive and negative release of N in soils. The relationships between C, N, and total phenolic acid content suggest that the phenolic acid concentrations in this soil were closely related to organic matter content of the soil. This latter relationship may also help to explain the observed soil phenolic acid content of the various treatments and the patterns observed in the principal component analysis, particularly the small distinction between fallow plots (FTH, FTC) and wheat–conventional till plots (WT2R, WT4R).

Concluding Remarks. Pools of "available" phenolic acids in these soils were small. The majority of phenolic acids recovered, by the procedure used, were released from "bound" forms, which were not necessarily recent in origin. The primary source of "available" phenolic acids would be from the action of microorganisms on plant litter and/or organic residues. Thus, the most likely place for allelopathic interactions in these soils would be in the soil surface of the no-till plots.

Since it appears that a substantial portion of the phenolic acids extracted by the autoclave procedure came from plant litter and/or soil organic residues, the procedure used here in conjunction with the FC method could be used to identify potential soil systems for more detailed study. Total C and/or N content of soil also may be helpful in this regard. However, data on phenolic acid pools ("available" and/or "bound") in soils are, by themselves, unlikely to provide much insight concerning allelopathic interactions unless flows in and out of these pools are determined and source–sink relationships within a given soil are understood.

Acknowledgments—The authors wish to thank Lynn D. Holappa for her technical assistance in the field and laboratory. This research was partially supported by the Southern Region Low-Input Sustainable Agricultural Research and Extension Program of USDA.

REFERENCES

BARNES, J.P., and PUTNAM, A.R. 1983. Rye residues contribute weed suppression in no-tillage cropping systems. *J. Chem. Ecol.* 9:1045–1057.

BARNES, J.P., PUTNAM, A.R., and BURKE, A. 1986. Allelopathic activity of rye (*Secale cereale* L.), pp. 271–286, *in* A.R. Putnam and C.S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

BATES-SMITH, E.C. 1956. The commoner phenolic constituents of plants and their systematic distribution. *Proc. R. Dublin Sci. Soc.* 27:165–176.

BLUM, U., and SHAFER, S.R. 1988. Microbial populations and phenolic acids in soil. *Soil Biol. Biochem.* 20:793–800.

BLUM, U., WEED, S.B., and DALTON, B.R. 1987. Influence of various soil factors on the effects of ferulic acid on leaf expansion of cucumber seedlings. *Plant Soil* 98:111–130.

BOX, J.D. 1983. Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. *Water Res.* 17:511–525.

CHOU, C.H., and PATRICK, Z.A. 1976. Identification and phytotoxic activity of compounds produced during decomposition of corn and rye residues in soil. *J. Chem. Ecol.* 2:369–387.

DALTON, B.R., WEED, S.B., and BLUM, U. 1987. Plant phenolic acids in soils: A comparison of extraction procedures. *Soil Sci. Soc. Am. J.* 51:1515–1521.

GAUCH, H.G., JR. 1982. Multivariate Analysis in Community Ecology. Cambridge University Press, New York.

HAIDER, K., and MARTIN, J.P. 1975. Decomposition of specifically carbon-14 labeled benzoic and cinnamic acid derivatives in soil. *Soil Sci. Am. Proc.* 39:657–662.

HARBORNE, J.B. 1980. Plant phenolics, pp. 331–401, *in* E.A. Bell and B.V. Charlwood (eds.). Secondary Plant Products, Encyclopedia of Plant Physiology, Vol. 8. Springer-Verlag, Berlin.

HARBORNE, J.B. 1984. Phytochemical Methods. A Guide to Modern Plant Analysis. Chapman and Hall, New York.

HARMSEN, G.W., and VAN SCHREVEN, D.A. 1955. Mineralization of organic nitrogen in soil. *Adv. Agron.* 7: 299–395.

HARTLEY, R.D., and WHITEHEAD, D.C. 1985. Phenolic acids in soils and their influence on plant growth and soil microbial processes, pp. 109–150, *in* D. Vaughan and R.E. Malcolm (eds.). Soil Organic Matter and Biological Activity, Developments in Plant and Soil Sciences, Vol. 16. Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, Netherlands.

HUANG, P.M., WANG, T.S.C., WANG, M.K., WU, M.H., and HSU, N.W. 1977. Retention of phenolic acids by noncrystalline hydroxy-aluminum and -iron compounds and clay minerals of soils. *Soil Sci.* 123:213–219.

IRITANI, W.M., and ARNOLD, C.Y. 1960. Nitrogen release of vegetable crop residues during incubation as related to their chemical composition. *Soil Sci.* 89:74–82.

KUITERS, A.T., and DENNEMAN, C.A.J. 1987. Water-soluble phenolic substances in soils under several coniferous and deciduous tree species. *Soil Biol. Biochem.* 19:765–769.

LIEBL, R.A., and WORSHAM, A.D. 1983. Inhibition of morningglory (*Ipomoea lacunosa* L.) and certain other weed species by phytotoxic components of wheat (*Triticum aestivum* L.) straw. *J. Chem. Ecol.* 9:1027–1043.

LODHI, M.A.K. 1978. Allelopathic effects of decaying litter of dominant trees and their associated soil in a lowland forest community. *Am. J. Bot.* 65:340–344.

PUTNAM, A.R., and DE FRANK, J. 1983. Use of phytotoxic plant residues for selective weed control. *Crop Prot.* 2:173–181.

PUTNAM, A.R., DE FRANK, J., and BARNES, J.P. 1983. Exploitation of allelopathy for weed control in annual and perennial cropping systems. *J. Chem. Ecol.* 9:1001–1010.

RICE, E.L. 1984. Allelopathy, 2nd ed. Academic Press, Orlando, Florida.

SAS INSTITUTE INC. 1988. SAS/STAT User's Guide Release 6.03 Edition. SAS Institute Inc., Cary, North Carolina.

SHILLING, D.G., LIEBL, R.A., and WORSHAM, A.D. 1985. Rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) mulch: The suppression of certain broadleaved weeds and the isolation and identification of phytotoxins, pp. 243–271, *in* A.C. Thompson (ed.). The Chemistry of Allelopathy: Biochemical Interactions Among Plants. ACS Symposium Series 268, American Chemical Society, Washington, D.C.

SHILLING, D.G., JONES, L.A., WORSHAM, A.D., PARKER, C.E., and WILSON, R.F. 1986a. Isolation and identification of some phytotoxic compounds from aqueous extracts of rye (*Secale cereale* L.). *J. Agric. Food Chem.* 34:633–638.

SHILLING, D.G., WORSHAM, A.D., and DANEHOWER, D.A. 1986b. Influence of mulch, tillage, and diphenamid on weed control, yield, and quality in no-till flue-cured tobacco (*Nicotiana tabacum*). *Weed Sci.* 34:738–744.

SKUJINS, J.J. 1967. Enzymes in soils, pp. 371–414, *in* A.D. McLaren and G.H. Peterson (eds.). Soil Biochemistry. Marcel Dekker, New York.

STEEL, R.G.D., and TORRIE, J.H. 1980. Principles and Procedures of Statistics, A Biomedical Approach. McGraw-Hill, New York.

STEVENSON, F.J. 1982. Humus Chemistry. John Wiley & Sons, New York.

SUMMERELL, B.A., and BURGESS, L.W. 1989. Decomposition and chemical composition of cereal straw. *Soil Biol. Biochem.* 21:551–559.

TURNER, J.A., and RICE, E.L. 1975. Microbial decomposition of ferulic acid in soil. *J. Chem. Ecol.* 1:41–58.

WHITEHEAD, D.C., BUCHAN, H., and HARTLEY, R.D. 1979. Composition and decomposition of roots of ryegrass and red clover. *Soil Biol. Biochem.* 11:619–628.

WHITEHEAD, D.C., DIBB, H., and HARTLEY, R.D. 1981. Extractant pH and the release of phenolic compounds from soils, plant roots and leaf litter. *Soil Biol. Biochem.* 13:343–348.

WHITEHEAD, D.C., DIBB, H., and HARTLEY, R.D. 1982. Phenolic compounds in soil as influenced by the growth of different plant species. *J. Appl. Ecol.* 19:579–588.

WILD, A. 1988. Russell's Soil Conditions and Plant Growth, 11th ed. Longman Scientific and Technical, Essex, England.

WORSHAM, A.D. 1989. Current and potential techniques using allelopathy as an aid in weed management, pp. 275–291, *in* C.H. Chou, and G.R. Waller (eds.). Phytochemical Ecology: Allelochemicals, Mycotoxins and Insect Pheromones and Allomones. Academia Sinica Monograph Series No. 9, Taipei, Taiwan.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

RESPONSE TO PREY CHEMICAL CUES BY HATCHLING PINE SNAKES (*Pituophis melanoleucus*): EFFECTS OF INCUBATION TEMPERATURE AND EXPERIENCE

JOANNA BURGER

Department of Biological Sciences
Rutgers University
Piscataway, New Jersey 08855-1059

(Received October 24, 1990; accepted February 4, 1991)

Abstract—The ability of hatchling pine snakes (*Pituophis melanoleucus*) to select and follow or avoid chemical odors of prey (mice, *Mus musculus*) on a shavings and paper substrate was investigated in Y-maze experiments, as a function of incubation temperature and experience. Incubation temperature affected behavior in the maze, and the maze choices of naive snakes, but not of snakes that had already eaten a mouse. The data indicate that snakes that have eaten, preferentially enter the arm bearing chemical stimuli from mice, whereas those that have not eaten show no preference.

Key Words—Chemical cues, incubation temperature, experience, predation, pine snake, *Pituophis melanoleucus*, mice, *Mus musculus*, learning, olfaction.

INTRODUCTION

Pheromonal communication, as demonstrated in controlled laboratory experiments on trailing of conspecifics or prey by chemical means, occurs in snakes (Brown and Maclean, 1983; Chiszar et al., 1986; Ford, 1982; Ford and O'Bleness, 1986; Gehlback et al., 1971; Heller and Halpern, 1981) and lizards (Cooper and Vitt, 1986a). Reptiles use chemical sensory abilities in several important contexts (Von Achen and Rakestraw, 1984; Mason 1991), including recognition of prey (Burghardt, 1973, 1980; Chiszar et al., 1986; Cooper, 1989, 1991; Cooper and Alberts, 1990; Cooper and Vitt, 1989; Krekorian, 1989), detection of conspecifics (Cooper and Vitt, 1984) and related congeners (Cooper

0098-0331/91/0600-1069$06.50/0

and Vitt, 1986c,d), trailing of conspecifics to hibernacula (Brown and Maclean, 1983) or to mates (Ford and Schofield, 1984; Ford and O'Bleness, 1986), and recognition of ophiophagous snakes (Weldon and Burghardt, 1979; Weldon, 1982; Burger, 1989a). Reptiles often respond to chemical stimuli by increased tongue flicks (Cooper and Vitt, 1984, 1985, 1986b; Cooper et al., 1986). Most of the studies dealing with prey detection have relied on examinations of tongue-flicking (i.e., Cooper, 1989; Krekorian, 1989) rather than examination of choice behavior in individuals.

In this report I examine the behavior of hatchling pine snakes (*Pituophis melanoleucus*) experimentally exposed to shavings with and without the odor of mice (*Mus musculus*). I was particularly interested in whether hatchling snakes could discriminate between odors and no odors and whether incubation temperature or prior experience with live mice affected their responses. In nature, pine snake eggs experience differences in temperature similar to those used in this experiment (Burger and Zappalorti, 1986).

METHODS AND MATERIALS

Under appropriate state permits, pine snake eggs were collected from the Pine Barrens of southern New Jersey (Ocean, Cumberland, and Monmouth counties) in 1986 (247 hatchlings), 1987 (221 hatchlings), and 1988 (230 hatchlings). In 1986 the clutches from each female were divided in thirds; one third was incubated at 23°C, another at 28°C and a third at 33°C. In 1987 and 1988 all eggs were incubated at 28°C.

Date of hatching was noted. Snakes were maintained in individual plastic (30 × 15 × 19 cm) cages at 24–26°C. Cages contained paper for shelter. They were given the opportunity to drink water daily. Snakes were sexed by eversion of the hemipenes (Fitch, 1963; Gregory, 1983; Gutzke et al., 1985).

To determine the response of hatchlings to potential prey, I performed two experiments: one to examine the effect of both incubation temperature and experience on response (1986), and the other to examine the effect of experience alone (1987 and 1988). All experiments were conducted at 24–26°C.

In 1986 all hatchlings were exposed to a live mouse, whereas in 1987 and 1988 some hatchlings were exposed to a live mouse and others were not. Hatchlings exposed to a mouse either caught and ate the mouse or they did not. This design (Table 1) allowed evaluation of the effects of different types of experiences on hatchling response in the maze. Because incubation temperature affected behavior in the Y maze in 1986, all eggs were incubated at the same temperature in 1987 and 1988.

In 1986, hatchlings that had shed (15–20 days of age) were individually placed in an aquarium with a live laboratory mouse (12–18 g) for 20 min and

TABLE 1. EXPERIMENTAL DESIGN FOR HATCHLING PINE SNAKE EXPOSURE TO MOUSE ODORS—EXPOSURE TO LIVE MICE AND NUMBER OF HATCHLINGS IN EACH CATEGORY

Prior experience	1986	1987 and 1988
Exposed to live mouse		
Ate	96	130
Did not eat	151	75
Not exposed to mice (post-shed)[a]	—	202
Not exposed to mice (pre-shed)[b]	—	44

[a] Snake tested in maze after initial shedding.
[b] Snake tested prior to shedding.

were allowed to eat the mouse if it was caught within this time. Ten days following their exposure to a mouse, the hatchlings were tested in a Y-maze apparatus (after Burger, 1989a). The base arm of the Y maze was 1 m long and 15 cm wide with 15-cm-high wooden sides. The two experimental arms extended at a 45° angle from the base and were of the same dimensions as the base arm. Plexiglas was placed over the maze to prevent the hatchlings from escaping, but the ends of the maze were open, allowing snakes to crawl out. The base arm had shavings with no odor, the experimental arms had shavings with no odor on one side and shavings from a mouse cage in which three mice had lived for five days on the other. The floor of all three arms was covered with paper. After every test the paper with the shavings was removed, and the arm with the mouse shavings was switched (to avoid possible position effects).

In 1987 and 1988 all snakes were incubated at the same temperature, but only half the snakes were exposed to a live mouse prior to experimentation (Table 1). The remaining hatchlings were tested without exposure to a live mouse, either before shedding (10–12 days of age) or after shedding (25–30 days of age). Pine snakes normally do not eat until they have shed (Burger, 1989b). In 1987 and 1988 hatchlings were randomly assigned to condition (whether to be exposed to a mouse or not), but some hatchlings within each clutch were assigned to each of the exposure categories.

In all test experiments the hatchlings had a choice of selecting the experimental arm with shavings with no odor or the experimental arm with shavings from a mouse cage. We did not touch the shavings with our hands. Each snake was tested only once. Following experimentation all pine snake hatchlings were returned to their original nest sites in the field, together with their siblings and egg shells.

For each trial the hatchling was placed by hand into the base arm with its head facing the entrance. The hatchling was allowed to move freely up the base arm to the intersection, where it usually moved into one of the experimental

arms. The trial ended when the hatchling left the Y maze through one of the experimental arms. Two experimenters were necessary to time various behaviors. The time each hatchling was in the base and experimental arm, time at the Y junction (where the two experimental arms diverge from the base arm), and the number of tongue flicks when the snake was in each location were recorded.

Contingency table χ^2 tests were used to determine differences in responses, and two-tailed Kruskal-Wallis χ^2 tests were used to determine differences in the distribution of response time and tongue flicks under several conditions. The percent of hatchlings that entered the arm with the mouse shavings as a function of experience did not differ significantly between 1987 and 1988 (contingency table χ^2 test), so these data were combined. Data are presented as means $\pm$ 1 SE. Probability levels of less than 0.05 were accepted as significant.

RESULTS

In the 1986 experiment, 71% (68 of 96) of the hatchlings that had eaten a mouse selected the experimental arm with mouse shavings ($\chi^2 = 16.6$, $df = 1$, $P < 0.001$), whereas only 33% (38 of 114) of those that had not eaten a mouse did so ($\chi^2 = 12.6$ $df = 1$, $P < 0.001$), In the above analysis, I tested the null hypothesis that there were no differences in response; thus, half the hatchlings should choose each experimental arm. Hatchlings that ate when exposed to a mouse selected the experimental arm with mouse odor significantly more often than did those that had not eaten ($\chi^2 = 29.2$, $df = 1$, $P < 0.001$).

Incubation temperature (28°C vs. 33°C) did not affect the frequency of mouse eating ($\chi^2 = 2.7$, not significant), but did affect Y-maze choices (Table 2). Hatchlings incubated at 28°C showed no significant difference in their response when they had eaten a mouse, but showed a significant difference when they had not previously eaten. Hatchlings incubated at 33°C showed the opposite response; significantly more hatchlings that had eaten chose the experimental arm with mouse shavings whereas there was no difference if they had not eaten (Table 2). I could not examine a sufficient sample incubated at 23°C in 1986 for a two-way analysis.

In the Y maze, hatchlings normally moved quickly down the base arm, stopped momentarily at the Y junction (called time motionless hereafter), explored in the junction by moving their head back and forth with rapid tongue flicking, and then moved down one of the experimental arms. The time hatchlings spent in the base arm and at the choice point in the Y junction, and the number of tongue flicks in the base arm varied significantly by incubation temperature (Table 3). Movement time in the base arm and time motionless were inversely related to incubation temperature.

The number of tongue flicks in the base arm was directly related to incu-

TABLE 2. EFFECT OF INCUBATION TEMPERATURE ON HATCHLING PINE SNAKE BEHAVIOR (1986)[a]

Presented with mouse	28°C	33°C
Ate mouse[b]		
Chose mouse shavings	24	44
Chose no-odor shavings	16	12
Contingency χ^2 (*P*)	1.6 (NS)	9.1 (0.001)
Did not eat mouse[c]		
Chose mouse shavings	13	25
Chose no-odor shavings	47	28
Contingency χ^2 (*P*)	19.2 (0.001)	0.8 (NS)

[a] Shown are number in each category. Snakes incubated at 22°C are not included because none initially ate a mouse.
[b] χ^2 for 28° vs. 33°C = 1.2 (NS).
[c] χ^2 for 28° vs. 33°C = 10.5 (0.001).

TABLE 3. BEHAVIOR OF HATCHLING PINE SNAKES AS FUNCTION OF INCUBATION TEMPERATURE. (MEANS ± SE; TIME IN SECONDS)

	Incubation temperature			Kruskal-Wallis
	23°C	28°C	33°C	χ^2 (*P*)
Number of Snakes	38	106	103	
Base arm				
Time	5.4 ± 0.6	4.1 ± 0.3	3.4 ± 0.3	9.71 (0.02)
Flicks	16.9 ± 0.3	17.9 ± 0.4	18.0 ± 0.5	9.91 (0.03)
Choice site				
Motionless				
Time	23.1 ± 5.6	12.4 ± 3.9	7.1 ± 2.2	7.79 (0.05)
Flicks	18.9 ± 1.6	17.1 ± 2.1	17.9 ± 2.6	0.86 (NS)
Explore				
Time	17.9 ± 7.9	9.8 ± 4.6	26.6 ± 14.3	0.49 (NS)
Flicks	2.3 ± 0.8	3.2 ± 1.2	3.9 ± 1.5	0.89 (NS)
Experimental arm				
Time	8.1 ± 3.4	5.6 ± 1.2	4.7 ± 1.3	3.99 (NS)
Flicks	15.6 ± 0.5	16.6 ± 0.4	16.9 ± 0.4	3.81 (NS)

bation temperatures. Although there were differences in the number of tongue flicks in the base arm, they were not large, and there were no differences in the number of tongue flicks in the other parts of the maze. These data may suggest that regardless of the time spent in different parts of the maze, the hatchlings

TABLE 4. RESPONSE OF HATCHLINGS AS FUNCTION OF EXPERIENCE (TIME IN SECONDS AND TONGUE FLICKS)

	Ate	Exposed	Not exposed		Kruskal-Wallis χ^2 (P)
			Shed	Preshed	
Number	130	75	202	44	
Base arm					
Time	4.5 ± 2.5	4.8 ± 2.1	9.9 ± 17.4	8.0 ± 14.5	43.9 (0.0001)
Flicks	16.8 ± 3.5	17.3 ± 3.9	17.0 ± 4.3	17.0 ± 3.9	7.2 (NS)
Choice site					
Motionless	4.9 ± 11.0	4.2 ± 8.1	10.4 ± 22.7	7.2 ± 17.0	11.6 (.02)
Flicks	3.2 ± 3.3	6.3 ± 4.3	6.3 ± 5.0	5.5 ± 4.7	42.7 (0.0001)
Explore					
Time	3.1 ± 8.2	2.1 ± 5.5	6.1 ± 15.4	5.0 ± 16.5	8.5 (0.07)
Flicks	10.8 ± 5.6	8.0 ± 6.1	12.0 ± 4.8	11.4 ± 5.4	7.3 (NS)
Experimental arm					
Time	6.1 ± 6.0	6.8 ± 4.4	12.4 ± 19.7	11.4 ± 17.3	66.3 (0.0001)
Flicks	16.4 ± 2.7	16.1 ± 3.0	16.4 ± 3.4	16.4 ± 3.3	0.5 (NS)
% Entered mouse choice arm	75.7	36.9	39.6	34.1	46.1 (0.0001)

used a similar number of tongue flicks to assay the situation when they encountered mouse odor.

There were significant differences in their choices as a function of experience ($\chi^2 = 46.1$, $df = 6$, $P < 0.001$). Over 75% of the hatchlings that had eaten chose the experimental arm with the shavings, whereas only 34–40% of the snakes with all other experiences (those exposed to mice but did not eat and those not exposed) did so.

Hatchling behavior in the maze also varied significantly with respect to experience (Table 4). The time in the various parts of the Y maze varied significantly (except for exploring in the Y junction), even though this was a novel situation for all hatchlings. In general, hatchlings exposed to live mice spent less time in every section than those not exposed.

DISCUSSION

Role of Experience. Pine snake eggs in the Pine Barrens normally hatch from late August to late September (Burger and Zappalorti, 1986). Most pine snakes travel to hibernacula in October, and by early November have entered the hibernacula or are near its entrance (Burger et al., 1988). Thus, pine snake hatchlings have only a few weeks to find food and a suitable hibernaculum.

Presumably, snakes that find food have higher overwintering success than those that do not find food. Thus, it would be adaptive to be able to recognize rodent runs or burrows by chemical means, increasing the likelihood of encountering prey. Field observations of hatchlings released in the New Jersey Pine Barrens indicate that 12 individuals encountered mouse runs and immediately changed their direction of movement, following them below ground (J. Burger, unpublished data). Indeed, in two cases the hatchlings found, caught, and ate *Peromyscus leucopus*.

I had initially predicted that hungry snakes (i.e., those that had not eaten) would show the highest preference for the mouse shavings. Yet the experiments indicate that experience with eating a mouse leads to the preference for prey odors. Hatchling pine snakes that have already eaten a mouse detect and follow the odor of mice, whereas those that have not eaten show no preference for a mouse trail.

Some hatchlings may never learn to forage, an experience amateur herpetologists and zoo keepers report for some snakes. With repeated exposure to live mice in the laboratory, about 15% of hatchlings fail to eat by 50 days of age.

Role of Incubation Temperature. In a variety of reptiles incubation temperature affects mortality and body structure (Burger et al., 1987; Deeming and Ferguson, 1989a; Gutzke et al., 1985; Vinegar, 1974), thermal selection (Deeming and Ferguson, 1989b, Lang, 1987; Lang et al., 1989), adult sexuality (Gutzke and Crews, 1988), secondary sex ratios (Burger and Zappalorti, 1988; Webb and Cooper-Preston, 1989), sexual differentiation (Charnier, 1966; Ferguson and Joanen, 1982; Yntema, 1976, 1979) and locomotion, agility, and antipredator behavior (Burger, 1989b, 1990). Thus it is no surprise that incubation temperature can affect chemoreception or feeding behavior.

In these experiments hatchlings incubated at 33°C showed a greater preference for the experimental arm with the mouse shavings than did those incubated at 28°C. These results suggest that hatchlings incubated at higher temperatures will respond more quickly to mouse trails or runs than those incubated at lower temperature. Even if these differences are developmental (i.e., in another week the 28°C hatchlings would behave similarly), the slight advantage suggested by these data for snakes that hatch from warmer nests may be enough to effect overall survival since hatchlings have only limited time to find their hibernacula before cold weather sets in.

Pine snake hatchlings incubated at low temperature (23°C) moved more slowly in the Y maze than did hatchlings from eggs incubated at higher temperatures. This corraborates my previous findings with pine snakes where locomotion was examined directly (Burger, 1989b). Similarly, black racer (*Coluber constrictor*) hatchlings incubated at 22°C moved more slowly than those incubated at 28°C (Burger, 1990).

More generally, however, the added time hatchlings incubated at low temperatures spend motionless or exploring at a decision point in nature may cause the difference between catching a mouse or not. That is, hatchlings hunt by searching for mouse burrows (J. Burger, unpublished data), and the extra time may allow prey to escape. Thus hatchlings from nests incubated at low temperatures may be at a disadvantage with respect to hunting compared to other hatchlings. In these experiments, previous experience with catching and eating a mouse affected responses to the chemical odor trials of mice, confirming that experience sharpens the hunting ability of pine snakes.

Acknowledgments—I thank R.T. Zappalorti for companionship in the field; M. Gochfeld, J. Sollazzo, M. Mikovsky, and B. Lauro for help with the experiments; and W. Cooper and M. Gochfeld for valuable suggestions on an earlier draft of the manuscript.

REFERENCES

BROWN, W.S., and MACLEAN, F.M. 1983. Conspecific scent-trailing by newborn timber rattlesnakes, *Crotalus horridus*. *J. Herpetol* . 39:430-436.

BURGER, J. 1989a. Following of conspecific and avoidance of predator chemical cues by pine snakes (*Pituophis melanoleucus*). *J. Chem. Ecol* . 15:799-806.

BURGER, J. 1989b. Incubation temperature has long-term effects on behavior of young pine snakes (*Pituophis melanoleucus*). *Behav. Ecol. Sociobiol.* 24:201-207.

BURGER, J. 1990. Effects of incubation temperature on behavior of young black racers (*Coluber constrictor*) and king snakes (*Lampropeltis getulus*). *J. Herpetol.* 24:158-163.

BURGER, J., and ZAPPALORTI, R.T. 1986. Nest site selection by pine snakes, *Pituophis melanoleucus*, in the New Jersey Pine Barrens. *Copeia* 1986:116-121.

BURGER, J., and ZAPPALORTI, R.T. 1988. Effects of incubation temperature on sex ratios in pine snakes: Differential vulnerabilities of males and females. *Am. Nat.* 132:492-585.

BURGER, J., ZAPPALORTI, R.T., and GOCHFELD, M. 1987. Developmental effects of incubation temperature on hatchling pine snakes *Pituophis melanoleucus*. *Comp. Biochem. Physiol.* 87A:727-732.

BURGER, J., ZAPPALORTI, R.T., GOCHFELD, M., BOARMAN, W.I., CAFFREY, M., DOIG, V., GARBER, S.D., LAURO, B., MIKOVSKY, M., SAFINA, C., and SALIVA, J. 1988. Hibernacula and summer den sites of pine snakes *Pituophis melanoleucus* in the New Jersey Pine Barrens. *J. Herpetol.* 22:425-433.

BURGHARDT, G.M. 1973. Chemical release of prey attack: Extension to naive newly hatched lizards, *Eumeces fasciatus*. *Copeia* 1973:178-181.

BURGHARDT, G.M. 1980. Behavioral and stimulus correlates of vomeronasal functioning in reptiles: Feeding, grouping, sex, and tongue use, pp. 275-301, D. Muller-Schwarze, and R.M. Silverstein (eds.). *in* Chemical Signals in Vertebrates and Aquatic Invertebrates. Plenum Press, New York.

CHARNIER, M. 1966. Action de la temperature sur la sex-ratio chez l'embryon d'*Agama agama* (Agamidae, Lacertilien). *Soc. Biol. Quest Afr.* 160:620-622.

CHISZAR, D., RADCLIFF, C., BOYD, R., RADCLIFF, A., YUN, H., SMITH, H.M., BOYER, R., ATKINS, B., and FEILER, F. 1986. Trailing behavior in cottonmouths (*Agkistrodon piscivorus*). *J. Herpetol.* 20:269-272.

COOPER, W.E. 1989. Strike-induced chemosensory searching occurs in lizards. *J. Chem. Ecol.* 15:1311-1320.

COOPER, W.E., 1991. Chemical detection of predators by a lizard, the broad-headed skink (*Eumeces laticeps*). *J. Exp. Zool.* In press.

COOPER, W.E., and ALBERTS, A.C. 1990. Responses to chemical food stimuli by an herbivorous actively foraging lizard, *Dipsosaurus dorsalis. J. Herpetol.* 46:259–266.

COOPER, W.E., and VITT, L.J. 1984. Detection of conspecific odors by the female broad-headed skink, *Eumeces laticeps. J. Exp. Zool.* 229:49–54.

COOPER, W.E., and VITT, L.J. 1985. Responses of the skinks, *Eumeces fasciatus* and *E. laticeps*, to airborne conspecific odors: Further appraisal. *J. Herpetol.* 19:481–486.

COOPER, W.E., and VITT, L.J. 1986a. Tracking of female conspecific odor trails by male broad-headed skinks (*Eumeces laticeps*). *Ethology* 71:242–248.

COOPER, W.E., and VITT, L.J. 1986b. Interspecific odor discrimination by a lizard (*Eumeces laticeps*). *Anim. Behav.* 34:367–376.

COOPER, W.E., and VITT, L.J. 1986c. Interspecific odor discrimination among syntopic congeners in scincid lizards (genus *Eumeces*). *Behavior* 97:1–9.

COOPER, W.E., and VITT, L.J. 1986d. Thermal dependence of tongue-flicking and comments on use of tongue flicking as an index of squamate behavior. *Ethology* 71:177–186.

COOPER, W.E., GARSTKA, W.R., and VITT, L.J. 1986. Female sex pheromone in the lizard *Eumeces laticeps. J. Herpetol.* 42:361–366.

DEEMING, D.C., and FERGUSON, M.W.J. 1989a. Effect of incubation temperature on growth and development of embryos of *Alligator mississippiensis. J. Comp. Physiol. B.* 159:183–193.

DEEMING, D.C., and FERGUSON, M.W.J. 1989b. The mechanism of temperature dependent sex determination in Crocodilians: A hypothesis. *Am. Zool.* 29:347–383.

FERGUSON, M.W.J., and JOANEN, T. 1982. Temperature of egg incubation determines sex in *Alligator mississippiensis. Nature* 296:850–853.

FITCH, H.S. 1963. Criteria for determining sex and breeding maturity in snakes. *Herpetologia* 16:49–51.

FORD, N.B. 1982. Species specificity of sex pheromone trails of sympatric and allopatric garter snakes (*Thamnophis*). *Copeia* 1982:10–13.

FORD, N.B., and O'BLENESS, M.L. 1986. Species and sexual specificity of pheromone trails of the garter snake, *Thamnophis marcianus. J. Herpetol.* 20:259–262.

FORD, N.B., and SCHOFIELD, C.W. 1984. Species specificity of sex pheromone trails in the plains garter snake, *Thamnophis radix. Herpetologica* 40:51–55.

GEHLBACK, F.R., WATKINS, J., and KROLL, J. 1971. Pheromone trail following studies of typhlopid, leptotyphlopid, and colubrid snakes. *Behavior* 40:282–294.

GREGORY, P.T. 1983. Identification of sex of small snakes in the field. *Herpetol. Rev.* 14:42–43.

GUTZKE, W.H.N., and CREWS, D. 1988. Embryonic temperature determines adult sexuality in a reptile. *Nature* 332:832–834.

GUTZKE, W.N., PAUKSTIS, G.L., and MCDANIEL, L.C. 1985. Skewed sex ratios of adult and hatchling bull snakes, *Pituophis melanoleucus*, in Nebraska. *Copeia* 1985:649–652.

HELLER, S., and HALPERN, M. 1981. Laboratory observations on conspecific and congeneric scent trailing in garter snakes (*Thamnophis*). *Behav. Neurol. Biol.* 33:372–377.

KREKORIAN, C.O. 1989. Field and laboratory observations on chemoreception in the desert iguana, *Dipsosaurus dorsalis. J. Herpetol.* 23:267–273.

LANG, J. 1987. Crocodilian thermal selection, pp. 301–307, *in* G.J.W. Webbs, S.C. Manolis, and P.J. Whitehead (eds.). Wildlife Management: Crocodiles and Alligators. Surry, Baetty and Sons, London.

LANG, J.W., ANDREWS, H., and WHITAKER, R. 1989. Sex determination and sex ratios in *Crocodylus palustris. Am. Zool.* 29:935–952.

MASON, R. 1991. Reptilian pheromones, *in* C. Gans and D. Crews (eds.). Biology of the Reptilia, Vol. 18. Academic Press, New York. In press.

VINEGAR, A. 1974. Evolutionary implication of temperature induced anomaly of development in snake embryos. *Herpetologica* 30:73–74.

VON ACHEN, P.H., and RAKESTRAW, J.L. 1984. The role of chemoreception in the prey selection of neonate reptiles, *in* Vertebrate Ecology and Systematics—A Tribute to Harry S. Fitch. R.A. Seigel, L.E. Hunt, J.L. Knight, L. Malaret, and N.L. Zuschlag (eds.). Museum of Natural History, University of Kansas, Lawrence, Kansas.

WEBB, G.J.W., and COOPER-PRESTON, H. 1989. Effects of incubation temperature on crocodiles and the evolution of reptilian oviparity. *Am. Zool.* 29:953–971.

WELDON, P.J. 1982. Responses to ophiophagous snakes by snakes of the genus *Thamnophis*. *Copeia* 1982:788–794.

WELDON, P.J., and BURGHARDT, G.M. 1979. The ophiophage defensive response in crotaline snakes: Extension to new taxa. *J. Chem. Ecol.* 5:141–151.

YNTEMA, C.L. 1976. Effects of incubation temperature on sexual differentiation in the turtle *Chelydra serpentina*. *J. Morphol.* 150:453–462.

YNTEMA, C.L. 1979. Temperature levels and periods of sex determination during incubation of eggs of *Chelydra serpentina*. *J. Morphol.* 159:17–27.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

PHYTOALEXIN INDUCTION IN RUBIACEAE[1]

MARCIA R. BRAGA,[2] M. CLAUDIA M. YOUNG,[2]
SONIA M.C. DIETRICH,[2,*] and OTTO R. GOTTLIEB[3]

[2]*Seção de Fisiologia e Bioquimica de Plantas*
Instituto de Botánica de São Paulo
C.P. 4005, São Paulo, SP 01051 Brazil

[3]*Instituto de Quimica*
Universidade de São Paulo
C.P. 20780, São Paulo, SP 01498 Brazil

(Received July 30, 1990; accepted February 4, 1991)

Abstract—Phytoalexin responses were measured by modified drop-diffusate and facilitated diffusion techniques after fungal inoculation of leaves of 32 Rubiaceae species from Brazilian forest and savanna. Such responses presented a trend similar to that previously observed for a broad sample of dicotyledonous plants and are more frequently positive for the more primitive (or slower growing) trees than for the advanced (or faster growing) herbs. Fifteen of these species analyzed during a one-year period showed that positive phytoalexin responses are stronger for the rainy (and hotter) than for the dry (and cooler) season. Species that contain relatively large quantities of phenolics gave invariably negative responses. Positive responses are not necessarily associated with the appearance of new substances within leaf tissue and are thus caused by inhibitins rather than by phytoalexins. These results are discussed recognizing that the tested plants are subject to the multifarious influences of their natural environment and of a possible conjugate-caused compartmentation of plant metabolites.

Key Words—Phytoalexins, fungitoxins, inhibitins, Rubiaceae, seasonal variations, leaf diffusates, leaf extractives, fungitoxin–phenol conjugation.

INTRODUCTION

Observation of phytoalexin induction is strongly influenced by the experimental techniques used. Application of the drop-diffusate (dd) procedure results in the

*To whom correspondence should be addressed.
[1]Taken in part from the MSc thesis presented by M.R.B. to Universidade de São Paulo (1988).

0098-0331/91/0600-1079$06.50/0

predominance of positive phytoalexin responses in cultivated (rather than in wild) species, while a modified facilitated diffusion (fd) procedure leads to comparable results for cultivated and wild species. Positive phytoalexin responses by both procedures are more common in summer, the rainy season, than in winter, the dry season. These correlations were encountered during a survey of phytoalexin formation involving 169 species belonging to 43 dicotyledonous families (Braga et al., 1986). Such broad sampling answered the main query of the investigation: the evaluation of the ubiquity of the phenomenon, but was of course not suited to reveal phylogenetic and environmental constraints.

It was the purpose of the present work to verify if morphologically closely related species could reveal clearly interpretable trends. To this end, sampling was reduced to 33 specimens of 32 species of the family Rubiaceae, substantially all of which had already been carefully marked and identified in a previous work (Braga et al., 1986). The number of species, even if minute in comparison with the ca. 7000 recognized species of the family, is nevertheless representative since it includes 44% and 48% of the species known to exist in the examined habitats, a forest (Jung-Mendaçolli, 1984) and a savanna (Mantovani, 1983), respectively, of southeastern Brazil.

METHODS AND MATERIALS

Recently collected leaves from adult plants were used in all tests. The plants occur either in the Reserva Biológica, Instituto de Botánica, São Paulo, SP (forest) or in the Estação Ecológica e Experimental de Moji Guaçu, Fazenda Campininha, SP (savanna) and are listed in Table 1. The data on climate were obtained from Instituto Astronómico e Geofísico, Universidade de São Paulo, SP (forest) or from Estação Ecológica e Experimental de Moji Guaçu, SP (savanna).

All experimental details, including collection of leaves, nature and administration of *Trichoderma pseudokoningii* Rifai, the inducer fungus, and *Cladosporium cladosporioides* (Fresen) de Vries, the detector fungus by the modified drop-diffusate (dd) and facilitated diffusion (fd) techniques have been described in a previous paper (Braga et al., 1986). After the initial screening of 32 species for phytoalexin response, 15 individuals of 15 species (eight from forest and seven from savanna) were selected in order to follow up these responses during a one-year period. Phenolic content in leaf diffusates of 14 of these plants assayed in autumn was measured by the Folin-Denis procedure (Swain and Hillis, 1959), using phenol as the standard.

Fungitoxic substances were extracted from leaves of eight of these species by Keen's technique (Keen, 1978). Aliquots of extracts were submitted to thin-layer chromatography, using hexane–ethyl acetate–methanol (60:40:1) fol-

TABLE 1. CLASSIFICATION OF SPECIES OF RUBIACEAE (KIRKBRIDE, 1982) AND RELATION TO PHYTOALEXIN RESPONSE MEASURED BY DIAMETERS OF INHIBITION HALOS OF *Cladosporium cladosporioides* OBTAINED BY DROP DIFFUSION (dd) AND FACILITATED DIFFUSION (fd) IN LEAVES INDUCED BY *Trichoderma pseudokoningii*[a]

Subfamily	Tribe	Species	Habitat	Habit	Season	*Phytoalexin response* (halo diam, mm)	
						dd	fd
Cinchonoideae	II Rondeletieae	*Bathysa meridionalis*	Forest	Tree	Spring	24	10–58
	VI Gardenieae	*Tocoyena formosa*	Savanna	Shrub	Autumn	0	23–42
		Alibertia myrcifolia	Forest	Tree	Summer	0	0
		A. macrophylla	Savanna	Shrub	Autumn	0	0
		A. sessilis	Savanna	Shrub	Autumn	0	0
		Amaioua intermedia var. *bras.*	Forest	Shrub	Summer	23	
	VII Coffeeae	*Chomelia catharinae*	Forest	Shrub	Summer		0
		Chomelia sp.	Savanna	Shrub	Autumn	0	0
Rubioideae	XI Coccocypseleae	*Coccocypselum condalia*	Forest	Herb	Summer	0	15
		C. lanceolatum	Savanna	Herb	Autumn	0	0
	XVI Psychotrieae	*Psychotria ruelliaefolia*	Forest	Herb	Autumn	0	0
		P. malaneoides	Forest	Shrub	Autumn	0	0–42
		P. stachioides	Forest	Shrub	Autumn		0
		P. cephalanta	Forest	Tree	Winter	0	0
		P. suterella	Forest	Shrub	Summer	0	15–40
		P. sessilis	Forest	Tree	Autumn	0	40–38
		P. tricholoba	Savanna	Shrub	Summer	0	40
		P. barbiflora	Savanna	Shrub	Summer	0	42–40
		Palicourea marcgravii	Forest	Shrub	Spring	26	28–45
			Savanna	Shrub	Autumn	0	33
		P. rigida	Savanna	Tree	Autumn	0	25–35
		Rudgea gardenioides	Forest	Tree	Summer	18	23–30

TABLE 1. Continued

Subfamily	Tribe	Species	Habitat	Habit	Season	*Phytoalexin response* (halo diam, mm) dd	fd
		R. jasminoides	Forest	Tree	Summer	22	20–30
		R. viburnioides	Savanna	Shrub	Autumn	0	40–35
	XV Coussareae	*Coussarea hydrangeaefolia*	Savanna	Tree	Autumn	0	30–38
		Faramea montevidensis	Forest	Tree	Summer	0	0
	XVII Spermacoceae	*Borreria latifolia*	Forest	Herb	Autumn		0
		B. verticillata	Forest	Herb	Autumn		0
		B. eupatorioides	Savanna	Herb	Autumn		0
		B. suaveolens	Savanna	Herb	Autumn		0
		Richardia brasiliensis	Forest	Herb	Autumn		0
		Diodia brasiliensis	Forest	Herb	Autumn	0	0
		D. gymnocephala	Forest	Herb	Autumn	0	0
		D. radula	Forest	Herb	Autumn	0	0

[a] Data partly taken from a previous survey (Braga et al., 1986) complemented with the seasonal analyses performed in the present work. Subfamilies and tribes within subfamilies arranged from top to bottom phylogenetically.

lowed by chloroform–methanol (50 : 1) as solvent systems. The fungitoxic zones were detected using a spore suspension of *Cladosporium sphaerospermum* Pemzig according to the bioautography technique (Homans and Fuchs, 1970).

RESULTS

Table 1 shows the taxonomic arrangement of the tested species. Data were taken partly from our previous work (Braga et al., 1986) and partly from the seasonal analyses performed in the present work. Inspection of the data in Table 1 show the following concerning phytoalexin induction in leaves of Rubiaceae: (1) The phenomenon is practically absent from herbs [11 species, positive responses by dd 0 (0%), by fd 1 (9%)], but generally present in shrubs [14 species, positive responses by dd 2 (14%), by fd 8 (57%)] and chiefly in trees [9 species, positive responses by dd 3 (33%), by fd 6 (67%)]. (2) Responses are equally negative for the species belonging to the more advanced tribes (see Table 1, footnote) of the two subfamilies. (3) The dd technique is applicable only to some of the forest species; savanna species gave positive results only by the fd technique. (4) Reproducibility of data is higher in autumn or in spring, when most measurements were taken. At the height of the rainy season (summer), even a herb (*Coccocypselum condalia*) shows a slightly positive result; at the height of the dry season (winter) even a forest tree (*Psychotria cephalanta*) may show a negative result. (5) For at least one genus (*Alibertia*) results are negative even for trees.

Extending the measurements to different monthly periods (Figure 1) in 15 selected species, revealed other facts: (1) Forest and savanna species usually are highly variable with season, tending towards positive response maxima in summer and minima in winter. (2) Responses for forest and savanna species of *Alibertia* are negative all year round. This situation is summarized most clearly observing either the means of the inhibition halos registered for all species providing positive phytoalexin responses per month (Figure 2) or the percentage of positive phytoalexin responses based on all species analyzed per month (Figure 3). Minimum values coincide with the slightly cooler (temperature difference 10°C) and drastically drier (pluviometric difference 200 mm) weather (Figure 3).

Analyses of the phenolic content of the diffusates obtained by the dd technique led to the following observations (Table 2): (1) In most cases fungal induction alters the amount of phenolic substances only slightly. No correlation of this alteration and phytoalexin response was noted. Increase in phenolic content is no guarantee for a positive response. (2) The species of *Alibertia*, for which only negative phytoalexin responses had been obtained under all conditions, contain considerable quantities of phenolics. (3) With respect to the vicar-

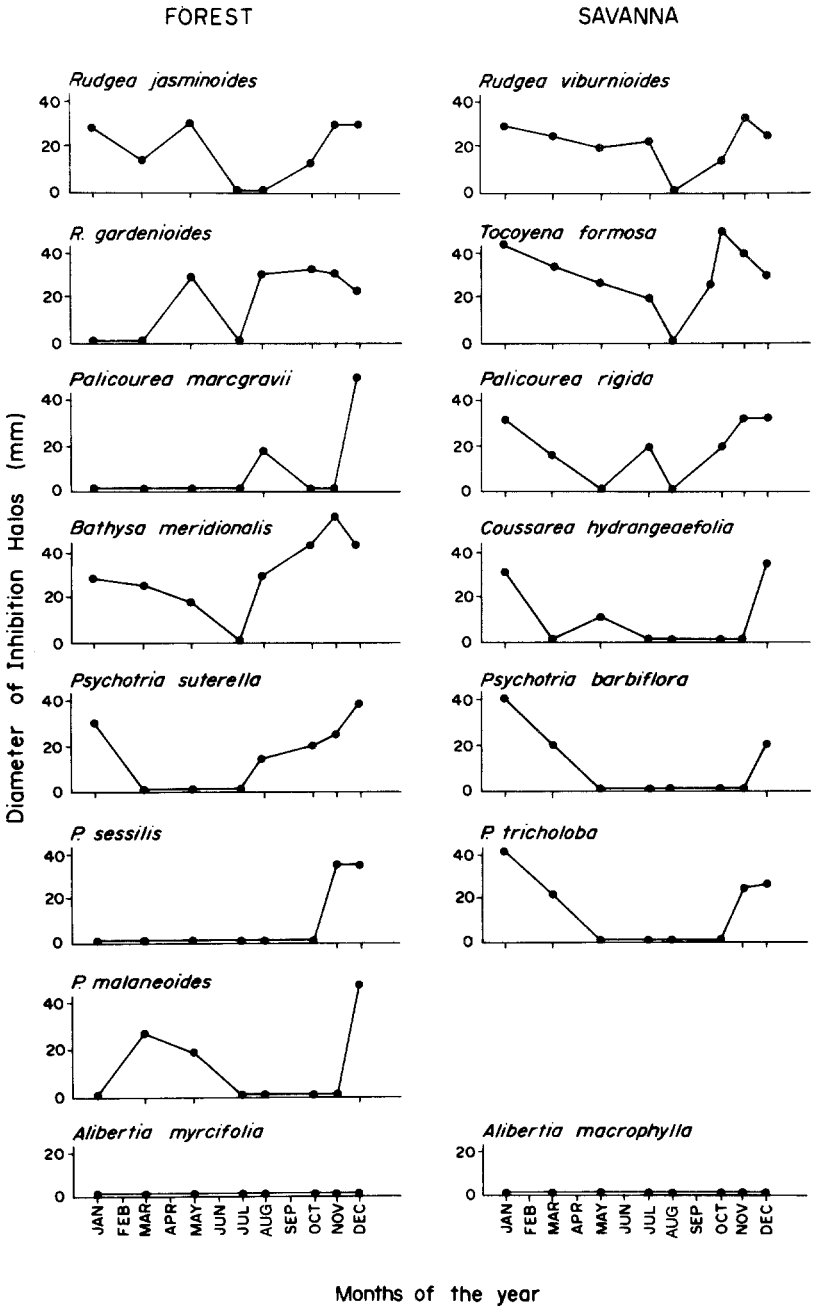


FIG. 1. Variation of phytoalexin induction (recorded as mean diameter in mm of the inhibition halos, fd technique) in leaves of 15 species of Rubiaceae from forest and savanna in months of 1985.

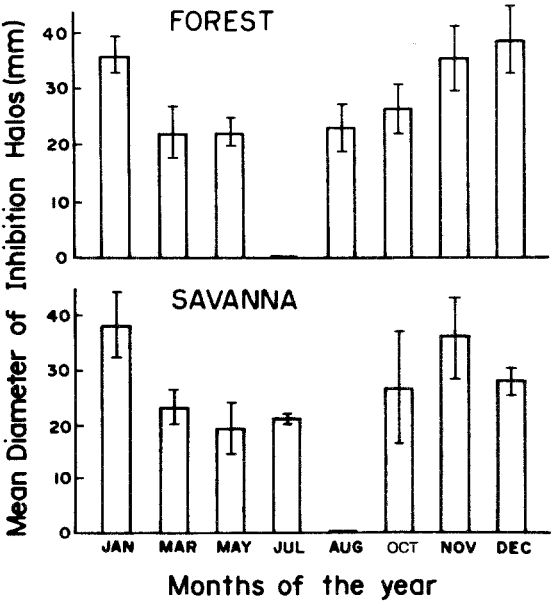


FIG. 2. Mean variation of phytoalexin induction (recorded as the mean diameter for all species providing positive responses in mm of the inhibition halos, fd technique) and standard deviation of the mean in leaves of Rubiaceae from forest and savanna in months of 1985.

ious *Alibertia*, the savanna species is much richer in diffusable phenols than the forest species.

Examination of extracts from leaves, inoculated with spores of the inducer fungus, by TLC revealed (Table 3): (1) The same fungitoxic compounds exist in extracts of inoculated leaves and their non-inoculated controls. (2) Among the five species with positive phytoalexin responses, only *Bathysa meridionalis* and *Palicourea rigida* possessed one fungitoxic compound not found in control leaves. (3) Fungitoxins were extracted even from the *Alibertia* species, which had given solely negative phytoalexin responses by the fd technique.

DISCUSSION

The results suggest that phytoalexin responses are subject to exogenous and endogenous factors. With respect to the former, it is well known that tropical plants, in contrast to plants in temperate regions, are highly sensitive to small environmental variations (Figueiredo-Ribeiro and Dietrich, 1983). Thus, although summer and winter temperatures differ by only a few degrees, they

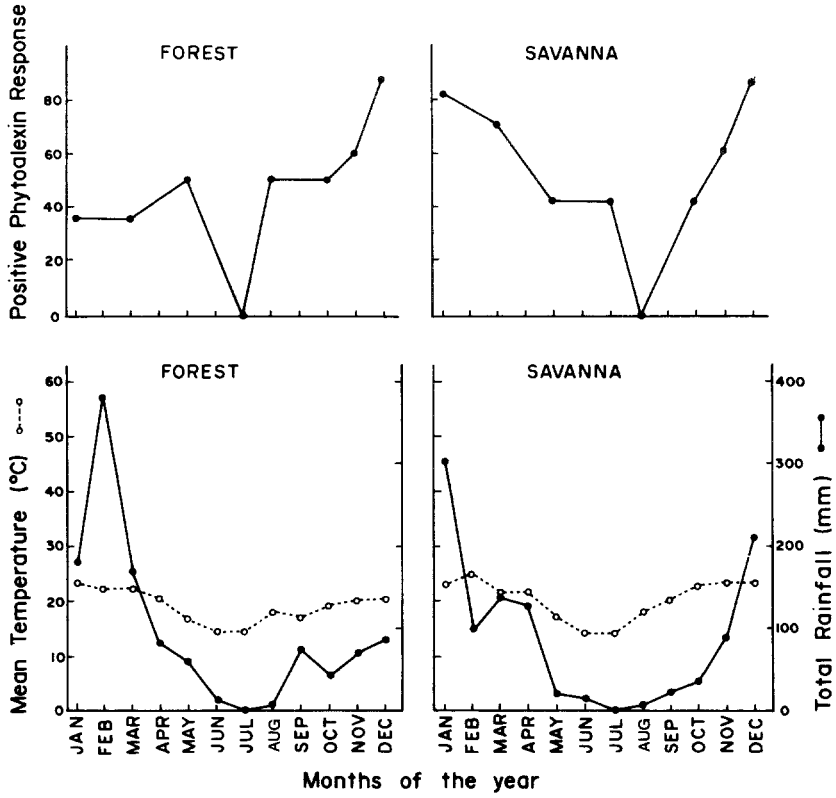


FIG. 3. Top: Mean variation of phytoalexin induction (recorded as the percentage of positive phytoalexin responses, fd technique) for leaves of Rubiaceae from forest and savanna in months of 1985. Bottom: Variations of mean temperature (°C) and rainfall (mm) in months of 1985.

may well play a decisive role. It also must be remembered that the readings represent mean temperatures and cooler or hotter periods would have compensated for each other and escaped registry. Furthermore, although the results are seen to depend dramatically on atmospheric humidity, this may be affected by an additional superimposed variable, the developmental stage of the specimen. However, preliminary analyses of phenological stages seem to suggest this to be a minor factor, which only accentuates positive responses for plants in the flowering and fruiting stage. Lack of phytoalexin response in winter is most probably due to difficulty of metabolite synthesis or diffusion through the dry tissue towards the leaf surface.

The evolutionary trend in angiosperms is usually considered to involve transition from woody to herbaceous habit. Because phytoalexin response is

TABLE 2. PHENOLIC CONTENT (MEAN VALUE OF TWO DETERMINATIONS) OF DIFFUSATES FROM LEAVES OF FOREST AND SAVANNA SPECIES OF RUBIACEAE BEFORE (CONTROL) AND AFTER INOCULATION WITH SUSPENSION OF SPORES OF *Trichoderma pseudokoningii* (+FUNGUS) VS. NEGATIVE OR POSITIVE PHYTOALEXIN RESPONSE BY dd TECHNIQUE

Forest species	dd response	Phenols (mg/ml) +Fungus	Phenols (mg/ml) Control	Savanna species	dd response	Phenols (mg/ml) (mg/ml) +Fungus	Phenols (mg/ml) (mg/ml) Control
Alibertia myrcifolia	Negative	77.8	75.4	*Alibertia macrophylla*	Negative	308.7	277.6
Rudgea jasminoides	Positive	8.1	7.2	*Tocoyena formosa*	Negative	20.1	20.1
Rudgea gardenioides	Positive	6.0	2.8	*Psychotria tricholoba*	Negative	7.7	4.8
Palicourea marcgravii	Positive	5.2	3.2	*Coussarea hydrangeaefolia*	Negative	5.6	3.6
Psychotria sessilis	Negative	4.8	5.6	*Psychotria barbiflora*	Negative	5.2	2.0
Bathysa meridionalis	Positive	4.4	3.6	*Palicourea rigida*	Negative	4.4	1.2
Psychotria suterella	Negative	4.0	6.8				
Psychotria malaneoides	Negative	0.0	0.0				

TABLE 3. TLC SPOTS (R_f VALUES) OF FUNGITOXINS FOR EXTRACTS FROM LEAVES OF FOREST AND SAVANNA RUBIACEAE SPECIES BEFORE (CONTROL) AND AFTER INOCULATION WITH SUSPENSION OF SPORES OF *trichoderma pseudokoningii* (+FUNGUS) VS. NEGATIVE OR POSITIVE PHYTOALEXIN RESPONSE BY fd TECHNIQUE

Forest species	fd response	+Fungus	Control	Savanna species	fd response	+Fungus	Control
Alibertia myrcifolia	Negative	0.54[a]	0.54[a]	*Alibertia macrophylla*	Negative	0.15	0.15
						0.29[a]	0.29[a]
						0.63	0.63
Palicourea marcgravii	Negative	0.59	0.59	*Palicourea rigida*	Positive	0.16	—
		0.75	0.75				
Rudgea jasminoides	Positive	0.62	0.62	*Rudgea viburnioides*	Positive	0.19[a]	0.19[a]
		0.69	0.69			0.31	0.31
Bathysa meridionalis	Positive	0.29		*Tocoyena formosa*	Positive	0.25[a]	0.25[a]
		0.48[a]	0.48[a]				
		0.77	0.77				

[a] Fluorescent spot.

consistently negative in herbs, it should thus be a primitive phenomenon in Rubiaceae. Indeed, the tribes Coffeeae and Spermacoceae, for which thus far only negative results have been reported (Table 1), are placed near the terminals of the tribal lists of their subfamilies (Kirkbride, 1982). Presumably these terminals incorporate the more highly evolved plant groups. However, it has been reported that fast rate of growth, as would be typical of herbaceous rather than of arboreous species, represses the production of defense compounds (Coley et al., 1985), a phenomenon that at least partly may well account for the observed facts.

The two *Alibertia* species are ligneous and hence would be expected to show positive phytoalexin responses. This is not the case. Thus again for *Alibertia*, as already suggested by the uniformity of the data for Spermacoceae (Table 1), phylogenetic factors seem to predominate. Nevertheless, the *Alibertia* species possess a totally different chemical makeup than all other analyzed species, accumulating tannins, i.e., immobile defense material sensu Coley et al. (1985). It is consequently surprising to find that both also contain constitutional fungitoxins (Table 3). However, these are not revealed as tannins in the dd and fd tests. It is hence highly probable that strong hydrogen bonding between the large quantities of macromolecular tannin and the relatively small quantity of micromolecular fungitoxin obstructs diffusion of the latter, which is of course essential for the manifestation of a positive response. Absence of exudated fungitoxins also occurs in *Palicourea marcgravii* (Table 3). *Rudgea jasminoides*, *R. viburnioides*, and *Tocoyena formosa* gave positive phytoalexin responses, although in this case none of the extracted fungitoxins could be defined as responsible for the effect, since they were present prior to fungal infection under these experimental conditions. Of course it must be considered that, in contrast with the plants used in most previous studies on phytoalexins, which were cultivated in greenhouses (Ingham, 1982), our plant material comes from the wild where the leaves are exposed to natural populations of fungi (Bailey, 1971) since their inception. Although de novo synthesis of fungitoxins in wild plants must occur, such true phytoalexins (Harborne, 1988) may not be detected by diffusion tests due to conjugate formation or compartimentalization (Barz and Koster, 1981). However, if an inducer fungus is subsequently supplied experimentally, an additional quantity of the same fungitoxins may be formed. Now, unless retention continued to be caused by excess of phenolic material, a positive phytoalexin response, at this time better designated inhibitin response (Harborne, 1988), will appear.

CONCLUSION

The analyses of phytoalexin responses in a group of taxonomically related species belonging to the family Rubiaceae reinforced the trends observed for a large sample of dicotyledonous species previously studied (Braga et al., 1986)

in terms of influence of environmental conditions and systematic relationships. Furthermore, leaves of wild Rubiaceae species contain phytoalexins probably as the result of interaction with naturally occurring fungi. Experimental inoculation of an inducer fungus frequently increases the quantity of these fungitoxins beyond the retention limit and leads to their diffusion onto the leaf surface, exhibiting positive phytoalexin or, more precisely, inhibitin (Harborne, 1988) responses. When the retention limit is high, due to strong conjugation with relatively large quantities of phenolics, responses continue negative.

Acknowledgments—The authors wish to thank Dr. S.L. Jung-Mendaçolli, Instituto de Botánica de São Paulo, for the identification of plant material. This work was partially supported by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil.

REFERENCES

BAILEY, J.A. 1971. Phytoalexins and the ability of leaf tissues to inhibit fungal growth, pp. 519–528, *in* T.F. Preece and C.H. Dickinson (eds.). Ecology of Leaf Surface Microorganisms. Academic Press, New York.

BARZ, W., and KOSTER, J. 1981. Turnover and degradation of secondary (natural) products, pp. 35–84, *in* E.E. Conn (ed.). The Biochemistry of Plants, Vol. 7. Academic Press, New York.

BRAGA, M.R., YOUNG, M.C.M., PONTE, J.V.A., DIETRICH, S.M.C., EMERENCIANO, V. de P., and GOTTLIEB, O.R. 1986. Phytoalexin induction in plants of tropical environment. *Biochem. Syst. Ecol.* 14:507–514.

COLEY, P.D., BRYANT, J.P., and CHAPIN, F.S. 1985. Resource availability and plant antiherbivore defense. *Science* 230:895–899.

FIGUEIREDO-RIBEIRO, R.C.L., and DIETRICH, S.M.C. 1983. Sugar content and metabolic activities in cold-stored fragmented xylopodium of *Ocimum nudicaule* Benth. var. *anisifolia* Giul. (Labiatae). *J. Exp. Bot.* 34:476–483.

HARBORNE, J.B. 1988. Introduction to Ecological Biochemistry, 3rd ed. Academic Press, London. 356 pp.

HOMANS, A.L., and FUCHS, A. 1970. Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. *J. Chromatogr.* 51:325–327.

INGHAM, J.L. 1982. Phytoalexins from Leguminosae, pp. 21–80, *in* J.A. Bailey and J.W. Mansfield (eds.). Phytoalexins. Blackie, London.

JUNG-MENDAÇOLLI, S.L. 1984. Contribuicão ao estudo palinológico das Rubiaceae. PhD thesis. Universidade de São Paulo, São Paulo. xi + 191 pp.

KEEN, N.T. 1978. Phytoalexins: Efficient extraction from leaves by a facilitated diffusion technique. *Phytopathology* 68:1237–1239.

KIRKBRIDE, M.C.G. 1982. Preliminary phylogeny for the neotropical Rubiaceae. *Plant Syst. Evol.* 141:115–121.

MANTOVANI, W. 1983. Composição e similaridade florística, fenologia e espectro biológico do cerrado da Reserva Biológica de Moji Guaçú Estado de São Paulo. MSc dissertation. Universidade Estadual de Campinas, Campinas. 147 pp.

SWAIN, T., and HILLIS, W.E. 1959. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* 10:63–68.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

CHEMICAL AND NUTRITIONAL DIFFERENCES BETWEEN TWO BIRD-DISPERSED FRUITS: *Ilex opaca* AND *Ilex verticillata*

MARGARET B. GARGIULLO* and EDMUND W. STILES

Department of Biological Sciences
Rutgers University
Piscataway, New Jersey 08855-1059

(Received October 18, 1990)

Abstract—*Ilex opaca* and *Ilex verticillata* are woody species bearing low-quality, bird-dispersed fall fruits that persist for several months. Tests of secondary chemistry show that fruits of both species contain phenolics and saponins as major classes of secondary metabolites. Soluble carbohydrates are the major nutrients in both species. Ripe fruits from ten trees in both wild and cultivated populations of each species were collected during 1986, 1987, and 1988 and analyzed for phenolic and carbohydrate content using Folin-Denis and anthrone methods, respectively. Fruits from 1987 and 1988 were analyzed for saponin content using hemanalysis. Significant differences were found in the average content of the above chemical classes between the two species and among individuals within each species. The fruits of *Ilex opaca* are more persistent, higher in phenolics, and lower in saponins and carbohydrates than *I. verticillata* fruits. Generally, there are greater differences in fruit chemistry among plants within a year than within one individual between years. Mechanical defenses were tested by puncturing fruits in situ. No differences in decay were found between punctured and unpunctured fruits.

Key Words—Saponins, phenolics, carbohydrates, *Ilex opaca*, *Ilex verticillata*, bird-dispersed fruit, Folin-Denis, secondary chemistry, plant–herbivore interaction.

INTRODUCTION

Secondary compounds in green fruits are generally changed or diminished during ripening, rendering ripe fruits more susceptible to attacks by microbes and

*To whom correspondence should be addressed.

0098-0331/91/0600-1091$06.50/0

herbivores. The fruit pulp of *Heteromeles arbutifolia*, a desert shrub, contains a cyanogenic glycoside which is relocated to seeds upon ripening (Dement and Mooney, 1974). Tannins in persimmons, grapes, and bananas become more highly polymerized and so less astringent upon ripening (Joslyn and Goldstein, 1964; Palmer, 1971; Peynaud and Ribereau-Gayon, 1971). Apples become more susceptible to fungal attack as chlorogenic acid content falls during ripening (Hulme and Rhodes, 1971) and antibacterial activity of preclimactic avocado fruits disappears in postclimactic fruit (Biale and Young, 1971). Some factors, however, such as thick epidermal cells, sclerified tissues, and low moisture content, may afford protection to ripe fruits against fungal infection (Moore-Landecker, 1982; Agrios, 1988).

Most of the bird-dispersed fruits in eastern deciduous forests are low-quality fall fruits (Stiles, 1980). These fruits ripen in late summer or early fall and persist for several months, sometimes remaining on the plants until the following spring. Chemical defenses in persistent ripe fruits must deter fungi and insects without deterring dispersers (Kear, 1968; Freeland and Janzen, 1974; Janzen, 1977; Herrera, 1982).

In this study we: (1) tested for differences in secondary chemistry and nutrient content between two congeneric species that have dissimilar fruit persistence patterns. We hypothesized that species with more persistent fruits would have higher levels of secondary compounds and lower levels of nutrients including water. (2) We also examined the variations in secondary chemical content among fruits of individuals within a species. (3) To test for differences between chemical and mechanical defenses, we punctured fruit skins and observed rates of decay. We hypothesized that the species more dependent on physical defenses would have higher rates of decay in punctured than in unpunctured fruits.

METHODS AND MATERIALS

Research Species. We studied two species of *Ilex* (holly) as models of differential fruit persistence in plants with low-quality fruit. *Ilex opaca* (American holly) is a long-lived, evergreen tree found in the eastern United States (Art, 1976; Stalter, 1980). Fruits ripen in November and may be retained until the following June (Hume, 1953; personal observation). Fruits are usually red, occasionally yellow. Fruits and leaves of *I. opaca* contain saponins (West et al., 1977; Potter and Kimmerer, 1986) and fruit skin contains two layers of sclereids (Ives, 1923).

Ilex verticillata (common winterberry, black alder), is a deciduous shrub found in swampy areas throughout the Northeast, Midwest, and into Canada (Sakai and Sulak, 1985). Fruits are usually red, rarely yellow (Hume, 1953). They ripen in September and generally become wrinkled and brown by late

January (personal observation). Secondary chemistry of *I. verticillata* has not been studied.

We evaluated differences in total soluble phenolics, hemolytic saponins, and soluble carbohydrates between species and among fruits within species. Phenolic compounds contain a hydroxylated aromatic ring and occur universally among plants. Many are known to act as antifungal agents (Friend, 1979; Swain, 1979). Saponins are glycosylated polycyclic compounds with soaplike properties. They are toxic to some insects, act as fungistatic agents, and bind to cell membranes, causing hemolysis of red blood cells (Applebaum et al., 1969; Applebaum and Birk, 1979). The major soluble carbohydrates found in most fruit include glucose, fructose, and sucrose (Whiting, 1970).

Methods. Ten plants of each species were studied for three years: 1986, 1987, and 1988. Six of each were in wild populations, *I. opaca* at Sandy Hook National Recreation Area on the New Jersey coast and *I. verticillata* at Cheesequake State Park, New Jersey. The other four plants of each species were in the display gardens at Cook College, Rutgers University.

Two of the *I. opaca* trees (02 and 03) at Cook bear yellow fruit. There is a single yellow-fruited *I. verticillata* shrub at Cook (VY), but it did not bear fruit during 1986. Another, red-fruited shrub (V3) has a yellow-fruited ramet, and these fruits were analyzed in 1988.

Ripe fruits from several areas of each plant were collected each year. Fruit ripeness was assured by taking samples several weeks after fruit skin was fully red and fruit pulp was yellow and soft. Studies of fruit chemistry during ripening show drops of over 90% in saponin content and 50% in phenolic content as fruits of both species change color (Gargiullo and Stiles, unpublished data). Fruits were frozen in liquid nitrogen, freeze-dried, and then stored at $-10°C$. Before extraction, seeds were removed and fruit pulp was ground to powder using a glass mortar and pestle.

Seed Loads and Pulp Water. Seed loads of fresh fruits were measured using 10 or 20 fruit from each of seven *I. verticillata* shrubs and eight *I. opaca* trees. Fruits were weighed, fruit pulp was rubbed off seeds, and seeds from each plant were weighed. Seeds were freeze-dried and reweighed to measure seed water content. This averaged $18.27 \pm 1.68\%$ for both species together (*I. opaca* 17.60 ± 1.33, *I. verticillata* 19.02 ± 1.82). Percent seed load was calculated as fresh seed weight divided by fresh fruit weight times 100. Fruit pulp water was estimated as follows: a fruit sample was weighed before and after freeze drying. Pulp then was removed by grinding fruit, and dry seeds were weighed. The following formula was used to calculate percent fruit pulp water: $[(W - F - 0.22D)/W] \times 100$ where W = fresh fruit weight, F = dry fruit weight, and D = dry seed weight. The constant, 0.22, results from back-calculating 18% water lost from seeds.

Puncture Test. Mechanical defenses of ripe fruit were tested by puncturing

10 fruit in situ on five different branches of each plant. This was done to simulate damage by insects or other trauma to fruit skins. The numbers of punctured and unpunctured control fruit that turned brown during the following three weeks were counted. Ten *I. verticillata* and eight *I. opaca* plants were tested during the autumn of 1989. Data were analyzed using *t* tests (SAS Institute, 1985).

Chemical Tests. Extracts for phenolic and saponin analysis were made using 40% methanol. Five hundred milligrams of dry, ground fruit pulp was extracted with 4 ml of solvent three times for 30 min each time. Methanol was removed from pooled extract under reduced pressure, and the volume was brought to 10 ml with distilled water. Residual fruit pulp was brown, indicating the presence of insoluble, oxidized phenolic compounds.

Tannins were sought using the radial diffusion method of Hagerman (1987), which involves precipitation of protein in an agarose gel. The method can detect as little as 0.025 mg of tannin. Tannic acid was used as a standard.

Total soluble phenolic content of fruit extracts was determined using the Folin-Denis test (Folin and Denis, 1912; Swain and Hillis, 1959, Schultz and Hollis, personal communication). Phenolics were reported as percent dry weight tannic acid.

The Folin-Denis test has been criticized because it only measures aromatic hydroxyls and gives results that differ with molecular structure (Martin and Martin, 1982; J.C. Schultz, personal communication). However, it does measure the degree to which a phenolic can be oxidized (Folin and Denis, 1912), and oxidized phenolics have been shown to be more toxic than unoxidized phenolics (Van Buren, 1970; Byrde et al., 1960).

Saponins were analyzed by combining the hemolytic methods of Wall et al. (1952), O'Dell et al. (1959), and Segelman and Farnsworth (1969). For each saponin analysis 1 ml of fruit extract was mixed with 4 ml of a 1% suspension of washed human erythrocytes (pH 7.4) and incubated for 2.5 hr at 31°C ± 1°C. Tubes were remixed every 15 min to keep cells in suspension. Unlysed blood cells were spun down in a desktop centrifuge and the absorbance of hemoglobin remaining in the supernatant was read at 520 nm. Hemolytic saponins were reported as percent dry weight Sigma saponin.

Hemanalysis of saponins, like the Folin-Denis, test, is not qualitative. However, it does measure the capacity of saponins to lyse cell membranes and is, therefore, relevant as a measure of fruit defense against fungi and bacteria.

Carbohydrates were analyzed by the anthrone method (Yemm and Willis, 1954; Allen, 1974) using a 2-hr hot-water extraction, 10-min color development, and glucose standards. This test measures mono- and disaccharides known to be assimilated by birds (Ziswiler and Farner, 1972). Soluble carbohydrates were reported as percent dry weight glucose.

Data Analysis. Data for phenolics, saponins, and carbohydrates were ana-

lyzed using analysis of variance (SAS Institute, 1985). Differences among fruits of individual plants were analyzed using Scheffe's tests (Day and Quinn, 1989). Data were also analyzed by ANOVAs using years as replicates to see if differences between fruits of individual plants were greater than differences within one tree over time. Linear regressions were used to evaluate correlations between carbohydrates, phenolics, and saponins. Data for saponins from 1986 were not analyzed due to inadequate freeze drying.

RESULTS

Tannin Analysis and Puncture Test. The analysis for tannins was performed on 24 samples of fruit using sufficient extract to detect 0.5% tannin in dry fruit pulp, but none was found in fruit of either species. A preliminary study, using the dye-labeled BSA method of Asquith and Butler (1986) also showed no tannins. Tannins are known to interfere with saponin hemolysis (Segelman et al., 1969) and, if present, should have prevented hemolysis, but this did not occur.

There were no significant differences in discoloration between punctured and unpunctured fruits on any plant of either species.

Differences between Species. Seed loads and fruit pulp water content of the two species were significantly different from one another (Table 1) with *I. opaca* having a higher seed load and lower water content than *I. verticillata*.

The ANOVAs showed highly significant ($P < 0.0001$) differences for carbohydrates, phenolics, and saponins between the two species for all years (Table 2).

Soluble carbohydrate content of dry fruit pulp ranged from 45.8% to 66.6% dry wt for *I. opaca* and from 51.7% to 86.7% dry wt for *I. verticillata* for all three years. Fruit pulp of *I. verticillata* had significantly more carbohydrate than did *I. opaca*.

Total soluble phenolics ranged from 1.67% to 3.64% dry wt for *I. opaca* and from 0.86% to 2.26% dry wt for *I. verticillata* for three years. *Ilex opaca* fruit had significantly higher phenolic content than did *I. verticillata* fruit. How-

TABLE 1. MEANS ± SD OF PULP WATER AND SEED LOADS FOR BOTH SPECIES[a]

Percent	*N*	*I. opaca*	*N*	*I. verticillata*	*P*
Seed load	8	17.19 ± 1.49	7	14.56 ± 2.45	0.024
Pulp water	7	50.26 ± 3.00	5	63.51 ± 5.11	0.0001

[a] Seeds from 10 or 20 fruit per plant. *N* = number of plants.

TABLE 2. MEANS ± SD OF PERCENT DRY WEIGHT SOLUBLE CARBOHYDRATES, SOLUBLE PHENOLICS, AND HEMOLYTIC SAPONINS RELATIVE TO GLUCOSE, TANNIC ACID, AND SIGMA SAPONIN, RESPECTIVELY, BY YEAR[a]

	Ilex Opaca	*Ilex verticillata*
Carbohydrates		
1986	54.6 ± 4.13	59.2 ± 5.96
1987	61.8 ± 3.36	67.3 ± 4.97
1988	54.3 ± 6.14	64.8 ± 6.14
Phenolics		
1986	2.36 ± 0.50	1.64 ± 0.41
1987	2.75 ± 0.59	1.42 ± 0.37
1988	2.68 ± 0.25	1.58 ± 0.44
Saponins		
1987	0.42 ± 0.58	1.14 ± 1.32
1988	0.80 ± 1.14	1.06 ± 1.26

[a] All means are significantly different from one another at $P > 0.0001$.

ever, differences in results between the two species may be caused by qualitative as well as quantitative differences in phenolic compounds (Goldstein and Swain, 1963).

Saponin content of fruit was more variable than either phenolic or carbohydrate content. Hemolytic saponins ranged from 0.00% (undetectable by this method) to 3.41% dry wt for *I. opaca* and from 0.00% to 3.37% dry wt for *I. verticillata* for two years. *Ilex verticillata* had a significantly higher mean saponin content than *I. opaca*. As with phenolics, differences in saponins between species may be qualitative as well as quantitative.

Differences within Species. There were significant differences in chemistry among fruits of some plants within species in each year (Table 3).

Carbohydrates. Scheffe's tests (Figures 1 and 2) for carbohydrate content of *I. opaca* fruits showed one tree with consistently high carbohydrate content (03) and one tree with low carbohydrate content (07) across all years. There was no significant difference between carbohydrate content of fruit from the two sites for *I. opaca* except in 1986 when Cook trees had significantly ($P < 0.006$) higher carbohydrate content than those from Sandy Hook.

Scheffe's tests for fruits of *Ilex verticillata* (Figures 1 and 2) showed fruit of VY (Cook) high in carbohydrates. Shrubs V5 and V9 (Cheesequake) had fruit with low carbohydrates. There were significantly more carbohydrates in *I. verticillata* fruit from Cook ($P < 0.0001$) than from Cheesequake. Yellow fruit of both species tended to be relatively high in carbohydrates.

Phenolics. There were more variations in phenolic content than in carbo-

TABLE 3. *F* VALUES AND *P* VALUES FOR DIFFERENCES AMONG FRUITS OF INDIVIDUAL TREES WITHIN SPECIES

	Ilex opaca		*Ilex verticillata*	
	F	*P*	*F*	*P*
Carbohydrates				
1986	9.66	<0.0008	14.65	<0.0003
1987	8.27	<0.0015	6.77	<0.0034
1988	13.69	<0.0002	11.19	<0.0002
Phenolics				
1986	856.93	<0.0001	6367.81	<0.0001
1987	365.22	<0.0001	5783.65	<0.0001
1988	413.90	<0.0001	112.06	<0.0001
Saponins				
1987	99999.99	<0.0	21045.70	<0.0001
1988	95659.42	<0.0001	1755.91	<0.0001

hydrate content among fruits of individual plants of both species (Figures 3 and 4). Differences between the two *I. opaca* sites were significant ($P < 0.0001$) for all three years, with fruit at Cook having higher phenolic content than those at Sandy Hook.

Fruits of *I. verticillata* shrubs at Cheesequake were significantly ($P < 0.0001$) higher in phenolic content than those at Cook during all three years.

Saponins. Saponin content of fruit was more variable than either carbohydrate or phenolic content for both species in both years (Figures 5 and 6). There was a highly significant difference between sites for *I. opaca* (Figure 6, $F = 99999.9$, $P = 0.0$) in both years, with fruits of trees at Sandy Hook having greater saponin content (means 0.82% in 1987, 1.25% in 1988) than those at Cook (means 0.17% in 1987, 0.12% in 1988).

Saponin content of *I. verticillata* fruits (Figures 5 and 6) were significantly different between sites ($P < 0.0001$) with fruits of trees at Cheesequake having more saponins (means 1.49% in 1987, 1.48% in 1988) than those at Cook (means 0.34% in 1987, 0.17% in 1988). Yellow fruits of both species tended to be low in saponins.

Additional Findings. Analysis of variance for each class of compounds within species, using years as replicates, had significant *F* statistics for everything except carbohydrates in *I. opaca* (Table 4), indicating greater variation among individuals than within an individual plant.

There were no significant correlations between carbohydrates and phenolics, carbohydrates and saponins, or between saponin and phenolics for *I. opaca* fruits. There were no significant correlations between carbohydrates and sapo-

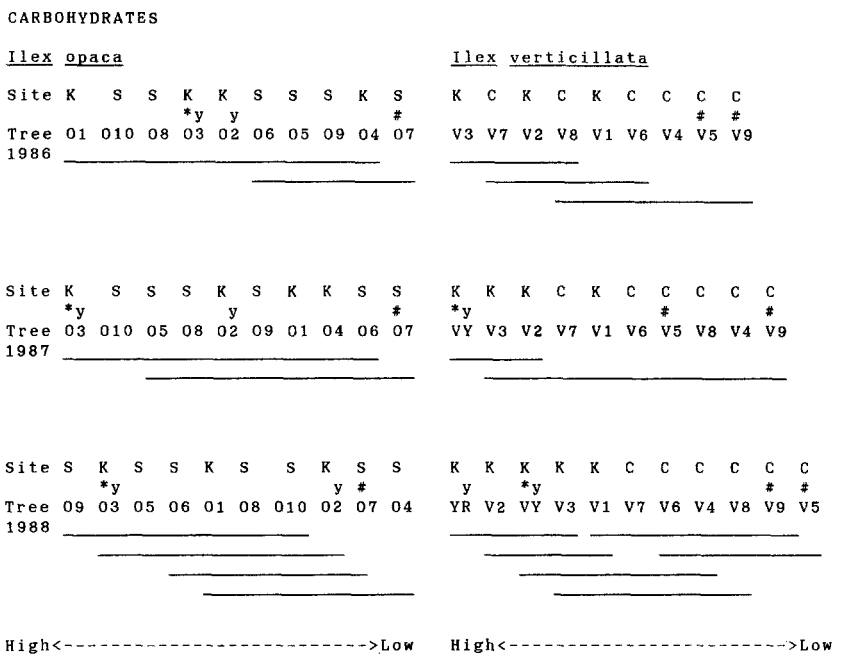


FIG. 1. Scheffe's tests for differences in carbohydrate content among fruits of individual plants. All $P < 0.005$. Plants are listed in descending order of carbohydrate content from left to right. Plants connected by the same line are not significantly different from one another. Sites: K = Cook, S = Sandy Hook, C = Cheesequake. Plants with yellow fruit are labeled "y," YR is the yellow fruited ramet of plant V3. *high carbohydrates, #low carbohydrate in all three years.

nins or between saponins and phenolics for *I. verticillata* fruits. However, the correlation coefficient for carbohydrates and phenolics for *I. verticillata* was negative and significant at $P < 0.001$ ($r = -0.639$, Table 1 and 3).

DISCUSSION

Differences between Species. Persistent fruits must maintain barriers to fungal invasion and frost damage for weeks or months. Fruits of the two species in this study are both persistent, but they have clear differences in secondary chemical content, nutrient content, and longevity. Given the results of the puncture experiment, chemical rather than mechanical factors appear to be most important for defense against pathogens in both species.

Our hypothesis, that more persistent fruit should be higher in secondary compounds and lower in nutrients, was partially supported by this study. *Ilex*

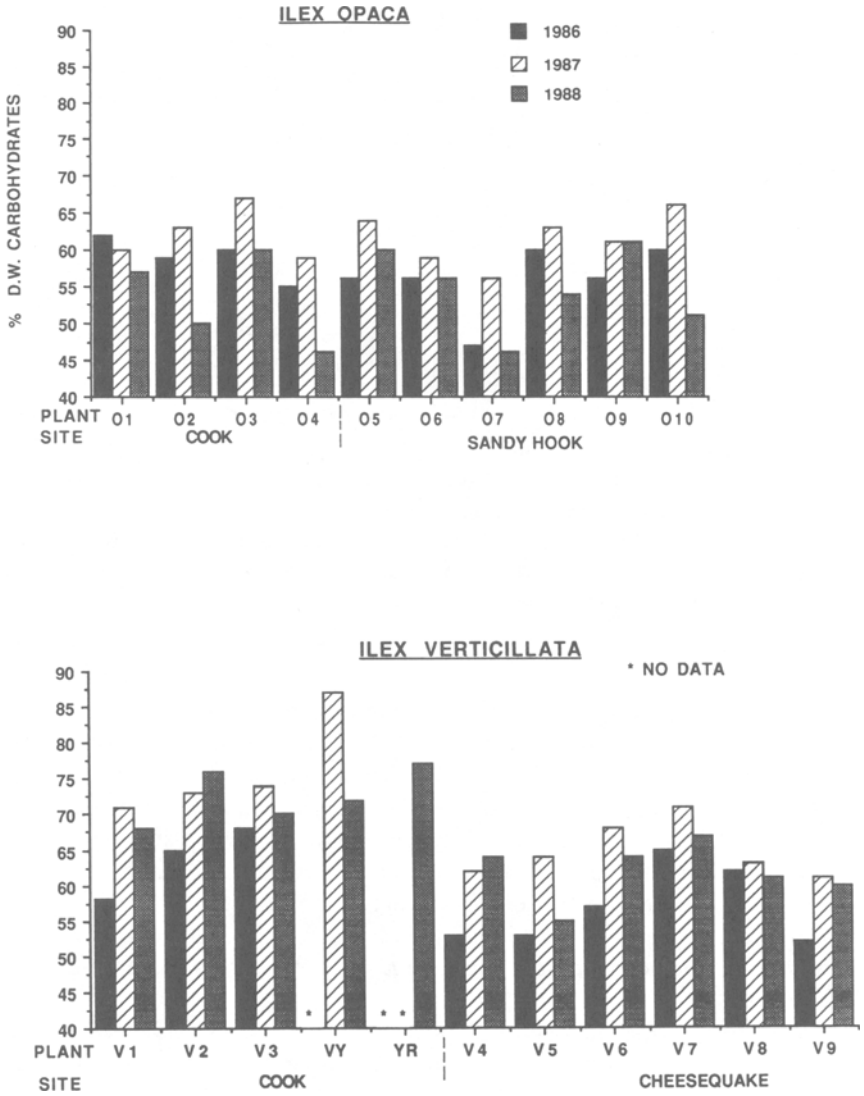


FIG. 2. Percent dry weight (D.W.) of carbohydrates for three years for fruit from each plant. O = *I. opaca*, V = *I. verticillata*.

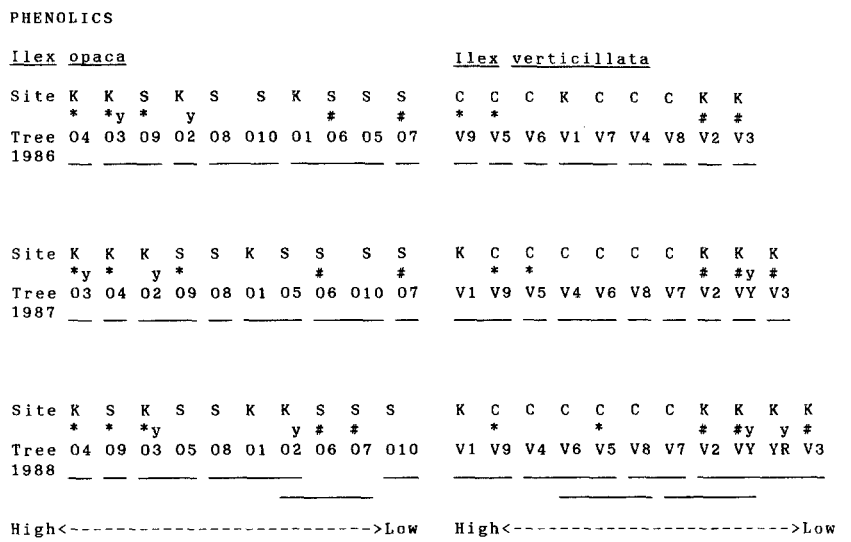


FIG. 3. Scheffe's tests for differences in phenolic content among fruits of individual plants. All $P < 0.005$. Plants are listed in descending order of phenolic content from left to right. Plants connected by the same line are not significantly different from one another. Sites: K = Cook, S = Sandy Hook, C = Cheesequake. Plants with yellow fruit are labeled "y," YR is the yellow fruited ramet of plant V3. *high phenolics, #low phenolics in all three years.

opaca, the more persistent fruit, was lower in water and soluble carbohydrates, and higher in phenolics than *I. verticillata*. These factors, high phenolics, low nutrients and low water, have all been shown to inhibit fungal infections (Agrios, 1988; Moore-Landecker, 1982). Another study has shown that frugivorous birds prefer *I. verticillata* over *I. opaca* (Gargiullo and Stiles, unpublished data). This seems to indicate a trade-off between fruit persistence and palatability. *Ilex opaca* fruits are ripe from November through winter into the following spring, making them available to resident birds in late winter and to spring migrants. However, the characteristics that preserve *I. opaca* fruits also make them poor-quality food.

Ilex verticillata fruits ripen in September, are generally removed or brown and desiccated by late January, and so must be dispersed principally by late fall migrants and winter residents. Although *I. verticillata* fruits have a higher mean saponin content than *I. opaca* fruits (Table 2), saponin content is very variable in ripe fruits of both species and lower saponin content is not correlated with shorter life-span in *I. opaca* fruits. The shorter life-span of *I. verticillata* fruit limits time available for dispersal. High carbohydrate, low phenolic content and

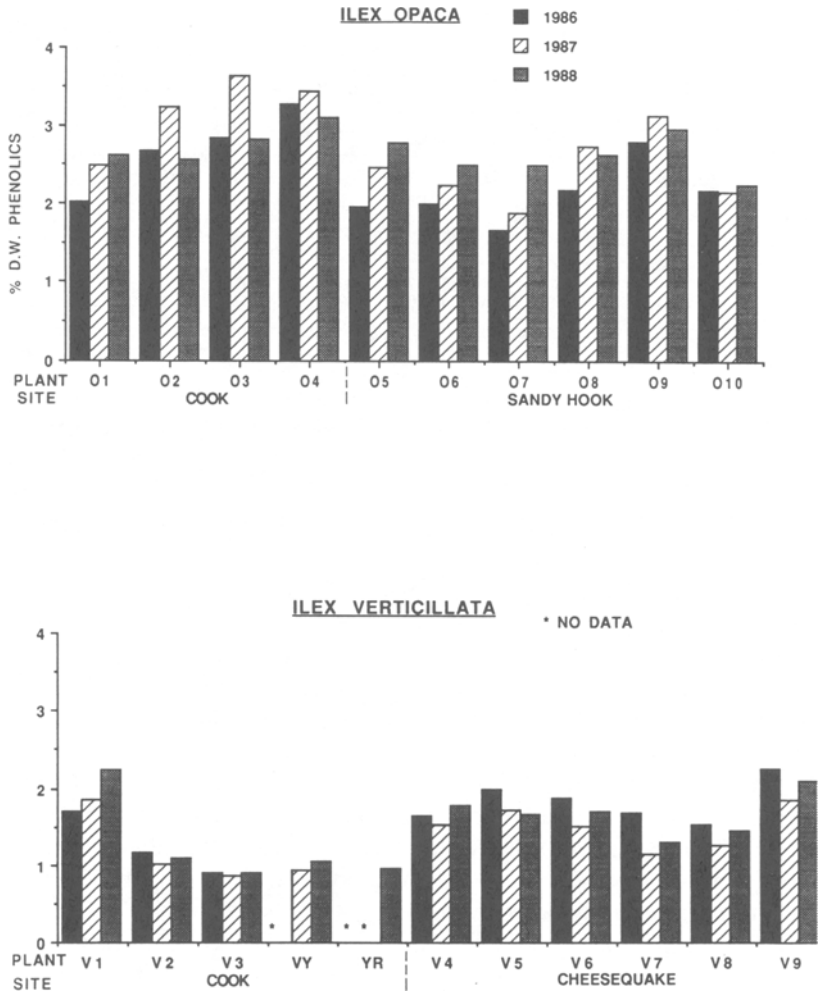


FIG. 4. Percent dry weight (D.W.) of phenolics for three years for fruit from each plant.

low seed load (Herrera, 1981) make them more attractive to dispersers than *I. opaca* fruits.

Differences within Species. Carbohydrate content in fruits of these two species varied considerably, indicating dependence on both environmental and genetic factors (Figure 2). Mean carbohydrate contents of *I. opaca* fruits were significantly different between sites only for 1986, so environmental factors such as soils were probably not directly related to carbohydrate production. *Ilex verticillata* fruits did have significant differences in carbohydrate content

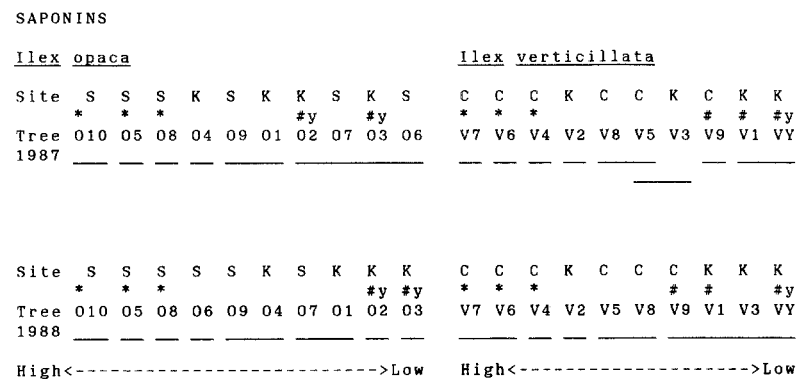


FIG. 5. Scheffe's tests for differences in saponin content among fruits of individual plants. All $P < 0.005$. Plants are listed in descending order of saponin content from left to right. Plants connected by the same line are not significantly different from one another. Sites: K = Cook, S = Sandy Hook, C = Cheesequake. Plants with yellow fruit are labeled "y." *high saponins, #low saponins in both years.

between sites. Shrubs at Cheesequake were shaded more heavily by other vegetation than those at Cook, and this may have influenced carbohydrate content of fruits.

Phenolic content of *I. opaca* fruits was significantly higher at Cook than at Sandy Hook. This may have been related to infestation of all four of trees at Cook by holly midges (*Asphondylia ilicicola*). The larva of this insect lives inside the carpellary cavity and prevents fruit ripening (Highland, 1964). It is possible that chronic infestation may have induced an increase in phenolic content of all fruit (Bell, 1981). Only a few fruit on two trees at Sandy Hook (08 and 09) were affected (Gargiullo, personal observation). None of the trees at either site was shaded by other vegetation.

Yellow fruit lacks anthocyanins and may have other differences in phenolics that influence fruit longevity. The pigments in yellow fruits are probably phenolics (Vickery and Vickery, 1981) rather than carotenoids, as the yellow color appeared in the fruit extracts and carotenoids are not water soluble.

Differences between sites were especially evident in fruit saponin content of both species ($P < 0.0001$ for both species in both years) (Figures 5 and 6). Plants with the highest saponin content in both species were from the two wild populations. It is likely that plants in these wild populations are related, whereas those at Cook are all different cultivars. Trees studied on Sandy Hook grow in a single large population and are within about 500 m of one another. Shrubs studied at Cheesequake are within about 150 m of one another. Although mean saponin content was higher for *I. verticillata* than for *I. opaca*, overall saponin content was highly variable among individuals (Table 1).

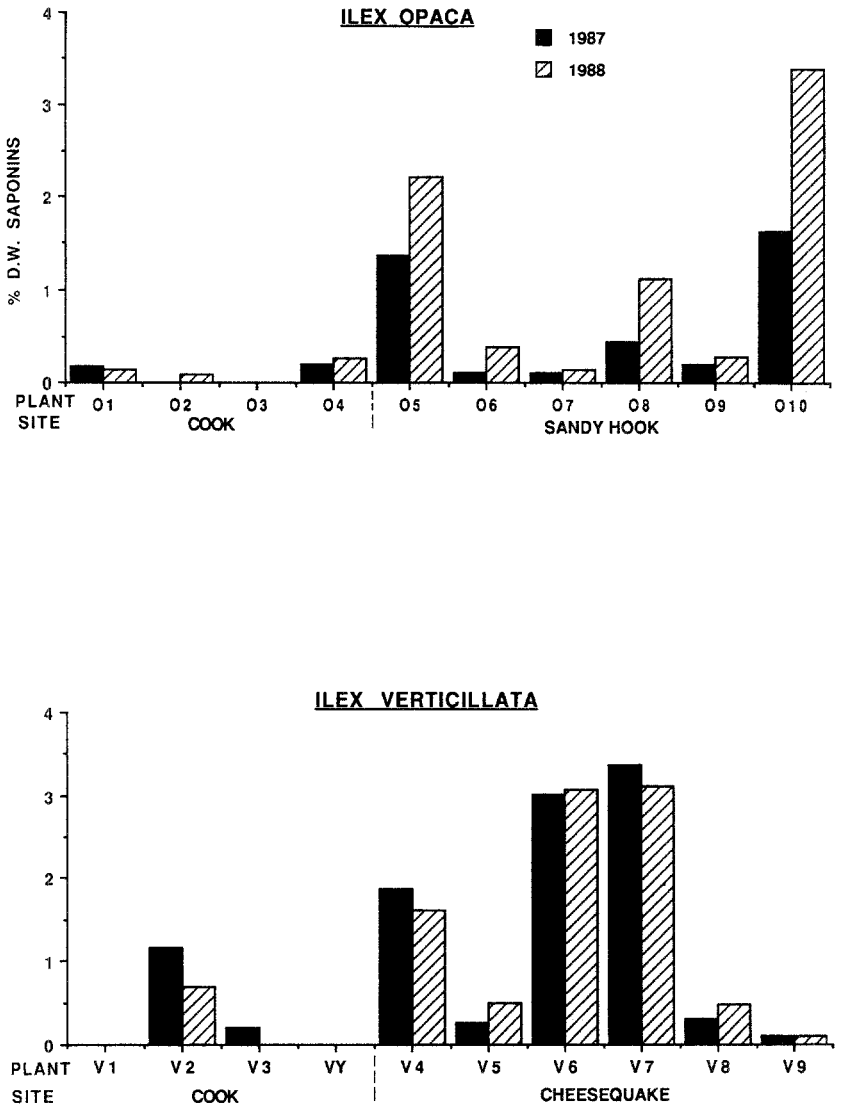


FIG. 6. Percent dry weight (D.W.) of saponins for two years for fruit from each plant.

These two species have disparate sets of characters to attract dispersers. *Ilex verticillata*, with lower chemical and higher nutrient content, is less persistent and available only in early winter. *Ilex opaca* is more highly defended, less nutritious, more persistent, and available at a time of scarcity in late winter. The wide variations between fruits of individual trees appear to be due to both

TABLE 4. ANALYSES OF VARIANCE WITHIN SPECIES USING YEARS AS REPLICATES[a]

	F	*P*
Ilex opaca		
Carbohydrates	1.63	0.174
Phenolics	5.26	0.001
Saponins	21.74	0.0001
Ilex verticillata		
Carbohydrates	3.48	0.013
Phenolics	13.65	0.0001
Saponins	46.74	0.0001

[a]Groups = number of groups in Scheffe's test with significantly different means.

genetic and environmental causes as fruit varies with year but is more consistent for an individual between years than among individuals within one year.

Acknowledgments—This study was supported by grants from the Anne B. and James H. Leathem fund. Field work was carried out on wild plants with permission from Cheesequake State Park and Sandy Hook Unit, National Recreation Area. We would also like to thank Elwin Orton for his permission to use trees in the holly collection at Cook display gardens and for sharing his expertise on hollies, David Gant for his assistance during the use of the display garden, and Jack Schultz for advice and assistance on analysis of phenolics.

REFERENCES

AGRIOS, G.N. 1988. Plant Pathology. Academic Press, New York.

ALLEN, S.E. 1974. Chemical Analysis of Ecological Materials. Wiley, New York.

APPLEBAUM, S.W., and BIRK, Y. 1979. Saponins, pp. 539–562, *in* G.A. Rosenthal and D.H. Janzen, (eds). Herbivores, Their Interactions with Secondary Plant Metabolites. Academic Press, New York.

APPLEBAUM, S.W., MARCO, S., and BIRK, Y. 1969. Saponins as possible factors of resistance of legume seeds to the attack of insects. *J. Agric. Food Chem.* 17:618–622.

ART, H.W. 1976. Ecological studies of the Sunken Forest, Fire Island National Seashore, New York. National Park Service Scientific Monographs.

ASQUITH, T.N., and BUTLER, L.G. 1986. Use of a dye-labeled protein as a spectrophotometric assay for protein precipitants such as tannin. *J. Chem. Ecol.* 11:1535–1544.

BELL, A.A. 1981. Biochemical mechanisms of disease resistance. *Annu. Rev. Plant Physiol.* 32:21–81.

BIALE, J.B., and YOUNG, R.E. 1971. The avocado pear, pp. 2–60, *in* A.C. Hulme (ed.). The Biochemistry of Fruits and Their Products, Vol. 2. Academic Press, New York.

BYRDE, R.J.W., FIELDING, A.H., and WILLIAMS, A.H. 1960. The role of oxidized polyphenols in the varietal resistance of apples to brown rot, *in* J.B. Pridham (ed.). Phenolics in Plants in Health and Disease. Pergamon Press, New York.

DAY, R.W., and QUINN, G.P. 1989. Comparisons of treatments after an analysis of variance in ecology. *Ecol. Monogr.* 59(4):433–463.

DEMENT, W.A., and MOONEY, H.A. 1974. Seasonal variation in the production of tannins and cyanogenic glucosides in the chaparral shrub. *Heteromeles arbutifolia. Oecologia* 15:65–76.

FOLIN, O., and DENIS, W. 1912. On phosphotungstic–phosphomolybdic compounds as color reagents. *J. Biol. Chem.* 12:239–243.

FREELAND, W.J., and JANZEN, D.H. 1974. Strategies in herbivory by mammals: The role of plant secondary compounds. *Am. Nat.* 108:269–289.

FRIEND, J. 1979. Phenolic substances and plant diseases, pp. 557–588, *in* T. Swain, J.B. Harborne, and C.F. Van Sumere (eds.). Recent Advances in Phytochemistry, Vol. 12. Plenum Press, New York.

GOLDSTEIN, J.L., and SWAIN, T. 1963. Changes in tannins in ripening fruits. *Phytochemistry* 2:371–383.

HAGERMAN, A.E. 1987. Radial diffusion method for determining tannin in plant extracts. *J. Chem. Ecol.* 13:437–449.

HERRERA, M.C. 1981. Fruit variation and competition for dispersers in natural populations of *Smilax aspera. Oikos* 36:51–58.

HERRERA, C.M. 1982. Defense of ripe fruit from pests: Its significance in relation to plant–disperser interactions. *Am. Nat.* 120:218–241.

HIGHLAND, H.A. 1964. Life history of *Asphondylia ilicicola* (Diptera: Cecidomyiidae), a pest of American holly. *J. Econ. Entomol.* 57:81–83.

HULME, A.C., and RHODES, M.J.C. 1971. Pome fruits, pp. 333–369, *in* A.C. Hulme (ed.). The Biochemistry of Fruits and Their Products, Vol. 2. Academic Press, New York.

HUME, H.H. 1953. Hollies. Macmillan, New York.

IVES, S.A. 1923. Maturation and germination of seeds of *Ilex opaca. Bot. Gaz.* 76:60–77.

JANZEN, D.H. 1977. Why fruits rot, seeds mold and meat spoils. *Am. Nat.* 111:691–713.

JOSLYN, M.A., and GOLDSTEIN, J.L. 1964. Changes in phenolic content in persimmons during ripening and processing. *J. Agric. Food Chem.* 12:511–520.

KEAR, J. 1968. Plant poisons in the diet of wild birds. *Bull. B.O.C.* 88:98–102.

MARTIN, J.S., and MARTIN, M.M. 1982. Tannin assays in ecological studies: Lack of correlation between phenolics, proanthocyanidins and protein-precipitating constituents in mature foliage of six oak species. *Oecologia* 54:205–211.

MOORE-LANDECKER, E. 1982. Fundamentals of the Fungi. Prentice-Hall, Englewood Cliffs, New Jersey.

O'DELL, B.L., REGAN, W.O., and BEACH, T.J. 1959. A study of the toxic principle in red clover. University of Missouri Agricultural Experimental Station Research Bulletin 702.

PALMER, J.K. 1971. The banana, pp. 65–101, *in* A.C. Hulme (ed.). The Biochemistry of Fruits and Their Products, Vol. 2. Academic Press, New York.

PEYNAUD, E., and RIBEREAU-GAYON, P. 1971. The grape, pp. 172–204, *in* A.C. Hulme (ed.). The Biochemistry of Fruits and Their Products, Vol. 2. Academic Press, New York.

POTTER, D.A., and KIMMERER, T.W. 1986. Seasonal allocation of defense investment in *Ilex opaca* Ait. and constraints on a specialist leaf miner. *Oecologia* 69:217–224.

SAKAI, A.K., and SULAK, J.H. 1985. Four decades of secondary succession in two lowland permanent plots in northern lower Michigan. *Am. Midl. Nat.* 113:146–157.

SAS INSTITUTE. 1985. SAS User's Guide: Statistics, 1985 edition. SAS Institute Inc., Cary, North Carolina.

SEGELMAN, A.B., and FARNSWORTH, N.R. 1969. Biological and phytochemical evaluation of plants. IV. A new rapid procedure for the simultaneous determination of saponins and tannins. *Lloydia* 32:59–65.

SEGELMAN, A.B., FARNSWORTH, N.R., and QUIMBY, M.W. 1969. Biological and phytochemical evaluation of plants. III. False-negative saponin test results induced by the presence of tannins. *Lloydia* 32:52–58.

STALTER, R. 1980. Some ecological observations on an *Ilex* forest. Sandy Hook, New Jersey. *Castanea* 44:202–207.

STILES, E.W. 1980. Patterns of fruit presentation and seed dispersal in bird disseminated woody plants in the eastern deciduous forest. *Am. Nat.* 116:670–688.

SWAIN, T. 1979. Phenolics in the environment, pp. 617–640, *in* T. Swain, J.B. Harborne, and C.F. Van Sumere (eds.). Recent Advances in Phytochemistry, Vol. 12. Plenum Press, New York.

SWAIN, T., and HILLIS, W.E. 1959. The phenolic constituents of *Prunus domestica* I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* 10:63–68.

VAN BUREN, J. 1970. Fruit phenolics, pp. 269–304 *in* A.C. Hulme (ed.). The Biochemistry of Fruits and Their Products, Vol. 2. Academic Press, New York.

VICKERY, M.L., and VICKERY, B. 1981. Secondary Plant Metabolism. University Park Press, Baltimore, Maryland.

WALL, M.E., EDDY, C.R., MCCLENNAN, M.L., and KLUMPP, M.E. 1952. Detection and estimation of steroidal sapogenins in plant tissue. *Anal. Chem.* 24:1337–1341.

WEST, L.G., MCLAUGHLIN, J.L., and EISENBEISS, G.U. 1977. Saponins and triterpenes from *Ilex opaca*. *Phytochemistry* 16:1846–1847.

WHITING, G.C. 1970. Sugars, pp. 1–31, *in* A.C. Hulme (ed.). The Biochemistry of Fruits and Their Products, Vol. 1. Academic Press, New York.

YEMM, E.W., and WILLIS, A.J. 1954. The estimation of carbohydrates in plant extracts by anthrone. *Biochem. J.* 57:508–514.

ZISWILER, V., and FARNER, D.S. 1972. Digestion and the digestive system, pp. 343–430, *in* D.S. Farner, J.R. King, and K.C. Parkes (eds.). Avian Biology, Vol. II. Academic Press, New York.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

EFFECTS OF COTTON PLANT ALLELOCHEMICALS AND NUTRIENTS ON BEHAVIOR AND DEVELOPMENT OF TOBACCO BUDWORM

PAUL A. HEDIN,* WILLIAM L. PARROTT, AND JOHNIE N. JENKINS

Crop Science Research Laboratory
USDA-ARS
Mississippi State, Mississippi 39762-5367

(Received November 9, 1990; accepted February 7, 1991)

Abstract—Female moths of the tobacco budworm, *Heliothis virescens* (F.), oviposit in the terminals of the cotton plant, *Gossypium hirsutum* (L.). The hatched larvae migrate to the terminal area and then to small squares (buds), on which they feed, finally burrowing into the anthers where they grow and develop. They attempt to avoid gossypol glands as they feed. Chemically related evidence explains, in part, these observations. The calyx crown of resistant lines (which is avoided) is high in the terpenoid aldehydes (TAs) including gossypol. HPLC data showed that the gossypol content of both susceptible and resistant glanded lines is equal, while the hemigossypolone and heliocides H_1 and H_2 are greatly increased in resistant lines and presumably are more closely associated with resistance. Analysis for total amino acids in cotton square tissues showed that there was a gradation from the calyx and calyx crown, which were lowest, to the anthers, the site of final insect development, which were highest. Synthetic diets mimicking amino acid distribution in anthers were found to be successful for larval growth and development.

Key Words—Tobacco budworm, *Heliothis virescens*, Lepidoptera, Noctuidae, cotton, *Gossypium hirsutum*, plant resistance, plant-insect interaction, terpenoids, gossypol, allelochemicals.

INTRODUCTION

The female moths of the tobacco budworm, *Heliothis virescens* (F.), oviposit in the terminals of the cotton plant, *Gossypium hirsutum* L. There is no evi-

*To whom correspondence should be addressed.

dence that they are specifically attracted to the terminals by chemical cues, but rather that they are most proximate to the terminals during an overflight. Most of the eggs are placed singly on the small terminal leaves approximately 12 cm^2 in size (Ramalho, 1983). Three days after oviposition, the eggs hatch. Immediately after hatch, the young larvae will feed on the leaf tissue for a brief period before they migrate into the terminal area, which is comprised of meristematic tissue (immature squares and leaves). The young larvae spend about three days feeding on small squares with the potential of destroying a maximum of four squares each during this feeding period (Parrott, unpublished data).

During this period, gossypol is toxic to the larvae (Hedin et al., 1988), which also are observed to avoid consuming glands that contain gossypol. However, when the larvae molt into the second instar at about 72 hr, they can nonselectively consume the glands (Parrott et al., 1983). Earlier, Shaver and Parrott (1970) reported young larvae to be more sensitive to gossypol than older larvae. This finding was later supported by laboratory studies in which gossypol and two other allelochemicals were fed in diets to 1-, 3-, and 5-day-old tobacco budworm (TBW) larvae. All three allelochemicals were toxic to 1- and 3-day-old larvae, but they were not toxic to 5-day-old insects. Evidence was obtained that the insects biosynthesized detoxifying enzymes, mixed function oxidases (MFOs), because piperonyl butoxide, a known inhibitor of MFOs, inhibited growth when added to the diets (Hedin et al., 1988).

When the larvae leave the terminal, they move onto small squares that arise at position one of the fruiting branch (Ramalho et al., 1984). They then prefer to feed along the margin area of the calyx crown. Using laboratory feeding tests in which larvae fed on squares from several experimental lines, Parrott et al. (1989) found that they fed less on squares from plants that have high gland density in the calyx crown, a normally preferred feeding site of young larvae.

During the later second and third instars, the larvae burrow through the calyx or petals of the cotton squares into the anthers. Once they reach the anthers, their growth rate immediately accelerates. Work by Shaver et al. (1977) showed that the major source of nutrients for tobacco budworm larvae in detached cotton buds is in the anthers. The nutritional requirements, however, have never been defined chemically, in part because the tobacco budworm has never been reared on synthetic amino acid diets. However, the expectation is that if this could be achieved, it would provide the basis for more reliable evaluation of the effects of natural and synthetic constituents.

The objectives of this study were to determine whether the migration of the tobacco budworm from the terminal leaf, terminal area (meristematic tissue), calyx crown, and finally to the anthers can be at least partly explained by, or associated with, the gradient concentrations of allelochemicals and nutrients.

METHODS AND MATERIALS

Cultivars and Agronomic Practices. In 1988, three lines with demonstrated resistance to the TBW—DH-118, DH-126, and DH-118 × ST 213 H—were planted on the Plant Science Farm, Mississippi State, Mississippi. For comparison, four susceptible lines, DH-118 × ST-213 L, ST-213, ST-825, and ST-213 gl, were also planted. Not all lines were used in every test. Standard cultural practices were followed during the growing season, except that some of the replicated plots were not protected from insects. All plant material was collected from cotton plants of similar maturity.

Gathering and Processing of Plant Tissue. Terminal leaves, squares, and square parts were harvested, frozen, freeze-dried, and then ground through a 40-mesh screen. The powders were stored in vials at −20°C until they were evaluated. A portion of the freshly gathered squares was dissected into the following parts: anthers, petals, bracts, calyx, and calyx crown. These tissues were also freeze-dried, ground to a powder, and stored at −20°C to await subsequent evaluations.

Insects and Diets. Neonate tobacco budworm larvae used in the various tests were obtained from a colony at the Crop Science Research Laboratory, Mississippi State, Mississippi, maintained as described by Jenkins et al. (1982). Larvae (20 per test) in some tests were placed in 30-ml clear plastic cups containing 10 g/cup of a Nutrisoy–wheat germ diet (Bioserv, Frenchtown, New Jersey).

In tests where the synthetic amino acids replaced the Nutrisoy–wheat germ protein as a source of nitrogen, the amino acids were procured either from Bioserv, or from Sigma Chemical Co. (St. Louis, Missouri). The amino acid distribution and levels in these diets were based on protein and amino acid analyses of the soy flour, wheat germ, and cotton anthers (methods and results described later in the text). The nonprotein dietary ingredients [and a separate vitamin mixture (Pack C)] were also provided as dry mixes by Bioserv. The dietary ingredients are listed in Table 1.

Analytical Procedures. The Association of Official Analytical Chemists (AOAC) method (Horwitz, 1975) for total protein, 2.049 (percent nitrogen × 6.25) was used to determine that the crude protein content of the Bioserv Pack B containing soy flour and wheat germ as protein sources was 22.7%; i.e., the quantity of amino acids that was required for addition to the Pack B less protein to provide an equivalent amount to that supplied by the soy flour and wheat germ.

Analysis of Allelochemicals. Analyses for gossypol and related terpenoid aldehydes were performed on cyclohexane–ethyl acetate–acetic acid (500:500:1; CHEA) extracts of plant (whole square and anther) tissue by the

TABLE 1. INGREDIENTS IN STANDARD LABORATORY AND SYNTHETIC AMINO ACID DIETS FOR TOBACCO BUDWORM

Ingredient	Percent of total content	
	Soy–wheat germ	Amino acid
Amino acids	0	18.9
Soy flour	24.8	0
Wheat germ	21.0	0
Wesson salts	0.6	0.6
Sucrose	24.8	22.3
Methyl paraben	0.8	1.3
Sorbic acid	0.8	0.4
Aureomycin, 14%	0.8	0.1
Cholesterol	0	0.1
Linseed oil	0	1.1
Corn cob grits	0	32.8
Agar	13.6	14.3
Vitamins[a]	5.8	6.1
Acids[b]	1.7	1.8

[a] Pack C from Bioserv, Inc., Frenchtown, NJ. Ascorbic acid, vitamin E acetate, biotin, calcium pantothenate, choline citrate, folic acid, inositol, niacin, pyridoxine HCl, riboflavin, thiamine HCl, vitamin B_{12} in sucrose; 1:1.
[b] Propionic acid–Phosphoric Acid: 9:1.

phloroglucinol reaction [2% in absolute EtOH-concentrated HCl (1:1)]; let stand 1 hr, with subsequent spectrometric analysis at 550 nm. The concentration was determined by comparison with data obtained from authentic gossypol and is expressed as gossypol equivalents. Condensed tannin analyses were performed on 70% aqueous methanol extracts of tissue. The anthocyanidin chromophore was developed from the tannin by boiling 1 hr with 1-butanol–HCl (95:5) (Hedin et al., 1983b). The concentration was determined by comparison with the color obtained at 550 nm from a purified cotton condensed tannin sample, the structure of which was elucidated by Collum et al. (1981). The anthocyanin content was determined by measuring the absorbancy at 540 nm of freeze-dried tissue extracted with methanol–water–HCl (79:19:3), using the molar extinction coefficient (E) of cyanidin 3-β-glucoside (Hedin et al., 1967). Flavonoids were determined after extraction of freeze-dehydrated tissue with 70% aqueous acetone. Diphenylboric acid–ethanolamine complex (Natural Product Reagent A, Aldrich Chemical Co., 1%) in methanol was added, and the chromophore absorptivity at 440 nm was determined and compared to that obtained from a purified sample of isoquercitrin, the most prevalent flavonoid in cotton.

HPLC Analyses of Terpenoid Aldehydes and Amino Acids. HPLC analysis

of the gossypol-type terpenoid aldehydes was performed on a reverse-phase C_{18} column according to the procedure of Stipanovic et al. (1988), who also kindly provided samples of hemigossypolone, and heliocides H_1 and H_2 for column calibration. Amino acids were determined by HPLC analyses after acid hydrolysis, employing their phenylthiocarbamyl derivatives (Cohen et al., 1986).

Procurement of Chemicals. All commercially available chemicals (other than the amino acids) were obtained from Sigma Chemical Co. Gossypol was a gift from the Southern Regional Research Center, USDA, New Orleans, Louisiana.

Statistical Procedures. Data obtained from the various analyses and measurements were subjected to the analysis of variance, and LSD values were calculated using SAS (1985) except for the HPLC analyses on the terpenoid aldehydes where standard errors of the mean are provided. No statistical analyses were obtained on the custom-procured amino acid data.

RESULTS AND DISCUSSION

Allelochemicals in Cotton Leaves, Squares, and Their Parts. The whole squares and square parts, anthers and stigma, bracts, calyx, calyx crown, and petals were analyzed for the major cotton allelochemicals (Table 2). The strains most resistant to TBW, DH 118 and DH 118 × ST 213 H, were highest in gossypol in the whole square and in the square parts. The DH 118 × ST 213 L strain was very similar to ST 213 in the level of gossypol and level of resistance to TBW, whereas the DH 118 × ST 213 H strain was very similar to DH 118 in the level of gossypol and the level of resistance to TBW in all square parts.

The tannins were highest in ST 213 glandless and in ST 213, the two strains most susceptible to TBW. This agrees with previous data of Hedin et al. (1983a,b) where tannin of glandless (susceptible) lines was about 20% higher than that of glanded resistant lines. The anthocyanin content should be somewhat correlated with gossypol because it often occurs as a halo around gossypol glands (Hedin et al., 1983a,b), but this was the case only in the anthers and bracts. The flavonoids were higher in petals and bracts of the resistant DH 118 and the DH × ST 213 H lines than in the DH 118 × ST 213 L and ST 213 lines. The same was true for flavonoids in whole squares.

Terpenoid Aldehydes (TAs) in Cotton Squares. Because gossypol is known to coexist in the plant with a number of related TAs, the gossypol fractions of three susceptible and two resistant double haploid lines were investigated by HPLC. Of the six major TA components listed in Table 3, the identities of four had previously been established, and their HPLC elution times were identical

TABLE 2. ALLELOCHEMICALS IN COTTON LEAVES, SQUARES, AND SQUARE PARTS OF TBW RESISTANT AND SUSCEPTIBLE LINES

Line	Percent content			
	Gossypol[a]	Tannins	Anthocyanin	Flavonoids
Terminal leaves				
ST 213 gl	0.13	8.90	0.13	4.58
ST 213	0.35	11.60	0.17	4.38
DH 126	0.39	11.67	0.19	4.42
LSD 0.05	0.02	0.65	0.02	0.62
Whole square				
ST 213 gl	0.12	10.10	0.16	2.14
ST 213	0.43	10.76	0.14	2.18
DH 118 × ST 213 L	0.48	7.51	0.11	2.04
DH 118 × ST 213 H	0.60	7.37	0.11	3.14
DH 118	0.63	6.86	0.11	2.66
LSD 0.05	0.18	0.74	0.02	0.33
Petals				
ST 213 gl	0.14	6.35	0.05	3.46
ST 213	0.69	4.55	0.05	2.09
DH 118 × ST 213 L	0.95	4.30	0.04	2.02
DH 118 × ST 213 H	1.25	4.40	0.05	2.16
DH 118	1.49	4.73	0.05	2.26
LSD 0.05	0.15	0.44	0.01	0.50
Anthers and stigma				
ST 213 gl	0.20	6.14	0.07	0.90
ST 213	1.14	5.12	0.08	0.30
DH 118 × ST 213 L	1.11	5.26	0.07	0.34
DH 118 × ST 213 H	1.37	4.56	0.09	0.30
DH 118	1.29	5.69	0.09	0.29
LSD 0.05	0.10	0.58	0.02	0.06
Bracts				
ST 213 gl	0.13	8.07	0.11	1.57
ST 213	0.13	8.37	0.17	1.33
DH 118 × ST 213 L	0.13	5.38	0.12	1.30
DH 118 × ST 213 H	0.22	6.56	0.17	1.42
DH 118	0.22	6.49	0.15	1.38
LSD 0.05	0.02	0.37	0.02	0.08
Calyx crown[b]				
ST 213 gl	0.09			
ST 213	0.12			
DH 118 × ST 213 L	0.11			
DH 118 × ST 213 H	0.12			
DH 118	0.15			
LSD 0.05	0.02			
Calyx[b]				
ST 213 gl	0.09			
ST 213	0.11			
DH 118 × ST 213 L	0.11			
DH 118 × ST 213 H	0.14			
DH 118	0.31			
LSD 0.05	0.02			

[a] Gossypol analyzed by the phloroglucinol procedure.
[b] Insufficient calyx tissue precluded analyses for tannins, anthocyanin, and flavonoids.

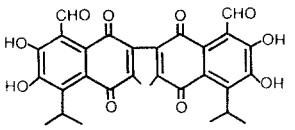
with those of available authentic samples (gossypol, hemigossypolone, and heliocides H_1 and H_2). Furthermore, the identity of the eluates was confirmed by EI-MS via solid probe analyses.

Tentative identifications of the other two maxima were obtained via EI-MS analysis. Comparison of the eluate at 8.2 min with that of an authentic

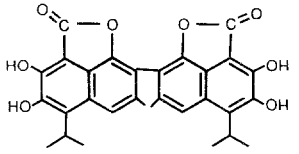
TABLE 3. TERPENOID ALDEHYDE CONTENT (%) IN SELECTED LINES OF COTTON SQUARES AND LEAVES

Line	Percent content						
	HGQ	GQ	GL	G	H_1	H_2	Total[a]
Buds							
ST-213 gl	0.01	0.02	0.01	0.02	0.01	0.01	0.08 ± 0.02
ST-213	0.04	0.09	0.03	0.14	0.06	0.07	0.43 ± 0.04
ST-825	0.02	0.08	0.03	0.15	0.04	0.05	0.37 ± 0.03
DH-118	0.14	0.06	0.03	0.12	0.17	0.11	0.63 ± 0.07
DH-126	0.16	0.07	0.03	0.14	0.20	0.13	0.73 ± 0.06
Leaves							
ST-213	0.10	0.02	0.01	0.03	0.12	0.12	0.40 ± 0.04
DH-118	0.17	0.03	0.02	0.05	0.16	0.18	0.61 ± 0.05

[a] Mean ± standard error of the mean.



a. Gossypolone



b. Gossypol Lactone

FIG. 1. Structures of gossypolone and gossypol lactone.

sample of gossypolone (Figure 1a) prepared by us (Phillips and Hedin, 1990) showed that the fragmentation patterns were nearly identical.

EI-MS analysis of the maximum at 10.7 min gave an apparent $[M]^+$ of 514. The most prominent ion fragments were as follows: *m/z* 514(100), 499(75), 482(65), 465(35), and 439(15). Repeated attempts to isolate sufficient quantities of this component for NMR and IR by liquid chromatography using silica gel and Sephadex LH-20 were unsuccessful because of degradation.

It was proposed that the apparent molecular ion could be accounted for if the gossypol aldehydic functions were oxidized to the dicarboxylic acid and subsequently dehydrated to form the dilactone. Therefore, gossypol was oxidized by stirring in air with MnO_2 in acetone or benzene. Both preparations dehydrated rapidly to the presumed dilactones, without the use of a dehydrating agent, and gave fragmentation patterns essentially identical to that of the isolate. Again, efforts to prepare sufficient samples in pure form for spectral work were unsuccessful. The presumed structure (Figure 1b) is 6,6′,7,7′-tetrahydroxy-3,3′-dimethyl, 5,5′-bis(methylethyl)-2,2′-binaphthalene-8,8′-γ-dibutyrolactone. The trivial name "gossypol lactone" is suggested.

The total TA content was lower for each of the three susceptible lines than in the two resistant lines (Table 3). It is noteworthy that the gossypol content was essentially the same in squares of all of the glanded lines (0.12–0.15%). This suggests that resistant and susceptible lines both biosynthesize equal (basal) amounts of gossypol, and thus resistance is determined by other factors, perhaps other terpenoid aldehydes.

Hemigossypolone (Table 3) accounts for less than 10% of the TAs in squares of susceptible lines but over 20% of the TAs in resistant lines. The heliocides H_1 and H_2 are also increased sharply in resistant lines (two to fourfold). On the other hand, neither square gossypolone or gossypol lactone (M^+514) is increased above the "basal" levels in resistant lines. While they, along with gossypol, can be presumed to contribute some resistance, they do not appear to account for the increased resistance of the DH lines.

Gossypol lactone is probably an artifact arising from the oxidation and subsequent lactonization of gossypol during processing and/or storage. The distribution of TAs in leaves (Table 3) is somewhat different. The gossypol content is low compared to hemigossypolone and heliocides H_1 and H_2 in both a susceptible and a resistant line. The latter three are again higher in the resistant line than the susceptible line as compared with gossypol.

Chan et al. (1978), Stipanovic et al. (1977, 1978), and Hedin et al. (1983a,b) determined the inhibition of TBW larval growth expressed as ED_{50}, percent of diet, for several TAs including gossypol, hemigossypolone, and heliocide H_1. They averaged about 0.10%. Therefore, at least when reconstituted in diets, they have about the same level of toxicity. If this relationship

also exists in the plant sites that are encountered by the insect, resistance as mediated by TAs is related to the concentration in the tissue of importance.

Relationships of Cotton Plant Amino Acids to TBW Growth and Development. For background information, the total amino acids, free and bound, were determined by HPLC analysis (Cohen et al., 1986). Amino acid analysis data for ST 213 leaves, squares, and their parts; ST 213 gl anthers; DH 118 calyx crown; and the TBW Pack B (soy flour plus wheat germ) are summarized in Table 4. The total amino acid contents of calyx and calyx crown are the lowest (13.1% and 14.8%). Bracts and petals are intermediate (17.0% and 17.3%), and anthers are highest (22.5% and 23.7%). It is of apparent significance that the larvae migrate to, and complete their growth and development in, the tissue of highest protein content.

The total sugar content of anthers is known to be high relative to other square tissues and also is higher in lines susceptible to boll weevil oviposition (Hedin and McCarty, 1990). Nutritionally, therefore, anthers appear to be the most favorable cotton tissue development site for insects.

The distribution of the amino acids in the various tissues is similar, and the ratio of essential to nonessential amino acids (45–47%) has been found adequate for larval growth of the fall armyworm [*Spodoptera frugiperda* (J.E. Smith)] (Hedin et al., 1990). The only likely growth constraints may be that methionine (6.8%) in DH 118 calyx crown and arginine (9.9%) in Pack B are high relative to the other essential amino acids for optimum growth. The high arginine of Pack B is contributed mostly by the wheat germ. Wheat germ amino acids, as a synthetic amino acid diet, failed to support fall armyworm larval growth (Hedin et al., 1990). Analysis of cotton plant amino acids has been reported on numerous occasions in the literature, including Parrott et al. (1969) and Lindig et al. (1980). The results are similar.

Tables 5 and 6 provide information about the synthetic amino acid contents of diets that were formulated and used to investigate whether they could successfully support growth and development of TBW larvae. Using synthetic amino acids as the sole source of nitrogen is not novel. Evidently the first report of the use of synthetic amino acids for the growth of a cotton insect was that of Vanderzant (1965) for the boll weevil *Anthonomus grandis* Boh. Dadd (1985) reported in a review article on the rearing of several other insects on amino acid diets. We recently reported on the rearing of the fall armyworm with synthetic amino acid diets (Hedin et al., 1990). The replacement of plant protein with amino acids in diets being tested for effects of allelochemicals removes from conjecture the possibility that some unknown factor, perhaps bound to the protein, also may be affecting growth and development.

In these tests (Table 5) amino acid diets were formulated at the same nitrogen level supplied by the protein (diets 1 and 4), at a level increased 50% to

TABLE 4. AMINO ACIDS IN SELECTED COTTON PLANT TISSUES AND TBW LABORATORY DIET

Amino acid	Amino Acids (% of total)									
	Terminal leaves, ST 213	Squares, ST 213	Calyx crown, ST 213	Calyx, ST 213	Bracts, ST 213	Petals, ST 213	Anthers, ST 213	Anthers, ST 213 gl	Calyx crown, DH 118	TBW Pack B
Arginine	7.3	6.0	7.9	6.6	7.4	7.2	6.9	6.6	7.0	9.9
Histidine	1.9	3.5	1.8	2.0	2.0	2.1	2.0	2.0	1.8	2.1
Isoleucine	5.5	5.5	5.3	5.0	5.4	4.6	5.5	5.5	4.8	4.5
Leucine	8.8	9.1	7.2	7.4	9.0	7.2	7.8	7.7	7.9	7.3
Lysine	7.1	8.6	8.5	6.9	7.1	6.5	6.8	6.9	7.2	6.4
Methionine	2.9	3.0	2.8	2.8	2.8	2.4	2.8	2.8	6.8	2.8
Phenylalanine	5.6	4.7	5.0	4.5	5.8	4.2	4.4	4.4	4.7	4.9
Threonine	4.0	4.2	4.2	3.2	3.9	3.3	4.7	4.7	4.4	3.5
Tryptophan[a]	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Valine	6.4	6.8	6.2	5.7	6.3	5.4	5.6	5.7	5.5	5.2
Alanine	7.0	6.1	6.4	6.4	6.9	6.0	5.6	5.7	5.7	5.4
Aspartic acid	8.3	10.3	11.2	15.5	8.2	17.7	11.1	11.3	11.5	11.0
Cysteic acid	1.8		0.3	1.9	1.8	1.9	0.2	0.2	0.2	0.3
Cystine	0.2	0.8	0.1	0.2	0.2	0.4	0.3	0.3	0.2	0.3
Glutamic acid	12.4	12.9	12.4	12.9	12.5	12.3	12.3	11.2	11.9	17.4
Glycine	5.9	6.0	5.6	5.1	5.8	4.9	5.1	5.1	5.2	4.8
Proline	6.3	5.7	5.8	6.3	6.3	5.8	8.1	7.5	5.6	5.6
Serine	3.9	3.5	4.8	3.9	3.9	3.9	5.5	5.5	5.0	4.5
Tyrosine	3.3	1.9	3.1	2.3	3.3	2.8	3.9	3.5	3.2	2.7
Amino acids in sample (%)	17.4	18.7	14.8	13.1	17.3	17.0	23.7	22.5	17.4	26.8

[a] Analysis for tryptophan was obtained for ST 213 squares only and included as an estimate of its content in the other tissues.

TABLE 5. FORMULATIONS OF SYNTHETIC AMINO ACID DIETS FOR TBW BASED ON ANALYSIS OF CONTENTS IN COTTON ANTHERS AND LABORATORY SOY–WHEAT GERM DIET[a]

	Cotton anthers			Soy–wheat germ		
Amino acid	g/14.01 g	g/21.01 g	g/14.16 g	g/14.01 g	g/21.01 g	g/14.35 g
Diet	1	2	3	4	5	6
Arginine	0.92	1.38	0.92	1.43	2.15	1.43
Histidine	0.28	0.42	0.28	0.31	0.47	0.31
Isoleucine	0.78	1.17	0.78	0.66	0.99	0.66
Leucine	1.12	1.68	1.12	1.06	1.59	1.06
Lysine	0.95	1.43	0.95	0.92	1.38	0.92
Methionine	0.14	0.21	0.28	0.06	0.09	0.31
Phenylalanine	0.63	0.95	0.63	0.70	1.05	0.70
Threonine	0.66	0.99	0.66	0.50	0.75	0.50
Tryptophan	0.20	0.30	0.28	0.19	0.29	0.31
Valine	0.81	1.22	0.81	0.76	1.14	0.76
Alanine	0.81	1.22	0.81	0.78	1.17	0.78
Aspartic acid	1.79	2.69	1.79	1.60	2.40	1.60
Cysteic acid	0.03	0.05	0.03	0.04	0.06	0.04
Cystine	0.04	0.06	0.06	0.06	0.09	0.06
Glutamic acid	1.88	2.82	1.88	2.55	3.83	2.55
Glycine	0.71	1.07	0.71	0.70	1.05	0.77
Proline	1.16	1.74	1.16	0.81	1.22	0.81
Serine	0.77	1.16	0.77	0.66	0.99	0.66
Tyrosine	0.52	0.78	0.52	0.41	0.62	0.41

[a] Diets 2 and 5 were increased in the amino acid content by 50%; diets 3 and 6 were increased only with methionine and tryptophan.

determine whether the amino acid concentration was limiting (diets 2 and 5), and at the base level of diets 1 and 4 but with increased amounts of methionine and tryptophan whose analysis was thought to be low. Diets 1–3 were based on the distribution of amino acids in cotton anthers, and diets 4–6 were based on the distribution in the soy–wheat germ diet (Table 4).

Tables 1 and 6 give the ingredients of the formulated diets. Table 6 also lists larval weights at nine days, pupal weights, and days to pupation, along with the statistical information. As expected, insects fed the standard laboratory diet grew more rapidly and pupated sooner. However, insects on the three cotton anther amino acid diets (diets 1–3) achieved pupation having pupal weights only somewhat lower than those on the standard diet. The days to pupation were longer. Increasing the level of amino acids by 50% (diet 2) had an adverse effect compared to diet 1. Increasing the methionine and tryptophan (diet 3) improved the growth rate through nine days, but the improvement was not sus-

TABLE 6. TBW GROWTH AND DEVELOPMENT ON SYNTHETIC AMINO ACID DIETS BASED ON CONTENT IN COTTON ANTHERS AND LABORATORY SOY-WHEAT GERM DIET

Ingredient	Cotton anthers			Soy-wheat germ			
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7[a]
Pack B (g)	0	0	0	0	0	0	61.8
Amino acids (g), Pack B	14.01	21.02	14.35	14.01	21.02	14.16	0
Less protein (g)	48.0	48.0	48.0	48.0	48.0	48.0	0
Agar (g)	10.6	10.6	10.6	10.6	10.6	10.6	10.6
Vitamins (g)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Acids (ml)	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Hot water (ml)	265	265	265	265	265	265	265
Cold water (ml)	177	177	177	177	177	177	177
Larval weight, 9 days, mg; LSD = 13.4	5.8 C[b]	8.9 B	33.9 B	0.6 C	0.7 C	34.9 B	317.7 A
Pupal weight, mg: LSD = 9.3	261.5 B	235.1 D	230.6 D	[c]	[c]	247.3 C	310.0 A
Days to pupation, LSD = 0.4	21.0 D	23.4 A	22.7 B	[c]	[c]	21.9 C	14.5 E

[a] Diet 7 is the standard laboratory diet.
[b] Means followed by the same letter are not significantly different.
[c] No survival.

tained through pupation. Larval growth on the soy-wheat germ-based amino acid diets 4 and 5 did not occur. With added methionine and tryptophan (diet 6), however, acceptable growth and development took place.

The growth lag can perhaps be explained by the need for insects to develop mechanisms to transport and metabolize the free amino acids. The somewhat poorer growth observed on the cotton anther-based amino acid diet 2 can probably be explained by the high amino acid-carbohydrate ratio. Phytophageous insects have been reported to grow best on diets in which the protein and carbohydrates each contribute about one third of the nutrients (Dadd, 1985). The poorer performance of the soy-wheat germ diets relative to the cotton anther diets may be in part explained by the high arginine content of diets 4–6.

The overall performance of these amino acid diets is similar to that observed with the fall armyworm (Hedin et al., 1990). In these present tests, all 10 amino acids reported as essential for rats were included in each of the tests. Dadd (1985), in reviewing the literature, reported that for specific insects additional

amino acids were found to be essential for insect growth. As previously stated, the growth and development observed in these tests was not as rapid as with the standard diet, but presumably could be optimized if desired. However, the present results established that the TBW can be reared successfully on synthetic amino acid diets, evidently for the first time. Moreover, the establishment of effective amino acid diets means that the effects of allelochemicals can be evaluated free from the effects of undefined growth factors.

SUMMARY

Chemical evidence has been obtained that explains, in part, observations about the migration of tobacco budworm larvae from terminal leaves to the square, their avoidance of calyx crowns that are high in gossypol glands, and their burrowing into the anthers where they develop.

The calyx crown of resistant lines was found to be higher in terpenoid aldehydes than in susceptible lines. HPLC data showed that the gossypol content of both susceptible and resistant lines is equivalent (basal), while the hemigossypolone and heliocides H_1 and H_2 are greatly increased in resistant lines and presumably are more closely associated with resistance.

Analysis for total amino acids in cotton square tissues showed that there was a gradation of levels from the leaves, calyx, and calyx crown, which were low; to the petals and bracts, which were intermediate; and to the anthers, the site of final development, which was highest. Amino acid diets based on their distribution in anthers were found to be successful for larval growth and development.

Acknowledgments—The authors thank Valeria Phillips, Joseph Mulrooney, Moderia Petty, and Eloise Sloan for technical support.

REFERENCES

CHAN, B.G., WAISS, A.C., BINDER, R.G., and ELLIGER, C.A. 1978. Inhibition of lepidopterous larval growth by cotton constituents. *Entomol. Exp. Appl.* 24:94–100.

COHEN, S.A., BIDLINGMEYER, B.A., and TARVIN, T.L. 1986. PITC derivatives in amino acid analysis. *Nature* 320: 769–770.

COLLUM, D.H., HEDIN, P.A., WHITE, W.H., PARROTT, W.L., JENKINS, J.N., and GRIMLEY, E.B. 1981. Studies on the structural properties of cotton tannin and its toxicity to the tobacco bud-

worm. Abstracts of Papers, 182nd National Meeting of the American Chemical Society, New York. American Chemical Society, Washington, D.C. Pest Abstract No. 54.

DADD, R.H. 1985. Nutrition: Organisms, pp. 313–390, *in* G.A. Kerbut and L.I. Gilbert (eds.). Insect Physiology, Biochemistry, and Pharmocology, Vol. 4, Regulation, Digestion, Nutrition, Excretion. Pergamon Press, Elmsford, New York.

HEDIN, P.A., and MCCARTY, J.C. 1990. Possible Roles of Cotton and sugars and terpenoids in oviposition by the boll weevil. *J. Chem. Ecol.* 16:757–772.

HEDIN, P.A., JENKINS, J.N., COLLUM, D.H., WHITE, W.H., PARROTT, W.L., and MACGOWN, M.W. 1983a. Cyanidin-3-β-glucoside, a newly recognized basis for resistance in cotton to the tobacco budworm. *Experientia* 39:799–801.

HEDIN, P.A., JENKINS, J.N., COLLUM, D.H., WHITE, W.H., and PARROTT, W.L. 1983b. Multiple factors in cotton contributing to resistance to the tobacco budworm. pp. 349–365, *in* P.A. Hedin (ed.). Plant Resistance to Pests. ACS Symposium Series 208, American Chemical Society, Washington, D.C.

HEDIN, P.A., PARROTT, W.L., JENKINS, J.N., MULROONEY, J.E., and MENN, J.J. 1988. Elucidating mechanisms of tobacco budworm resistance to allelochemicals by dietary tests with insecticide synergists. *Pest Biochem. Physiol.* 32:55–61.

HEDIN, P.A., WILLIAMS, W.P., DAVIS, F.M., and BUCKLEY, P.M. 1990. Roles of amino acids, protein, and fiber in leaf-feeding resistance of corn to the fall armyworm. *J. Chem. Ecol.* 16:1977–1995.

HORWITZ, W. (ed.). 1975. Methods of Analysis of the Association of Official Analytical Chemists, 12th ed. 1094 pp. Association of Official Analytical Chemists, Washington, D.C.

JENKINS, J.N., PARROTT, W.L., MCCARTY, J.C., JR., and WHITE, W.H. 1982. Breeding cotton for resistance to the tobacco budworm: Techniques to achieve uniform field infestation. *Crop Sci.* 22:400–404.

LINDIG, O.H., POE, W.E., and HEDIN, P.A. 1980. Essential amino acids in dietary protein sources and the nutritional status and oviposition of boll weevils. *J. Econ. Entomol.* 73:172–175.

PARROTT, W.L., MAXWELL, F.G., JENKINS, J.N., and MAULDIN, J.K. 1969. Amino acids in hosts and non-hosts of the boll weevil. *Ann. Entomol. Soc. Am.* 62:255–260.

PARROTT, W.L., JENKINS, J.N., and MCCARTY, J.C. 1983. Feeding behavior of first-stage tobacco budworm on three cotton cultivars. *Ann. Entomol. Soc. Am.* 76:167–170.

PARROTT, W.L., JENKINS, J.N., MULROONEY, J.E., MCCARTY, J.C., and SHEPHERD, R.L. 1989. Relationship between gossypol gland density on cotton squares and resistance to tobacco budworm larvae. *J. Econ. Entomol.* 82:589–592.

PHILLIPS, V.A., and HEDIN, P.A. 1990. Spectral techniques for structural analyses of the cotton terpenoid aldehydes gossypol and gossypolone. *J. Agric. Food Chem.* 38:525–528.

RAMALHO, F.S. 1983. Behavior of the tobacco budworm in cotton. PhD dissertation. Mississippi State University, Mississippi State, Mississippi.

RAMALHO, F.S., MCCARTY, J.C., JR., JENKINS, J.N., and PARROTT, W.L. 1984. Distribution of tobacco budworm (Lepidoptera: Noctuidae) larvae within cotton plants. *J. Econ. Entomol.* 77:591–594.

SAS. 1985. SAS User's Guide: Statistics, Version 5 Edition, SAS Institute, Inc., Cary, North Carolina. 956 pp.

SHAVER, T.N., and PARROTT, W.L. 1970. Relationship of larval age to toxicity of gossypol to bollworms, tobacco budworm, and pink bollworms. *J. Econ. Entomol.* 63:1802–1804.

SHAVER, T.N., GARCIA, K.A., and DILDAY, R.H. 1977. Tobacco budworm: Feeding and larval growth on component parts of cotton flowerbuds. *J. Envirn. Entomol.* 6:82–84.

STIPANOVIC, R.D., BELL, A.A., O'BRIEN, D.H., and LUKEFAHR, M.J. 1977. Heliocide H_2: An insecticidal sesterterpenoid from cotton (*Gossypium*). *Tetrahedron Lett.* 6:567–570.

STIPANOVIC, R.D., BELL, A.A., O'BRIEN, D.H., and LUKEFAHR, M.J. 1978. Heliocide H_1: A new insecticidal sesterterpenoid from cotton (*Gossypium hirsutum*). *J. Agric. Food Chem.* 26:115.

STIPANOVIC, R.D., ALTMAN, D.W., BEGIN, D.L., GREENBLATT, G.A., and BENEDICT, J.H. 1988. Terpenoid aldehydes in upland cottons: Analysis by aniline and HPLC methods. *J. Agric. Food Chem.* 36:509–515.

VANDERZANT, E.S. 1965. Axenic rearing of the boll weevil on defined diets: amino acid, carbohydrate and mineral requirements. *J. Insect Physiol.* 11:659–670.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

PROCESSING OF IRIDOID GLYCOSIDE ANTIRRINOSIDE FROM *Maurandya antirrhiniflora* (Scrophulariaceae) BY *Meris paradoxa* (GEOMETRIDAE) AND *Lepipolys* SPECIES (NOCTUIDAE)[1]

CHRISTIE A. BOROS,[2] FRANK R. STERMITZ,[2,*] and NOEL McFARLAND[3]

[2]*Department of Chemistry, Colorado State University*
Fort Collins, Colorado 80523
[3]*P.O. Box 1404*
Sierra Vista, Arizona 85636

(Received November 9, 1990; accepted February 11, 1991)

Abstract—The iridoid glycoside antirrinoside was found to be sequestered by highly aposematic larvae of the geometrid moth *Meris paradoxa* and two noctuid moth *Lepipolys* species feeding on *Maurandya antirrhiniflora* (Scrophulariaceae), a natural food plant from southern Arizona. The antirrinoside content of leaves and petioles being consumed, early-instar larvae, late-instar larvae, larval frass, regurgitant, reflex-bleeding emission (*Meris paradoxa*), cocoons, pupae, meconium emitted upon eclosion, and adult moths was determined. Larvae, other than the earliest instars, did not excrete antirrinoside in the frass, but sequestered it in amounts of 3–11% of the dry weight. Small amounts of antirrinoside remained in various pupal or cocoon parts and some was emitted in the meconium upon eclosion. The total antirrinoside accounted for was, however, considerably below that expected based upon the remarkably high 20% content of the leaves and petioles being consumed. The adult cryptic moths of both species contained little or no antirrinoside. This is the first report of a natural food plant and larval stages for *M. paradoxa* and a previously undescribed *Lepipolys* species. It is also the first report of antirrinoside sequestration and utilization by insects.

Key Words—*Meris paradoxa, Lepipolys perscripta*, Lepidoptera, Geometridae, Noctuidae, *Maurandya antirrhiniflora, Antirrhinum majus*, Scrophulariaceae, antirrinoside, iridoid glycosides, herbivory, sequestration.

*To whom correspondence should be addressed.

[1] Chemistry of the Scrophulariaceae. 20. Paper 19: Stermitz, F.R., Ianiro, T.T., Robinson, R.D., and Gardner, D.R. 1991. *J. Nat. Prod.* 54:626–628.

0098-0331/91/0600-1123$06.50/0

INTRODUCTION

Specialist Lepidoptera whose larvae feed on plants containing iridoid glycosides ("iridoids") process and sequester these bitter substances in a variety of ways (Bowers and Puttick, 1986; Stermitz et al., 1986, 1988; Franke et al., 1987; Bowers, 1988; Gardner and Stermitz, 1988; L'Empereur and Stermitz, 1990a,b). In previous studies, sequestration of the iridoids catalpol, aucubin, and macfadienoside in several percent of the dry weight of the insect has been established. In cases where esters of catalpol are the major food-plant iridoids (Gardner and Stermitz, 1988; L'Empereur and Stermitz, 1990b), larvae hydrolyze the esters, sequester the resultant catalpol, and excrete or metabolize the acylating acid. Where both larval and adult stages of the insect are aposematic (boldly patterned and/or colored), both contain sequestered iridoids. Where the larval stage is aposematic and the adult stage is cryptic, the adults contained little or no iridoids, having left most in the meconium emitted upon eclosion.

In one such case (Stermitz et al., 1988), the larvae of two different taxa, *Meris alticola* and *Neoterpes graefiaria* (both geometrids), were both black and white striped with gold–yellow spots; it was suggested that Müllerian mimicry might be involved. Recently, similarly colored black and white banded geometrid larvae were observed utilizing *Maurandya antirrhiniflora* (Scrophulariaceae; Antirrhineae) in southern Arizona. These larvae were determined to be the previously undescribed immature stage of the moth *Meris paradoxa*, whose food plant was also previously unknown. The same plant species also supported noctuid larvae exhibiting a pattern of bright yellow, black-ringed spots on a gray background. These were determined to be larval stages of a previously undescribed moth, closely related to *Lepipolys perscripta*. Finally, a winter-collected adult female of *L. perscripta* was collected at a UV light, and larvae from its eggs were found to readily consume *Maurandya antirrhiniflora*. *Maurandya* belongs to the tribe Antirrhineae of the Scrophulariaceae, and the tribe is known to characteristically contain the iridoid antirrinoside. We therefore undertook a study designed to identify iridoid(s) of *Maurandya*, to determine if any were sequestered and, if so, to quantify the patterns of processing and sequestration among the various life stages of the hosted moth species.

METHODS AND MATERIALS

Organisms. The original collections of larvae on the food plant were made on August 23, 1986, above the town of Bisbee, Cochise County, Arizona, on the north slope of Higgins Hill. The food plant, determined to be the perennial *Maurandya antirrhiniflora* Willd., is also cultivated in Arizona gardens and is known as the snapdragon vine. In a recent revision of the tribe Antirrhineae (Sutton, 1988), readoption of its previous designation as *Maurandella antir-*

rhiniflora (Willd.) Rothm. was suggested. The taxon will simply be called *Maurandya* in this report. Additional plants were grown from seed in Ash Canyon, south of Sierra Vista, Arizona, and in the greenhouse at Colorado State University.

One collected group of 3- to 4-cm-long larvae were boldly patterned with black and white bands and black and yellow–orange spots (Figure 1). These were fed on *Maurandya* until pupation. Gray, slightly dark-spotted adults emerged from hanging, hammocklike cocoons 25–30 days after pupation. These were identified as *Meris paradoxa* Rindge by Frederick H. Rindge of the American Museum of Natural History, New York. The moth had been described from collected adult males as a new species (Rindge, 1981), whose food plant, larval stages, and female adult stage were unknown. Three Arizona field-collected moths (one female and three males) also were available for analysis.

The second group of collected larvae, also 3–4 cm long, were boldly patterned in gray, yellow, and black (Figure 2). Gravid adult females were collected at a UV light in the SE Huachuca mountains (5354 Ash Canyon Road, Cochise County, Arizona) on September 20, 1989. Their progeny were raised

FIG. 1. Larva of *Meris paradoxa*.

FIG. 2. Larva of *Lepipolys* (undescribed).

from eggs on *Maurandya*. Pupae were bare if last-instar larvae were placed in empty jars but were contained within a sand-covered capsule if the larvae were placed in a jar containing sand or sandy soil. The emergent adults were identified as an undescribed species of *Lepipolys* (Noctuidae) by Robert W. Poole, Systematic Entomology Laboratory, United States Department of Agriculture, Washington, D.C. The larvae were identical to those field-collected on *Maurandya*. The moth will be denoted as *Lepipolys* (undescribed), awaiting a complete description by Poole.

True *L. perscripta* Gn. (also identified by Poole) were obtained from eggs from a gravid female collected at the UV light on March 20, 1988. Larvae were colored and patterned considerably differently from those of *Lepipolys* (undescribed) but corresponded to the original description of *L. perscripta* (Dyar, 1903). The larvae were virtually identical with those depicted in a black-and-white drawing (Comstock, 1958). They were raised to adults on *Maurandya*.

Because of the large number of September 1989 brood larvae, insufficient *Maurandya* was available to carry all larvae to pupation on that food plant. Larvae did, however, readily consume leaves and flowers of the common garden snapdragon, *Antirrhinum majus* L., and this proved to be a useful alternate host.

Larvae were held in Petri dishes with fresh *Maurandya*, and frass was collected and air-dried before weighing and analysis. Larvae and pupae were weighed, crushed into methanol, and the mixture allowed to stand for 24 hr at room temperature (or more extended periods in the refrigerator or freezer). After the mixture was filtered, the methanol was evaporated to dryness and the residue used directly for iridoid analyses. Adult moths were treated similarly. Agitation of larvae of *Meris paradoxa* resulted in emission of yellow droplets from intersegmental membranes. Reflex-bleeding droplets were collected in a microliter pipet and the volume recorded. The material was washed out with a little methanol and the residue, after evaporation (unweighed), was analyzed for total antirrinoside. Pupae were placed in a large glass vial containing filter paper in the bottom, which collected meconium emitted upon eclosion or shortly thereafter. The paper was extracted with methanol, the methanol evaporated, and the residue (unweighed) analyzed for total antirrinoside.

Iridoid Glycoside Analyses. Quantitative iridoid glycoside analyses of plants or plant parts, larvae, excretion products, pupae, and adult moths were by gas chromatography (GC) of the trimethylsilyl ether derivative as previously described in detail (Gardner and Stermitz, 1988).

In a large-scale determination, 100 g fresh weight of *Maurandya antirrhiniflora*, collected in Arizona, was soaked in methanol for 48 hr, filtered, and the methanol evaporated to leave a gum, which was triturated with water. The water layer was washed with diethyl ether, evaporated, and the residue triturated with absolute methanol. The methanol was evaporated to leave 2.0 g of

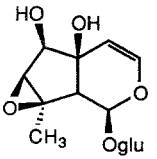
a semisolid "crude" iridoid preparation, the content of which was examined by NMR and GC analysis.

RESULTS

Plant Analyses. [^{1}H]NMR analysis of the "crude" iridoid residue from field-collected *Maurandya* showed that this preparation was at least 95% antirrinoside (Scheme 1). This was confirmed by [^{13}C]NMR and GC analysis. Pure antirrinoside for a GC standard was obtained by vacuum liquid chromatography (VLC). The large-scale isolation indicated an approximate antirrinoside content of 2% wet weight or about 10% dry weight based on 80% volatiles (see below).

More detailed analyses of the antirrinoside content of two large greenhouse-grown plants (whose leaves were used for larval feeding) were conducted at various times during the course of the study, using the quantitative GC method of analysis. Individual green stems contained 14–20%, petioles 12–31%, and leaves 4–36% dry weight antirrinoside. Older living, but not green stems contained 1–9%. Larvae consumed only petioles and leaves, with the petioles (N = 5) averaging 22% and the leaves (N = 11) averaging 18% antirrinoside. Since larvae consumed many dozens of leaves and petioles during their growth, the average antirrinoside content of about 20% dry weight is probably a reasonable estimate of intake in spite of a high variability among individual leaves and petioles. The percent volatiles (loss of weight on drying) were 69–75% for stems, 71–83% for petioles, and 77–87% for leaves. The garden snapdragon, *A. majus*, used to supplement larval feeding in one experiment, showed a leaf analysis of 5.8% dry weight antirrinoside.

Lepipolys perscripta. Data from an April 1988 study was limited to six larvae raised on *Maurandya* and is not complete through all life stages because of the small number available. Some useful data were obtained, however, particularly those resulting from frass analysis (Table 1). Early-instar larvae were not completely efficient at sequestration or at plant digestion, since considerable antirrinoside was found in the frass. Insignificant antirrinoside was found in frass throughout the remainder of the instars, except for the final, reddish-col-



antirrinoside

SCHEME 1.

TABLE 1. PROCESSING OF ANTIRRINOSIDE FROM *Maurandya antirrhiniflora* BY *Lepipolys perscripta*

Organism	Analyte	Dry wt. (mg)	Antirrinoside	
			mg	% Dry wt.
1–6	frass			
	days 10–12	56.7	4.0	7.1
	days 12, 13	145.8	0.3	0.2
	days 14, 15	202.9	0.05	<0.1
	days 15, 16	157.7	0.07	<0.1
	days 17, 18	343.2	0.05	<0.1
	days 18, 19	703.7	0.05	<0.1
	day 20	395.2	0.0	0.0
4–6	day 21	43.8	0.05	0.1
	red-tinted[a]	48.0	0.9	1.9
	red[b]	5.0	0.0	0.0
1–6	last skin casts	8.3	0.8	9.6
1–6	oral secretion[c]	18.5	0.8	4.3
1	pupa[d]	58.2	0.6	1.0
2	pupa[d]	62.2	0.7	1.1
3	pupa[e]	70.7	1.5	2.1
4	pupa[e]	60.1	1.4	2.3
5	pupal case	1.8	0.1	5.6
	moth	23.2	0.2	0.9
	meconium	5.0	1.8	
6	moth (female)[f]	16.3	0.0	0.0
	eggs (130)	3.1	0.0	
7	moth (female) + eggs[f]	25.4	trace	
8	moth[f]	43.8	0.03	<0.1
9	moth[f]	46.6	trace	

[a] Next-to-last frass.
[b] Last frass excreted before pupation.
[c] Orange fluid left on filter paper by larvae "chewing" paper immediately prior to pupation.
[d] Frozen 3–4 days after pupation.
[e] Pupae died prior to eclosion.
[f] Wild-caught.

ored frass excreted just before entering diapause. When larvae would no longer consume plant material and began to wander, they were transferred to vials containing filter paper on the bottom. Larvae continued to wander rapidly for several days before pupating. During this time they attempted to chew the filter paper (but would not consume plant material), and deposited mouth secretions on the paper. This secretion or regurgitant contained antirrinoside, as did the last instar skin molt (Table 1). Four pupae (Nos. 1–4, Table 1; two living and

then frozen and two that did not develop) contained small amounts of antirrinoside as did the pupal case and meconium of the one adult (No. 5) that emerged. The adult (from which the abdomen had been removed for dissection and identification) contained a trace amount of antirrinoside. Four adult moths (Nos. 6–9, Table 1), collected in the field in March 1988, showed at most traces of antirrinoside, as did the eggs from two.

Lepipolys (undescribed). Of two last-instar larvae obtained from *Maurandya* in the field (September 1987), one was analyzed for antirrinoside after it was removed from food for 24 hr. The larva (114 mg dry weight) contained 11.6 mg (10%) antirrinoside. The second larva was placed on sandy soil for pupation, but did not survive. The same site was searched in 1988 and 1989, but no larvae were found. Subsequent studies were performed on insects lab-raised from eggs laid by wild-caught females.

TABLE 2. ANTIRRINOSIDE PROCESSING BY *Lepipolys* (UNDESCRIBED) LARVAE FED ON *Maurandya antirrhiniflora* AND *Antirrhinum majus*

Organism	Analyte	Dry wt. (mg)	Antirrinoside	
			mg	% Dry wt.
Early instar				
1	larva[a]	25.8	2.7	10.5
2	larva[a]	18.7	2.0	10.7
3	larva[a]	14.2	0.7	4.9
4	larva[a]	13.7	0.8	5.8
5	larva[a]	13.4	0.5	3.7
Late instar				
6	larva[a]	68.9	2.2	3.2
7	larva[a]	95.1	8.2	8.6
8	larva[a]	103.5	6.0	5.8
9	larva[b]	85.8	9.6	11.2
10	larva[b]	98.9	5.4	5.5
11	larva[b]	105.3	9.4	8.9
12	last skin casts ($N = 9$)	21.6	1.7	7.9
13–16	pupae[b,c]	66.4 (ave.)	0.1	0.1
17–22	pupae[a]	51.0 (ave.)	0.3	0.6
23	pupa[a,c]	57.6	0.0	0.0
18–23	sand capsules	226.2	0.0	0.0
24–32	moths	23.6 (ave.)	0.0	0.0
	meconium		0.2 (ave.)	

[a] Larvae fed *M. antirrhiniflora.*
[b] Larvae fed *A. majus.*
[c] Pupae died and desiccated before eclosing.

Five larvae (Nos. 1–5, Table 2, fed on *Maurandya*) contained 3.7–10.7% dry weight antirrinoside, while three larvae (Nos. 9–11, Table 2, fed on *A. majus*) contained 5.5–11.2% antirrinoside.

Last-instar larvae, which had been raised on greenhouse *Maurandya*, were provided with jars containing sand and a few leaves for pupation. For pupation, larvae constructed capsulelike pupal cells of sand particles, which enclosed the pupae proper. Pupae contained only traces of antirrinoside, whether they died and desiccated (Nos. 13–16) or were analyzed while still apparently viable (Nos. 17–22). The pupal cells contained no antirrinoside (Nos. 18–23) nor did the emergent adult moths (Nos. 24–32). The combined meconium from these moths contained a small amount of antirrinoside.

Meris paradoxa. Data on this moth (Table 3) were limited since gravid

TABLE 3. PROCESSING OF ANTIRRINOSIDE BY *Meris paradoxa* WHOSE LARVAE WERE RAISED ON *Maurandya antirrhiniflora*

			Antirrinoside	
Organism	Analyte	Dry wt. (mg)	mg	% Dry wt.
1	last larval skin cast	1.5	trace	trace
	pupal case and silk	10	0.32	3.2
	adult male moth[a]	9.9	trace	trace
	1st (milky) meconium		0.18	
	2nd (orange) meconium		0.10	
2	larval reflex-bleed		0.67[b]	
	larva	52	4.6	8.9
3	larval reflex-bleed		0.33[c]	
	larva	51	4.8	9.4
2 + 3	combined frass	415	2.2	0.5
4	larval reflex bleeding excretion		0.63[d]	
	last larval skin cast	1.1	0.04	3.6
	pupal case and silk	8.3	trace	trace
	1st meconium		0.20	
	2nd meconium		0.09	
5	adult female[e]	51.6	0.3	0.6
6	adult male[e]	26.0	0.9	3.5
7	adult male[e]	18.0	0.02	0.1
8	adult male[e]	6.2	0.01	0.2

[a] Minus abdomen removed for dissection.
[b] Content of a 10-μl emission; no wt. obtained.
[c] Content of a 7.5-μl emission.
[d] Content of an 11-μl emission.
[e] Field collected.

females were not captured. Six larvae, collected in the field from *Maurandya* in September 1987, were available for study. No larvae were found in 1988 or 1989. Of the six larvae, two succumbed before data collection. Of the four remaining, two yielded larval data (Nos. 2 and 3, Table 3), while two were raised through to adulthood. Of the two adults, only part of one (No. 1, Table 3) was available for antirrinoside analysis. The abdomen of that one and the entire other (No. 4) were needed for taxon identification. Adults from earlier McFarland field collections however, were available for analysis (Nos. 5–7).

DISCUSSION

With this work, we have established *Maurandya antirrhiniflora* as another lepidopteran larval food plant that serves as a source for a sequestered iridoid glycoside. Antirrinoside has been identified as an additional sequesterable iridoid. Highly aposematic larvae of a geometrid moth (*Meris paradoxa*) and two noctuid moths (*Lepipolys* species) have been shown to contain large amounts of antirrinoside, while their cryptic adult stages contain at the most traces of the iridoid. This aposematic larva/cryptic adult correlation with the presence/absence of an iridoid glycoside is similar to those previously identified for the sphingid *Ceratomia catalpae* (Bowers and Puttick, 1986) and the two geometrids *Meris alticola* and *Neoterpes graefiaria* (Stermitz et al., 1988).

Leaves and petioles of *Maurandya* were found to contain remarkably high concentrations of antirrinoside, averaging 20% dry weight, while the highest concentrations observed in any insect life stages were around 10% in larvae. Total plant material and total iridoids consumed by larvae were not measured in any of the experiments, but rough estimates of food intake can be made from the weight of frass excreted and the weight of larvae or newly formed pupae. These estimates (along with the average plant iridoid content) suggest that individual larvae are consuming on the order of 30–80 mg of iridoid glycoside. The most found (sequestered, excreted, and emitted) is of the order of 5–10 mg per individual. This indicates that consumed iridoids are being metabolized. How much of this is due to the larvae themselves or to gut microflora is not known. It seems likely that iridoids continue to be metabolized during the pupal stage, but since none are taken in, the concentration drops markedly.

Catalpol was found previously in a reflex-bleeding secretion emitted by disturbed larvae of *N. graefiaria* (Stermitz et al., 1987). A similar secretion by *Meris paradoxa* was found in the present case, with about 0.7 mg of antirrinoside being emitted in a typical 10-μl drop. Whether the secretion represents hemolymph or that of a special organ is not known, but the high iridoid concentration should give the secretion a bitter taste. The secretion is usually a clear yellow–orange color, and a droplet is nearly identical in both size and

color to the series of lateral spots on the larvae. Although we have no direct evidence for the possible deterrence of the secretion, one might hypothesize that the larval spots are mimics of the secretion.

Larvae fed on *A. majus* (about 6% dry weight antirrinoside) appeared to contain slightly more iridoid than those fed on *Maurandya* (20% dry weight antirrinoside), but the number of larvae analyzed (three of each; Nos. 7–12, Table 2) was small and a statistical analysis was not warranted.

The larval stage and food plant of *Meris paradoxa* were not previously known nor was the larval stage for the undescribed species of *Lepipolys*. An official description of this taxon, whose adults are present in a number of collections, will be published elsewhere (R. Poole, personal communication). *L. perscripta* larvae were reported previously to utilize *Linaria canadensis* Dum. (Dyar, 1903) and *Antirrhinum nuttallianum* Benth. (Comstock, 1958). These belong to the tribe Antirrhineae, many of whose taxa contain antirrinoside (Sutton, 1988, pp. 54–56). It seems likely that other lepidopteran sequestrations of this iridoid glycoside will be discovered.

Acknowledgments—This work was supported by National Science Foundation Grant CHE-8521382 and a Colorado State University Fellowship to C.B. We thank Robert W. Poole and Frederick H. Rindge for moth identifications and Dr. Poole for valuable discussions.

REFERENCES

BOWERS, M.D. 1988. Chemistry and coevolution: Iridoid glycosides, plants, and herbivorous insects, pp. 133–166, *in* K. C. Spencer, (ed.). Chemical Mediation of Coevolution, Academic Press, San Diego.

BOWERS, M.D., and PUTTICK, G.M. 1986. The fate of ingested plant allelochemicals: Iridoid glycosides and lepidopteran herbivores. *J. Chem. Ecol.* 12:169–178.

COMSTOCK, J.A. 1958. The early stages of *Oncocnemis perscripta* (Guenee). *Bull. So. Calif. Acad. Sci.* 57:81–84.

DYAR, H.G. 1903. New North American Lepidoptera with notes on larvae. *Proc. Entomol. Soc. Washington* 5:290–298.

FRANKE, A., RIMPLER, H., and SCHNEIDER, D. 1987. Iridoid glycosides in the butterfly *Euphydryas cynthia* (Lepidoptera, Nymphalidae). *Phytochemistry* 26:103–106.

GARDNER, D.R., and STERMITZ, F.R. 1988. Host-plant utilization and iridoid glycoside sequestration by *Euphydryas anicia* (Lepidoptera: Nymphalidae). *J. Chem. Ecol.* 14:2147–2168.

L'EMPEREUR, K.M., and STERMITZ, F.R. 1990a. Iridoid glycoside content of *Euphydryas anicia* (Lepidoptera: Nymphalidae) and its major hostplant, *Besseya plantaginea* (Scrophulariaceae), at a high plains Colorado site. *J. Nat. Prod.* 16:187–197.

L'EMPEREUR, K.M., and STERMITZ, F.R. 1990b. Iridoid glycoside metabolism and sequestration by *Poladryas minuta* (Lepidoptera: Nymphalidae) feeding on *Penstemon virgatus* (Scrophulariaceae). *J. Chem. Ecol.* 16:1495–1506.

RINDGE, F.H. 1981. A revision of the moth genera *Meris* and *Nemeris* (Lepidoptera, Geometridae). *Am. Mus. Novit.* No. 2710.

STERMITZ, F.R., GARDNER, D.R., ODENDAAL, F.J., and EHRLICH, P.R. 1986. *Euphydryas anicia*

(Lepidoptera: Nymphalidae) utilization of iridoid glycosides from *Castilleja* and *Besseya* (Scrophulariaceae) hostplants. *J. Chem. Ecol.* 12:1459–1468.

STERMITZ, F.R., GARDNER, D.R., and MCFARLAND, N. 1988. Iridoid glycoside sequestration by two aposematic *Penstemon*-feeding geometrid larvae. *J. Chem. Ecol.* 14:435–441.

SUTTON, D.A. 1988. A Revision of the Tribe Antirrhineae. British Museum and Oxford University Press, London.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

PRODUCTION AND BIOLOGICAL ACTIVITY OF SAPONINS AND CANAVANINE IN ALFALFA SEEDLINGS

PIOTR M. GORSKI,[1] JÜRGEN MIERSCH,[2,*] and MICHAL PLOSZYNSKI[1]

[1]*Department of Biochemistry and Crop Quality*
Institute of Soil Science and Plant Cultivation
24-100 Pulawy, Poland

[2]*Section of Biosciences, Department of Biochemistry*
Martin-Luther-University
Weinbergweg 16a
0-4050 Halle (S.)
Germany

(Received October 23, 1990; accepted February 12, 1991)

Abstract—The saponin and canavanine concentrations and pattern were analyzed in growing alfalfa seedlings (*Medicago sativa* L.). Accumulation of saponins and canavanine was found to follow different time courses. During the first eight days, saponin concentration rose from zero in alfalfa seeds to 8.7% in roots and 1.8% in shoots on eighth day and then slowly decreased to 7.6% in roots and 0.8% in shoots present on the 24th day. Canavanine was found in seeds at a concentration of 1% then increased to 3.2% in seedlings on the sixth day and rapidly decreased to 0.2% per dry mass in roots and shoots on the 24th day. The effect of saponins—medicagenic acid sodium salt and medicagenic acid glycosides—on the growth of *Amaranthus* and *Lepidium* in Petri dishes and tomato (*Lycopersicon*) cell growth in tissue culture also was investigated. In contrast to medicagenic acid glycosides, a very strong inhibition of plant and cell growth was found as an effect of medicagenic acid.

Key Words—Saponins, medicagenic acid, medicagenic acid glycosides, canavanine, biological activity, alfalfa.

INTRODUCTION

Alfalfa contains some biologically active substances like saponins (Pfüller, 1976; Birk, 1969; Oleszek and Jurzysta, 1987), toxic amino acids (Bell, 1980),

*To whom correspondence should be addressed.

0098-0331/91/0600-1135$06.50/0

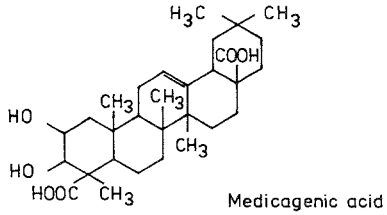
phenolics (Abdul-Rahman, 1989), and quaternary ammonia bases (Pfüller, 1976), some of which are recognized as allelochemicals (Wilson and Bell, 1978; Rosenthal, 1983; Oleszek and Jurzysta, 1987). Saponins were found to show inhibitory effects on plant germination (Jurzysta, 1970; Ream et al., 1977; Oleszek and Jurzysta, 1986; Read and Jensen, 1989) and fungal growth (Assa et al., 1975; Polacheck et al., 1986). These compounds also act as antifeedants to animals (Birk, 1969) and cause hemolysis of red blood cells (Gestetner et al., 1971). Saponins were found to be present in the soil, and their allelochemical function was shown. Alfalfa contains biologically active saponins of medicagenic acid which, among other effects, causes blood hemolysis and fungal growth inhibition (Oleszek and Jurzysta, 1987).

Another plant constituent, canavanine, also exhibits high biological activity affecting microorganisms, plants, animals, and especially insects. This toxic secondary plant product is related structurally to arginine and can be activated by arginine-tRNA synthetase yielding aberrant proteins in insects as well as in other animal organisms (Rosenthal, 1977; 1982). In plants canavanine acts as an allelochemical and reduces, e.g., the growth of radicles (Wilson and Bell, 1978), but the biochemical cause is still unknown. Chemical structures of both compounds are presented in Figure 1.

The present paper deals with changes of saponin and canavanine concentrations in growing alfalfa seedlings, and we attempt to determine whether these compounds are released into the environment. In addition the effect of pure saponins on germination of test plants and growth as well as the effect on tomato suspension culture growth was investigated.

METHODS AND MATERIALS

Plant Material. Seeds of alfalfa (*Medicago sativa* L. var. Verko), collected in 1987, were purchased from the Station of Plant Production, Langenstein, Germany. For cultivation of seedlings under sterile conditions the seeds were surface sterilized for 1 min in an aqueous solution of 0.1% of mercury chloride and washed six times with 5 ml of double-distilled water. Subsequently, the seeds were germinated for two days in darkness at 27–29°C and 80% humidity on paper disks in Petri dishes of 8 cm in diameter. After three to four days two seedlings were transferred to one plastic holder in centrifuge tubes of 50 ml containing 6 ml of Knop solution (per liter double-distilled water: 1 g $Ca(NO_3)_2$; 0.25 g $MgSO_4 \cdot H_2O$; 0.25 g KH_2PO_4; 0.25 g KNO_3; 50 mg $FeSO_4$, and 1 ml of trace element solution: 55 mg $Al_2(SO_4)_3$; 28 mg KJ; 28 mg KBr; 55 mg TiO_2; 28 mg $SnCl_2 \cdot H_2O$; 28 mg LiCl; 389 mg $MnCl_2 \cdot 4H_2O$; 614 mg $Ba(OH)_2$; 55 mg $ZnSO_4$; 59 mg $Co(NO_3)_2$; 55 mg $CuSO_4 \cdot 5H_2O$ per liter). Furthermore, the seedlings were cultivated for 24 days at 27–29°C and 80%



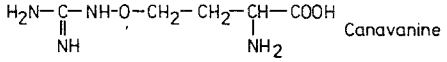


FIG. 1. Chemical structure of medigagenic acid and canavanine.

humidity with a 12 : 12hr light–dark period. After 10 days 2 ml of Knop solution was added to each tube. The seedlings were grown under sterile as well as nonsterile conditions in plastic pots of 7 × 9 cm with a sandy bed 2.5 cm high (mesh size 0.6–1.9 mm, at 20°C, in daylight) and 10 ml Knop solution.

Biotests. The influence of canavanine, medicagenic acid, and medicagenic acid glycosides on cell growth of tomato suspension culture (*Lycopersicon esculentum* Mill cv. Lukullus) was measured as described by Grancharov et al. (1985). The experiment was begun by inoculation of cells and the applications of a filter-sterilized solution of the compounds at concentrations of 10^{-3}–10^{-7} M. Inhibition of radicle growth of amaranth (*Amaranthus caudatus* L.) and cress (*Lepidium sativum* L.) was estimated during a period of seven days at 20°C and 6000 lux. The plant seeds were numbered on a paper disk in Petri dishes of 9 cm diameter (*Lepidium*) or 5 cm diameter (*Amaranthus*) saturated with 5 ml of water (control) or 5 ml water containing the compounds. After two to four days, the radicle length was measured and analyzed statistically by F, t, and χ^2 test.

Chemical Analysis. Canavanine sulfate was purchased from Serva (Heidelberg, Germany) and isolated according to Rosenthal (1973) and Natelson and Bratton (1984). Canavanine-containing fractions obtained from biological materials were purified by cation-exchange chromatography and calibrated by colorimetric estimation with pentacyanoammonium ferrate according to Natelson and Bratton (1984). Canavanine was analyzed by TLC of dansylated canavanine (Miersch et al., 1990). Medicagenic acid glycosides were extracted from alfalfa shoots with 80% ethanol. The ethanol was largely evaporated and the water phase extracted five times with *n*-butanol. The butanol was removed, and the crude saponins contained in residue were dissolved in water. Subsequently, saponins were precipitated with cholesterol and isolated after centri-

fugation. After decomposition of the saponin–cholesterol complex with hot pyridine, the saponins were precipitated from pyridine with ethyl ether. The precipitate contained a mixture of some medicagenic acid glycosides. Pure medicagenic acid was obtained by acidic hydrolysis of the glycosides. Because this compound is practically insoluble in water, the disodium salt of medicagenic acid was prepared (Gorski et al., 1984). Saponin concentrations were determined by *Trichoderma viride* biotest measured the inhibition of fungal growth (Jurzysta, 1979a) and hemolytic test (hemolysis of red blood cells) according to Jurzysta (1979b).

RESULTS AND DISCUSSION

Saponin and Canavanine Content and Pattern in Alfalfa Seedlings. In alfalfa seedlings, the accumulation of medicagenic acid and canavanine was found to follow different time courses. During the first eight days, the amount of medicagenic acid rose from zero in seeds to 2 mg per 10 seedlings of alfalfa, whereas canavanine increased from 0.2 to 0.4 mg. In older seedlings, the saponin reached 11 mg present on the 24th day. In this time range, the amount of the toxic amino acid canavanine decreased to 0.25 mg (Table 1). If we use the terms in relation to dry mass, the concentration of canavanine was about 1% in alfalfa seeds, while no biologically active saponins were present. During the time of seedling growth, the concentration of these two compounds changed rapidly (Figure 2). The most important finding is that the maxima of saponin and canavanine concentrations are different in different stages of seedling development.

TABLE 1. AMOUNT OF CANAVANINE AND MEDICAGENIC ACID DURING DEVELOPMENT OF ALFALFA SEEDLINGS[a]

Age (days)	Material	Dry mass* (mg ± SD)	Canavanine[b] (μg ± SD)	Medicagenic acid (mg)
0	seeds	18 ± 0.41	195 ± 14	0
1	seeds	18 ± 0.44	280 ± 17	0
2	seedlings	18 ± 0.39	300 ± 19	0.81 ± 0.16
3	seedlings	18 ± 0.35	310 ± 24	1.35 ± 0.20
5	seedlings	19 ± 0.40	400 ± 23	1.48 ± 0.18
8	seedlings	22 ± 0.32	400 ± 19	2.07 ± 0.23
16	seedlings	50 ± 0.63	340 ± 20	4.90 ± 0.59
24	seedlings	130 ± 1.06	250 ± 17	10.90 ± 1.12

[a] Data obtained are given per 10 seeds or seedlings.
[b] Calculated according to Miersch et al. (1988, 1990).

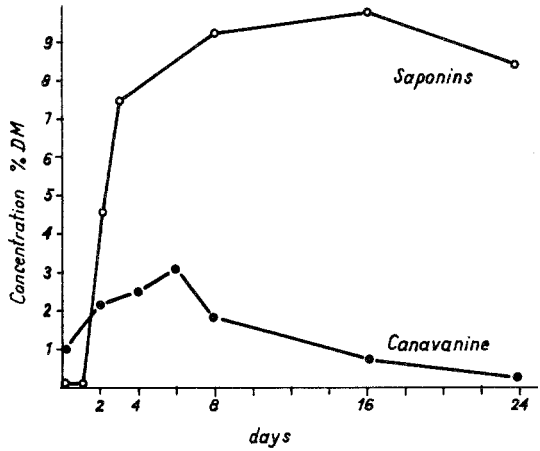


FIG. 2. Concentration of saponin and canavanine in alfalfa seedlings.

Concentrations of saponins and canavanine in alfalfa seedlings were analyzed under both sterile and nonsterile conditions. With respect to chemical composition, no differences were found in the two samples.

During the experiment we also analyzed the release of saponin and canavanine from seedling to culture medium and paper disks. In the nutrient solution of 8-, 16-, and 24-day-old healthy seedlings, no saponin was found. Hemolysis tests with seedlings also proved that saponins did not penetrate from roots as long as they were intact. In contrast, saponin efflux was detected from cut and dead sections of both roots and shoots. As shown previously, canavanine penetrates from alfalfa roots into the nutrient solution; however, when the seedlings grew in water, no canavanine was found (Miersch et al., 1988).

Effect of saponins on growth of radicle of Lepidium *and* Amaranthus. Medicagenic acid sodium salt has a very strong effect on the growth of *Lepidium* and *Amaranthus*. The results obtained are shown in Table 2. *Lepidium* proved to be more sensitive than *Amaranthus*, especially at higher concentrations. Saponins have a similar influence on the growth of cereal, cotton, and some other plants (Jurzysta, 1970) and animal cell cultures (Slotwinska, 1983).

Growth inhibition on *Lepidium* and *Amaranthus* caused by pure canavanine sulfate is remarkable: 21% and 13%, respectively, were found at concentrations of 10 μM. Statistical analysis of these data showed high significance: *Lepidium* $\chi^2 = 17$ (table = 15, 0.05%) and *Amaranthus* $\chi^2 = 47$ (table = 28, 0.01%). The sulfate ion in canavanine sulfate has no effect in the used concentration of 10 μM as was controlled by potassium sulfate. In contrast to the findings with seedlings of amaranth and cress, the growth inhibition of tomato cell suspen-

TABLE 2. EFFECT OF MEDICAGENIC ACID SALT ON RADICLE GROWTH OF *Lepidium* AND *Amaranthus*

Plant	Concentration (M)	Length of radicle ± SD (mm)	Inhibition of growth (%)
Lepidium sativum 3 days	0	21.24 ± 7.81	0
	5×10^{-6}	20.95 ± 6.05	0
	10^{-5}	24.88 ± 5.21	−17[a]
	2.5×10^{-4}	9.50 ± 7.30	55
	5×10^{-4}	4.51 ± 1.79	78
	10^{-3}	3.24 ± 1.22	85
	2.5×10^{-3}	2.43 ± 0.73	88
Amaranthus caudatus 2 days	0	24.98 ± 6.85	0
	5×10^{-6}	21.71 ± 7.04	13
	10^{-5}	20.43 ± 5.12	18
	2.5×10^{-4}	22.10 ± 5.17	11
	5×10^{-4}	21.18 ± 5.04	13
	10^{-3}	16.09 ± 4.38	35
	2.5×10^{-3}	12.08 ± 4.23	53

[a] = 17% increase.

sion, measured to be 35% in four days, was more sensitive (Miersch et al., 1988).

In experiments with alfalfa seedlings grown together with other plants (e.g., *Brassica oleracea*), we also found growth inhibition (Miersch et al., 1988). The release of canavanine from roots of alfalfa was observed and a concentration of 3–57 μM was calculated under experimental conditions (Miersch et al., 1990). Because canavanine was found in the solution, sand, or on the paper disks (Miersch et al., 1990), these inhibitory effects seem to be caused by canavanine or some other substances rather than by saponins.

Effects of Saponins on Tomato Tissue Culture. In the tissue culture experiment, we used aqueous solutions of medicagenic acid as its sodium salt (because of better water solubility) and medicagenic acid glycosides isolated from alfalfa. In contrast to medicagenic acid, its sodium salt and glycosides are highly water soluble.

As shown in Table 3 and Figure 3, a very strong inhibitory effect on tomato cell growth was found. At 10^{-5} M, all cells were lysed on fourth day of the experiment. In contrast, for unknown reasons, medicagenic acid glycosides caused only low inhibition (Table 4).

During seedling development (see Figure 2 and Table 1), different amounts

TABLE 3. EFFECT OF MEDICAGENIC ACID SALT ON GROWTH OF TOMATO CELLS

Concentration (M)	Growth (dry mass ± SD in mg/ml)			
	1 day	2 day	3 day	4 day
0	1.67 ± 0.09	2.24 ± 0.18	3.71 ± 0.55	4.78 ± 0.59
10^{-7}	1.31 ± 0.47	1.50 ± 0.66	2.16 ± 0.78	2.60 ± 0.69
5×10^{-7}	0.89 ± 0.03	0.75 ± 0.06	0.75 ± 0.16	0.73 ± 0.05
10^{-6}	0.91 ± 0.19	0.73 ± 0.05	0.81 ± 0.07	0.83 ± 0.13
10^{-5}	1.13 ± 0.06	1.06 ± 0.06	1.10 ± 0.10	n.d.[a]

[a] Not detected—lysis of cells.

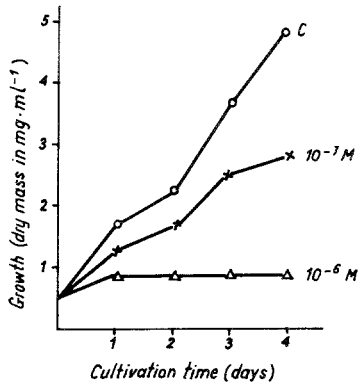


FIG. 3. The effect of medigagenic acid sodium salt on the growth of tomato suspension culture.

TABLE 4. EFFECT OF MEDICAGENIC ACID GLYCOSIDES ON TOMATO CELL GROWTH

Concentration (M)	Growth (dry mass ± SD in mg/ml)			
	1 day	2 day	3 day	4 day
0	1.25 ± 0.33	2.34 ± 0.30	3.26 ± 0.79	4.99 ± 1.35
10^{-8}	1.50 ± 0.14	2.45 ± 0.07	2.90 ± 0.14	4.60 ± 0.14
10^{-7}	1.43 ± 0.06	2.50 ± 0.10	3.20 ± 0.10	4.03 ± 0.15
10^{-6}	1.29 ± 0.26	2.16 ± 0.37	3.27 ± 0.97	4.66 ± 1.52
5×10^{-6}	1.25 ± 0.26	2.46 ± 0.74	3.31 ± 1.04	4.82 ± 1.70
10^{-5}	1.20 ± 0.25	2.21 ± 0.32	3.13 ± 0.96	4.70 ± 1.65

of saponins and canavanine were observed. In addition, penetration of both substances into the root space is different. Therefore, we assume that under our experimental conditions: (1) There is no release of saponins from intact alfalfa seeds and seedlings to the environment, and no allochemical influence of saponins on other plants surrounding the alfalfa seedlings may be expected. Oleszek and Jurzysta (1987) found medicagenic acid to be present and active in soil after cultivation of alfalfa; however, these saponins were released into the soil from decomposed roots and stems after harvesting. (2) Further experiments are needed to clarify the release of small amounts of other phytotoxic and nontoxic compounds present in the green parts of alfalfa (Pfüller, 1976; Read and Jensen, 1989). (3) Furthermore, the question arises whether there is any synergistic effect of canavanine and saponins with other compounds, as was discussed for canavanine and amino acids during the growth of *Lemna* by Rosenthal et al. (1975).

Acknowledgments—The authors wish to thank Dr. M. Jurzysta of Institute of Soil Science and Plant Cultivation for the donation of the sample of medicagenic acid glycosides and Mrs. M. Dübler for her skillful assistance. We are grateful to Prof. Dr. W. Francke of Institute of Organic Chemistry, University Hamburg, for kindly reading the manuscript.

REFERENCES

ABDUL-RAHMAN, A.A. 1989. Allelopathic effect of alfalfa (*Medicago sativa*) on bladygrass (*Imperata cylindrica*) *J. Chem. Ecol.* 15:2289–2301.

ASSA, Y., CHET, J., GESTETNER, B., GOVRIN, R., BIRK, Y., and BONDI, A. 1975. The effect of alfalfa saponins on growth and lysis of *Physarum polycephalum. Arch. Microbiol.* 103:77–80.

BELL, E.A. 1980. The non-protein amino acids of higher plants. *Endeavour N.S.* 4:102–107.

BIRK, Y. 1969. Saponins, pp. 169–210, *in* I.E. Liener (ed.). Toxic Constituents of Plant Foodstuffs. Academic Press New York.

GESTETNER, B., ASSA, Y., HENIS, Y., BIRK, Y., and BONDI, A. 1971. Lucerne saponins. IV. Relationship between their chemical constitution and haemolytic and antifungal activity. *J. Sci. Food. Agric.* 22:168–172.

GORSKI, P.M., JURZYSTA, M., BURDA, S., OLESZEK, W., and PLOSZYNSKI, M. 1984. Studies on *Medicago lupulina* saponins. 2. Isolation, chemical characterization and biologically activity of saponins from *M. lupulina* tops. *Acta Soc. Bot. Pol.* 53:527–533.

GRANCHAROV, K., KRAUSS, G.-J., SPASSOVSKA, N., MIERSCH, J., MANEVA, L., MLADENOVA, L., and GOLOVINSKY, E. 1985. Inhibitory effect of pyruvic acid semi- and thiosemicarbazone on the germination of bacteria, yeasts, experimental tumors and plant cells. *Pharmazie* 40:574–575.

JURZYSTA, M. 1970. Effect of saponins isolated from seeds of lucerne on germination and growth of cereal seedlings. *Zesz. Nauk UMK Torun* 13:253–256.

JURZYSTA, M. 1979a. Simple method of quantification of biologically active alfalfa saponins by *Trichoderma viride* growth. *Biul. Branz. Hod. Roslin* 1:16–18.

JURZYSTA, M. 1979b. Haemolytic micromethod for rapid estimation of toxic alfalfa saponins. *Acta Agrob.* 32:5–11.

MIERSCH, J., JÜHLKE, C., and SCHLEE, D. 1988. Canavaninmetabolismus während der Sämlingsentwicklung von Luzerne (*Medicago sativa* L. cv. Verko). *Wiss. Beitr. Martin-Luther-Univ. Halle-Wittenberg* 33(S 65):62–71.

MIERSCH, J., JÜHLKE, C., KABAT VEL JOB, C., and SCHLEE, D. 1990. Canavanine as an allelochemical in the seedlings of alfalfa. Proc. 14th Intern. Congr. of Biochemistry, Prague, 1988. In press.

NATELSON, S., and BRATTON, G.R. 1984. Canavanine assay of some alfalfa varieties (*Medicago sativa*) by fluorescence: Practical procedure for canavanine preparation. *Microchem. J.* 29:26–43.

OLESZEK, W., and JURZYSTA, M. 1986. Isolation, chemical characterization and biologically activity of alfalfa (*Medicago media* Pers.) root saponins. *Acta Soc. Bot. Pol.* 55:23–33.

OLESZEK, W., and JURZYSTA, M. 1987. The allelopathic potential of alfalfa root medicagenic acid glycosides and their fate in soil environments. *Plant Soil* 98:67–80.

PFÜLLER, K. 1976. Isolierung, Analytik und Biochemie toxischer Inhaltsstoffe der Luzerne (*Medicago sativa* L.). Dissertation A, Math.-Nat.-Fak. University of Leipzig.

POLACHECK, J., ZEHAVI, U., NAIM, M., LEVY, M., and EVRON, R. 1986. Activity of compound G2 isolated from alfalfa roots against medically important yeasts. *Antimicrob. Agents Ther.* 30:290–294.

READ, J.J., and JENSEN, E.H. 1989. Phytotoxicity of water-soluble substances from alfalfa and barley soil extracts on four crop species. *J. Chem. Ecol.* 15:619–628.

REAM, H.W., SMITH, D., and WALGENBACH, R.P. 1977. Effect of deproteinized alfalfa juice applied to alfalfa-bromograss, bromegrass and corn. *Agron. J.* 69:685–689.

ROSENTHAL, G.A. 1973. The preparation and colorimetric analysis of L-canaline. *Anal. Biochem.* 51:354–361.

ROSENTHAL, G.A. 1977. The biological effects and mode of action of L-canavanine a structural analogue of L-arginine. *Rev. Biol.* 52:155–178.

ROSENTHAL, G.A. 1982. Plant Non-protein Amino- and Imino Acids. Biological, Biochemical, and Toxicological Properties. Academic Press New York, 273 pp.

ROSENTHAL, G.A. 1983. A seed-eating beetle's adaptations to a poisonous seed. *Sci. Am.* 249:138–345.

ROSENTHAL, G.A., GULATI, D.K., and SABHARWAL, P.S. 1975. Studies on the growth effects of the canaline urea cycle amino acids with *Lemna minor* L. *Plant Physiol.* 56:420–424.

SLOTWINSKA, M. 1983. The effect of alfalfa saponins on L cells cultured in vitro. *Ann. Univ. Maria Curie-Slodowska Sect. C Biol.* 38:177–182.

WILSON, M.F., and BELL, E.A. 1978. The determination of the changes in the free amino acid content of the eluate from germinating seeds of *Glycine wightii* (L.) and its effect on the growth of lettuce fruits. *J. Exp. Bot.* 29:1243–1247.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

SEASONAL VARIABILITY IN RESPONSE OF *Ips pini* (COLEOPTERA: SCOLYTIDAE) TO IPSDIENOL IN NEW YORK

STEPHEN A. TEALE* and GERALD N. LANIER[1]

Department of Environmental and Forest Biology
College of Environmental Science and Forestry
State University of New York
Syracuse, New York 13210

(Received October 29, 1990; accepted February 12, 1991)

Abstract—In May, *Ips pini* in New York did not respond in the field to 50–98.5% (*R*)-(−)-ipsdienol (synthetic). In September, beetles responded strongly to 50–60% (*R*)-(−)-ipsdienol (synthetic). In May and June, New York beetles showed marked preference for their own males over Arizona males, which produce an average of 94.1% (*R*)-(−)-ipsdienol. This suggested that ipsdienol stereochemistry alone does not ensure activity and that an additional compound is necessary for attraction in May. In the second year of field tests, attraction to synthetic ipsdienol and male beetles was tested in the spring, summer, and fall. There was response only to males in the spring and mid-summer and to both males and synthetic ipsdienol in the late summer and fall, causing a significant treatment × sampling period (date) interaction. Laboratory-reared beetles were not significantly more attracted to ipsdienol than to a blank airstream in laboratory assays, while male volatiles were significantly more attractive than ipsdienol and the blank. These data demonstrate that there is one or more unknown semiochemicals necessary for pheromonal response and that the behavioral activity of synthetic ipsdienol varies seasonally.

Key Words—Insecta, Coleoptera, Scolytidae, *Ips pini*, pheromone, ipsdienol, seasonal variation, bioassay.

INTRODUCTION

Several bark beetle species undergo seasonal changes in their pheromone systems. Female *Dendroctonus brevicomis* LeConte were totally unresponsive to

*To whom correspondence should be addressed.
[1]Deceased.

0098-0331/91/0600-1145$06.50/0

their pheromones from October to January in laboratory bioassays (Silverstein et al., 1968). Laboratory studies also found that *Ips paraconfusus* Lanier were least responsive to their pheromones during January and February (Wood and Bushing 1963; Borden 1967). Birch (1974) found that in *Ips pini* (Say) the intensity of both response in laboratory bioassays and pheromone attractiveness decreased in winter. Hagen and Atkins (1975) reported that overwintered *I. paraconfusus* contained significantly more fat and were less responsive to attractants than the spring generation. Likewise, *D. pseudotsugae* with high fat content tend to be unresponsive to attractants, and those with low fat content tend to be more responsive to attractants (Atkins 1966). Hagen and Atkins (1975) predicted that the overwintered generation of *I. paraconfusus* would be less amenable to semiochemically based management compared to the spring generation due to their lower responsiveness to attractants.

The terpene alcohol ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) was reported as the male-produced "aggregation" pheromone of the pine and spruce infesting bark beetle *I. pini* (Lanier et al., 1980; Birch et al., 1980). More recently, we observed lack of responsiveness of *I. pini* to synthetic ipsdienol in the field in the early summer. Previous field tests in which synthetic ipsdienol was attractive to *I. pini* in New York were done in August and September (Lanier et al., 1980). At least two changes occur in the biology of *I. pini* between spring and late summer. First, there is typically an increase in population density in late summer as the spring and early summer broods emerge (Schenk and Benjamin 1969). Second, in the late summer, *I. pini* frequently engage in high-density feeding attacks in the western states (Sartwell et al., 1971) and in New York (authors' personal observation). The changes in population density and colonization behavior may be related, and both may be related to an intergenerational difference in fat content such as that reported by Hagen and Atkins (1975). It appeared to us that there may be seasonal changes in the pheromone system of *I. pini* associated with seasonal changes in biology. We report a series of experiments that measured the response of *I. pini* to synthetic ipsdienol and natural attractants in the field in the spring, summer, and fall, and laboratory bioassays comparing response to synthetic ipsdienol and natural attractants.

METHODS AND MATERIALS

Field Sites. In 1987, experiments were conducted in a mixed stand of red (*Pinus resinosa* Ait.) and Scots (*P. sylvestris* L.) pine in the southwestern Adirondack Mountains, near Forestport, New York. Thinning in parts of the stand in late 1986 and throughout 1987, and previous logging in adjacent stands, provided abundant host material in which a large population of *I. pini* devel-

oped. In 1988, an experiment was conducted in a mixed red and Scots pine stand 25 km south of Syracuse, New York, which had been thinned the previous summer and fall. A large amount of slash was available for colonization by *Ips*.

Field Test of Synthetic Ipsdienol Enantiomers in Two Seasons. This experiment was designed to determine if the specificity of response to ipsdienol enantiomeric blends varied with season. The enantiomeric compositions were 98.5%, 90%, 80%, 70%, 60%, and 50% (*R*)-(−)-ipsdienol (Phero Tech Inc., Delta, British Columbia) and a solvent (ethanol) control. Each dispenser contained 10 mg ipsdienol in ethanol, except for an additional treatment of 10 mg 50% (−)-ipsdienol in pentane. The optical purity of the ipsdienol enantiomers was 98.5% (*R*)-(−) and 98% (*S*)-(+). The dispensers were 3.7-ml polyethylene vials (Olympic Plastics), which released 35 μg/day. The release rate was determined by aerating the release device ($N = 3$), collecting volatiles on Porapak Q over seven days at 22°C, and quantifying on capillary GC. Sixteen eight-unit multiple-funnel traps (Lindgren, 1983) (Phero Tech Inc.), were spaced 10 m apart in two rows. Each row received one of each treatment in randomized order. The experiment started on May 26, 1987, and insects were removed from the traps on May 29. The treatment positions were rerandomized and new attractants with the same type of dispenser and release rate were placed in the traps on September 3, 1987. Insects were removed from the traps and treatment positions were rerandomized on September 6, 8, 11, 14, and 17. Treatments of 25% and 40% (−)-ipsdienol were used in May and September, respectively, to determine if there was greater response below 50%, but were not included in the analysis.

Field Test of Beetle-Produced Ipsdienol Enantiomers. This experiment compared the specificity of response to natural ipsdienol enantiomers with that to synthetic enantiomers. If the specificity of response was different for synthetic ipsdienol and natural material with the same range of enantiomers, then compounds other than ipsdienol must play a role in specificity. A range of enantiomers could be obtained by using various proportions of males from eastern and western populations of *I. pini* (Stewart, 1975; Birch et al., 1980; Miller et al., 1989). The treatments were the following numbers of emergent New York and Arizona male *I. pini* introduced into 15 × 30-cm fresh red pine logs from Tully, New York (NY–AZ): 20–0, 15–5, 10–10, 5–15, and 0–20 (20 males/log/trap). The Arizona beetles were taken from a laboratory colony originating in *Pinus ponderosa* Laws. in Young, Arizona, in August 1986 and reared on red pine in the laboratory. Males from the Arizona laboratory colony produce an average of 94.1% (−)-ipsdienol based on aerations of individual males on red pine, collection of volatiles on Porapak Q (Byrne et al., 1975), and enantiomeric determination (Slessor et al., 1985). The New York beetles were collected from red pine near Tully, New York. Beetles were introduced into the phloem through 5-mm-diam. holes and covered with a patch of aluminum win-

dow screen. The logs were enclosed in fiberglass mesh bags to prevent feral attacks. An uninfested log served as a control. The logs were nailed to plywood covers (40 × 40 cm) and suspended in hardware cloth cylinders (30 cm diam. × 40 cm high) coated with insect trapping adhesive (Tangle-trap, Tanglefoot Company, Grand Rapids, Michigan), which were placed on the ground 10 m apart on plywood bases. The experiment was arranged in a 6 × 6 Latin square design with two sampling periods: May 8–18 and May 18–June 9, 1987. The lengths of the sampling periods were determined by periodically inspecting the numbers of trapped beetles to ensure that the catches from the two sampling periods were approximately equal. This compensated for the effects of changes in the numbers of flying beetles due to variable weather and phenology. The traps and baits were rotated among positions between sampling periods. At the end of the experiment, the logs were peeled to determine the actual numbers of actively boring males.

Field Test of Trap Type and Source of Volatiles. Five treatments were tested to determine if volatile and trap types were significant factors. Male and host volatiles were collected by introducing 150 male New York beetles from a laboratory colony into a red pine log (8 cm diam. × 50 cm) in a glass cylinder (10 cm diam. × 55 cm) and collecting the volatiles on Porapak Q for 72 hr. The Porapak Q was extracted with pentane and the solution concentrated to contain 10 μg ipsdienol/μl, as determined by capillary gas chromatography. The extract contained male-produced volatiles and host odors, which volatilize from both the pine log and beetle frass. The treatments were: (1) 1 mg synthetic racemic ipsdienol, (2) 10 mg synthetic racemic ipsdienol, (3) 1 mg natural ipsdienol (Porapak Q extract), (4) a red pine log with 20 New York male *I. pini* hung adjacent to funnel traps, and (5) a red pine log baited with 20 New York male *I. pini* in a sticky trap. Treatments 1–3 were in ethanol (total volume 100 μl each), were eluted from polyethylene vials (3.7 ml, 1-mm hole in cap), and were suspended in hardware cloth sticky traps. Fifteen traps were placed 10 m apart in three rows. Each row received a complete set of treatments in randomized order. The experiment started on August 12, and insects were taken from the traps and trap positions were rotated on August 25 and 31 and September 6, 1987.

Field Test of Synthetic, Racemic Ipsdienol across Seasons. The response of *I. pini* to synthetic ipsdienol was also measured in the spring, summer, and fall of 1988. The treatments were (1) 1 mg, (2) 10 mg synthetic racemic ipsdienol in ethanol, (3) 20 male New York beetles boring in a red pine log, and (4) ethanol. Synthetic stimuli were placed in polyethylene vials (3.7 ml) and were hung on eight-unit funnel traps. Twelve traps were placed 30 m apart in three rows. Each received a complete set of treatments in randomized order. All stimuli were replaced and their positions were rerandomized on May 6, July 22, and August 22. The male beetle treatments were also replaced on September

14. Insects were removed from the traps and treatment positions were rerandomized on May 10, 13, and 17; July 27; August 8, 12, and 31; September 9, 16, and 28; and October 17.

When trap catches were large (over 500), the numbers of beetles and sex ratios were estimated by sexing and counting a subsample and counting the remainder, or sexing, counting, and weighing a subsample and weighing the remainder. This method was accurate within 6.8% based on weighing and counting a subset of the samples.

Laboratory Bioassay of Synthetic, Racemic Ipsdienol. This experiment tested synthetic ipsdienol in laboratory bioassays using a modification of the Wood and Bushing (1963) olfactometer. Odor sources were placed in 14.6-cm disposable Pasteur pipets placed 30° apart, 10 cm from the point of convergence of the airstreams. The odors were removed by a vacuum at the downwind end of the olfactometer. Stimulants were deposited on filter paper strips (4 × 30 mm) placed within a pipet in position 2 and tested singly. The airflow through each pipet was 120 ml/min. Laboratory reared beetles were allowed to emerge from their brood host and were tested within 24 hr. The laboratory colony originated in Tully, New York, in 1973 and had been maintained on red pine and kept at approximately 21°C in constant darkness. Periodically, field collected beetles were added to the colony to prevent inbreeding. Bioassays were conducted in December 1987. Previous bioassays with beetles from this laboratory colony (unpublished data) indicated that beetle response specificity was uniform throughout the year. This is not surprising because *I. pini* is multivoltine, does not exhibit obligate diapause, and shows no signs of seasonal activity in our laboratory colony. Only females were used due to their higher level of response. Beetles were tested in groups of 10 at 21°C under red light to minimize spontaneous flight. Each group of 10 beetles was tested against all three stimuli at each concentration. Seventy beetles were tested at each stimulus concentration. Beetles were kept on the arena for up to 5 min, or until they responded by clear orientation upwind to the point of contacting the odor-bearing pipet. The paper substrate of the arena was changed between assays.

To determine if ipsdienol alone was attractive, we tested four concentrations (10^{-3} μg, 10^{-2} μg, 10^{-1} μg, and 1 μg) of synthetic racemic ipsdienol, male and host volatiles (see above: Field Test of Trap Type and Source of Volatiles) and a blank airstream. Dilutions of male and host volatiles were determined based on the quantity of ipsdienol in the extract, as determined by capillary gas chromatography. The enantiomeric composition of natural (from male volatiles) and synthetic ipsdienol was determined (Slessor et al., 1985).

Statistics. Data from the first, second, and fourth experiments were log(rank) transformed (Conover and Iman, 1981) and analyzed by multifactor ANOVA. This allowed us to partition the variance due to temporal replication and to test for interaction effects. The data from the third experiment were log

$(Y + 1)$ transformed and analyzed by one-way ANOVA. Sex ratios of trap catches with over 50 individuals were compared as population proportions (Mendenhall, 1983). The bioassay data were log $(Y + 1)$ transformed and analyzed by one-way ANOVA.

RESULTS

Field Test of Synthetic Ipsdienol Enantiomers in Two Seasons. Significantly ($P < 0.001$) more beetles were trapped in September (4.6 ± 1.6 beetles/trap/sampling period) than in May (0.0 beetles/trap/sampling period). There were no significant ($P > 0.05$) effects of replication over time in September, nor was there a significant difference between the mean trap catch of the 50% (−)-ipsdienol treatment in ethanol (14.7 ± 7.8) and in pentane (8.6 ± 3.1) ($P > 0.05$). The effect of ipsdienol enantiomeric composition was highly significant ($P << 0.001$). Only the 50% and 60% treatments attracted significantly more beetles than the controls (Figure 1). Because the same range of enantiomers [50–98.5% (−)-ipsdienol] was covered in the spring and late summer, these data suggest that the lack of attraction in the spring was not due to the enantiomeric composition of ipsdienol. Additional monitoring with ipsdienol enantiomers in May, June, and July using release rates of 26–74 μg/day also found no attraction to synthetic ipsdienol.

Field Test of Beetle-Produced Ipsdienol Enantiomers. In the field test of

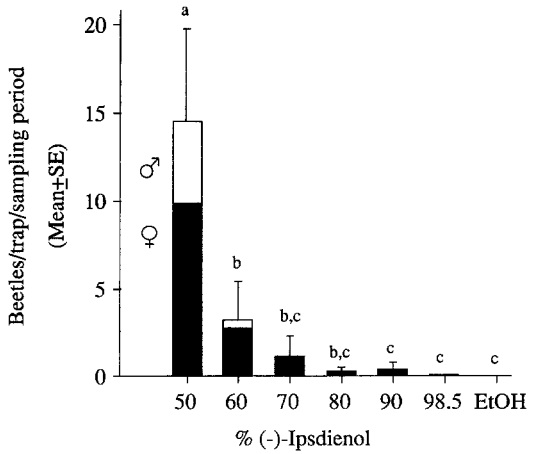


FIG. 1. Mean number of *I. pini* trapped (±SE) at different proportions of ipsdienol enantiomers near Forestport, New York, September 3–9, 1987. Different letters indicate significant differences between means ($P < 0.05$; LSD test).

actively boring New York and Arizona male beetles during May and June 1987, treatments with at least half New York beetles attracted significantly more beetles than all other treatments (Figure 2). Traps with all Arizona beetles did not attract significantly more than the uninfested log, while the 5:15 New York–Arizona baits attracted significantly more than the uninfested log.

Examination of the bait logs after termination of the experiment revealed that 70% of the New York beetles and 83% of the Arizona beetles had constructed nuptial chambers. Most of the males that did not construct nuptial chambers were dead. The proportions of ipsdienol enantiomers may have been slightly different from those expected based on the proportions of beetles introduced but were consistent across treatments. The actual enantiomeric composition of ipsdienol produced was not directly measured, but we estimate that the 15–5, 10–10, 5–15 (New York–Arizona) treatments produced disproportionately more (−)-ipsdienol due to the higher mortality of New York beetles and the greater production by western populations compared to the New York population (Miller et al., 1989).

This experiment established the presence of flying, responsive beetles in the area during the spring portion of the synthetic enantiomer experiment reported above. Furthermore, the enantiomeric composition of natural ipsdienol affected catches, but stereochemistry was not the basis for lack of attraction to synthetic ipsdienol in May in the experiment with synthetic enantiomers.

Field Test of Trap Type and Source of Volatiles. The 10-mg ipsdienol treatment attracted significantly ($P < 0.05$) more beetles than all other treat-

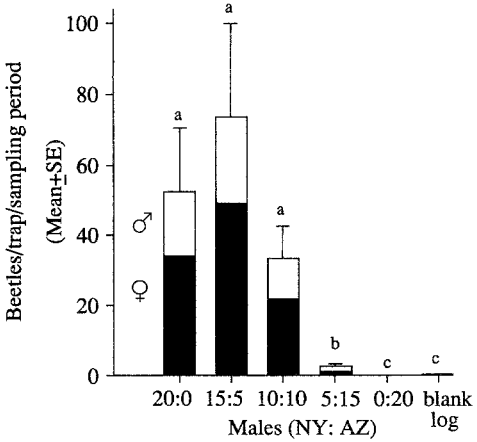


FIG. 2. Mean number of *I. pini* trapped (±SE) at red pine logs with ratios of New York and Arizona male *I. pini* near Forestport, New York, May 8–June 9, 1987. Different letters indicate significant differences between means ($P < 0.05$; confidence intervals).

ments except the funnel trap with 20 males (Figure 3). The mean of the sticky trap/20 males treatment was inflated by a single replicate and was slightly higher than that of the funnel trap/20 males treatment, yet it was significantly less attractive than the 10-mg ipsdienol treatment. This experiment excludes both trap design and release rate as explanations for the disparity of results between the first two experiments.

Field Test of Synthetic, Racemic Ipsdienol across Seasons. From May 6 through August 8, the 10-mg ipsdienol treatment caught 4% of the total, and from August 9 through October 17 it caught 31% of the total (Figure 4), causing a significant treatment × sampling period interaction ($P = 0.002$). The 1-mg ipsdienol treatment was never significantly more attractive than the control ($P > 0.05$) and the 20 male beetle treatment was always more attractive than the control ($P < 0.01$). The mean trap catches were two to three orders of magnitude higher from August 8 through October 17 than from May 6 through August 8 (Table 1). Although the means of the 1.0 mg, 10 mg, and male beetle treatments from August 8 to October 17 varied considerably, there were no significant differences among them due to large variances. We monitored attraction to ipsdienol with a lower dose (0.1 mg) in May and a higher dose (100 mg) from July through October. Both doses attracted fewer beetles than the 1.0 mg and 10 mg treatments.

In the field experiments, the mean sex ratios for treatments ranged from

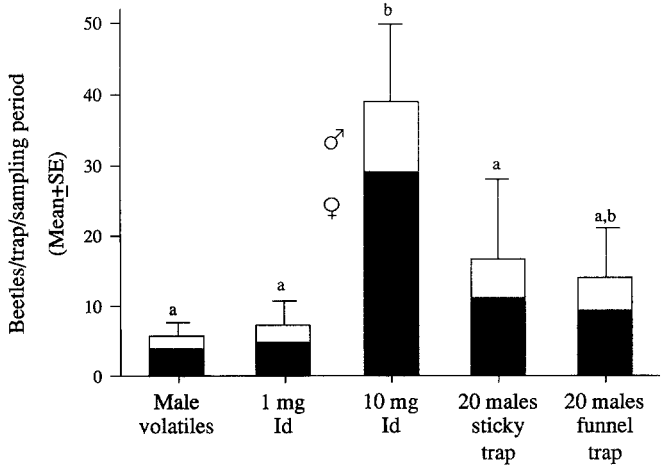


FIG. 3. Mean number of *I. pini* trapped (±SE) at male *I. pini* (New York) frass volatiles collected on Porapak, synthetic racemic ipsdienol, and male *I. pini*, near Forestport, New York, August 25–September 6, 1987. Except where noted, all treatments utilized sticky traps. Different letters indicate significant differences between means ($P < 0.05$; LSD test). Id = ipsdienol.

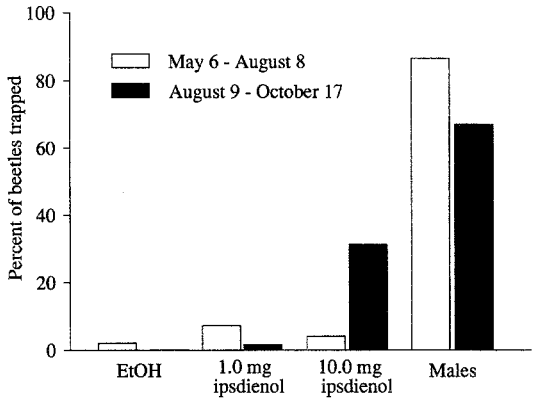


FIG. 4. Percent of *I. pini* trapped within each of two time periods, near Syracuse, New York, May 6–October 17, 1988. Attractants were synthetic ipsdienol, logs with 20 male *I. pini* from New York, and an ethanol control. Different letters indicate significant differences between means ($P < 0.01$; confidence intervals).

TABLE 1. SEASONAL VARIATION IN RESPONSE BY *Ips pini* TO SYNTHETIC RACEMIC IPSDIENOL NEAR SYRACUSE, NEW YORK, MAY 6–OCTOBER 17, 1988

Time period	Treatment	*N*	Mean ± SE[a]	Males (%)
May 6–August 8	Ethanol	18	1.0 ± 0.5a	50
	1 mg ipsdienol	18	3.4 ± 2.3a	52
	10 mg ipsdienol	18	1.9 ± 0.7a	34
	Males	18	40.9 ± 9.3b	39
August 9–October 17	Ethanol	18	2.3 ± 2.3a	39
	1 mg ipsdienol	18	73.1 ± 32.6ab	37
	10 mg ipsdienol	18	1373.1 ± 774.5b	33
	Males	18	2945.7 ± 1565.8b	39

[a] Beetles/trap/sampling period; means within a column for each time period followed by the same letter are not significantly different ($P < 0.01$, Tukey's multiple mean comparison test).

25.5% to 41.8% males. There were no significant differences among sex ratios within any of the experiments ($P > 0.05$).

Laboratory Bioassay of Synthetic, Racemic Ipsdienol. In the laboratory bioassays, ipsdienol was not significantly more attractive than the blank airstreams at any concentration tested (Figure 5). The male volatiles were significantly more attractive than both the blank airstream and synthetic ipsdienol at the three higher concentrations. The enantiomeric composition of ipsdienol from male beetles and the synthetic formulation was 1:1 (±1%), which excludes

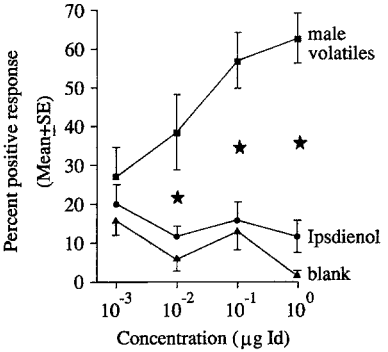


FIG. 5. Percent of female *I. pini* responding positively to male *I. pini* frass volatiles, synthetic ipsdienol, and blank airstreams in the laboratory olfactometer. Stars indicate significant differences between means ($P < 0.05$; LSD test).

stereochemistry as an explanation for the lack of attraction to synthetic ipsdienol.

DISCUSSION

These data demonstrate that one or more compounds in addition to ipsdienol are necessary for behavioral response by *I. pini* in New York and that the role of this compound varies seasonally. The origin of this semiochemical may be the host (a kairomone) or the insect (a pheromone). (*E*)-Myrcenol is produced by *I. pini* in British Columbia (Greis et al., 1988); when placed on fresh logs it increases the number of attacks, but because it is inhibitory when combined with ipsdienol (Miller et al., 1990), it is probably not the compound that enhances attraction to ipsdienol in New York. Other reports that found attraction to ipsdienol alone in the field were conducted in the late summer or fall. Lanier et al. (1980) tested synthetic ipsdienol in August and September in New York. Birch et al. (1980) began their tests in late July in California, and Miller et al. (1990) conducted experiments in July and August. Field tests of western populations in the spring are needed to determine if compounds in addition to ipsdienol are required for response.

In our laboratory bioassays, response by female beetles was similar to the response of beetles in the field in the spring, i.e., ipsdienol was not significantly more attractive than blank controls. However, Lanier et al. (1980) reported that ipsdienol was attractive in laboratory bioassays. Lanier et al. (1980) used ipsdienol extracted and isolated from beetles, which could have contained small amounts of impurities that enhanced attraction to ipsdienol. Positive response to synthetic ipsdienol in the lab bioassays of Lanier et al. (1980) is more difficult

to reconcile. It is possible that their scoring method (Lanier et al., 1977) produced false positives, that their release rates could have been very high (no quantitative measure of ipsdienol delivery rate or dosage was given), or that their synthetic ipsdienol was contaminated with a synergist. The use of walking beetles in laboratory bioassays for testing bark beetle attractants was criticized for being an oversimplified representation of a complex series of behavioral steps (Vité, 1967). However, when coupled with field data, laboratory bioassays are an indispensable tool in studies of bark beetle pheromones (Wood et al., 1967). The variability in response to ipsdienol in our field tests at different times of the year indicates that simple field tests alone should not be uncritically accepted as yielding a more correct or meaningful result than laboratory bioassays.

The enantiomeric composition of ipsdienol has been implicated (Lanier et al., 1980) as the basis for geographic variation in response specificity in three populations of *I. pini* (Lanier et al., 1972). Populations in California (Birch et al., 1980; Miller et al., 1989) and Idaho (Plummer et al., 1976) produce predominantly the (−) enantiomer, while New York populations have been found to produce racemic ipsdienol (Stewart, 1975; Miller et al., 1989). Our results, comparing the attractiveness of New York and Arizona males in New York show substantial interpopulational differences (Figure 2). The enantiomeric composition of ipsdienol varies between these populations, but in our tests synthetic enantiomers did not attract beetles in the spring, indicating that more than stereochemistry is involved in eliciting response. The relative roles of additional compounds and ipsdienol stereochemistry in interpopulational specificity remains to be investigated.

The seasonal variation in the response to ipsdienol by *I. pini* is qualitatively different from seasonal variation in pheromone systems previously reported in *I. pini* as well as other species. Other reports involve decreases in the level of response of field-collected bark beetles to complete pheromone blends in laboratory bioassays during adverse seasons when the beetles are not normally active (Wood and Bushing, 1963; Borden, 1967; Silverstein et al., 1968; Birch, 1974; Roberts et al., 1982). Birch (1974) found that the level of attractiveness of male frass of *I. pini* also declined during the fall and winter. During diapause and quiescence insects undergo physiological changes, including the diminution of reproductive functions (Tauber et al., 1986). Apparently, reduced reproductive activity includes diminished use of the pheromone system.

In contrast, the qualitative change in the response to ipsdienol by *I. pini* in New York occurs during the active season. Beginning in early to mid-August in New York, *I. pini* generally do not initiate breeding attacks. Instead, they engage in feeding attacks during which beetles continue to produce and respond to pheromones (as evidenced by the aggregation itself), but the density of arriving beetles in the host is about 10 times higher than that of breeding attacks.

The beetles' primary activity in feeding attacks is feeding, while attempts at reproduction are generally unsuccessful (unpublished data). These feeding attacks have been reported from a population in Idaho (Sartwell et al., 1971) and probably occur throughout the range of *I. pini*. Interestingly, they coincide with the onset of response to synthetic ipsdienol alone. Whether there is a direct relationship between the change in response to ipsdienol and attack behavior has yet to be determined.

The change in response to ipsdienol may also be associated with seasonal changes in the fat content of beetles and their general level of responsiveness. In the late summer there is an increase in population density after two or, under favorable conditions, three generations have developed and emerged (Clemens, 1916; Thomas, 1961). Hagen and Atkins (1975) showed that overwintered *I. paraconfusus* had a higher mean fat content and were less responsive to attractants than the spring generation. Seasonal variation in fat content also has been found in *D. frontalis* (Hedden and Billings, 1977; Roberts et al., 1982). Hagen and Atkins (1975) suggested that seasonal variation in fat content may be due to variability in the conditions under which different generations develop. Population and colonization densities increase between overwintered and summer generations of *I. pini* in Wisconsin (Schenk and Benjamin, 1969). A negative correlation between colonization density and the fat content of offspring has been demonstrated in *I. typographus* (Anderbrant et al., 1985; Botterweg, 1983) and *D. pseudotsugae* (Atkins, 1975). Fat content is negatively correlated with responsiveness to attractants in *D. pseudotsugae* (Atkins, 1966) and *I. paraconfusus* (Hagen and Atkins, 1975).

We hypothesize that, in *I. pini*, intergenerational variability in host abundance and population density can lead to variability in intraspecific larval competition, fat content, and responsiveness to semiochemicals. Responsiveness to semiochemicals in our investigation was represented as specificity of response to synthetic ipsdienol compared to male and perhaps host odors. The decrease in specificity that we saw in the late summer and fall may be a manifestation of increased responsiveness.

Acknowledgments—We thank J.R. West and R.M. Silverstein for determinations of enantiomeric compositions and related GC work and D.K. Jewett for assistance in handling field-trapped beetles. The New York State Department of Environmental Conservation assisted in locating suitable pine stands and granted permission for their use. We are grateful to T.W. Phillips, S.J. Seybold, D.L. Wood, R.A. Norton, S. Palmer, and anonymous reviewers for critical reviews of the manuscript. This research was funded by NSF grant DCB8608077 and USDA-CSRS grant 8802317.

REFERENCES

ANDERBRANT, O., SCHLYTER, F., and BIRGERSSON, G. 1985. Intraspecific competition affecting parents and offspring in the bark beetle *Ips typographus*. *Oikos* 45:89–98.

ATKINS, M.D. 1966. Laboratory studies on the behavior of the Douglas-fir beetle, *Dendroctonus pseudotsugae* Hopkins. *Can. Entomol.* 98:953–991.

ATKINS, M.D. 1975. On factors affecting the size, fat content and behavior of a scolytid. *Z. Angew. Entomol.* 78:209–218.

BIRCH, M.C. 1974. Seasonal variation in pheromone-associated behavior and physiology of *Ips pini*. *Ann. Entomol. Soc. Am.* 67:58–60.

BIRCH, M.C., LIGHT, D.M., WOOD, D.L., BROWNE, L.E., SILVERSTEIN, R.M., BERGOT, B.J., OHLOFF, G., WEST, J.R., and YOUNG, J.C. 1980. Pheromonal attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. *J. Chem. Ecol.* 6:703–717.

BORDEN, J.H. 1967. Factors influencing the response of *Ips confusus* (Coleoptera: Scolytidae) to male attractant. *Can. Entomol.* 99:1164–1193.

BOTTERWEG, P.F. 1983. The effect of attack density on size, fat content and emergence of the spruce bark beetle *Ips typographus* L.*Z. Angew. Entomol.* 96:47–55.

BYRNE, K.J., GORE, W.E., PEARCE, G.T., and SILVERSTEIN, R.M. 1975. Porapak-Q collection of airborne organic compounds serving as models for insect pheromones. *J. Chem. Ecol.* 1:1–7.

CLEMENS, W.A. 1916. The pine bark beetle (*Ips pini* Say). *Cornell Univ. Agric. Exp. Stn. Bull.* 383:287–298.

CONOVER, W.J., and IMAN, R.L. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. *Am. Stat.* 35: 124–129.

GREIS, G., PIERCE, H.D., JR., LINDGREN, B.S., and BORDEN, J.H. 1988. New techniques for capturing and analyzing semiochemicals for scolytid beetles (Coleoptera: Scolytidae). *J. Econ. Entomol.* 81:1715–1720.

HAGEN, B.W., and ATKINS, M.D. 1975. Between generation variability in the fat content and behavior of *Ips paraconfusus* Lanier. *Z. Angew. Entomol.* 79:169–172.

HEDDEN, R.L., and BILLINGS, R.F. 1977. Seasonal variations in fat content and size of the southern pine beetle in east Texas. *Ann. Entomol. Soc. Am.* 70:876–880.

LANIER, G.N., BIRCH, M.C., SCHMITZ, R.F., and FURNISS, M.M. 1972. Pheromones of *Ips pini* (Coleoptera: Scolytidae): Variation in response among three populations. *Can. Entomol.* 104:1917–1923.

LANIER, G.N., GORE, W.E., PEARCE, G.T., PEACOCK, J.W., and SILVERSTEIN, R.M. 1977. Response of the European elm bark beetle, *Scolytus mulitstriatus* (Coleoptera: Scolytidae), to isomers and components of its pheromone. *J. Chem. Ecol.* 3:1–8.

LANIER, G.N., CLAESSON, A., STEWART, T., PISTON, J.J., and SILVERSTEIN, R.M. 1980. *Ips pini:* The basis for interpopulational differences in pheromone biology. *J. Chem. Ecol.* 6:677–687.

LINDGREN, B.S. 1983. A multiple funnel trap for scolytid beetles (Coleoptera). *Can. Entomol.* 115:299–302.

MENDENHALL, W. 1983. Introduction to Probability and Statistics. Prindle, Weber and Schmidt Publ., Boston.

MILLER, D.R., BORDEN, J.H., and SLESSOR, K.N. 1989. Inter- and intrapopulation variation of the pheromone, ipsdienol, produced by male pine engravers, *Ips pini* (Say) (Coleoptera: Scolytidae). *J. Chem. Ecol.* 15:233–247.

MILLER, D.R., GREIS, G., and BORDEN, J.H. 1990. (*E*)-Myrcenol: A new pheromone for the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). *Can. Entomol.* 122:401–406.

PLUMMER, E.L., STEWART, T.E., BYRNE, K., PEARCE, G.T., and SILVERSTEIN, R.M. 1976. Determination of the enantiomeric composition of several insect pheromone alcohols. *J. Chem. Ecol.* 2:307–331.

ROBERTS, E.A., BILLINGS, P.M., PAYNE, T.L., RICHERSON, J.V., BERISFORD, C.W., HEDDEN, R.L., and EDSON, L.J. 1982. Seasonal variation in laboratory response to behavioral chemicals of the southern pine beetle. *J. Chem. Ecol.* 8:641–652.

SARTWELL, C., SCHMITZ, R.F., and BUCKHORN, W.J. 1971. Pine engraver, *Ips pini*, in the western states. *USDA For. Serv. Pest Lfl.* 122:1-5.

SCHENK, J.A., and BENJAMIN, D.M. 1969. Notes on the biology of *Ips pini* in central Wisconsin jack pine forests. *Ann. Entomol. Soc. Am.* 62:480-485.

SILVERSTEIN, R.M., BROWNLEE, R.G., BELLAS, T.E., WOOD, D.L., and BROWNE, L.E. 1968. Brevicomin: Principle sex attractant in the frass of the female western pine beetle. *Science* 159:889-891.

SLESSOR, K.N., KING, G.G.S., MILLER, D.R., WINSTON, M.L., and CUTFORTH, T.L. 1985. Determination of chirality of alcohol or latent alcohol semiochemicals in individual insects. *J. Chem. Ecol.* 11:1659-1667.

STEWART, T.E. 1975. Volatiles isolated from *Ips pini:* Isolation, identification, enantiomeric composition and biological activity. MS thesis. State University of New York, College of Environmental Science and Forestry, Syracuse. 135 pp.

TAUBER, M.J., TAUBER, C.A., and MASAKI, S. 1986. Seasonal Adaptations of Insects. Oxford University Press, New York.

THOMAS, J.B. 1961. The life history of *Ips pini* (Say) (Coleoptera: Scolytidae). *Can. Entomol.* 93:384-390.

VITÉ, J.P. 1967. Sex attractants in frass from bark beetles. *Science* 156:105.

WOOD, D.L., and BUSHING, R.W. 1963. The olfactory response of *Ips confusus* (LeConte) (Coleoptera: Scolytidae) to the secondary attraction in the laboratory. *Can. Entomol.* 95:1066-1078.

WOOD, D.L., SILVERSTEIN, R.M., and RODIN, J.O. 1967. *Science* 156:105.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

LANIERONE: A NEW PHEROMONE COMPONENT FROM *Ips pini* (COLEOPTERA: SCOLYTIDAE) IN NEW YORK

STEPHEN A. TEALE,[1,*] FRANCIS X. WEBSTER,[2] AIJUN ZHANG,[2] G.N. LANIER[1,3]

[1]*Department of Environmental and Forest Biology*

[2]*Department of Chemistry*
College of Environmental Science and Forestry
State University of New York
Syracuse, New York 13210

(Received October 29, 1990; accepted February 12, 1991)

Abstract—A new pheromone component, lanierone, (2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one) was isolated and identified from a Porapak Q collection of volatiles from male *Ips pini* from New York through GC fractionation, bioassay, and spectrometry. In both the laboratory and the field, synthetic lanierone, in a 1 : 100 ratio with synthetic ipsdienol, is as attractive as natural pheromone sources. Synthetic ipsdienol alone is not attractive in the laboratory and only weakly attractive in the field. Varying the ratio of lanierone to ipsdienol in the field from 10^{-4} : 1 to 1 : 1 in 10-fold increments resulted in an increased number of beetles trapped at the three lower ratios, but also in an increase in the proportion of males trapped. In the field, all combinations of lanierone to ipsdienol attracted proportionately fewer males than did pheromone-producing male beetles. GC and GC-MS analyses of Porapak Q-trapped volatiles revealed that lanierone is produced in an amount equal to about 0.2% of that of ipsdienol and is produced exclusively by males. The small amount of lanierone produced, together with a GC retention time similar to that of ipsdienol on a nonpolar column, probably confounded its detection in earlier studies.

Key Words—Insecta, Coleoptera, Scolytidae, *Ips pini*, pheromone, isolation, identification, bioassay, ipsdienol, lanierone.

*To whom correspondence should be addressed.
[3]Deceased.

0098-0331/91/0600-1159$06.50/0

INTRODUCTION

The pine engraver, *Ips pini* (Say), breeds in pines and spruces throughout most of North America. Upon locating a host tree, males bore into the phloem–cambium region and release a chemical attractant that mediates further colonization of the host by responding males and females. The pheromone was reported to consist of a single compound, ipsdienol (Birch et al., 1980; Lanier et al., 1980). More recently, Greis et al. (1988) reported that *I. pini* in British Columbia produce (*E*)-myrcenol, and Miller et al. (1990) reported that (*E*)-myrcenol was behaviorally active for *I. pini* in British Columbia. In addition, β-phellandrene, a major constituent of the terpene fraction of lodgepole pine oleoresin (*Pinus contorta* var. *latifolia* Engelmann) is attractive to *I. pini* in British Columbia (Miller and Borden, 1990).

Interpopulational pheromonal specificity (Lanier et al., 1972) has been reported to be based on ipsdienol enantiomeric composition (Birch et al., 1980; Lanier et al., 1980). In general, western populations produce and respond to blends of over 90% (−)-ipsdienol [less than 10% (+)] and eastern populations produce and respond to a nearly racemic mixture of ipsdienol [roughly 50:50 (−):(+)]. The specificity of response to pheromones can also vary seasonally within a population. Teale and Lanier (1991) reported that a New York population of *I. pini* responded in the spring and early summer to natural pheromone sources but not to racemic ipsdienol, while in the late summer and fall, the numbers of beetles trapped by ipsdienol and natural sources were not significantly different. In addition, laboratory-reared beetles originating in New York were attracted to a pheromone extract, but not to ipsdienol in laboratory bioassays (Teale and Lanier, 1991). This demonstrated that one or more additional compounds were required for response. Here we report the isolation and identification of a new pheromone component for *I. pini* through the use of the fractionation, spectrometry, and bioassay method of Silverstein et al. (1967). The new component is also a new natural product, and we give it the name "lanierone" in recognition of the late Gerald N. Lanier and his valuable contributions to bark beetle chemical ecology.

METHODS AND MATERIALS

Insect Rearing. Beetles used for pheromone collection, laboratory bioassays, and bait logs for field experiments were reared in a laboratory colony originating in red pine (*Pinus resinosa* Ait.) 25 km south of Syracuse, New York, in 1973. Beetles captured at the original locality were periodically added to the colony to prevent inbreeding. Fresh red pine logs were sealed at the ends with hot wax and placed in the colony weekly. Beetles were collected by placing infested logs in metal containers (40 cm diam. × 60 cm) fitted with glass col-

lection jars (1 liter). For bioassay, beetles were removed and used within 24 hr of emergence. For pheromone collections, beetles were collected daily, sexed, and then stored on damp toweling at 5°C for up to two weeks.

Pheromone Collection. Two large collections of volatiles were made by introducing individual male beetles into the phloem of fresh red pine logs (about 15 cm diam. × 1 m) through cork borer holes (5 mm diam.) covered with aluminum screen patches. The infested logs were placed in a metal container (152 × 43 × 69 cm) and aerated with charcoal-filtered air. Volatiles were trapped on two Porapak Q (Byrne et al., 1975) filters (50 ml each) connected in series. The volatiles were extracted from the Porapak Q with pentane (100 ml, combined) and the pentane solution was concentrated by distillation.

The purpose of the first aeration (aeration 1) was to obtain a large amount of active material for fractionation and bioassay (Silverstein et al., 1967). Male beetles (1040) were aerated (4 liters/min) for 168 hr on five logs (15 cm diam. × 120 cm). The second aeration (aeration 2) was used to quantify production of ipsdienol and lanierone and for use in laboratory and field bioassays comparing natural and synthetic materials. Male beetles (1250) were aerated (1 liter/min) for 80 hr on five logs (15 cm diam. × 120 cm). To determine the origin of lanierone, males and females (150 each) were simultaneously aerated for 105 hr in separate glass columns (15 cm ID × 50 cm) while boring in red pine logs (12 cm diam. × 48 cm), which were cut from adjacent sections of a single tree. Volatiles were collected separately on Porapak Q (50 ml). Due to the small quantity of lanierone in the male aeration, both extracts were analyzed by GC-MS using selective ion monitoring (*m/z* 109).

Fractionation. The concentrated Porapak Q extract from the first aeration was sequentially fractionated on four packed columns in a Varian Aerograph Series 2700 gas chromatograph with a splitter (Brownlee and Silverstein, 1968) and flame-ionization detector. The four columns and the retention times of the fractions were as follows: (1) 5% OV-101 (Applied Science, State College, Pennsylvania), on Chromosorb WAW-DMCS 100/120 mesh, 3 m × 7.5 mm (OD) glass, 90°C for 30 min, then 4°C/min until 140°C, F1 5–25 min, F2 25–27 min, F3 27–60 min; (2) 4% Carbowax 20 M (Applied Science) on Chromosorb WAW-DMCS 100/120 mesh, 6.1 m × 6.4 mm (OD) glass, 120°C for 20 min, then 4°C/min until 140°C, F3.1 0–9 min, F3.2 9–15 min, F3.3 15–24 min; (3) 5% FFAP (Supelco, Inc., Bellefonte, Pennsylvania) on Chromosorb G-HP, 100/120 mesh, 3 m × 6.4 mm (OD) glass, 130°C isothermal, F3.2.1 0–24.5 min, F3.2.2 24.5–26.5 min, F3.2.3 26.5–29 min, F3.2.4 29–40 min; (4) 6% TCEP (Supelco, Inc.) on Chromosorb WAW-DMCS 100/120 mesh, 80°C initial, then 4°C/min until 110°C, F3.2.3.1 33–38 min, F3.2.3.2 38–43 min, F3.2.3.3 43–56 min, F3.2.3.4 56–66 min. For all the columns, the carrier gas was nitrogen (60 ml/min), the injector temperature was 150°C, and the FID temperature was 160°C. Active fractions were analyzed by injection

on BP-1, 50 m × 0.33 mm (ID), at 45°C for 1 min, then 5°C/min until 200°C in a Hewlett Packard 5890 gas chromatograph to determine the number of compounds present. The enantiomeric composition of ipsdienol in F2 (OV-101) was determined by the method of Slessor et al. (1985).

Chemical Analyses. Volatiles were analyzed by injection on BP-1, 50 m × 0.33 mm (ID), at 45°C for 1 min, then 1°C/min until 200°C in a Hewlett Packard 5890 gas chromatograph. Ipsdienol and lanierone peaks were identified by coinjection with synthetic samples and by GC-MS using selective ion monitoring (*m/z* 109) in a Finnigan model 4500 fitted with a 30-m SPB-5 capillary column (Supelco, Inc.) and with the injector temperature set at 250°C. Coupled GC-MS was performed on a Finnigan model 4500 fitted with a 30-m SPB-5 capillary column (Supelco, Inc.), run in splitless mode, and with the injector temperature set at 250°C. All samples were run at 70 eV.

An infrared spectrum of natural lanierone was obtained on a Hewlett Packard 5965A Fourier transform infrared spectrometer coupled to a Hewlett Packard 5890 gas chromatograph fitted with an HP-5 capillary column (25 m × 0.3 mm ID).

A proton nuclear magnetic resonance spectrum of about 5 μg of pure natural lanierone was obtained on a GE GN-500 (500 MHz) NMR spectrometer in C_6D_6 with a pulse sequence to suppress solvent and water peaks.

Synthesis of Lanierone: Preparation of 2-Hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one. Lanierone was synthesized using the method of Frimer et al. (1989). Isophorone (Aldrich Chemical Co.) (3,5,5-trimethyl-2-cyclohexen-1-one), (3.0 g, 21.74 mmol), 18-crown-6 (Aldrich Chemical Co.) (8.62 g, 32.60 mmol) and potassium superoxide (Aldrich Chemical Co.) (6.18 g, 86.96 mmol) were dissolved in 30 ml benzene and stirred under dry argon for 2 hr. Water (50 ml) was added and the solution was acidified with 6 N hydrochloric acid. The product was extracted with methylene chloride, washed with water followed by saturated sodium bicarbonate, and dried over sodium sulfate. The solvent was removed in vacuo to yield the crude product (3.55 g) as an oil, which was purified by flash column chromatography (Still et al., 1978) using 20% ethyl acetate in hexane to yield lanierone (1.14g, 7.5 mmol, 35%). The enol was recrystallized from hexane as white needles with a melting point of 59–60°C. MS *m/z* (% rel. inten.): 152 (M^+, 30.20), 137 (M^+-CH_3, 28.10), 124 (M^+-CO, 52.00), 109 (M^+-CH_3-CO, 100). [^{1}H]NMR (C_6D_6): δ 6.71 (s, 1H, OH), 6.10 (m, J = 2.8 Hz, $J_{allylic}$ = 1.4 Hz, 1H), 5.85 (d, J = 2.8 Hz, 1H), 1.85 (d, $J_{allylic}$ = 1.4 Hz, 3H), 0.80 (s, 6H) [^{13}C]NMR (C_6D_6): δ 182.17, 154.87, 145.94, 130.85, 124.40, 36.60, 26.96, 15.26.

Laboratory Bioassays. Female beetles were tested in groups of 10 in a modified Wood and Bushing (1963) choice olfactometer (Teale and Lanier, 1991). Positive responses were scored when a beetle followed an airstream for at least 3 cm before coming within 5 mm or contacting the pipet. When testing

fractions, each group of 10 beetles was used for each stimulus or combination of stimuli being compared. The order in which stimuli were presented was randomized between groups of beetles to minimize possible interaction effects. The raw bioassay data were analyzed by ANOVA and decisions were made at the $P < 0.05$ level. The OV-101 fractions were assayed at dilutions equal to 1.0, 0.1, 0.01, and 0.001 of an amount of starting material containing 1 μg ipsdienol. All subsequent fractions were assayed with 1 μg synthetic racemic ipsdienol added. The concentrations of test fractions were increased at two steps in the sequence to compensate for material lost during collection.

When synthetic materials were tested, each group of 10 beetles was used once, then discarded. The test stimuli were pentane (negative control), synthetic racemic ipsdienol (1 μg), lanierone (10 ng), ipsdienol plus lanierone (1 μg + 10 ng), and male volatiles (second aeration; 1 μg + 10 ng). The raw data (homoscedastic; Bartlett's test, $P > 0.05$) were analyzed by one-way ANOVA followed by the LSD range test.

Field Experiments. Field experiments were conducted in a mixed stand of red and Scots (*P. sylvestris* L.) pine, 25 km south of Syracuse, New York. The purpose of the first experiment was to determine the attractiveness of lanierone using treatments similar to those of the laboratory bioassays. The treatments were: (1) hexane, (2) male volatiles (aeration 2; 1.0 mg natural ipsdienol, 10 μg lanierone), (3) synthetic racemic ipsdienol (1.0 mg) and synthetic lanierone (10 μg), (4) synthetic lanierone (100 μg), (5) synthetic racemic ipsdienol (10 mg), (6) synthetic racemic ipsdienol (10 mg) and synthetic lanierone (100 μg), and (7) 20 male beetles in a red pine log. Treatments 1–6 were placed in 3.7-ml polyethylene vials with a 1-mm hole in the cap, and the total volume including solvent (hexane) was 1 ml. The release rate of ipsdienol in treatments 5 and 6 was estimated to be about 67μg/day. The release rate was determined by aerating the release device ($N = 3$), collecting volatiles on Porapak Q over seven days at 22°C, and quantifying by injection on capillary GC. Baits were covered with aluminum foil for protection from sunlight. Chemical baits were hung in eight-unit multiple-funnel traps (Lindgren, 1983) (supplied by Phero Tech Inc., Delta, British Columbia) from the lower middle funnel; logs with male beetles were hung adjacent to the lower middle funnels. The treatments were randomized in 10 rows. Traps were spaced at a minimum distance of 20 m. Due to the limited quantity of male volatiles (5 mg ipsdienol) only five replicates of treatment 2 were possible; all other treatments received 10 replicates. The experiment was conducted from July 26 to August 1, 1990.

The purpose of the second field experiment was to determine the effect of varying the amount of lanierone relative to a fixed amount of ipsdienol. The treatments were the following amounts of lanierone in combination with synthetic racemic ipsdienol (10 mg): 10 mg, 1.0 mg, 0.1 mg, 0.01 mg, and 0.001 mg. In addition, there were two controls: hexane, and 20 male *I. pini* boring

in a red pine log. The seven treatments were arranged in a randomized complete block design with 10 replicates. Traps were spaced at a minimum distance of 20 m. The experiment was conducted from August 10 to 16, 1990, at the same location as the first field experiment.

RESULTS

Isolation. The concentrated Porapak Q extract from aeration 1 contained 20 mg of ipsdienol as determined by comparison with a synthetic standard on capillary GC. A dose of the extract containing 1 μg ipsdienol elicited 67% positive response in the laboratory bioassay (Figure 1). Fractionated material for the laboratory bioassays was quantified in the following manner. Because the starting material contained 20 mg ipsdienol and because an aliquot of the starting material containing 1 μg (or 5×10^{-5} of the total) ipsdienol elicited maximal response, each fraction was diluted and tested in a fraction of 5×10^{-5} of the total, so that the ratio of ipsdienol to the fraction of starting material was 5×10^{-5}:5×10^{-5}, or 1:1.

The three fractions from OV-101 were collected so that F1 contained all peaks eluting before ipsdienol, F2 contained one large ipsdienol peak, and F3 contained all peaks eluting after ipsdienol. A GC-MS analysis of F2 detected no significant (<1.0%) impurities. In bioassays comparing the extract, F2, and synthetic racemic ipsdienol, F2 was not significantly less attractive than the

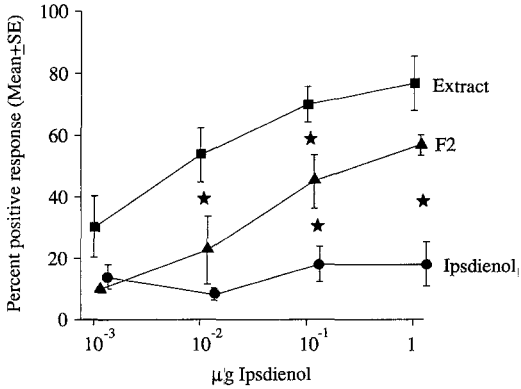


FIG. 1. Percent positive response by female *I. pini* in laboratory bioassays of OV-101 fraction 2 (F2), starting extract (aeration 1), and synthetic racemic ipsdienol. $N = 30$ beetles at each concentration; concentrations are μg ipsdienol. Stars indicate significant differences between means ($P < 0.05$; LSD test).

extract ($P < 0.05$, ANOVA) at all but the 10^{-2} dilution (Figure 1). Ipsdienol was significantly less attractive than the extract at all but the lowest concentration and was significantly less attractive than F2 at the two higher concentrations. The enantiomeric composition of ipsdienol in F2 was 46%(−):54%(+). There are no significant differences in response in the field to 30, 40, 50, and 60% (−)-ipsdienol (unpublished data). Therefore, we feel that the small difference in enantiomeric composition between F2 and synthetic racemic ipsdienol is insufficient to account for the difference in laboratory responses. This suggests that F2 contained an amount of a synergist below the limits of GC detection, but detectable by beetles. Lanierone may have been split between F2 and F3.

Further bioassays of the OV-101 fractions (Figure 2) showed that F3 and recombined F1 + F3, each in combination with ipsdienol in a 1:1 ratio, were not significantly less attractive than the extract at all dilutions. Fraction 1 in combination with ipsdienol was significantly less attractive than the extract at the 10^{-2} and 10^{-1} dilutions and less attractive ($P = 0.07$, ANOVA) than the extract at the highest concentration. The recombined F1 + F3 without ipsdienol added was significantly less attractive than the extract at all but the lowest concentration. Because the largest differences between stimuli occurred at the higher concentrations, subsequent bioassays utilized the single highest concentration (i.e., 1μg ipsdienol).

Only one Carbowax 20 M (C20M) fraction (F3.2) in combination with

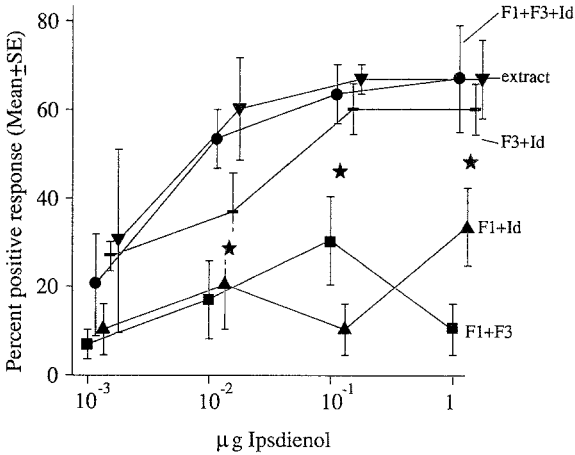


FIG. 2. Percent positive response by female *I. pini* in laboratory bioassays of starting extract (aeration 1) and OV-101 fractions 1 (F1) and 3 (F3). Stars indicate significant differences between stimuli and the extract ($P < 0.05$; LSD test). $N = 30$ beetles at each concentration; concentrations are μg ipsdienol. Id = ipsdienol.

ipsdienol elicited a level of response (47%) that was not significantly less attractive than that of the recombined fractions (68%) (Figure 3). When this fraction was refractionated on FFAP, only one fraction (F3.2.3) was not significantly less attractive (70%) than the recombined fractions (58%). The concentration of the fractionated material used in the assays was increased 10-fold in order to maintain levels of response comparable to those of the extract. We attributed the need to increase concentration to material losses incurred in the GC fractionation.

Of the TCEP fractions, F3.2.3.1, F3.2.3.2, and F3.2.3.4, when recombined, elicited a significantly lower level of response (7%) than all four fractions recombined (47%) (Figure 3). Fraction 3.2.3.3 elicited the same level of response (47%) as all four fractions recombined. Fraction 3.2.3.3 alone and the recombined F3.2.3.1, F3.2.3.2, and F3.2.3.4 were assayed in this configuration because F3 manifested a characteristic odor that we had detected in all previous attractive fractions and the extract. [There is a report in the patent literature that lanierone masks unpleasant phenolic flavors for humans (Demole, 1975)]. Again, the concentration was increased (sixfold) in order to maintain a satisfactory level of response. Capillary GC analysis of F3.2.3.3 revealed the presence of a single peak.

Identification. In the mass spectrum of the compound in F3.2.3.3 (Figure 4) the M^+ ion was judged to be *m/z* 152. The next highest fragment ion was 137, $M^+ - CH_3$. Assuming that there is only C, H, and O present, three likely molecular formulae were $C_{10}H_{16}O$, $C_9H_{12}O_2$, and $C_8H_8O_3$.

The FT-IR spectrum (Figure 5) indicated absorptions at 3462 ν OH, 2974 ν CH_3, 1659 ν C=O, 1434 δ_{as} CH_3, 1367 δ_s CH_3, and 1264 ν C—O, cm^{-1}.

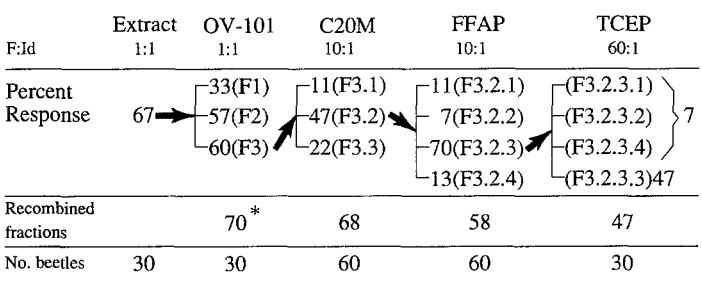


FIG. 3. Summary of laboratory bioassay data on four sequential fractionations of Porapak Q-collected volatiles from 1040 male *Ips pini* (aeration 1). The numbers in parentheses are referenced in the text. F:Id = ratio of fraction to ipsdienol. Quantification of fractions is based on the quantity of ipsdienol (20 mg) in the starting material (i.e., 1 μg ipsdienol is equal to 1 μg/20 mg, or 5×10^{-5} of the total; see text). *GC total.

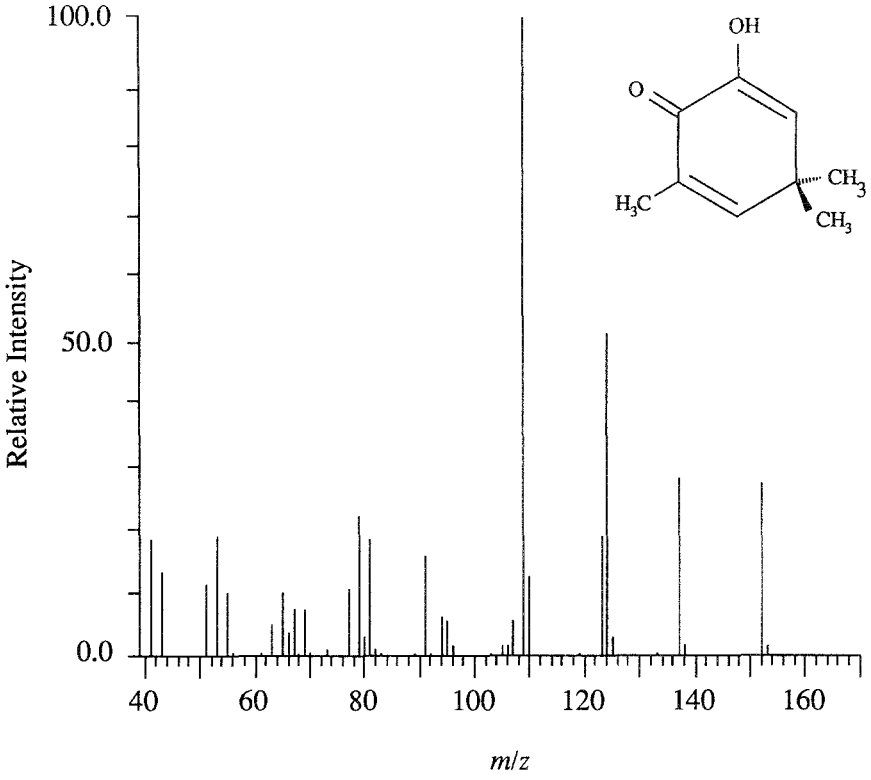


FIG. 4. Mass spectrum of natural lanierone isolated from Porapak Q-collected volatiles from 1040 male *Ips pini* (aeration 1).

The presence of two types of oxygen-containing functional groups excludes the molecular formula $C_{10}H_{16}O$.

The [^{1}H]NMR spectrum is shown in Figure 6. The singlet at δ 0.70 (6H) indicates that there are two identical methyl groups on a quaternary carbon (no coupling); the doublet at δ 1.80 (3H) indicates a methyl group on an olefinic carbon; the doublet at δ 5.78 (1H) and the multiplet at δ 6.03 (1H) indicate protons on olefinic carbons; and the singlet at δ 6.53 (1H) is a hydrogen-bonded hydroxyl proton. The indication of three methyl groups (9H) excludes the molecular formula $C_8H_8O_3$. On the basis of the MS, FT-IR, and NMR spectra of the natural and the synthetic compound, the structure was determined to be 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one (Figure 4).

Analyses of Beetle Aerations. On the normal-scale GC trace (Figure 7) of aeration 2, ipsdienol was represented by a peak that was small relative to several others, representing host volatiles. Only when the scale was increased 100-fold

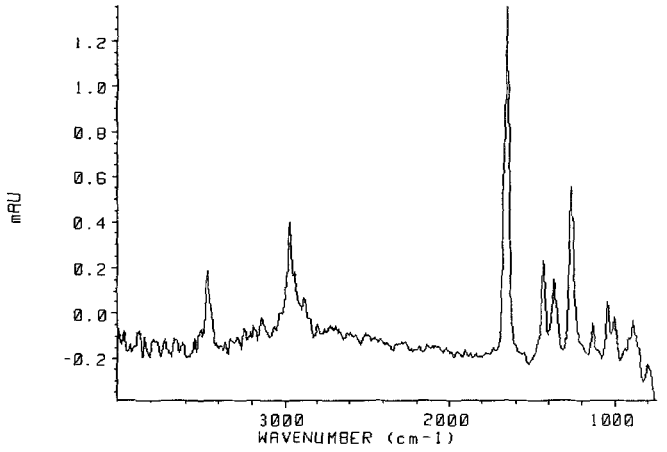


FIG. 5. Infrared spectrum of lanierone isolated from Porapak Q-collected volatiles from 1040 male *Ips pini* (aeration 1).

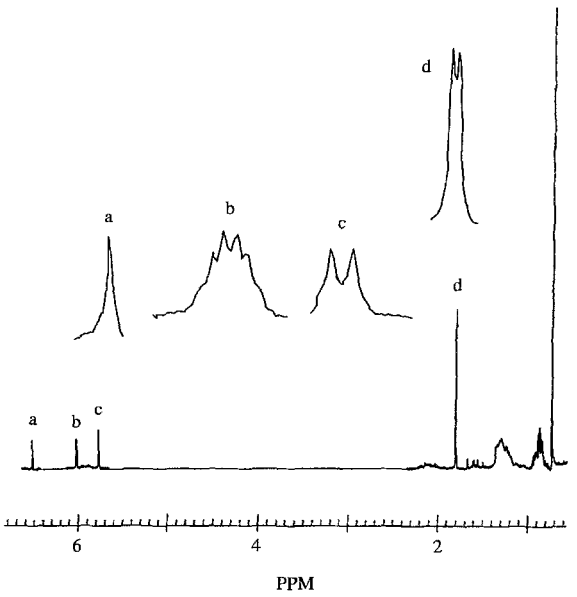


FIG. 6. NMR spectrum of natural lanierone isolated from Porapak Q-collected volatiles from 1040 male *Ips pini* (aeration 1).

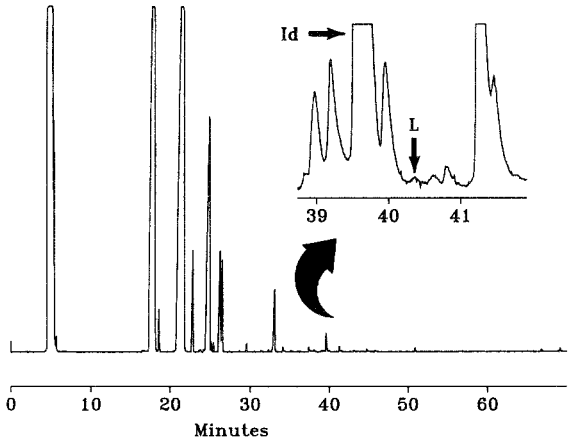


FIG. 7. GC tracings of volatiles from 1250 male *I. pini* boring in red pine collected on Porapak Q (aeration 2; see text for details). Normal scale and 100 × (inset).

The selective ion monitoring mass spectra (*m/z* 109) of the male and female aerations (Figure 8) revealed both ipsdienol and lanierone in the male extract but neither compound in the female extract. Because the female (and male) aeration was made with beetles feeding on host material (red pine), and no lanierone was collected, lanierone must not be produced by the host or females. Rather, lanierone is exclusively male-produced.

Laboratory Bioassays. In laboratory bioassays, neither synthetic ipsdienol nor lanierone alone was significantly more attractive to females than the solvent (pentane) control (Figure 9) ($P > 0.05$; ANOVA followed by LSD range test). The male volatiles and the combination of synthetic ipsdienol (1 μg) and synthetic lanierone (10 ng) were significantly more attractive than the solvent control, ipsdienol, and lanierone alone. There was no significant difference between the levels of attraction elicited by the male volatiles and the combination of synthetic ipsdienol and synthetic lanierone. These data indicate that ipsdienol and lanierone function in a synergistic manner.

Field Experiments. The treatments of the first field experiment fell into three homogeneous groups [Figure 10; $P < 0.05$; ANOVA on $\log(Y + 1)$ transformed data followed by LSD range test]: (1) hexane control, male volatiles and lanierone, (2) ipsdienol plus lanierone (low dose) and ipsdienol (high dose), and (3) ipsdienol plus lanierone (high dose) and males. The large number of beetles attracted by ipsdienol plus lanierone (high dose) compared to the small numbers attracted by ipsdienol and lanierone alone supports our laboratory result that these two compounds function in a synergistic, rather than an additive, manner. The sex ratios varied significantly between the ipsdienol plus

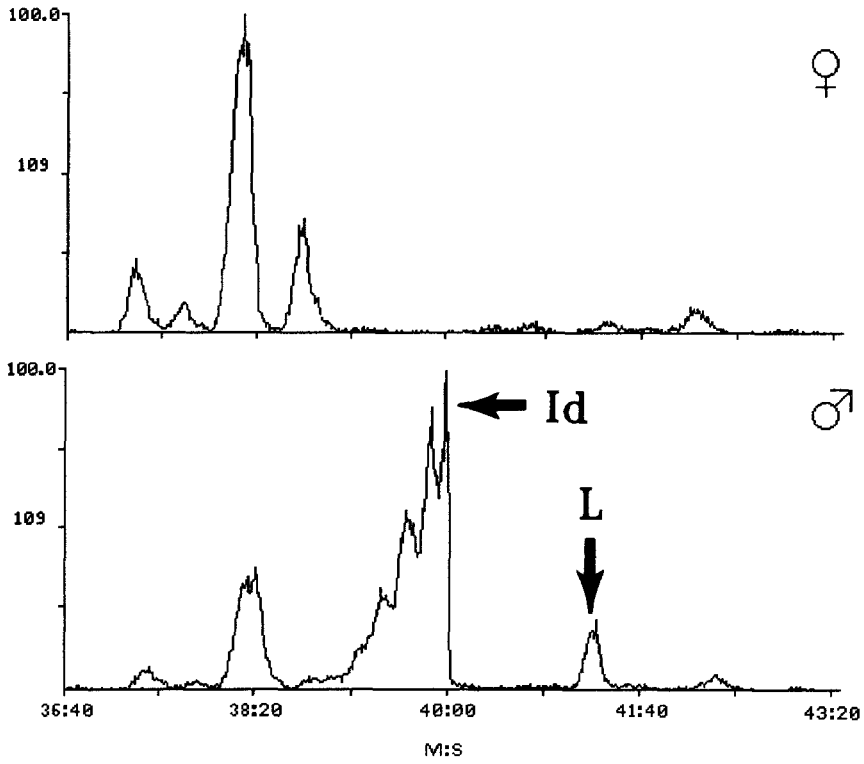


FIG. 8. Selective ion monitoring mass spectra (*m/z* 109) of male volatiles (bottom) and female volatiles (top). Note that peak sizes are not proportionate because *m/z* 109 is the base peak for lanierone, but only a minor fragment for ipsdienol. See text for details.

lanierone (high dose) ($m/f + m = 0.38$) and the male beetle ($m/f + m = 0.49$) treatments [$P < 0.01$, test for population proportions (Mendenhall, 1983)].

The male volatiles collected by aeration on Porapak Q (aeration 2) contained amounts of ipsdienol and lanierone equal to those of the synthetic mixture at low dose. Therefore, the small number of beetles attracted by the male volatiles must have been due to either the presence of large quantities of terpenes from the aerated host material, the presence of repellent microbial oxidation products, insufficient replication ($N = 5$ for the male volatile treatment, for all others $N = 10$), or other factors.

In the second field experiment, the amount of lanierone had a significant effect on the numbers of beetles trapped (Figure 11). The three higher doses together with the male treatment formed a homogeneous group [$P > 0.05$; ANOVA on $\log(Y + 1)$ transformed data followed by LSD range test]. The treatment with 0.01 mg lanierone in combination with 10 mg ipsdienol attracted

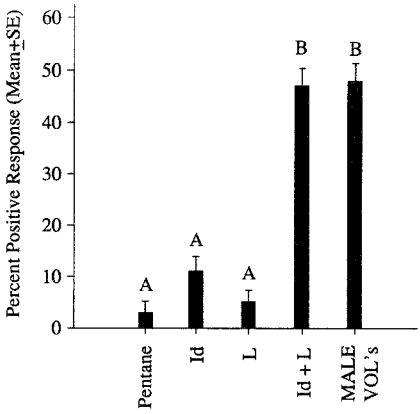


FIG. 9. Percent positive response in the laboratory bioassay by female *I. pini* to synthetic lanierone and ipsdienol. Id = ipsdienol; L = lanierone; MALE VOL's = volatiles from aeration 2 (see text for details). Different letters over bars indicate significant differences (ANOVA on raw data followed by LSD range test).

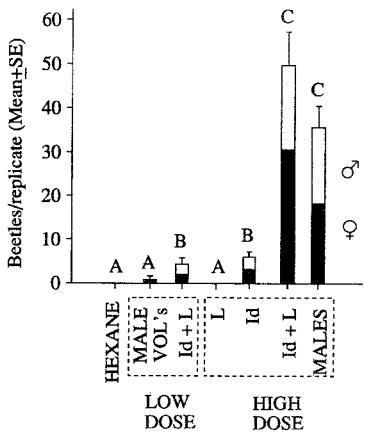


FIG. 10. Field test of lanierone at two doses 25 km south of Syracuse, New York, July 26–August 1, 1990. Id = ipsdienol; L = lanierone; MALE VOL's = male volatiles (aeration 2, see text); MALES = 20 males boring in a red pine log. LOW DOSE: Id = 1 mg; L = 0.01 mg. HIGH DOSE: Id = 10 mg; L = 0.1 mg. Different letters over bars indicate significant differences (ANOVA on log $(Y + 1)$ transformed data followed by LSD range test).

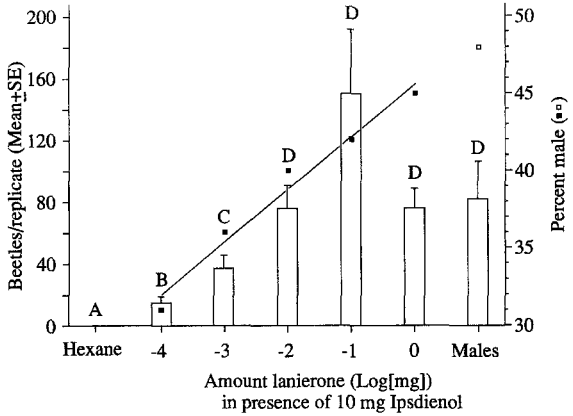


FIG. 11. The effect of varying the proportion of lanierone with a fixed amount (10 mg) of ipsdienol on the mean number (left axis, bars) and percent male (right axis, regression) of *I. pini* trapped in multiple-funnel traps 25 km south of Syracuse, New York, August 10–16, 1990. Different letters over bars indicate significant differences among mean numbers of beetles trapped (ANOVA on log $(Y + 1)$ transformed data followed by LSD range test). For the regression of sex ratio on proportion of lanierone, $R^2 = 0.97$, $P = 0.002$, $Y = 3.4 \log X + 45.6$.

fewer beetles than the higher doses, but more than the solvent control and the lowest dose. The lowest dose (0.001 mg lanierone) attracted significantly more than the solvent control. There was a close positive relationship between the percentage of males in the responding beetles and the quantity of lanierone in the presence of 10 mg ipsdienol ($R^2 = 0.97$, $P = 0.002$; Figure 11).

DISCUSSION

Our results demonstrate that the principal male-produced pheromone components of *Ips pini* in New York are racemic ipsdienol and lanierone (2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one). The bioassay of the starting extract showed that we had collected attractive volatiles in the aeration of male beetles on Porapak Q. The attractiveness was retained during fractionation, and the fraction that contained lanierone (F3) was attractive in combination with synthetic ipsdienol but not alone. Furthermore, laboratory bioassays and field experiments demonstrated that only the combined mixture was as attractive as natural pheromone. Previously, we found that ipsdienol alone is not attractive in laboratory bioassays (Teale and Lanier, 1991). Thus, lanierone and ipsdienol have a synergistic, or multiplicative, effect.

The amount of lanierone produced is small relative to ipsdienol. We iso-

lated only about 5 μg lanierone and about 20 mg ipsdienol from the starting material. However, because our GC collection efficiency was less than 100%, there must have been more lanierone present in the starting material. The GC analysis of aeration 2 (Figure 7), which was not fractionated, showed that the proportion of lanierone to ipsdienol is about 1 : 500. Byers et al. (1990) reported a similar situation in the scolytid *Pityogenes chalcographus* (L.) in which one component, methyl decadienoate [(*E*,*Z*)-MD], is present as a minute fraction of another, chalcogran. The cryptic nature of pheromone components like (*E*,*Z*)-MD and lanierone emphasizes the importance of the fractionation and bioassay method (Silverstein et al., 1967) for identifying pheromone components from complex mixtures of plant and insect compounds. Another method is the differential diagnosis of Vité and Renwick (1970) or variations thereof (e.g., Smith et al., 1985; Greis et al., 1988). In this method, GC traces of attractive and nonattractive extracts are compared and the peaks that are unique to the attractive extract are considered potential pheromone components. This method can be used to detect pheromone components if they do not coelute with other peaks and if the peaks are reasonably large. Lanierone in *I. pini* presents an ideal example of a situation that would be extremely difficult to resolve by differential diagnosis methods. Lanierone coelutes with ipsdienol on at least one nonpolar GC column and its peak is only about 0.2% as large as that of ipsdienol.

Our isolation of lanierone represents the first isolation of this compound from a natural source, and lanierone is the only known scolytid pheromone component with a carotenoid-ring structure. Birgersson et al. (1984) reported the presence of a reduced form of lanierone, β-isophorone, in the hindguts of *I. typographus* females. However, its behavioral significance, if any, is unknown.

The sex ratio differences among responders to synthetic (lanierone plus ipsdienol) and natural (20 males in red pine) baits (Figure 10 and 11) suggest that other factors may be important in the host colonization process. Using a differential diagnosis technique, Greis et al. (1988) found that (*E*)-myrcenol is produced by *I. pini* in British Columbia. Miller et al. (1990) found that (*E*)-myrcenol had an inhibitory effect on attraction by *I. pini* in British Columbia when released with ipsdienol. In contrast to the New York population (Teale and Lanier, 1991) *I. pini* in British Columbia are attracted to synthetic ipsdienol in July (Miller et al., 1990). Higher release rates of (*E*)-myrcenol, in combination with a fixed amount of ipsdienol, attracted more males than the lower release rates, but the experiments were conducted consecutively so it was not possible to exclude the possibility that the sex ratios of flying beetles were different during the two experiments (Miller et al., 1990). In addition, when (*E*)-myrcenol was placed on freshly cut lodgepole pine logs, the increase in the number of *I. pini* colonizing the log was proportional to the amount of (*E*)-myrcenol released (Miller et al., 1990). More experiments are needed to eval-

uate interactions between ipsdienol, lanierone, (*E*)-myrcenol, and the kairomone, β-phellandrene (Miller and Borden, 1990) as well as geographic variation in the use of these compounds.

The fact that neither of two previous investigations (Birch et al., 1980; Lanier et al., 1980) into the chemical identification of the pheromone of *I. pini* had found lanierone is perplexing. In addition, our findings that ipsdienol alone was not attractive in laboratory bioassays or in the field in the spring and early summer appears contradictory with the reports of Birch et al. (1980), Lanier et al. (1980), and Miller et al. (1990), in which ipsdienol alone was reported to be attractive. Birch et al. (1980) and Lanier et al. (1980) utilized the rigorous fractionation and bioassay method (Silverstein et al., 1967) that is designed to find synergistically active behavioral components. This made the apparent incongruity between our results and those of Lanier et al. (1980) and Birch et al. (1980) especially difficult to reconcile.

Three factors interacted to make the detection and isolation of lanierone difficult: (1) the similar retention times of ipsdienol and lanierone, (2) the small quantity required for response, and (3) seasonal variation in response to pheromone. Together, they can explain why lanierone was not detected by Lanier et al. (1980). First, the incomplete separation of ipsdienol and lanierone that we observed on OV-101, as indicated by laboratory bioassay of fraction 2, suggests that Lanier et al. (1980) may not have achieved complete separation of ipsdienol and lanierone. Lanier et al. (1980) used apparently pure, natural ipsdienol in the laboratory, but we now have reason to suspect that it could have contained lanierone, which would have caused an apparent response to ipsdienol. Positive response to synthetic ipsdienol in the lab bioassays of Lanier et al. (1980) is more difficult to explain. It is possible that the less stringent scoring method (Lanier et al., 1977) produced false positives; that the airflow rates of the Moeck (1970) olfactometer were not equal, which could cause differential anemotaxis; that their release rates could have been very high (no quantitative measure of ipsdienol delivery rate or dosage was given); or that their synthetic ipsdienol was inadvertently contaminated with natural lanierone.

Lanier et al. (1980) tested the attractiveness of synthetic ipsdienol in the field and observed a level of response equivalent to 20 male beetles boring in red pine. Attraction to synthetic material under natural field conditions is often considered the final, conclusive test of a pheromone identification (Silverstein et al., 1967; Vité, 1967). Yet, it has been shown that the response of *I. pini* to pheromone can vary during the flight period (Teale and Lanier, 1991). In the spring and early summer, *I. pini* only responded to male beetles and not to synthetic ipsdienol. In the late summer and fall, there was no significant difference between the numbers of beetles attracted to synthetic ipsdienol and male beetles. This seasonal variation in response to pheromone is associated with population density, which is usually highest in late summer and fall (unpub-

lished data). Lanier et al. (1980) conducted field tests of synthetic ipsdienol in August and September when *I. pini* frequently responds to an incomplete component blend (i.e., ipsdienol). Stewart (1975) described the field site of Lanier et al. (1980) as having been thinned in the spring of the year previous to the field test. If Lanier et al. (1980) had conducted their field tests in the spring or at a lower density site (undisturbed), they probably would not have observed attraction to synthetic ipsdienol. The role of lanierone is the key to understanding seasonal variation in response to ipsdienol in New York. Because variation in response to ipsdienol is dependent on population density (unpublished data), production of lanierone probably does not vary significantly with season. This can now be determined by direct measurement.

There is no a priori contradiction between our results and those of Birch et al. (1980) and Miller et al. (1990). In view of the differences in ipsdienol stereochemistry between New York and western populations, the possibility of geographic variation in the presence of lanierone cannot be ruled out. It is not known if lanierone is produced by the California population, and unless this is demonstrated, there is no inherent incongruity between our results and those of Birch et al. (1980).

Acknowledgments—We thank D. Kiemle, Department of Chemistry, College of Environmental Science and Forestry, SUNY, and G. Levy, Department of Chemistry, Syracuse University for NMR spectra; R.M. Silverstein and J.R. West for GC fractionations during preliminary stages of this work; H.M. Fales and T. Spande, National Institutes of Health, Bethesda, Maryland, for GC-FT-IR spectrometry; and S.J. Seybold, Department of Entomology and Parasitology, University of California, Berkeley, and anonymous reviewers for critical reviews of the manuscript. This research was funded by NSF grant DCB8608077 and USDA-CSRS grant 8802317.

REFERENCES

BIRCH, M.C., LIGHT, D.M., WOOD, D.L., BROWNE, L.E., SILVERSTEIN, R.M., BERGOT, B.J., OHLOFF, G., WEST, J.R., and YOUNG, J.C. 1980. Pheromonal attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. *J. Chem. Ecol.* 6:703–717.

BIRGERSSON, G., SCHLYTER, F., LÖFQVIST, J., and BERGSTROM, G. 1984. Quantitative variation of pheromone components in the spruce bark beetle *Ips typographus* from different attack phases. *J. Chem. Ecol.* 10:1029–1055.

BROWNLEE, R.G., and SILVERSTEIN, R.M. 1968. A micro-preparative gas chromatograph and modified carbon skeleton determinator. *Anal. Chem.* 40:2077–2079.

BYERS, J.A., BIRGERSSON, G., LÖFQVIST, J., APPELGREN, M., and BERGSTROM, G. 1990. Isolation of pheromone synergists of bark beetle, *Pityogenes chalcographus*, from complex insect-plant odors by fractionation and subtractive-combination bioassay. *J. Chem. Ecol.* 16:861–876.

BYRNE, K.J., GORE, W.E., PEARCE, G.T., and SILVERSTEIN, R.M. 1975. Porapak Q collection of airborne organic compounds serving as models for insect pheromones. *J. Chem. Ecol.* 1:1–7.

DEMOLE, E.P. 1975. Use of cycloaliphatic compounds as perfumes in foods and drinks. *Chem. Abs.* 82:394.

BYRNE, K.J., GORE, W.E., PEARCE, G.T., and SILVERSTEIN, R.M. 1975. Porapak Q collection of airborne organic compounds serving as models for insect pheromones. *J. Chem. Ecol.* 1:1–7.

DEMOLE, E.P. 1975. Use of cycloaliphatic compounds as perfumes in foods and drinks. *Chem. Abs.* 82:394.

FRIMER, A.A., GILINSKY-SHARON, P., ALJADEFF, G., GOTTLIEB, H.E., HAMEIRI-BUCH, J., MARKS, V., PHILOSOF, R., and ROSENTAL, Z. 1989. Superoxide anion radical ($O_2 \cdot^-$) mediated base catalyzed autoxidation of enones. *J. Org. Chem.* 54:4853–4866.

GREIS, G., PIERCE, H.D., JR., LINDGREN, B.S., and BORDEN, J.H. 1988. New techniques for capturing and analyzing semiochemicals for scolytid beetles (Coleoptera: Scolytidae). *J. Econ. Entomol.* 81:1715–1720.

LANIER, G.N., BIRCH, M.C., SCHMITZ, R.F., and FURNISS, M.M. 1972. Pheromones of *Ips pini* (Coleoptera: Scolytidae): variation in response among three populations. *Can. Entomol.* 104:1917–1923.

LANIER, G.N., GORE, W.E., PEARCE, G.T., PEACOCK, J.W., and SILVERSTEIN, R.M. 1977. Response of the European elm bark beetle, *Scolytus mulitstriatus* (Coleoptera: Scolytidae), to isomers and components of its pheromone. *J. Chem. Ecol.* 3:1–8.

LANIER, G.N., CLAESSON, A., STEWART, T., PISTON, J., and SILVERSTEIN, R.M. 1980. *Ips pini:* The basis for interpopulational differences in pheromone biology. *J. Chem. Ecol.* 6:677–687.

LINDGREN, B.S. 1983. A multiple funnel trap for scolytid beetles (Coleoptera). *Can. Entomol.* 115:299–302.

MENDENHALL, W. 1983. Introduction to Probability and Statistics. Prindle, Weber and Schmidt Publ., Boston.

MILLER, D.R., and BORDEN, J.H. 1990. β-Phellandrene: Kairomone for the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). *J. Chem. Ecol.* 16:2519–2531.

MILLER, D.R., GREIS, G., and BORDEN, J.H. 1990. (*E*)-Myrcenol: A new pheromone for the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). *Can. Entomol.* 122:401–406.

MOECK, H.A. 1970. An olfactometer for the bio-assay of attractants for scolytids. *Can. Entomol.* 102:792–796.

SILVERSTEIN, R.M., RODIN, J.O., and WOOD, D.L. 1967. Methodology for isolation and identification of insect pheromones with reference to studies on California five-spined ips. *J. Econ. Entomol.* 60:944–949.

SLESSOR, K.N., KING, G.G.S., MILLER, D.R., WINSTON, M.L., and CUTFORTH, T.L. 1985. Determination of chirality of alcohol or latent alcohol semiochemicals in individual insects. *J. Chem. Ecol.* 11:1659–1667.

SMITH, A.B., III, BELCHER, A.M., EPPLE, G., JURS, P.C., and LAVINE, B. 1985. Computerized pattern recognition: A new technique for the analysis of chemical communication. *Science* 228:175–177.

STEWART, T.E. 1975. Volatiles isolated from *Ips pini:* Isolation, identification, enantiomeric composition, biological activity. MSc thesis. College of Environmental Science and Forestry, SUNY, Syracuse, New York.

STILL, W.C., KAHN, M., and MITRA, A. 1978. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 43:2923–2925.

TEALE, S.A., and LANIER, G.N. 1991. Seasonal variability in response of *Ips pini* (Coleoptera: Scolytidae) to ipsdienol in New York *J. Chem. Ecol.* 17:1145–1158.

VITÉ, J.P. 1967. Sex attractants in frass from bark beetles. *Science* 156:105.

VITÉ, J.P., and RENWICK, J.A.A. 1970. Differential diagnosis and isolation of population attractants. *Contrib. Boyce Thompson Inst.* 24:323–328.

WOOD, D.L., and BUSHING, R.W. 1963. The olfactory response of *Ips confusus* (LeConte) (Coleoptera: Scolytidae) to the secondary attraction in the laboratory. *Can. Entomol.* 95:1066–1078.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

CONDENSED TANNINS, ATTINE ANTS, AND THE PERFORMANCE OF A SYMBIOTIC FUNGUS

COLIN NICHOLS-ORIANS[1,2]

[1]*Department of Entomology*
Pennsylvania State University
University Park, Pennsylvania 16802

(Received November 26, 1990; accepted February 13, 1991)

Abstract—Field experiments indicate that the foliar concentration of condensed tannin affects the selection of leaf material of *Inga oerstediana* Benth., a tropical legume tree, by leaf cutter ants. In one study an increase in tannin concentration was correlated with a decrease in the acceptability of leaves to leaf-cutter ants, except at low tannin concentrations. Protein concentration was not correlated with acceptability nor was the ratio of protein to tannin. Results from a second study suggest that when the concentration of tannin was low the ants appear to select leaves on the basis of nutrient availability. Laboratory assays with the ants indicated that quebracho tannin, a commercially available condensed tannin, inhibits foraging ants. Again, at lower concentrations, quebracho tannin appeared to have little affect on the ants.The fungus the ants cultivate is a wood-rotting Basidiomycete that produces enzymes, such as polyphenol oxidase (PPO), that are capable of inactivating tannins. The activity of these PPOs may explain why leaf-cutter ants are undeterred by low concentrations of condensed tannins. I hypothesized that PPO activity would be absent from fungal cultures without tannin and that only high concentrations of tannin would inhibit the fungus. Cultures with and without tannin showed similar PPO activity. Thus PPO activity is constitutive. In fact, as fungal biomass increased, so did PPO activity. As hypothesized, only high concentrations of quebracho tannin inhibited PPO activity and fungal growth. However, it is not clear whether the ants can discriminate between concentrations that do and do not inhibit the fungus.

Key Words—*Atta cephalotes*, attine ants, Hymenoptera, Formicidae, *Inga oerstediana*, plant-herbivore interactions, host selection, condensed tannins, fungal performance, polyphenol oxidase.

[2]Present address: Department of Biology, Vassar College, Poughkeepsie, New York 12601.

0098-0331/91/0600-1177$06.50/0

INTRODUCTION

The concentrations of nutrients and phenolics typically differ in leaves of plants growing under different environmental conditions and in leaves of different ages, and these chemical traits may influence patterns of herbivory (Fox and Macauley, 1977; Chandler and Goosem, 1982; Coley, 1983a,b; Waterman et al., 1984; Meyer and Montgomery, 1987; Mole and Waterman, 1988; Denslow et al., 1990; Nichols-Orians and Schultz, 1990). Tannins are one class of phenolics that may influence selection of leaves by herbivores (Waltz, 1984; Mole and Waterman, 1988; Schultz, 1989). However, Wint (1983) and Mole and Waterman (1988) suggest that selection would not depend upon the concentration of nutrients or tannins, but upon the ratio of the two.

Leaf-cutter ants exhibit strong intraspecific preferences for host foliage (Fennah, 1950; Cherrett, 1968, 1972; Rockwood, 1976, 1977; Nichols-Orians and Schultz, 1990), and this appears to be related to the availability of nutrients and the concentrations of secondary chemicals (Barrer and Cherrett, 1972; Howard, 1990; Nichols-Orians, 1991a,b). Although terpenoids are widely recognized for their ability to deter leaf-cutter ants (Hubbell and Wiemer, 1983), some plant species appear to contain tannins in sufficient quantities to deter foraging ants (Howard, 1990; Nichols-Orians and Schultz, 1990).

Leaves of sun plants growing in nutrient-poor soils are generally less susceptible to leaf-cutter ants than leaves of plants growing in nutrient-rich soils (Nichols-Orians, 1991b; R. Marquis, personal communication). This may be because these growth conditions result in elevated concentrations in some plant species of carbon-based secondary compounds like tannins (Chandler and Goosem, 1982; Nichols-Orians, 1991b). However, leaves of plants growing in the sun and in nutrient-rich soil often have higher concentrations of both condensed tannins and foliar nutrients than the leaves of plants growing in the forest understory (Field and Mooney, 1986; Denslow et al., 1990; Nichols-Orians, 1991a), and these sun leaves have been found to be more acceptable to leaf-cutter ants than understory leaves (Nichols-Orians, 1991a; R. Marquis, personal communication). Thus, moderate concentrations of condensed tannins do not appear to deter foraging ants.

In addition to environmentally based differences in chemistry and acceptability, there may be phenological differences as well. Young leaves usually contain higher concentrations of nutrients and usually are more acceptable to leaf-cutter ants than mature leaves (Barrer and Cherrett, 1972; Waller, 1982). However, in one study (Nichols-Orians and Schultz, 1990), we found young leaves to be less acceptable chemically to leaf-cutter ants than mature leaves, despite higher concentrations of nutrients. These young leaves had much higher concentrations of condensed tannins. Young leaves of neotropical trees often have higher concentrations of tannins and other phenolics than mature leaves (Coley, 1983a,b), which suggests that leaf-cutter ants often may find young

leaves less acceptable. Recently, Coley and Aide (1989) found that as anthocyanin concentration increases leaves are less acceptable to leaf-cutter ants. Anthocyanin is structurally similar to tannins and, like tannins, has antibiotic properties. All these studies suggest that the chemical acceptability of leaves to leaf-cutter ants may depend upon both the concentrations of nutrients and tannins and perhaps upon the ratio of the two.

Here, I evaluate trade-offs in host plant acceptability with respect to levels of foliar protein and condensed tannin. I divided this study into two parts. First, using leaves from plants growing in different light and soil nutrient conditions, I investigated how quantitative differences in the concentrations of protein and condensed tannins influence the selection of leaves by leaf-cutter ants, and compare these results to those of previous studies on this system (Nichols-Orians and Schultz, 1990; Nichols-Orians, 1991a,b). Second, I determined how varying the concentration of a commercially available condensed tannin, quebracho tannin, influences selection by the ants and performance of the symbiotic fungus the ants cultivate. The first part of the study was done with field colonies in Costa Rica and the second part with laboratory colonies at Pennsylvania State University.

The symbiotic fungus is a Basidiomycete fungus that produces enzymes, polyphenol oxidases and tannases, which are capable of inactivating tannins via polymerization (Cherrett et al., 1989). The ability of the fungus to inactivate tannins may explain why leaf-cutter ants appear insensitive to relatively low concentrations of tannin (Nichols-Orians, 1991a). As with many fungal enzymes (Cooper and Wood, 1973, 1975), polyphenol oxidases (PPOs) can be induced by the presence of certain chemicals, such as gallic acid, a precursor of tannin (Mayer, 1987). The PPOs produced by the ants' fungus may be induced by the presence of condensed tannin and, once produced, polymerize and inactivate the tannins. Therefore, I hypothesized that PPO activity would only be present in fungal cultures containing tannin. Furthermore, I predicted that low concentrations of tannin would be quickly inactivated and so would not inhibit PPO activity or fungal growth. Since high concentrations of tannin may inhibit PPOs (Lyr, 1962), fungal growth may be inhibited as well. Because PPOs are produced extracellularly, as evident from the formation of colored oxidative products following addition of fungus to media containing tannins and other phenolics (Bavendamm's test) (Kirk and Kelman, 1965; unpublished data), it is easy to quantify PPO activity.

METHODS AND MATERIALS

Site

The study was conducted at the Organization for Tropical Studies' La Selva Biological Station (10°26′N, 83°59′W) in the Atlantic lowland near Puerto Viejo de Sarapiqui, Costa Rica. This forest is a tropical wet premontane forest

(Holdridge et al., 1971) and receives a mean annual rainfall of 4000 mm (Hartshorn, 1983), with a short dry season from late January to April (La Selva, unpublished records). The field portion of this study was conducted from January to July 1987.

Laboratory assays with the ants and the fungus were conducted in 1989–1990 with colonies maintained at Pennsylvania State University. Colonies were collected from La Selva in January 1987 and maintained on a diet of frozen *Forsythia* and fresh cabbage during the experiments.

Leaf-Cutter Ants/Fungus

Colonies of a leaf-cutter ant, *Atta cephalotes* (L.) (Hymenoptera: Formicidae: Attini), are abundant within La Selva and naturally encounter plants growing under different environmental conditions (personal observation). Leaf-cutter ants use the harvested leaf material to support the growth of a symbiotic fungus, which is the sole food source of the developing larvae (Quinlan and Cherrett, 1979). Hydrolyzable tannins have been shown to inhibit the growth of an attine fungus (Seaman, 1984), but condensed tannins appear to be even more effective inhibitors of the fungi (Cherrett et al., 1989). This result is consistent with the observation that condensed tannins are very effective inhibitors of fungi and their enzymes (Zucker, 1983). Therefore leaf-cutter ants would be expected to avoid leaves with tannin levels sufficient to inhibit the fungus. If tannin levels are low, then nutrients would be expected to dictate acceptability (Berish, 1986; Waltz, 1984).

Plant Species

The tropical legume tree, *Inga oerstediana* Benth. [formerly *Inga edulis* var. *minutula* Schery, (Nichols-Orians and Schultz, 1990)] (Fabaceae: Mimosoideae) is naturally attacked by leaf-cutter ants (personal observation). Yet *Inga* is regarded as being relatively resistant to leaf-cutter ants (Arckoll, 1984). *Inga* spp. produce high concentrations of tannins (Koptur, 1985; Nichols-Orians, 1990, 1991a,b), so the low susceptibility of *Inga* to leaf-cutter ants may be due to these tannins.

Plant Treatments

In a previous study I showed that light and soil nutrient conditions influence foliar chemistry and the acceptability of leaves to leaf-cutter ants (Nichols-Orians, 1991b). For a subset of the plants used in the above study ($N = 48$), I measured foliar chemistry and conducted bioassays with the leaves to determine their acceptability to the ants. This study evaluates the relationship between chemistry and acceptability.

The following is a brief description of the growth conditions. Initially, field-collected seedlings were grown in pots containing either nutrient-rich alluvial or nutrient-poor residual soils (Vitousek and Denslow, 1987) at 20%-of-full-sunlight. After 13 weeks half the seedlings were transferred to 2%-of-full-sunlight and grown for an additional seven weeks. At the same time I altered light conditions, I initiated fertilization treatments. Thus, there were eight treatments with six seedlings in each treatment (Figure 1). I noted when leaves matured throughout the experiment so that at the end of the 20 weeks two different leaf types were harvested from each plant: (1) those that had matured during the first 13 weeks (PRE leaves) and (2) those that developed and matured during the last seven weeks (POST leaves).

Leaf Material

Both PRE and POST leaves were collected seven weeks (±4.5 days) after light and nutrient manipulation. Seedlings were first transported in their pots to the study colony and a minimal amount of leaf material was used during the bioassays with the ants. *I. oerstediana* leaves are compound. Bioassays to determine how leaves differed in acceptability were conducted with disks from the distal leaflets only in order to avoid confounding leaflet position effects (Gall, 1987). Although I have not detected position effects on acceptability (personal observation), Gall (1987) found that basal leaflets in a number of Juglandaceae species were less acceptable to various *Catocala* larvae (Noctuidae) than either lateral or terminal leaflets.

Once bioassays were complete (approximately 30 min), all remaining leaf material was removed, immediately frozen, freeze-dried, and ground to a powder, with a UDY Cyclone Mill, at La Selva before being transported back to Pennsylvania State University for chemical analyses. Because of the large amount of leaf material required for chemical analyses, chemistry was deter-

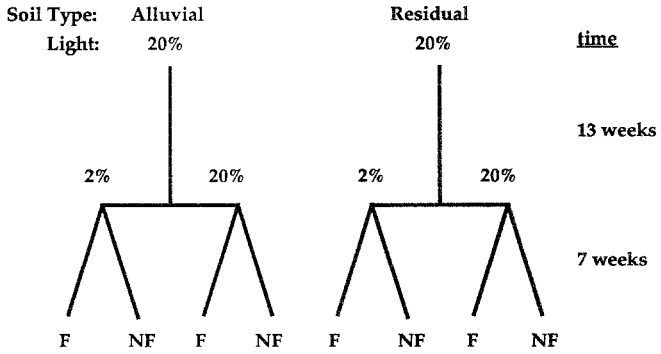


FIG. 1. Schematic diagram of treatments. F = fertilized, NF = not fertilized.

mined from the analysis of entire leaves minus the petiole. Although removal of leaf material during the bioassays could conceivably cause a change in chemistry, I found no change in chemistry 24 hr after damaging *I. oerstediana* seedlings (Nichols-Orians, 1991a).

Unfortunately there was not enough leaf material from some plants to conduct bioassays and do chemical analyses. Therefore, the correlation between chemistry and acceptability was done on a subset of leaves ($N = 37$). Even with the reduced sample size, there was extensive variability in leaf chemistry. In fact, the range of values reported here very nearly expand the full range measured previously in *I. oerstediana* leaves (Nichols-Orians, 1990, 1991a,b).

Chemical Analyses

For analysis of protein concentration, I extracted approximately 20 mg of leaf powder in 10 ml of 0.1 N NaOH for 2 hr in a boiling water bath and used Bio-Rad reagent to quantify protein content (Snyder and Desborough, 1978; Compton and Jones, 1985). This extraction technique is designed to measure the concentration of protein in leaves high in tannin (Jones et al., 1989).

For the analysis of the various phenolic traits, leaf powder (300–400 mg) was washed with ether for 30 min. Phenolics were extracted with 70% acetone for 3 hr at 40°C. The acetone was removed under reduced pressure, and the extracts were diluted to 10 ml with distilled water. Extracts were analyzed for proanthocyanidin condensed tannins (butanol–HCl method; Bate-Smith, 1977), leucoanthocyanin condensed tannins (vanillin method; Broadhurst and Jones, 1978; Butler et al., 1982), and protein-binding capacity using hemoglobin as the substrate (Schultz et al., 1981). Relative concentrations are expressed as wattle tannin (*Acacia* sp. from Leon Monnier, Inc., Peabody, Massachusetts) equivalents per milligram of dry weight (%WTE) for the two condensed tannins and as tannic acid (Sigma, Lot No. 11F-0559) equivalents per milligram of dry weight (%TAE) for protein-binding capacity. I also measured total phenolics (Folin-Denis assay; Swain and Hillis, 1959), but because they were over 90% correlated with the two condensed tannins I am restricting my discussion to the two tannins and protein-binding capacity. Thus far, surveys indicate that tannins are the only secondary compounds in *Inga* (Koptur, 1985), and only condensed tannins are abundant in *I. oerstediana* (unpublished data).

Bioassays with Leaves

All bioassays were done with a single colony. Previous work has shown that the relative acceptability of leaf types of *I. oerstediana* to leaf-cutter ants is similar among ant colonies (Nichols-Orians and Schultz, 1990; Nichols-Orians, 1991a).

I conducted the "pickup" assay to assess the chemical acceptability of

each leaf type to leaf-cutter ants (Howard, 1987; Nichols-Orians and Schultz, 1990). The pickup assay is designed to determine preferences based on chemical differences, defensive or nutritional, between leaf types (Howard, 1987). Two leaf disks produced with a standard paper punch, were placed beside two disks of a highly preferred control, *Hamelia patens* (Rubiaceae). When one disk was removed it was replaced by a like disk. To ensure that the assays with the various leaf types were independent, I separated them by 9 in. and randomized the order of presentation each day. The chemical acceptability of a given leaf type was expressed as the number of leaf disks removed when 20 control disks had been removed. The final acceptability of each leaf type was the average number of leaf disks removed during two replicate trials.

The use of a control indicated whether ants were willing to pick up leaf disks on any given day and standardized for fluctuations in ant activity during each assay replication. *H. patens* was used as the control because of its high acceptability to leaf-cutter ants and because numerous plants can be found growing under similar conditions. It is abundant in a clearing near the study colony. The use of *H. patens* as a control yields very consistent results (Nichols-Orians and Schultz, 1990; Nichols-Orians 1991a,b).

Physical features of a leaf (e.g., trichomes) could influence disk removal, but I did not identify any such physical features in *I. oerstediana*. Conceivably, leaf mass could influence disk removal as well. However, the leaf disks were small enough that all foraging ants could easily pick up these leaf disks. In fact, the toughest and heaviest leaves assayed to date were highly acceptable to the ants (Nichols-Orians and Schultz, 1990). Thus, leaf mass differences among these treatments would not have influenced the results.

Researchers often employ a "cutting" assay when testing whether physical features interfere with the harvesting of leaves by leaf-cutter ants (Howard, 1988; Nichols-Orians and Schultz, 1989, 1990). I did not employ this assay because I was only interested in determining the relationship between nutrients and tannins on the initial selection of leaves by leaf-cutter ants.

Laboratory Bioassays

Bioassays with Ants. I determined whether quebracho tannin influences foraging ants using two laboratory-maintained colonies (collected from La Selva, Costa Rica, in January 1987). Quebracho tannin was obtained from the Van Dyke Supply Co. (Woonsocket, South Dakota). Seven different tannin treatments (concentrations) were tested: 10, 5, 2.5, 1, 0.5, 0.25 and 0.1% (w/v). Distilled water was used at all times. Organic rye (1 g) flakes were soaked for 2 min in 2 ml of solution (one of the seven tannin treatments or a water only control). The flakes were drained and dried at 25°C overnight.

During each assay, 50 treatment flakes and 50 control flakes were placed

on a glass plate above a randomly generated grid on a foraging platform (design similar to Hubbell and Wiemer, 1983). Treatment and control flakes were placed on one of two patterned boxes. Ants were allowed access to the foraging platform and the number of control and treatment flake types remaining was determined every 2 min. The assay was terminated when the ants removed 25 of either flake type. After each run the access holes were plugged and the ants removed before initiating another assay. Because control flakes often were placed during subsequent assays where treatment flakes had been, and visa versa, the glass plate was wiped with acetone to prevent pheromone carry-over. The acceptability of each treatment is expressed as the number of treatment flakes removed when 25 control flakes had been removed. (Occasionally 25 treatment flakes were removed first. For these assays the number treatment flakes/25 control flakes was calculated algebraically.)

All treatments were tested on both colonies on the same day. The order in which the different concentrations were tested was randomized each day for each colony. Also treatment flakes were randomly assigned to one of the two patterned boxes for each assay. In all, there were five replicate assays for each colony. On the first day, only three treatments (10%, 1%, and 0.1%) were tested.

Fungal Bioassays. The fungus from one of the two colonies tested above was isolated, maintained on potato dextrose agar (PDA) plates, transferred to Sabaroud's dextrose broth (SDB), and later grown in a liquid medium designed to test for the effects of tannins on fungal performance (Seaman, 1984). This FPT medium contained the following components per liter of distilled water: (1) major components (g)—KH_2PO_4 (1), $MgSO_4 \cdot 7H_2O$ (0.5), NaCl (0.1), $CaCl_2 \cdot 2H_2O$ (0.1), NH_4 tartrate (5.0), and dextrose (20); (2) minor components (mg)—$ZnSO_4 \cdot 4H_2O$ (4), $MnSO_4 \cdot 4H_2O$ (4), and ferric tartrate (4); (3) organic supplement (mg)—4-methyl-5-thiazole ethanol (0.180), and 4-amino-5-aminomethyl-2-methylpyrimidine dihydrochloride (0.250). In these experiments, 500 ml of distilled water was replaced with 500 ml of 0.05 M dimethyl glutaric acid buffer (pH 5.5) (Powell and Stradling, 1986).

Three basic in vitro experiments were conducted. First, at each tannin concentration I determined the effect of tannin on polyphenol oxidase (PPO) activity. Second, I measured changes in PPO activity in liquid cultures of fungi incubated with quebracho tannin for different lengths of time (10, 30, 60, or 120 min, and 2, 7, or 21 days). In a separate experiment, there were three replications per treatment at day 2. Finally, I measured differences in fungal biomass after 21 days of incubation and regressed PPO activity against biomass.

Fungal Growth Assays. Fungus obtained from PDA plates was placed in 65 ml SDB and blended for 1 min in a Vertis blender at low speed. Five milliliters of the fungal suspension were pipetted into Erlenmeyer flasks containing 45 ml of SDB. The cultures were then grown for 10 weeks in the dark. After

10 weeks, the fungus was filtered, resuspended in modified FPT (50 ml FPT/flask of fungus), and blended for 1 min at low speed. The fungus was kept evenly suspended using a stir bar and magnetic plate.

One milliliter of fungal suspension was added to 8 ml of modified FPT in sterile test tubes and placed for four days in the dark at room temperature. After four days 1 ml of one of four quebracho tannin solutions was added to the suspension to give a final concentration of 0, 0.0025, 0.025, and 0.25% quebracho tannin. At various time intervals from 10 min to 21 days, the activity of polyphenol oxidase (PPO) was determined (using the protocol described below), and at 21 days the dry mass of the fungus in each treatment was determined.

PPO Activity. Catechol is commonly used as a substrate to measure PPO activity (Flurkey and Jen, 1978). Liquid cultures of fungus were centrifuged at 1000 rpm for 10 min and the supernatants placed on ice. In test tubes, 0.75 ml of the supernatant from the fungal cultures were combined with 2 ml 0.1 M citric acid–sodium citrate buffer (pH 5.5). Following the addition of 0.25 ml of catechol (or distilled water for controls), the test tubes were vortexed and incubated at 30°C for 30 min, and then the final absorbance was determined (420 nm) with a spectrophotometer. After subtracting the absorbance of the controls, a final PPO activity was obtained.

Finally, because the concentration of tannin in solution influences pH, I also determined whether differences in pH would influence PPO activity. Using a 0.1 M citric acid–sodium citrate buffer, the effects of three different pH solutions (4.0, 5.0, 6.0) on PPO activity were tested. Solutions were incubated for two days before measuring PPO activity.

Statistical Analyses

All statistical analyses were done using SAS (SAS Institute, 1985). Chemistry data were natural log transformed prior to analyses.

Bioassays with Leaves. The number of leaf disks removed during the pickup assay was regressed against each of the chemical traits measured. In the process, three outliers were identified (based on studentized residuals) and removed. Because Mole and Waterman (1988) suggested that the ratio of protein to tannin may be the key to the susceptibility of plants to herbivores, I also divided the concentration of protein by each of the three phenolic traits (the two condensed tannins and protein-binding capacity) and regressed the number of leaf disks removed against each of these ratios.

Laboratory Bioassays with Ants. Neither the order in which the treatments were tested nor the colony tested had a significant effect on leaf pickup assays. Therefore the effects of increasing tannin concentration on the acceptability of rye flakes was determined using the following one-way ANOVA model: flakes removed ($\bar{X}$) = constant + ln (tannin concentration). A Bonferroni test was

used to compare differences in acceptability among the different tannin concentrations.

Laboratory Fungal Bioassays. The effect of increasing tannin concentration on PPO activity also was analyzed with an ANOVA model with tannin concentration as the main effect. A Bonferroni test was used to compare treatment means. Unfortunately, the quebracho tannin bound to the fungus, so I was unable to determine the effects of tannin concentration on fungal growth. A regression analysis was used to determine the relationship between fungal biomass and PPO activity.

RESULTS

Bioassays with Leaves

Results from the regression analysis indicate that the acceptability of leaves to leaf-cutter ants was negatively correlated with protein-binding capacity (PBC) and the two condensed tannins (Figure 2A–C). The fact that all three traits gave similar negative correlations was because PBC and the two condensed tannins were all highly correlated ($P \geq 0.91$). For both condensed tannin traits, proanthocyanidin (PA) and leucoanthocyanin (VN) condensed tannins, a quadratic model provided the highest correlation. This suggests that lower concentrations of tannins did not deter the ants. However, linear and quadratic models provided identical correlation coefficients for PBC. Protein (PRT) concentration was not correlated with leaf acceptability ($R^2 = 0.02$, $P = 0.651$) and the ratio of PRT to PBC, PA, or VN did not improve the correlation (PRT/PBC, $R^2 = 0.31$; PRT/PA, $R^2 = 0.23$; PRT/VN, $R^2 = 0.24$).

Laboratory Bioassays

Bioassays with Ants. An analysis of variance indicates that the concentration of quebracho tannin (QT) affected the acceptability of rye flakes to leaf-cutter ants (Figure 3). Lower concentrations did not deter the ants, while higher concentrations did deter them.

Fungal Bioassays. Quebracho tannin did inhibit fungal PPO activity. Although the addition of QT to the fungal suspensions lowered pH, the inhibitory effect was not due to a change in pH (Table 1). I found no difference in the PPO activity of fungal cultures buffered at pH 5.0 and 6.0. Only at pH 4 was there a reduction in PPO activity (PPO = 0.075).

Incubation of QT with the fungus culture for two days demonstrated that PPO activity is inhibited only at the highest concentration (Figure 4). Only high concentrations appeared to inhibit the fungus as well. After 21 days, fungal cultures in 0.25% QT had died, whereas those at the lower concentrations were still growing vigorously.

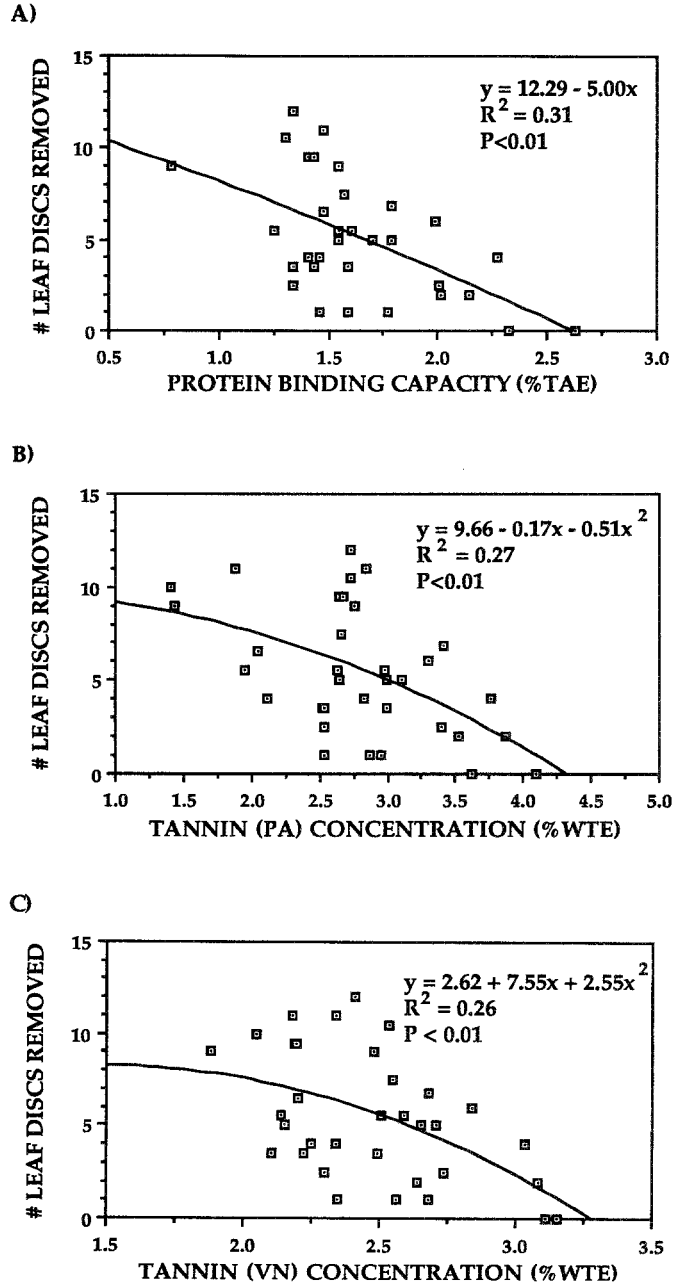


FIG. 2. Regression analysis showing the relationship between the acceptability of leaf disks to leaf-cutter ants and three phenolic traits: (A) protein-binding capacity, (B) proanthocyanidin (PA) condensed tannin, (C) leucoanthocyanin (VN) condensed tannin. Chemical data were natural log transformed.

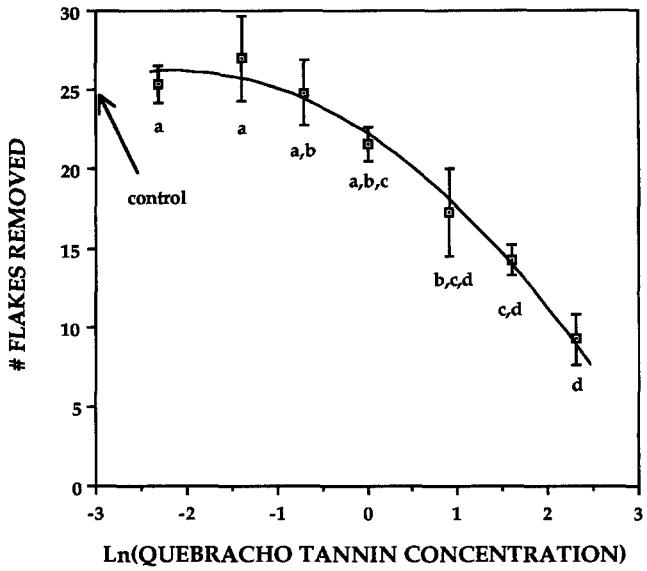


FIG. 3. Analysis of variance results showing the relationship between the acceptability of rye flakes (± standard error) to leaf-cutter ants and the concentration of quebracho tannin. Chemical data were natural log transformed.

TABLE 1. EFFECTS OF QUEBRACHO TANNIN (QT) AND pH ON PPO ACTIVITY[a]

Concentration (%)	pH	PPO
A. Quebracho tannin		
0	5.32	0.105
0.0025	5.28	0.114
0.025	5.28	0.093
0.25	5.16	0.015
B. Buffer		
	6.0	0.085
	5.0	0.086
	4.0	0.075

[a] PPO activity was measured as the change in absorbance following incubation of fungal suspension with catechol for 30 minutes. (A) The effects of QT on pH and PPO activity, and (B) the effects of pH on PPO activity. In (B), a 0.10 M citric acid–sodium citrate buffer was used to manipulate pH.

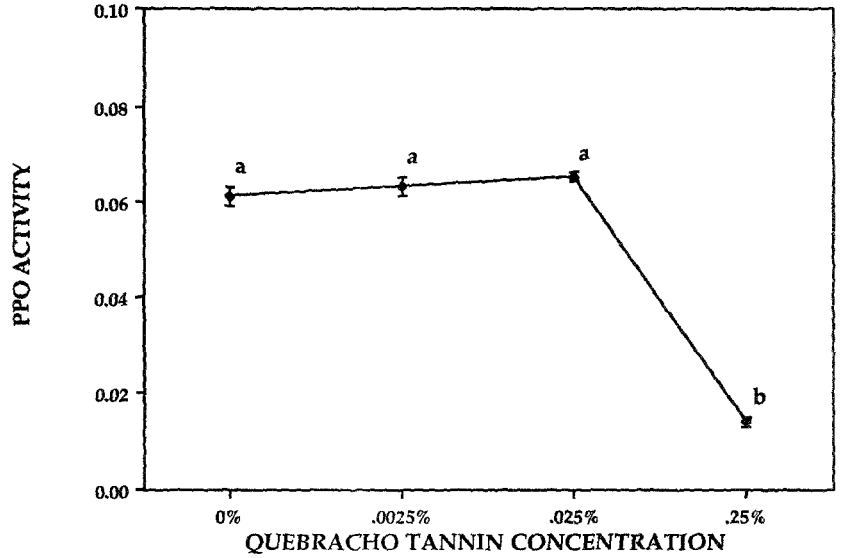


FIG. 4. Polyphenol oxidase activity (± standard error) after two days in fungal cultures containing different concentrations of quebracho tannin. *x* axis is log scaled.

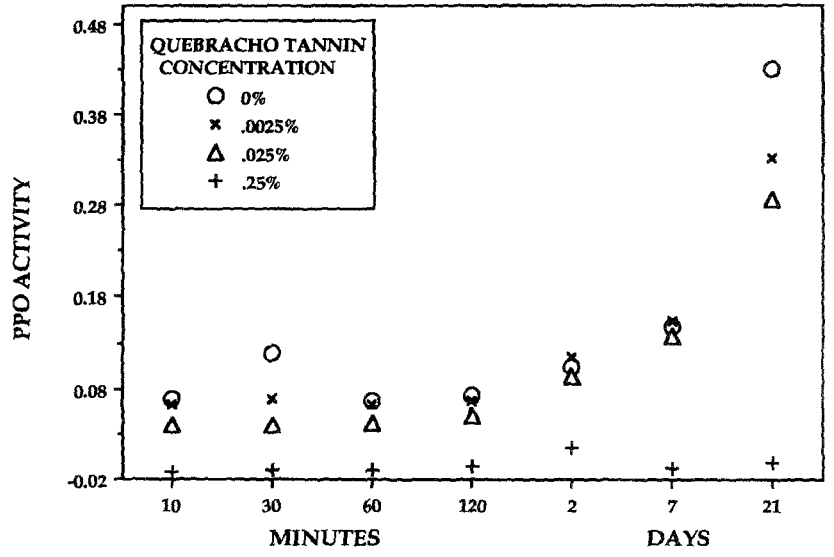


FIG. 5. The effects of different concentrations of quebracho tannin on the polyphenol oxidase activity through time.

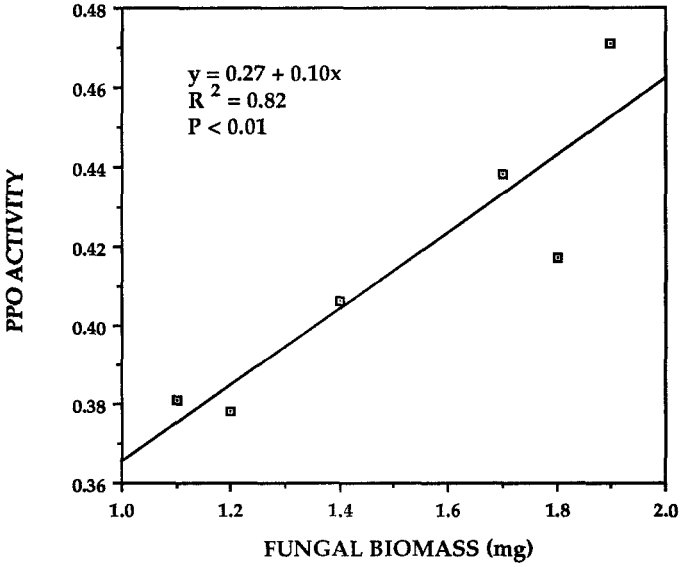


FIG. 6. The effects of fungal biomass on polyphenol oxidase activity. All measures taken from 21- day-old tannin-free fungal cultures.

There was no evidence that the presence of tannin was necessary for the production of PPO (Figures 4 and 5). PPO activity in supernatants without tannin was as high if not higher than in supernatants with tannin at several sampling periods (Figure 5). Rather, PPO activity is correlated with fungal biomass (Figure 6). In 21-day-old control (0% QT) cultures, the greater the biomass the higher the PPO activity.

DISCUSSION

Tannins do not deter herbivores uniformly (Bernays, 1978, 1981; Schultz, 1989). Some herbivores appear adapted for consuming high tannin leaves (see Schultz, 1989). For others, some dietary tannin may actually be beneficial (Bernays, 1978; Keating et al., 1988). Previous research on the interactions between *I. oerstediana* and leaf-cutter ants suggests that only higher concentrations of tannin deter the ants (Nichols-Orians and Schultz, 1990; Nichols-Orians, 1991a,b). Since the fungus the ants cultivate produces enzymes that facilitate the inactivation of phenolic substances (Cherrett et al., 1989), the fungus may be adapted to handle lower concentrations of tannin. I had hypothesized that leaf-cutter ants may not be sensitive to lower concentrations of tannin because the fungus is capable of inactivating the tannins.

Results of the bioassays with leaves and rye flakes indicate that the condensed tannins of *I. oerstediana* and QT inhibited leaf–fungal substrate selection by leaf-cutter ants (Figures 2 and 3). I should note that the protein-binding capacity of *I. oerstediana* leaves was as high or higher than the flakes (unpublished data), which suggests that the concentrations of QT selected were appropriate. Consistent with previous research (Nichols-Orians, 1991a), very low concentrations did not deter the ants (Figures 2 and 3).

I had hypothesized that only in the presence of QT would there be PPO activity (sensu Mayer, 1987). I further suggested that only higher concentrations of tannin would inhibit PPO activity and, therefore, the fungus. Contrary to my hypothesis, fungal cultures without QT showed PPO activity (Figures 4 and 5). In fact, PPO activity increases with fungal biomass (Figure 6). Thus, the production of PPO is constitutive and does not appear to be induced by QT. However, the results are not definitive because QT can inhibit the enzymes directly (unpublished data). This could have masked an increase in PPO activity above constitutive levels at the lower tannin concentrations. Studies of gene activation are needed to determine whether QT or other phenolics can induce PPO production.

As hypothesized, only a high concentration of quebracho tannin strongly inhibited PPO activity (Figures 4 and 5). This may explain why leaf-cutter ants were most inhibited by higher concentrations of condensed tannins. Unfortunately, it was not possible to compare directly the concentration of QT in the liquid media to the concentration applied to the flakes. Therefore, it is not clear from these results whether leaf-cutter ants can discriminate between a tannin concentration that does and does not inhibit the fungus. Future experiments should investigate this possibility.

Tannins are not the only chemicals that vary among leaves of different ages and among plants in different environments; nutrients vary as well. Both nutrients and secondary chemicals may be responsible for differential acceptability of leaves to herbivores (Fox and Macauley, 1977; Mattson, 1980; Wint, 1983; Waltz, 1984; Mole and Waterman, 1988). Wint (1983) and Mole and Waterman (1988) hypothesized that the selection of leaves by herbivores would not depend upon the concentration of protein or tannin but upon the ratio of protein to tannin. The data presented here do not provide support for their hypothesis.

In a previous study (Nichols-Orians, 1991a), leaves of seedlings growing in full sun had a higher concentration of protein and were more acceptable to leaf-cutter ants than were leaves of understory seedlings, despite a higher concentration of tannin (Figure 7). [Since the concentration of other nutrients, such as nonstructural carbohydrates, may also increase with increasing light availability (Nichols-Orians, 1991a), the preference for sun leaves may not have been due changes in protein concentration specifically.] In another study

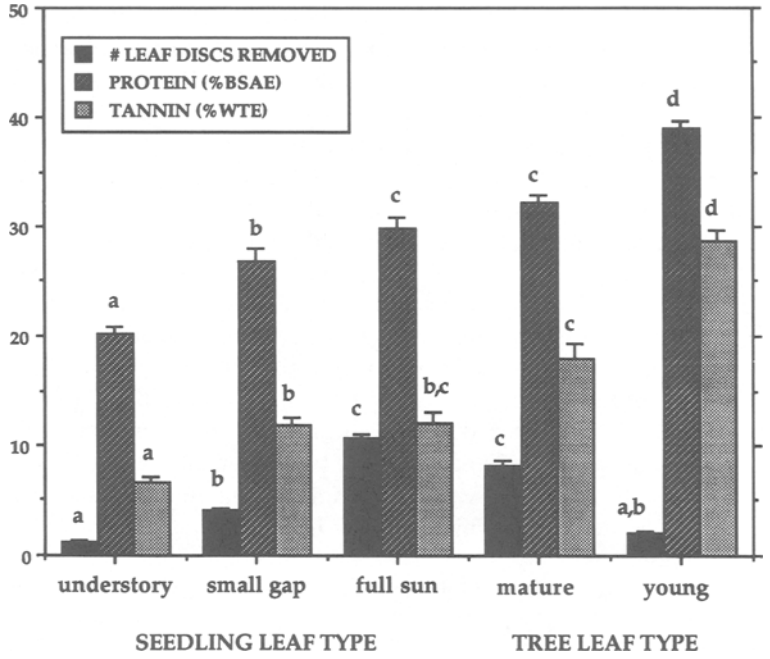


FIG. 7. Relationship between the acceptability of leaf disks to leaf-cutter ants (± SE) and the protein and condensed tannin (leucoanthocyanin) concentration (± SE) in various leaf types. (Included are mature leaves of seedlings growing in different light environments, and young and mature leaves of trees growing in full sun. Chemical data were natural log transformed prior to statistical analyses. Bars (within category) with different letters are different at $P \leq 0.05$. (Data from Nichols-Orians and Schultz, 1990; Nichols-Orians, 1991a).

(Nichols-Orians and Schultz, 1990), young leaves of juvenile trees growing in full sun had a higher protein concentration than mature leaves, yet were less acceptable to the ants. The rejection of the young leaves appeared due to the higher concentration of tannin in young leaves as compared to mature leaves (Figure 7). These studies suggest that, although the ratio of nutrients to tannin appears unimportant, both nutrients and condensed tannins do influence leaf selection by leaf-cutter ants.

Researchers have not always found tannins to deter leaf-cutter ants (Littledyke and Cherrett, 1976; Kawanashi and Raffauf, 1986; Howard, 1987, 1988, 1990). Most of these studies have been qualitative. Since most woody plants produce tannins, yet many produce only a low concentration (Coley, 1983a), qualitative studies may not detect tannin effects on acceptability. Relative to other species (Denslow et al., 1990), *I. oerstediana* produces a high concentra-

tion of tannin (Nichols-Orians, 1990), yet even in this species an increase in tannin does not necessarily lead to a decrease in the acceptability of leaves to leaf-cutter ants (Nichols-Orians, 1991a). Clearly, the inhibition of ants and their fungus by condensed tannins is concentration dependent.

Acknowledgments—H. Appel, J. Cazin, W. McCarthy, C. Mullin, and D. Wiemer provided suggestions for the experimental protocol. The Organization for Tropical Studies (OTS) helped make this research possible. A special thanks go to David and Deborah Clark and Victor Chavarria of OTS for making it possible to live in Puerto Viejo. Marc Pacchioli helped me conduct laboratory bioassays with the ants. Early versions of this paper benefited from the comments of J. Schultz, H. Appel, J. Howard, S. Keating, and two anonymous reviewers. This work was supported by a Jesse Smith Noyes Fellowship Grant awarded by OTS, and by three National Science Foundation Grants [BSR-8605106 to J. Schultz, BSR-8815648 to C. Nichols-Orians (Dissertation Improvement Grant), and BSR-8917752 to R. Fritz.]

REFERENCES

ARKCOLL, D.B. 1984. A comparison of some fast growing species suitable for woodlots in the wet tropics. *Pesquisa Agr. Brasil.* 19:61–68.

BARRER, P.M., and CHERRETT, J.M. 1972. Some factors influencing the site and pattern of leaf-cutting activity in the ant *Atta cephalotes* (L.). *J. Entomol.* 47:15–27.

BATE-SMITH, E.C. 1977. Astringent tannins of *Acer* species. *Phytochemistry* 16:1421–1426.

BERISH, C.W. 1986. Leaf-cutting ants (*Atta cephalotes*) select nitrogen rich forage. *Am. Midl. Nat.* 115:268–276.

BERNAYS, E.A. 1978. Tannins: an alternate viewpoint. *Entomol. Exp. Appl.* 24: 44–53.

BERNAYS, E.A. 1981. Plant tannins and insect herbivores: An appraisal. *Ecol. Entomol.* 6:353–360.

BROADHURST, R.B., and JONES, W.T. 1978. Analysis of condensed tannins using acidified vanillin. *J. Sci. Food Agric.* 29:788–794.

BUTLER, L.G., PRICE, M.L., and BROTHERTON, J.E. 1982. Vanillin assay for proanthocyanidins (condensed tannins): Modification of the solvent for estimation of the degree of polymerization. *J. Agric. Food Chem.* 30:1087–1089.

CHANDLER, G., and GOOSEM, S. 1982. Aspects of rainforest regeneration. III. The interaction of phenols, light and nutrients. *New Phytol.* 92:369–380.

CHERRETT, J.M. 1968. The foraging behavior of *Atta cephalotes* L. (Hymenoptera, Formicidae). 1. Foraging pattern and plant species attacked in tropical rain forest. *J. Anim. Ecol.* 37:387–403.

CHERRETT, J.M. 1972. Some factors involved in the selection of vegetable substrate by *Atta cephalotes* (L.) (Hymenoptera: Formicidae) in tropical rain forest. *J. Anim. Ecol.* 41:647–660.

CHERRETT, J.M., POWELL, R.J., and STRADLING, D.J. 1989. The mutualism between leaf-cutting ants and their fungus, pp. 93–120, *in* N. Wilding, N.M. Collins, P.M. Hammond and J.F Webber (eds.). Insect–Fungus Interactions. Academic Press, London.

COLEY, P.D. 1983a. Herbivory and defensive characteristics of tree species in a lowland tropical forest. *Ecol. Monogr.* 53:209–233.

COLEY, P.D. 1983b. Intraspecific variation in herbivory on two tropical tree species. *Ecology* 64:426–433.

COLEY, P.D., and AIDE, T.M. 1989. Red coloration of tropical young leaves: A possible antifungal defence? *J. Trop. Ecol.* 5:293–300.

COMPTON, S.J., and JONES, C.G. 1985. Mechanisms of dye response and interference in the Bradford protein assay. *Anal. Biochem.* 151:369–374.

COOPER, R.M., and WOOD, R.K.S. 1973. Induction of synthesis of extracellular cell-wall degrading enzymes in vascular wilt fungi. *Nature* 246:309–311.

COOPER, R.M., and WOOD, R.K.S. 1975. Regulation of synthesis of cell wall degrading enzymes by *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*. *Physiol. Plant Pathol.* 5:135–156.

DENSLOW, J.S., SCHULTZ, J.C., VITOUSEK, P.M., and STRAIN, B.R. 1990. Growth responses of tropical shrubs to treefall gap environments. *Ecology* 71:165–179.

FIELD, C., and MOONEY, H.A. 1986. The photosynthesis-nitrogen relationship in wild plants, pp 25–56, *in* T. Givnish (ed.). On the Economy of Plant Form and Function. Cambridge University Press, Cambridge, England.

FENNAH, R.G. 1950. Parasol ants, their life history and methods for their control. *Proc. Agric. Soc. Trinidad* 50:312–326.

FLURKEY, W.H., and JEN, J.J. 1978. Peroxidase and polyphenoloxidase activities in developing peaches. *J. Food Sci.* 43:1828–1831.

FOX, L.R., and MACAULEY, B.J. 1977. Insect grazing on *Eucalyptus* in response to variation in leaf tannins and nitrogen. *Oecologia (Berlin)* 29:145–162.

GALL, L.F. 1987. Leaflet position influence caterpillar feeding and development. *Oikos* 49:172–176.

HARTSHORN, G.S. 1983. Plants: Introduction, pp 136–140, *in* D.H. Janzen (ed.). Costa Rican Natural History. University of Chicago Press, Chicago, IL.

HOLDRIDGE, L.R., GREMEKE, W.C., HATHEWAY, W.H., LIANG, T., and TOSE, J. JR. 1971. Forest Environments in Tropical Life Zones: A Pilot Study. Pergamon Press, Oxford. 747 pp.

HOWARD, J.J. 1987. Leaf-cutting ant diet selection: The role of nutrients, water and secondary chemistry. *Ecology* 68:503–515.

HOWARD, J.J. 1988. Leaf-cutting ant diet selection: The relative influence of leaf chemistry and physical features. *Ecology* 69:250–260.

HOWARD, J.J. 1990. Infidelity of leafcutting ants to host plants: Resource heterogeneity or defense induction? *Oecologia* 82:394–401.

HUBBELL, S.P., and WEIMER, D.F. 1983. Host plant selection by an attine ant, pp. 133–154, *in* P. Jaisson (ed.). Social Insects in the Tropics. University of Paris Press, Paris, France.

JONES, C.G., HARE, J.D., and COMPTON, S.J. 1989. Measuring plant protein with the Bradford assay. 1. Evaluation and standard method. *J. Chem. Ecol.* 15:979–992.

KAWANASHI, K., and RAFFAUF, R.F. 1986. *Caryocar microcarpum:* An ant repellent and fish poison of the northwest Amazon. *J. Nat. Prod.* 49:1167–1169.

KEATING, S.T., YENDOL, W.G., and SCHULTZ, J.C. 1988. Relationship between susceptibility of gypsy moth larvae (Lepidoptera: Lymantriidae) to baculovirus and host plant foliage constituents. *Environ. Entomol.* 17:952–958.

KIRK, T.K., and KELMAN, A. 1965. Lignin degradation as related to the phenoloxidases of selected wood-decaying Basidiomycetes. *Phytopathology* 55:739–745.

KOPTUR, S. 1985. Alternative defenses against herbivores in *Inga* (Fabaceae: Mimosoideae) over an elevational gradient. *Ecology* 66:1639–1650.

LITTLEDYKE, M., and CHERRETT, J.M. 1976. Direct ingestion of plant sap from cut leaves by the leaf-cutting ants *Atta cephalotes* (L.) and *Acromyrmex octospinosus* (Reich) (Formicidae, Attini). *Bull. Entomol. Res.* 66:205–217.

LYR, H. 1962. Detoxification of heartwood toxins and chlorophenols by higher fungi. *Nature* 195:289–230.

MATTSON, W.J. 1980. Herbivory in relation to plant nitrogen content. *Annu. Rev. Ecol. System.* 11:119–161.

MAYER, A.M. 1987. Polyphenol oxidases in plants—recent progress. *Phytochemistry* 26:11–20.

MEYER, G.A., and MONTGOMERY, M.E. 1987. Relationships between leaf age and the food quality of cottonwood foliage for the gypsy moth, *Lymantria dispar. Oecologia (Berlin)* 72:527–532.

MOLE, S., and WATERMAN, P.G. 1988. Light-induced variation in phenolic levels in foliage of rain-forest plants. II. Potential significance to herbivores. *J. Chem. Ecol.* 14:23–34.

NICHOLS-ORIANS, C.M. 1990. Interactions among plant environment, foliar traits, and leaf selection by an attine ant. PhD thesis. The Pennsylvania State University, University Park, Pennsylvania.

NICHOLS-ORIANS, C.M. 1991a. The effects of light on foliar chemistry, growth and susceptibility of seedlings of a canopy tree to an attine ant. *Oecologia* In press.

NICHOLS-ORIANS, C.M. 1991b. Environmentally induced differences in plant traits: Consequences for susceptibility to a leaf-cutter ant. *Ecology*, 72(5).

NICHOLS-ORIANS, C.M., and SCHULTZ, J.C. 1989. Leaf toughness affects leaf harvesting by the leaf-cutter ant, *Atta cephalotes* (L.) (Hymenoptera: Formicidae). *Biotropica* 21:80–83.

NICHOLS-ORIANS, C.M., and SCHULTZ, J.C. 1990. Interactions among leaf toughness, chemistry and harvesting by attine ants. *Ecol. Entomol.* 15:311–320.

POWELL, R.J., and STRADLING, D.J. 1986. Factors influencing the growth of *Attamyces bromatifucus*, a symbiont of attine ants. *Trans. Br. Mycol. Soc.* 87:205–213.

QUINLAN, R.J., and CHERRETT, J.M. 1979. The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L.) .*Ecol. Entomol.* 4:151–160.

ROCKWOOD, L.L. 1976. Plant selection and foraging patterns in two species of leaf-cutting ants (*Atta*). *Ecology* 57:48–61.

ROCKWOOD, L.L. 1977. Foraging patterns and plant selection in Costa Rican leaf-cutting ants. *J. NY Entomol. Soc.* 85:222–233.

SAS INSTITUTE. 1985. SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina.

SCHULTZ, J.C. 1989. Tannin–insect interactions, pp. 417–433, *in* R.W. Hemingway and J.J. Karchesy (eds.). Chemistry and Significance of Condensed Tannins. Plenum Press, New York.

SCHULTZ, J.C., BALDWIN, I.T., and NOTHNAGLE, P.J. 1981. Hemoglobin as a binding substrate in the quantitative analysis of plant tannins. *J. Agric. Food Chem.* 29:823–826.

SEAMAN, F.C. 1984. The effects of tannic acid and other phenolics on the growth of the fungus cultivated by the leaf-cutting ant, *Myrmicocrypta buenzlii. Biochem. System. Ecol.* 12:155–158.

SNYDER, J.C., and DESBOROUGH, S.L. 1978. Rapid estimation of potato tuber total protein content with coomassie brilliant blue G-250. *Theor. Appl. Genet.* 52:135–139.

SWAIN, T., and HILLIS, W.E. 1959. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* 10:63–68.

VITOUSEK, P.M., and DENSLOW, J.S. 1987. Differences in extractable phosphorous among soils of the La Selva Biological Station, Costa Rica. *Biotropica* 19:167–190.

WALLER, D.A. 1982. Leaf-cutting ants and live oak: The role of leaf toughness in seasonal and intraspecific host choice. *Entomol. Exp. Appl.* 32:146–150.

WALTZ, S.A. 1984. Comparative study of predictability, value, and defenses of leaves of tropical wet forest trees (Costa Rica). PhD Thesis. University of Washington, Seattle, Washington.

WATERMAN, P.G., ROSS, J.A.M., and MCKEY, D.B. 1984. Factors affecting levels of some phenolic compounds, digestibility, and nitrogen content of the mature leaves of *Barteria fistulosa* (Passifloraceae). *J. Chem. Ecol.* 10:387–401.

WINT, G. R. W. 1983. The effect of foliar nutrients upon the growth and feeding of a lepidopteran larva, pp. 301–320, *in* J.A. Lee, S. McNeill, and I.H. Rorison (eds.). Nitrogen as an Ecological Factor. Blackwell Scientific, Oxford, England.

ZUCKER, W.V. 1983. Tannins: Does structure determine function? An ecological perspective. *Am. Nat.* 121:335–365.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

CATTLE FEEDING DETERRENTS EMITTED FROM CATTLE FECES

H. DOHI,* A. YAMADA, and S. ENTSU

Department of Ecology
National Grassland Research Institute
Senbonmatu, Nishinasuno, Tochigi, 329-27 Japan

(Received October 4, 1990; accepted February 14, 1991)

Abstract—Cattle avoid grazing forage near their feces on pasture for more than a month. The relation of cattle feces odor to the rejection was studied in feeding choice tests using cattle. The feeding deterrent of feces odor was investigated using a trough partitioned to allow passage of feces odor through test food to the cattle. The cattle did not feed from the trough containing feces 0–35 days after excretion. Volatile chemicals isolated from feces 0–35 days after excretion by a cold trap method also inhibited cattle from feeding. These results demonstrated that feces odor is a major cause of the unpalatability of forage around cattle feces. The volatile chemicals were separated into ether and aqueous fractions. The ether fraction inhibited the feeding behavior of cattle. Furthermore, the ether fraction was separated into neutral, acidic, and basic fractions. The neutral fraction of the three was bioactive. These results suggested that specific volatile chemicals emitted from cattle feces were deterrents to the feeding behavior of cattle.

Key Words—Cattle, dung patch, feces odor, feeding deterrents, volatiles.

INTRODUCTION

On pasture, it is widely observed that cattle selectively graze plants and their olfactory sense appears to be involved in that selection. In particular, the phenomenon that cattle reject eating grass near their feces is frequently observed (Tayler and Large, 1955; MacLusky, 1960; Marten and Donker, 1964a, b; Tayler and Rudman, 1966; Greenhalgh and Reid, 1968). The ungrazed area is called a dung patch. It is also known that cattle readily eat grass cut from dung

*To whom correspondence should be addressed.

0098-0331/91/0600-1197$06.50/0

patches, reject dung-affected grass by night as much as by day, and reject the grass after their marked audible inhalations near the feces. Thus, Voisin (1959) and Arnold and Hill (1972) concluded that undesirable odor from cattle feces is probably a cause of the rejection by cattle. Cattle accept forage clipped from dung patches (Marten and Donker, 1966), and the grazing pattern between blindfolded and non-blindfolded cows on a pasture containing dung patches is similar (MacDiarmid and Watkin, 1972). However, there is very little evidence for the direct deterrent activity of feces odors and which classes of chemicals are involved.

We studied the direct feeding deterrent activity of feces odor and preliminarily separated the deterrent chemicals from cattle feces.

METHODS AND MATERIALS

The cattle used for all feeding choice tests were Japanese Black cattle and were allowed to graze on pasture unless they were used in the feeding choice tests. A total of 5 kg of fresh feces, collected immediately after the excretion during grazing, was put on an isolated pasture for the fecal sampling. A series of seven samplings was made at 0, 1, 7, 14, 21, 28, and 35 days, collecting a total of 1 kg feces from the simulated dung for the feeding choice tests and the isolation of volatile chemicals. After those experiments, all feces were put back onto the same simulated dung. This sampling was repeated five times sequentially in late autumn to avoid worm contamination in the feces. For the separation of isolates, feces more than two weeks after excretion was collected.

Test of Odors from Feces. Figure 1 shows the troughs used to investigate the inhibitory effect of cattle feces odor on feeding. The trough consisted of two boxes. The bottom of the top box had many holes to allow passage of the feces odor. The upper box contained 300 g of artificial diet, which the cattle liked, and the lower box contained 1 kg of feces 0, 1, 7, 14, 21, 28, or 35 days after excretion. The trough without feces in the lower box was prepared as a

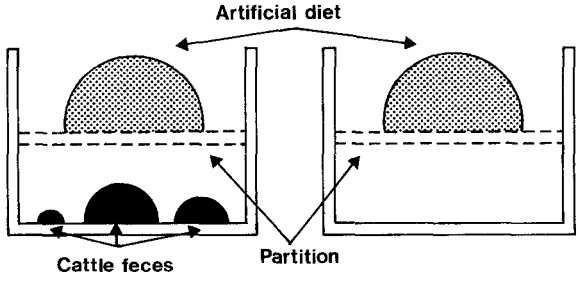


FIG. 1. Trough used to investigate deterrent effects of odor from cattle feces.

control. The two troughs were simultaneously presented to cattle for 2 min and the time cattle fed from each trough was measured. The artificial diet for the choice tests was in pellet form, and its ingredients were milo, maize, barley, rice bran, soybean oilcake, and minerals.

Test of Volatile Chemicals. The solution of volatile chemicals was isolated from 1 kg of feces 0, 1, 7, 14, 21, 28, or 35 days after excretion with an apparatus as shown in Figure 2 and then sprayed on the artificial diet. Distilled water in place of feces was sprayed on the artificial diet in the desiccator as the control. The two choices were presented to cattle for 2 min, and the amount of time the cattle fed on the two choices was measured.

Test of Isolate Fractions. Ether from each fraction of isolates was removed by flowing nitrogen gas at −10°C, and volatile chemicals in the fraction were dissolved in 20 ml distilled water. Artificial diets sprayed with these fractions were presented to cattle and their responses to two or three choices were observed. The degree of feeding was ranked on a scale of −, ± or +. The − represented no feeding or a trace of feeding on diet sprayed with the fraction, ± represented feeding on diet after the end of feeding on other diets, and + represented feeding readily.

Isolation and Separation of Volatile Chemicals. Volatile chemicals of feces were isolated with the apparatus shown in Figure 2. The feces in the desiccator were maintained at 50°C. The volatile chemicals of feces or distilled water were swept from the desiccator by the air filtered through silica gel and a 5 Å molecular sieve and then condensed in two cold traps, which were cooled with iced water and liquid nitrogen. The air was pumped at a flow rate of 500 ml/min and the total volume of air pumped was 500 liters. The isolates obtained by two repeated operations for feces on the day after excretion were combined and used for the feeding choice tests.

For the separation of isolates, a desiccator (25 × 30 cm, ID × H) was filled with 1.5 kg of feces two or more weeks after excretion. Volatile chemicals

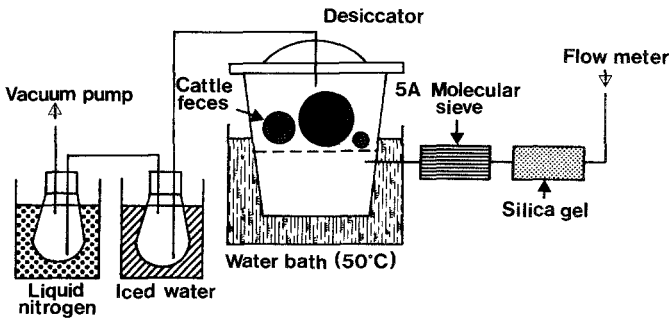


FIG. 2. Apparatus used to isolate volatile chemicals from cattle feces.

were isolated by the same method as described above. The isolates obtained from six repeated operations were combined, extracted with diethyl ether, and separated into ether and water fractions. The solution was also separated into neutral, acidic, and basic fractions by adjusting pH with HCl and NaOH.

RESULTS AND DISCUSSION

The inhibitory effect of feces odor on feeding by cattle is shown in Figures 3 and 4. The cattle almost totally rejected feeding from the trough containing feces 0–35 days after excretion and on food sprayed with the volatile chemicals

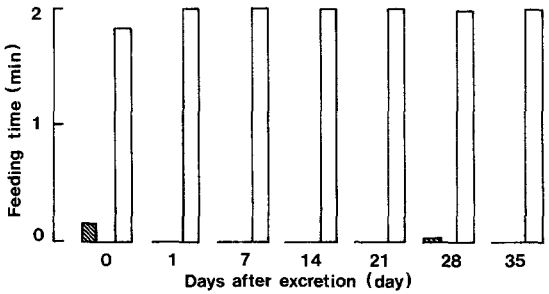


FIG. 3. The amount of time cattle fed on diet from trough with feces (▨) and trough without feces (□) was measured after the two choices were simultaneously presented to cattle for 2 min. Feces were used 0, 1, 7, 14, 21, 28, and 35 days after excretion. Five different cattle were tested for each age of feces. Results are the mean of five determinations.

FIG. 4. The amount of time cattle fed on diet sprayed with the solution of volatile chemicals from feces (▨) and diet sprayed with the control solution (□) was measured. The solution of volatile chemicals was isolated from feces 0, 1, 7, 14, 21, 28, or 35 days after excretion and the control solution from distilled water. Five different cattle were tested for each age of feces. Results are the mean of five determinations.

that were isolated from feces 0–35 days after excretion. These results demonstrated the feces odor had an inhibitory effect on feeding. Grass affected by feces remains unacceptable to cattle over a long period of time (Norman and Green, 1958; Marten and Donker, 1964a; Weeda, 1967; Castle and MacDaid, 1972; MacDiarmid and Watkin, 1972). For an explanation of the long-term rejection by cattle, Norman and Green (1958) hypothesized that the initial refusal of herbage near droppings was due to the dung itself and that the ungrazed herbage then became unpalatable due to its increased maturity. Plice (1951) reported that changes in chemical composition of grass heavily fertilized with cattle dung caused the forage to become unpalatable. However, our studies showed that odors and volatile isolates from feces 35 days after excretion were deterrent to cattle. This suggested that odors from dung are a major cause of the rejection by cattle over the period when dung patches were maintained.

In various phytophagous insects, it is well known that stimulants in plants cause their selective feeding. Many chemicals that stimulate insects have been identified from plants. Arnold (1966a,b) and Arnold et al. (1980) reported that the olfactory sense is involved in food selection using sensory impaired sheep and that the food intake by sheep was regulated by some chemicals found in plants that sheep grazed. The food intake of cattle was increased by flavoring agents (Weller and Phipps, 1989), and feeding by cattle on pine trees was prevented by repellents (Marnane, 1982). Their results led us to the possibility that the selective grazing behavior of cattle, like the behavior of insects, is partially caused by specific stimulant chemicals contained in natural products on pasture. Thus, we hypothesized that some specific chemicals of all volatile chemicals emitted from feces are responsible for the reluctance of cattle to feed in a dung patch. In order to confirm the hypothesis, the volatile chemicals isolated from

TABLE 1. FEEDING RESPONSE OF CATTLE TO FRACTIONS OF VOLATILE CHEMICALS ISOLATED FROM FECES[a]

Fraction	Degrees of feeding				
(Test 1)					
Ether	−	−	−	±	−
Water	+	+	+	+	+
(Test 2)					
Neutral	−	±	−	−	−
Acidic	+	+	+	+	+
Basic	+	+	+	+	+

[a] (−) represents no feeding or a trace of feeding. (±) represents feeding after the end of feeding on another diets. (+) represents feeding readily. Each fraction was tested with five different animals.

feces were separated and the deterrent activity of fractions was investigated in feeding choice tests (Table 1). In the fraction with diethyl ether, feeding deterrents appeared in the ether fraction (test 1). Among acidic, neutral, and basic fractions obtained by further separation with diethyl ether, the neutral fraction had an inhibitory effect (test 2). These results suggested the presence of specific feeding deterrents to cattle in the odors of cattle feces.

Acknowledgments—We are grateful to Dr. R. Ishida and Dr. H. Tanaka for helpful suggestions. This work was supported in part by a Grant-in-Aid (Bio Cosmos Program) from the Ministry of Agriculture, Forestry and Fisheries. (BCP 90-II-A-3)

REFERENCES

ARNOLD, G.W. 1966a. The special senses in grazing animals. I. Sight and dietary habits in sheep. *Aust. J. Agric. Res.* 17:521–529.

ARNOLD, G.W. 1966b. The special senses in grazing animals. II. Smell, taste, and touch and dietary habits in sheep. *Aust. J. Agric. Res.* 17:531–542.

ARNOLD, G.W., and HILL, J.L. 1972. Chemical factors affecting selection of food plants by ruminants, pp. 71–101, *in* J.B. Harbourne (ed.). Phytochemical Ecology. Academic Press, London.

ARNOLD, G.W., DE BOER, E.S., and BOUNDY, C.A.P. 1980. The influence of odour and taste on the food preferences and food intake of sheep. *Aust. J. Agric. Res.* 31:571–587.

CASTLE, M.E., and MACDAID, E. 1972. The decomposition of cattle dung and its effect on pasture. *J. Br. Grassl. Soc.* 27:133–137.

GREENHALGH, J.F.D., and REID, G.W. 1968. The effects of grazing intensity on herbage consumption and animal production. III. Dairy cows grazed at two intensities on clean or contaminated pasture. *J. Agric. Sci. Camb.* 71:223–228.

MACDIARMID, B.N., and WATKIN, B.R. 1972. The cattle dung patch. 3. Distribution and rate of decay of dung patches and their influence on grazing behaviour. *J. Br. Grassl. Soc.* 27:48–54.

MACLUSKY, D.S. 1960. Some estimates of the areas of pasture fouled by the excreta of dairy cows. *J. Br. Grassl. Soc.* 15:181–188.

MARNANE, N.J., MATTHEWS, L.R., KILGOUR, R., and HAWKE, M. 1982. Prevention of bark chewing of pine trees by cattle: The effectiveness of repellents. *Proc. N.Z. Soc. Anim. Prod.* 42:61–63.

MARTEN, G.C., and DONKER, J.D. 1964a. Selective grazing induced by animal excreta. I. Evidence of occurrence and superficial remedy. *J. Dairy Sci.* 47:773–776.

MARTEN, G.C., and DONKER, J.D. 1964b. Selective grazing induced by animal excreta. II. Investigation of a causal theory. *J. Dairy Sci.* 47:871–874.

MARTEN, G.C., and DONKER, J.D. 1966. Animal excrement as a factor influencing acceptability of grazed forage, pp. 359–363, *in* Proceedings, 10th International Grassland Congress, Helsinki.

NORMAN, M.J.T., and GREEN, J.O. 1958. The local influence of cattle dung and urine upon the yield and botanical composition of permanent pasture. *J. Br. Grassl. Soc.* 13:39–45.

PLICE, M.J. 1951. Sugar versus the intuitive choice of foods by livestock. *Agron. J.* 43:341–343.

TAYLER, J.C., and LARGE, R.V. 1955. The comparative output of two seeds mixtures. *J. Br. Grassl. Soc.* 10:341–351.

TAYLER, J.C., and RUDMAN, J.E. 1966. The distribution of herbage at different heights in grazed

and dung patch areas of a sward under two methods of grazing management. *J. Agric. Sci.* 66:29–39.

VOISIN, A. 1959. Grass productivity. Trans. Herriot, C.T.M. Philosophical Library, New York.

WEEDA, W.C. 1967. The effect of cattle dung patches on pasture growth, botanical composition, and pasture utilization. *N.Z. J. Agric. Res.* 10:150–159.

WELLER, R.F., and PHIPPS, R.H. 1989. Preliminary studies on the effect of flavouring agents on the dry-matter intake of silage by lactating dairy cows. *J. Agric. Sci. Camb.* 112:67–71.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

SEX PHEROMONE OF FEMALE AFRICAN WHITE RICE STEM BORER, *Maliarpha separatella* (LEPIDOPTERA: PYRALIDAE) FROM SIERRA LEONE: IDENTIFICATION AND FIELD TESTING

A. CORK,[1,*] M. AGYEN-SAMPONG,[2] S.J. FANNAH,[2] P.S. BEEVOR,[1] and D.R. HALL[1]

[1]*Natural Resources Institute*
Central Avenue, Chatham Maritime
Chatham, Kent, ME4 4TB, UK

[2]*West African Rice Development Association*
Regional Mangrove Swamp Rice Research Station
P.M.B. 678, Freetown, Sierra Leone

(Received October 9, 1990; accepted February 14, 1991)

Abstract—Analysis of ovipositor washings of female *Maliarpha separatella* from Sierra Leone by high-resolution gas chromatography (GC) linked to a male electroantennograph (EAG) indicated the presence of three electrophysiologically active compounds. The GC retention times of these compounds were consistent with those of (*Z*)-9-tetradecen-1-ol, (*Z*,*E*)-9,12-tetradecadien-1-ol, and (*E*,*E*)-10,12-tetradecadien-1-ol. Analysis by EI and CI mass spectrometry of ovipositor washings confirmed these identifications and also indicated the presence of the saturated analog, tetradecan-1-ol. There was no evidence, from these analyses, of the corresponding aldehydes or acetates. The EAG-active compounds were present in the ovipositor washings in a ratio of approximately 2:3.5:1, with the major component constituting approximately 0.4 ng per ovipositor. GC-MS analysis of entrained female effluvia confirmed that the EAG-active compounds were released by virgin females. Field testing of the EAG-active compounds indicated that (*Z*,*E*)-9,12-tetradecadien-1-ol and (*E*,*E*)-10,12-tetradecadien-1-ol were attractive to male moths in ratios of between 9:1 and 39:1, while (*Z*)-9-tetradecen-1-ol was found to reduce trap catch when added to blends of the other two compounds.

Key Words—*Maliarpha separatella*, (*Z*)-9-tetradecen-1-ol, (*Z*,*E*)-9,12-

*To whom correspondence should be addressed.

tetradecadien-1-ol, (*E,E*)-10,12-tetradecadien-1-ol, sex pheromone, Lepidoptera, Pyralidae.

INTRODUCTION

The African white rice stem borer, *Maliarpha separatella*, is the predominant rice stem borer in West Africa. It is specific to the genus *Oryza* (Akinsola and Agyen-Sampong, 1984) and is capable of inflicting considerable economic damage with the number of rice grains reduced by 70% (Akinsola, 1984) and yield losses of up to 1000 kg/ha being reported (WARDA, 1977). In addition secondary damage can be caused by rice blast, *Pyricularia oryzae*, which gains entrance through the emergence holes of adult moths (Pollet, 1978).

The presence of stem borer larvae of other species usually can be assessed by the number of "white heads" or empty rice panicles formed in a crop. However, the larvae of *M. separatella* rarely cause this condition in West Africa, nor do they cause "dead heart," presumably because of their habit of residing in the lower internodes of the rice culm (Akinsola, 1979; Akinsola and Agyen-Sampong, 1984). The absence of visual damage makes detection difficult, and although adult populations have been monitored by use of paraffin lamp traps (Agyen-Sampong and Fannah, 1987) these traps are not selective. Sex pheromone traps could provide an inexpensive, sensitive, and selective trapping method for monitoring populations of the adult moths (Campion and Nesbitt, 1983, Campion et al., 1987). The presence of a female-produced sex pheromone in *M. separatella* had been demonstrated by Ho and Seshu Reddy (1983), and this paper describes the results of our investigation into the chemical composition of the sex pheromone of *M. separatella* from Sierra Leone and the preliminary field trials with the electrophysiologically active compounds identified with a view of producing a species-specific lure for monitoring this pest species.

METHODS AND MATERIALS

Insect Material. Pupae were collected from susceptible rice plants grown at the WARDA Research Station, Rokupr, Sierra Leone, and dispatched by air to NRI. There they were placed in individual test tubes and maintained on a reversed 12-hr light, 12-hr dark cycle with temperatures alternating from 28°C to 24°C, and a relative humidity of approximately 90%. Emerging adults were sexed and kept in groups of up to 20 in Perspex containers (28 × 15 × 9 cm) with access to a 10% sucrose solution.

Pheromone Collection. Ovipositor washings were prepared in hexane 1–6 hr into the scotophase using virgin females 0–2 days old as described by Sower

et al. (1973). Effluvia from individual virgin females in glass vessels were collected on charcoal filters (5 mg) by air entrainment for 24-hr periods, as previously described (Grob and Zurcher, 1976; Nesbitt et al., 1979; Tumlinson et al., 1982).

Gas Chromatography (GC). GC analyses of natural and synthetic compounds were conducted on a Carlo Erba 4160 instrument with flame ionization detector. Fused silica capillary columns were used throughout the study, coated with either nonpolar CP Sil 5CB (chemically bonded methyl silicone, 50 m × 0.32 mm ID, 0.12 μm film thickness; Chrompack) or polar CP Wax 57CB (chemically bonded Carbowax 20 M; 25 and 50 m × 0.32 mm ID, 0.2 μm film thickness; Chrompack). The carrier gas was helium with a linear velocity of 23 cm/sec. Injections were made with a Grob split–splitless injector at 220°C in the splitless mode, with the valve closed for 40 sec. The analytical column was held at 70°C for 2 min, then temperature programmed as indicated in the tables. GC retention times for isothermal analyses are quoted in minutes, while retention times from temperature-programmed analyses are quoted in equivalent chain lengths (ECLs) relative to the retention times of straight-chain saturated acetates; thus, for example tetradecyl acetate = 14.00 (Harris and Habgood, 1966).

Mass Spectrometry (GC-MS). Electron impact mass spectra (EI-MS) were obtained from a Finnigan MAT Ion Trap Detector (ITD) model 700 under continuous ion monitoring between *m/z* 30 and 250 at 220°C with a filament emission current of 70 μA. The ITD was connected to a Carlo Erba Mega Series 5160 GC via a 1-m fused silica capillary column coated with a chemically bonded, nonpolar methyl silicone liquid phase, heated to 220°C and 1 : 1 open-split interface. Samples were introduced into the GC through a Grob type split–splitless injector held at 220°C with the split valve closed for 40 sec after injection. Chromatographic separations were conducted on fused silica capillary columns coated with CP Wax 52CB (25 m × 0.32 mm ID, 0.2 μm film thickness, Chrompack), a polar chemically bonded Carbowax 20 M equivalent, and CP-Cyclodex B (50 m × 0.25 mm ID, 0.25 μm film thickness, Chrompack), a cyclodextrin-β-2,3,6-M-19 column. The GC columns were held at 70°C for 2 min, then the CP Wax 52CB column was temperature programmed to 120°C at 20°C/min and subsequently at 4°C/min to 210°C, while the CP-Cyclodex B column was temperature programmed at 20°C/min to 190°C. The carrier gas was helium, with an inlet pressure of 0.4 kg/cm^2 for the CP Wax 52CB and 1.0 kg/cm^2 for the CP-Cyclodex B column. Chemical ionization mass spectra (CI-MS) also were obtained with the ITD using isobutane as reagent gas, but holding the ion trap at 200°C and reducing the filament emission current to 35 μA. Scans were either made over the range *m/z* 60–250 or under selective ion monitoring.

Electroantennography (EAG). Combined GC-EAG analyses were recorded essentially as described by Cork et al. (1990). EAG responses elicited by syn-

thetic pheromone analogs were recorded from different doses of compounds applied to filter papers (1.5 cm diameter circle, Whatman No. 1) in 1 μl of pentane and placed in a disposable Pasteur pipet. Nitrogen (250 ml/min) was passed through the Pasteur pipet for 10 sec to remove the solvent. Samples then were presented to the EAG preparation in 1-sec pulses of charcoal-filtered nitrogen (250 ml/min). Chemicals were tested in a randomized order, although the lowest doses were tested first. The EAG preparation was given a 1-min recovery period between stimuli, and each sample was tested five times on each of three males. Samples were stored under nitrogen at −30°C when not in use.

Synthetic Chemicals. (*Z*,*E*)-9,12-Tetradecadien-1-ol was obtained from Food Industries, Ltd., in 1978 and contained 99.4% *Z*,*E*, <0.1% *E*,*Z*, 0.2% *Z*,*Z*, and 0.36% *E*,*E* isomers and 0.01% of the related (*Z*,*E*)-9,12-tetradecadienal.

(*E*,*E*)-10,12-Tetradecadien-1-ol was prepared by Grignard coupling of 1-bromo-10-(2-tetrahydropyranyloxy)decane and (*E*,*E*)-2,4-hexenyl acetate in THF in the presence of $LiCuCl_4$. The reaction product was further purified by recrystallization twice from hexane at −20°C to give material containing 99% *E*,*E* and 1% *E*,*Z* isomers. The mother liquors were isomerized to the thermodynamically most stable mixture with iodine in sunlight and recycled.

Monounsaturated alcohols were prepared by standard acetylenic and Wittig coupling reactions (Henrick, 1977) and were at least 97% isomerically and chemically pure by GC analysis. (*Z*)-9-Tetradecen-1-ol was further purified by argentation chromatography (Houx et al., 1974) to give 99.9% stereochemically pure material.

(*Z*,*E*)-9,12-Tetradecadienal, (*E*,*E*)-10,12-tetradecadienal, and (*Z*)-9-tetradecenal were prepared from the corresponding alcohols by oxidation with pyridinium chlorochromate in dichloromethane using sodium acetate as a buffer (Corey and Suggs, 1975). Traces of alcohol in the products were removed by liquid chromatography on Florisil (80–200 mesh) using gradient elution with hexane and diethyl ether followed by short-path Kugelrohr distillation. Subsequent analysis by GC-EAG and GC-MS did not detect any of the corresponding alcohol with a limit of detection of 0.01%. A similar procedure was followed to remove trace amounts of the aldehydes in samples of the corresponding alcohols.

Field Trials. Field trials of synthetic compounds were conducted in rice paddy fields at the WARDA Rice Research Station, Rokupr, Sierra Leone. Pheromone dispensers were white rubber septa (Aldrich, catalog No. Z10,072-2) impregnated with 0.1 ml of a hexane solution containing 200 μg of the chemical blend and an equivalent weight of 2,6-di-*tert*-butyl-4-methylphenol (BHT) as antioxidant.

White delta shaped traps (20 × 22 cm base, 12 cm height) with sticky bases (Agrisense-BCS, UK) were used throughout the trials fastened to an

inverted L-shaped pole positioned in such a way that the trap was just above crop height. Traps were placed out in lines containing one replicate of each treatment with each trap in a line separated by at least 25 m, and the lines were separated by at least 50 m. Trap catch was recorded daily and discarded. Traps in a replicate were moved one position in the line daily in order to minimize the effect of trap position on catch. All trials were run for a number of nights equal to a multiple of the number of treatments in the trial so that each treatment occupied all trap sites an equal number of times. Trap catches for each treatment were totaled for each complete rotation, converted to log $(x + 1)$ and subjected to analysis of variance. If this indicated there was a significant difference between the treatments at better than the 5% level of confidence, then the means were ranked by Duncan's new multiple-range test (DNMRT) (Duncan, 1955).

RESULTS AND DISCUSSION

Structure Determination. Ovipositor washings were initially analyzed by GC-EAG using the polar CP Wax 57CB column under temperature programmed conditions. A typical GC-EAG analysis was conducted with between two and five ovipositor equivalents.

EAG responses elicited by compounds eluting from the GC column were consistently observed at 15.3, 16.1, and occasionally at 16.8 ECL on the polar column with relative amplitudes in the ratio of approximately 1:2:0.5. However, the GC peaks associated with these responses were highly variable in amount and rarely constituted more than 1 ng per compound (Table 1). The GC ECL values were indicative of mono-, nonconjugated di-, and conjugated diunsaturated straight-chain, 14-carbon primary alcohols, respectively. Analysis of the same ovipositor washings on a nonpolar CP Sil 5CB column under isothermal conditions confirmed these findings with EAG responses observed to components at 12.49, 12.56, and 13.08 ECL (Table 1).

Comparison of the GC retention times of the EAG-active compounds with a range of synthetic straight-chain, 14-carbon primary alcohols on both GC phases under isothermal GC conditions indicated that the retention times of the active components were consistent with those of (*Z*)-9-tetradecen-1-ol (*Z*9–14:OH), (*Z,E*)-9,12-tetradecadien-1-ol (*ZE*9,12–14:OH) and (*E,E*)-10,12-tetradecadien-1-ol (*EE*10,12–14:OH) (Table 2). However, *ZE*9,12–14:OH could not be separated from the *E,E* isomer on either of these liquid phases.

Analysis of the ovipositor washings by GC-MS on a polar CP Wax 52CB column in EI mode indicated compounds with GC retention times and mass spectra that were consistent with those of synthetic *Z*9–14:OH, *ZE*9,12–14:OH and *EE*10,12–14:OH, although none of the compounds gave molecular ions and only *EE*10,12–14:OH produced $M-H_2O$ ions at *m/z* 192. However, the

TABLE 1. GC RETENTION TIMES AND COMPOSITION OF EAG-ACTIVE COMPOUNDS IDENTIFIED IN OVIPOSITOR WASHINGS OF *Maliarpha separatella* ORIGINATING FROM SIERRA LEONE

GC phase, compound	Retention times (ECL)[a]		Composition	
	Synthetic	Natural	ng/female	Ratio
CP Wax 57CB[b]				
*Z*9-14:OH	15.30	15.31	0.22 ± 0.08	2.2
*ZE*9, 12-14:OH	16.08	16.10	0.38 ± 0.08	3.8
*EE*10, 12-14:OH	16.78	16.86	0.1 ± 0.06	1
CP Sil 5CB[b]				
*Z*9-14:OH	12.47	12.49	0.46 ± 0.37	1.9
*ZE*9, 12-14:OH	12.55	12.56	0.98 ± 0.36	3.3
*EE*10, 12-14:OH	13.03	13.08	0.29 ± 0.19	1

[a]Retention times in equivalent chain length units relative to the retention times of straight-chain saturated acetates.
[b]Oven temperatures 70°C for 2 min then programmed at 20°C/min to 140°C, and then at 2°C/min to 220°C.

m/z 31 (CH_2OH^+) ions that Attygalle et al. (1988) have shown to be produced by long-chain primary alcohols were observed at the GC retention times of *Z*9-14:OH and *ZE*9,12-14:OH, but the abundance of *EE*10,12-14:OH was too low to confirm the presence of *m/z* 31 ions in that compound. The ratio of the three compounds obtained on the MS in EI mode was 2:3.5:1 respectively. Further analysis of the ovipositor washings was undertaken in CI mode with isobutane as reagent gas. Strong ions consistent with M+1 were observed at *m/z* 213 for *Z*9-14:OH and *m/z* 211 for *ZE*9,12-14:OH and *EE*10,12-14:OH. These GC-MS analyses also showed trace quantities of a compound with the same ECL value (14.7 ECL) and EI and CI mass spectra as those of tetradecan-1-ol. However, as this compound had not elicited an EAG response in the GC-EAG analyses, it was not investigated further.

CI-MS analysis of effluvia collected from entrainment of virgin females confirmed that 14:OH, *Z*9-14:OH, *ZE*9,12-14:OH and *EE*10,12-14:OH were released, but their abundance was too low for quantitation.

Recent work by Hall et al. (unpublished) has shown that the cyclodextrin-β-2,3,6-M-19 liquid phase, originally developed by Nowotny et al. (1989) for the separation of optical isomers, is also effective at separating *Z* and *E* isomers of long-chain primary alcohols and their analogs. Under the GC conditions used, the *Z,E* and *E,E*-isomers of 9,12-14:OH were readily resolved (GC retention times of the *E,E*, *Z,E*, *E,Z*, and *Z,Z* isomers were 32.80, 33.17, 34.43, and 34.51 min, respectively). GC-MS analysis of ovipositor washings on this col-

TABLE 2. GC RETENTION TIMES OF SYNTHETIC C-14 PRIMARY ALCOHOLS AND EAG-ACTIVE COMPOUNDS

Compound	Retention times (min)	
	CP Wax57CB[a]	CP Sil 5CB[b]
Natural EAG-active compounds		
Z9-14:OH	27.01	29.91
*ZE*9, 12-14:OH	35.71	31.14
*EE*10, 12-14:OH	50.00	38.45
Synthetic compounds		
14:OH	23.81	31.69
13-14:OH	28.59	31.27
Z12-14:OH	31.91	33.23
*E*12-14:OH	29.85	31.88
Z11-14:OH	28.63	31.19
*E*11-14:OH	27.61	30.80
Z10-14:OH	27.73	30.56
*E*10-14:OH	26.78	30.38
Z9-14:OH	27.05	29.93
*E*9-14:OH	26.56	30.09
Z8-14:OH	26.59	29.52
*E*8-14:OH	26.27	29.78
Z7-14:OH	26.17	29.24
*E*7-14:OH	26.03	29.69
Z6-14:OH	26.21	29.25
*E*6-14:OH	25.96	29.62
Z5-14:OH	26.53	29.47
*E*5-14:OH	26.24	29.86
Z4-14:OH	25.73	29.33
*E*4-14:OH	25.69	29.81
Z3-14:OH	25.27	29.53
*E*3-14:OH	23.32	28.97
Z2-14:OH	28.63	31.15
*E*2-14:OH	28.49	30.52
*ZE*8, 10-14:OH	42.45	34.51
*EZ*8, 10-14:OH	44.11	35.45
*ZZ*8, 10-14:OH	45.80	36.81
*EE*8, 10-14:OH	47.35	37.75
*ZE*9, 11-14:OH	44.05	35.18
*EZ*9, 11-14:OH	45.46	36.30
*ZZ*9, 11-14:OH	46.61	37.14
*EE*9, 11-14:OH	48.18	37.94
*ZE*10, 12-14:OH	48.10	36.85
*EZ*10, 12-14:OH	51.05	39.01
*ZZ*10, 12-14:OH	51.79	39.34
*EE*10, 12-14:OH	50.00	38.35
Z11, 13-14:OH	44.05	34.46
*E*11, 13-14:OH	44.05	34.46
*ZE*9, 12-14:OH	35.71	31.14
*EZ*9, 12-14:OH	37.73	33.14
*ZZ*9, 12-14:OH	37.88	32.42
*EE*9, 12-14:OH	35.63	31.38

[a] Oven temperature 70°C for 2 min then programmed at 20°C/min to 135°C, held for 38 min, then programmed at 20°C/min to 140°C.
[b] Oven temperature 70°C for 2 min, then programmed at 20°C/min to 135°C.

umn showed a peak with retention time and mass spectrum identical with that of *ZE*9,12–14:OH and no peak at the retention time of the *E,E* isomer.

Comparison of EAG responses elicited by the three unsaturated alcohols identified from female ovipositor washings at different doses indicated that the responses were dose dependent with the magnitude of responses elicited increasing in the order of *EE*10,12–14:OH < *Z*9–14:OH < *ZE*9,12–14:OH for a given dose (Figure 1). The small amplitude of the EAG responses elicited by *EE*10,12–14:OH was probably indicative of a small number of olfactory receptors sensitive to this compound. It would also explain why EAG responses to this compound in the GC-EAG analyses were not observed from all the extracts analyzed.

The related aldehydes, (*Z*)-9-tetradecenal (*Z*9–14:Ald) and (*Z,E*)-9,12-tetradecadienal (*ZE*9,12–14:Ald) have been identified in the sex pheromone of *M. separatella* from Madagascar (H. Arn, personal communication). Thus the EAG responses elicited by *Z*9–14:Ald, *ZE*9,12–14:Ald, and (*E,E*)-10,12-tetradecadienal (*EE*10,12–14:Ald) were compared with those elicited by the corresponding alcohols identified in this study at a single dose of 1000 ng. The results (Table 3) showed that the aldehydes elicited responses of similar ampli-

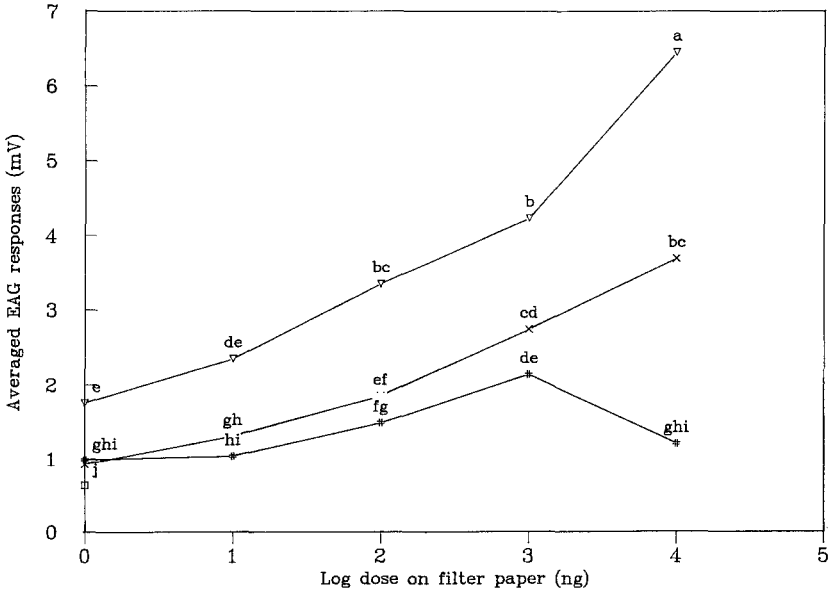


FIG. 1. EAG responses from male *M. separatella* to *Z*9–14:OH (X), *ZE*9,12–14:OH (∇), and *EE*10,12–14:OH (#). Each value represents the mean of five replicates from each of three males. Means followed by different letters are significantly different at the 5% level by DNMRT.

tude to those elicited by the related alcohols, indicating that if the aldehydes had been present in the ovipositor washings or female effluvia in quantities comparable to the alcohols, then they would have been detected in the GC-EAG analyses.

Field Tests. Initially all three EAG-active alcohols were assumed to be required for the pheromone lure, and the first trial was undertaken to optimize the ratio of *Z*9–14:OH and *ZE*9,12–14:OH, while keeping *EE*10,12–14:OH fixed at 10% of the total loading. The results of this trial (Table 4) showed that the compounds were attractive to male moths and that, as the proportion of *ZE*9,12–14:OH was increased with respect to *Z*9–14:OH over the range tested, trap catch also increased significantly.

TABLE 3. EAG RESPONSES RECORDED FROM MALE *M. separatella* TO EAG-ACTIVE COMPOUNDS IDENTIFIED IN OVIPOSITOR WASHINGS AND RELATED ALDEHYDES

Compound[a]	EAG responses (average of 10 responses ± SD mV)
Pentane, 1, μl	1.02
*Z*9–14:OH	3.11 ± 0.32
*ZE*9, 12–14:OH	4.33 ± 1.27
*EE*10, 12–14:OH	1.99 ± 0.20
*Z*9–14:Ald	3.22 ± 0.66
*ZE*9, 12–14:Ald	3.54 ± 0.46
*EE*10, 12–14:Ald	1.82 ± 0.14

[a] 1000-ng samples applied in 1 μl of pentane.

TABLE 4. CATCHES OF MALE *M. separatella* MOTHS IN TRAPS BAITED WITH DIFFERENT RATIOS OF *Z*9–14:OH, *ZE*9, 12–14:OH AND *EE*10, 12–14:OH (5 REPLICATES, 10 NIGHTS)

Ratio of compounds (200 μg loading)			
*Z*9–14:OH	*ZE*9, 12–14:OH	*EE*10, 12–14:OH	Total catch/treatment[a]
8	1	1	2c
6	3	1	8bc
4.5	4.5	1	14bc
3	6	1	32ab
1	8	1	52a

[a] Total catches followed by the same letter are not significantly different at the 1% level by DNMRT using log $(x + 1)$ transformed data.

In order to determine whether all three EAG-active compounds identified in the female-derived extracts were required to attract male moths, the effect of removing individual components from the three-component mixture was tested (Table 5). This demonstrated that both *ZE*9,12–14:OH and *EE*10,12–14:OH were essential for catching male moths and also that the presence of *Z*9–14:OH significantly reduced catch at the level tested.

Different ratios of *ZE*9,12–14:OH and *EE*10,12–14:OH were then tested in order to optimize the ratio of these compounds in the lure. No significant differences were observed between the treatments tested, although the highest catches were obtained with between 5% and 10% *EE*10,12–14:OH in *ZE*9,12–14:OH (Table 6).

To demonstrate that the *Z,E* isomer, not the *E,E* isomer, of 9,12–14:OH

TABLE 5. CATCHES OF MALE *M. separatella* MOTHS IN TRAPS BAITED WITH 3:6:1 MIXTURE OF ALCOHOLS COMPARED TO MIXTURE IN WHICH ONE COMPOUND HAD BEEN REMOVED (4 REPLICATES, 12 NIGHTS)

Ratio of compounds (200 μg loading)			
*Z*9–14:OH	*ZE*9, 12–14:OH	*EE*10, 12–14:OH	Total catch/treatment[a]
3	6	1	37b
0	6	1	48a
3	0	1	1c
3	6	0	0c

[a]Total catches followed by the same letter are not significantly different at the 1% level by DNMRT using log $(x + 1)$ transformed data.

TABLE 6. CATCHES OF MALE *M. separatella* IN TRAPS BAITED WITH DIFFERENT RATIOS OF *ZE*9, 12–14:OH AND *EE*10, 12–14:OH (4 REPLICATES, 12 NIGHTS)

Percentage composition (200 μg loading)		
*ZE*9, 12–14:OH	*EE*10, 12–14:OH	Total catch/treatment[a]
97.5	2.5	31ns
95	5	43ns
90	10	42ns
80	20	21ns

[a]No significant difference between treatments with log $(x + 1)$ transformed data.

was a natural pheromone component, the attractiveness of binary blends containing one of the 9,12–14 : OH isomers and *EE*10,12–14 : OH were compared. Irrespective of whether lures were changed every four days or not changed throughout the duration of the trial, blends containing the *E,E* isomer of 9,12–14 : OH were essentially unattractive to male moths, while lures containing the *Z,E* isomer were significantly attractive (Table 7).

In view of Arn's results from Madagascar (personal communication) and the ease with which some species of Lepidoptera are known to convert alcohols to aldehyde or acetate functionalities (Morse and Meighen, 1984; Silk et al., 1980; Silk and Kuenen, 1988; Teal and Tumlinson, 1986; Tumlinson and Teal, 1987), ternary blends of the corresponding aldehydes and acetates were compared with the alcohols in a 3 : 6 : 1 ratio at 200-μg loading. The results showed that only the blend of alcohols caught male moths (Table 8), as would have been expected from the CI-MS analysis of the entrained female effluvia.

However, the GC-EAG and GC-MS studies could not rule out the possibility that very small quantities of the corresponding aldehydes were released by female moths, so 9 : 1 ratios of *ZE*9,12–14 : OH and *EE*10,12–14 : OH were field tested with between 0% and 1% of the corresponding aldehydes added. This field trial was repeated in two successive seasons without showing any clear evidence that the aldehydes influenced trap catch at the levels tested (Table 9).

Numerous attempts were made to catch male moths with caged virgin females. However, despite a high level of survival among the female moths used, no male moths were caught in the traps used. The reason for this is uncer-

TABLE 7. CATCHES OF MALE *M. separatella* WITH BINARY MIXTURES OF *EE*10, 12—14 : OH AND EITHER *E*, *E* OR *Z*, *E* ISOMER OF 9, 12—14 : OH (4 REPLICATES, 24 NIGHTS)

Ratio of compounds (200 μg loading)			
*ZE*9, 12-14 : OH	*EE*9, 12-14 : OH	*EE*10, 12-14 : OH	Total catch/treatment[a]
Lures changed every four days			
9	0	1	153a
0	9	1	1c
Lures not changed during trial			
9	0	1	37b
0	9	1	0c

[a] Total catches followed by the same letter are not significantly different at the 1% level by DNMRT using log $(x + 1)$ transformed data.

TABLE 8. CATCHES OF MALE *M. separatella* MOTHS IN TRAPS BAITED WITH 3:6:1 MIXTURE OF ALCOHOLS COMPARED TO RELATED MIXTURES CONTAINING EQUIVALENT ACETATES AND ALDEHYDES, AND A 6:1 MIXTURE OF *ZE*9, 12–14:OH AND *EE*10, 12–14:OH (4 REPLICATES, 8 NIGHTS)

Ratio of compounds (200 μg loading)					Total catch/treatment[a]
3	:	6	:	1	
*Z*9–14:OH		*ZE*9, 12–14:OH		*EE*10, 12–14:OH	15b
*Z*9–14:Ald		*ZE*9, 12–14:Ald		*EE*10, 12–14:Ald	0c
*Z*9–14:Ac		*ZE*9, 12–14:Ac		*EE*10, 12–14:Ac	0c
0	:	6	:	1	
		*ZE*9, 12–14:OH		*EE*10, 12–14:OH	30a

[a] Total catches followed by the same letter are not significantly different at the 5% level by DNMRT using log $(x + 1)$ transformed data.

TABLE 9. CATCHES OF MALE *M. separatella* IN TRAPS BAITED WITH 9:1 MIXTURE OF *ZE*9, 12–14:OH AND *EE*10, 12–14:OH AND 0–1% OF CORRESPONDING ALDEHYDES ADDED (30 NIGHTS)

Ratio of compounds (200 μg loading)				Total catch/treatment[a]	
*ZE*9, 12–14:OH	*EE*10, 12–14:OH	*ZE*9, 12–14:Ald	*EE*10, 12–14:Ald	Trial A 5 reps.	Trial B 4 reps.
9	1	0	0	85ab	32bc
9	1	0.0009	0.0001	134a	40b
9	1	0.009	0.001	90ab	98a
9	1	0.09	0.01	39b	29bc

[a] Total catches followed by the same letter are not significantly different at the 5% level by DNMRT using log $(x + 1)$ transformed data.

tain, although light and synthetic pheromone-baited traps did catch significant numbers of moths during the same periods. However, Ho and Seshu Reddy (1983) only caught a maximum of 11.25 moths/trap/night with virgin female-baited traps during a period when a high population level would have been anticipated, as indicated by a 70% tiller infestation.

On the basis of the above field trials, it was evident that although three EAG-active compounds were present in the extracts prepared from female *M. separatella* only *ZE*9,12–14:OH and *EE*10,12–14:OH could be considered to

be components of the female sex pheromone. *Z*9–14:OH has been identified in the female sex pheromones of other Lepidoptera (Arn et al., 1986), notably in the Noctuidae *Spodoptera exigua* (Tumlinson et al., 1981; Wakamura, 1987) and *Euxoa plagigera* (Steck et al., 1982). It also has been reported to be a component of the pheromones of some Pyralidae, and the Phycitinae in particular. Examples include the lesser cornstalk borer, *Elasmopalpus lignosellus* (Lynch et al., 1984), the sunflower moth, *Homoeosoma electellum* (Underhill et al., 1979) and the driedfruit moth, *Vitula edmandsae serratilineella* (Struble and Richards, 1983).

The most commonly identified nonconjugated tetradecadien-1-ol in the sex pheromones of the Lepidoptera is the methylene-interrupted diene *ZE*9,12–14:OH (Arn et al., 1986) identified in this study. It is particularly prevalent in the pheromones of other Phycitinae, such as *H. electellum* (Underhill et al., 1979), Indian meal moth *Plodia interpunctella* (Sower et al., 1974) and *V. e. serratilineella* (Struble and Richards, 1983). To our knowledge the conjugated tetradecadien-1-ol *EE*10,12–14:OH has not been identified in any other lepidopterous sex pheromone to date, although the corresponding acetate was identified by McDonough et al. (1982) in the female sex pheromone of the western avocado leafroller, *Amorbia cuneana*, together with (*E*)-10-tetradecenyl acetate.

As a result of the differences found in the female sex pheromone of *M. separatella* originating from Sierra Leone and Madagascar (H. Arn, personal communication), the taxonomic status of the adults is being investigated at the British Museum (Natural History). Differences have been found in the genitalia of both males and females, but at present it is uncertain whether they should be reclassified as subspecies, *M. separatella separatella* from Sierra Leone, and *M. separatella vectiferella* from Madagascar or as separate species (M. Shaffer, personal communication).

While blends of *ZE*9,12–14:OH and *EE*10,12–14:OH are effective attractants of male *M. separatella* when formulated on white rubber septa, they only have an effective field life of approximately one week. The reason for this is not known, although no change in the amounts of other isomers was found in analyses of field exposed septa. Despite this drawback, preliminary experiments suggest that pheromone trap catches can be related to subsequent larval infestations (Fannah, unpublished), so that trapping systems based on the synthetic pheromone lure could provide farmers with a monitoring system for predicting potentially damaging infestations of this pest.

Acknowledgments—The authors would like to thank Mrs. J.L. Smith and Dr. P.H. Adams for technical assistance with the laboratory studies. This study was partially supported by EEC DG XII contract TSD.A.204.UK(H). The insects were imported under MAFF licence No. PHF 1172/24.

REFERENCES

AGYEN-SAMPONG, M., and FANNAH, S.J. 1987. Seasonality of the white borer, *Maliarpha separatella* Rag. (Lepidoptera: Pyralidae) in mangrove swamp rice ecology of northwest Sierra Leone. *WARDA Tech. Newsl.* 7:4–6.

AKINSOLA, E.A. 1979. The biology and ecology of rice stem-borer in Nigeria. PhD thesis. University of Ibadan, Nigeria. 217 pp.

AKINSOLA, E.A. 1984. Effect of rice stem-borer infestation on grain yield and yield components. *Insect Sci. Appl.* 5:91–94.

AKINSOLA, E.A., and AGYEN-SAMPONG, M. 1984. The ecology, bionomics and control of rice stem-borers in West Africa. Mini-review. *Insect Sci. Appl.* 5:69–77.

ARN, H., TOTH, M., and PRIESNER, E. 1986. List of sex pheromones of Lepidoptera and related attractants. OILB-SROP/IOBC-WPRS, France.

ATTYGALLE, A.B., CAI-HONG, W., SCHWARZ, J., VOSTROWSKY, O., HASENFUSS, I., and BESTMANN, H.J. 1988. Sex pheromone of female *Myelois cribella* Hubner (Lepidoptera: Pyralidae). Chemical identification, electrophysiological evaluation, and field attractancy tests. *J. Chem. Ecol.* 14:485–494.

CAMPION, D.G., and NESBITT, B.F. 1983. The utilization of sex pheromones for the control of stem-borers. *Insect Sci. Appl.* 4:191–197.

CAMPION, D.G., HALL, D.R., and PREVETT, P.F. 1987. Use of pheromones in crop and stored products pest management: Control and monitoring. *Insect Sci. Appl.* 8:737–741.

COREY, E.J., and SUGGS, J.W. 1975. Pyridinium chlorochromate. An efficient reagent for oxidation of primary and secondary alcohols to carbonyl compounds. *Tetrahedron Lett.* 1975:2647–2650.

CORK, A., BEEVOR, P.S., GOUGH, A.J.E., and HALL, D.R. 1990. Gas chromatography linked to electroantennography: A versatile technique for identifying insect semiochemicals, pp. 271–279, *in* A.R. McCaffery and I.D. Wilson (eds.). Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press. London.

DUNCAN, D.B. 1955. Multiple range and multiple *F* tests. *Biometrics* 11:1–42.

GROB, K., and ZURCHER, F. 1976. Stripping of organic trace substances from water. Equipment and procedure. *J. Chromatogr.* 117:285–294.

HARRIS, W.E., and HABGOOD, H.W. 1966. Programmed temperature gas chromatography. John Wiley & Sons, New York.

HENRICK, C.A. 1977. The synthesis of insect sex pheromones. Tetrahedron Report no. 34. *Tetrahedron* 33:1–45.

HO, D.T., and SESHU REDDY, K.V. 1983. Monitoring of lepidopterous stem-borer population by pheromone and light traps. *Insect Sci. Appl.* 4:19–23.

HOUX, N.W.H., VOERMAN, S., and JONGEN, W.M.F. 1974. Purification and analysis of synthetic insect sex-attractants by liquid chromatography on a silver-loaded resin. *J. Chromatogr.* 96:25–32.

LYNCH, R.E., KLUN, J.A., LEONHARDT, B.A., SCHWARZ, M., and GARNER, J.W. 1984. Female sex pheromone of the lesser cornstalk borer, *Elasmopalpus lignosellus* (Lepidoptera: Pyralidae). *Environ. Entomol.* 13:121–126.

MCDONOUGH, L.M., HOFFMANN, M.P., BIERL-LEONHARDT, B.A., SMITHHISLER, C.L., BAILEY, J.B., and DAVIS, H.G. 1982. Sex pheromone of the avocado pest, *Amorbia cuneana* (Walsingham) (Lepidoptera: Tortricidae): Structure and synthesis. *J. Chem. Ecol.* 8:255–265.

MORSE, D., and MEIGHEN, E., 1984. Aldehyde pheromones in Lepidoptera: Evidence for an ester precursor in *Choristoneura fumiferana. Science* 226:1434–1436.

NESBITT, B.F., BEEVOR, P.S., LESTER, R., DAVIES, J.C., and SESHU REDDY, K.V. 1979. Com-

ponents of the sex pheromone of the spotted stalk borer, *Chilo partellus*. (Swinhoe) (Lepidoptera: Pyralidae): Identification and preliminary field trials. *J. Chem. Ecol.* 5:153–163.

NOWOTNY, H.P., SCHMALZING, D., WISTUBA, D., and SCHURIG, V. 1989. Extending the scope of enantiomer separation on diluted methylated β-cyclodextrin derivatives by high resolution gas chromatography. *J. High Resol. Chromatogr.* 12:383–393.

POLLET, A. 1978. Les ravageurs du riz en Cote d'Ivoire. V. Interactions entre *Maliarpha separatella* (Lepidoptera: Pyralidae) et *Pyricularia oryzae* (Fungi). *Z. Angew Entomol.* 85:324–327.

SILK, P.J., and KUENEN, L.P.S. 1988. Sex pheromone and behavioural biology of the coniferous Choristoneura. *Annu. Rev. Entomol.* 33:83–101.

SILK, P.J., TAN, S.H., WIESNER, C.J., ROSS, R.J., and LONERGAN, G.C. 1980. Sex pheromone chemistry of the eastern spruce budworm, *Choristoneura fumiferana. Environ. Entomol.* 9:640–644.

SOWER, L.L., COFFELT, J.A., and VICK, K.W. 1973. Sex pheromone: A simple method of obtaining relatively pure material from females of five species of moths. *J. Econ. Entomol.* 66:1220–1222.

SOWER, L.L., VICK, K.W., and TUMLINSON, J.H. 1974. (*Z,E*)-9,12-Tetradecadien-1-ol: A chemical produced by female *Plodia interpunctella* that inhibits the sex pheromone response of male *Cadra cautella. Environ. Entomol.* 62: 264–265.

STECK, W.F., UNDERHILL, E.W., and CHISHOLM, M.D. 1982. Structure–activity relationships in sex attractants for north American noctuid moths. *J. Chem. Ecol.* 8:731–754.

STRUBLE, D.L., and RICHARDS, K.W. 1983. Identification of sex pheromone components of the female driedfruit moth, *Vitula edmandsae serratilineella*, and a blend for attraction of male moths. *J. Chem. Ecol.* 9:785–801.

TEAL, P.E.A., and TUMLINSON, J.H. 1986. Terminal steps in pheromone biosynthesis by *Heliothis virescens* and *H. zea. J. Chem. Ecol.* 12:353–366.

TUMLINSON, J.H., and TEAL, P.E.A. 1987. Relationship of structure and function to biochemistry in insect pheromone systems, pp.3–26, *in* G.D. Prestwich and G.J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, London.

TUMLINSON, J.H., MITCHELL, E.R., and SONNET, P.E. 1981. Sex pheromone components of the beet armyworm, *Spodoptera exigua. J. Environ. Sci. Health A* 16:189–200.

TUMLINSON, J.H., HEATH, R.R., and TEAL, P.E.A. 1982. Analysis of chemical communications systems of Lepidoptera, pp. 1–25, *in* B.A. Leonhardt and M. Beroza (eds.). Insect Pheromone Technology: Chemistry and Applications, ACS Symposium Series 190. American Chemical Society, Washington, D.C.

UNDERHILL, E.W., ARTHUR, M.D., CHISHOLM, M.D., and STECK, W.F. 1979. Sex pheromone of the sunflower moth, *Homoeosoma electellum: Z*-9,*E*-12-tetradecadienol and *Z*-9-tetradecenol. *Environ. Entomol.* 8:740–743.

WAKAMURA, S. 1987. Sex pheromone of the beet armyworm, *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae): Field attraction of male moths in Japan to (*Z,E*)-9,12-tetradecadienyl acetate and (*Z*)-9-tetradecen-1-ol. *Appl. Entomol. Zool.* 22:348–351.

WARDA. 1977. Regional Mangrove Swamp Rice Research Station, Rokupr, Annual Report. WARDA, Monrovia, Liberia.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

EVIDENCE FOR MALE-PRODUCED AGGREGATION PHEROMONE IN AMERICAN PALM WEEVIL, *Rhynchophorus palmarum* (L.) (COLEOPTERA: CURCULIONIDAE):

DIDIER ROCHAT,[1,*] ARIEL GONZÁLEZ V.,[2] DOMINIQUE MARIAU,[3] ALEXANDER VILLANUEVA G.,[2] and PIERRE ZAGATTI[1]

[1]*Laboratoire des Médiateurs Chimiques, INRA*
Domaine de Brouessy, 78114 Magny-les-Hameaux, France

[2]*Palmeras de la Costa S.A.*
Subgerencia técnica y Departamiento de sanidad vegetal
Calle 54 No. 10-81, AA 050364, Bogotá, Colombia

[3]*Institut de Recherche pour les Huiles et les Oléagineux, CIRAD*
Département Entomologie
Avenue du Val de Montferrand, 34032 Montpellier Cedex, France

(Received November 21, 1990; accepted February 14, 1991)

Abstract—Field trapping of the American palm weevil (APW), *Rhynchophorus palmarum*, showed that the combination of caged male APWs and palm stem was much more attractive to APWs of both sexes than palm stem alone. Caged female APWs did not enhance the attractiveness of the palm. Caged APWs without palm stem were not attractive. Virgin laboratory-bred males were highly attractive to APWs of both sexes in a two-choice pitfall olfactometer, whereas virgin laboratory-bred females were not. Adsorbent-trapped volatiles from virgin laboratory-bred males reproduced the effect of living males, giving evidence for a male-produced aggregation pheromone in this species. Wild-mated APWs of both sexes were as responsive to the aggregation pheromone as virgin laboratory-bred APWs. This is the first record of chemical communication in this species. These results have prompted investigations into the chemical identification of the aggregation pheromone.

Key Words—Aggregation pheromone, field trapping, laboratory bioassay, Coleoptera, Curculionidae, *Rhynchophorus palmarum*.

*To whom correspondence may be addressed.

0098-0331/91/0600-1221$06.50/0

INTRODUCTION

The American palm weevil (APW), *Rhynchophorus palmarum* (L.), is a serious pest on coconut, *Cocos nucifera* L., and oil palm, *Elaeis guineensis* Jacq., trees in the neotropical region (Lepesme et al., 1947; Genty et al., 1978). It is occasionally a pest on sugarcane, *Saccharum officinarum* L. (Restrepo et al., 1982). The APW adult is the major known vector of red ring disease caused by the nematode *Rhadinaphelenchus cocophilus* Goodey (Griffith, 1968, 1987). APW larvae develop in the stems, buds, and rachis of leaves and inflorescences within a large variety of Palmae species (Lepesme et al., 1947; Hagley, 1965). The only way to prevent red ring disease is to eliminate the adults and larvae of the APW, since the difficulty and cost of nematicide treatments are extreme.

Extensive studies have been conducted on the development and larval rearing of palm weevils from the genus *Rhynchophorus* (Wilson, 1963; Giblin-Davis et al., 1989). In contrast, not much is known about the chemical ecology of the adults. They have been caught in traps baited with palm pieces (Morin et al., 1988; Rochat, 1990). It has been observed for a long time that wounds on palms are attractive to the APW (Mariau, 1968), but nothing is known about the role of the APW itself in the aggregation of the weevils on trees.

This study reports investigations carried out in the field and in the laboratory and presents evidence to document the existence of a male-produced aggregation pheromone in the APW.

METHODS AND MATERIALS

Field Experiments

The attractiveness of seven combinations of living APWs and oil palm stem was studied (Table 1). The experiment was conducted in an oil palm plantation of northern Colombia. The bait weevils were caught on oil palm trees felled before the experiment. These weevils were mated as shown by the hatching of the eggs laid by females trapped in the field. Four insects (four males or four females or two males + two females) were placed in wire mesh cages (8 × 5 × 3 cm). The traps consisted of plastic jerricans (38 × 30 × 17 cm) from which one side had been removed. Palm-baited jerricans were filled with stem pieces from the same freshly felled oil palm tree. Other jerricans were filled with wood shavings as shelters for the insects. Cages containing weevils were placed at the bottom of the jerricans onto a half mango as food for the weevils. The bait weevils could also feed on palm stem pieces in palm-baited traps. Wire cages were hidden by the stem pieces or the shavings. No killing agent was added to the trap because of the use of living weevils as bait, and

TABLE 1. APW CAPTURES OBTAINED WITH DIFFERENT COMBINATIONS OF 4 LIVING APW AND PALM STEM

Treatment	Mean captures ($\Sigma n_i/4$) per 4-day period (range)[a]	Sex ratio (Male : Female)
4 males without stem	0.5 (0–2)a	2:0
4 females without stem	0.5 (0–1)a	1:1
2 males + 2 females without stem	0.8 (0–1)a	1:2
Stem without insects	3.0 (1–4)b	1:0.6
Stem with 4 females	3.3 (3–4)b	1:0.9
Stem with 2 males + 2 females	3.0 (2–9)b	1:2.6
Stem with 4 males	12.3 (5–18)c	1:1.0
$F(6, 21)$	13.66 ($P < 0.0001$)	—

[a] Data were subjected to a log ($n_i + 1$) transformation for homoscedasticity purposes. Statistical reference is to a one-way analysis of variance on the transformed variate. Treatments followed by the same letter do not differ significantly at 5% level according to Newman-Keuls test on the means of transformed values.

lured weevils could freely leave the jerricans. Seven traps were baited with each of the different combinations of palm and APW. The 49 traps were placed 80 m apart on the ground according to a Latin-square design, each one at the base of a palm tree. The weevils caught in the traps were collected twice a day at ca. 2 hr after dusk and ca. 3–4 hr after sunrise. This regime took into account the daily rhythm of the APW flights (Hagley, 1965; Lucchini and Morin, 1985; Rochat, 1990). Weevils were collected for only four consecutive days since we observed that older pieces of palm stem were not attractive to APWs (Rochat, 1990).

The experiment consisted of four four-day trapping periods with, each time, random permutation of the Latin-square design, random redistribution of bait weevils into the wire cages, and replacement of the decaying palm material by fresh material. Two consecutive trapping periods were separated by three days.

The data were subjected to a one-way analysis of variance on the transformed variate log ($n_i + 1$), n_i being the total number of insects caught in the seven traps of the i^{th} palm–APW combination during one four-day period. Differences between means were tested using the Newman-Keuls test at the 5% level (Dagnelie, 1975).

Laboratory Bioassays

Insects. Weevils of two distinct histories were used: wild-mated weevils from Equador or Colombia and virgin weevils reared on sugarcane in the laboratory from a strain that originated in Equador. Sexes were separated on arrival

of the wild insects or when the reared adults eclosed. These large weevils (35–45 mm long) were kept individually in cylindrical plastic boxes (105 × 75 mm) with a wire mesh cover and fed with sugarcane. They were maintained at 27 ± 2°C and 85 ± 10% relative humidity on a 12 : 12 light–dark regime.

Volatile Collections. Five virgin APW males were placed in a cylindrical glass jar (6 × 22 cm) with three sugarcane pieces. Three pieces of the same size and from the same stem were placed in a similar jar as a control. Volatiles emitted by APWs and/or sugarcane were simultaneously trapped onto two glass cartridges connected downstream to the jars during a two-day period. Each cartridge was filled with 0.5 g of Supelpak-2 adsorbent (16–50 mesh; purified Amberlite XAD-2 resin, Supelco). The volatiles were collected in a room maintained at 27 ± 2°C with a 12 : 12 light–dark regime. A regulated airflow (500 ml/min) passed through the glass systems. The cartridges were eluted with 2.5 ml of methylene chloride (HPLC grade, Waters). The eluates were then concentrated at 100 μl under a nitrogen flow (150 ml/mn) and stored at −30°C until use.

Insect Bioassays. Insects were bioassayed using a two-choice pitfall olfactometer adapted from Pierce et al. (1981). The olfactometer consists of a circular arena (40 × 14 cm) connected to two glass jars. The floor of the arena is made of Plexiglas and covered with white paper to allow the weevils to walk. A filter paper disk closes each jar at 4 cm from below the floor of the arena to prevent any visual stimulus when weevils are introduced into the jar as bait. Filter papers also close the two jars when volatiles are used as bait. The pitfall olfactometer is placed at the center of a 70 × 70-cm arena surrounded by white walls and homogeneously lighted with four red painted fluorescent tubes (18 W, daylight type D) giving a 40-lux lighting at the olfactometer level.

Bioassays were run during the first half of the scotophase at 27 ± 2°C. The weevils were transferred to the testing room in their individual boxes half an hour prior to an experiment. A single weevil was released at the center of the olfactometer arena and its position was recorded after 30 min. Weevils were classified as responding only if they fell into a jar (stimulus or control) or clung to the underside of the arena floor in a jar.

Male and female individual responses to three different stimuli versus a control were recorded: (1) three virgin laboratory-bred males versus no insects; (2) three virgin laboratory-bred females versus no insects; (3) virgin laboratory-bred male effluvia + sugarcane volatiles versus sugarcane volatiles alone. One microliter of methylene chloride extract containing the volatiles to be tested (0.1 male-day-equivalent) was applied to a filter paper (17 × 17 mm) placed into a glass jar just prior to the test.

The responses of APWs also were recorded in a control treatment with two empty jars. Twenty to 35 of both wild-mated and virgin laboratory-bred weevils were bioassayed in each of the different cases.

Data Analysis

Two criteria were used to quantify the APW responses. First, the proportion of tested weevils that responded after 30 min; the proportions of weevils that responded in presence of a stimulus (living males, living females, male + sugarcane volatiles) were compared with that obtained in the absence of a stimulus (control treatment) using the χ^2 test for two independant samples (Siegel, 1956). Secondly, the number of APW choices for the stimulus jar was compared with the number of choices for the associated control jar using the nonparametric binomial test (Siegel, 1956) with the null hypothesis of an equal probability of choosing the control or the stimulus.

Comparisons between sexes for the same situation and between wild-mated and virgin laboratory-bred insects were analyzed by χ^2 tests.

RESULTS

Field Experiments (Table 1)

The bait of palm stem + four males was very attractive to APWs, significantly more so than all other baits. The baits of palm stem + four females and palm stem without caged APWs were equally attractive to APWs and significantly more attractive than the three baits that did not contain palm stem but only caged APWs.

Laboratory Bioassays

Few APWs responded in the absence of an experimental stimulus (control treatment with two empty jars), and the responses occurred randomly between the two empty jars (Table 2).

The introduction of three virgin laboratory-bred males as bait induced a significant response by both virgin laboratory-bred and wild-mated APWs after 30 min. Weevil choice for the male-baited jar was highly significant (Table 2).

There was very little response to three virgin laboratory-bred females as bait (Table 2). Only virgin laboratory-bred females had a slightly heightened activity level. No significant choices occurred to the female-baited jar by any category of test weevils.

The volatiles from males and sugarcane induced high numbers of weevils to respond (Table 2). The effect was slightly stronger in virgin females than males. Both laboratory-bred and virgin APWs preferred the male + sugarcane volatiles to the sugarcane volatiles alone.

TABLE 2. APW RESPONSES TO VARIOUS STIMULI IN TWO-CHOICE PITFALL OLFACTOMETER AFTER 30 MINUTES

Source of test weevils	Treatment	Sex tested	Number tested	Number of responses[a]	Percent response		Binomial probability stimulus vs. control[b]
					Stimulus	Control	
Laboratory-bred, virgin	2 empty jars (control)	Male	30	8	50	50	.637
		Female	20	2	50	50	—
	3 living males vs. no insects	Male	30	21***	90	10	.0002
		Female	22	17***	94	6	.0002
	3 living females vs. no insects	Male	28	11 ns	73	27	.113
		Female	20	8*	38	62	.363
	Virgin male + food volatiles vs. food volatiles	Male	31	21**	71	29	.021
		Female	21	14***	93	7	.001
Wild, mated	2 empty jars (control)	Male	30	17	47	53	.395
		Female	30	8	63	37	.363
	3 living males vs. no insects	Male	30	21 ns	81	19	.004
		Female	30	19**	89	11	.0004
	3 living females vs. no insects	Male	23	7 ns	57	43	.363
		Female	25	12 ns	67	33	.194
	Virgin male + food volatiles vs. food volatiles	Male	35	17 ns	82	18	.006
		Female	35	27***	81	19	.0007

[a] Difference from the control (χ^2 test) indicated by: ns: not significant, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.
[b] Under the null hypothesis to respond to the stimulus and to the control with an equal probability of 1/2.

The percentages of wild-mated males responding to any stimulus were not statistically greater than the responses obtained in the control treatment (Table 2), probably because many males entered unbaited jars in the control treatment.

No significant differences appeared between responses of wild-mated and virgin laboratory-bred APWs within each sex.

DISCUSSION

The requirement for palm stem to be a component of an attractive stimulus for APWs agreed with previous observations that palm tissues were attractive to the palm weevils (Nadarajan, 1984; Morin et al., 1988). The male effect in combination with palm stem volatiles was aggregative since male as well as female APWs were caught in a nearly 1 : 1 sex ratio. Synergy for attraction between beetle-produced and host-plant chemicals has been found frequently in Coleoptera, especially in bark beetles and stored-product insects (Borden, 1985). A similar synergistic relationship was shown for the pea and bean weevil, *Sitona lineatus* L., (Blight and Wadhams, 1987). Meksongsee and Sakulpanich (1975) showed that coconut + male *Rhynchophorus vulneratus* Panzer baits were much more attractive than coconut + female *R. vulneratus* baits, but synergy between males of this species and the host plant has not been proved.

The presence of APW females with males seemed to modify the male-produced aggregating effect, but the lower number of males (two instead of four) used for this bait could explain in part the weak catches. However, recent field trapping data (Lacerda Moura,[1] personal communication) support the hypothesis that females modify the release by males of an aggregation pheromone or that females release a antiaggregative stimulus.

The few weevils caught in traps baited only with caged APWs might indicate either that males do not release the aggregation stimulus in the absence of host-plant volatiles or that flying weevils might be lured only by the simultaneous perception of host and male APW stimuli. The hypothesis of sonic communication (Dumortier, 1963; Haskell, 1974; Rudinsky and Ryker, 1977) in APWs was refuted by the laboratory observations in which attraction was induced solely by captured host-plant plus male volatiles.

Among the numerous olfactometers described in the literature to investigate aggregation pheromones in adults of coleopteran species (Hardee et al., 1967; Payne et al., 1976; Borden et al., 1979; Phillips and Burkholder, 1981; Pierce et al., 1981; Faustini et al., 1982b), the two-choice pitfall olfactometer

[1] CEPLAC/CEPEC, Km 22, Rod. Ilheus/Itabuna, CX. Postal 7, 45.600-Itabuna-Ba., Brazil.

proved to be highly discriminative. This type of olfactometer gave accurate responses by the APW too. Our single-insect laboratory bioassay eliminated the need to work with large olfactometers adapted for multiple-insect tests with these very large beetles. Furthermore it avoided any possibility that bioassayed weevils could have emitted pheromones that biased the results. It avoided also any tactile and visual interaction between individuals, which are extremely strong in APW group formation, especially under artificial conditions (Rochat, 1987).

The attractiveness of the living males without palm in pitfall bioassays appears to contradict the results in the field. However, the time and space scales were totally different in the two experiments, as was the physiological stage of the bait weevils.

Mating seemed not to modify the response by APWs to the aggregation pheromone since the responses of mated and virgin APWs did not differ. This result is consistent with the fact that APWs have considerable longevity and are able to copulate several times during their life (Wilson, 1963). The retention of a high response capability would ensure that weevils are able to aggregate on favorable sites throughout their life. Phillips and Burkholder (1981) reported that mated and virgin *Sitophilus oryzae* L. were equally responsive to aggregation pheromone. O'Ceallachain and Ryan (1977) also demonstrated that mating did not affect the response of *Tribolium confusum* L. to its aggregation pheromone.

The APW (*Rhynchophorus palmarum*) belongs the subfamily Rhynchophorinae as do the *Sitophilus* spp. Male-produced aggregation pheromones have been reported in three species from this latter genus (Phillips and Burkholder, 1981; Faustini et al., 1982a; Walgenbach et al., 1983). The existence of a male-produced aggregation pheromone in the APW is a new report of such a pheromone system in this Curculionidae subfamily.

This first record of chemical communication in this pest has opened new fields of investigation for a potential improvement of APW and red ring disease control. These results have prompted chemical investigations to identify the APW aggregation pheromone. Synthetic pheromone could be used as a weevil lure in combination with either palm stems or synthetic host-plant kairomones. Studies on the chemical identification and synthesis of the APW aggregation pheromone are underway.

Acknowledgments—We thank Palmeras del Ecuador S.A. (Equador) for providing wild weevils. We thank the Institut de Recherche pour les Huiles et les Oléagineux (France), Fedepalma (Colombia), and P. Genty (INDUPALMA, Colombia) for financial and technical assistance. We also thank R. Vallée for insect rearing; J.I. Lacerda Moura (CEPLAC, Brazil) for communicating unpublished results; J.H. Borden, B. Frérot, B. Lalanne-Cassou, G. Lemperière, and M. Renou for critical readings of the manuscript; and A. Bishop for English revision.

REFERENCES

BLIGHT, M.M., and WADHAMS, L.J. 1987. Male-produced aggregation pheromone in pea and bean weevil, *Sitona lineatus* (L.). *J. Chem. Ecol.* 13:733–739.

BORDEN, J.H. 1985. Aggregation pheromones, pp. 257–285, *in* G.A. Kerkut and L.I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 9. Pergamon Press, Oxford.

BORDEN, J.H., DOLINSKI, M.G., CHONG, L., VERIGIN, V., PIERCE, H.D., JR., and OEHLSCHLAGER, A.C. 1979. Aggregation pheromone in the rusty grain beetle, *Cryptolestes ferrugineus* (Coleoptera: Cucujidae). *Can. Entomol.* 111:681–688.

DAGNELIE, P. 1975. Théorie et méthodes statistiques, applications agronomiques, Vol. 2. Les presses agronomiques de Gembloux, Belgium. pp. 107–252.

DUMORTIER, B. 1963. Morphology of sound emission apparatus in Arthropoda, pp. 277–304, *in* R.G. Busnel (ed.). Acoustic Behaviour of Animals. Elsevier, Publishing Co., Amsterdam.

FAUSTINI, D.L., GIESE, W.L., PHILLIPS, J.K., and BURKHOLDER, W.E. 1982a. Aggregation pheromone of the male granary weevil, *Sitophilus granarius* (L.). *J. Chem. Ecol.* 8:679–687.

FAUSTINI, D.L., ROWE, J.R., and BURKHOLDER, W.E. 1982b. A male-produced aggregation pheromone in *Tribolium brevicornis* (Leconte) (Coleoptera: Tenebrionidae) and interspecific responses of several *Tribolium* species. *J. Stored Prod. Res.* 18:153–158.

GENTY, P., DESMIER DE CHENON, R., and MORIN, J.P. 1978. Les ravageurs du palmier à huile en Amérique Latine. *Oléagineux* 33:325–419.

GIBLIN-DAVIS, R.M., GERBER, K., and GRIFFITH, R. 1989. Laboratory rearing of *Rhynchophorus cruentatus* and *R. palmarum* (Coleoptera: Curculionidae). *Fla. Entomol.* 72:480–488.

GRIFFITH, R. 1968. The relationship between the red ring nematode and the palm weevil. *J. Agric. Soc. Trin. Tob.* 68:342–356.

GRIFFITH, R. 1987. Red ring disease of coconut palm. *Plant Dis.* 71:193–196.

HAGLEY, E.A.C. 1965. On the life history and habits of the palm weevil, *Rhynchophorus palmarum. Ann. Entomol. Soc. Am.* 58:22–27.

HARDEE, D.D., MITCHELL, E.B., and HUDDLESTON, P.M. 1967. Procedure for bioassaying the sex attractant of the boll weevil. *J. Econ. Entomol.* 60:169–171.

HASKELL, P.T. 1974. Sound Production, pp. 353–410, *in* M. Rockstein (ed.). The Physiology of Insecta, Vol. 2, 2nd ed. Academic Press, New York.

LEPESME, P., GHESQUIÈRE, J., BOURGOGNE, J., CAIRASCHI, E., PAULIAN R., and VILLIERS, A. 1947. Les insectes des palmiers. Paul Lechevalier, Paris. pp. 611–617.

LUCCHINI, P., and MORIN, J.P. 1985. Estudos e controle de *Rhynchophorus palmarum* (L.) em dendê no Estado de Bahia, Manaus. Relatorio de projecto de pesquisa, EMBRAPA/CNPSD. 15 pp.

MARIAU, D. 1968. Méthodes de lutte contre le Rhynchophore. *Oléagineux* 23:443–446.

MEKSONGSEE, B., and SAKULPANICH, U. 1975. Field study of trapping coconut weevil, *Rhynchophorus vulneratus* (Panzer), with sex attraction. 4th Session of the FAO technical working party on Coconut production, protection and processing. Kingston Jamaica, September 14–25, 1975. 5 pp.

MORIN, J.P., LUCCHINI, F., ARAUJO, J.C., FERREIRA, J.M., and FRAGA, L.S. 1988. Le contrôle de *Rhynchophorus palmarum* par piégeage à l'aide de morceaux de palmier. *Oléagineux* 41:57–59.

NADARAJAN, L. 1984. Studies on trapping the palm weevil, *Rhynchophorus phoenicis* F. pp. 12–38, *in* Coconut and Oil Palm Entomology, Training report, IRHO, Abidjan, Ivory Coast.

O'CEALLACHAIN, D.P., and RYAN, M.F. 1977. Production and perception of pheromones by the beetle *Tribolium confusum. J. Insect Physiol.* 23:1303–1309.

PAYNE, T.L., HART, E.R., EDSON, L.J., MCCARTY, F.A., BILLINGS, P.M., and COSTER, J.E.

1976. Olfactometer for assay of behavioral chemicals for the southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Scolytidae). *J. Chem. Ecol.* 2:411–419.

PHILLIPS, J.K., and BURKHOLDER, W.E. 1981. Evidence for a male-produced aggregation pheromone in the rice weevil. *J. Econ. Entomol.* 74:539–542.

PIERCE, A.M., BORDEN, J.H., and OEHLSCHLAGER, A.C. 1981. Olfactory response to beetle-produced volatiles and host-food attractants by *Oryzaephilus surinamensis* and *O. mercator*. *Can. J. Zool.* 59:1980–1990.

RESTREPO, G.L., RIVERA, A.F., and RAIGOSA, B.J. 1982. Ciclo de vida, hábitos y morfometría de *Metamasius hemipterus* Olivier y *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae) en caña de azucar (*Saccharum officinarum* L.). *Acta Agron. Colombia* 32:33–44.

ROCHAT, D. 1987. Etude de la communication chimique chez un Coléoptère Curculionidae: *Rhynchophorus palmarum* L. DEA report, Université Paris VI. 30 pp.

ROCHAT, D. 1990. *Rhynchophorus palmarum* L. (Coleoptera, Curculionidae): Nuevos datos sobre el comportamiento del insecto y su control por trampeo olfactivo. Perspectivas. *Palmas* 10:69–79.

RUDINSKY, J.A., and RYKER, L.C. 1977. Olfactory and auditory signals mediating behavioral patterns of bark beetles. *Coll. Int. C.N.R.S.* 265:195–207.

SIEGEL, S. 1956. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York. 312 pp.

WALGENBACH, C.A., PHILLIPS, J.K., FAUSTINI, D.L., and BURKHOLDER, W.E. 1983. Male-produced aggregation pheromone of the maize weevil, *Sitophilus zeamais*, and interspecific attraction between three *Sitophilus* species. J. Chem. Ecol. 9:831–841.

WILSON, M.E. 1963. Investigations into the development of the palm weevil *Rhynchophorus palmarum* (L.). *Trop. Agric. Trin. Tob.* 40:185–196.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSES OF APHIDS TO HOST AND NONHOST PLANT VOLATILES

STEPHEN F. NOTTINGHAM,[1,*] JIM HARDIE,[1] GLENN W. DAWSON,[2] ALASTAIR J. HICK,[2] JOHN A. PICKETT,[2] LESTER J. WADHAMS,[2] and CHRISTINE M. WOODCOCK[2]

[1]*Agricultural and Food Research Council Linked Research Group in Aphid Biology*
Department of Biology
Imperial College of Science, Technology and Medicine
London SW7 2AZ, UK

[2]*AFRC Institute of Arable Crops Research*
Rothamsted Experimental Station
Harpenden, Herts AL5 2JQ, UK

(Received December 3, 1990; accepted February 19, 1991)

Abstract—Alate and apterous virginoparae of *Aphis fabae* Scop. and alate virginoparae of *Brevicoryne brassicae* (L.), walking in a linear track olfactometer, were attracted by odor from leaves of their host plants. *A. fabae* responded to odor from undamaged but not damaged bean leaves. Gynoparae (autumn migrants) of *A. fabae*, however, did not respond to their host plant (spindle, *Euonymus europaeus*) odor. Odors of certain nonhost plants masked the attractiveness of the host plant leaves, but tansy (*Tanacetum vulgare*) and summer savory (*Satureja hortensis*) volatiles repelled *B. brassicae* and *A. fabae*, respectively. 3-Butenyl isothiocyanate attracted *B. brassicae* and *Lipaphis erysimi* (Kalt.), the latter species being more sensitive in both behavioral and electrophysiological studies. Isothiocyanate receptors were found on the antennae of *A. fabae*, which was repelled by these compounds, 4-pentenyl isothiocyanate being the most active.

Key Words—Aphid, *Aphis fabae*, *Brevicoryne brassicae*, Homoptera, Aphididae, *Lipaphis erysimi*, olfaction, plant volatiles, isothiocyanate, electrophysiology, repellent, odor masking.

*To whom correspondence should be addressed at: AFRC Linked Research Group in Aphid Biology, Imperial College at Silwood Park, Ascot, Berks SL5 7PY UK.

0098-0331/91/0600-1231$06.50/0

INTRODUCTION

The role that host plant odor plays in aphid host finding and selection is still uncertain. For many years, odor was thought not to be involved in long-range host finding. Field studies demonstrated equal landing rates on host and nonhost plants by gynoparae and alate virginoparae of *Myzus persicae* and *Aphis fabae* and alate virginoparae of *Brevicoryne brassicae* (Kennedy et al., 1959a,b; Müller, 1962), suggesting that hosts were only selected after landing, with aphids having differential leaving rates from hosts and nonhosts. However, electrophysiological responses to plant volatiles have been shown in *Nasonovia ribis-nigri* (Bromley and Anderson, 1982), *Sitobion avenae* (Yan and Visser, 1982), *Lipaphis erysimi* (Dawson et al., 1987), and *A. fabae* (Wadhams, 1990), and behavioral responses have been shown in olfactometers (*A. fabae*, Alikhan, 1960; *B. brassicae* and *Rhopalosiphum padi*, Pettersson, 1970, 1973; *Aphis gossypii*, Pospisil, 1972), in the field (*Cavariella aegopodii*, Chapman et al., 1981; *Phorodon humuli*, Campbell et al., 1990), and in a wind-tunnel experiment on walking *Cryptomyzus korschelti* (Visser and Taanman, 1987), which demonstrated an odor-conditioned upwind anemotaxis. In addition, herbs have been recommended in the popular and organic gardening literature for many years as a means of repelling or deterring aphid pest species. For example, savory (*Satureja* spp.) and tansy (*Tanacetum vulgare*) are suggested as companion plants to deter aphids (e.g., Yepson, 1984) and reductions in *M. persicae* populations on bell peppers (*Capsicum luteus*) were reported after interplanting with tansy (Matthews et al., 1983). Thus, there is evidence that volatiles are implicated in host finding and that this behavior can be modified by nonhost odors.

This study was designed to reassess the role of host and nonhost plant odors in the orientation behavior of the black bean aphid, *A. fabae*, the cabbage aphid, *B. brassicae*, and the turnip aphid, *Lipaphis erysimi*. *A. fabae* alternates between a primary host, usually spindle (*Euonymus europaeus* L.), and a range of herbaceous secondary hosts, including broad bean (*Vicia faba* Moench). *B. brassicae* and *L. erysimi* complete their life-cycles on plants in the Cruciferae (Brassicaceae) and Resedaceae, families characterized by the presence of glucosinolates. These compounds have been implicated in the attraction of *B. brassicae* to rape buds and flowers (Pettersson, 1973); they are also reported to stimulate feeding in *B. brassicae* (Wensler, 1962) and *L. erysimi*, but to deter feeding in other species, including *A. fabae* (Nault and Styer, 1972). Glucosinolates are decomposed by enzymic action to produce volatile isothiocyanates (Kjaer, 1960; Ju et al., 1982). Behavioral responses of *B. brassicae*, *L. erysimi*, and *A. fabae* to a range of isothiocyanates were therefore examined in an olfactometer. As *L. erysimi* is known to possess olfactory receptors for isothiocyan-

ates (Dawson et al., 1987), such receptors were also sought in *A. fabae* and *B. brassicae*.

METHODS AND MATERIALS

Insects. A. fabae were reared on tick beans (*Vicia faba*) in environmental cabinets held at 15°C. Apterae were obtained from continuous cultures, while alate virginoparae (summer migrants) were produced by crowding early instar aphids in long-day (light–dark 16 : 8) conditions. Gynoparae (autumn migrants) were induced by short days (light–dark 12 : 12). *B. brassicae* and *L. erysimi* were reared on Brussels sprouts (*Brassica oleracea* L., Bedford Darkmar) and turnip (*Brassica campestris* var. *rapifera* Metz.), respectively, in long days, with alates again being induced by crowding. All experimental aphids were young adults, starved for 24 hr, and used only once.

Plant Material. Host plants, bean (*Vicia faba*: Sutton dwarf bean or tick bean) and Brussels sprouts (*B. oleracea*: Bedford Darkmar) were greenhouse-grown, while spindle (*Euonymus europaeus*) leaves were field-picked. Non-hosts, winter savory (*Satureja montana* L.), summer savory (*Satureja hortensis* L.), tansy (*Tanacetum vulgare* L.), basil (*Ocimum basilicum* L.), thyme (*Thymus vulgaris* L.), and sage (*Salvia officinalis* L.) were grown in the greenhouse. All host and nonhost plant leaves were freshly picked and weighed prior to experiments.

Olfactometer. A linear track olfactometer, based on a design by Sakuma and Fukami (1985) and modified for aphids (Hardie et al., 1990), was constructed from transparent Plexiglas tubing and steel rods. The rods formed a T junction at the point where equal airstreams met from two side arms in the olfactometer. Twenty-five adult aphids were placed in a Fluon-lined dish at the base of the vertical rod and those that climbed up were scored for their direction of turn along the horizontal rod. All subsequent movement was ignored. An airflow of 1 liter/min was maintained in all experiments, with the air being subsequently exhausted from the room. The apparatus was housed within a black box and aphids were attracted upwards by a diffuse light. When plant material was used as a treatment, filter papers (Whatman No. 1; 4.25 cm) saturated with water were included to create equivalent humidities. Treatment and control sides were alternated for each new replicate and runs lasted for 10 min. Experiments were conducted at 19–23°C; between replicates the olfactometer was washed with methanol and soapy water. All data were analyzed using paired t tests, with means quoted $\pm$ SE and significance taken as $P < 0.05$.

Responses of alate and apterous *A. fabae* to three different levels of host plant leaf damage were tested: (1) undamaged, where leaves were gently picked

off the plant; (2) light damage, where leaves were rolled between the fingers; and (3) heavy damage, where leaves were crushed prior to use. All treatments employed whole leaves weighing 2 g (± 0.2 g). In bioassays with *B. brassicae*, and all host and nonhost experiments, undamaged leaf material was used. Host and nonhost leaves were presented together, and separately, in experiments with alate virginoparae of *A. fabae* and *B. brassicae*. Two ratios of host to nonhost leaf weight were used (2.0:2.0 g and 2.0:0.5 g).

Chemicals. Allyl, 3-butenyl, 4-pentenyl, and phenylethyl isothiocyanates (Figure 1) were obtained commercially or synthesized by standard methods. Isothiocyanates in hexane (10 μl) were applied to filter paper (Whatman No. 1; 4.25 cm) and placed in the treatment side of the olfactometer. Hexane (10 μl) alone served as the control.

Electrophysiology. Recordings from cells associated with the olfactory receptors on the proximal primary rhinaria of alate virginoparous *A. fabae* and *B. brassicae* were obtained using tungsten microelectrodes. Signals were amplified and recorded by standard methods (Wadhams et al., 1982; Dawson et al., 1987). The stimulus was delivered into a purified airstream (1 liter/min) that flowed continuously over the preparation. The delivery system, which utilized a filter paper in a disposable Pasteur pipet cartridge, has been described previously (Wadhams et al., 1982). The impulse frequency was determined as the number of impulses elicited during the first 1 sec after stimulus application.

RESULTS AND DISCUSSION

There was no preferred direction of turn for apterous or alate *A. fabae* or for alate *B. brassicae* when the two chambers of the olfactometer contained only damp filter paper. However, *A. fabae* virginoparae were attracted to undamaged, but not to damaged, bean leaves (Table 1); apterae were attracted

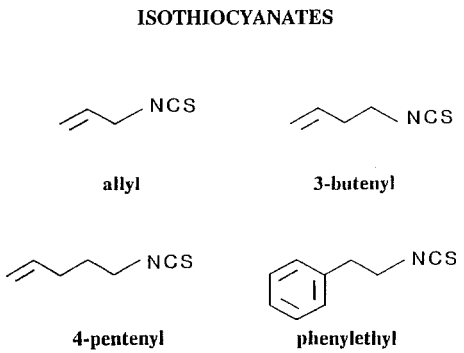


FIG. 1. Structures of isothiocyanates.

TABLE 1. RESPONSE (±SE) OF APTEROUS AND ALATE VIRGINOPARAE OF *Aphis fabae* TO BEAN LEAVES[a]

	Apterous virginoparae		Alate virginoparae	
	Treatment	Control	Treatment	Control
1. Undamaged tick bean	7.1 ± 0.5	4.6 ± 0.5 A	8.3 ± 0.8	6.4 ± 0.5 ns
2. Undamaged Sutton bean	6.9 ± 0.5	4.3 ± 0.5 A	7.9 ± 0.8	5.4 ± 0.4 A
3. Lightly damaged bean	4.6 ± 0.6	5.4 ± 0.8 ns	6.5 ± 0.6	6.3 ± 0.7 ns
4. Heavily damaged bean	4.3 ± 0.5	5.5 ± 0.7 ns	6.4 ± 0.8	6.5 ± 1.0 ns

[a] 3 and 4: apterous virginoparae with tick beans and alate virginoparae with Sutton dwarf beans, respectively. A = attraction, ns = not significantly different at $P < 0.05$, two-tailed paired t tests (eight replicates).

to both undamaged tick beans and Sutton dwarf beans, while alates were only significantly attracted by the Sutton dwarf beans. The lack of response to damaged plants may be due to an inability to recognize the altered volatile profile as that of a host plant. The degree of leaf damage, however, would have been greater than that normally encountered in the field. Alate virginoparae were not attracted to odor from mature leaves of spindle, the primary host (6.8 ± 0.4 cf. 6.4 ± 0.6; $N = 8$), and gynoparae did not respond to bean leaves or to mature (7.4 ± 0.7 cf. 6.1 ± 1.1; $N = 8$) or scenescent spindle leaves (6.5 ± 0.5 cf. 6.8 ± 0.4; $N = 8$). No response to spindle was observed even after 72 hr of starvation (3.6 ± 0.5 cf. 2.8 ± 0.3; $N = 8$), which should have ensured that the insects had completed any migratory or host-ignoring behavioral phase associated with olfactory cues, i.e., comparable to that observed with visual cues (Nottingham and Hardie, 1989). Previous olfactometer studies have produced variable results for responses of *A. fabae* to host plant odor. Alikhan (1960) reported attraction of apterous and alate virginoparae to bean and sugar beet leaf extracts, while Jones (1944) found no evidence for olfactory responses of alate virginoparae to bean, spindle, or leaf extracts. The present results suggest that plant volatiles play a role in host location by some aphid morphs.

Although alate virginoparae of *A. fabae* were attracted to Sutton dwarf beans alone and to beans in 4:1 ratios with winter savory and tansy and in a 1:1 ratio with sage, no significant attraction was elicited from 1:1 ratios of beans with winter savory, summer savory, tansy, thyme, or basil (Table 2). Summer savory alone appeared to be repellent. Similarly, alate virginoparae of *B. brassicae* were attracted to Brussels sprouts (host) odor, but combinations

of sprouts and winter savory or tansy odor were either unattractive or repellent (Table 3). Tansy alone also proved to be repellent.

It thus appears that attraction of aphids to host leaves can be disrupted by the presence of nonhost plant odor. This may be due to repellency, i.e., oriented movement away from an odor source (Dethier et al., 1960) or to odor masking,

TABLE 2. RESPONSE (±SE) OF ALATE VIRGINOPARAE OF *Aphis fabae* TO COMBINATIONS OF HOST AND NONHOST PLANT LEAVES[a]

	Ratio	Treatment	Control
Sutton beans		7.0 ± 0.4	4.0 ± 0.6 A
Sutton beans and winter savory	4:1	8.2 ± 0.5	5.5 ± 0.7 A
Sutton beans and winter savory	1:1	5.5 ± 0.8	7.3 ± 1.1 ns
Winter savory		6.5 ± 1.2	6.7 ± 0.6 ns
Sutton beans and tansy	4:1	7.5 ± 0.5	5.2 ± 0.5 A
Sutton beans and tansy	1:1	5.8 ± 0.8	6.3 ± 1.1 ns
Tansy		5.2 ± 0.9	8.2 ± 1.0 ns
Sutton beans and summer savory	1:1	7.7 ± 0.8	6.5 ± 0.6 ns
Summer savory		4.2 ± 0.6	6.2 ± 0.8 R
Sutton beans and thyme	1:1	6.3 ± 1.1	6.3 ± 0.8 ns
Thyme		5.0 ± 0.9	5.7 ± 0.6 ns
Sutton beans and basil	1:1	5.5 ± 0.6	7.3 ± 1.0 ns
Basil		5.8 ± 1.0	5.7 ± 0.6 ns
Sutton beans and sage	1:1	8.3 ± 0.5	6.0 ± 0.6 A
Sage		5.7 ± 0.5	5.8 ± 0.6 ns

[a] A = attraction, R = repulsion, ns = not significantly different at $P < 0.05$, two-tailed paired t tests (six replicates).

TABLE 3. RESPONSE (±SE) OF ALATE VIRGINOPARAE OF *Brevicoryne brassicae* TO COMBINATIONS OF HOST AND NONHOST LEAVES[a]

	Ratio	Treatment	Control
Brussels sprouts		7.3 ± 0.6	4.7 ± 0.4 A
Brussels sprouts and winter savory	4:1	3.7 ± 0.3	4.7 ± 0.7 ns
Brussels sprouts and winter savory	1:1	3.7 ± 0.7	6.3 ± 1.2 R
Winter savory		5.0 ± 0.9	6.7 ± 0.7 ns
Brussels sprouts and tansy	4:1	3.8 ± 0.6	4.5 ± 0.8 ns
Brussels sprouts and tansy	1:1	4.0 ± 0.6	7.5 ± 0.7 R
Tansy		4.0 ± 0.6	7.2 ± 0.7 R

[a] A = attraction, R = repulsion, ns = not significantly different at $P < 0.05$, two-tailed paired t tests (six replicates).

implying neutralization of an insect's orientation response without repellency (Thiery and Visser, 1986). Both effects were probably operating in this study; for example, tansy proved repellent to *B. brassicae*, while winter savory and thyme masked the attraction of *A. fabae* to host plant odor without in themselves being repellent. It is also of interest that the repellent action of tansy was masked by Brussels sprout odor.

Electrophysiological recordings from the proximal rhinaria on the antenna showed the presence of cells in *B. brassicae* (Figure 2) and *L. erysimi* (Dawson et al., 1987) that responded to isothiocyanates. Both species displayed strong electrophysiological responses to 3-butenyl isothiocyanate and were attracted by this compound in the olfactometer (Table 4). However, the electrophysiological threshold concentration was considerably lower for *L. erysimi* (ca. 10^{-11} g) than for *B. brassicae* (10^{-7} g), and this is reflected in its greater behavioral sensitivity. In *L. erysimi* isothiocyanates have the additional function of synergizing the action of the alarm pheromone, (*E*)-β-farnesene (Dawson et al.,

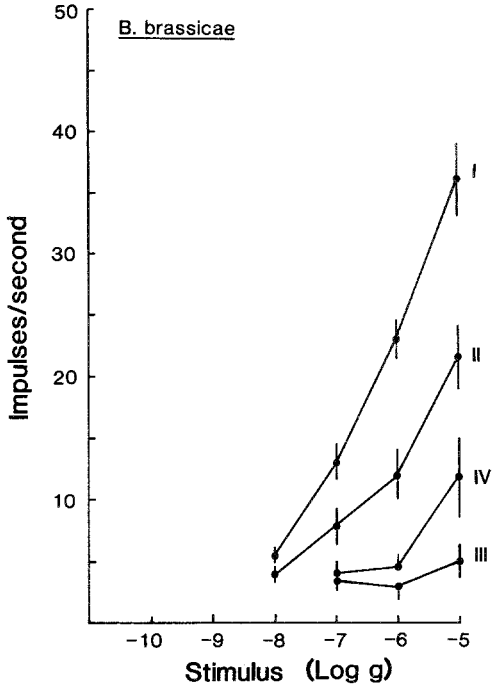


FIG. 2. Dose–response curves of *B. brassicae* olfactory cells to (I) 4-pentenyl, (II) 3-butenyl, (III) allyl, and (IV) phenylethyl isothiocyanates (means of five preparations ± SE). Where standard errors overlap, only half the SE bar is shown.

TABLE 4. RESPONSE (±SE) OF ALATE VIRGINOPARAE OF *Brevicoryne brassicae* AND *Lipaphis erysimi* TO 3-BUTENYL ISOTHIOCYANATE[a]

	B. brassicae		*L. erysimi*	
Amount (μg)	Treatment	Control	Treatment	Control
100	7.2 ± 0.4	4.8 ± 0.3 A	9.0 ± 0.7	5.5 ± 0.8 A
10	7.2 ± 0.8	5.2 ± 0.7 ns	8.8 ± 0.9	5.7 ± 0.4 A
1	6.7 ± 0.4	6.0 ± 0.7 ns	7.8 ± 1.0	5.3 ± 0.8 A
0.1	6.2 ± 0.9	6.2 ± 0.5 ns	8.3 ± 0.9	7.3 ± 1.2 ns
Hexane	5.8 ± 0.6	5.3 ± 0.6 ns	6.8 ± 0.6	6.5 ± 0.7 ns

[a] A = attraction, ns = not significantly different at $P < 0.05$, two-tailed paired t tests (six replicates).

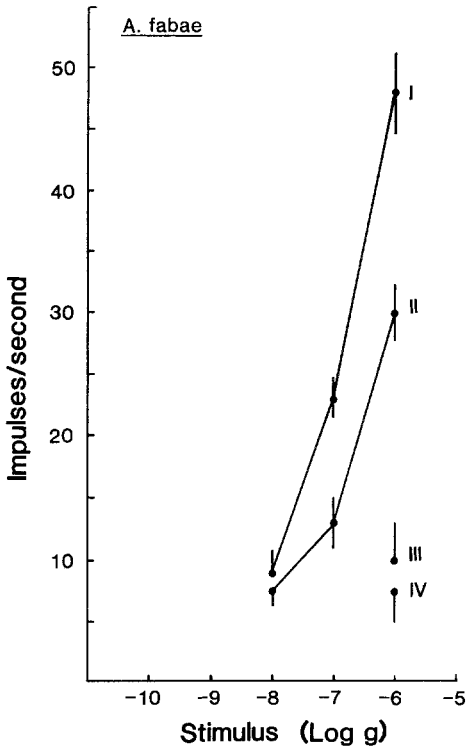


FIG. 3. Dose–response curves of *A. fabae* olfactory cells to (I) 4-pentenyl, (II) 3-butenyl, (III) allyl, and (IV) phenylethyl isothiocyanates (means of five preparations ± SE). Where standard errors overlap, only half the SE bar is shown.

FIG. 4. Olfactory cell in *A. fabae* proximal primary rhinarium: response to 1 μg of 4-pentenyl isothiocyanate. The stimulus duration (1 sec) is shown by the black bar.

TABLE 5. RESPONSE (±SE) OF ALATE VIRGINOPARAE OF *Aphis fabae* TO ISOTHIOCYANATES[a]

Amount (μg)	4-Pentenyl		3-Butenyl		Allyl	
	T	C	T	C	T	C
1,000	4.2 ± 0.7	6.8 ± 0.5 R	5.0 ± 0.7	7.8 ± 0.7 R	4.8 ± 0.7	7.3 ± 0.8 R
100	5.7 ± 0.7	8.0 ± 0.8 ns	4.3 ± 0.7	7.5 ± 0.9 R	5.5 ± 0.4	7.7 ± 0.6 ns
10	4.0 ± 0.4	6.7 ± 1.0 R	6.2 ± 0.9	6.2 ± 0.6 ns	4.5 ± 0.6	6.5 ± 0.8 ns
1	6.7 ± 0.4	5.5 ± 0.8 ns	5.8 ± 0.7	6.0 ± 0.7 ns	6.5 ± 0.7	6.5 ± 0.8 ns
Hexane	4.7 ± 0.4	5.0 ± 0.4 ns				

[a] T = treatment, C = control. R = repulsion, ns = not significantly different at $P < 0.05$, two-tailed paired t tests (six replicates).

TABLE 6. RESPONSES (±SE) OF ALATE VIRGINOPARAE OF *Aphis fabae* TOWARD SUTTON DWARF BEANS ALONE AND BEANS WITH 3-BUTENYL OR 4-PENTENYL ISOTHIOCYANATE[a]

Amount (μg)	3-Butenyl		4-Pentenyl	
	Treatment	Control	Treatment	Control
100	5.5 ± 0.6	4.9 ± 1.1 ns	5.0 ± 0.7	6.6 ± 1.0 ns
10	6.3 ± 0.8	6.1 ± 0.6 ns	6.5 ± 0.8	6.1 ± 0.7 ns
1	8.8 ± 0.5	5.9 ± 0.7 A	7.9 ± 0.7	5.9 ± 0.5 A
Beans only	7.3 ± 0.6	5.1 ± 0.4 A	—	—

[a] A = attraction, ns = not significantly different at $P < 0.05$, two-tailed paired t tests (eight replicates).

1987). Thus, alone they are attractants, but in combination with (*E*)-β-farnesene their effect is to initiate an alarm response.

Alate virginoparae of *A. fabae* also were shown to have olfactory receptors sensitive to isothiocyanates (Figures 3 and 4), although these compounds are not associated with their host plants. The response characteristics of the cells were similar to those observed for *B. brassicae*, but the behavioral responses were markedly different. While 100 μg of 3-butenyl isothiocyanate attracted *B. brassicae*, it repelled *A. fabae* (Tables 4 and 5). Allyl isothiocyanate was also repellent to *A. fabae*, but only at high concentrations (Table 5). The low behavioral activity of this compound correlates well with its relatively low electrophysiological activity (Figure 3). 4-Pentenyl isothiocyanate was the most active compound in electrophysiological studies (Figure 3) and proved repellent at a

level of 10 μg (Table 5). Phenylethyl isothiocyanate did not elicit electrophysiological responses in either *A. fabae* or *B. brassicae* (Figures 2 and 3). Both 3-butenyl and 4-pentenyl isothiocyanates masked the attractant response of *A. fabae* to Sutton bean leaves at levels of 100 and 10 μg (Table 6). No repellency was observed. Therefore, the presence of host plant volatiles reduced the repellency of the isothiocyanates, presumably in a similar way to the interaction of tansy and host volatiles for *B. brassicae* described above.

It must now be accepted that plant volatiles play a role in host location and selection by aphids. The current study demonstrates behavioral responses of alate and apterous aphids, with attraction to host and repulsion by some nonhost odors. In addition, it reveals that nonhost volatiles can mask host attraction. In some cases, individual volatiles have been implicated, but the interaction of different odor components is undoubtedly complex. Although evidence of olfactory responses by flying aphids is restricted to *C. aegopodii* (plant volatiles; Chapman et al., 1981) and *P. humuli* (sex pheromone and plant volatiles; Campbell et al., 1990), these observations demonstrate a role for olfaction at a distance. After landing, local odor fields may also prove important. The present results indicate that host and nonhost odor interactions may provide a degree of crop protection in companion planting programs and suggest that manipulating behavior via plant volatiles or mimics may be a way forward for aphid control.

Acknowledgments—We thank Jill Nicholas and Nicola Taylor for technical assistance.

REFERENCES

ALIKHAN, M.A. 1960. The experimental study of the chemotactic basis of host-specificity in a phytophagous insect, *Aphis fabae* Scop. (Aphididae: Homoptera). *Ann. Univ. Mariae Curie-Sklodowska Sect. C* 15:117–159.

BROMLEY, A.K., and ANDERSON, M. 1982. An electrophysiological study of olfaction in the aphid *Nasonovia ribis-nigri*. *Entomol. Exp. Appl.* 32:101–110.

CAMPBELL, C.A.M., DAWSON, G.W., GRIFFITHS, D.C., PETTERSSON, J., PICKETT, J.A., WADHAMS, L.J., and WOODCOCK, C.M. 1990. The sex attractant pheromone of the damson-hop aphid *Phorodon humuli* (Homoptera, Aphididae). *J. Chem. Ecol.* 16:3455–3465.

CHAPMAN, R.F., BERNAYS, E.A., and SIMPSON, S.J. 1981. Attraction and repulsion of the aphid, *Cavariella aegopodii*, by plant odors. *J. Chem. Ecol.* 7:881–888.

DAWSON, G.W., GRIFFITHS, D.C., PICKETT, J.A., WADHAMS, L.J., and WOODCOCK, C.M. 1987. Plant-derived synergists of alarm pheromone from turnip aphid, *Lipaphis (Hyadaphis) erysimi* (Homoptera, Aphididae). *J. Chem. Ecol.* 13:1663–1671.

DETHIER, V.G., BARTON-BROWNE, L., and SMITH, C.N. 1960. The designation of chemicals in terms of the responses they elicit from insects. *J. Econ. Entomol.* 53:135–136.

HARDIE, J., HOLYOAK, M., NICHOLAS, J., NOTTINGHAM, S.F., PICKETT, J.A., WADHAMS, L.J., and WOODCOCK, C.M. 1990. Aphid sex pheromone components: Age-dependent release by females and species-specific male response. *Chemoecology* 1:63–68.

JONES, M.G. 1944. The structure of the antennae of *Aphis (Dorsalis) fabae* Scopoli, and of *Melan-*

oxantherium salicis L. (Hemiptera), and some experiments on olfactory responses. *Proc. R. Entomol. Soc. London (A)* 19:13–22.

JU, H.-Y., CHONG, C., MULLIN, W.J., and BIBLE, B.B. 1982. Volatile isothiocyanates and nitriles from glucosinolates in rutabaga and turnip. *J. Am. Soc. Hortic. Sci.* 107:1050–1054.

KENNEDY, J.S., BOOTH, C.O., and KERSHAW, W.J.S. 1959a. Host finding by aphids in the field. I. Gynoparae of *Myzus persicae* (Sulzer). *Ann. Appl. Biol.* 47:410–423.

KENNEDY, J.S., BOOTH, C.O., and KERSHAW, W.J.S. 1959b. Host finding by aphids in the field. II. *Aphis fabae* Scop. (gynoparae) and *Brevicoryne brassicae* L.; with a re-appraisal of the role of the host-finding behaviour in virus spread. *Ann. Appl. Biol.* 47:424–444.

KJAER, A. 1960. Naturally derived isothiocyanates (mustard oils) and their parent glucosides. *Fortschr. Chem. Org. Naturst.* 18:122–176.

MATTHEWS, D.L., NICHALAK, P.S., and MACRAE, R.J. 1983. The effect of traditional insect-repellent plants on insect numbers in a mixed planting system. Proceedings of the 4th International Federation of Organic Agricultural Movements Conference, Massachusetts Institute of Technology, Cambridge, Massachusetts, August 18–20, 1982. Praeger, Boston. pp. 117–127.

MÜLLER, H.J. 1962. Über die Ursachen der unterschiedlichen Resistenz von *Vicia faba* L. gegenüber der Bohnenblattlaus, *Aphis (Dorsalis) fabae* Scop. VIII. Das Verhalten geflügelter Bohnenläuse nach der Landung auf Wirten und Nichtwirten. *Entomol. Exp. Appl.* 5:189–210.

NAULT, L.R., and STYER, W.E. 1972. Effects of sinigrin on host selection by aphids. *Entomol. Exp. Appl.* 15:423–437.

NOTTINGHAM, S.F., and HARDIE, J. 1989. Migratory and targeted flight in seasonal forms of the black bean aphid, *Aphis fabae*. *Physiol. Entomol.* 14:451–458.

PETTERSSON, J. 1970. Studies on *Rhopalosiphum padi* (L.). I. Laboratory studies on olfactometric responses to the winter host *Prunus padus* L. *Lantbrukshegsk. Ann.* 36:381–399.

PETTERSSON, J. 1973. Olfactory reactions of *Brevicoryne brassicae* (L.) (Hom.: Aph.). *Swed. J. Agric. Res.* 3:95–103.

POSPÍŠIL, J. 1972. Olfactory orientation of certain phytophagous insects in Cuba. *Acta Entomol. bohemoslov.* 69:7–17.

SAKUMA, M., and FUKAMI, H. 1985. The linear track olfactometer: An assay device for taxes of the german cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae) toward their aggregation pheromone. *Appl. Ent. Zool.* 20:387–402.

THIERY, D., and VISSER, J.H. 1986. Masking of host plant odour in the olfactory orientation of the Colorado potato beetle. *Entomol. Exp. Appl.* 41:165–172.

VISSER, J.H., and TAANMAN, J.W. 1987. Odour-conditioned anemotaxis of apterous aphids (*Cryptomyzus korschelti*) in response to host plants. *Physiol. Entomol.* 12:473–479.

WADHAMS, L.J. 1990. The use of coupled GC-electrophysiological techniques in the identification of insect pheromones, pp. 289–298, *in* A.R. McCaffery and I.D. Wilson (eds.) Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, New York.

WADHAMS, L.J., ANGST, M.E., and BLIGHT, M.M. 1982. Responses of the olfactory receptors of *Scolytus scolytus* (F.) (Coleoptera: Scolytidae) to the stereoisomers of 4-methyl-3-heptanol. *J. Chem. Ecol.* 8:477–492.

WENSLER, R.J.D. 1962. Mode of host selection by an aphid. *Nature* 195:830–831.

YAN, F.-S., and VISSER, J.H. 1982. Electroantennogram responses of the cereal aphid *Sitobion avenae* to plant volatile compounds. Proceedings 5th International Symposium Insect-Plant Relationships, Wageningen, 1982. Pudoc, Wageningen. pp. 387–388.

YEPSON, R.B., JR. 1984. The Encyclopedia of Natural Insect and Disease Control. Rodale Press, Emmaus, Pennsylvania. pp. 490.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

STABILITY OF PHENOLIC AND PROTEIN MEASURES IN EXCISED OAK FOLIAGE

KARL W. KLEINER

Department of Entomology, Pesticide Research Lab
The Pennsylvania State University
University Park, Pennsylvania 16802

(Received December 12, 1990; accepted February 19, 1991)

Abstract—The stability of protein and phenolic measures in excised foliage from two oak species was measured under conditions that simulated the handling and treatment of foliage during insect rearing trials. Excised foliage kept hydrated under refrigeration or insect-rearing conditions maintained stable levels of protein content, proanthocyanidins, gallotannins, total phenolics, and protein-binding capacity for up to 48 hr following field sampling. Measures of protein content, total phenolics, protein-binding capacity, and proanthocyanidins were significantly greater 48–72 hr after field sampling, followed by declines to near field levels within 120 hr.

Key Words—*Quercus rubra, Quercus prinus*, phenolics, tannins, foliage quality, insect-rearing trials.

INTRODUCTION

Investigations of the suitability of food plants for herbivorous insects usually require rearing trials with foliage. Ideally, such trials should be performed in situ, but logistical constraints (access to foliage on mature trees, location of food plants) and environmental variability (temperature, humidity) often make laboratory rearing with excised foliage more practical or useful. The stems or petioles of excised foliage used in laboratory trials are often inserted into a source of water to maintain leaf turgor and leaf shape (Scriber, 1977, 1978; Hough and Pimentel, 1978; Lawson et al., 1982, 1984). Although this procedure maintains the visible aspects of leaf freshness, stability in the dietary components of excised foliage has received little consideration (Miller, 1987). Changes in quantities or composition of leaf constituents, e.g., allelochemicals, in excised foliage could influence the validity of laboratory results.

0098-0331/91/0600-1243$06.50/0

Phenolics comprise a class of putative allelochemicals that is virtually ubiquitous in plants. They have been shown to have both positive (Bernays, 1981; Bernays et al., 1983) and negative (Bernays, 1981; Berenbaum, 1983; Manuwoto et al., 1985; Karowe, 1989) effects on the feeding behavior and performance of insect herbivores. For example, the secondary chemistry of oaks and other tree species favored by the gypsy moth (*Lymantria dispar* L.) is comprised primarily of phenolics (Gibbs, 1974), and several classes of these phenolics have been shown to influence gypsy moth growth, fecundity, and mortality (Keating et al., 1988; Kleiner, 1989).

In this study, stability in four phenolic measures and one protein measure in foliage from two oak species was examined over a range of handling and treatment stages identical to those used in laboratory rearing trials.

METHODS AND MATERIALS

Sampling

Experiment 1 (June 1986). One branch (0.5–1.0 m) was cut from each of five mature chestnut oaks (*Quercus prinus* L.) and five mature red oaks (*Q. rubra* L.) and recut under water in 19-liter buckets. Prior to transporting the branches to the lab, one foliage sample (four to five leaves) was taken from each branch (=tree) and flash frozen in liquid nitrogen. In the lab, one bouquet of foliage was made per tree by inserting the petioles of four to five leaves into a florists' Aqua-pic (Syndicate Sales, Kokomo, Indiana). These foliar bouquets are identical to those used in our lab for gypsy moth rearing trials (e.g., Kleiner, 1989). In addition, four pairs of single leaf bouquets were made per tree in the same manner and fed to gypsy moth larvae for 42 hr, then frozen for chemical analysis. All bouquets used in this experiment were kept at ambient conditions (26°C). After a portion of the foliage from each branch had been removed to make the foliar bouquet, the remaining branch was kept for additional foliage sampling. These branches were kept in the sample buckets in an unlit cooler at 5°C. Samples were frozen for analysis in the order shown in Table 1.

Multileaf bouquets were chemically analyzed as separate samples. Combining the paired single leaf bouquets (sample 7) for chemical analysis produced 20 samples per species (four per tree).

Experiment 2 (June 1987). One branch (0.5–1.0 m) was cut from each of five mature chestnut oak and five mature red oak trees and two samples of five to six leaves each were taken from each branch (day 1, zero hr). The branches were recut under water and transported to the lab in 19-liter buckets. Ten bouquets of foliage (five to six leaves in each bouquet) were prepared from each branch as described above. Foliar bouquets from each branch were placed in separate covered 31 × 17 × 9-cm plastic boxes in a growth chamber under

TABLE 1. DESCRIPTION OF OAK LEAF SAMPLES FOR EXPERIMENT 1

Sample	Source	N/species	Day	Hours
1	Field	5	1	0
2	Buckets	5	1	5
3	Aqua-pics	5	2	18
4	Buckets	5	2	18
5	Buckets	5	2	30
6	Buckets	5	3	42
7	Aqua-pics (fed to larvae)	20	3	42

conditions used for gypsy moth rearing (24°C with a 14:10 light–dark photoperiod). Two foliar bouquets were sampled from each branch every day for five days (days 2–6) for a total of 50 bouquets per species sampled throughout a 120-hr period. All samples were flash frozen in liquid nitrogen and stored at −34°C.

Statistical Analyses

To avoid pseudoreplication with sample 7 in experiment 1, the mean of the four samples per tree was used for statistical analysis, resulting in a balanced design of $N = 5$ per species. In experiment 2, red oak samples 2 and 3 were lost due to mishandling in the laboratory. For both experiments, differences among samples were analyzed by species, using one-way ANOVA (GLM, SAS Institute) with individual trees as the randomized blocks and sample time as the main effect. Samples different from sample 1 (the field sample) were detected with Tukey's studentized range test.

Chemical Analyses

Frozen leaf samples were lyophilized and thoroughly ground in a UDY Cyclone Sample Mill and a subsample was extracted in 0.1 N NaOH to reduce interference from phenolics (Jones et al., 1989). Protein concentration was analyzed using a modification of the micro method of Bradford (1976) (0.04 ml of extract was diluted with 0.76 ml of deionized water, then mixed with 0.20 ml of Coomassie brilliant blue G-250; Biorad dye reagent). Samples were read within 10 min at 595 nm against a distilled water blank, which resulted in higher protein values than reported elsewhere (Faeth, 1985; Jones et al., 1989). A portion of the remaining ground sample was extracted in 70% acetone, reduced to an aqueous extract via rotary evaporation, and analyzed for proanthocyanidins (vanillin assay; Swain and Hillis, 1959; Schultz and Baldwin 1982), gal-

lotannins (potassium iodate; Bate-Smith, 1977, as modified by Schultz and Baldwin, 1982), total phenolics (Folin-Denis), and protein-binding capacity using hemoglobin as a substrate (Schultz et al., 1981). Protein concentration is reported as percent dry weight bovine serum albumin equivalents (%BSAE); protein binding, Folin-Denis active phenolics, and hydrolyzable tannins are reported as percent dry weight tannic acid equivalents (%TAE; Sigma, Lot 11F-0559); and proanthocyanidins as percent dry weight wattle tannin equivalents (%WTE; *Acacia* sp., from Leon Monnier, Inc. Peabody, Massachusetts), derived from standard curves.

RESULTS AND DISCUSSION

Experiment 1. For both red and chestnut oak foliage, there was only one significant change in the five measures of leaf quality made among the seven samples taken over a 42-hr period (Figure 1). After 42 hr, chestnut oak foliage

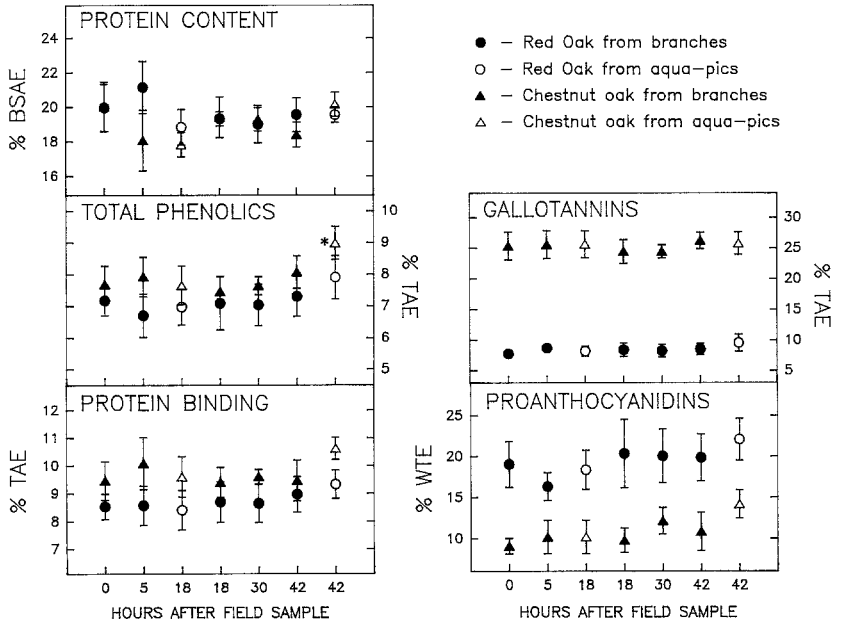


FIG. 1. Levels of protein content and phenolics in excised foliage of red oak and chestnut oak subjected to laboratory treatment to simulate handling during rearing trials. Each point is mean ± SE of five trees. * indicates sample is significantly different from sample 1 at $P < 0.05$. % BSAE = percent dry weight bovine serum albumin equivalents; % TAE = percent dry weight tannic acid equivalents; WTE = dry weight wattle tannin equivalents. See text for explanation of samples.

in Aqua-pics that had been fed to gypsy moth larvae had greater measures of total phenolics than the initial field sample (8.99% TAE vs. 7.68% TAE; $P \leq 0.05$). Interestingly, the smallest difference among means that the statistical analysis could have detected (regardless of species) ranged from low (1.27% TAE, protein binding; 1.29% TAE, total phenolics; 1.9% TAE gallotannins) to medium (4.4% BSAE protein) to high (8.17% WTE proanthocyanidins). In this study, significant differences between sample times only indicates changes in the measures of foliage quality. Because of the differential tolerance to tannins by various herbivores (Bernays, 1978; Berenbaum, 1983; Karowe, 1989), the biological impact of even the smallest change in phenolics will be species specific.

The results of experiment 1 show that after 42 hr of gypsy moth feeding, only small increases in phenolics were observed in the remaining foliage. This is surprising in light of many studies that have shown increases in secondary metabolites within hours of damage or insect feeding on intact foliage (Baldwin and Schultz, 1983; Tallamy, 1985; Niemeyer et al., 1989). Some inducible responses are limited to the site of damage, while others occur systemically and may require the response of the whole plant (Bowles, 1990). The limited response of excised foliage observed in this study suggests the latter, but without proper controls, this conclusion cannot be assumed.

Experiment 2. Gallotannin was the only measure for both red and chestnut oak that did not change significantly over a five-day period (120 hr). The measures of protein content, proanthocyanidin, total phenolics, and protein-binding capacity all displayed increases (with the exception of red oak protein content and protein-binding capacity) within 48–72 hr, followed by subsequent declines (Figure 2). Protein measures in chestnut oak foliage were significantly greater 72 and 96 hr following the field sample ($P < 0.0001$ from ANOVA), but were below field sample measures by 120 hr. The measure of red oak protein content displayed a marked decrease at 72 hr, a considerable increase at 96 hr ($P < 0.0001$ from ANOVA), and a return to near field sample levels by 120 hr. The measures of total phenolics in red oak and chestnut oak foliage were significantly greater 72, 96, and for red oak, 120 hr after field sampling ($P < 0.0001$ from ANOVA for each species).

The results of experiment 1 indicate that oak foliage can be collected, returned to the laboratory, and stored under refrigeration for up to 42 hr without significantly changing these measures of protein content and phenolics. Both experiments indicate that the most stable period for the measures of protein and phenolics in excised foliage is during the first 24 hr following field sampling. In experiment 2, some of these measures (protein content, total phenolics) did not differ significantly until 48 or 72 hr.

The patterns of change observed in this study are not readily explained by the natural variation among samples. Although there was significant variation

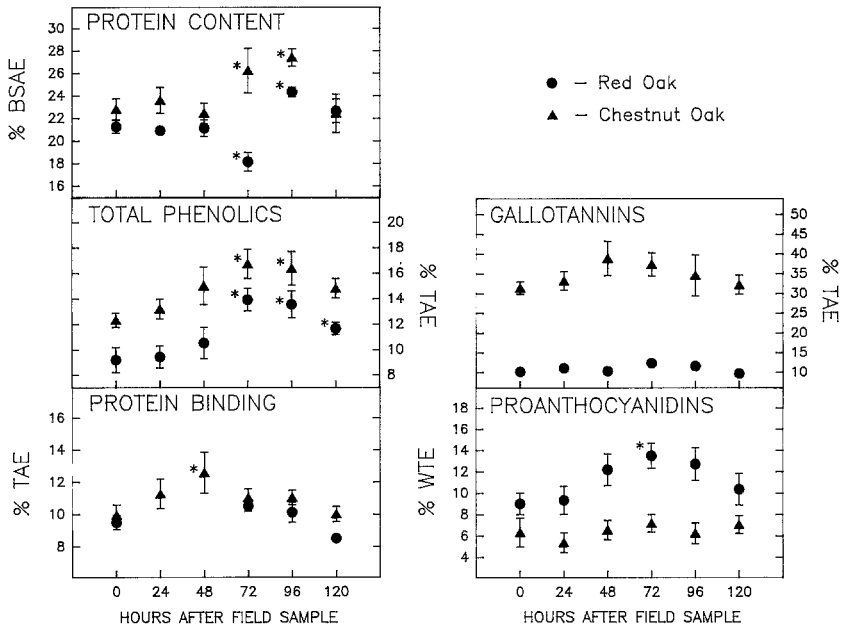


FIG. 2. Levels of protein content and phenolics in excised foliage of red oak and chestnut oak from the field (0 hr) and at five 24-hr intervals while under insect rearing conditions. Each point is mean ± SE of 10 trees. * indicates sample is significantly different from field sample at $P < 0.05$. Axis labels defined in Figure 1.

among trees of both species within any sample period, this variation was exceeded by the magnitude of change in the measures over the 120-hr period (Figure 2).

The increases in the measures of total phenolics after 120 hr were similar to those observed by Miller (1987) in excised birch and willow foliage after 144 hr. However, the increases in some of the phenolic measures cannot be interpreted simply as changes in the quantity of the compounds (proanthocyanidins and gallotannins) that were measured in the initial field samples. The results of assays that are specific for a class of tannins (potassium iodate and vanillin assays) indicate that no change occurred in the quantity of red oak gallotannins or chestnut oak proanthocyanidins. However, the measures of total phenolics and protein-binding capacity, which are sensitive to both proanthocyanidins and gallotannins, suggest that these tannins did increase. Because the Folin-Denis and protein binding assays are sensitive to a wide array of phenolics, they may reflect changes in other foliar constituents not measured in the more specific assays. These changes could include the synthesis and/or restructuring in excised foliage of nontannin phenolics or tannins not measured by the

analyses used here. Alternatively, the patterns observed in this study may reflect a process of phenolic degradation, which may involve oxidation and restructuring, followed by the complete breakdown of these products. This would result in the greater measures of phenolic activity observed at 72–96 hr, followed by the lower phenolic measures observed by 120 hr. As a consequence, it would appear that after 120 hr, hydrated, excised foliage is quantitatively the same as samples taken from the first 48 hr. However, it cannot be assumed that such foliage is qualitatively the same.

The maintenance of leaf turgor for insect-rearing trials cannot assure the integrity of some dietary constituents for more than 48 hr. Such an assumption may lead to uninterpretable or incorrect biological indices, particularly when results are compared across different host species or foliage treatments (Scriber, 1977; Hough and Pimentel, 1978). Cautious interpretations should be made from larval performance and/or leaf quality studies in which foliage used for chemical analyses may have been handled differently from foliage for rearing trials (Fox and MacCauley, 1977; Wint, 1983; Barbosa et al., 1983, 1986; Puttick, 1986). Researchers who hydrate excised foliage and change it within 48 hr or use refrigerated foliage within 48 hr (Raupp et al., 1988) are likely to maintain the foliage traits that were measured in this study.

Acknowledgments—This study was supported by United States Forest Service Cooperative Agreement FS-23-155 and National Science Foundation grants BSR 8813433 and BSR 8400284. M. Briggs helped analyze samples. Thanks to J. Schultz and M. Hunter and two anonymous reviewers for comments on earlier drafts of this manuscript.

REFERENCES

BALDWIN, I.T., and SCHULTZ, J.C. 1983. Rapid changes in tree leaf chemistry induced by damage: Evidence for communication between plants. *Science* 221:277–279.

BARBOSA, P., WALDVOGEL, M., MARTINAT, P., and DOUGLASS, W. 1983. Development and reproductive performance of the gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), on selected hosts common to mid-atlantic and southern forests. *Environ. Entomol.* 12:1858–1862.

BARBOSA, P., MARTINAT, P., and WALDVOGEL, M. 1986. Development, fecundity and survival of the herbivore *Lymantria dispar* and the number of plant species in its diet. *Ecol. Entomol.* 11:1–6.

BATE-SMITH, E.C. 1977. Astringent tannins of *Acer* species. *Phytochemistry* 16:1421–1426.

BERENBAUM, M.R. 1983. Effects of tannins on growth and digestion in two species of papilionids. *Entomol. Exp. Appl.* 34:245–250.

BERNAYS, E.A. 1978. Tannins: An alternative viewpoint. *Entomol. Exp. Appl.* 24:44–53.

BERNAYS, E.A. 1981. Plant tannins and insect herbivores: An appraisal. *Ecol. Entomol.* 6:353–360.

BERNAYS, E.A., CHAMBERLAIN, D.J., and WOODHEAD, S. 1983. Phenols as nutrients for a phytophagous insect *Anacridium melanorhodon*. *J. Insect Physiol.* 29:535–539.

BOWLES, D. 1990. Signals in the wounded plant. *Nature* 343:314–315.

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principles of protein-dye binding. *Anal. Biochem.* 72:248–254.

FAETH, S.H. 1985. Quantitative defense theory and patterns of feeding by oak insects. *Oecologia* 68:34–40.

FOX, R.L., and MACAULEY, B.J. 1977. Insect grazing on *Eucalyptus* in response to variation in leaf tannins and nitrogen. *Oecologia* 29:145–162.

GIBBS, R.O. 1974. Chemotaxonomy of Flowering Plants. McGill-Queens University Press, Montreal, Quebec, Canada. 1200 pp.

HOUGH, J.A., and PIMENTEL, D. 1978. Influence of host foliage on development, survival and fecundity of the gypsy moth. *Environ. Entomol.* 7:97–102.

JONES, C.G., HARE, T.D., and COMPTON, S.J. 1989. Measuring plant protein with the Bradford assay. *J. Chem. Ecol.* 15:979–992.

KAROWE, D.N. 1989. Differential effect of tannic acid on two tree-feeding Lepidoptera: Implications for theories of plant anti-herbivore chemistry. *Oecologia* 80:507–512.

KEATING, S.T., YENDOL, W.G., and SCHULTZ, J.C. 1988. Relationship between susceptibility of gypsy moth larvae (Lepidoptera: Lymantriidae) to a baculovirus and host plant foliage constituents. *Environ. Entomol.* 17:952–958.

KLEINER, K.W. 1989. Sources of variation in oak leaf quality as food for the gypsy moth: implications for forest stand susceptibility. Unpublished PhD thesis. Pennsylvania State University, University Park, Pennsylvania.

LAWSON, D.L., MERRITT, R.W., KLUG, M.J., and MARTIN, J.S. 1982. The utilization of late season foliage by the orange striped oakworm, *Anisota senatoria. Entomol. Exp. Appl.* 32:242–248.

LAWSON, D.L., MERRITT, R.W., MARTIN, M.M., and MARTIN, J.S., and KUKOR, J.J. 1984. The nutritional ecology of larvae of *Alsophila pometaria* and *Anisota senatoria* feeding on early- and late-season oak foliage. *Entomol. Exp. Appl.* 35:105–114.

MANUWOTO, S., SCRIBER, J.M., HSIA, M.T., and SUNARJO, P. 1985. Antibiosis/antixenosis in tulip tree and quaking aspen leaves against the polyphagous southern armyworm, *Spodoptera eridania. Oecologia* 67:1–7.

MILLER, W.E. 1987. Changes in nutritional quality of detached aspen and willow foliage used as insect food in the laboratory. *Great Lakes Entomol.* 20:41–45.

NIEMEYER, H.M., PESEL, E., COPAJA, S.V., BRAVO, H.R., FRANKE, S., and FRANCKE, W. 1989. Changes in hydroxamic acid levels of wheat plants induced by aphid feeding. *Phytochemistry* 28:447–449.

PUTTICK, G.M. 1986. Utilization of evergreen and deciduous oaks by the Californian oak moth *Phryganidia californica. Oecologia* 68:589–594.

RAUPP, M.J., WERREN, J.H., and SADOF, C.S. 1988. Effects of short-term phenological changes in leaf suitability on the survivorship, growth and development of gypsy moth (Lepidoptera: Lymantriidae) larvae. *Environ. Entomol.* 17:316–319.

ROSSITER, M.C., SCHULTZ, J.C. and BALDWIN, I.T. 1988. Relationships among defoliation, red oak phenolics, and gypsy moth growth and reproduction. *Ecology* 69:267—277.

SCHULTZ, J.C., and BALDWIN, I.T. 1982. Oak leaf quality declines in response to defoliation by gypsy moth larvae. *Science* 217:149–151.

SCHULTZ, J.C., BALDWIN, I.T., and NOTHNAGLE, P.J. 1981. Hemoglobin as a binding substrate in the quantitative analysis of plant tannins. *J. Agric. Food Chem.* 29:823–826.

SCRIBER, J.M. 1977. Limiting effects of low leaf-water content on the nitrogen utilization, energy budget, and larval growth of *Hyalophora cecropia* (Lepidoptera: Saturniidae). *Oecologia* 28:269–287.

SCRIBER, J.M. 1978. The effects of larval feeding specialization and plant growth form on the

consumption and utilization of plant biomass and nitrogen: An ecological consideration. *Entomol. Exp. Appl.* 24:494–510.

SWAIN, T., and HILLIS, W.E. 1959. The phenolic constituents of *Prunus domestica. J. Sci. Food Agric.* 10:63–68.

TALLAMY, D.W. 1985. Squash beetle feeding behavior: An adaptation against induced cucurbit defenses. *Ecology* 66:1574–1579.

WINT, G.R.W. 1983. The effect of foliar nutrients upon the growth and feeding of a Lepidopteran larva, pp. 301–320, *in* J.A. Lee, S. McNeill, and I.H. Rorison (eds.). Nitrogen as an Ecological Factor. 22nd Symposium of the British Ecological Society, Oxford 1981. Blackwell Scientific, Oxford.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

CHEMICAL CHARACTERIZATION OF FRUIT AND FUNGAL VOLATILES ATTRACTIVE TO DRIED-FRUIT BEETLE, *Carpophilus hemipterus* (L.) (COLEOPTERA: NITIDULIDAE)

P. LARRY PHELAN* and HENGCHEN LIN

Department of Entomology
Ohio Agricultural Research & Development Center
The Ohio State University
Wooster, Ohio 44691

(Received October 1, 1990; accepted February 19, 1991)

Abstract—The chemical basis underlying orientation to fruit and fungal odors was investigated for the dried-fruit beetle, *Carpophilus hemipterus* (L.). In wind-tunnel bioassays of walking and flight response from 1.8 m, beetles were attracted to odors of the yeast *Saccharomyces cerevisiae* on agar, aseptic banana, or banana inoculated with *S. cerevisiae*, although both banana substrates elicited greater response than the yeast alone. When presented in a two-choice bioassay, the yeast-inoculated banana attracted approximately twice as many beetles as did the aseptic banana. GC-MS analysis of the headspace volatiles above these odor sources revealed a somewhat more complex and concentrated volatile profile for yeast-inoculated banana than for aseptic banana. The odor from yeast on agar had fewer components, and these were present at lower concentrations than the odors of either banana substrate. By blending mineral-oil or aqueous solutions of the 18 components of inoculated-banana odor in varying concentrations, it was possible to mimic closely the headspace profile of the natural odor. This synthetic odor also elicited beetle attraction in the wind tunnel at levels comparable to the inoculated banana. Through a series of bioassays in which individual components were subtracted from or added to a synthetic odor blend, it was determined that ethyl acetate, acetaldehyde, 2-pentanol, and 3-methylbutanol comprised the simplest blend of compounds evoking full behavioral response. However, 2-methylpropanol or butanol were apparently interchangeable with 3-methylbutanol in this blend, and comparable response could also be elicited by replacing acetaldehyde with a combination of both 2-pentanone and 3-hydroxy-2-butanone. Thus, our results suggest that this generalist insect

*To whom correspondence should be addressed.

0098-0331/91/0600-1253$06.50/0

herbivore locates its hosts by a long-range response to a variety of blends of common fruit volatiles, whose concentrations are enhanced by fungi.

Key Words—Coleoptera, Nitidulidae, *Carpophilus hemipterus*, dried-fruit beetle, host-finding, yeast, wind tunnel, attraction, volatiles, headspace.

INTRODUCTION

The dried-fruit beetle, *Carpophilus hemipterus* (L.), is a cosmopolitan species that causes damage to a large number of agricultural products by direct infestation or by transmission of phytopathogenic fungi (Hinton, 1945). Fruit baits are used commonly to monitor this and other nitidulid beetles in the field, and response to fruits can be enhanced in *C. hemipterus* if the fruits are inoculated with fungi (Wildman, 1933; Blackmer and Phelan, 1991); however, monitoring populations using fruit baits is made difficult by the confounding variability in volatile release from living tissue, especially during the chemically dynamic period of ripening. Smilanick et al. (1978) demonstrated that a synthetic mixture of ethanol, ethyl acetate, and acetaldehyde could be used to attract *C. hemipterus* in the field, and propyl propionate or butyl acetate can be used to selectively trap another nitidulid, *Glischrochilus quadrisignatus* (Alm et al., 1986); however, in both cases the release rates used for the lures were much higher than that expected from a plant, and thus it is difficult to determine the role that these compounds may play in host-finding for these species. To date, no attempt has been made to investigate systematically the components of a natural odor source to determine which constituents may be mediating long-range host-finding in any nitidulid species. In this study, we: (1) measure the relative attractiveness of aseptic banana, the yeast *Saccharomyces cerevisiae* Hanson, and banana inoculated with yeast in a wind-tunnel bioassay; (2) chemically characterize the odor profiles in the headspace above these substrates; and (3) determine the relative activity of odor components for *C. hemipterus* host finding by their systematic elimination from and addition to synthetic odor blends.

METHODS AND MATERIALS

Insects. C. hemipterus was maintained in the laboratory on artificial diet using the methods of Hall et al. (1978) under the conditions of 24 ± 1°C, 16:8 light–dark, and 75–85% relative humidity. After emergence from the pupae, adults were kept as mixed sexes in 12-oz. plastic cups with moisture, without contact with food or food odors.

Natural Odors. Banana (var. Cavendish) was used to investigate host-finding in *C. hemipterus* because it was found to elicit high levels of upwind flight

in wind-tunnel studies (Blackmer and Phelan, in preparation) and because fresh samples were available on a year-round basis in local grocery stores. The role of fungi in host finding was measured using *Saccharomyces cerevisiae* var. Montrachet, which was maintained in the laboratory on YM agar (Difco Laboratories, Detroit, Michigan) at 24 ± 1°C. Four natural-odor combinations were prepared for chemical analysis and behavioral bioassays: YM agar, *S. cerevisiae* on yeast medium, aseptic banana, and banana inoculated with *S. cerevisiae*. Samples of 30 g freshly peeled banana or 30 ml yeast medium were prepared in 240 ml Qorpak straight-sided jars that had been sterilized with 1% NaOCl and rinsed with sterile doubly deionized water. The lid of each jar was equipped with a 0.20 μm Bacterial Air Vent (Gelman Sciences, Ann Arbor, Michigan), to allow free air exchange while precluding microbial contaminants, and with a sleeve-type rubber septum (Thomas Scientific) for collection of headspace samples. Banana samples were placed in the jars, sterilized by treatment with 70% ethanol for 5 min, and then rinsed five times with 100–150 ml doubly deionized water passed through the Bacterial Air Vent. In rare instances where sterilization was unsuccessful (<1%), contamination became visible within three to four days and these samples were discarded; otherwise, substrates remained aseptic for greater than a month. To determine the degree to which the ethanol used in sterilizing the banana may have contributed to the occurrence of ethanol in the headspace of aseptic substrates, we compared the headspace profiles of bananas sterilized using the above procedures with those of bananas sterilized with 1% NaOCl. For inoculated samples, *S. cerevisiae* was transferred in a sterile transfer hood to sterilized banana samples by scratching the surface of the fungal culture with a flame-sterilized wire loop and then smearing the surface of the banana. All samples were incubated in the jars for seven days prior to testing in the wind tunnel and headspace analysis.

Headspace Volatile Identifications. Headspace volatiles were trapped using a device integral to a Hewlett-Packard 5890A gas chromatograph (GC) that allowed thermal desorption of constituents directly to the GC column (Phelan, unpublished). Briefly, the device consisted of a six-port two-position capillary valve (Valco Instruments, Houston, Texas) and a 60-cm × 0.1-cm-ID capillary volatile trap filled with Tenax GC (60/80 mesh). When placed in the trapping position, the valve allowed volatiles to be carried via a helium flow to the capillary trap. After a designated collection period, the trap was ballistically heated to 200°C and the valve position was switched to reverse the helium flow in the trap such that retained volatiles were desorbed and back-flushed to the GC analytical column. For this study, jars containing odor sources were opened for 5 min, resealed, and a 1-ml headspace sample immediately collected through the rubber septum using a gas-tight syringe. The 1-ml sample was then slowly injected onto the volatile trap and desorbed as above. Because the headspace profile was greatly dominated by ethanol, a second method was used to reduce

the amount of ethanol desorbed to the analytical column while enriching minor components of the headspace samples. A 20-ml headspace sample was collected and injected onto the trap as described; however, at this point the trap was flushed with helium for 6 min at a flow of 20 ml/min. This flush period allowed the preponderance of ethanol and acetaldehyde to be removed from the trap, which we have determined to have breakthrough volumes of approximately 90 ml and 45 ml, respectively; the next smallest breakthrough volume for the components encountered in this study was 450 ml for propanol, so that all other components were concentrated on the trap without breakthrough for desorption to the analytical column.

Headspace components were chromatographed on either DB-1 (30 m × 0.32 mm ID, 5.0-μm film thickness) or DB-FFAP (30 m × 0.25 mm ID, 0.25-μm film thickness) capillary columns (J & W Scientific, Folsom, California), with a temperature program of 30–200°C increased at 10°C/min. Helium flow was 0.9 ml/min on the DB-1 column and 0.7 ml/min on the DB-FFAP column. Tentative identifications of the components were made using a HP 5970C Mass Selective Detector with a direct interface to the GC. Chemical assignments were confirmed by comparison of GC retention times on the two columns and mass spectra with those of authentic samples; no attempts were made to determine enantiomeric identity of chiral compounds. The concentration of each component in the headspace was quantified by comparison of its peak areas with that of synthetics.

Synthetic Odors. Synthetic odors were created by blending compounds identified from *S. cerevisiae* -inoculated banana. All chemicals were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin) and were prepared individually as mineral-oil solutions, except ethanol, which was diluted with double-deionized water due to low solubility in mineral oil. Mineral-oil solutions were applied to cotton wicks in 240-ml Qorpak straight-sided jars. Solution concentrations and volumes were adjusted to mimic the natural odor by comparing the concentration of each component in the headspace above the synthetic blend with that of the natural source. A total combination of 18 synthetic compounds was used in the first three bioassays, but this blend was reduced to 13 components for subsequent bioassays as the elimination of five minor compounds (four esters and 2-butanone) did not significantly affect the attractiveness of the synthetic blend. Synthetic odor blends were used for bioassay immediately after preparation, and new mixtures were prepared for each replicate.

Wind-Tunnel Bioassays. Odor-modulated responses of *C. hemipterus* were evaluated in a previously described wind tunnel that measured 2.5 m long × 1 m wide × 0.5 m high (Phelan et al., 1991). Bioassays were conducted 4–0 hr prior to lights out at 35–65% relative humidity, 24–27°C, with a wind speed of 0.5 m/sec. The tunnel was illuminated from above by three 60-W bulbs

operated at 80 V to provide 17 lux on the tunnel floor. During wind-tunnel bioassays, two jars were placed on the floor 10 cm away from the center line of the upwind end of the tunnel. Unless otherwise indicated, one of the jars was empty and served as a negative control for possible odor contamination. The position of the jars was randomized for each treatment, and the test beetles were released on the floor at the center of the downwind end of the tunnel at a distance of 180 cm from the odor sources. Smoke tests showed that plumes from the two jars merged before reaching the beetle-release site. Ten 6- to 7-day-old beetles were released per replicate, and beetles were used only once. Beetles that oriented to test odors within 10 min and that walked or flew up the odor plume to within 10 cm downwind of the jar were counted as responders. The 10-cm criterion was used since the top of the jar, and thus the source of the odor, was 8.7 cm above the floor, and those beetles that walked to within 10 cm of the jar would lose the odor plume. A final bioassay measuring flight response alone was conducted to confirm that the synthetic blends eliciting the greatest walking response also evoked a flight response equivalent to that to inoculated banana. For this bioassay, the sample jars were set on 15-cm-high tripods and beetles were released from a platform on a similar tripod. The locations of jars and distances between odor and beetle-releasing site were the same as in previous bioassays. A positive response was recorded for those individuals that flew up the odor plume and landed on the jar. Since beetles were never observed to respond to an empty jar, these bioassays effectively were operated as single-choice tests, with a randomized complete-block presentation of treatments. Beetle response was measured as the percent of each group of 10 beetles released. Response data were analyzed by two-way ANOVA after transformation by $\sin^{-1}\sqrt{X}$, with mean separations performed using LSD on data sets where ANOVA indicated a significant F value (SAS Institute, 1985). In addition to the single-choice bioassays, beetle response to *S. cerevisiae*-inoculated banana was compared with that to aseptic banana as a two-choice bioassay in the wind tunnel.

RESULTS

Attraction to Natural Odors. All four natural odor sources elicited significant levels of upwind orientation by *C. hemipterus* (Figure 1). The *S. cerevisiae*-inoculated and the aseptic bananas elicited comparable beetle responses, both of which were significantly greater than that to the yeast on agar medium or the medium alone; however, when given a choice between the inoculated- and aseptic-banana odors, beetles showed a strong preference for the former, with more than twice as many orienting to the inoculated substrate (Figure 1).

Headspace Volatile Identifications. A total of 18 compounds (eight esters,

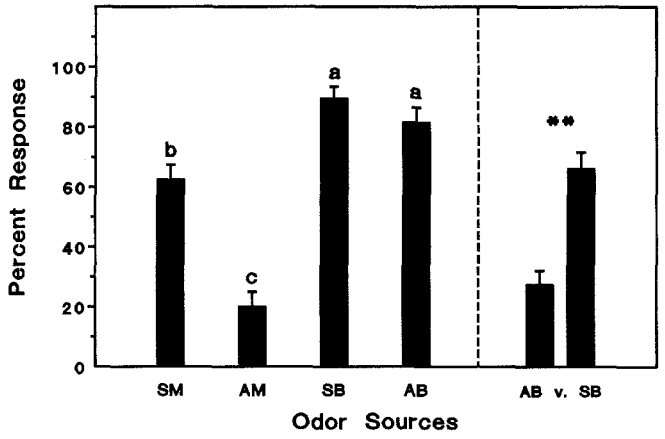


FIG. 1. Orientation of *Carpophilus hemipterus* to four natural odor sources in a wind tunnel: *Saccharomyces cerevisiae* on agar medium (SM), aseptic agar medium (AM), *S. cerevisiae*-inoculated banana (SB), or aseptic banana (AB). Response bars (±SEM) marked by the same letter are not significantly different (LSD, $N = 8$ groups of 10 beetles, $P < 0.05$). Orientation to SB versus AB compared in a two-choice experimental design ($\chi^2 = 17.62$, 7 *df*, $P < 0.02$).

six alcohols, three ketones, and one aldehyde) were identified from the four substrates, as listed in Table 1 with the major mass ions used for their identification; ethanol was by far the dominant component in the odor of all four substrates. The possibility that ethanol in the headspace was a result of the use of ethanol for sterilizing substrates was ruled out by the fact that the headspace of 7-day-old aseptic banana sterilized by 1% NaOCl contained ethanol at levels identical to that sterilized with 70% ethanol [peak areas ($\times 10^7$) ± SEM = 2.41 ± 0.32 and 2.48 ± 0.21, respectively; $t = 0.19$; $P = 0.86$; $N = 5$]. Table 2 shows the relative abundance of these constituents in the volatile profiles of the four substrates, with the odor of yeast-inoculated banana possessing all 18 components (Figure 2a). The aseptic-banana odor was qualitatively similar, with 15 of these compounds present (Table 2); however, the two substrates were quantitatively distinct, with the inoculated banana producing roughly twice the concentration of volatiles overall and with the largest increase occurring in the production of alcohols. The concentration of nonethanol alcohols was about four times greater in the headspace of inoculated banana, and the concentration of propanol increased by more than 40-fold (Table 2). The headspace profile of *S. cerevisiae* on yeast medium was both qualitatively and quantitatively depauperate relative to that of the yeast on banana, with only seven compounds detected in headspace samples of yeast on agar medium.

Attraction to Synthetic Odors. When the 18 components of yeast-inocu-

TABLE 1. CHEMICALS IDENTIFIED FROM HEADSPACE OF *Saccharomyces cerevisiae*-INOCULATED BANANA

Peak No.[a]	Component		Mass ions (m/z)[b]
	Abbr.	Identity	
1	AcAl	Acetaldehyde	29 , 42, 43, 44
2	EtOH	Ethanol	27, 29, 31 , 45, 46
3	PrOH	Propanol	27, 29, 31 , 42, 59, 60
4		2-Butanone	27, 29, 43 , 57, 72
5	EtAc	Ethyl acetate	28, 29, 43 , 45, 61, 70, 73, 88
6	MPrOH	2-Methylpropanol	27, 31, 33, 41, 42, 43 , 55, 74
7	BuOH	Butanol	27, 29, 31 , 41, 42, 43, 56
8	PeKe	2-Pentanone	27, 29, 43 , 57, 71, 86
9	2PeOH	2-Pentanol	29, 31, 39, 41, 43, 45 , 55, 73
10	HBuKe	3-Hydroxy-2-butanone	27, 29, 43, 45 , 88
11	MBuOH	3-Methylbutanol	29, 31, 41, 42, 43, 55 , 57, 70
12	iBuAc	Isobutyl acetate	27, 29, 39, 41, 43 , 56, 62, 73
13	EtBu	Ethyl butyrate	29, 41, 43 , 45, 60, 71, 73, 88
14		Butyl acetate	27, 29, 41, 43 , 56, 61, 73
15		Ethyl isovalerate	29 , 41, 43, 57, 60, 61, 85, 88
16	iPeAc	Isopentyl acetate	39, 43 , 55, 61, 70, 87
17		Isopentyl butyrate	31, 40, 41, 43, 55, 70, 71 , 89
18		Isopentyl isovalerate	41, 43, 55, 57, 70 , 85, 103

[a] See Figure 2 for total ion chromatogram.
[b] Major ions of mass spectra used for identification, with base ions underlined.

lated banana odor were blended in the amounts and concentrations given in Table 2, a synthetic-odor profile was generated that closely mimicked the natural source both in overall quantity and in the ratio of individual components (Figure 2b). This synthetic banana odor also closely mimicked the natural substrate in behavioral activity, eliciting upwind attraction in *C. hemipterus* at levels comparable to the inoculated banana [87.8 $\pm$ 2.5% ($\bar{X}$ $\pm$ SEM) for the synthetic odor and 67.9 $\pm$ 6.3% for the natural odor, $N = 4$ groups of 10 beetles, $P > 0.05$). In addition, both the synthetic blend and the inoculated banana elicited beetle response at a level several times greater than that to a blend of ethanol, acetaldehyde, and ethyl acetate (9.5 $\pm$ 4.8%, $P < 0.05$), a combination previously reported to be an effective attractant for *C. hemipterus* in the field (Smilanick et al., 1978).

In the first of a series of bioassays of synthetic blends, the 18-component blend again was very similar to the inoculated banana in eliciting upwind orientation; however, the elimination of all members of any of the three groups of functionalities resulted in a reduction in beetle response (Figure 3). The per-

TABLE 2. PERCENT COMPOSITION OF VOLATILE CONSTITUENTS IN HEADSPACE OF *Saccharomyces cerevisiae*-INOCULATED AGAR MEDIUM (SM), ASEPTIC AGAR MEDIUM (AM), *S. cerevisiae*-INOCULATED BANANA (SB), AND ASEPTIC BANANA (AB); RATIO OF ABUNDANCE OF EACH COMPONENT IN SB VERSUS AB HEADSPACE; AND COMPOSITION OF SYNTHETIC ODOR BLENDS

Component	Percent composition (non-EtOH)[a]				SB:AB (absolute quantity)[b]	Synthetic odor composition[c]	
	SM	AM	SB	AB		μl	%
Alcohols							
Ethanol	NQ[d]	NQ	(85.05)	(86.53)	1.83	500	25
Propanol			10.30	0.49	43.80	200	1
2-Methylpropanol	25.73		8.49	7.61	2.30	100	1
Butanol	1.13	87.14	0.72	0,84	1.77	100	0.1
2-Pentanol			0.44	0.94	0.97	50	0.1
3-Methylbutanol	55.98		10.63	6.27	3.50	200	1
Aldehydes/ketones							
Acetaldehyde	NQ	NQ	55.36	54.13	2.11	500	1
2-Butanone[e]			0.09		>1.00	10	0.1
2-Pentanone			0.65	1.36	1.00	20	0.1
3-Hydroxy-2-butanone			0.27		>1.00	100	0.1
Esters							
Ethyl acetate	14.99	12.86	11.78	22.05	1.10	100	1
Isobutyl acetate			0.25	0.84	0.62	10	0.1
Ethyl butyrate			0.45	2.59	0.36	20	0.1
Butyl acetate[e]			<0.01		>1.00	10	0.1
Ethyl isovalerate[e]			0.15	1.27	0.25	100	0.1
Isopentyl acetate	2.17		0.32	1.30	0.50	50	0.1
Isopentyl butyrate[e]			0.08	0.02	1.00	20	0.1
Isopentyl isovalerate[e]			<0.01	0.01	<1.00	20	0.1
Totals (non-EtOH)							
Alcohols			30.58	16.14	3.91		
Esters			13.03	28.38	0.95		
Aldehydes/ketones			56.38	55.48	2.10		

[a] Percent composition is relative to all nonethanol headspace components; ethanol is given as a percentage of the total profile.
[b] Ratio between absolute abundance of each component in the headspace of SB relative to AB.
[c] Solutions (v/v) in mineral oil except ethanol, which was diluted with distilled water.
[d] Ethanol and acetaldehyde not quantified for SM and AM substrates.
[e] Compound not included in 13-component total blend.

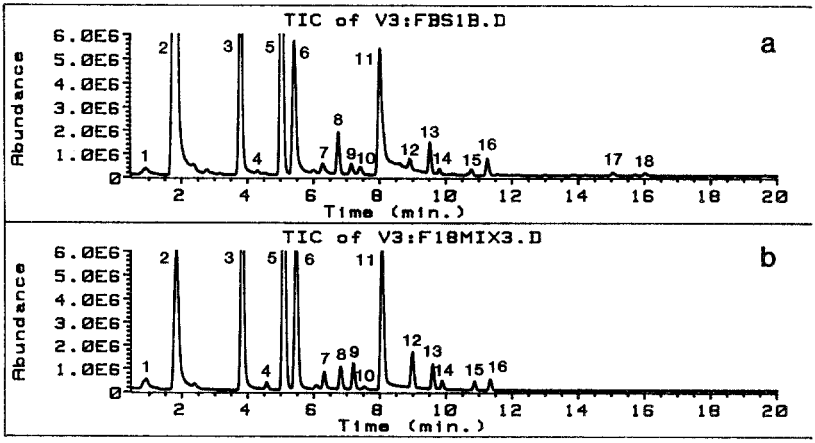


FIG. 2. Headspace volatile profiles for banana inoculated with *Saccharomyces cerevisiae* (a) and for a synthetic blend of constituents formulated to mimic the inoculated banana (b). Methods for collection and analysis are explained in the text; note that the collection method intentionally reduces the amount of ethanol and acetaldehyde relative to other components. See Table 1 for the identity of peaks and for the amount of each compound used in the synthetic odor.

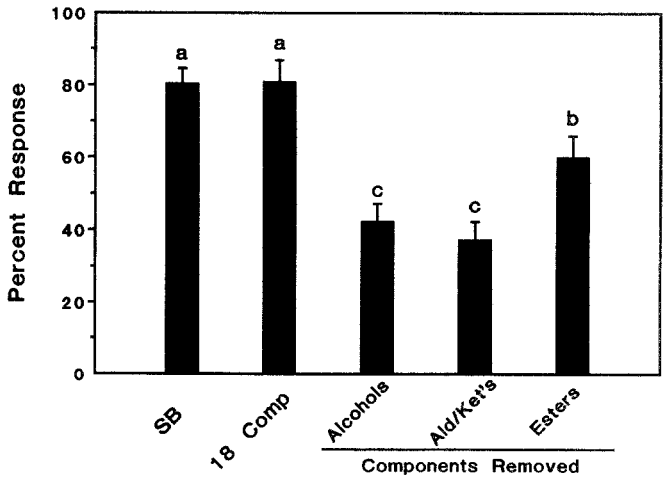


FIG. 3. Orientation of *Carpophilus hemipterus* to the odor of *Saccharomyces cerevisiae*-inoculated banana (SB), an 18-component synthetic blend, or synthetic blends with all members of each of three groups of functionalities removed: six alcohols, four aldehydes/ketones, or eight esters. Response bars (±SEM) marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$).

centage of beetles orienting to the source was lowest when either the alcohols or aldehyde/ketones were excluded, responses that were significantly lower than that to a blend that was lacking in esters. Focusing on the alcohol constituents, removal of five of the six alcohols individually did not significantly affect beetle response (Figure 4), only the synthetic blend that was lacking 2-pentanol attracted significantly fewer beetles than did the total combination of 18 components. Similarly, when esters were individually excluded from a 13-component total blend, which elicited response equivalent to that of inoculated banana, only the combination that omitted ethyl acetate evoked a significantly lower response than either the inoculated banana or the total blend (Figure 5). The response when ethyl acetate was removed was comparable to that elicited by a blend that lacked all the esters. Results from a bioassay of blends that individually eliminated acetaldehyde or the two ketones were less straight-forward. Although a blend lacking all three compounds was significantly less attractive than the total 13-component blend, the removal of any of the three individually did not significantly diminish response (Figure 6). In this group, acetaldehyde appeared to be the most important, as the removal of this component resulted in a beetle response not different from that to the blend lacking all three compounds, while blends that included acetaldehyde were significantly more active than the latter. In addition, the blend excluding acetaldehyde was significantly less active than the inoculated banana, as was the total synthetic blend in this

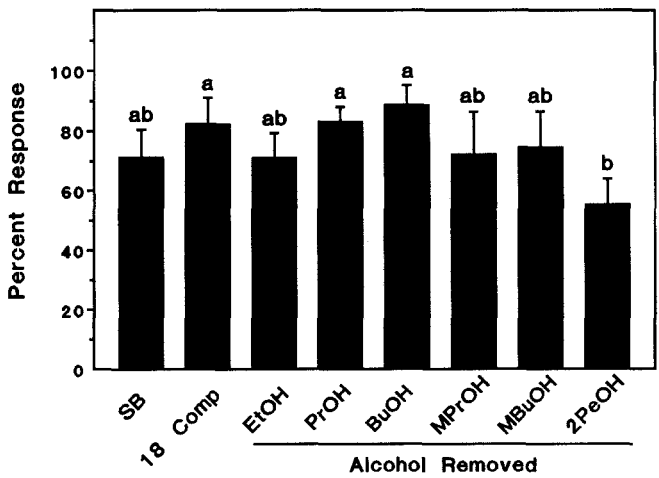


FIG. 4. Orientation of *Carpophilus hemipterus* to the odor of *Saccharomyces cerevisiae*-inoculated banana (SB), an 18-component synthetic blend, or synthetic blends with one alcohol removed. Response bars (±SEM) marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$). See Table 1 for explanation of compound abbreviations.

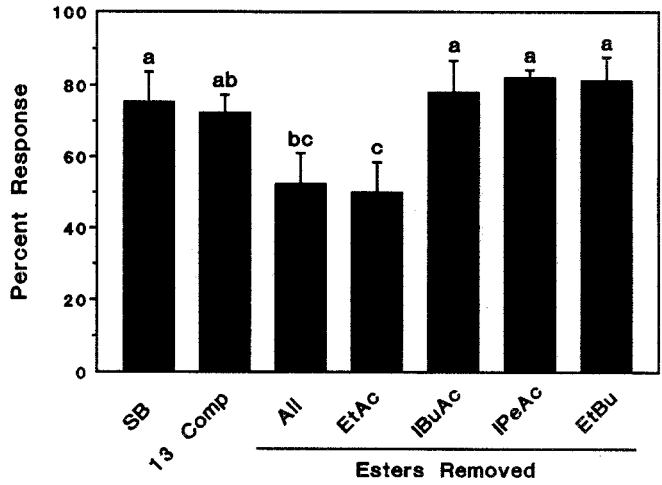


FIG. 5. Orientation of *Carpophilus hemipterus* to the odor of *Saccharomyces cerevisiae*-inoculated banana (SB), a 13-component synthetic blend, a synthetic blend with all ester components removed, or synthetic blends with one ester removed. Response bars (±SEM) marked by the same letter are not significantly different (LSD, $N = 5$ groups of 10 beetles, $P < 0.05$). See Table 1 for explanation of compound abbreviations.

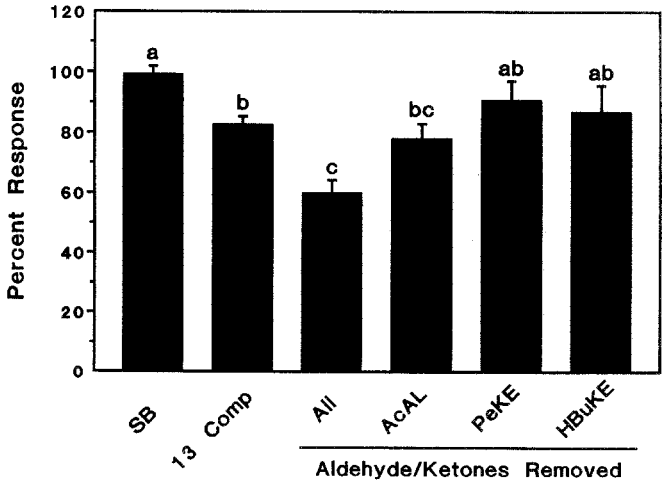


FIG. 6. Orientation of *Carpophilus hemipterus* to the odor of *Saccharomyces cerevisiae*-inoculated banana (SB), a 13-component synthetic blend, a synthetic blend with all aldehydes/ketones removed, or synthetic blends with one member of the latter group removed. Response bars (±SEM) marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$). See Table 1 for explanation of compound abbreviations.

test. The greater role of acetaldehyde in beetle attraction was confirmed by testing blends that added acetaldehyde or the ketones individually to the alcohols and esters. A blend that included only acetaldehyde from this group was significantly more active than a blend of alcohols and esters alone and was not significantly different from the total synthetic combination (Table 3). Similar blends with either 2-pentanone or 3-hydroxy-2-butanone were not significantly more active than the blend of alcohols and esters. In this bioassay, it also was confirmed that adding ethyl acetate as the only ester to a blend of alcohols, ketones, and acetaldehyde was sufficient for full beetle response (Table 3); however, a blend of acetaldehyde, ketones, and esters with 2-pentanol as the only alcohol was significantly less attractive than the total synthetic blend. This result suggested the requirement of additional alcohols for optimal response; thus a subsequent bioassay was conducted in which the other alcohols were added individually to a blend of acetaldehyde, ketones, ethyl acetate, and 2-pentanol. The addition of either ethanol or propanol did not significantly improve the attractiveness of the five-component base mixture (Figure 7). On the other hand, blends that included 1-butanol, 2-methylpropanol, or 3-methylbutanol were significantly more active than the base mixture, and beetle response to any of these blends was not significantly different from that to a mixture of acetaldehyde, ketones, alcohols, and ethyl acetate, to the 13-component blend, or to yeast-inoculated banana.

In the final bioassay based primarily on a walking response, the simplest combination of synthetic components to elicit attraction comparable to that to either the 13-component mixture or the natural odor source was a blend of ethyl acetate, 2-pentanol, 3-methylbutanol, and acetaldehyde (Figure 8). The addi-

TABLE 3. ORIENTATION OF *Carpophilus hemipterus* IN WIND TUNNEL TO ODOR OF *Saccharomyces cerevisiae*-INOCULATED BANANA, 13-COMPONENT SYNTHETIC BLEND, OR SYNTHETIC BLENDS WITH VARIOUS GROUPS OF COMPONENTS REMOVED

Odor sources[a]	Percent response ($\bar{x} \pm$ SEM)[b]
Inoculated banana	95.2 ± 3.8a
13 Component blend	89.0 ± 5.1ab
Alcohols + esters	52.0 ± 3.8c
Alcohols + esters + AcAl	81.0 ± 5.5b
Alcohols + esters + PeKe	56.0 ± 2.5c
Alcohols + esters + HBuKe	50.0 ± 6.3c
Ketones + AcAl + alcohols + EtAc	88.3 ± 2.0ab
Ketones + AcAl + esters + 2PeOH	60.2 ± 4.7c

[a] See Table 1 for explanation of compound abbreviations.
[b] Means marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$).

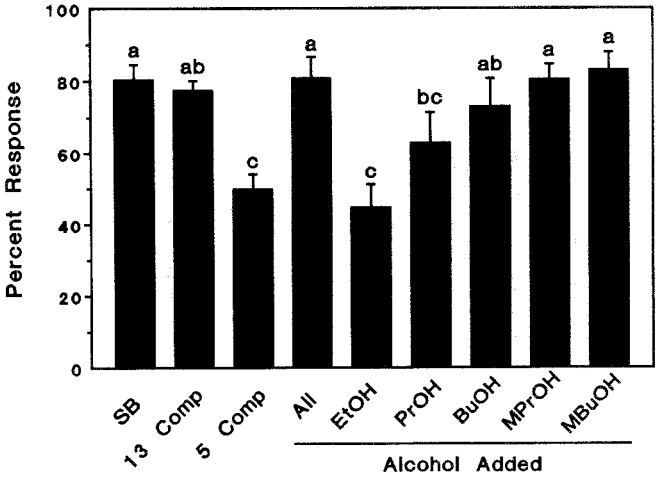


FIG. 7. Orientation of *Carpophilus hemipterus* to the odor of *Saccharomyces cerevisiae*-inoculated banana (SB), a 13-component synthetic blend, a five-component synthetic blend (acetaldehyde, two ketones, ethyl acetate, and 2-pentanol), or synthetic blends composed of the five-component blend with one of five remaining alcohols or all alcohols added. Response bars (±SEM) marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$). See Table 1 for explanation of compound abbreviations.

tion of either 2-pentanone or 3-hydroxy-2-butanone to this four-component blend did not significantly increase activity; however, a five-component blend that included both of these ketones in lieu of acetaldehyde did evoke activity comparable to the four-component and to the 13-component blends, as was also suggested by the bioassay of Figure 6. Unlike in previous bioassays, a six-component blend that included acetaldehyde and the ketones was significantly more attractive than either the four- or 13-component blends.

The bioassay based on flight-only response confirmed the previous findings on walking-and-flight response. The four-component and 13-component blends elicited a level of flight response comparable to that to the inoculated banana, while a three-component blend that lacked acetaldehyde was significantly less active (Figure 9). In addition, a blend that included acetaldehyde and the two ketones was no more active than the four-component blend.

DISCUSSION

Various fruit substrates commonly have been used as baits for trapping nitidulid species in the field (Foott and Timmins, 1977; Smilanick et al., 1978; Alm et al., 1986). In a wind-tunnel investigation of flight response to several

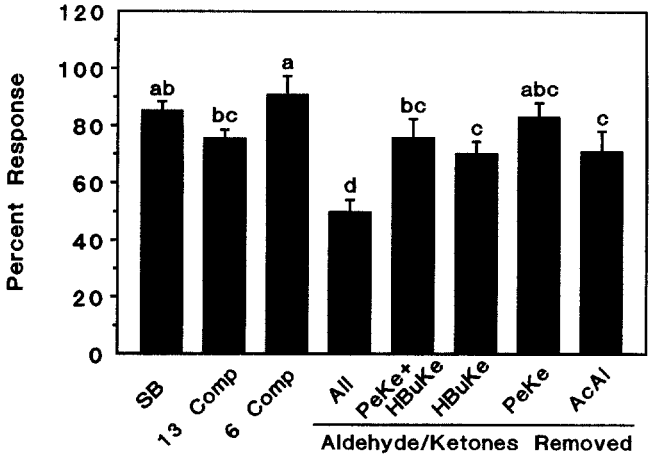


FIG. 8. Orientation of *Carpophilus hemipterus* to the odor of *Saccharomyces cerevisiae*-inoculated banana (SB), a 13-component synthetic blend, a six-component synthetic blend (acetaldehyde, two ketones, ethyl acetate, 2-pentanol, and 3-methylbutanol), or synthetic blends composed of the six-component blend with all aldehydes/ketones, both ketones, each ketone, or acetaldehyde removed. Response bars (±SEM) marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$). See Table 1 for explanation of compound abbreviations.

fruit substrates inoculated with one of two fungi or maintained asceptically, Blackmer and Phelan (1991) found *C. hemipterus* to exhibit a very broad response, with all substrates eliciting some level of attraction, but with most fungal-inoculated materials, particularly those inoculated with *S. cerevisiae*, eliciting significantly higher response than those maintained aseptically. One difficulty with using fruit baits to monitor insect activity in the field stems from the chemically dynamic nature of plant tissue, with the quantitative and qualitative aspects of the odor profile highly dependent on the stage of fruit ripening and on the degree and nature of microbial contamination of the substrate, as well as other environmental factors. This variability also was evidenced in our own wind-tunnel studies even though the condition of the substrate could be controlled to a much greater degree than in the field. During this series of bioassays, beetle response to *S. cerevisiae*-inoculated banana ranged from 68% to 99%, about twice the variability of that measured for the 13- or 18-component synthetic blends (73–89%), even though the mean responses across all experiments were virtually identical for the natural and synthetic odors, 82% and 81%, respectively.

In spite of the variability in inoculated banana as an odor stimulus, it is essential that a natural host be used as a benchmark for measuring the behavioral

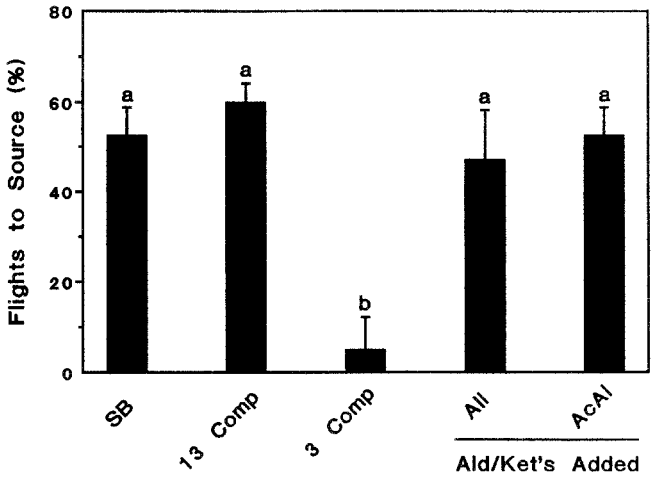


FIG. 9. Upwind flight orientation of *Carpophilus hemipterus* to the odor of *Saccharomyces cerevisiae*-inoculated banana (SB), a 13-component synthetic blend, a three-component synthetic blend (ethyl acetate, 2-pentanol, and 3-methylbutanol), or synthetic blends composed of the three-component blend with either all aldehydes/ketones or only acetaldehyde added. Response bars (±SEM) marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$).

activity of synthetic chemical blends (Miller and Strickler, 1984). Likewise, it is also important in determining the activity of a synthetic odor that the components be presented at quantitative levels corresponding to those of the natural source. For example, although Finch and Skinner (1982) found allylisothiocyanate to be as attractive as host-plant extracts in trapping *Delia brassicae*, the cabbage root fly, the concentration necessary for this response was far greater than that released from a plant. Similarly, Alm et al. (1986) found either propyl propionate or butyl acetate to be effective for trapping the nitidulid *G. quadrisignatus*; however, since a release rate of >1 g/day was needed for this response, we do not know what role this compound plays in host-finding for the species. By closely simulating the release rate for each of the volatiles identified from *S. cerevisiae*-inoculated banana in the present study, we were better able to examine the relative role played by these components in attracting *C. hemipterus* to the natural substrate and to deduce the complexity of interactions between the components in this host-finding system.

The role of the esters identified from yeast-inoculated-banana odor was clear: beetle orientation to the odor source was reduced only when ethyl acetate was removed (Figure 5); thus no combination of the other components could duplicate the behavioral activity of ethyl acetate, at least using the release rates measured in the natural odor. Furthermore, in later bioassays when ethyl acetate

was the only ester included in a synthetic blend, activity was comparable to the 13-component blend (Figures 7–9, Table 3). Among the banana-odor aldehyde/ketones, full behavioral activity was elicited by synthetic blends that included acetaldehyde as the sole member of this group (Figures 8 and 9, Table 3). Although the addition of either 2-pentanone or 3-hydroxy-2-butanone did not elicit a higher level of response than a blend that included no aldehyde/ketones (Table 3), when both of these compounds were present, response was increased to a level not significantly different from the 13-component blend (Figures 6 and 8). These bioassays also indicated that 2-pentanol was an alcohol essential for full beetle response, although an additional alcohol also was required. Three of the five alcohols identified from the natural odor led to a significant increase in beetle response: 3-methylbutanol, 2-methylpropanol, and apparently to a lesser extent, butanol (Figure 7). In total, of the 18 volatiles characterized from *S. cerevisiae*-inoculated banana: (1) ethyl acetate and 2-pentanol were essential for full upwind response; (2) acetaldehyde was important but could be replaced to some extent by the inclusion of both 2-pentanone and 3-hydroxy-2-butanone; and (3) one additional alcohol was also needed, with 2-methylpropanol, 3-methylbutanol, and possibly butanol interchangeable in this role. There apparently was no interchangeability among the three functionality groupings, because when all of the members of any functional group were removed, activity was diminished (Figure 3).

The bulk of the bioassays in this study were based primarily on a long-range walking response; across all these bioassays, 89% of beetles responded by walking the 1.8-m distance to the odor source and 11% responded by flying. The final bioassay, which only allowed a flight response, produced results that were congruent with those conclusions based primarily on a walking response. The four-component blend characterized as being the minimal blend sufficient for a full walking response also evoked levels of flight orientation similar to a six-component blend, the 13-component blend, or the natural odor source, and significantly higher flight response was elicited than by a three-component blend (Figure 9). Thus, orientation by flight or by walking appear to be mediated by similar blends of chemicals, although more subtle differences may exist between flight and walking responses to blends with intermediate levels of activity. In parallel studies of chemically mediated food finding in the congener, *C. lugubris*, results from a long-range walking bioassay in the wind tunnel were confirmed by trapping studies conducted in the field (Lin and Phelan, 1991, and in preparation). In total, these results suggest the long-range walking bioassay to be a good indicator of the blends of chemicals used by these beetles for host-finding in nature.

The results of our bioassays are in some ways congruent with, but also conflict with, the findings of Smilanick et al. (1978), who determined that none of a list of 17 fruit volatiles attracted *C. hemipterus* in significant numbers in

the field when used alone, but that a blend of acetaldehyde, ethyl acetate, and ethanol (1 : 1 : 1) did elicit high trap catch. Although they did not attempt to measure the response to various combinations of the 17 components, they did find that the three-component blend was at least as active as a natural substrate, fig paste. Furthermore, when any of the three compounds was removed from the blend, including ethanol, they measured a significant reduction in beetle response. Similarly, Bartelt et al. (1990) found that combining the three-component blend (1 : 1 : 1) with an aggregation pheromone for *C. hemipterus* increased the response to the pheromone in a synergistic fashion and that the three-component blend performed at least as well as yeast-inoculated banana even in the absence of the pheromone. In contrast, we found that when presented in the ratio found in the odor of yeast-inoculated banana (125 : 5 : 1), a blend of ethanol, acetaldehyde, and ethyl acetate was significantly less active than the natural odor; also we could find no evidence that the addition of ethanol increased behavioral response (Figures 4 and 7). Although we do not know the basis for this disparity, it may be due to differences in chemical release rates or ratios between the components, or due to intraspecific variation in beetle response.

Concerning the role that fungi may play in insect-plant interactions, there is considerable information linking fungi with nitidulid host-associations, as well as with the host preferences of other insects. For example, in dates, *Carpophilus* spp. were found preferentially in those fruits that were infested with fungi (Lindgren and Vincent, 1953). *C. hemipterus* is a transmitting agent of fig smut caused by *Aspergillus niger* (Caldis, 1930) and has been incriminated as a vector of brown rot in stone fruits and pome fruits, as well (Tate and Ogawa, 1975). A role of fungi in food-finding by *C. hemipterus* also was suggested by Miller and Mrak (1953), who found that short-range orientation in a multi-choice arena was greater to fig tissue inoculated with any of three fungi, but interestingly not to that inoculated with a bacterium. Similarly, flight response of this species in a wind tunnel was greater to various food substrates inoculated with *S. cerevisiae* than to aseptic substrates (Blackmer and Phelan, 1991).

The results of the present study confirm a preference in *Carpophilus hemipterus* for fermenting fruit and suggest a mechanism for the increased activity. In addition to the yeast-inoculated banana, both yeast alone and aseptic banana also elicited beetle attraction, although at a level below that of the combination of the two. This is distinct from the onion fly, *Delia antiqua*, for whom rot-causing bacteria elicit a higher response when inoculated in onion, but which elicit no attraction on their own (Dindonis and Miller, 1981). Honda et al. (1988) measured an increased ovipositional response in the yellow peach moth, *Conogethes punctiferalis*, to rice cakes contaminated with fungi, but oviposition was deterred by contamination with bacteria. Analysis of the volatiles from *S.*

cerevisiae on agar and from aseptic banana suggested that the attraction of beetles to these substrates was modulated by the same components as that in inoculated banana, as they produced similar volatiles but generally at lower rates than in the inoculated banana (Table 2). Yeast reared on agar had no volatile constituents that were unique or in a higher concentration than those in either aseptic- or inoculated-banana odor. Although the two banana substrates were more similar in their volatile profiles, of the six constituents with behavioral activity, acetaldehyde and 2-methylpropanol were released from inoculated banana at a rate more than twice as high as that from aseptic banana, 3-methylbutanol was 3.5 times higher, ethyl acetate and 2-pentanone were released at comparable levels, and 3-hydroxy-2-butanone was not detected in aseptic banana. The biggest change in volatile release between aseptic and inoculated banana was with propanol, which was more than 40 times greater in the latter substrate; however, this component appeared to contribute little behavioral activity to synthetic blends (Figures 4 and 7).

Thus, the yeast apparently increased beetle response by increasing the production of behaviorally active constituents rather than through a qualitative change in the odor profile. The constituents deemed to play a role in *Carpophilus hemipterus* host-finding are common volatiles of plants, produced through the catabolic pathways of fatty acid metabolism, which is accelerated during the postclimacteric phase of fruit ripening (Schreier, 1984). Despite the strong association of *C. hemipterus* with fermenting materials, the volatile constituents to which it was responsive in the present study are no more strongly associated with fermentation products than are the other inoculated-banana volatiles that had no behavioral activity (Maarse and Visscher, 1988). The yeast increased the production of these volatiles probably either through the combined effect of its own metabolism with that of the fruit or simply by causing an increase in fruit cell lysis.

As a final point, it is noteworthy that despite considerable interest in the field of insect–plant interactions, there is relatively little information on the chemical mediation of long-range host-finding. A number of studies can be cited that catalog specific compounds that influence insect host-finding behavior (Visser, 1986), but there is a notable paucity of studies in which a synthetic blend of chemicals has been determined to elicit orientation comparable to a natural host when presented at release rates found in the plant. Addressing the ecological pressures that may direct the evolution of host-finding systems, May and Ahmad (1983) and Lance (1983) have suggested that chemically mediated host finding should be much more prominent among specialist insect herbivores and that generalists would be likely to find their food plants by more random processes. However, long-range chemoorientation to hosts now has been documented for more generalists than for specialists, and it is questionable that breadth of host range has been a significant factor in whether insects evolve a

mechanism for locating potential hosts from a distance (Phelan et al., 1991). On the other hand, breadth of host range probably has determined the types of chemicals to which an herbivore does orient from a distance, or possibly the inverse is true; that the range of hosts utilized by an herbivore is determined by the chemical blends to which it is responsive (Jermy, 1988). Host-finding by insect herbivores with broad host ranges could be mediated by a response to ubiquitous plant volatiles or by a response to any of a number of more specific components. Food finding by *C. hemipterus* appears to represent a combination of both, as they respond to compounds commonly released by ripening fruit, but rather than respond to a specific combination of these, *C. hemipterus* and probably many other generalist feeders locate food and ovipositional sites by orientation to a number of different combinations of common volatiles. The conclusions drawn here concerning the relative behavioral roles of each of the 18 volatile constituents identified from yeast-inoculated banana odor for *C. hemipterus* are based on the concentration found in the natural odor profile. Expanding the investigation of chemically modulated host-finding in this species to other plant volatiles and to different ratios of those volatiles would very likely reveal an even broader and more complex response to plant odor blends.

Acknowledgments—We thank C.J. Roelofs for rearing the beetles and J.L. Blackmer for maintaining the yeast colony. Critical reviews of the manuscript were made by J.L. Blackmer and Drs. R.E. Treece and R.N. Williams. Salaries and research support were provided by state and federal funds, as well as by a grant from the USDA Competitive Research Grants Office (87-CRCR-1-2294), appropriated to the Ohio Agricultural Research & Development Center, The Ohio State University, Journal Article No. 268-90.

REFERENCES

ALM, S.R., HALL, F.R., MCGOVERN, T.P., and WILLIAMS, R.N. 1986. Attraction of *Glischrochilus quadrisignatus* (Coleoptera: Nitidulidae) to semiochemicals: Butyl acetate and propyl propionate. *J. Econ. Entomol.* 79:654–658.

BARTELT, R.J., DOWD, P.F., PLATTNER, R.D., and WEISLEDER, D. 1990. Aggregation pheromone of driedfruit beetle, *Carpophilus hemipterus*: Wind-tunnel bioassay and identification of two novel tetraene hydrocarbons. *J. Chem. Ecol.* 16:1015–1039.

BLACKMER, J.L., and PHELAN, P.L. 1991. Effect of physiological state and fungal inoculation on chemically modulated host-plant finding by *Carpophilus hemipterus* and *Carpophilus lugubris. Entomol. Exp. Appl.* In press.

CALDIS, P.D. 1930. Souring of the figs by yeasts and the transmission of the disease by insects. *J. Agric. Res.* 40:1031–1051.

DINDONIS, L.L., and MILLER, J.R. 1981. Onion fly and little house fly host finding selectively mediated by decomposing onion and microbial volatiles. *J. Chem. Ecol.* 7:421–428.

FINCH, S., and SKINNER, G. 1982. Trapping cabbage root flies in traps baited with plant extracts and with natural and synthetic isothiocyanates. *Entomol. Exp. Appl.* 31:133–139.

FOOTT, W.H., and TIMMINS, P.R. 1977. Biology of *Glischrochilus quadrisignatus* (Coleoptera: Nitidulidae) in southwestern Ontario. *Proc. Entomol. Soc. Ont.* 108:37–44.

HALL, R.W., SMILANICK, J.M., and EHLER, L.E. 1978. Laboratory rearing and field observation on *Carpophilus mutilatus*. *Ann. Entomol. Soc. Am.* 71:408–410.

HINTON, H.E. 1945. A Monograph of the Beetles Associated with Stored Products. Jarrold and Sons, Norwich, UK. 443 pp.

HONDA, H., ISHIWATARI, T., and MATSUMOTO, Y. 1988. Fungal volatiles as ovipositional attractants for the yellow peach moth, *Conogethes punctiferalis* (Guenée) (Lepidoptera: Pyralidae). *J. Insect Physiol.* 34:205–211.

JERMY, T. 1988. Can predation lead to narrow food specialization in phytophagous insects? *Ecology* 69:902–904.

LANCE, D.R. 1983. Host-seeking behavior of the gypsy moth: The influence of polyphagy and highly apparent host plants, pp. 201–224, *in* S. Ahmad (ed.). Herbivorous Insects. Academic Press, New York.

LIN, H., and PHELAN, P.L. 1991. Identification of food volatiles attractive to the dusky sap beetle, *Carpophilus lugubris* (Coleoptera: Nitidulidae). *J. Chem. Ecol.* In press.

LINDGREN, D.L., and VINCENT, L.E. 1953. Nitidulid beetles infesting California dates. *Hilgardia* 22:97–118.

MAARSE, H., and VISSCHER, C.A. 1988. Volatile Compounds in Food: Quantitative Data, Vol. 7. TNO-CNO Food Analysis Institute, Zeist, The Netherlands.

MAY, M.L., and AHMAD, S. 1983. Host location in the Colorado potato beetle: searching mechanisms in relation to oligophagy, pp. 173–199, *in* S. Ahmad (ed.). Herbivorous Insects. Academic Press, New York.

MILLER, J.R., and STRICKLER, K.L. 1984. Finding and accepting host plants, pp. 127–157 *in* W.J. Bell and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

MILLER, M.W., and MRAK, E.M. 1953. Yeasts associated with dried-fruit beetles in figs. *Appl. Microbiol.* 1:174–178.

PHELAN, P.L., ROELOFS, C.J., YOUNGMAN, R.R., and BAKER, T.C. 1991. Characterization of chemicals mediating ovipositional host-plant finding by *Amyelois transitella* females. *J. Chem. Ecol.* 17:599–613.

SAS INSTITUTE. 1985. SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina.

SCHREIER, P. 1984. Chromatographic Studies of Biogenesis of Plant Volatiles. Hüthig, New York. 171 pp.

SMILANICK, J.M., EHLER, L.E., and BIRCH, M.C. 1978. Attraction of *Carpophilus* spp. (Coleoptera: Nitidulidae) to volatile compounds present in figs. *J. Chem. Ecol.* 4:701–707.

TATE, K.G., and OGAWA, J.M. 1975. Nitidulid beetles as vectors of *Monilinia fructicola* in California stone fruits. *Phytopathology* 65:977–983.

VISSER, J.H. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol.* 31:121–144.

WILDMAN, J.D. 1933. Note on the use of microorganisms for the production of odors attractive to the dried fruit beetle. *J. Econ. Entomol.* 26:516–517.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

IDENTIFICATION OF FOOD VOLATILES ATTRACTIVE TO DUSKY SAP BEETLE, *Carpophilus lugubris* (COLEOPTERA: NITIDULIDAE)

HENGCHEN LIN* and P. LARRY PHELAN

Department of Entomology
Ohio Agricultural Research & Development Center
The Ohio State University
Wooster, Ohio 44691

(Received December 7, 1990; February 25, 1991)

Abstract—The chemical mediation of host-finding was investigated for the dusky sap beetle, *Carpophilus lugubris* Murray. GC-MS analysis of the headspace volatiles above whole-wheat bread dough inoculated with baker's yeast, a substrate previously determined to be an effective attractant, revealed seven major components in the following order of decreasing concentration: ethanol, acetaldehyde, 2-methylpropanol, 3-methylbutanol, propanol, 2-methylbutanol, and ethyl acetate. Solutions of these seven compounds blended so as to mimic the odor of whole-wheat bread dough elicited upwind orientation from 1.8 m in a wind tunnel by *C. lugubris* at a level comparable to that elicited by the bread dough. A series of bioassays investigating the role of individual components from the synthetic blend determined that all seven compounds contributed to behavioral activity; however, the simplest blend evoking attraction comparable to bread dough included acetaldehyde, ethyl acetate, 2-methylpropanol, and 3-methylbutanol. Of these compounds, acetaldehyde was essential, ethyl acetate was interchangeable with ethanol or partially replaceable with propanol, and 2-methylpropanol and 3-methylbutanol were partially replaceable with a combination of the other alcohols. Headspace volatiles above aseptic or fungus-inoculated tomato, banana, sweet corn, and strawberry were also qualitatively and quantitatively analyzed. This study suggests that *C. lugubris* locates its food sources by response to variable blends of common volatile constituents of plants and fungi.

Key Words—Coleoptera, Nitidulidae, *Carpophilus lugubris*, dusky sap beetle, host finding, chemical attractants, wind-tunnel bioassay, attraction.

*To whom correspondence should be addressed.

0098-0331/91/0600-1273$06.50/0

INTRODUCTION

The dusky sap beetle, *Carpophilus lugubris* Murray, occurs throughout the temperate and tropical regions of North, Central and South America (Sanford and Luckmann, 1963), and infests a variety of fruits and vegetables, especially sweet corn (Connell, 1975). *C. lugubris* infestation is increased when corn plants are damaged by other insects, and adults are attracted to fermenting and decaying plant materials (Harrison, 1962). Economically, *C. lugubris* is considered one of the three major insect pests of sweet corn along with the corn ear worm and the European corn borer in some areas (Harrison, 1974). Furthermore, nitidulid beetles are considered the primary vectors of the oak wilt disease (Appel et al., 1986); *C. lugubris* has been demonstrated to be capable of transmitting the disease to healthy oaks (Dorsey et al., 1953) and can be one of the predominant species feeding on tree wounds (Dorsey and Leach, 1956).

Field traps with food baits including bread dough are commonly used to monitor this and other nitidulid beetles (Alm et al., 1985; Bartelt et al., 1990; Peng and Williams, 1991). In a wind-tunnel bioassay, *C. lugubris* exhibited stronger response to whole-wheat bread dough and to fungus-inoculated fruits than to aseptic substrates (Blackmer and Phelan, 1991). To better understand the mechanisms mediating host-finding in this species and to determine the potential for using semiochemicals in its control, the role of food odors attractive to the beetle need to be fully elucidated. In this study, headspace volatiles above whole-wheat bread dough were qualitatively and quantitatively characterized; the relative activity of the identified compounds in *C. lugubris* host-finding was determined; and the odors of aseptic and fungus-inoculated tomato, banana, sweet corn, and strawberry were analyzed to relate the relative attractiveness of these substrates to the presence of specific odor components.

METHODS AND MATERIALS

Insects. A laboratory colony of *C. lugubris* was maintained on an artificial diet (Hall et al., 1978) under the conditions of 24 $\pm$ 1°C, 16:8 hr light–dark and 75–85% relative humidity. After eclosion from the pupae, both sexes were held with moisture in 12-oz. plastic cups, but without contact with food or food odors.

Natural Odors. Thirty-gram samples of whole-wheat bread dough inoculated with baker's yeast or similar samples of fruits were incubated in 240-ml Qorpak straight-sided jars at room temperature for about 24 hr or five to seven days, respectively, before being used for volatile collections or for bioassays. Fresh tomato, banana, sweet corn, and strawberry were maintained aseptically or inoculated using a previously described method (Phelan and Lin, 1991) with either *Saccharomyces cerevisiae* or *Candida krusei*, two yeasts that are ubiq-

uitous in nature (Alexopoulos, 1979). The lid of each jar was installed with a 37-mm Bacterial Air Vent (Gelman Science, Ann Arbor, Michigan) for ventilation, and a sleeve-type rubber septum (Thomas Scientific) for collection of headspace samples.

Headspace Volatile Collection and Identification. Headspace volatiles were trapped on Tenax GC and then thermally desorbed to a capillary gas chromatograph (Phelan and Lin, 1991). Due to the high concentration and volatility of ethanol and acetaldehyde relative to the other odor components, two volatile collection methods were used. In the first method, a 20-ml headspace sample was injected into the volatile trap, which was then flushed with helium (20 ml/min) for 6 min at room temperature before desorption. During this time, most of acetaldehyde and ethanol were removed due to their low breakthrough volume on the trap (Phelan and Lin, 1991). The second method was used to quantify these two components by injection of a 1-ml headspace sample, followed by a helium flow for 0.5 min, which allowed the trap to retain all the volatile constituents for desorption to the capillary column. Jars containing natural samples were opened for 3 min before taking air samples, and those containing synthetic odor sources were sampled at 3 min after they were prepared.

Volatile constituents from natural substrates were quantified and tentatively identified using a Hewlett-Packard 5890A capillary gas chromatograph and a HP 5970C Mass Selective Detector. A capillary column, DB-1 (30 m × 0.32 mm ID, 5.0-μm film thickness), was used with a temperature program of 30–200°C increased at 10°C/min, and a helium flow of 0.7 ml/min. Chemical identifications were confirmed and concentrations were quantified by comparing retention times, mass spectra, and peak areas with those of known quantities of authentic compounds.

Synthetic Odors. Synthetic odors were prepared by applying compounds as mineral-oil solutions to cotton wicks at the concentrations and quantities listed in Table 1. Ethanol was the exception in that it was diluted with double-deionized water due to its insolubility in mineral oil. The synthetic odors were used for bioassay immediately after preparation, and fresh samples were prepared for each replicate. All chemicals were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin).

Wind-Tunnel Bioassay. The behavioral responses of *C. lugubris* to natural and synthetic odors were assessed in a horizontal wind tunnel (2.5 m long × 1 m wide × 0.5 m high) (Phelan et al., 1991). The conditions of the wind tunnel were 35–65% relative humidity, 24–27°C, 17 lux light at the level of wind-tunnel floor and a wind speed of 0.5 m/sec. Bioassays were conducted at 4–0 hr before scotophase using 6- to 8-day-old adults of mixed sex.

The bioassays were set up with two jars placed on the floor 10 cm apart from the center of the upwind end of the tunnel. One of the two jars was empty to serve as a check for possible odor contamination, and the other contained

TABLE 1. CHEMICAL CHARACTERIZATION AND QUANTIFICATION OF HEADSPACE VOLATILES FROM WHOLE-WHEAT BREAD DOUGH AND THEIR RELATIVE COMPOSITION IN SYNTHETIC BREAD-DOUGH ODOR

Component[a]				Bread dough		Synthetic odor[c]	
No.	Abbr.	Identity	Mass spectra (*m/z*)	Quantity[b]	Percent	%	μl
1	AcAl	Acetaldehyde	29, 41, 43, 44	39.6	7.7	1	500
2	EtOH	Ethanol	27, 29, 31, 45, 46	402.8	78.8	25	500
3	PrOH	1-Propanol	27, 29, 31, 42, 59, 60	8.3	1.7	1	100
4	EtAc	Ethyl acetate	43, 45, 61, 70, 73, 88	6.1	1.2	1	50
5	MPrOH	2-Methylpropanol	33, 41, 42, 43, 55, 74	31.6	6.2	1	100
6	3MBOH	3-Methylbutanol	41, 42, 43, 55, 56, 57, 70	15.2	3.0	1	30
7	2MBOH	2-Methylbutanol	39, 41, 42, 55, 56, 58, 70	7.6	1.5	1	20

[a] Peak numbers were used in Figure 1 and the underlined numbers in mass spectra indicate the base ions.
[b] Quantity measured as ng/ml of headspace.
[c] Concentrations (v/v) and volumes of mineral-oil solutions, except ethanol, which was diluted with distilled water.

odor samples; the positions of the two jars were rotated between replicates. Beetles were released on the floor at the center of the downwind end of the tunnel at a distance of 180 cm from the odor sources. Smoke tests showed that plumes from the two jars merged before reaching the beetle-release site. Ten beetles were released each time and were used only once. Beetles that oriented to odor sources by walking or flying up the odor plume to within 10 cm downwind of the jar in 8 min were counted as responders, and no beetle response to the empty jar was observed. Although both walking and flying responses were observed during bioassays, the majority of beetles (>95%) responded by walking to odor sources. The 10-cm criterion was set since the top of the jar, and thus the source of odors, was about 9 cm above the floor, and the beetles that walked to within 10 cm of the jar would lose the odor plume. Eight minutes were needed for most beetles to reach odor source, to which they were responding.

A randomized complete-block design was used in all bioassays with the sequence of odors in a block randomized. Data were recorded as percent response of each group of 10 beetles released, and these percentages were subjected to transformation by $\sin^{-1}(X^{1/2})$ before analysis of variances. LSD tests were used for comparing means of percent response to odor treatments where the ANOVA indicated a significant F value (SAS Institute, 1985).

RESULTS

Identification of Headspace Volatiles. A total of seven compounds (acetaldehyde, ethyl acetate, and five alcohols) was identified from the headspace of whole-wheat bread dough (Figure 1A) as listed in Table 1 with their retention times and mass spectra used for their identification. Quantification of this volatile profile showed that ethanol was the most abundant component (402.8 ng/ml) with the others present at 6.1–39.6 ng/ml (Table 1).

The volatile composition of fruit substrates was analyzed focusing on the components present in the bread-dough odor (Table 2). In this analysis only the first volatile collection method was used, so that acetaldehyde and ethanol were not quantified. The concentration of ethyl acetate in banana and tomato odor was not affected by fungus inoculation; however, in sweet corn and strawberry ethyl acetate was increased to about 1.4 times. Propanol was detected only in fungus-inoculated banana and the bread dough, and 2-methylbutanol was not detected in any of the fruit substrates. The increase in 2-methylpropanol production due to fungus inoculation ranged from onefold in *Candida*-inoculated sweet corn to 22-fold in *Candida*-inoculated strawberry. Inoculation also resulted in increased production of 3-methylbutanol, ranging from 1.6 times higher in *Candida*-inoculated banana to 14 times higher in inoculated straw-

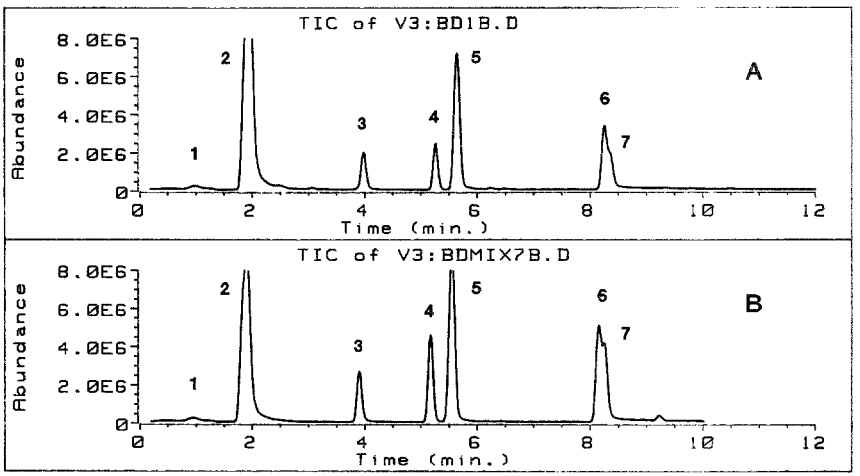


FIG. 1. Headspace volatile profiles from whole-wheat bread dough (A) and from a synthetic blend of constituents formulated to mimic bread-dough odor (B). See Table 1 for identity of peaks and amounts applied in synthetic blend, and see text for explanation of methods for collection and analysis. Note that the collection method intentionally reduced the amount of ethanol and acetaldehyde relative to other components.

TABLE 2. RELATIVE ABUNDANCE OF VOLATILE CONSTITUENTS (EXCLUDING ETHANOL AND ACETALDEHYDE) IN HEADSPACE OF BREAD DOUGH AND FRUIT SAMPLES MAINTAINED ASEPTICALLY OR INOCULATED WITH EITHER *Saccharomyces cerevisiae* OR *Candida krusei*

Volatile compound	Bread dough	Banana					Tomato			Sweet Corn			Strawberry		
		A[a]	S	C	S:A[b]	C:A	A	C	C:A	A	C	C:A	A	C	C:A
EtAc	10.6	50.1	33.1	41.2	0.96	1.01	50.9	65.5	0.94	62.1	51.6	1.42	84.2	52.3	1.40
PrOH	12.2	0	24.8	7.6			0	0		0	0		0	0	
MPrOH	47.5	10.0	10.2	19.6	1.48	2.40	0.7	4.0	4.04	2.8	2.6	1.58	1.8	17.9	22.96
3MBOH	19.8	14.3	19.6	18.0	1.98	1.55	0.9	5.5	4.47	3.3	3.2	1.63	2.5	15.3	13.97
2MBOH	9.9	0	0	0			0	0		0	0		0	0	
Others	0	25.0	12.3	13.5	0.70	0.65	47.4	25.0	0.38	31.7	42.9	2.30	11.5	14.5	1.15
Total					1.34	1.15			0.82			1.29			1.64

[a] Percent composition of headspace components other than ethanol and acetaldehyde in substrates maintained aseptically (A), or inoculated with *S. c.* (S) or *C. k.* (C).

[b] Ratio between absolute concentration of each component in the headspace of inoculated vs. aseptic substrate.

berry. The concentrations of non-bread-dough components were reduced by fungus inoculation in banana and tomato and increased in sweet corn.

Beetle Attraction to Bread Dough and Synthetic Odors. The synthetic blend of seven components closely mimicked bread dough both in its odor profile (Figure 1) and in its attractiveness to beetles; the level of *Carpophilus lugubris* attraction to a blend of the seven compounds (97.4 $\pm$ 2.9%, $\bar{X} \pm$ SEM) was not significantly different from that to bread dough (93.8 $\pm$ 9.5%, $\bar{X} \pm$ SEM) (t test, $P < 0.05$). Beetles were never observed responding to an empty jar.

In the first of a series of subtractive and additive bioassays, the removal of either acetaldehyde alone or all five alcohols together resulted in a significant reduction in beetle attraction, while the elimination of ethyl acetate or any one of the five alcohols did not significantly affect beetle response (Figure 2). To determine the relative importance of each alcohol in attracting *C. lugubris*, the alcohols were next added individually to a base blend of acetaldehyde and ethyl acetate. The addition of any alcohol significantly increased beetle response above that of the base blend, with the order of decreasing activity being 2-methylpropanol, 2-methyl and 3-methylbutanol, propanol, and ethanol (Figure 3). Only the addition of 2-methylpropanol increased the beetle response to the level of the seven-component blend.

The next two bioassays focused on the role of ethyl acetate and its relationship with alcohols. The addition of this component to acetaldehyde and 2-methylpropanol significantly improved beetle attraction (Figure 4); however, the elimination of ethyl acetate from the total blend of seven compounds did

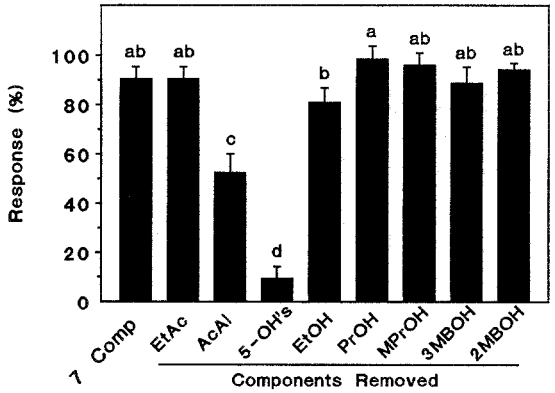


FIG. 2. Percent ($\bar{X} \pm$ SEM) upwind orientation in a wind tunnel by *C. lugubris* to the seven-component blend, to blends with individual components removed, or to a blend with all alcohols removed (5-OH's). Bars marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$). See Table 1 for explanation of compound abbreviations.

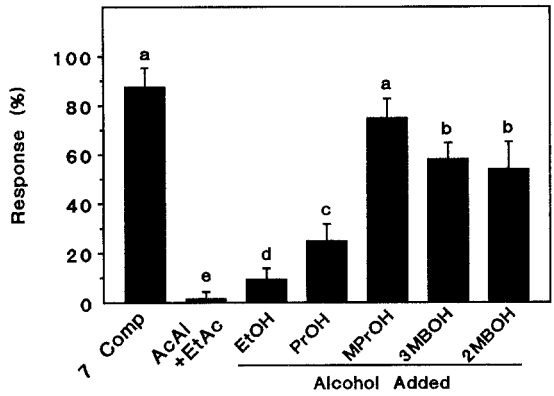


FIG. 3. Percent ($\bar{X} \pm$ SEM) upwind orientation in a wind tunnel by *C. lugubris* to the seven-component blend, a two-component blend of acetaldehyde and ethyl acetate, or three-component blends that added individual alcohols to the two-component blend. Bars marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$). See Table 1 for explanation of alcohol abbreviations.

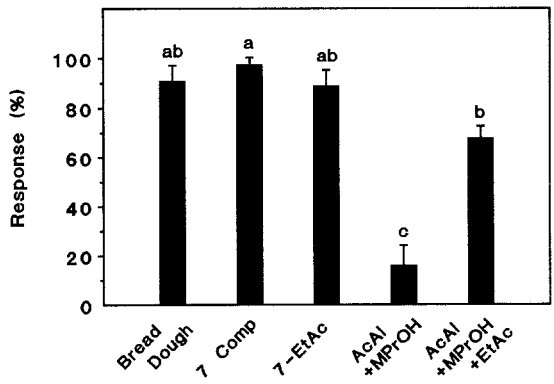


FIG. 4. Percent ($\bar{X} \pm$ SEM) upwind orientation in a wind tunnel by *C. lugubris* to whole-wheat bread dough, the seven-component blend, a six-component blend lacking ethyl acetate, a two-component blend of acetaldehyde and 2-methylpropanol, and the two-component blend with ethyl acetate added. Bars marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$). See Table 1 for explanation of compound abbreviations.

not affect the beetle response, confirming the result of Figure 2. Response to the seven-component blend again was similar to that to bread dough, as was that to the synthetic blends lacking ethyl acetate or to the combination of acetaldehyde, 2-methylpropanol, and ethyl acetate, although the latter was signifi-

cantly less active than the seven-component blend (Figure 4). The addition of ethyl acetate to a blend of acetaldehyde and 2-methylpropanol again significantly increased beetle response (Figure 5), this time to a level not significantly different from that to the seven-component blend. The addition of either ethanol or propanol to the base blend also significantly increased attraction; however, blends containing ethanol and propanol were less attractive than the seven-component blend. The addition of both 3-methylbutanol and 2-methylbutanol to acetaldehyde and 2-methylpropanol did not significantly enhance response.

In the final bioassay, which used a base blend of acetaldehyde, 2-methylpropanol, and 3-methylbutanol, the interchangeability between ethyl acetate and ethanol that was suggested by the data of Figure 5 was confirmed (Figure 6). A four-component blend containing either of these compounds possessed activity comparable to the seven-component blend and to bread dough. The addition of propanol to the three-component base blend did not significantly increase beetle response, and this combination was significantly less active than either of the other two four-component blends, the total synthetic blend, or bread dough.

DISCUSSION

This study demonstrated that a blend of seven compounds simulating the odor of whole-wheat bread dough could evoke a level of attraction in *Carpophilus lugubris* comparable to the natural odor. At the concentrations found in

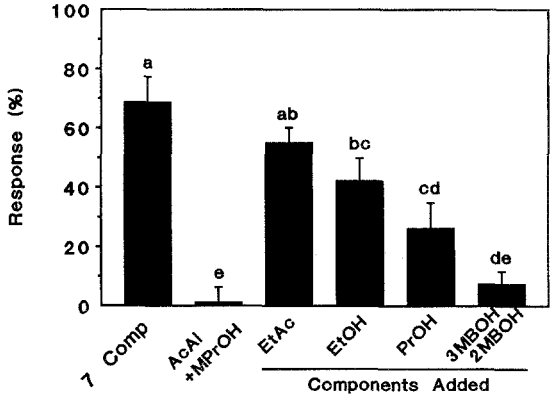


FIG. 5. Percent ($\bar{X} \pm$ SEM) upwind orientation in a wind tunnel by *C. lugubris* to the seven-component blend, a two-component blend of acetaldehyde and 2-methylpropanol, or three-component blends that added individual components to the two-component blend. Bars marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$). See Table 1 for explanation of compound abbreviations.

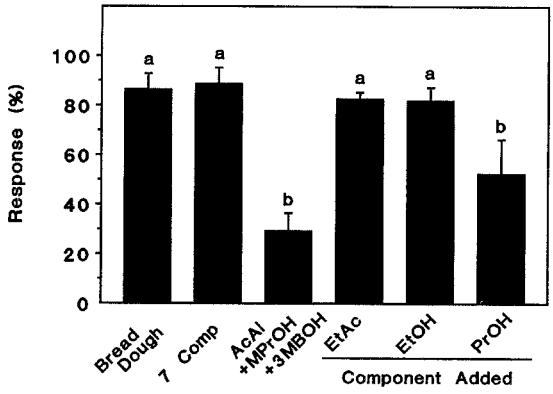


FIG. 6. Percent ($\bar{X} \pm$ SEM) upwind orientation in a wind tunnel by *C. lugubris* to whole-wheat bread dough, the seven-component blend; a three-component blend of acetaldehyde, 2-methylpropanol, and 3-methylbutanol; or four-component blends that added individual components to the three-component blend. Bars marked by the same letter are not significantly different (LSD, $N = 4$ groups of 10 beetles, $P < 0.05$). See Table 1 for explanation of compound abbreviations.

bread-dough odor, all seven components contributed to beetle attraction, but not all were needed for full response. The seven compounds could be clustered into three groups based on their interchangeability with other components in blends eliciting full host-finding response. Acetaldehyde represented the first group and was not replaceable with any other components identified from bread-dough odor; the elimination of this compound from the total synthetic blend resulted in a significant reduction in beetle response (Figure 2). Acetaldehyde also plays a role in the host-finding of another nitidulid beetle, *C. hemipterus* (Smilanick et al., 1978; Phelan and Lin, 1991) and was partially replaceable with other carbonyl components identified from banana odor (Phelan and Lin, 1991). Since acetaldehyde was the only carbonyl compound identified from bread-dough odor in the present study, its relationship to other carbonyl components of food odors attractive to *C. lugubris* is not known. The second group included ethyl acetate, ethanol, and possibly propanol, although to lesser extent. Of these three components, the removal of ethyl acetate from the total synthetic blend did not reduce beetle attraction (Figures 2 and 4), but its addition to a blend of acetaldehyde and 2-methylpropanol or to a blend of acetaldehyde, 2-methylpropanol, and 3-methylbutanol significantly enhanced beetle response (Figures 4–6). The replacement of ethyl acetate with ethanol did not significantly affect beetle response, while replacement with propanol reduced beetle attraction (Figure 5 and 6), although when added to acetaldehyde and 2-methylpropanol, propanol did enhance activity. Ethyl acetate also was determined

to be important to *C. hemipterus* host-finding (Smilanick et al., 1978; Phelan and Lin, 1991), but it was not replaceable by any of the other 17 banana-odor components tested (Phelan and Lin, 1991). The third active constituent group for *C. lugubris* included the five alcohols of bread-dough odor. The most important member of this group was 2-methylpropanol, which was only partially interchangeable with the other alcohols (Figure 3), but fully replaceable by some combination of these alcohols (Figure 2). The alcohols in bread-dough odor, with exception of 2-methylbutanol, also are emitted by fungus-inoculated banana. All of these except ethanol are active in attracting *C. hemipterus* (Phelan and Lin, 1991), although ethanol was shown to be attractive to *C. hemipterus* in combination with acetaldehyde and ethyl acetate at 1 : 1 : 1 ratio (Smilanick et al., 1978). Among the banana-odor alcohols, 2-pentanol plays a separate role from the others in attracting *C. hemipterus* (Phelan and Lin, 1991). Although 2-pentanol was not identified from bread-dough odor and not tested in this study, it is unlikely to play a separate role from the other bread-dough alcohols, since bread dough was more attractive than fungal-inoculated banana to *C. lugubris* (Blackmer and Phelan, 1991). In summary, based on a long-range walking response to the seven major components of bread-dough odor in the wind tunnel, maximum host orientation appears to be elicited by a blend of acetaldehyde, ethyl acetate, 2-methylpropanol, and 3-methylbutanol, of which ethyl acetate is interchangeable with ethanol, and 3-methylbutanol plus 2-methylpropanol are replaceable with some combination of the other alcohols. Field studies, which are necessary to confirm these results in nature, are planned.

Although it appears that plant odors play an important role in insect host-plant finding as a whole, there are few species for which we have characterized a synthetic blend of compounds to mimic the full behavioral activity of a natural attractive odor. Although a list of plant compounds may be cited that are attractive to a number of phytophagous insects (Visser, 1986), the fact that these chemicals have rarely been investigated at release rates comparable to that of a host plant makes interpretation of their ecological and behavioral significance difficult. Simulating the natural odor with the appropriate concentration of authentic components provides a more accurate method for elucidating the relative role of individual odor constituents in host-finding. The systematic investigation of the chemical constituents of food odors attractive to *C. lugubris* in this study and for *C. hemipterus* by Phelan and Lin (1991) has demonstrated considerable overlap in the blends of components responsible for chemically mediated host-finding in these congeners. Both species appear to possess broad host ranges and are attracted to a variety of food odors (Blackmer and Phelan, 1991), and not surprisingly the compounds eliciting attraction in these species are common to a wide variety of plant substrates (Maarse and Visscher, 1988). Nevertheless, differences are evident in the relative responsiveness of these two species to different plant odors, which probably are due more to quantitative

variations in the relative activity of individual components or in the ratio of those components rather than the presence or absence of specific volatiles. For example, it has long been suggested that fungus inoculation of plant substrates increases their attractiveness to many nitidulid species (Wildman, 1933; Neel et al., 1967); however, Blackmer and Phelan (1991) found a much greater level of discrimination between inoculated and aseptic substrates by *C. lugubris* than by *C. hemipterus*. Long-range response of *C. lugubris* measured in a wind tunnel was 3.2 times and 3.9 times higher for substrates inoculated with *Candida krusei* and *S. cerevisiae*, respectively, than for aseptic substrates, while these fungi only increased response in *Carpophilus hemipterus* by 1.3-fold and 1.5-fold, respectively. Whether this differential response has an adaptive basis, e.g., because *C. lugubris* has a more strongly saprophagous feeding habit, is not known.

Nitidulids are certainly not unique in their enhanced attraction due to the action of microorganisms. Similar responses have been documented for a number of phytophagous insects, mites, and nematodes (Dicke, 1988; Blackmer and Phelan, 1991, Phelan and Lin, 1991). Despite the widespread nature of microbially mediated response to plants, one of the questions needing to be addressed is whether increased attraction is due to microbes simply elevating the release of plant-produced volatiles or is due to a qualitative change in the odor blend resulting from the addition of volatile microbial metabolites. In this and our previous studies, increased attraction of both *C. hemipterus* and *C. lugubris* was probably due to a quantitative increase in fruit-derived volatiles rather than to novel compounds generated by fungal metabolism, as evidenced by our GC-MS analysis of inoculated and aseptic substrates. The only component unique to a fungal-inoculated substrate was propanol, which was found in inoculated banana; however, this compound was not associated with any other inoculated substrate. On the other hand, many of the behaviorally active components found in aseptic substrates showed increased production when these substrates were fungal-inoculated. Enhanced production was most dramatic for 2-methylpropanol and 3-methylbutanol in *Candida*-inoculated strawberry, where the absolute concentrations of these components in the headspace were 23 and 14 times higher, respectively, than in the odor of aseptic strawberry. Although the change due to fungal inoculation in the two most prominent odor components, ethanol and acetaldehyde, was not measured in this study, Phelan and Lin (1991) found these compounds were released at levels 1.8 and 2.1 times higher, respectively, in *Saccharomyces*-inoculated banana than in aseptic banana. Whether the role of microbes in enhanced attraction of other phytophagous insects is predominantly quantitative as well is not known. We do not suggest quantitative odor change and qualitative change to be mutually exclusive categories, but rather view these as two ends of a continuum. A model that we would propose concerning the mode of action of microbes in animal–plant interactions would be

that those animals with broad feeding habits might be more likely mediated by quantitative changes in an odor profile, while those animals with stronger microbial associations, especially those with obligatory relations, would be more narrowly responsive to compounds unique to or greatly enhanced by microbial activity. The extent of this proposed relationship between strength of microbial association and the nature of chemical signals eliciting attraction remains to be determined by conducting chemical and behavioral analyses in other systems similar to those now carried out for *Carpophilus hemipterus* and *C. lugubris*.

Acknowledgments—The authors would like to thank C. Roelofs for assistance in maintaining the insect colony and J.L. Blackmer for maintaining the fungal colonies. Critical reviews of the manuscript were made by A. Wayadandi and Drs. R.E. Treece and R.N. Williams. Salaries and research support were provided by state and federal funds, as well as by a grant from the USDA Competitive Research Grants Office (87-CRCR-1-2294), appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University, Journal Article No. 310-90.

REFERENCES

ALEXOPOULOS, C.J. 1979. Introductory Mycology, 3rd ed. John Wiley & Sons, New York.

ALM, S.R., HALL, F.R., LADD, T.L., JR., and WILLIAMS, R.N. 1985. A chemical attractant for *Glischrochilus quadrisignatus* (Coleoptera: Nitidulidae). *J. Econ. Entomol.* 78:839–843.

APPEL, D.N., ANDERSEN, K., and LEWIS, R. 1986. Occurrence of nitidulid beetles (Coleoptera: Nitidulidae) in Texas oak wilt centers. *J. Econ. Entomol.* 79:1276–1279.

BARTELT, R.J., DOWD, P.F., and PLATTNER, R.D. 1990. The aggregation pheromone of *Carpophilus lugubris*: Toward new pest management tools for the nitidulid beetles, pp. 27–40, *in* P.A. Hedin (ed.). Naturally Occurring Pest Bioregulators. American Chemical Society, Washington, D.C.

BLACKMER, J., and PHELAN, P.L. 1991. Effect of physiological state and fungal inoculation on chemically modulated host-plant finding by *Carpophilus hemipterus* and *Carpophilus lugubris*. *Entomol. Exp. Appl.* 58:137–148.

CONNELL, W.A. 1975. Hosts of *Carpophilus dimidiatus*. *J. Econ. Entomol.* 68:279–280.

DICKE, M. 1988. Microbial allelochemicals affecting the behavior of insects, mites, nematodes, and protozoa in different trophic levels, pp. 125–162, *in* P. Barbosa and D.K. Letourneau (eds.). Novel Aspects of Insect-Plant Interactions. John Wiley & Sons, New York.

DORSEY, C.K., and LEACH, J.G. 1956. The bionomics of certain insects associated with oak wilt with particular reference to the Nitidulidae. *J. Econ. Entomol.* 49:219–230.

DORSEY, C.K., JEWELL, F.F., LEACH, J.G., and TRUE, R.P. 1953. Experimental transmission of oak wilt by four species of nitidulidae. *Plant Dis. Rep.* 37:419–420.

HALL, R.W., SMILANICK, J.M., and EHLER, L.E. 1978. Laboratory rearing and field observation on *Carpophilus mutilatus*. *Ann. Entomol. Soc. Am.* 71:408–410.

HARRISON, F.P. 1962. Infestation of sweet corn by the dusky sap beetle, *Carpophilus lugubris*. *J. Econ. Entomol.* 55:922–925.

HARRISON, F.P. 1974. Chemical control of ear-infesting insects of sweet corn. *J. Econ. Entomol.* 67:548–550.

MAARSE, H., and VISSCHER, C.A. 1988. Volatile Compounds in Food: Quantitative Data, Vol. 7. TNO-CNO Food Analysis Institute, Zeist, The Netherlands.

NEEL, W.W., GLICK, B.D., MAY, L.L., and TRUE, R.P. 1967. Attractiveness to Nitidulidae

(Coleoptera) of natural attrahents of tree and fungus origin supplemented with vinegar and water in an Appalachian hardwood forest. *J. Econ. Entomol.* 60:1104–1109.

PHELAN, P.L., and LIN, H. 1991. Chemical characterization of fruit and fungal volatiles attractive to dried-fruit beetle, *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae). *J. Chem. Ecol.* 17:1253–1272.

PHELAN, P.L., ROELOFS, C.J., YOUNGMAN, R.R., and BAKER, T.C. 1991. Characterization of chemicals mediating ovipositional host-plant finding by *Amyelois transitella* females. *J. Chem. Ecol.* 17:599–613.

PENG, C., and WILLIAMS, R.N. 1991. Effect of trap design, trap height and habitat on the capture of sap beetles (Coleoptera: Nitidulidae) using whole wheat bread dough. *J. Econ. Entomol.* In press.

SAS INSTITUTE. 1985. SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina.

SANFORD, J.W., and LUCKMANN, W.H. 1963. Observations on the biology and control of the dusky sap beetle in Illinois. *Proc. North Central Branch E.S.A.* 18:39–43.

SMILANICK, J.M., EHLER, L.E., and BIRCH, M.C. 1978. Attraction of *Carpophilus* spp. (Coleoptera: Nitidulidae) to volatile compounds present in figs. *J. Chem. Ecol.* 4:701–707.

VISSER, J.H. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol.* 31:121–144.

WILDMAN, J.D. 1933. Note on the use of microorganisms for the production of odors attractive to the dried fruit beetle. *J. Econ. Entomol.* 26:516–517.

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

Book Review

Insect Defenses. Adaptive Mechanisms and Strategies of Prey and Predators. David L. Evans and Justine O. Schmedt. Albany: State University Press, 1990. 482 pp. $73.50 Hb; $20.95 Pb.

The scope of this interesting and stimulating book is broad since it includes not only reviews of various insect defensive strategies, but also, to a lesser extent, predator strategies, as pointed out by its subtitle. This broad scope has obvious merits, but also unavoidable limitations. Indeed, defense and predation must be considered together, although these two sides of the same interaction are too often analyzed more or less independently.

The editors must be congratulated for their attempt to assemble reviews on both aspects. However, defensive and predatory strategies are so diverse that a single volume of a little more than 400 pages cannot give an exhaustive overview of the subject. This is certainly not the aim of the book and was never claimed by the editors. Each chapter should be taken as a state of the art report on one facet of the complex interaction between predator and prey, and as an illustration of the diversity of the whole field.

The book is organized in four sections, each introduced briefly by one of the two editors: Evolution of Major Defensive Ensembles, Predatory Strategies and Tactics, Predation Prevention: Avoidance and Escape Behaviors, Predation Prevention: Chemical and Behavioral Counterattack.

The first part includes three excellent chapters by Edmunds, Guilford, and Sakaluk. Edmunds aptly comments on the evolution of cryptic coloration, considering successively the point of view of the predators and the evolutionary responses of the prey. Guilford gives the most cunning and subtle account of the evolution of aposematism I have ever read. He discusses the concept itself, how it works, and how it could have evolved, taking into account the complexities of predator psychology. He presents alternative hypotheses carefully, and makes clear distinctions between intuitively evident mechanisms and those that are demonstrated by unambiguous experiments. Sakaluk's interesting chapter discusses the increasing risks due to sexual activities, conflicting aspects of sexual and natural selection, and how predation pressure has influenced the evolution of arthropod mating systems.

In the second section, two chapters describe predatory tactics in two specific groups of predators, spiders and birds. Uetz's review of spider predatory behavior is restricted to web-building spiders, with some emphasis on the

0098-0331/91/0600-1287$06.50/0

behavior of *Micrathena gracilis* studied by the author's group in North American deciduous forest. Both selection of prey and avoidance of predators by prey (flies and moths) are considered. Schuler describes the behavior of insectivorous birds in response to prey density and prey profitability, discussing concepts such as optimal foraging, area concentrated search, or search image. In the last chapter of this section, Robinson advocates the idea that the complexity of prey–predator interactions in tropical rain forest, rather than the complexity of social interactions, was at the origin of the evolution of memory and "intelligence."

The third section is devoted to avoidance and escape behavior. Lederhouse describes primary defenses in lepidopteran caterpillars, i.e., defenses operating independently of predator presence. These include predator–prey allopatry, physical barriers (borers, miners, and galls, protective retreats), elimination of evidence of activity, mimesis, aposematism, and Batesian mimicry. Evans emphasises phenology (temporal distribution) as part of defensive strategy, whereas Fullard discusses possible coevolution between bats' echolocation mechanism and moths' tympanic organ structure and function.

The last six chapters in the fourth section are assembled under the subheading of counterattack by prey. Sargent gives a thorough critical analysis of startle as an antipredator mechanism. As in most other chapters throughout the book, a strong emphasis is placed on the few careful experiments demonstrating the mechanism as opposed to simple descriptions made by naturalists. The same attitude, critical and full of nuances, is adopted by Vulinec in her analysis of aggregation or gregariousness as possible defensive counterattack. Whitman, Blum, and Alsop's chapter is probably the best available introduction to the diversity of chemical defense in arthropods, considering not only the chemical diversity of defensive compounds, but also the diversity of modes of storage and release and the structures and behaviors related to these functions. They also discuss the modes of action of the secretions in deterring predators and other possible roles of these secretions. Bowers complements this overview of chemical defense in insects by considering how some herbivores are able to recycle plant natural products for defense. Schmidt details defense by stinging in Hymenoptera. He compares in different taxa the efficiency and the various modes of action of the venoms, and also describes counteradaptations in predators. In the last chapter, Starr gives a stimulating account of the various defensive strategies in primitive social wasps, e.g., primary defenses, including nest structures and locations, and individual and social defenses. An interesting discussion of threat as defensive strategy in wasps is included in this chapter, mainly based on his own research.

There are obvious overlaps among the different parts of the book and even some redundancies. For example, crypsis, aposematism, and various aspects of bird foraging behavior are considered several times. This is, however, a minor

fault, since, depending on the authors, the emphasis is different, thus enlightening theoretical controversies.

As stated above, some aspects of defense and predation are not discussed at all. For example, the selective pressure of important natural enemies, e.g., parasitoids or ants, and possible defensive counteradaptations to them, are not or only incidently considered. The complexity of defense in social insects is only described in primitive wasps. Again, these are shortcomings unavoidable without extending the scope and the volume of the book.

The absence throughout the book of an overall ecological perspective is, striking, however. Are predation risks and defenses different in various habitats? Is it possible to recognize different patterns in the defensive strategies adopted by, say, insect predators or herbivores, by insects living on the ground or on plants, by feeders on herbs in open habitats or on trees in forests? This seems to be more a drawback of the entire field than of the book itself. Very seldom are these questions addressed, and it is perhaps time to try to do so, despite the obvious difficulties in answering them.

However, the book covers a large diversity of topics, giving a fair idea of the main development in the study of insect defenses. The different chapters are uneven in their scope, but on average are good or even first-rate reviews of various aspects of insect defense. Undoubtedly, this book will be of great interest not only for those working on insect defense or predation, but also for those wishing to be informed of the present status of this field.

Jacques M. Pasteels
Department of Biology,
Université libre de Bruxelles,
Bruxelles, Belgium

Journal of Chemical Ecology, Vol. 17, No. 6, 1991

Book Review

Chemistry and Significance of Condensed Tannins. Richard W. Hemingway and Joseph J. Karchesy. New York: Plenum Press, 1989. 553 pp.

All chemists and ecologists who deal with, or have an interest in, tannins need to be aware of this book. As its title suggests, the focus is on chemistry but the more ecologically minded reader will find much of interest and perhaps inspiration. The coverage is diverse, with some 33 chapters organized into seven sections covering biogenesis, structure, analytical methods, reactions, complexation, biological significance, and the use of condensed tannins as specialty chemicals. The book was developed from the inaugural North American Tannin Conference held in August 1988 and reflects this in its organization. The majority of contributions are authoritative and well-written reviews, while a minority report new data or are short overviews.

The first section covers the biogenesis of tannins, which is timely in counterpoint to recent ecological literature on resource allocation and the general question of what regulates the accumulation of tannins in plants. At the biochemical level, we are ignorant about condensed tannin biosynthesis, to the point that it is still controversial as to whether an enzyme is involved in the condensation step. An understanding of regulatory control clearly lies in the distant future. Hergert's overview and Stafford's exposition of what is known about the enzymology of condensed tannin biosynthesis are valuable accounts of this area.

Hergert also begins the book with an introductory chapter on the development of the North American tanning industry and, with it, chemical studies of condensed tannins. This is a fascinating account and should dispel any lingering ideas among biologists that the tannins are single, uniform, and easily defined chemical entities. The present state of the art on condensed tannin structure is presented in four chapters, of which Hemingway's will be particularly useful as an eye-opening exposition of their chemical diversity. With this diversity exposed, chemical ecologists are now in the position to find out whether it has any ecological significance.

After structure, four chapters cover analytical methods including new NMR and FAB-MS methods. At the "low tech" end of the spectrum, an excellent chapter by Karchesy et al. covers chromatographic techniques from HPLC down to TLC and LC. This chapter should stimulate new research on tannins as these techniques are accessible to a wide range of biological laboratories. The follow-

0098-0331/91/0600-1291$06.50/0

ing section covers the reactions of condensed tannins. Much of the writing is from the perspective of exploiting tannins in commercial applications, but there is still material that will attract readers of this journal, such as the underlying chemistry behind the colorimetric assays used in ecological studies. "Complexation" is distinguished as a separate topic from "reactions," and here three chapters are devoted in turn to the interaction of tannins with carbohydrates, proteins, and synthetic biopolymers. The single chapter on tannin–protein interactions seems slight relative to their significance for biological systems. However, Hagerman provides an account that deserves to be read well.

Seven diverse chapters are devoted to the biological significance of tannins. Schultz provides a compelling account of how advances in plant–herbivore ecology can depend on chemical tools: This should signal ecologists that chemistry is worth learning and also inform chemists about the complexities of ecology and evolutionary biology. An interesting example of structural analyses for tannins in an ecosystem study is provided by Tiarks et al.

For all the sophistication in chemical analysis documented in this book, it is frustrating to see that the carcinogenicity of "tannin" is still being documented with epidemiological studies of bush-tea consumption and that histological studies on the distribution of "tannin" in plant tissues depend largely on nonspecific stains. Readers ought to worry about what the word "tannin" means chemically in these chapters. More explicit lapses from the title theme of condensed tannins are found in the discussion of hydrolyzable tannins in several chapters.

Some readers of this journal may also do a double-take at the final five chapters, which cover such topics as wood glue and other industrial uses of tannins. The one chapter of more than passing interest will be that of Laks, who reviews their use as substrates for the synthesis of novel biocides. In summary, not every page of this book will be relevant to chemical ecologists, but there is much here that is worth exploring.

Simon Mole
School of Biological Sciences
University of Nebraska at Lincoln
Lincoln, Nebraska 68588

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

CHEMICAL ORIENTATION OF LOBSTERS, *Homarus americanus*, IN TURBULENT ODOR PLUMES

PAUL A. MOORE,* NAT SCHOLZ, and JELLE ATEMA

Boston University Marine Program
Marine Biological Laboratory
Woods Hole, Massachusetts 02543

(Received December 13, 1990; accepted February 27, 1991)

Abstract—The lobster, *Homarus americanus*, relies upon its lateral antennules to make initial directional choices in a turbulent odor plume. To determine whether chemical signals provide cues for source direction and distance during orientation, we studied the search patterns of the lobster orienting within a turbulent odor plume. In an odor plume, animals walked significantly more slowly and most often up the middle of the tank; control animals (no odor present) walked rapidly in straight lines, frequently along a wall. Search patterns were not stereotyped either for the population of experimental animals or for individuals. Three different phases of orientation were evident: an initial stage during which the animals increased their walking speeds and decreased their heading angles; an intermediate stage where both the walking speed and headings were constant; and the final stage close to the source, where heading angles increased while walking speed decreased. During this last stage the animals appear to be switching from a distance orientation (mediated by the antennules) to a local food search (mediated by the walking legs) as evidenced by a great increase in leg-raking behavior.

Key Words—Chemotaxis, chemoreception, orientation, lobster, *Homarus americanus*, odor plume.

INTRODUCTION

Animals use many different behavioral mechanisms to orient to a chemical source. Various authors have categorized and labeled these behaviors during

* Present address and to whom correspondence should be addressed: University of Colorado Health Science Center, Department of Pharmacology, Campus Box C236, 4200 East Ninth Ave., Denver, Colorado 80262.

0098-0331/91/0700-1293$06.50/0

the last century (Loeb, 1891, 1913, 1918, Kühn, 1919; Fraenkel and Gunn, 1961). Categories for different orientation behaviors have been established using a combination of criteria, including behavioral output, type of sensory receptors and sampling, and information available to and used by the animal. This has spawned a debate on the role of a chemical signal in controlling orientation, resulting in a proliferation of new terms (Schöne, 1984; preface for Bell and Cardé, 1984; Bell, 1984; Kennedy, 1986). To avoid ambiguity, we will use two broad categories of orientation: either the chemical signal plays a direct guidance role (e.g., tropotaxis in *Panulirus argus*, Reeder and Ache, 1980; nurse sharks, *Ginglymostoma cirratum*, Hodgson and Matthewson, 1971), or it serves to initiate maneuvers that depend on nonchemical stimuli (internally controlled motor program, i.e., counterturning in *Adoxophyes orana*, Kennedy, 1982; chemically-stimulated rheotaxis in lemon sharks, *Negaprion brevirostris*, Hodgson and Matthewson, 1971).

Turbulent odor plumes have two closely related sensory signals associated with them: odor patches and velocity gradients. We do not want to belabor here the fluid dynamics involved and refer to other literature (Murlis and Jones, 1981; Murlis, 1986; Atema 1985, 1988). Chemoreceptors respond to chemical concentration gradients and patches, while mechanoreceptors monitor velocity gradients. The term chemotaxis refers to the use of purely chemical information to orient to a source; anemotaxis and rheotaxis are terms referring to using velocity gradients in air or water, respectively. Further classifications have been used for animals that sample chemical signals temporally (klinotaxis) and spatially (tropotaxis). Until recently (Moore et al., 1989), it has been difficult to quantify, at the spatial scale of the receptor, the instantaneous odor distributions that exist in turbulent odor plumes. Therefore, the correlation of behavioral responses to patterns of turbulence has not been accomplished, and determining in detail the role of chemical signals in guiding orientation has not been possible for macroscopic animals.

Internal control refers to a motor program that produces the observed orientation path; the program is initiated and can be switched on and off by chemical stimuli (Bell and Tobin, 1982; Havukkala and Kennedy, 1984; Kennedy, 1978). It remains ambiguous whether control over orientation is completely internal or if external nonchemical signals are used (Kennedy, 1986). Counterturning is frequently cited as an example of internal control, and animals orienting by anemo- or rheotaxis may fit this description. For example, animals orienting under internal control may use wind (Kramer, 1986; Preiss and Kramer, 1986) but employ visual cues (David, 1986) as an external frame of reference.

The instantaneous structure of an odor plume, at the size scale of macroscopic animals (>1cm), is intermittent in structure (Moore et al., submitted; Murlis, 1986; Murlis and Jones, 1981; Atema, 1985). This patchy distribution

can result in packets of high odor concentration far away from the source and areas of low odor concentration near the source. Animals using the local odor profile for directional or distance information will need to extract information from the plume either by spatial/temporal sampling and/or by receptor filtering (Moore and Atema, 1988).

Chemical cues play an important role in food searching and manipulation for the lobster, *Homarus americanus*, but the sensory information that provides directional cues for orientation to the odor source is not known. Previous studies have shown that antennular chemoreception is necessary for initial directional cues and search efficiency within a turbulent odor plume (Devine and Atema, 1982), that chemosensory input from the walking legs is needed to locate and to recognize food, and that chemosensory input from the maxillipeds is needed to feed (Derby and Atema, 1982). The purpose of this study was to investigate the role of the two classes of orientation (internal vs. external chemical control) in a lobster's search for an odor source and to determine how that search pattern may reflect responses to the patchy distributions of odor signals typical of turbulent odor plumes.

METHODS AND MATERIALS

Lobsters ranging from 8.5 to 11.2 cm carapace length were caught from waters around Woods Hole and maintained in ambient running seawater tanks for at least two weeks before testing. Each lobster was fed twice weekly a mussel and squid diet, and deprived of food for 3–10 days prior to each trial.

Orientation Arena. All tests were performed in a flow-through flume (250 $\times$ 90 $\times$ 20 cm, Figure 1). Water depth varied from 19 cm at the front to 21 cm at the rear due to an intentional downward tilt of the flume. Unfiltered seawater entered through 15 holes at the head of the tank. Sheets of fluorescent light grating (egg crates, 16.9 mm^2 holes) wrapped with plastic screens (1 mm^2 holes) served as collimators (four upstream, one downstream). The carrier flow rate was 1.8 $\pm$ 0.1 liters/sec. Average flow velocity in the tank was 1.0 $\pm$ 0.1 cm/sec; velocities in the center were somewhat greater. The stimulus was gravity fed through a 1-mm Pasteur pipet at a rate of 50 $\pm$ 5 ml/min. The nozzle of the pipet was placed in the cross-sectional center of the tank 9 cm off the bottom, roughly at the antennular sampling plane of a 0.5-kg lobster. As a frame of reference for digitizing, the source was set at $x, y, z = 0$. Water temperature varied between 20 and 24°C. Illumination was provided by three 150-W floodlights mounted 1.2 m above the water surface. Light level was controlled with a dimmer switch to provide just enough illumination for low-light video taping. A camera (Panasonic WV 1850) equipped with a wide-angle lens was mounted on a track system 1.5 m above the water surface. This allowed

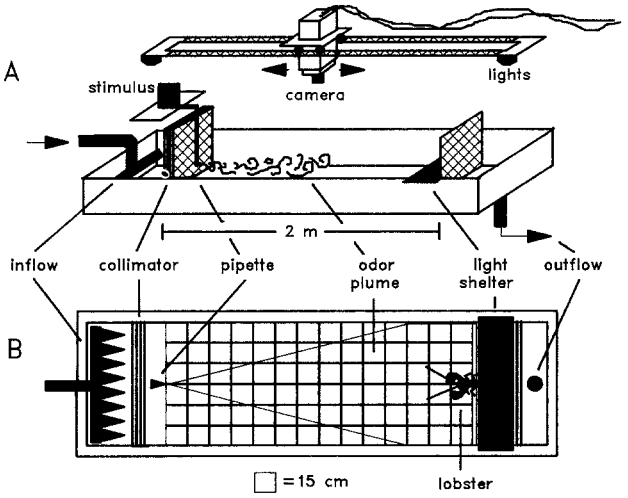


FIG. 1. Diagram of the behavioral arena. (A) Perspective drawing, showing flow (from left to right) in the flume, stimulus introduction (pipet), and position of camera and lights. Raw seawater flowed in through 15 holes in a T-section of PVC pipe and out through a single drain downstream. Both ends of the tank had flow collimators (downstream end not labeled). (B) Top view, showing scale of the lobster relative to tank and pipet (plume source). Grids (15 cm) were drawn on the bottom of the flume to serve as a reference for digitizing.

the camera to move with the animal, minimizing parallax problems that might have interfered with digitizing the responses. A large board was placed over the far end of the tank to provide a darkened shelter area for the lobsters.

The stimulus was an extract of homogenized and centrifuged mussel (*Mytilus edulis*) at a concentration of 5 g/liter wet weight in raw seawater (see Borroni et al., 1986, for further details). One batch was frozen to serve the entire experiment. For each test aliquots of 10 ml of mussel juice were diluted in 90 ml of seawater for a final concentration of 0.5 g/liter. Control tests were done under identical conditions, with raw seawater substituted for the mussel extract.

This stimulus and flow system was identical to the system used in the characterization of the three-dimensional structure of an odor plume (Moore et al., submitted). Electrochemical and dye tests showed that the stimulus released from the pipet reached the far end of the arena within 2 min.

Testing Methods. All orientation responses were recorded in a darkened room between 0900 and 1700 hr. Preliminary trials showed that low-level lighting did not prevent lobsters from leaving the shelter to search for food. Animals were placed within the tank and allowed to settle anywhere beneath the light

shelter (Figure 1). If the lobster did not settle within 30 min, it was removed and replaced with another lobster.

Once the lobster was settled, stimulus was introduced into the arena. A trial was stopped if the lobster did not move from the shelter within 10 min after the stimulus was introduced. A trial was considered successful only if the lobster approached within 20 cm of the pipet tip within 20 min after the start of stimulus introduction, and if it did not run into the up-current or side walls. Since we were interested in directional information extracted from odor plumes without additional cues provided by other physical features, such as solid walls, we excluded animals that found the source by walking along the arena walls. These quantitative criteria were reinforced by qualitative observations of changes in lobster behavior, e.g., raising themselves up, increased flicking of lateral antennules, or sweeping with legs. While orienting, most lobsters walked up the middle of the flume. Control animals were tested without chemical stimulus present. For appropriate comparison of orientation parameters, tracks were chosen specifically for those animals that walked up the middle of the flume without touching the walls and whose tracks passed through the area of the source.

Data Analysis. Orientation paths were digitized at 1/sec with the tip of the rostrum serving as the single reference point. The x and y values were determined from the 15 × 15-cm grids drawn on the bottom of the tank. Since lobsters can walk sideways and backwards without changing overall body orientation, a single reference point allowed us to follow the walking path of the animal without regard to body orientation.

Parameters chosen to quantify movement patterns included walking speed, turn angles, and headings (e.g., Akers, 1989; Kleerekoper, 1967; Kleerekoper et al., 1969). Walking speed was based on distance travelled (cm) per unit time (sec). Turn angle was defined as the angle between the path connecting the previous ($t = -1$) position to the present ($t = 0$) position and the path connecting the present and the next ($t = +1$) position (Figure 2). Right-hand turn angles were defined as positive. A lobster's heading was defined as the angle between a straight line towards the source and the direction from the present position to the next position on the track. Absolute turn angles and headings were defined as their absolute (positive) values.

Leg Raking Experiments. To test for the importance of walking legs in locating an odor source, we used the same flume with the same flow and odor source conditions. Lobsters were released at the downstream area of the flume and allowed to acclimate for 15 min, after which the odor was turned on. Every 5 sec the animal's location and the number of "rakes" over the preceding 5-sec interval were recorded. Raking is the lateral swinging movement of the first two pairs of walking legs described in Atema and Cobb (1980). Each trial lasted 3 min. Four animals were tested five times each with three different odor

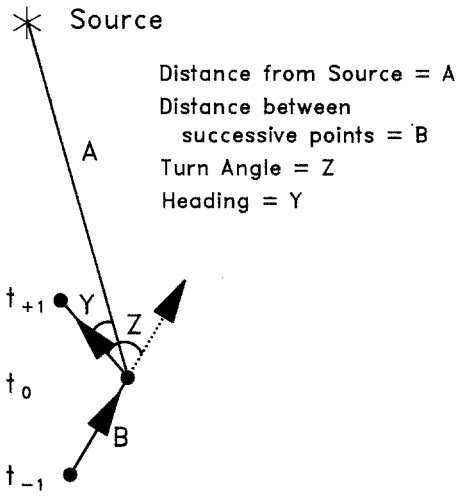


FIG. 2. Three successive positions ($t = -1$, 0, and $+1$; solid circles) on a hypothetical orientation path used to define behavioral parameters. The dotted line represents the lobster's projected path (if it had continued in a straight line), from which a turn angle value (z) at point $t = 0$ is calculated. A indicates distance to the source (asterisk), from which a heading value (y) is calculated at point $t = 0$. Walking speed at $t = 0$ is B cm/sec, since time between successive digitizing points on the path was set at 1 sec.

source concentrations—1, 2.5, 7.5 $\times$ 10^{-1}—of mussel extract prepared as the supernatant of 200 g/liter wet weight mussel flesh in artificial seawater.

RESULTS

Our criteria for orientation resulted in rejection of a large number of trials (140 of 166 trials or 84.3%; 62 different animals). Most of the rejections (122 of 140) resulted from the lack of any movement of the animals. Of the 18 animals that moved in the presence of stimulus but were rejected by our criteria, 15 walked slowly partway up the arena (a behavior characteristic of orienting animals, see below) but did not come within 20 cm of the source and returned to the shelter, and three hit one of the walls. The 26 successful trials were from 16 different animals. Repeated-measures ANOVA showed no difference between animals for walking speed, heading, and turn angles, so we group the trials for individual animals together for further analysis.

Detection of the odor caused the animal to switch into a different state. This is shown by a significantly lower walking speed in the experimental animals compared with controls (t test $P < 0.001$). Experimental animals walked at 5.94 $\pm$ 0.09 (SEM) cm/sec and control animals at 10.81 $\pm$ 0.35 cm/sec.

Other behaviors, such as increased antennular flicking and leg sweeping rates and animals raising themselves high up on their legs, also exemplified search behavior but were not quantified for this study. While orienting, experimental animals would frequently stop and walk backwards and sideways (Figure 3, left column). In contrast, control animals frequently would walk rapidly in a straight line without stopping until the up-current wall was approached or hit (Figure 3, right column). This also is seen in significantly lower heading and turn values for the control animals (Table 1). For experimental animals, the frequency distributions of heading and turn angle values were unimodal with normal distributions centered around 0–10 degrees and 10–20 degrees, respectively (Figure 4).

A search pattern characteristic for the population or for any one individual does not seem to be present. This can be seen qualitatively in the variation in orientation patterns both within the population (Figure 3, left column) and within individuals (Figure 5). Pattern variability also is reflected in large standard deviations for the orientation parameters. Experimental animals had significantly higher variability than control animals in all four orientation parameters quantified (Table 1). This large variability argues against a single characteristic search pattern that would result in low standard deviations around mean values. Furthermore, variability within single individuals suggests that individuals also do not have a characteristic search pattern. Since turbulent plumes tend to be quite different even under similar starting conditions, it is not unreasonable to think that observed differences are the result of different odor distributions.

To examine how orientation parameters changed with distance to the source, we calculated the mean and SEM for heading, turn angle, and walking speed within 5 cm bins (e.g., 0–5 cm, 5–10 cm, etc). These distances are the linear distance between the animal and the source (A in Figure 2). A second-order polynomial regression was fit to these values, with r values for heading and walking speed of 0.84 and 0.86, respectively. Animals appear to be orienting to the source in three different phases. These phases occur in all three parameters at the same distances from the source. Between 240 cm and 110 cm from the source, the walking speed gradually increased from 2.3 cm/sec to 7.8 cm/sec (Figure 6). At this point, the walking speed stayed fairly constant at 7 cm/sec until 40 cm from the source. From 40 to 5 cm, the walking speed decreased from 7 cm/sec to 4.7 cm/sec. A mirror image of these values is seen in the heading values (Figure 6). Between 240 and 110 cm, the headings gradually decrease from 98.8° to 20°. There was a constant heading of about 20° in the intermediate distances, and at 40 cm the headings began to increase again to 37° at 5 cm. Turn angle values followed the patterns of heading values, and this occurred at the same distances; however, the turn angle regression is not significant.

Mean raking rates for all animals and source concentrations increased dra-

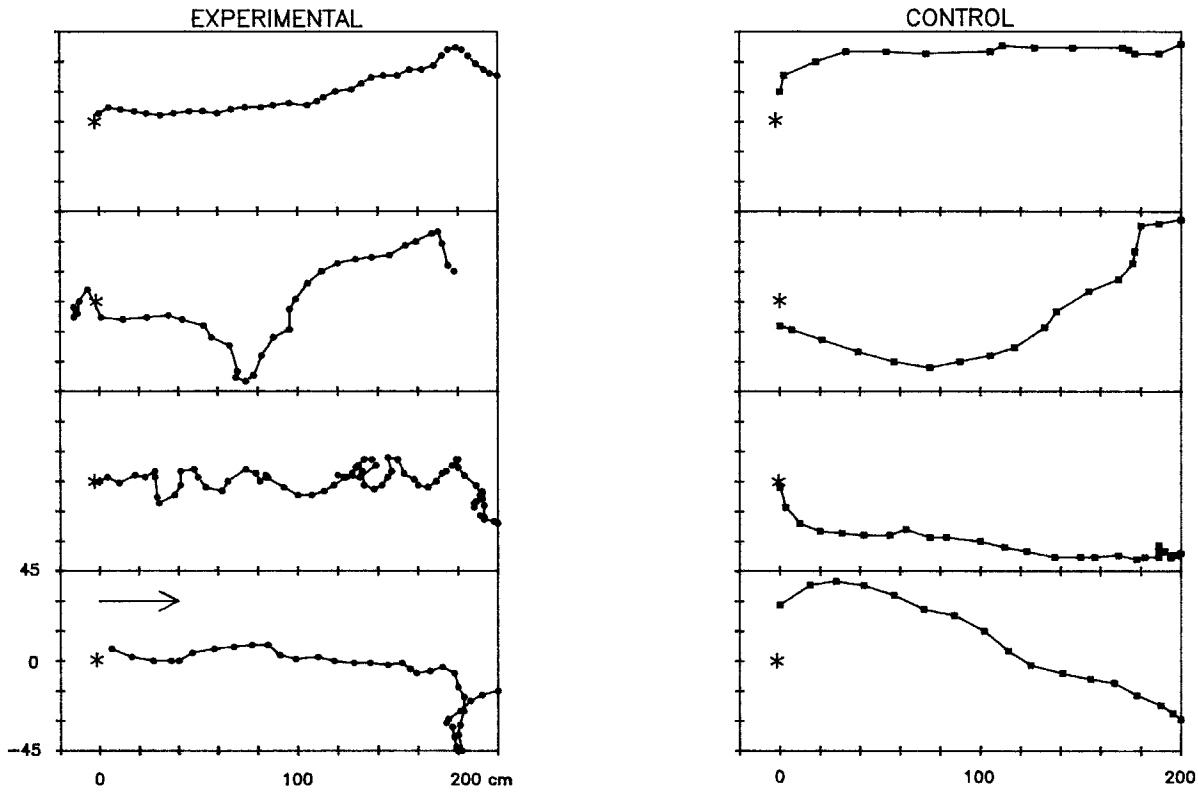


FIG. 3. Eight representative tracks (demonstrating variety) as lobsters move to the plume's source (asterisk). Left column, experimental animals; right column, control animals. (For control animals: plotting of tracks was stopped at level of the source.) Direction of flow is from left to right (arrow). Lines are derived from pathways digitized at 1/sec. The *x*-axis ticks indicate 20-cm intervals; *y*-axis ticks are 15 cm.

TABLE 1. VARIABILITY OF ORIENTATION PARAMETERS (IN DEGREES)

	Experimental (N = 1,173[a])	Control (N = 213[a])	Significance
Heading			
Mean	0.61	−13.96	**[b]
SD	56.34	42.74	**
Turn angle			
Mean	3.40	−0.22	N.S.
SD	45.87	34.95	**
Abs. heading			
Mean	40.33	33.34	*
SD	39.35	30.16	**
Abs. turning			
Mean	31.11	21.80	**
SD	33.89	27.32	**

[a] N refers to the number of digitized data points.
[b] *$P < 0.005$: Means test with t test; variances with F test; **$P < 0.001$.

matically as distance to the source decreased; from 3.4/5-sec interval in the area nearest the source (r = 7 cm, radial distance) to 2.6 per interval in the next area (r = 7–15 cm) to 1.15 (r = 15–22 cm) and to 0.1 in the area beyond 22 cm from the source. More than three quarters of all rakes observed (79%) were in the three closest areas, i.e., less than 22 cm from the source. This total near-source area is only about 3% of the total tank bottom surface.

DISCUSSION

The results of this study indicate that lobsters use chemical signals to locate a food source. Other orientation mechanisms using the chemical stimuli only to turn on an internal motor program or to start a rheotactic orientation appear less important for the lobster.

An example of an internal motor program is seen in the counterturning of some moth species (e.g., Baker et al., 1984). These moths initiate left–right turns around 180–200° with a relatively high degree of accuracy and consistency. This type of orientation mechanism would produce a bimodal distribution of turn angles, with each mode centered on the positive and negative values of the repeated turn angle or heading. The lobsters used in this study did not show this type of orientation behavior. The turn angles and headings were both unimodal distributions centered around 15° and 5°, respectively (Figure 4). The coefficient of variance was greater than 100% for all parameters, indicating a

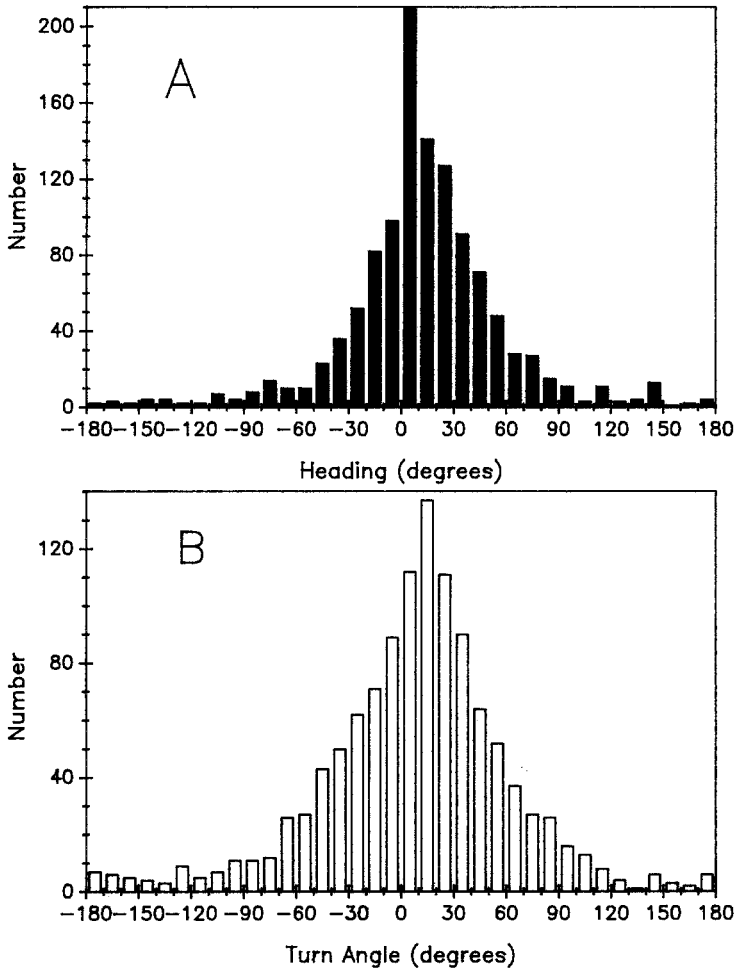


FIG. 4. Frequency distributions for 26 experimental runs (based on 1173 data points) for (A) heading and (B) turn angles. Bin width equals 10 degrees.

large degree of variability among the population. This indicates that for the experimental population there was not an "internally set" motor program that guides the orientation search pattern.

Another possible orientation mechanism is rheotaxis (or, in air, anemotaxis) where mean current direction guides the search path. Animals orienting rheotactically move directly up the mean water current. This mean is not the grand mean for the day but the local mean the animal can determine from inte-

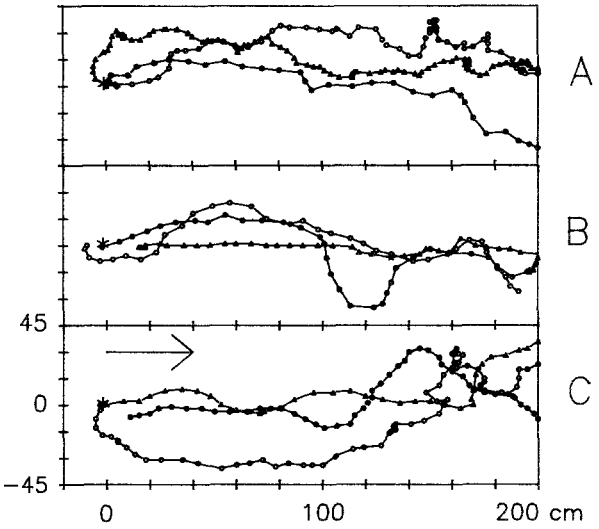


FIG. 5. Variability in orientation paths for three individual lobsters. Three repeated trials by three different lobster (A, B, and C) are shown. Direction of flow is from left to right in each figure. Vertical and horizontal distances are plotted in cm. Source at* (0,0 cm).

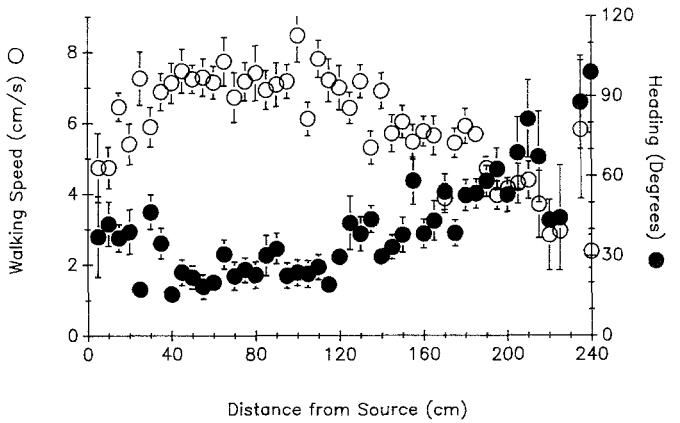


FIG. 6. Changes in behavioral parameters: walking speed (open circles) and heading (closed circles) along the track to the source. Distance from the source is distance between animal and source not distance along the x axis. Circles are mean values ($\pm$SEM) over 5 cm distances (N = 26 trials, 1173 data points).

gration over an unknown length of time. The animal will then stop and, depending upon the presence or absence of odor, will either move cross-current, further up-current, or turn around and move down-current. Evidence from tsetse flies in the field (Bursell, 1984) and nurse sharks in a laboratory setting (Hodgson and Matthewson, 1971) demonstrate this strategy. If the lobsters oriented rheotactically in our unidirectional flow tank, their straight up-current path would have resulted in an increase in heading values with decreasing distance from the source approaching 90° as it reaches the up-current wall (Figure 7). At the downstream end of the tank, heading values would have been small when facing directly into the current. If a lobster happened to start directly in front of the stimulus pipet, the heading values would have started and stayed around 0°. In no instance would a lobster approaching the source rheotactically have shown a decrease in heading values. However, since observed headings did decrease when the lobster approached the source (Figure 7), it is unlikely that the lobsters are using a rheotactic orientation in our system.

In a tropotactic behavior, the stimulus pattern arriving at (bilateral) receptors plays a direct role in steering the motor output of the orienting animal (Fraenkel and Gunn, 1961). Under this mechanism, the local stimulus profile is sampled spatially and/or temporally, and subsequent changes in the orientation path are made in response to the changes in certain features of the signal. Examples of this exist in the chemoreception literature (see Payne et al., 1986; Bell and Cardé, 1984, for reviews). The observed changes in heading values and walking speeds with distance together with the need for bilateral compari-

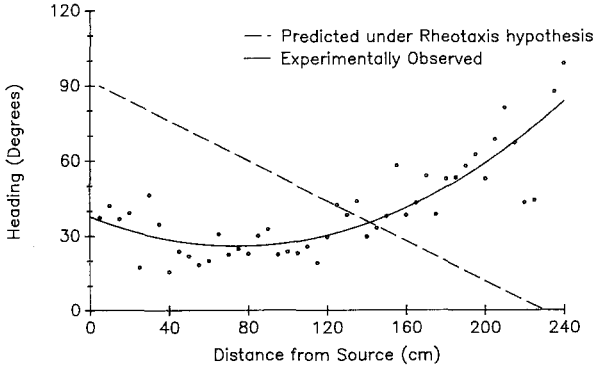


FIG. 7. Changes in heading values with decreasing distance from source. Data points are mean values from Figure 6. Experimental headings (solid line) vs. those predicted for a chemically mediated rheotactic response (dashed line). The solid line is a second-order polynomial regression fit to the observed headings from this study (least squares method; $r = 0.86$, $P < 0.01$ for $N = 26$ experimental runs). Distance from source as in Figure 6.

son (results from Devine and Atema, 1982) are most consistent with a chemotropotactic behavior for the lobster under the conditions tested. Ultimately, the changes in heading and walking speed with distance (and thus odor) may suggest a combination of taxis and kinesis. Further manipulations with both the water current and odor dispersal are needed to firmly establish the degree to which different sensory cues control orientation of the lobster.

The present results indicate that chemotactic orientation occurred in three different phases. Initially (far away from the source), the odor cue switches the lobster into a different state: sampling the local area an initial source direction is determined. At this stage, heading values are large while walking velocity is low. During this initial phase, lobsters accelerate and begin to walk more directly toward the source as seen in a decrease in heading values (Figure 6, 240–110 cm). At an intermediate range, it appears that the lobster has "locked on" to the source direction. A walking velocity of 7.5 cm/sec (triple that of the initial rate) is maintained, as well as a rather precise heading towards the source of approximately 20°. The final approach phase takes place relatively close to the source (40 cm or less) and seems to indicate a local food searching behavior. During this last stage, the animal slows down considerably (from 7.5 to 5 cm/sec) while increasing heading values 20° to 45°. We suggest that this may indicate a switch from a distance orientation, controlled by lateral (and possibly medial) antennular input, to a local food searching behavior, controlled by walking leg chemoreceptors.

Although in both *H. americanus* and *P. argus* the lateral antennules play a primary role in distance olfaction and orientation (Devine and Atema, 1982; Reeder and Ache, 1980), it is the walking legs of *H. americanus* that are used primarily for local food searching and recognition (Derby and Atema, 1982). The walking legs also play a secondary role in search path control (Devine and Atema, 1982). The leg behavior data obtained under these conditions of flow and odor distribution show that legs become increasingly involved in responses to odor plumes at distances less than 22 cm from the odor source. This distance corresponds to the area in which search paths begin to meander again close to the source. It may be at these distances the odor concentration reaches a threshold at which legs begin to take over from antennules in guiding the lobster to its food odor source.

The observed orientation pattern and the hypothetical switch from antennular to leg chemoreception may be controlled by information about the distance between the odor source and the lobster that is contained within the spatial patterns of the odor plume. This information does not necessarily have to be associated with average odor intensity; other parameters characteristic of turbulent odor plumes contain spatial information that may be useful for orientation (Moore and Atema, 1988; Murlis, 1986).

Although our experimental flow was purely unidirectional, the turbulent

odor signals generated within the arena (Moore et al., submitted) are similar to those signals that have been measured in natural environments (Atema et al., submitted; Murlis and Jones, 1981). Therefore, we suggest that similar orientation mechanisms could take place in the lobster's natural environment.

Acknowledgments—Special thanks to Lynn Lacomis for her assistance in data collecting; to Don Tremel, Kirsten Poling, and Pam Sexton for use of their data on leg clasping behavior resulting from their course projects during the Woods Hole Marine Semester 1989; and to Drs. Ring Cardé and Fred Wasserman for critically reviewing the manuscript as a chapter in P.M.'s PhD thesis at the Boston University Marine Program (BUMP). This research was supported by a grant from NSF (BNS 90-12952) to J.A. and a BUMP alumni award to P.M.

REFERENCES

AKERS, R.P. 1989. Counterturns initiated by decrease in rate of increase of concentration: possible mechanism of chemotaxis by walking female *Ips paraconfusus* bark beetle. *J. Chem. Ecol.* 15:183–208.

ATEMA, J. 1985. Chemoreception in the sea: adaptation of chemoreceptors and behavior to aquatic stimulus conditions. *Soc. Exp. Biol. Symp.* 39:387–423.

ATEMA, J. 1988. Distribution of chemical stimuli, pp. 29–56, *in* J. Atema, A.N. Popper, R.R. Fay, and W.N. Tavolga (eds.). Sensory Biology of Aquatic Animals, Springer-Verlag, New York.

ATEMA, J., and COBB, J.S. 1980. Social behavior, pp. 409–450, *in* J.S. Cobb, and B.F. Phillips (eds.). The Biology and Management of Lobster, Vol. I, Academic Press, New York.

BAKER, T.C., WILLIS, M.A., and PHELAN, P.L. 1984. Optomotor anemotaxis polarizes self-steered zigzagging in flying moths. *Physiol. Entomol.* 9:365–376.

BELL, W.J. 1984. Chemo-orientation in walking insects, pp. 93–106, *in* W.J. Bell, and R.T. Cardé (eds.). Chemical Ecology of Insects, Sinauer Associates, Inc., Sunderland, Massachusetts.

BELL, W.J. and CARDÉ, R.T. 1984. Chemical Ecology of Insects. Sinauer Associates, Inc., Sunderland, Massachusetts.

BELL, W.J., and TOBIN, T.R. 1982. Chemo-orientation. Biol. Rev. 57:219–260.

BORRONI, P., HANDRICH, L., and ATEMA, J. 1986. The role of narrowly tuned taste cell populations in lobster (*Homarus americanus*) feeding behavior. *Behav. Neurol.* 100:206–212.

BURSELL, E. 1984. Observations on the orientation of tsetse flies (*Glossinia pallipdipes*) to windborne odours. *Physiol. Entomol.* 9:133–137.

DAVID, C.T. 1986. Mechanisms of directional flight in wind, pp. 49–58, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction, Clarendon Press, Oxford. 364 pp.

DERBY, C.D., and ATEMA, J. 1982. The function of chemo- and mechanoreceptors in lobster (*Homarus americanus*) feeding behavior. *J. Exp. Biol.* 98:317–327.

DEVINE, D.V., and ATEMA, J. 1982. Function of chemoreceptor organs in spatial orientation of the lobster, *Homarus americanus*: Differences and overlap. *Biol. Bull.* 163:144–153.

FRAENKEL, G.S., and GUNN, D.L. 1961. The Orientation of Animals, ; 2nd ed. Dover Publishers, New York.

HAVUKKALA, I.J., and KENNEDY, J.S. 1984. A programme of self-steered turns as a humidity response in *Tenebrio*, and the problem of categorizing spatial manoeuvres. *Physiol. Entomol.* 9:157–164.

HODGSON, E.S., and MATTHEWSON, R.F. 1971. Chemosensory orientation in sharks. *Ann. N.Y. Acad. Sci.* 188:175–182.

KENNEDY, J.S. 1978. The concepts of olfactory "arrestment" and "attraction." *Physiol. Entomol.* 3:91–98.

KENNEDY, J.S. 1982. Mechanism of moth attraction: a modified view based on wind tunnel experiments with flying male *Adoxophyes. les Colloq. INRA* 7:189–192.

KENNEDY, J.S. 1986. Some current issues in orientation to odour sources, pp. 11–25, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction, Clarendon Press, Oxford. 364 pp.

KLEEREKOPER, H. 1967. Some aspects of olfaction in fish, with special reference to orientation. *Am. Zool.* 7:385–295.

KLEEREKOPER, H., TIMMS, A.M., WESTLAKE, G.F., DAVY, F.B., MALAR, T., ANDERSON, V.M. 1969. Inertial guidance system in the orientation of the goldfish (*Carassius auratus*). *Nature* 223:501–502.

KRAMER, E. 1986. Turbulent diffusion and pheromone-triggered anemotaxis, pp. 59–68, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction, Clarendon Press, Oxford. 364 pp.

KÜHN, A. 1919. Die Orientierung der Tiere im Raum. Gustav Fischer Verlag, Jena.

LOEB, J. 1891. Über Geotropismus bei Tieren. Pflügers Arch. 49:175–189.

LOEB, J. 1913. Die Tropismen. *Hdb. d. vergl. Physiol. Bd. IV.* pp. 451–511. Hrsg.: H. Winterstein. Gustav Fischer, Jena.

LOEB, J. 1918. Forced Movements, Tropisms and Animal Conduct. Philadelphia. 209 pp.

MOORE, P.A., and ATEMA, J. 1988. A model of a temporal filter in chemoreception to extract directional information from a turbulent odor plume. *Biol. Bull.* 174:355–363.

MOORE, P.A., GERHARDT, G.A., and ATEMA, J. 1989. High resolution spatio-temporal analysis of aquatic chemical signals using microelectrochemical electrodes. *Chem. Senses* 14:829–840.

MURLIS, J. 1986. The structure of odour plumes, pp. 27–38, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction, Clarendon Press, Oxford. 364 pp.

MURLIS, J., and JONES, C.D. 1981. Fine-scale structure of odour plumes in relation to insect orientation to distant pheromone and other attractant sources. *Physiol. Entomol.* 6:71–86.

PAYNE, T.L., BIRCH, M.C., and KENNEDY, C.E.J. (eds.). 1986. Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

PREISS, R., and KRAMER, E. 1986. Pheromone-induced anemotaxis in simulated free flight, pp. 69–80, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction, Clarendon Press, Oxford. 364 pp.

SCHÖNE, H. 1984. Spatial Orientation. Princeton University Press, Princeton, New Jersey.

REEDER, P.B., and ACHE, B.W. 1980. Chemotaxis in the Florida spiny lobster, *Panulirus argus*. *Anim. Behav.* 28:831–839.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

CHEMICALLY MEDIATED ASSOCIATIVE LEARNING: AN IMPORTANT FUNCTION IN THE FORAGING BEHAVIOR OF *Microplitis croceipes* (CRESSON)

W.J. LEWIS,[1,*] J.H. TUMLINSON,[2] and S. KRASNOFF[3]

[1]*Insect Biology & Population Management Research Laboratory*
USDA, ARS
P.O. Box 748
Tifton, Georgia 31793

[2]*Insect Attractants, Behavior and Basic Biology Research Laboratory*
USDA, ARS
P.O. Box 14565
Gainesville, Florida 32604

[3]*Boyce Thompson Institute*
Tower Road
Ithaca, New York 14853

(Received October 2, 1990; accepted March 1, 1991)

Abstract—When experienced by contact with feces from hosts feeding on cowpeas, laboratory-reared females of *Microplitis croceipes*, a larval parasitoid of *Heliothis* spp., orient and fly to odors of the same feces, whereas naive laboratory-reared females do not. Flight-tunnel studies revealed that associative learning occurs during female encounters with hosts and host products. When females antennate host feces, they learn to recognize the volatile odors associated with the feces. Females even can be conditioned to respond to novel and otherwise unattractive odors such as vanilla extract by exposure to these volatile substances in association with a water extract of the feces. They apparently link the volatile odors with a nonvolatile host-specific recognition chemical found in the feces. The antennating stimulant, 13-methylhentriacontane, was found to be a valuable ingredient, apparently as a facilitator of the initial antennation and subsequent linkage of the volatiles to the nonvolatile host recognition cue.

Key Words—*Microplitis croceipes*, Hymenoptera, Braconidae, parasitoid, host-finding, behavior, feces odors, attraction, 13-methylhentriacontane, associative learning.

*To whom correspondence should be addressed.

0098-0331/91/0700-1309$06.50/0

INTRODUCTION

The survival of an insect parasitoid species is dependent upon the ability of the ovipositing females to locate host insects for attack. The host insects are often highly mobile and sporadically distributed among massive amounts of plant foliage or other habitat material. Furthermore, the host insects employ various evasive techniques to minimize exposure, such as feeding in concealed crevices, feeding in bursts and then moving, leaving sites, and falling from the plant when the parasitoid approaches. The hosts may be found in a variety of habitats including different parts or growth stages of a plant as well as different plant species. Consequently, parasitoids can be expected to employ a sophisticated repertoire of search tactics that includes an acute perception of physical and chemical cues that increase their host-location prospects. Moreover, they need the ability to make effective strategy adjustments for varying situations. Vet and Janse (1984) clearly demonstrated the ability of the two parasitoids of Drosophilidae, *Asobara tabida* (Nees) and *A. rufescens* (Foerster), to vary their preference for different habitats through associative learning of the different habitat odors after oviposition experience. Turlings et al. (1990) and Ding et al. (1989) demonstrated how parasitoids exploit plant odors to locate herbivorous hosts. Wardle and Borden (1986) showed that a parasitoid's prior experience on an artificial system may diminish its tendency to respond to the habitat of a natural host. Vinson (1984) and Van Alphen and Vet (1986) reviewed numerous other studies that present evidence of learning in parasitoids. Yet, relatively little is known about the mechanisms and role of learning in parasitoid foraging.

A number of studies have demonstrated the importance of chemical cues in the host-searching behavior of *Microplitis croceipes* (Cresson), a parasitoid specific to the larvae of the genus, *Heliothis*. Lewis and Jones (1971) demonstrated that *Heliothis zea* (Boddie) feces elicited intense host-seeking antennation, and Jones et al. (1971) identified 13-methylhentriacontane as the compound eliciting the antennation response. Gross et al. (1975) demonstrated that exposure of *M. croceipes* at time of release to *H. zea* larval frass reduced their dispersal and prolonged their retention in the area of release. Drost et al. (1986) demonstrated that *M. croceipes* females reared from hosts fed artificial diet rarely flew upwind to odors of hosts feeding on cowpea seedlings unless they were provided a previous "experience" consisting of contact with host feces. Herard et al. (1988a) demonstrated similar results with females of *M. demolitor* Wilkinson. Drost et al. (1988), while working with *M. croceipes*, and Herard et al. (1988b), while working with *M. demolitor*, found that inexperienced parasitoids reared from hosts fed cowpeas responded significantly more often to *H. zea* larvae feeding on cowpeas than those from artificial-diet-reared hosts. Herard et al. (1988b) further demonstrated that *M. demolitor* reared from hosts fed cowpeas obtain an important experience from the cocoons before or at the

time of eclosion that improves their flight response, while parasitoids reared from hosts fed on artificial diet lack such experience at eclosion, apparently due to a lack of the proper chemicals on the cocoons. Drost et al. (1988) showed that the flight-tunnel responses of *M. croceipes* females varied significantly based on interactions of the parasitoid's age and experience with the species, growth phase, and part of the host plant provided as an odor source.

Studies by Eller et al. (1988) showed that feces from the *H. zea* larvae are an effective source of attractants for *M. croceipes* females. Studies by Drost et al. (1986, 1988) showed that *M. croceipes* which had experienced only the feces from *H. zea* larvae responded as well in the wind tunnel as those that had a more complete experience that included contact with the feces and stinging a host larva. Thus, *H. zea* feces are a primary source of factors for conditioning the parasitoids for flight tunnel responses.

In a previous paper, Lewis and Tumlinson (1988) reported the discovery that during encounters with host feces, *M. croceipes* females associatively learn and subsequently fly to volatile odors linked with hosts without need of actual host contact. Here we present more detailed and further studies of this associative learning process.

METHODS AND MATERIALS

Parasitoid Rearing. The parasitoids used in this study were reared on *H. zea* larvae according to the procedure of Lewis and Burton (1970) and held in 30 × 30 × 20-cm cages at 26°C, 70% relative humidity, and a 14 : 10 hr light–dark photoperiod until ready for bioassay.

Bioassay Procedure. The wind tunnel was described by Drost et al. (1986). All flight responses were tested at 26–28°C, windspeed of 56 cm/sec, and light intensity of 2500 *lux* . The material to be tested as an attractant source was pipetted onto a triangular (9.5 × 6.5 cm) piece of Whatman No. 1 filter paper suspended from the tip of a disposable pipet placed in an inverted vertical position at the center of the upwind end of the chamber. The females were released from a 4-dram shell vial 80 cm downwind and directly in line with the odorant source. The females were allowed to walk to the rim of the vial and their behaviors were observed and recorded. Females either dispersed, i.e., flew to the sides, top, or bottom of the wind tunnel or responded with anemotactic flight. A positive response consisted of a complete flight and landing on the target source.

Feces and feces extracts were tested both as attractive sources in the wind tunnel and as preflight conditioners. Unless otherwise stated, the material tested as an attractant source consisted of 2.8 mg of raw feces or extract of 1.4 mg of feces. (Preliminary data showed that larger quantities of raw frass are required

as an attractant source, perhaps due to a slower emission rate.) The extract was pipetted in a 14-μl aliquot of solvent onto the triangular piece of filter paper, described above, from which it was emitted.

The materials tested as preflight conditioners were placed on a sheet of Whatman No. 40 filter paper positioned in a Petri dish, and the females were allowed to walk directly from a shell vial onto the material. Unless otherwise stated, the females were allowed to antennate the material for 45 sec; also unless stated otherwise, the amount of material with which females were given preflight experience was ca. 2 mg of feces or the extract of 1.4 mg of feces. The extract was dispensed onto the filter paper in 28 μl of solvent.

Feces Collection. H. zea and *Trichoplusia ni* were reared on artificial diet (Guy et al., 1985) at the Insect Attractants, Behavior and Basic Biology Laboratory, Gainesville, Florida. Larvae were removed from their diet cups when they were 3–4 days old and placed in individual compartments (2.8 × 4.1 × 1.6 cm) in polystyrene trays. They were held at 27°C and 70% relative humidity on a 15-hr light–9-hr dark cycle throughout the period they were used to produce feces. Leaves from 7- to 14-day-old pink-eye, purple hull cowpea [*Vigna unguiculata* (L.)] seedlings grown in a greenhouse were placed in the compartments, and the larvae were allowed to feed for 24 hr to clean their guts of artificial diet. Then, at about 5–6 days of age, the larvae were transferred to clean containers and furnished fresh cowpea leaves. Feces were collected daily from these containers, taking care not to acquire bits of leaves and other debris, and stored in glass vials with Teflon-lined screw caps at −60°C until it was extracted. Feces were collected from larvae until they were about 10 days old. The quantity of feces produced ranged from 2.2 mg/day/5-day-old larva to about 17 mg/day/10-day-old larva. The feces were weighed immediately after collection while still moist. At no time during collection or extraction were feces allowed to dry.

To collect feces of *H. zea* larvae fed on artificial diet, larvae were held in the individual containers of diet in which they had hatched. Feces were removed daily with a small spatula, taking care to avoid collecting diet, and stored as described previously.

Larvae of *Trichoplusia ni* (Hübner) were collected when 5–6 days old, placed in a large Plexiglas cage and fed cowpea leaves for two days to clear their guts of artificial diet. Then feces were collected daily for two days and stored as previously described.

Extraction of Feces. Feces were extracted sequentially either with hexane and then with water (both HPLC grade, J.T. Baker), or in the opposite order. The procedure is illustrated in the following example. Feces (1.35 g) collected over a period of five days from ca. 80 larvae were combined and extracted with 8 ml of hexane by vigorously mixing for ca. 1 min in a glass vial and then letting the mixture stand for about 30 min. Then the hexane was removed by

vacuum filtration through Whatman No. 1 filter paper and the feces residue was scraped from the paper, mixed with a second aliquot of about 5 ml of hexane and, after 30 min, filtered again. The procedure was repeated a third time, and the residual feces on the filter paper were rinsed with sufficient hexane to give a final filtrate volume of 13.5 ml and thus a concentration of 0.1 mg equivalents of feces per microliter. Considerable hexane was lost during the extraction, apparently to evaporation during vacuum filtration, and thus the final volume of 13.5 ml was less than the amount of hexane used for the extraction (18.0 ml). The extract was colored a deep straw yellow. It was stored in a glass vial with a Teflon-lined screw cap at −60°C until used. The feces residue was scraped from the filter paper, mixed vigorously with 15 ml of hexane, and allowed to soak for 24 hr. The hexane then was removed by filtration and stored separately from the previous extract.

After hexane extraction the feces residue was scraped from the filter paper, mixed with about 9 ml of water in a glass vial, and allowed to soak for 30 min. The same procedure was used for the water extractions as for the hexane. However, more water was required for extraction because the feces seemed to swell slightly in water. Also, water was not lost to evaporation. Therefore, the residue was finally rinsed with sufficient water to give a final filtrate volume of 27 ml or 0.05 mg equivalents of feces per microliter.

The second batch of feces was extracted in exactly the same manner except that it was extracted first with water and then with hexane. After extraction with water, it was allowed to soak for 24 hr in 15 ml of water; the water was removed by filtration before it was extracted with hexane.

Although they had been filtered, the water extracts prepared by either of the above procedures still contained suspensions of fine particulate matter. These extracts were designated as H_2O—F and maintained separately according to whether they were extracted before or after hexane extraction. For some experiments the H_2O—F extracts were filtered again by forcing 3–4 ml through a 0.22-μm Millex-Gv filter unit (Millipore Corporation) with a glass syringe. Extracts filtered through Millipore filters were designated H_2O—MF.

Because the water extract (H_2O—F) of feces from *H. zea* larvae feeding on cowpea leaves attracted *M. croceipes* females in the wind tunnel (see later), we decided to attempt to remove all the attractive volatiles by exhaustive extraction with pentane. In a Soxhlet extraction apparatus, 1184 mg of feces from *H. zea* larvae fed cowpea leaves was held in a Whatman 10 × 50-mm cellulose extraction thimble and extracted with about 40 ml of HPLC grade pentane (J.T. Baker) for 24 hr. Then the pentane extract was removed, replaced with fresh pentane, and the extraction was continued for another 24 hr. This procedure was continued, with extraction periods lasting as long as 134 hr until 10 extractions were completed, and the feces had been extracted a total of 393 hr. The feces residue, after this exhaustive pentane extraction, amounted to only 183

mg. This residue was extracted with three aliquots of water of about 7 ml each, as described before, to give a final concentration of the combined filtrates of 0.05 mg equivalents of feces per milliliter based on the weight of the feces before pentane extraction. This water extract was designated H_2O-R.

RESULTS

Experiment 1. Conditioning and Attractive Properties of Selected Feces Extracts. In preliminary tests, batches of the feces from *H. zea* larvae fed leaves (seedlings of cowpea plants) were extracted with either hexane, ether, methylene chloride, methanol, or water. When *M. croceipes* females were allowed to contact these extracts on filter paper prior to flight tests in the wind tunnel, only experience with the H_2O extract resulted in subsequent flights to the hexane extract. However, the hexane extract was the most effective source of attraction. Furthermore, the conditioning and attractive properties of the water and hexane extracts of feces were not affected by whether the feces were extracted first with water or hexane.

In experiment 1 the water and hexane extracts of raw fresh feces were tested to compare their effectiveness in preflight conditioning and as attractants in a wind tunnel. As shown in Table 1, the preflight conditioning properties of water extract of *H. zea* feces (H_2O-F) were equal to those of fresh feces. In contrast, very few of the naive females and females conditioned on hexane extract flew to the hexane extract source. The attractant properties of the different materials were compared using individuals given a preflight exposure to H_2O-F extract. The hexane extract of the feces was the most attractive, while the water extract and fresh feces were significantly less attractive (Table 1). Only 4% of the individuals flew to the pure hexane (blank). These results demonstrate that water is an effective solvent for extracting the substances responsible for the conditioning properties of feces and that hexane is the best extractant for the volatile components that attract *M. croceipes* females. While the water extract was also somewhat attractive to the females, the hexane extract had no conditioning properties.

Experiment 2. Conditioning and the Need for Direct Contact. When provided with feces or water extract for preflight conditioning, the female parasitoid would immediately antennate the material and often (but not always) would probe the active spot with the tip of her abdomen in an apparent attempt to find the host insect for oviposition. If permitted, this intense examination would continue sometimes for several minutes.

To determine if actual contact with the fecal material was required for effective conditioning, individuals were placed in a Petri dish that contained the H_2O-F (28 μl) on a filter paper. The filter paper containing the extract was

TABLE 1. FLIGHT CHAMBER RESPONSE OF *M. croceipes* FEMALES WITH INDICATED MATERIALS USED AS PREFLIGHT CONDITIONERS AND ATTRACTANT SOURCES

Indicated material tested as	Response (%)[a]					
	Host feces	Hexane extract	H_2O-F extract	Naive	Hexane blank	H_2O-F extract + vanilla
Preflight conditioner (hex. extract as source)	92a	8b	96a	4b	—	—
Attractant source (H_2O extract as conditioner)	21b	96a	38b	—	4c	—
Preflight conditioner (vanilla as source)	—	—	3a	0a	—	47b

[a]Responses (%) in the same row followed by different letters are significantly different ($P = 0.01$,

covered with a piece of cheesecloth separated by approximately 1.0 mm from the extract by a thin cardboard spacer with a hole in the center to coincide with the location of the extract. By inverting and rotating the Petri dish, the parasitoid was induced to walk continuously and in close proximity to the material for 1 min. The parasitoids could not contact the extract. Other than an occasional quick antennal palpitation of the cheesecloth, the parasitoid gave little attention to the extract.

Twenty individuals were treated in this manner and subsequently tested for response to the hexane extract in the flight chamber. No positive responses to the hexane odorants were obtained. Twenty females from the same group of parasitoids were provided 30 sec of direct antennation contact with an equal amount of the water extract. Of these, 18 made a positive response to the hexane extract source in the flight chamber.

These results show that direct contact with the material is required for conditioning and indicates that the active chemical is probably very nonvolatile. Consequently, the chemicals that elicit conditioning would be necessarily different (at least partially) from the attractant material.

To determine if the conditioning could be accomplished solely by contact of the antennae with the water extract, individual females were allowed to walk to the rim of a glass vial to antennate filter paper containing an aliquot of the

water extract for 30 sec, while the observer used care so that no other part of the individual touched the material. Of the 20 individuals treated in this manner, 16 flew to the hexane extract source when tested in the flight chamber. Thus, the conditioning stimuli can be perceived and processed by the antennae without involvement of sensory organs located on other appendages.

Experiment 3. Test for Longevity of Conditioned Responses. Tests were conducted to establish whether or not the conditioning process that increases the response of the parasitoid is simply excitation. Parasitoid females were provided a preflight experience on H_2O—F extract and tested for flight responses to hexane extract at 0, 24, and 48 hr following this experience.

Although the highest response was obtained at 0 hr with significant loss at 24 and 48 hr (Figure 1), the results indicate that there is a significant effect up to 48 hr, thereby demonstrating that the conditioning is not just an excitation response.

Experiment 4. Purification of Water Extract. Results of experiment 1 demonstrated that the water extract of the feces, while serving as an excellent conditioner for subsequent flight responses to hexane extracts, also possessed significant attractiveness. It was assumed that this attraction could be at least partially the result of contamination from the same components serving as attractants in the hexane extract. Therefore, it was not clear whether the conditioning of flight responses to hexane extract was caused by separate components in the H_2O—F extract acting independently of the material(s) in the hexane extract or by true associative learning as a result of contaminating hexane-sol-

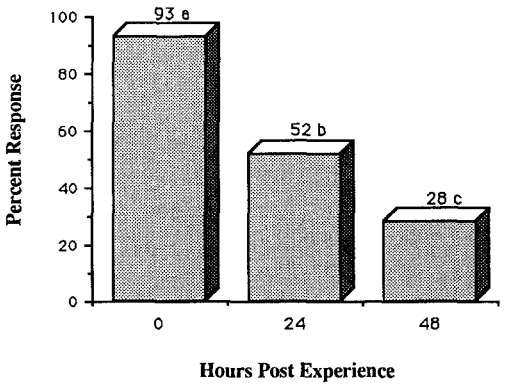


FIG. 1. Response of *M. croceipes* females to hexane extract of host feces at indicated time intervals after conditioning by exposure to water extract of host feces. Bars capped by different letters are significantly different ($P = 0.01$, Waller-Duncan k-ratio t test, $N = 40$ observations).

uble chemical(s) acting together with water-soluble chemical(s) in the water extract.

In an attempt to remove the attractant volatiles contaminating the H_2O-F extract, a batch of feces was extracted with pentane in a Soxhlet extractor. The feces residue was then extracted with water (H_2O-R) (see detailed procedure in Methods and Materials). Despite this exhaustive extraction, each successive pentane extract continued to remove attractant materials from the feces (14 of 22, or 64% of the females flew to the 10th pentane extract). Furthermore, the final after-pentane residue and the H_2O-R were still slightly attractive (four of 27 and three of 28 females flew to the after-pentane residue and H_2O-R extract, respectively).

However, comparison of the water extracts showed that fewer females responded to the hexane extract after preflight experience with H_2O-R (11 of 40, 28%) than after experience with H_2O-F (34 of 40, 88%). The conditioning property of H_2O-F remained stable for hours (38 of 54, 70% response after 2 hr on filter paper), whereas H_2O-R became essentially inactive within 30 min after application to filter paper (2 of 28, 7% response).

Since the H_2O-F extract contained a small amount of suspended fine particulates, it was filtered through a 0.22-μm Millipore filter in an attempt to remove contaminants that might be adsorbed to the particles. In preliminary tests conducted by the same procedure used above, the filtrate (H_2O-MF) appeared inactive as a preflight conditioner. However, H_2O-MF dried more rapidly than H_2O-F, and it was considered that moisture may be important to the conditioning process. Therefore, another bioassay procedure was used to compare the conditioning properties of H_2O-MF with those of H_2O-F.

An aliquot (40 μl) of either of the H_2O extracts was placed on a 2.3-cm-diam. semicircular filter paper held by the edge with a pair of spring-loaded forceps. The impregnated disk was kept lightly moist with small amounts of water applied with a Pasteur pipet. Parasitoids were allowed to examine the disk for 60 sec. Preflight exposure in this manner to H_2O-MF resulted in no flights to the hexane extract source compared with 53% flights by females exposed in a similar manner to H_2O-F (Figure 2).

These results indicate that some contaminant, perhaps the attractive hexane-soluble material, is involved in the conditioning of the parasitoids by the water extract. However, since the hexane extract does not show any conditioning properties and since this process requires direct contact with the antennae, it would seem that the conditioning is caused at least partially by a nonvolatile chemical present in the water extract and not in the hexane extract.

Experiment 5. Restoration of Conditioning Properties of Filtered Water Extract. A nonvolatile chemical vital to the conditioning process could remain present in the water extract after the activity apparently disappears; however, important volatile compounds and other materials necessary for antennation and

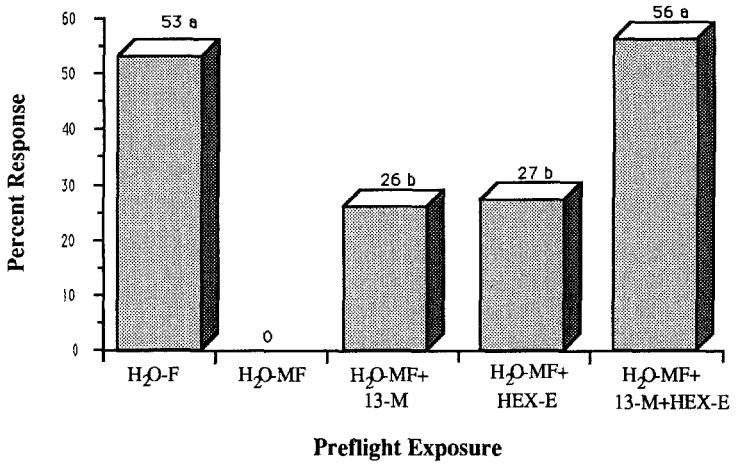


FIG. 2. Response of *M. croceipes* females to hexane extract of host feces after conditioning with indicated formulation of water extract of host feces. H_2O—F, water extract of host feces (40 μl aliquot); H_2O—MF, H_2O—F filtered through a Millipore filter (40-μl aliquot); 13-M, 13-methylhentriacontane (1 μg); Hex-E, hexane extract of host feces (10-μl aliquot). Bars capped by different letters are significantly different ($P = 0.01$, Waller-Duncan k-ratio, t test, $N = 30$ observations).

conditioning may be lost through filtering or extraction with hexane or pentane. A very low antennation response to H_2O—MF is obtained (10 sec or less) as compared with H_2O—F (30–60 sec).

This question was tested by use of an antennating stimulant. An aliquot (40 μl) of H_2O—MF was placed on 2.3-cm-diam. semicircular filter paper disks as described above. In addition, 1 μg of 13-methylhentriacontane (delivered in 10 μl hexane) was applied to the center of the disk and at least 2 min were allowed for the hexane to evaporate before testing. This compound was identified by Jones et al. (1971) as an antennating stimulant present in *H. zea* feces. Consistent 15- to 30-sec antennation responses were obtained by female parasitoids allowed to examine the disks.

Further, the antennating stimulant experiment was conducted to determine whether associative learning is a key part of the activation process and thus whether some amount of the volatile attractant must be present and antennated in conjunction with the nonvolatile water-soluble activation component. To answer this question, other disks were treated with 40 μl of H_2O—MF and 1 μg of 13-methylhentriacontane as above plus 10 μl of the hexane extract; Discs treated with 40 μl of H_2O—MF and 10 μl of hexane extract also were tested.

The results of these tests are presented in Figure 2. Antennation of these disks as induced by the addition of 13-methylhentriacontane to H_2O—MF

resulted in a significant increase in percentage of flights (26%). The addition of 10 μl of the hexane extract to H_2O—MF also resulted in a significant increase in percentage of flights (27%). However,the addition of both the hexane extract and 13-methylhentriacontane restored the flight percentage (56%) to the level obtained by H_2O—F (53%). The fact that the addition of a small amount of hexane extract along with 13-methylhentriacontane provided a much higher response than 13-methylhentriacontane alone, which induced antennation only, indicates that the conditioning process involves a nonvolatile chemical(s) that mediates responses to other chemicals, such as the hexane extract, through a type of associative learning. That is, the water-soluble nonvolatile compound, probably a host recognition material, serves as a type of unconditioned stimulus to which the parasitoid links various less-specific odorants to aid in finding its host. The low response to H_2O—MF plus hexane extract without the addition of 13-methylhentriacontane shows the need of the latter material as an antennation facilitator.

The significant response that resulted from the antennation induced by 13-methylhentriacontane could be from several possible causes. First, the induced antennation may facilitate detection of minute amounts of contaminating volatiles still present in the H_2O—MF. Alternatively, response tendencies to some key compound(s) in the hexane extract may be "hard-wired," and antennation of the water soluble material, prompted by the 13-methylhentriacontane, even in the absence of any hexane soluble material, may increase the central excitatory state and cause a response to these materials without their association.

Experiment 7. Assessment of Role of 13-Methylhentriacontane in Conditioning Process and Confirmation of Active Nonvolatile Chemical in H_2O—MF Extract. To further assess the role of 13-methylhentriacontane, two procedures were conducted. First, a test was conducted to determine whether 13-methylhentriacontane could effectively function together with the hexane extract to condition the flight response of female parasitoids. The females were given a preflight exposure to a range of doses of 13-methylhentriacontane in combination with the hexane extract. The 13-methylhentriacontane was applied at doses ranging from 0.1 to 100 μg to a moistened paper disk, as described earlier, along with 10 μl of the hexane extract. The individual parasitoids were exposed to the disks for 60 sec and subsequently tested for flights to the hexane extract. These responses were compared to those obtained after a preflight experience of the combination of these same two materials (1 μg 13-methylhentriacontane + 10 μl hexane extract) plus 40 μl of the H_2O—MF.

The 13-methylhentriacontane and hexane extract combination alone provided no significant conditioning at any dose. However, with the addition of H_2O—MF, significant conditioning was obtained (19 of 31, 61%).

The second procedure involved testing a range of doses of 13-methylhentriacontane in combination with both the hexane extract and filtered water

extract. Using the paper disk method, female parasitoids were given a 60-sec exposure to the three materials with doses of 13-methylhentriacontane ranging from 0–1 μg (hexane extract 10 μl, H_2O—MF 40 μl). The effect of 13-methylhentriacontane seemed to level out at dosages higher than 0.1 μg (Figure 3A). However, at dosages of 0.030 μg and lower, conditioning of the parasitoids was significantly reduced.

These data strongly support the hypothesis that 13-methylhentriacontane elicits antennation and thereby facilitates the associative learning interaction between the nonvolatile substances and the hexane odors.

As a final confirmation of the presence of a functional water-soluble component, the paper disk was used to compare the conditioning effect of a range of doses of the filtered H_2O in combination with the hexane extract and 13-methylhentriacontane. Doses of 0–2.0 mg feces equivalents of H_2O—MF (40-μl aliquot) were tested in combination with 1 μg of 13-methylhentriacontane and 10 μl of hexane extract.

The results presented in Figure 3B demonstrate a positive linear dose effect of the filtered water on conditioning for the flight response.

Experiment 8. Demonstration of Ability to Associatively Learn Novel Odors. The above results led to the question of whether the water extract could be used to condition the parasitoids to respond to novel compounds. Vanilla extract (Nielsen-Massey) was used as a novel odor. Females were provided with either no experience, preflight exposure to the standard H_2O—F extract, or exposure to H_2O—F plus 1 μl of the vanilla extract added to the center of the H_2O—F aliquot. These females then were tested for flights to 80 μl of the vanilla extract as the source attractant.

The results of these tests (Table 1) show that females conditioned on the H_2O—F extract made no response to the vanilla extract. However, significant responses to vanilla extract were obtained after they were given a preflight exposure to the vanilla extract in association with the H_2O—F extract. In a separate experiment, 10 females each were exposed to doses of 1, 5, and 10 μl of the vanilla extract and subsequently tested for flight responses to the vanilla extract source alone. None of the individuals would antennate or subsequently fly to the vanilla source. These results confirm that *M. croceipes* can learn to fly to odors by linking these materials to the water extract reference base (associative learning). Furthermore, this can be accomplished for novel odors that they would not encounter normally.

Experiment 9. Influence of Host Insect and Host Diet on Conditioning and Attractant Properties of Feces. Since the attractant and conditioning responses apparently were elicited by two different materials, it was important to test the interacting effects of these two responses when obtained from different host insects or diet sources. The conditioning and attractant properties of feces collected from *H. zea* feeding on cowpeas were compared with those of feces from

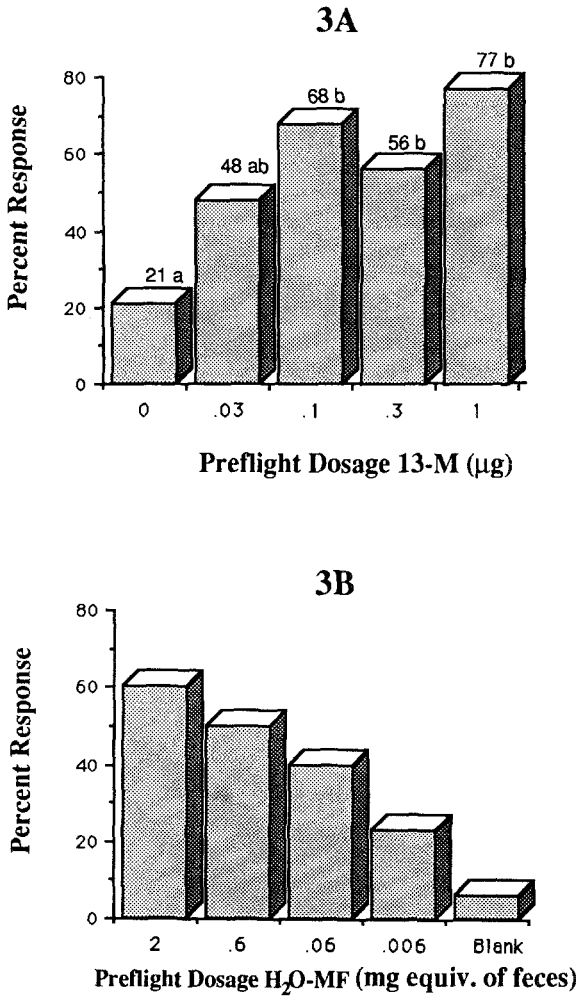


FIG. 3. Response of *M. croceipes* females to hexane extract of host feces. (A) After conditioning with indicated dosages of 13-methylhentriacontane (13-M) in formulation with Millipore-filtered water extract, H_2O—MF, (40-μl aliquot) and hexane extract (10-μl aliquot) of host feces. The linear dosage effect was not significant ($P = 0.05$) according to the general linear model procedure. Bars capped by different letters are significantly different ($P = 0.05$) as separated by the least square mean test, $N = 30$ observations. (B) After conditioning with indicated dosages (dilutions) of Millipore-filtered water extract, H_2O—MF (40-μl aliquot), of host feces formulated together with 13-methylhentriacontane (1 μl) and hexane extract (10-μl aliquot). There was a significant linear dosage effect of H_2O—MF ($P = 0.01$) according to the general linear model procedure.

the same species feeding on artificial diet. The properties of feces from *T. ni* and *H. zea*, both feeding on cowpeas, also were compared.

The results for feces of *H. zea* feeding on cowpeas versus artificial diet are presented in Figure 4A. Both conditioning and attractant properties were extracted from feces from both diets. However, the data show that the *H. zea* feces from cowpeas had stronger attractant properties, as would be expected since the artificial diet is lacking most green-plant compounds. The data also show that the parasitoids responded to the hexane extract attractant of a given diet best when they were conditioned on the water extract of the same diet. These findings fit earlier data that substantiate associative learning.

The results from studies of the comparative properties of feces from *H. zea* and *T. ni*, both fed on cowpeas, are presented in Figure 4B. As expected for *M. croceipes*, which is specific to *Heliothis*, the water extract of *T. ni* feces possessed no conditioning properties. However, the response to hexane extract of the *T. ni* feces was as a strong as that of *H. zea*, even though the parasitoid was conditioned on the water extract of *H. zea* feces.

The data indicate that the water-soluble conditioning material is host-specific and that the hexane-soluble attractant extract is predominantly host-diet associated. The question naturally arises as to whether *M. croceipes* would be equally attracted to *H. zea* and *T. ni* sites if both were feeding on the same plant. Preliminary tests indicate that there are differences that *M. croceipes* and other parasitoids distinguish under more discriminative conditions such as feeding naturally on a plant with all the host and plant components present (see Turlings et al., 1990).

CONCLUSIONS

These flight chamber studies show that the host-seeking behavior of *M. croceipes* is strongly influenced by feces from *H. zea* host larvae. Less than 5% of naive parasitoids reared from hosts fed on artificial diet would fly upwind to feces of hosts fed on cowpeas. However, over 90% of these females experienced by a brief (30-sec) contact with feces from a cowpea-fed host would subsequently orient and fly to cowpea feces. These effects on the parasitoid's behavior were found to be mediated by both a nonvolatile contact chemical and by volatile chemical cues. The nonvolatile substance is water-extractable and distinct from the hexane-soluble volatile cues that are highly effective in attracting the experienced females.

The nonvolatile, water-soluble compound is a host-specific recognition cue with which the parasitoid associates the volatile cues during antennal contact with the feces. Water extracts of *T. ni* frass failed to enhance subsequent flight responses. The antennation stimulant, 13-methylhentriacontane, previously

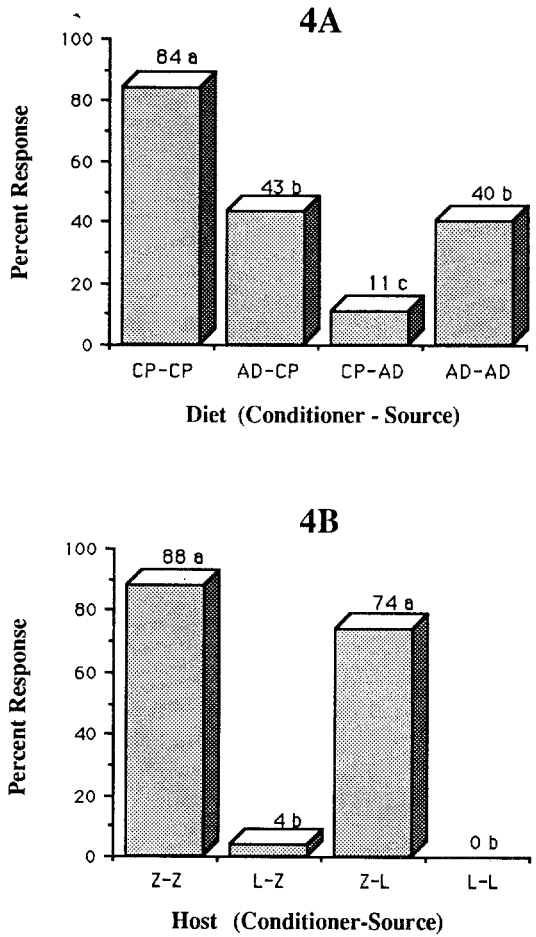


FIG. 4. (A) Influence of host diet on response of *M. croceipes* females under varying preflight conditioning and attractant source combinations. CP-CP (conditioner-source), water extract of feces from *H. zea* fed on cowpeas–hexane extract of feces from *H. zea* fed on cowpeas; AD-CP, water extract of feces from *H. zea* fed on artificial diet–hexane extract of feces from *H. zea* fed on cowpeas; CP-AD, water extract of feces from *H. zea* fed on cowpeas–hexane extract of feces from *H. zea* fed on artificial diet; AD-AD, water extract of feces from *H. zea* fed on artificial diet–hexane extract of feces from *H. zea* fed on artificial diet. (N = 30 observations). (B) Influence of host species (fed on cowpeas) on response of *M. croceipes* females under varying preflight conditioning and attractant source combinations. Z-Z (conditioner–source), water extract of *H. zea* feces–hexane extract of *H. zea* feces; L-Z, water extract of *T. ni* feces–hexane extract of *H. zea* feces; Z-L, water extract of *H. zea* feces–hexane extract of *T. ni* feces; L-L water extract of *T. ni* feces–hexane extract of *T. ni* feces. Bars capped by different letters are significantly different (P = 0.01, Waller-Duncan k-ratio t test, N = 40 observations).

identified for *M. croceipes* and present in *H. zea* feces, was shown to facilitate the associative learning process. The volatile attractants present in the hexane extract of host feces are diet related and thus would be primarily plant-derived. However, the parasitoid was conditioned to fly to a novel and otherwise unattractive odor, vanilla extract, when it was antennated in conjunction with the water extract of the feces.

Larvae of *H. zea* spp. feed on a wide variety of plants and plant parts of various ages, often in diverse vegetational settings, along with many other actively feeding herbivorous insects. Consequently, their trail odors vary considerably and occur in the context of a vast array of other chemical signals. Furthermore, in a high percentage of cases, when *M. croceipes* females find host sites, the hosts are feeding within a crevice or within a fruiting part so that contact with the host is limited, or the host has moved recently to a new location. Linkage of various odorants associated with the site to a dependable host-recognition chemical in a host by-product, independent of host contact, provides the parasitoid with a valuable alternative means for ongoing reinforcement or adjustments of its foraging behavior.

This phenomenon provides a valuable way of finding hosts or prey species and may be widespread in parasitic and predatory systems, particularly for cryptic hosts or prey where the target organisms (but not their by-products) often elude the searching parasitoid or predator.

Acknowledgments—We thank Thoris Green and Ara Manukian for valuable technical assistance throughout this study.

REFERENCES

DING, D., SWEDENBORG, P.D., and JONES, R.L. 1989. Plant odor preferences and learning in *Macrocentrus grandii* (Hymenoptera: Braconidae), a larval parasitoid of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *J. Kans. Entomol. Soc.* 2:164–176.

DROST, Y.C., LEWIS, W.J., ZANEN, P.O., and KELLER, M.A. 1986. Beneficial arthropod behavior mediated by airborne semiochemicals. I. Flight behavior and influence of preflight handling of *Microplitis croceipes* (Cresson). *J. Chem. Ecol.* 12:1247–1262.

DROST, Y.C., LEWIS, W.J., and TUMLINSON, J.H. 1988. Beneficial arthropod behavior mediated by airborne semiochemicals. V. Influence of rearing method, host plant, and adult experience on host-searching behavior of *Microplitis croceipes* (Cresson), a larval parasitoid of *Heliothis*. *J. Chem. Ecol.* 14:1607–1616.

ELLER, F.J., TUMLINSON, J.H., and LEWIS, W.J. 1988. Beneficial arthropod behavior mediated by airborne semiochemicals: Source of volatiles mediating the host-location flight behavior of *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae), a parasitoid of *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae). *Environ. Entomol.* 17:745–753.

GROSS, H.R., JR., LEWIS, W.J., JONES, R.L., and NORDLUND, D.A. 1975. Kairomones and their use for management of entomophagous insects. III. Stimulation of *Trichogramma achaeae*, *T. pretiosum*, and *Microplitis croceipes* with host-seeking stimuli at time of release to improve their efficiency. *J. Chem. Ecol.* 1:431–438.

GUY, R.H., LEPPLA, N.C., RYE, J.R., GREEN, C.W., BARRETTE, S.L., and HELLIENE, K.A. 1985. *Trichoplusia ni*, pp. 487–494, *in* P. Singh, and R.E. Moore (eds.). Handbook of Insect Rearing, Vol. 2. Elsevier Science Publishers, Amsterdam.

HERARD, F., KELLER, M.A., LEWIS, W.J., and TUMLINSON, J.H. 1988a. Beneficial arthropod behavior mediated by airborne semiochemicals. III. Influence of age and experience on flight chamber responses of *Microplitis demolitor* Wilkinson. *J. Chem. Ecol.* 14:1583–1596.

HERARD, F., KELLER, M.A., LEWIS, W.J., and TUMLINSON, J.H. 1988b. Beneficial arthropod behavior mediated by airborne semiochemicals. IV. Influence of host diet on host-oriented flight chamber responses of *Microplitis demolitor* Wilkinson. *J. Chem. Ecol.* 14:1597–1606.

JONES, R.L., LEWIS, W.J., BOWMAN, M.C., BEROZA, M., and BIERL, B.A. 1971. Host-seeking stimulant for parasite of corn earworm: isolation, identification, and synthesis. *Science* 173:842–843.

LEWIS, W.J., and BURTON, R.L. 1970. Rearing *Microplitis croceipes* in the laboratory with *Heliothis zea* as hosts. *J. Econ. Entomol.* 63:656–658.

LEWIS, W.J., and JONES, R.L. 1971. Substance that stimulates host-seeking by *Microplitis croceipes* (Hymenoptera: Braconidae), a parasite of *Heliothis* species. *Ann. Entomol. Soc. Am.* 64:471–473.

LEWIS, W.J., and TUMLINSON, J.H. 1988. Host detection by chemically mediated associative learning in a parasitic wasp. *Nature* 331:257–259.

TURLINGS, T.C.J., TUMLINSON, J.H., and LEWIS, W.J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250:1251–1253.

VAN ALPEN, J.J.M., and VET, L.E.M. 1986. An evolutionary approach to host finding and selection, pp. 23–61, *in* J. Waagem and D. Greathead (eds.). Insect Parasitoids, 13th Symposium, Royal Entomology Society of London. Academic Press, New York.

VET, L.E.M., and JANSE, C.J. 1984. Fitness of two sibling species of *Asobara* (Braconidae: Alysiinae), larval parasitoids of Drosophilidae in different microhabitats. *Ecol. Entomol.* 9:345–354.

VINSON, S.B. 1984. How parasitoids locate their hosts: A case of insect espionage, pp. 325–348, *in* T. Lewis (ed.). Insect Communications. Royal Entomology Society of London, London.

WARDLE, A.R., and BORDEN, J.H. 1986. Detrimental effect of prior conditioning on host habitat location by *Exerstes roborator*. *Naturwissenschaften* 73:559–560.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

A MODIFIED ALLOMONE COLLECTING APPARATUS

BRUCE A. SCHULTE,[1] ROCKY DE NYS,[2] GERALD J. BAKUS,[3,*] PHILIP CREWS,[4] CLARK EID,[4] STEPHEN NAYLOR,[4] and LAWRENCE V. MANES[4]

[1]*College of Environmental Science and Forestry*
State University of New York
Syracuse, New York 13210

[2]*Department of Chemistry and Biochemistry*
James Cook University of North Queensland
Townsville, Q 4812, Australia

[3]*Department of Biological Sciences*
University of Southern California
Los Angeles, California 90089-0371

[4]*Department of Chemistry, Institute of Marine Sciences*
University of California, Santa Cruz
Santa Cruz, California 95064

(Received May 17, 1990; accepted March 4, 1991)

Abstract—A modified allomone collecting apparatus was designed that could be used under water with a scuba tank. This apparatus provides a simple method of obtaining naturally secreted chemicals from benthic marine organisms at scuba depths, for the first time without the necessity of using a bilge pump. Organic material from Sep-paks in the allomone collector confirmed the release of secondary metabolites from a soft coral into the surrounding water.

Key Words—Allomone, Sep-pak, bioactive substances, secondary metabolites, thin-layer chromatography.

INTRODUCTION

In the last 20 years it has been recognized that secondary metabolite synthesis by marine organisms, previously considered only a by-product of detoxification or an avenue for metabolic wastes, is an evolutionary event in which biologi-

*To whom correspondence should be addressed.

0098-0331/91/0700-1327$06.50/0

cally active compounds, or bioactive substances, enhance individual survival (Sondheimer and Simeone, 1970; Kittredge et al., 1974; Fenical, 1982). These metabolites are often thought to play an allelopathic role and are known as allelochemicals or allomones.

Until the work by Coll et al. (1982), chemical evidence to confirm the presence of marine allomones was lacking. In their study a submersible allomone collecting apparatus, operated by a bilge pump with its battery aboard a boat, was constructed, utilizing Sep-paks (C-18, reverse-phase, silica gel columns) to sample water from around two species of alcyonaceans or soft corals (*Sarcophyton crassocaule* and *Sinularia flexibilis*). Using thin-layer chromatography (TLC), they concluded that the lipophilic secondary metabolites known to exist within these species (i.e., sarcophytoxide and sarcophine from *Sarcophyton crassocaule* and flexibilide and dihydroflexibilide from *Sinularia flexibilis*) were also present in the surrounding water. Interestingly, in noncontact transplant experiments involving *Sinularia flexibilis* with the soft corals *Nephthea* sp. or *Alcyonium molle*, no necrotic effects were evidenced. However, over a four-week period all colonies exhibited growth ("movement") away from each other (La Barre et al., 1986). Exudation of bioactive metabolites also was reported for the sponge *Aplysina fistularis* (Thompson, 1985).

The main objective of this study was to develop an allomone collecting apparatus that could be used by scuba divers to collect secondary metabolites secreted from benthic marine organisms. This would allow chemical ecologists a considerable advantage over the system developed by Coll et al. (1982) because the latter were limited to a depth of about 6 m (20 ft) and the apparatus required a battery-powered, submersible bilge pump.

METHODS AND MATERIALS

An allomone collecting apparatus with a hemispherical Plexiglas collecting chamber was designed by one of us (B.A.S.) that could be placed over a sponge or soft coral without additional weights or supports to keep it in position. After extensive laboratory trials at UCSC and field tests over sponges, the apparatus was found to leak. Rather than place large O-rings in the system to prevent the leakage, a simpler collecting chamber was designed (R.d.N.). Four 18-mm-long hollow stainless-steel tubes 3 mm in diameter with a central opening 1 mm in diameter were brazed to a basal tube 60 mm long. A single 18-mm-long tube was then brazed to the basal tube (Figure 1). This configuration was duplicated and the two pieces (adaptors) connected by four Sep-paks. The lower metal adaptor was connected to an inverted funnel (Figure 2). The upper metal adaptor was connected to an outlet hose that received compressed air from a scuba tank.

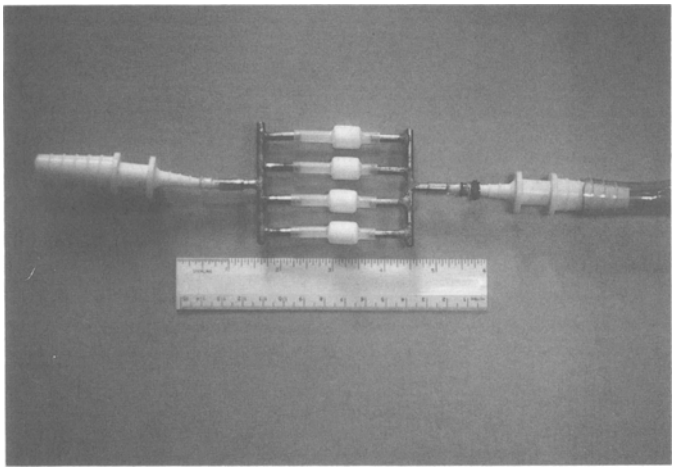


FIG. 1. Close-up photograph of the metal adaptors with Sep-paks *in situ*.

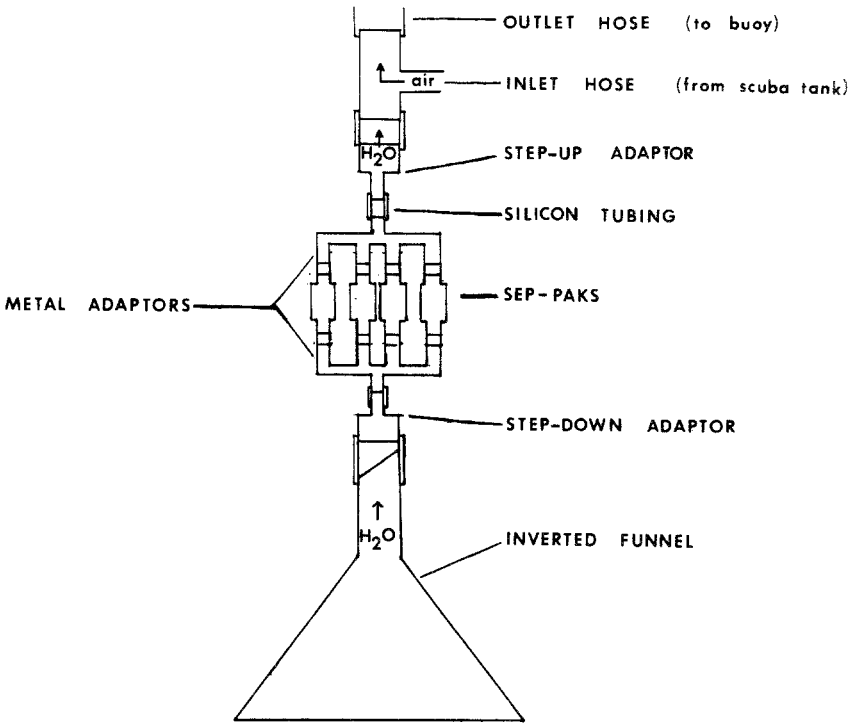


FIG. 2. Modified allomone collecting apparatus.

The allomone collecting apparatus is powered by compressed air from a scuba tank, whose flow is controlled by a needle valve in the high-pressure line connected to the low-pressure outlet of a scuba regulator. Water flow rates of 5–8 liters/hr are attainable by adjustment of this needle valve. At a rate of 7 liters/hr and a depth of 8 m (25 ft), the tank loses pressure at an average rate of 150 psi/hr. The allomone device was tested to depths of 13 m (40 ft) without any difficulties.

The modified allomone apparatus was first tested in a pool using neutral red. Field tests were carried out at Geoffrey Bay, Magnetic Island, near Townsville, Australia. A small colony of the soft coral *Sinularia flexibilis* was enclosed in the collector and cut to ensure release of metabolites. The Sep-paks were primed in MeOH (methanol), and the collector was operated for 4 hr. The Sep-paks were removed, returned to the laboratory, and eluted with 20 ml distilled water, 20 ml MeOH, and 20 ml dichloromethane (DCM). The MeOH extract was washed with DCM and the DCM extracts combined. The Sep-paks yielded 25 mg of yellow extract. A sample of the same colony of *Sinularia flexibilis* was also collected and extracted wet using MeOH and DCM. The MeOH extract was washed with DCM and the DCM extracts combined. A seawater control was run upstream of the soft coral. Comparison of the *S. flexibilis* extract, the Sep-pak extract, and the pure compounds flexibilide and dihydroflexibilide previously isolated and identified from the soft coral using TLC (Kazlauskas et al., 1978) allowed identification of compounds present in the Sep-pak extract. The TLC was carried out on silica gel (Keisel-gel 60F254) using a 30% petroleum ether–diethyl ether solvent system. The TLC fractions were visualized using a vanillin–concentrated sulfuric acid spray and the color developed on gentle warming. The Sep-pak extracts of *S. flexibilis* and the seawater control were examined by [^{1}H]-and [^{13}C]NMR.

RESULTS

When the modified apparatus was first tested in a pool using neutral red, the Sep-paks removed the neutral red from the water. The organic material eluted from the Sep-paks in the field experiments in Australia was compared with that of the soft coral tissue and the compounds flexibilide and dihydroflexibilide by TLC. Figure 3 confirms, by TLC, the release from *S. flexibilis* of flexibilide and a number of nonterpenoid compounds thought to be lipids and sterols into the water column. No dihydroflexibilide was detected. [^{1}H] and [^{13}C]NMR further confirmed the presence of lipids and sterols; however, no resonances for either flexibilide or dihydroflexibilide were detected because of low concentrations. TLC and [^{1}H]NMR of the Sep-pak extracts of the upstream seawater control show no flexibilide or dihydroflexibilide to be present. These

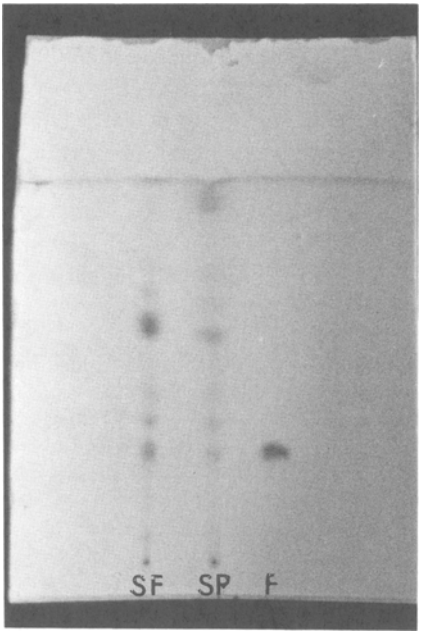


FIG. 3. Thin-layer chromatographic comparison of the tissue extract (SF), the Sep-pak eluent (SP), and flexibilide (F) of *Sinularia flexibilis;* solvent 30% petroleum ether–diethyl ether.

results confirm the release of metabolites from *S. flexibilis* and the ability of the modified allomone apparatus to concentrate organic compounds from the water column.

DISCUSSION

The modified allomone collecting apparatus will allow biologists and chemists to collect natural products (allomones and potential kairomones or pheromones) from marine organisms at any safe diving depth without the need of an accompanying boat containing a battery and cables connected to a submersible pump. The new model (Figures 1 and 2) is simpler and lighter than the original Plexiglas design and does not leak; however, weight does need to be added to the top of the chamber to stabilize it and keep the hood in position over the specimen being tested. The hood can be modified easily to accommodate organisms of different shapes and sizes. This apparatus continued to perform well during extensive field studies on soft corals in Australia conducted by one of us (R.d.N.). Through TLC, the presence of known bioactive second-

ary metabolites was confirmed in the whole-organism extracts of the soft coral species under study and in the water extracts surrounding the animal studied.

Examination of organisms other than soft corals would reveal whether allelopathy is a general marine phenomenon or not. Our failure to detect allomones from around marine sponges may be the result of leaks in the Plexiglas allomone model or some other cause (e.g., the sponges sampled may not release allomones). Further studies on sponges with the new model should clarify this issue. The modified allomone collecting apparatus will facilitate the study of waterborne chemical interactions by providing a simple means of sampling the water around aquatic organisms for bioactive substances.

Acknowledgments—We thank Drs. John Coll, Porfirio Aliño, Jim Kittredge, and Frederick Browand for making valuable suggestions on the design of the allomone apparatus. Drs. Richard Brusca, Russel Zimmer, and John Coll critically reviewed the manuscript, for which we are grateful. Support for this research was provided by a grant from the University of Southern California Institute for Marine and Coastal Studies (awarded to B.A.S.), by the U.S. Office of Naval Research (grant 00014-84-K-0375 awarded to G.J.B.), and by Sea Grant R/MP-33, U.R.E.P., and Syntex (awarded to P. Crews).

REFERENCES

COLL, J.C., BOWDEN, B.F., TAPIOLAS, D.M., and DUNLAP, W.C. 1982. In situ isolation of allelochemicals released from soft corals (Coelenterata: Octocorallia): A totally submersible sampling apparatus. *J. Exp. Mar. Biol. Ecol.* 60:293–299.

FENICAL, W. 1982. Natural products chemistry in the marine environment. *Science* 215:923–928.

KAZLAUSKAS, R., MURPHY, P.J., WELLS, R.J., SCHONHOLZER, P., and COLL, J.C. 1978. Cembranoid constituents from an Australian collection of soft coral *Sinularia flexibilis*. *Aust. J. Chem.* 31:1817–1824.

KITTREDGE, J.S., TAKAHASHI, F.T., LINDSEY, J., and LASKER, R. 1974. Chemical signals in the sea: Marine allelochemics and evolution. *Fish. Bull.* 72:1–11.

LA BARRE, S.C., COLL, J.C., and SAMMARCO, P.W. 1986. Competitive strategies of soft corals (Coelenterata: Octocorallia). III. Spacing and aggressive interactions between alcyonareans. *Mar. Ecol. Prog. Ser.* 28:147–156.

SONDHEIMER, E., and SIMEONE, J.B. 1970. Chemical Ecology. Academic Press, New York. 336 pp.

THOMPSON, J.E. 1985. Exudation of biologically active metabolites in a sponge (*Aplysina fistularis*) I. Biological evidence. *Mar. Biol.* 88:23–26.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

MEASUREMENT OF SHORT-TERM CHANGES IN HEART RATE AND IN PLASMA CONCENTRATIONS OF CORTISOL AND CATECHOLAMINE IN A SMALL MARSUPIAL

D. MICHAEL STODDART and A.J. BRADLEY*

Department of Zoology
University of Tasmania,
GPO Box 252C, Hobart, Tasmania 7001, Australia

(Received August 21, 1990; accepted March 4, 1991)

Abstract—Using a chronically placed jugular catheter and a silver electrode, it was possible to monitor short-term changes in the plasma concentration of cortisol and catecholamine in the marsupial sugar glider (*Petaurus breviceps*) and to monitor both heart and respiration rates. Males judged to be low in the social hierarchy of a particular group were exposed to the whole-body odor of a dominant male from the same social group, a foreign dominant male, or a castrate male. While there was no evidence of a change in any of the physiological parameters when a male was exposed to either a castrate male or a female, a rapid increase occurred in heart rate and plasma concentrations of cortisol, glucose and catecholamine when the donor was a dominant male from the same or a different social group.

Key Words—Sugar glider, *Petaurus breviceps*, Marsupialia, heart rate, respiration rate, stress, cortisol, catecholamine.

INTRODUCTION

A relationship between rank order and testosterone levels has been shown to be significantly correlated in several species of primate (Rose et al., 1971; Eberhart et al., 1980). A more specific relationship between olfactory signals and sexual inhibition in males has been described in a primate (Schilling et al., 1984) and mice, rats, and rabbits (Brain and Nowell, 1970; Mykytowycz, 1973). The mechanism by which this inhibition is achieved is not clear, but it has been

*To whom correspondence should be addressed.

0098-0331/91/0700-1333$06.50/0

known for some years that reproductive function in male primates, rodents, and domestic animals may be suppressed by stress (Rose and Sachar, 1982). It is accepted that glucocorticoids, β-endorphin, and prolactin, which are secreted during stress, are each capable of suppressing testosterone with the site of action varying within the hypothalamopituitary–gonadal axis. While the opioids appear to suppress testosterone concentration by inhibiting LHRH release (Hulse and Coleman, 1983), both glucocorticoids (Bambino and Hsueh, 1981; Johnson et al., 1982) and prolactin (McNeilly et al., 1983) are thought to act on the pituitary and testes to suppress testosterone secretion. In a study of the role of glucocorticoids in stress-induced suppression of testicular function in the baboon, Sapolsky (1985) concluded that most of the glucocorticoid action was exerted at the testicular level.

In studies using marsupials, few attempts have been made to investigate directly the link between social stimulation and physiological change. Scott (1986) used pheromonal cues from urine and feces to induce ovulation synchrony in isolated *Antechinus stuartii*. In this study social and pheromonal effects on plasma androgens were investigated in males, but the study did not investigate pituitary–adrenal effects. Other than the study of Scott (1987), which examined the effects of mating and agonistic experience on adrenal function of male *Antechinus stuartii*, we are not aware of any other work using marsupials that directly links stress response to social stimulation. More general aspects of stress response to social stimulation in small mammals are discussed by Lee and McDonald (1985).

Most of our knowledge of the physiological attributes of a stress response in marsupials is derived from studies on small dasyurid marsupials where, in several species, there occurs an annual total, synchronous stress-related male mortality (see Bradley 1987, 1990).

The object of this study was to investigate the feasibility of using repeated small blood samples as well as electrocardiography to assess the short-term pituitary adrenal and sympathetic nervous system effects induced by olfactory stimulation in a small mammal. The species used was the marsupial sugar glider (*Petaurus breviceps*, adult body mass 125–140 g).

METHODS AND MATERIALS

Surgical and Experimental Procedures. Anesthesia was induced with an intramuscular injection of 15 mg ketamine/kg (Ketalar; Parke Davis, Sydney, NSW, Australia) and maintained with further intraperitoneal injections of 250 mg chloral hydrate/kg. Salivation and nasopharyngeal secretion were minimized with an initial subcutaneous injection of 0.1 mg atropine sulfate/kg (Atrosine Mitis, Parnell Laboratories Pty. Ltd., Peakhurst, NSW, Australia).

Dual-lumen vinyl catheters (internal diameter 0.41 mm, external diameter 1.0 mm; Critchley Electrical Products, Auburn, NSW, Australia) were prepared by inserting a stainless-steel wire in one lumen and paring away the other channel to leave one side 10 mm shorter than the other. This procedure was repeated at the distal end of the catheter to facilitate the connection of separate tubing for infusion and blood sampling. The cannula was sterilized; then both channels were filled with sterile isotonic saline. The catheter was inserted into the right jugular vein and advanced until the aperture of the shorter lumen lay adjacent to the entry to the right atrium. The distance from point of insertion to the required position in the blood vessel was determined by measurements made at necropsy on an animal of similar size that had died of natural causes. The catheter was secured in the vein with a purse-string suture of 6-0 vascular silk, filled with 50,000 IU heparin/liter saline, and each lumen was plugged with a stainless-steel pin. The distal end of the catheter was passed subcutaneously to exit in the midline between the scapulae. Before closure of the incision, approximately 0.05 g amoxycillin sodium (Ibiomox, Protea Pharmaceuticals, Thornleigh, NSW, Australia) was puffed into the wound. No infections were ever seen as a result of this cannulation procedure. Animals were allowed two days to recover before commencement of experimental procedures. With daily flushing with saline and refilling with heparin–saline, some cannulae remained patent for up to 21 days.

Blood samples of 0.08–0.10 ml were collected using tubing of low dead space connected to the longer lumen of the catheter. Great care was taken during both intravenous injection and blood withdrawal since small pressure pulses were found to affect both heart and respiration rate.

A blood volume equivalent to the dead space of the sampling line was withdrawn before, and replaced after, the taking of the blood sample which was to be assayed for hormones and metabolites. Blood samples were collected into polypropylene centrifuge tubes and centrifuged at 4000 g for about 2 min in an Eppendorf 5414S centrifuge (Eppendorf, Hamburg, Germany) prior to removal of the plasma, which was stored at −25°C until later assay. Aliquots of plasma before freezing were flame sealed into plain hematocrit tubes (Blue Tip, Monoject Scientific, Sherwood Medical, St. Louis, Missouri) into which a sodium fluoride solution previously had been dried to give a concentration in the plasma sample of approximately 4 mg/ml.

To determine when the test animal had satisfactorily acclimatized to the testing chamber and monitoring equipment, plasma glucose concentrations were determined at intervals from a small drop of whole blood using Dextrostix (Miles Laboratories Australia, Pty. Ltd., Mulgrave North, Victoria, Australia) and a portable laboratory glucometer (Miles Laboratories). This provided a plasma glucose concentration with a known accuracy of at least 10%.

Recording and Odor Delivery. To facilitate recording of heart rate, a silver

electrode sheathed along its entire length with vinyl catheter tubing except for the 1-mm tip was placed subcutaneously in the left midthoracic position. A second electrode was provided by the tip of the saline-filled jugular catheter to which an electrical connection was made at the needle inserted into the distal end of the catheter. These electrodes were connected to a Gilson GME model M8PM Macro polygraph equipped with ECG and cardiotachygraph modules (Gilson Medical Electronics Inc. Middleton, Wisconsin) and to a Gould digital storage oscilloscope (model 0S1420, Hainault, England).

The odors used in this investigation were whole-body odors from sugar gliders deemed dominant or subordinate to the test animal. The donor animal was placed in a polypropylene chamber measuring 150 × 70 × 55 mm. In the control experiments air flowed through an identical, but empty, chamber.

Clean laboratory air was provided by a diaphragm-operated aquarium pump and delivered via Teflon tubing to either control or donor at a rate of 3.6 liters/min to the polypropylene recipient test chamber, which measured 200 × 50 × 40 mm. The recipient animal was loosely restrained in a clean calico bag with its nose adjacent to the inlet port at one end of the chamber. Connections were made to the jugular catheter and the silver electrode where they emerged from the skin in the midline suprascapular position. The recipient animal in the test chamber was placed in an earth-shielded cage and left undisturbed for at least 1 hr prior to the commencement of blood sampling; however, both heart rate and respiration rate were monitored during this period. The respiration rate could be determined by the effects of the alteration of intrathoracic pressure on the ECG trace during the respiratory cycles.

Determination of Social Rank. These studies were carried out between January and March prior to the commencement of the annual breeding season. Observations were made on each social group for 1 hr following the daily placement of a food container on each of five successive days. Individuals could be distinguished either by naturally occurring patches of white fur on the flank or tip of the tail or by the placement of a small piece of white insulating tape around the ear tag. The two stable social groups consisted of two females and three males and one female and four males. The social rank of each male within a group was judged by its response when approached by another male in the group. A male that invariably retreated from the food bowl when another male approached rapidly was designated the Ω male, while the male from which retreats were made by all other males was designated the α male. The relative social order of males of intermediate rank was less easily determined but is of little consequence for this particular study. In the group that consisted of one female and four males, the results of naturally occurring, rather than contrived, social encounters between males were scored as wins or losses. The results from highest to lowest in the hierarchy were 26/0, 11/5, 5/18, and 1/20, respectively. Males judged to be lowest in the social hierarchy of a particular group were

exposed to the whole-body odor of a dominant male from the same social group, a foreign dominant male, or a castrate male.

Hormone and Metabolite Analysis. The concentration of cortisol in the blood plasma was determined by RIA (intraassay variability 6.8%, interassay variability 6.9%), and the concentration of free cortisol was then determined. The methods employed have been described by Bradley (1990).

The plasma total catecholamine concentration was determined using the Cat-A-Kit assay system (Amersham, UK). Blood samples of 0.1 ml were collected into polypropylene centrifuge tubes which contained 5 μl of a solution (pH 6.0–7.4) of EGTA (ethyleneglycol-bis-*N*,*N*,*N*,*N*,-tetraacetic acid 45 mg/ml) and glutathione (30 mg/ml). These chemicals were purchased from the Sigma Chemical Co.

Plasma glucose concentration was measured by the glucose oxidase method of Hugget and Nixon (1957) using 10-μl plasma samples.

RESULTS

Odor of a Castrate Male (Figure 1). During this experiment a male of lowest social rank was placed in the test chamber and allowed a period of acclimatization during which the air flowed through an empty control chamber.

For 60 min the airflow was switched to expose the test animal to the whole-body odor of a castrate male, after which the air source was again switched back to the control chamber. The switching of the air supply did not result in any detectable changes in any of the parameters measured. When the airflow was passed to the test animal from an empty chamber, no change in any of the parameters was seen during the experiment.

Odor of Familiar Dominant Male (Figure 2). The exposure of a male of low social rank to the odor of a familiar dominant male from the same social group had some marked effects. Within 10 min of exposure to the test odor source, respiration rate increased from 64.5 $\pm$ 0.5 to 76.5 $\pm$ 1.5 (mean $\pm$ SEM) cycles/min ($N = 2$) while heart rate increased from 268 $\pm$ 7 to 393 $\pm$ 18 beats/min. The plasma concentration of glucose and catecholamine reached maximum values of 7.0 $\pm$ 0.1 nM and 4.5 $\pm$ 0.7 ng/ml, respectively, after 30 min of odor exposure. The plasma-free cortisol concentration did not reach the maximum concentration of 11.2 $\pm$ 1.1 nM until 10 min after switching the airflow source back to the control chamber.

Odor of Unfamiliar Dominant Male (Figure 3). The exposure of a male of lowest social rank to the odor of an unfamiliar dominant male had some marked effects. While respiration rate increased from 66 to 78 cycles/min within 10 min of the commencement of odor delivery from an unfamiliar dominant male, during this same period the heart rate increased from 270 to 465 beats/min. After

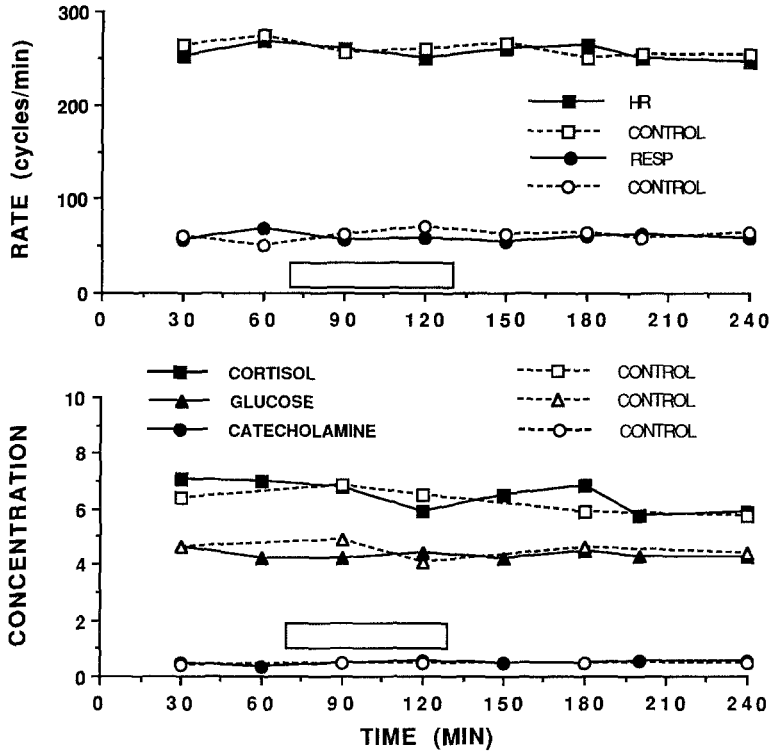


FIG. 1. The effect of the whole-body odor of a castrate male on heart and respiration rate, and on plasma concentration of cortisol, glucose, and catecholamine of a socially subordinate male sugar glider (*Petaurus breviceps*). The open rectangle denotes length of odor stimulation. (Concentration = nM cortisol; mM glucose; mg/ml catecholamine).

10 min of exposure to the donor odor, the plasma-free cortisol concentration had decreased slightly from 6.0 to 5.8 nM, plasma glucose concentration remained virtually unchanged, but catecholamine concentration increased from 0.46 to 3.2 ng/ml. The heart rate decreased from the maximum of 465 beats/min to reach the value in the control within 10 min of the airflow source being changed from donor animal chamber to control chamber. Plasma cortisol concentration continued to increase, reaching a peak value of 20.8 nM at 10 min after the diversion of the airflow to the control chamber. Plasma catecholamine concentration reached a maximum of 5.0 ng/ml after 40 min of exposure to the odor from the donor animal, thereafter decreasing to 0.77 ng/ml at 240 min. The plasma glucose concentration reached a peak of 6.8 nM 10 min after the

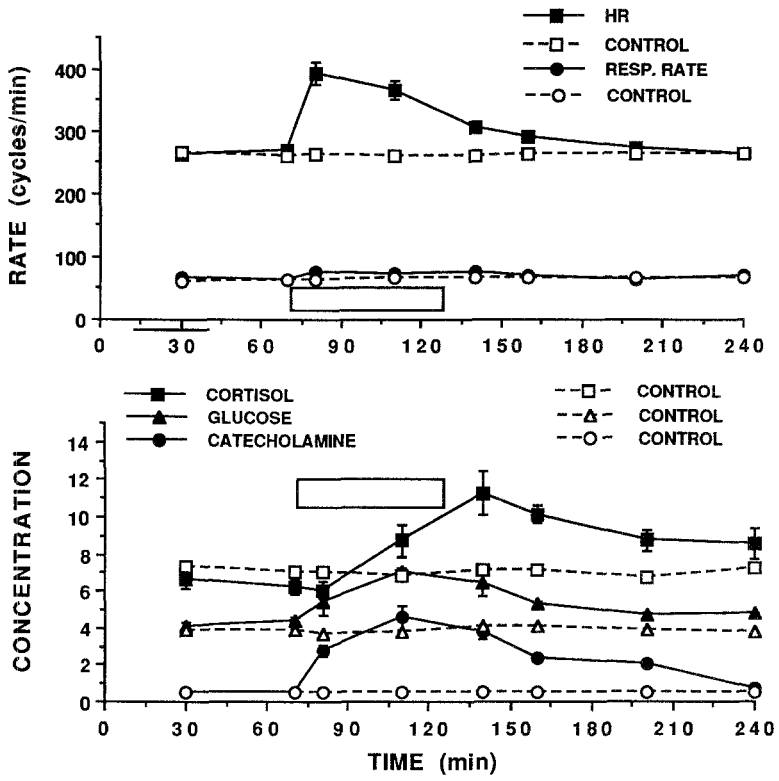


FIG. 2. As in Figure 1. Body odor of the socially dominant male from the test subject's colony (n = 2).

diversion of the airflow to the control chamber, thereafter declining to the preexposure concentration at 240 min.

DISCUSSION

Despite the preliminary nature of this investigation and the small number of replicates, it is apparent that serial blood sampling using a chronically implanted jugular catheter, together with electrocardiography, is a feasible technique for studying short-term physiological effects induced by various intraspecific odorants. The data suggest that socially subordinate males react differently to the whole-body odor of a castrated or a socially dominant intact male. A dominant male from an unfamiliar colony appeared to elicit a more intense reaction than one familiar to the test subject, but the significance of this observation cannot be assessed at this stage.

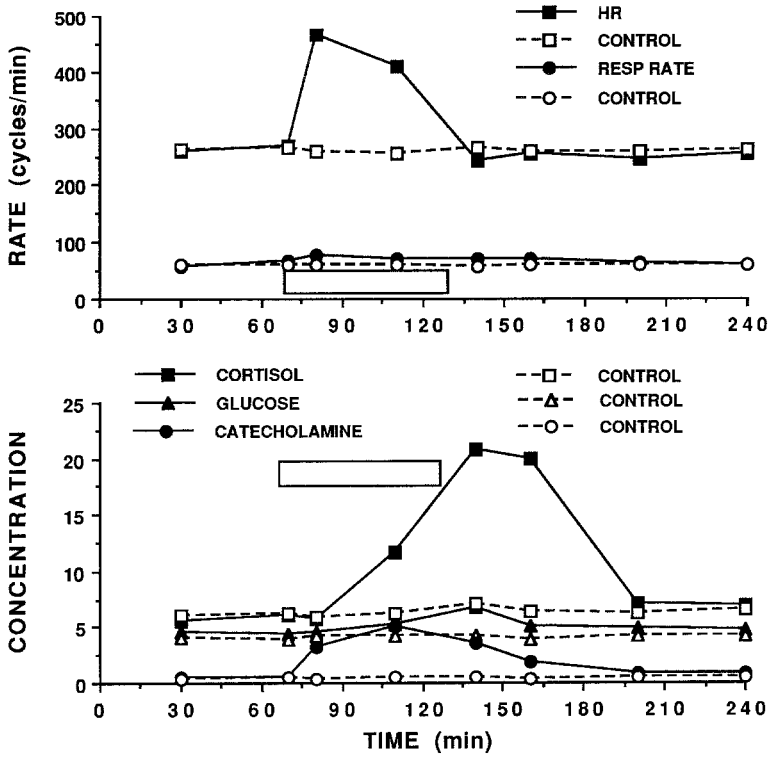


FIG. 3. As in Figure 1. Body odor of a socially dominant male from a colony unfamiliar to the test subject.

The most rapid and marked increases occurred in the heart rate; however, more delayed responses also occurred in the plasma concentration of free cortisol, catecholamine, and glucose. Since it has been shown previously that increases in the plasma concentration of either catecholamine or free cortisol will result in an elevation in the plasma glucose concentration in this species (Bradley and Stoddart, 1990), it is not surprising that rises in plasma glucose concentration were detected in this study. Because of this dual stimulatory effect, measurement of plasma glucose concentration would seem to be a useful means for assessing nonspecific psychosocial stress in *P. breviceps*.

The usefulness of this technique is that it allows repeated blood sampling in a small mammal. It is crucial, however, that the test animal be allowed adequate time, often up to 2 hr, to acclimatize to the loose restraint provided by the test chamber. From the physiological point of view, a nonstressed starting condition may be judged in the short term by measurement of the plasma glu-

cose concentration and in the longer term by determination of the plasma concentrations of free cortisol and catecholamine.

Acknowledgments—This investigation was assisted by grant number A18615564 from the Australian Research Council to DMS.

REFERENCES

BAMBINO, T., and HSEUH, A. 1981. Direct inhibitory effect of glucocorticoids upon testicular luteinizing hormone receptors and steriodogenesis in vivo and in vitro. *Endocrinology* 108:2142–2153.

BRADLEY, A.J. 1987. Stress and mortality in the red-tailed phascogale *Phascogale calura* (Marsupialia: Dasyuridae). *Gen. Comp. Endocrinol.* 67:85–100.

BRADLEY, A.J. 1990. Failure of glucocorticoid feedback during breeding in the male red-tailed phascogale *Phascogale calura* (Marsupialia: Dasyuridae). *J. Steroid Biochem.* 37:155–163.

BRADLEY, A.J., and STODDART, D.M. 1990. Metabolic effects of cortisol, corticotrophin and adrenalin in the marsupial sugar glider, *Petaurus breviceps* (Marsupialia: Petauridae). *J. Endocrinol.* 127:203–212.

BRAIN, P.F., and NOWELL, N.W. 1970. The effects of differential grouping on endocrine function in mature male albino mice. *Physiol. Behav.* 5:907–910.

EBERHART, J.A., KEVERNE, E.B., and MELLER, R.E. 1980. Social influences on plasma testosterone levels in male talapoin monkeys. *Horm. Behav.* 15:247–266.

HUGGET, A., and NIXON, D.A. 1957. Enzymic determination of blood glucose. *Biochem. J.* 66:12P.

HULSE, G., and COLEMAN, G. 1983. The role of endogenous opioids in the blockade of reproductive function in the rat following exposure to acute stress. *Pharmacol. Biochem. Behav.* 19:795–801.

JOHNSON, B., WELSH, T., and JUNIEWICZ, P. 1982. Suppression of luteinizing hormone and testosterone secretion in bulls following adrenocorticotropin hormone treatment. *Biol. Reprod.* 26:305–310.

LEE, A.K., and MCDONALD, I.R. 1985. Stress and population regulation in small mammals, pp. 261–304. *in* Oxford Reviews of Reproductive Biology. Oxford University Press, Oxford.

MCNEILLY, A., SHARPE, R., and FRASER, H. 1983. Increased sensitivity to the negative feedback effect of testosterone induced by hyperprolactinemia in the adult male rat. *Endocrinology* 112:22–28.

MYKYTOWYCZ, R. 1973. Reproduction in mammals in relation to environmental odours. *J. Reprod. Fertil. Suppl.* 19:433–446.

ROSE, R., and SACHAR, E. 1982. Psychoendocrinology, p. 1383, *in* R.H. Williams (ed.). Textbook of Endocrinology. W.B. Saunders, Philadelphia.

ROSE, R.M., HOLODAY, J.W., and BERNSTEIN, I.S. 1971. Plasma testosterone, dominance rank and aggressive behaviour in male rhesus monkeys. *Nature* 231:366–368.

SAPOLSKY, R.M. 1985. Stress-induced suppression of testicular function in the wild baboon: Role of glucocorticoids. *Endocrinology* 116:2273–2278.

SCHILLING, A., PERRET, M., and PREDINE, J. 1984. Sexual inhibition in a prosimian primate: A pheromone-like effect. *J. Endocrinol.* 102:143–151.

SCOTT, M.P. 1986. The timing and synchrony of seasonal breeding in the marsupial *Antechinus stuartii:* Interaction of environmental and social cues. *J. Mammal.* 67:551–560.

SCOTT, M.P. 1987. The effects of mating and agonistic experience on adrenal function and mortality of male *Antechinus stuartii* (Marsupialia). *J. Mammal.* 68:479–486.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

DODECYL PROPIONATE, ATTRACTANT FROM RAT PUP PREPUTIAL GLAND: CHARACTERIZATION AND IDENTIFICATION

I. BROUETTE-LAHLOU,[1] R. AMOUROUX,[2] F. CHASTRETTE,[2] J. COSNIER,[3] J. STOFFELSMA,[4] and E. VERNET-MAURY[1,*]

[1]*Laboratoire de Physiologie neurosensorielle*

[2]*Laboratoire de Chimie Organique Physique*
CNRS-Université Claude Bernard
Lyon, F-69622 Villeurbanne cedex, France

[3]*Laboratoire d'Ethologie des Communications*
Psychologie et Sciences Sociales
69676 Bron, France

[4]*P.F.W. Central Research Laboratory*
Box 3, 3800 A.A. Amersfoort, The Netherlands

(Received November 7, 1990; accepted March 4, 1991)

Abstract—A chemical agent contained in the rat pup preputial gland was found to regulate anogenital licking, a behavioral pattern crucial to pup survival. Combined gas chromatography and mass spectrometric analysis led to identification of four components in addition to high molecular alkanes (waxes). Independent synthesis added to behavioral observations were used to quantify the dam's response to these components. Among them, dodecyl propionate (DP), a pheromone-like compound, appears to be the only one efficient enough to induce, in the dam, behavior patterns similar to those observed with crude lipidic extract from the preputial gland secretion. Various esters previously were predicted in the adult rat preputial gland. DP is the first ester identified from mammalian infant tissue. These results, in combination with previous findings, allow discussion of the DP pheromonal role.

*To whom correspondences should be addressed.

0098-0331/91/0700-1343$06.50/0

Key Words—Dodecyl propionate, rat pup, preputial gland, maternal behavior, anogenital licking, pheromone.

INTRODUCTION

The anogenital area of Wistar rat pups was shown to be very attractive to the dam (Charton et al., 1971). Brouette-Lahlou et al. (1991) have demonstrated recently that this attraction is a result of preputial gland secretion in pups; olfactory cues alone support this. This attraction is an important link in the mother–young bond. It regulates anogenital licking, a fundamental pattern of maternal care, crucial to pup survival (Brouette-Lahlou et al., 1991). Can this secretion act as a pheromone-like factor?

In insects, many pheromones have been identified since bombykol was isolated in 1959. In mammals, pheromones have been studied to a lesser degree, and sexual odors are most generally thought to sustain pheromonal activity. Many of these odors have been described, but only a few have been isolated and identified. First results were reviewed by Wheeler (1976), Albone (1977), and Müller-Schwarze and Mozell (1976). The concept of pheromones in mammals is now reviewed (Brain et al., 1987). As far as maternal behavior is concerned, dimethyldisulfide (DMDS) was shown to act as a nipple attachment pheromone in pup rats (Pederson and Blass, 1982); odor cues releasing nipple search behavior and nipple attachment are known to be present on the mother's ventral skin and on the nipples. Leon (1983) hypothesized that pup saliva, maternal saliva, and amniotic fluid all contain DMDS and that the pups respond to a single cue throughout lactation. In rabbits, the pheromone induced in nipple searching behavior has not yet been identified (Hudson and Distel, 1983); it appears to form a gradient on the doe's ventral skin, increasing in strength toward the nipples. The possibility that responsiveness to the pheromone is acquired in utero is not excluded (Hudson and Distel, 1984). In the same way, a maternal pheromone from feces of the rat dam promotes brain development in rat pups (Lee and Moltz, 1984): when pups were denied ingesting maternal feces, the reciprocal weight of the whole brain, cerebrum and cerebellum to body weight was significantly lower than in 21-day control young. The retardation in brain development was reflected in experimental young at 21 days in retarded motor development. The chemical nature of the maternal pheromone is not yet known. Moreover, pheromonal emission by pregnant rats protects pups against infanticide (Menella and Moltz, 1989).

In the mother–young bond, a chemical substance from amniotic fluid plays an important role in some species; amniotic fluid influence was well demonstrated in parturient ewes by Levy and Poindron (1984). In goats (Stoddart,

1976), the doe's first reaction is to lick the traces of birth fluid and membranes from the kid; if the kids are taken away before she has a chance to establish any contact with them, rejection in the form of butting and biting of the kid occurs in almost all cases. In rats, both parturient rats and a large percentage of virgin rats avidly consume placenta (Kristal et al., 1981), but the elimination of the ingestion of placenta at parturition by a previously induced taste aversion did not lead to obvious deficits in maternal behavior (Engwall and Kristal, 1977). In fact, results leading to the conclusion that amniotic fluid plays a role in the acceptance of new born rats were obtained in virgins and not in the lactating female (Mayer and Rosenblatt, 1980). Our recent work (Brouette-Lahlou and Vernet-Maury, 1991) is in accordance with these results: in the rat, amniotic fluid is not involved in the mother–young bond. It shortens the maternal sensitization latency in virgins, but it seems to be a negligible stimulus in lactating female rats.

Chemosignals from pup urine were found to be effective on contact, picking-up, retrieval, licking, and nest building by dams and may act as infant signals (Londei et al., 1989): the authors suggested that the urine might leave a persistent chemical signal on the skin of the pup and that this signal could contribute to symbolizing the infant identity. In mammals, these chemical substances are produced mainly by cutaneous glands. In a recent work, Vernet-Maury and Brouette-Lahlou (1985) identified pups' preputial glands secretion as the source of chemosignals regulating dams' specific behavior consisting of licking pups' anogenital areas. These glands were established by Moore and Samonte (1986) as providing chemosignals for maternal discrimination of a pup's sex, thus confirming the authors' previous work (Vernet-Maury and Brouette-Lahlou, 1985; Vernet-Maury et al., 1987).

Unique hypertrophic apocrine sweat glands are described in the neck, perineal, and inguinal skin of mink kids (Yager et al., 1988). These glands enlarge only after birth, regress rapidly and become vestigial by weaning age. Behavioral studies indicate a possible role for the glandular secretion in maternal recognition of the young.

Preputial glands also were described in sympatric voles (Welsh et al., 1988). Gas-chromatographic analysis revealed that the preputial glands of *Microtus montanus* contain a series of species-typical lipids that are not found in *Microtus pennsylvanicus*. Using gas chromatography–mass spectrometry and nuclear magnetic resonance spectrometry, the species-typical lipids were identified as esters of branched, saturated, and unsaturated C-5 and C-4 alcohols and straight-chain C-16, and iso branched C-17 fatty acids.

It is well known that the pup rat, born immature, has to be licked to be able to defecate; therefore, anogenital licking constitutes a fundamental pattern of maternal behavior. Thus, the analysis of preputial gland secretions responsible for this behavior is of the highest importance.

The aim of the study is to identify the chemical structure of the active part of the preputial secretion and investigate its contingent identification.

METHODS AND MATERIALS

Animals

Forty litters of primiparous, lactating females (Wistar SPF), each with eight pups, were involved in the experiment. Each behavioral test was performed on 20 dams.

Preputial glands are bilaterally symmetrical subcutaneous organs located on each side of the midline genitalia. Their excretory ducts run along the lateral surface of the penis or clitoris and empty via a pinpoint opening on the side of the urethral meatus; they do not connect with the terminal urethra. The glands can easily be detected through the still hairless skin in young pups.

The secretions were obtained(1) By pressing the preputial glands of the oldest pups (from 7 days old).(2) By extraction with the aid of a special glass syringe for the younger pups; in this case, the sterile needle of a sterile syringe containing dichloromethane as a solvent was introduced into gland openings. The preputial secretions thus obtained were dissolved in a lipophilic solvent (dichloromethane), as a lipidic nature of the secretions was suspected (Vernet-Maury and Brouette-Lahlou, 1985). We thus obtained the lipidic extract. (3) By gently stroking pups' (male or female) genitalia with cotton swabs impregnated with distilled water anogenital smears were obtained from perineal secretions. Perineal secretions also were obtained, paying attention not to remove urine and feces. Stroking was stopped before urine and/or feces were released. The glandular secretion was obtained from pups of various ages from birth to weaning: 10–100 pups were used according to pups' ages.

Chromatography and GC-MS Analyses

Material Used. In a first experiment, analytical chromatography (Intersmat IGC 120 DFB with flame ionization detector) with a packed column silicone oil (SE 30) was used to study the preputial gland lipidic extract, as first described by Beaver (1960), for adult rats. In a second experiment, a far more efficient capillary column chromatograph (with a OV-101, silicone oil, 25-m-long column, flame ionization detector) was used. To identify the signals, a third experiment using combined gas chromatography and mass spectrometric analysis was undertaken. A Perkin Elmer Sigma 3B chromatograph with a CP SIL5, 25-m-long column and helium as the carrier gas, and a VG 70-70 F mass spectrometer were used (Figure 1).

Organic Compounds and Mass Analysis. Isopropyl myristate (IM),

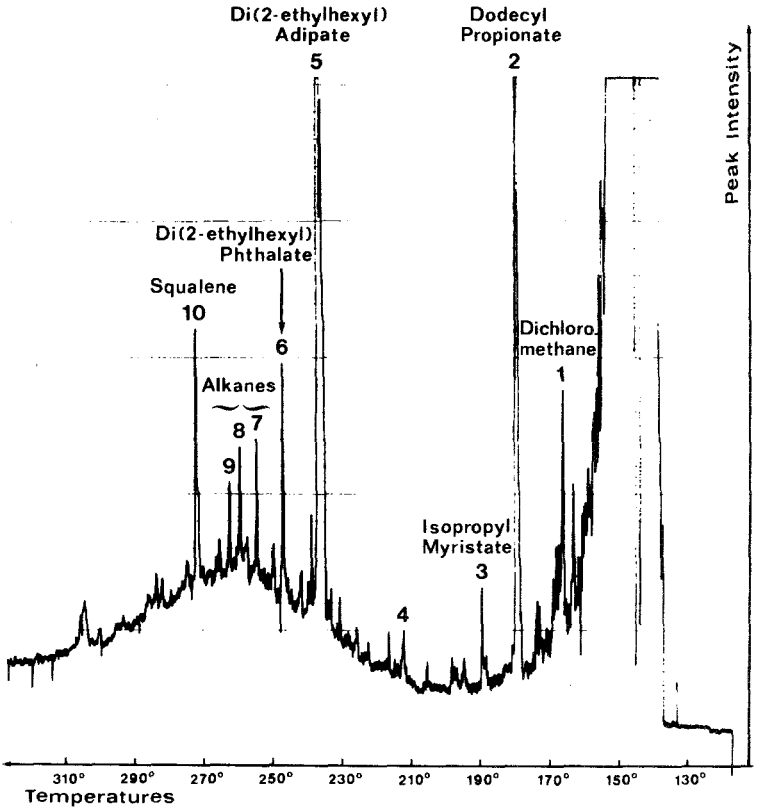


FIG. 1. Capillary column chromatogram (Perkin Elmer Sigma 3B); gas chromatography (GC): SIL 5, 25-m column, vector gas helium; injection temperature 250°C, four temperatures 100–310°C). Each compound giving raise to a signal is analyzed by mass spectrometry (coupled GC-MS) (see Figure 3).

di(2-ethylhexyl) adipate (EHA), and di(2-ethylhexyl)phthalate (EHP) are commercially available. Dodecylpropionate (DP) was synthesized by esterification of propionic acid with *n*-dodecanol (H_2SO_4, 12 hr, reflux), and distilled (bp_{0-1} = 77°C). Mass spectra were performed on a Varian MAT CH5 apparatus (70 eV) and compared with those of the natural extracts.

Behavioral Tests

Tested Odors. The above chemical compounds were compared with natural secretions (anogenital smears) or lipidic extracts obtained from preputial glands contents, using behavioral tests. Pure or mixed chemical compounds

were used according to the relative magnitude of their observed signals in the chromatographic analysis of the natural secretions.

Choice Behavior with Dual-Choice Apparatus. Two identical, perforated containers, depth 30 mm, diameter 50 mm with 6-mm holes at their facing extremities, were fitted to the opposite sides of a cage identical to dams' home cages. Dams were given access to this test cage 24 hr before the experimental test with a view to familiarizing them with these new surroundings (this experimental device has been fully described elsewhere (Brouette-Lahlou et al., 1991). For each test, these two perforated containers contained cotton swabs impregnated either with the sample to be tested or with (1) the control made up of evaporated dichloromethane used as a lipophilic solvent (organic compounds or lipidic extracts) or (2) distilled water (perineal smears). These containers were presented to 20 dams in a dual-choice test. Sniffing times toward the odorous or control container were recorded in a 3-min observation schedule.

Choice Behavior with Glass Dish Selection Test. In order to observe the choice and any ingestive behavior toward active compounds, 20 dams had access to three dishes containing preputial secretions: (A) from eight, 6-day-old pups (male or female), or (B) an equivalent male adult preputial secretion versus (control C) a 1-cm^2 filter paper impregnated with 0.5 ml distilled water. Similarly, after identifying the active compound, 20 dams were observed in the presence of the same dishes containing filter paper impregnated with (1) 0.5 ml dichloromethane with adequate concentration (8 ng) of the synthetic active identified component (D) or with (2) 0.5 ml dichloromethane (control E). Sniffing and any ingestion of contents (filter paper or glandular secretions) by dams from the glass dishes were recorded in a 3-min observation schedule.

RESULTS

Chromatographic Analyses

After preliminary analysis in which two signals were observed, capillary column chromatography separated more than 10 signals (Figure 1). GC-MS results were analyzed with the aid of computerized data bases. Excluding solvent and minor components, eight molecules were identified: dodecyl propionate (DP), (Figures 2 and 3); isopropyl myristate (IM), di(2-ethylhexyl)adipate (EHA), di(2-ethylhexyl)phthalate (EHP) (a well known chromatographic pollutant), and hydrocarbons of high molecular weight (waxes) (signals 7–10). As previously demonstrated (Vernet-Maury et al., 1987), only DP, IM, and EHA might have functioned as the active agents and would have to be subjected to further analysis.

The mass spectra of independently obtained DP, IM, EHA, and EHP were compared to those obtained from GC-MS analyses. The mass spectra were found

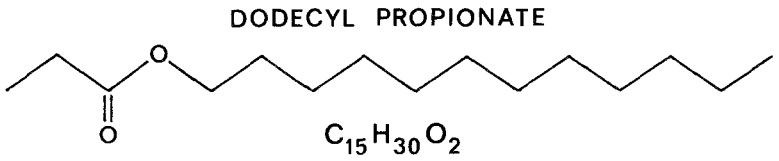


FIG. 2. Dodecyl propionate. Its molecular mass (242) is in agreement with typical values for pheromones (80–300).

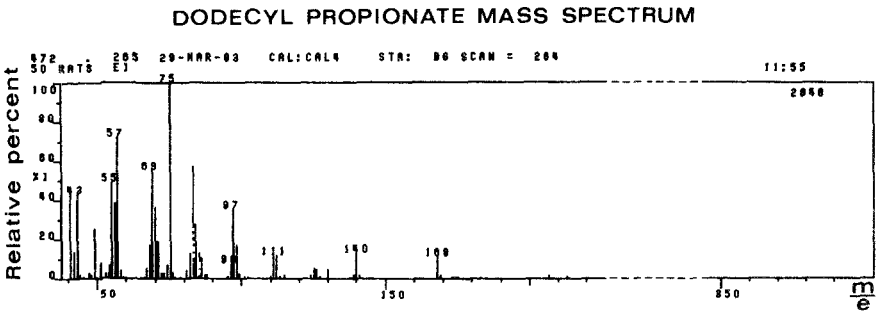


FIG. 3. Mass spectrum of dodecyl propionate (DP) as obtained from joint GC-MS analysis of the preputial extract, identical to the spectrum of the synthesized DP (VG 70-70F mass spectrograph, ionization energy 70 eV, intensity 200 μA, source temperature 200°C). Ion relative intensity (percent of the main ion) is plotted vs . ion ratio of charge to mass (m/e). The retention time of the corresponding signal on the chromatogram is indicated on the right (here 11 min, 55 sec). The main ions observed on the DP spectrum are typical of a propionate (m/e = 75) and of a $C_{12}H_{24}$ fragment (m/e = 168) that corresponds to a six-center rearrangement with expulsion of a neutral molecule of propionic acid and ion $[CH_3-(CH_2)_9-CH=CH_2]^+$. All mass spectra were analyzed and the chemical structures determined by comparison with computerized data bases from Dr. Stoffelsma's laboratory in Amersfoort.

to be clearly identical (the same ionized fragments were observed in both samples) (Figure 3 for DP). These results confirmed the chemical identification of DP, IM, EHA, and EHP. These four chemical compounds were then further studied in behavioral tests. According to the relative magnitude of the chromatographic signals, we used 8.3 ng of DP, 2 ng of IM, 8 ng of EHA, and 6 ng of EHP.

Behavioral Tests

The dams' responses in the dual-choice apparatus are plotted in Table 1. Only DP, either pure or mixed, attracted the dams. The other compounds isolated from pups' preputial glands were not detected significantly. The mixture

containing DP was efficient, but less so than pure DP (Table 1). With regard to dams' sniffing times, statistical differences between the odorous box and control box (Wilcoxon test) emerged for only three stimuli: anogenital smears, lipidic extracts from pups' preputial secretions, and DP; sniffing time was statistically longer toward these three stimuli. Moreover, from a Friedman analysis of variance, no difference was found between the dams' responses to these three stimuli ($\chi_r^2 = 2.9$; $P > 0.2$). In the same way, licking sequences toward the odorous box were observed only in response to DP and natural secretion, smears, or lipidic extract. In consequence, it can be concluded that DP (Figure 2) was the active part of all the extracts, and other tests were undertaken to justify this proposal.

As a main result obtained from the glass dish selection test, a specific ingestive behavior pattern on the part of the dam toward the contents of dishes A and D was observed: 14 dams from 20 actually ate the contents of dish A (pups' preputial gland secretion) (binomial test, $N = 20$, $X = 6$, $P = 0.058$) and 17 dams from 20 ate the contents of dish D (filter paper impregnated with DP) ($N = 20$, $X = 3$, $P = 0.001$). There was no significant difference in the dams' ingestive behavior toward DP (D) and natural secretions (A) (χ_r^2 test =

TABLE 1. RAT SNIFFING TIMES IN PRESENCE OF ODORANTS: SELECTED CHEMICAL COMPOUNDS COMPARED TO PREPUTIAL SECRETIONS[a]

Odor sources[b]	Sniffing time (sec)		
	Sample[c]	Control[d]	P[e]
DP: (S)	23.0(5.7)	4.5(1.7)	**
IM: (S)	4.5(3.0)	4.0(2.0)	ns
EHA: (S)	3.0(2.2)	4.0(3.0)	ns
EHP: (S)	3.0(3.0)	3.5(3.2)	ns
DP: mixture + IM + EHA (S)	12.0(5.2)	2.0(1.0)	*
Pups' anogenital smears (N)	16.0(5.5)	4.0(2.0)	**
Lipidic extract from pups preputial gland (N)	14.0(5.5)	2.5(2.0)	**

[a] Odorants were presented to rat dams as stimuli in a dual-choice test. During the 3-min observation schedule, the time spent by the dams sniffing around each stimulus box was recorded in seconds (sec).

[b] N-natural secretions. S-chemical compounds used according to the relative intensity of the chromatographic signals in the analysis of natural secretions: DP, dodecyl propionate, 8.3 ng; IM, isopropyl myristate, 2 ng; EHA, di(2-ethylhexyl)adipate, 8 ng; and EHP, di(2-ethylhexyl)phthalate, 6 ng.

[c] Sniffing time: median value (semiinterquartile range) in seconds.

[d] For control, evaporated solvent (versus S samples) or distilled water (versus N samples) was used.

[e] P: statistical significance; Wilcoxon, nonparametric test: * <0.05; ** <0.005; ns: nonsignificant.

0.29, $df = 1$, $P > 0.50$). No dam ate the contents of other dishes: adult male preputial gland secretions or control filter papers (B, C, E).

DISCUSSION

Combined gas chromatography–mass spectrometry allowed us to isolate the active part of pups' preputial gland secretion implicated in dams' specific behavior: anogenital licking (AGL). Results obtained from the dual-choice apparatus established that DP is significantly more attractive than the other compounds isolated from rat pups' preputial glands. This attractiveness equaled that of preputial secretions themselves. Dams' ingestive behavior in the dish selection test confirmed the presence and activity of DP within natural preputial gland secretions and suggested that DP is the active part of the preputial gland secretion for which a pheromonal action was originally postulated. The characteristic ingestive behavior induced by DP or natural secretions renders inapplicable the classical criticism addressed to behavioral observations in response to odorants: a specific chemical or blend of chemicals does not elicit behavioral responses other than aspecific sniffing in mammals (Johnston, 1983).

From GC-MS analysis, the DP chemical structure was elucidated and was proven to be in accordance with previous work on rat preputial gland secretion (Stacewicz-Saptuntzakis and Gawienowski, 1977): analysis of the male preputial gland volatiles indicated the presence of ethyl, propyl, isopropyl, pentyl, and decyl acetates among other compounds, i.e., *n*-aliphatic acetates. Pietras (1981) reported that in the lipid composition of rodent preputial glands, wax esters predominate in the neutral lipid fraction, and that other significant classes include glyceryl ether diesters, triglycerides, and sterol esters. The description of such esters from preputial gland secretions confirms our preliminary results (Vernet-Maury and Brouette-Lahlou, 1985) and was verified recently in voles' preputial glands by Welsh et al. (1988). It should be added that esters are widely described in the pheromonal activity of numerous insects, particularly in *Drosophila* (Schaner et al., 1989; Collins and Cardé, 1989; Kou et al., 1989).

Furthermore, Gawienowski et al. (1976) pointed out many preputial components with molecular weights up to approx. 200 from rat preputial gland attractants, while Pietras (1981) estimated these molecular weights from 80 to 300. DP molecular weight (242) is in accordance with these previous values. The low volatility of DP may explain dams' behavior in the dual-choice apparatus: characteristic sniffing behavior is observed only when the dam's nose is about 5 mm from the perforated cover of the test container; vibrisse movements could be seen, sometimes associated with box-cover licking by dams.

Taking into account our recent statement (Brouette-Lahlou et al., 1991) resulting from behavioral assays led us to argue in favor of DP pheromonal activity; DP may act typically as a regulator but not as a signaling substance.

It is well known that preputial gland secretion provides a vehicle for sex pheromones. In mice, they possibly promote an aggression odor (Mugford and Nowell, 1971). In adult rats, the pheromonal nature of preputial gland secretion was well established by Pietras (1981); rat preputial glands elaborate a sex attractant pheromone. Therefore, the preputial gland functions in pups during the neonatal period are different: they produce chemosignals that are used by dams to sustain AGL behavior.

It must be noted that this attractant consists of one active compound only. Unlike insect pheromones, in which a "bouquet phéromonal" has been described (Masson and Brossut, 1981), and also unlike DMDS, which acts in conjunction with other active compounds, DP is as efficient as the whole preputial gland extract. Our ontogenetic analysis (Vernet-Maury et al., 1987) of the pups' preputial secretions confirms this particularity: when the dam begins to perform AGL, about 3 hr after the pup's birth, DP is the only active compound present in the anogenital smears.

Could this identification of the active part of pups' preputial gland secretion be helpful in explaining the mechanisms involved in dam AGL behavior? This would be of major interest, since after removal of the pups' preputial glands, AGL is disorganized: licking time is increased and some pups are never licked, and die (Brouette-Lahlou et al., 1991). The identification of the active part of pups' preputial gland secretion is the first description of such an ester in mammalian infants.

Acknowledgments—We are most grateful to A.M. Brandon for her technical assistance and to M. Anderson and S. Economidès for help with translation.

REFERENCES

ALBONE, E.S. 1977. Ecology of mammals—a new focus for chemical research. *Chem. Br.* 13:92–99.

BEAVER, D. 1960. A re-evaluation of the rat preputial gland as a "dicrine" organ from the standpoint of its morphology, histochemistry and physiology. *J. Exp. Zool.* 143:153–173.

BRAIN, P.F., HOMADY, M.H., CASTANO, D., and PARMIGIANI, S. 1987. "Pheromones" and behaviour of rodents and primates. *Bull. Zool.* 4:279–288.

BROUETTE-LAHLOU, I., and VERNET-MAURY, E. 1991. Amniotic fluid and dodecyl propionate pheromone: Influence on the onset and regulation of maternal anogenital licking in the rat, *in* D.W. MacDonald, D. Müller-Schwarze, and S. Natynczuk (eds.). Chemical Signals in Vertebrates. Oxford University Press. Oxford.

BROUETTE-LAHLOU, I., VERNET-MAURY, E., and CHANEL, J. 1991. Is rat dam licking behavior regulated by pups preputial gland secretion? *Anim. Learn. Behav.* In press.

CHARTON, F., ADRIEN, J., and COSNIER, J. 1971. Déclencheurs chimiques du comportement de lèchage des petits par la ratte parturiente. *Rev. Comp. Anim.* 5:89–94.

COLLINS, R.D., and CARDÉ, R.T. 1989. Wing fanning as a measure of pheromone response on the

male pink bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechudae). *J. Chem. Ecol.* 15(12):2635–2645.

ENGWALL, D.B., and KRISTAL, M.B. 1977. Placentophagia is modifiable by taste aversion conditioning. *Physiol. Behav.* 18:495–502.

GAWIENOWSKI, A.M., ORSULAK, P.J., STACEWICZ-SAPUNTZAKIS, M., and PRATT, J.J. 1976. Attractant effect of female preputial gland extracts on the male rat. *Psychoneuroendocrinology* 1:411–418.

HUDSON, R., and DISTEL, H. 1983. Nipple location by newborn rabbits: Evidence for pheromonal guidance. Behaviour 82:260–275.

HUDSON, R., and DISTEL, H. 1984. Nipple-search pheromone in rabbits: Dependence on season and reproductive state. *J. Comp. Physiol. A.* 155:13–17.

JOHNSTON, R.E. 1983. Chemical signals and reproductive behavior, pp. 3–37, *in* J.G. Vandenberg (ed.). Pheromones and Reproduction in Mammals. Academic Press, New York.

KOU, R., TANG, D.S., and CHOW, Y.S. 1989. Alarm pheromone of pentatomid bug, *Erthesina fullothunberg* (Hemniptera: Pentatomidae). *J. Chem. Ecol.* 15(12):2695–2702.

KRISTAL, M.B., WHITNEY, J.F., and PETERS, L.C. 1981. Placenta on pups' skin accelerates onset of maternal behaviour in non-pregnant Rats. *Anim. Behav.* 29:81–85.

LEE, T.M., and MOLTZ, H. 1984. The maternal pheromone and brain development in the preweaning rat. *Physiol. Behav.* 33:385–390.

LEON, M. 1983. Chemical communication in mother-young interactions, pp. 39–77, *in* J.G. Vandenberg (ed.). Pheromones and Reproduction in Mammals. Academic Press, London.

LEVY, F., and POINDRON, P. 1984. Influence du liquide amniotique sur la manifestation du comportement maternel de la brebis parturiente. *Biol. Behav.* 9:271–278.

LONDEI, T., SEGALA, P., and LEONE, V.G. 1989. Mouse pup urine as an infant signal. *Physiol. Behav.* 45:579–583.

MASSON, C., and BROSSUT, R. 1981. La communication chimique chez les Insectes. La Recherche, 12(121):406–416.

MAYER, A.D., and ROSENBLATT, J.S. 1980. Hormonal interaction with stimulus and situational factors in the initiation of maternal behavior in non pregnant rats. *J. Comp. Physiol. Psychol.* 94:1040–1059.

MENELLA, J.A., and MOLTZ, H. 1989. Pheromonal emission by pregnant rats protects against infanticide by nulliparous conspecifics. *Physiol. Behav.* 46:591–595.

MOORE, C.L., and SAMONTE, B.R. 1986. Preputial glands of infant rats (*Rattus norvegicus*) provide chemosignals for maternal discrimination of sex. *J. Comp. Psychol.* 100(1):76–80.

MUGFORD, R.A., and NOWELL, N.W. 1971. The preputial glands as a source of aggression-promoting odors in mice. *Physiol. Behav.* 6:247–249.

MÜLLER-SCHWARZE, D., and MOZELL, M.M. 1976. Chemical Signals in Vertebrates. Plenum Press, New York.

PEDERSON, P.E., and BLASS, E.M. 1982. Prenatal and postnatal determinants of the 1st. suckling episode in albino rats. *Dev. Psychobiol.* 15(4):349–355.

PIETRAS, R.J. 1981. Sex pheromone production by preputial gland: The regulatory role of oestrogen. Chem. Senses 6(4):391–408.

SCHANER, A.M., TANICO-HOGAN, L.D., and JACKSON, L.L. 1989. (*S*)-2-Pentadecyl acetate and 2 pentadecanone. components of aggregation pheromone of *Drosophila buschii. J. Chem. Ecol.* 15(11):2577–2588.

STACEWICZ-SAPUNTZAKIS, M., and GAWIENOWSKI, A.M. 1977. Rat olfactory response to aliphatic acetates. *J. Chem. Ecol.* 3(4):411–417.

STODDART, H.D. 1976. Mammalian Odours and Pheromones. Camelot Press Ltd, Southampton.

VERNET-MAURY, E., and BROUETTE-LAHLOU, I. 1985. Characterization and identification of the

pheromone of the rat pup's preputial gland. Abstract from 19th international ethological conference. Université P. Sabatier, Toulouse, France.

VERNET-MAURY, E., BROUETTE-LAHLOU, I., and CHANEL, J. 1987. Ontogenetic analysis of the rat pup pheromone implicated in perigenital licking. *Chem. Senses* 12(1):186.

WELSH, C.J., MOORE, R.E., BARTELT, R.J., and JACKSON, L.L. 1988. Novel, species-typical esters from preputial glands of sympatric voles, *Microtus montanus* and *M. pennsylvanicus*. *J. Chem. Ecol.* 14(1):143–158.

WHEELER, J.W. 1976. Insect and mammalian pheromones. *Lloydia* 39(1):53–59.

YAGER, J.A., HUNTER, D.B., WILSON, M.R., and ALLEN, O.B. 1988. A source of cutaneous maternal semiochemicals in the mink? *Experientia* 44(1):79–81.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

WIND-TUNNEL STUDY ON ATTRACTION INHIBITOR IN MALE *Coleophora laricella* Hbn. (LEPIDOPTERA: COLEOPHORIDAE)

PETER WITZGALL [1] and ERNST PRIESNER

Max-Planck-Institut für Verhaltensphysiologie
8131 Seewiesen, Germany

(Received November 26, 1990; accepted March 4, 1991)

Abstract—Modifying effects of (*Z*)-5-decenyl acetate, an attraction-inhibitor of male *C. laricella*, were studied at different levels of the pheromonal behavioral sequence. When blended with the attractant alcohol (*Z*)-5-decen-1-ol, 0.001% acetate significantly reduced and 1% almost abolished orientation flights followed by landing at the source. By contrast, 1000% of the acetate placed 5 cm apart did not suppress orientation flights to the alcohol source. Differential modifying effects of the acetate in blends vs. separate sources also occurred with respect to orientation flight duration, landing position, close-range behavior at the source, and the rate of unsuccessful orientation attempts. Our findings support the view that, in *C. laricella*, pheromonal inhibition results from simultaneous (as opposed to intermittent) perception of attractant and inhibitor. The various effects of the inhibitory acetate observed in the wind tunnel compare to results of earlier field observations.

Key Words—Pheromone, attraction inhibitor, (*Z*)-5-decen-1-ol, (*Z*)-5-decenyl acetate, orientation behavior, wind tunnel, *Coleophora laricella*, Coleophoridae, Lepidoptera.

INTRODUCTION

The suppression of pheromonal orientation by structurally related chemicals is a well-known phenomenon in Lepidoptera. Such "attraction inhibitors" or "pheromone-response antagonists" have been widely demonstrated by field

[1] Present address: Department of Ecology, Lund University, Helgonavägen 5, 223 62 Lund, Sweden.

0098-0331/91/0700-1355$06.50/0

trapping experiments, but there are few attempts to analyze their mode of behavioral action.

Earlier field studies have shown that (Z)-5-decen-1-ol (Z5–10:OH) is a strong attractant for male larch casebearer moths *Coleophora laricella* Hbn. (Priesner et al., 1982). This compound induces a sequence of pheromonal actions in wild males: activation from rest, upwind orientation flight, and close-range behaviors near the source after landing (Witzgall and Priesner, 1984). The corresponding acetate (Z5–10:Ac) is an attractant for various other *Coleophora spp.* (Priesner, 1987), whereas in *C. laricella* this compound strongly reduces trap catches when added to the attractant alcohol (Priesner et al., 1982). Field observations indicated multiple behavioral effects of this inhibitor (Priesner and Witzgall, 1984) and prompted the present wind-tunnel study.

METHODS AND MATERIALS

Insects. Male *C. laricella* were collected as pupae at several locations in southern Germany and the Swiss Alps. The time lag in adult emergence between these different sites permitted us to conduct experiments for about eight weeks, although local flight periods in the field last only one to three weeks (Witzgall, 1985). Pupae and adults were held under a 16:8 light–dark photoperiod.

Wind Tunnel. The tunnel was the one used by Preiss and Priesner (1988). The upwind and downwind ends of the flight compartment (80 × 50 × 50 cm) were covered by black screen. Air (20–22°C) was pulled through the tunnel at 0.25 m/sec and blown out of the building. The tunnel was lit diffusely from above at about 1000 lux.

Chemicals. The two compounds used in this study, Z5–10:OH and Z5–10:Ac were obtained from Dr. S. Voerman, Wageningen. Isomeric purity was approx. 99%. The compounds were applied as hexane solutions to filter paper (1.5 × 1.5 cm).

Stimulus Application. Blends of Z5–10:OH and Z5–10:Ac, and the respective amount of Z5–10:OH alone were tested using a single filter paper held by thin wire inside the tunnel, 1 cm from the upwind screen and 30 cm above the tunnel floor. In tests with separate sources, the two filter papers were fixed, at this position, 5 cm from each other perpendicular to the airstream. For a dosage series of pure Z5–10:OH, the filter paper was positioned inside a conical glass tube, which touched the upwind screen from outside, as in Preiss and Priesner (1988).

Test Procedures. For each test, one to five males were placed in a cotton-screen cage (10 × 8 × 8 cm) at 70 cm downwind from the source(s). Males were exposed to the test chemicals for 1 min. After opening the cage, responses were recorded over 5 min. Countings were made of (1) males leaving the hold-

ing cage by flight; males flying upwind and landing at either (2) <5 cm or (3) >5 cm from the attractant source; and (4) males flying upwind without reaching the source (interrupted orientation flights). Duration of upwind flight (from initiation of oriented flight to landing at the source) and close-range behaviors (walking or sitting on the upwind screen at <5 cm from the source) were taped. Flying males were recorded by two video cameras placed above and at the side of the tunnel. Males were tested once, at the end of the light period.

Test Program. Responses to pure Z5–10:OH were studied over eight decadic dosages, 10^{-5}–10 μg (N = 57–125 males). Blend sources were made of 10^{-1} μg Z5–10:OH plus 10^{-6}–10^{-1} μg Z5–10:Ac (N = 41–94); separate sources of 10^{-1} μg Z5–10:OH and 10^{-6}–1 μg Z5–10:Ac (N = 47–85). Each treatment was tested on at least four days. After an arcsin transformation of the percentages of males showing a specific behavior during each test session, we calculated an analysis of variance, followed by a Duncan test (P = 0.05).

RESULTS

Sources of Z5–10:OH. Behavioral responses of *C. laricella* males to different amounts of pure Z5–10:OH were studied with the source placed in the glass tube applicator. For the seven test doses, 10^{-5}–10 μg, takeoffs from the holding cage during 5 min ranged from 47.4% to 94.1% (Figure 1), compared to 57.9% on stimulation with air alone. Oriented upwind flights followed by landing near the source were displayed by 9.3% of the test males at 10^{-4} μg, while the response rate raised to 68.8% at 10^{-1} μg, but declined to 46.2% at 10 μg. At 10 μg the rate of interrupted upwind flights rose to 11%. The lowest dose of 10^{-5} μg did not elicit upwind flight attempts (Figure 1).

Mean flight duration, from initiation of upwind orientation until landing, ranged from 17.0 $\pm$ 16.3 sec at 10^{-4} μg to 22.9 $\pm$ 14.7 sec at 10 μg. Of the males reaching the source, 79.2% landed within 5 cm from the applicator outlet at 10^{-1} μg, but only 40.0% at 10 μg (Figure 1). Their mean stay within this 5 cm range was longest (37.8 $\pm$ 22.2 sec) at 10^{-2} μg and shortest (17.2 $\pm$ 13.3 sec) at 10 μg.

In addition to the rate of complete orientation flights, three further effects of increasing the amount of Z5–10:OH are apparent from these data: a longer duration of upwind flight, a tendency to land at a greater distance from the source, and a shorter stay near the source after landing.

Z5–10:Ac additions, considered further below, were mainly made to 10^{-1} μg of Z5–10:OH. This amount of Z5–10:OH was accordingly also tested with the filter paper placed inside the tunnel (rather than in the applicator). Responses were as follows: 94.6% males left the holding cage by flight, 3.2% males initiated upwind flight without reaching the source, 66.7% oriented upwind and

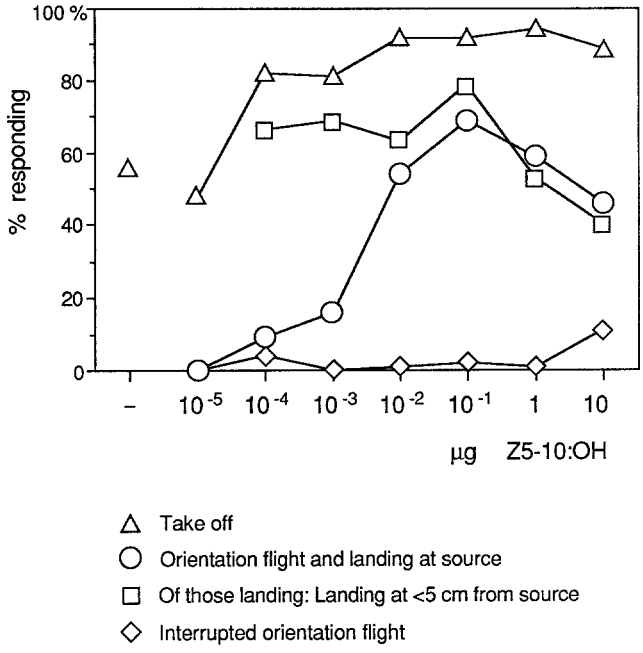


FIG. 1. Responses of *C. laricella* males to varying amounts of attractant alcohol Z5-10:OH in the wind tunnel.

landed near the filter paper; of those, 78.4% landed within 5 cm from the source (Figure 2). Mean flight duration to the source was 16.6 ± 11.3 sec and duration of walking within 5 cm from the source was 32.5 ± 22.5 sec.

Blend Sources of Z5-10:OH/Z5-10:Ac. Single filter papers containing Z5-10:OH (10^{-1} µg) plus Z5-10:Ac (10^{-6} µg to 10^{-1} µg) were placed inside the tunnel. For these six blends, male takeoffs ranged from 81.5% to 97.6%. The $10^{-1}/10^{-6}$ µg and $10^{-1}/10^{-5}$ µg blends elicited complete orientation flights from 39.2% and 40.4% males (significantly less than with 10^{-1} µg of Z5-10:OH alone), of these 56.9% and 42.6% landed at <5 cm from the source (Figure 2A). Flight durations in response to these two blends were 22.1 ± 11.3 sec and 23.7 ± 15.2 sec; the mean stays within the 5 cm range were reduced to 15.7 ± 13.7 sec and 18.4 ± 16.7 sec.

Only 9.6% to 3.7% test males successfully oriented to either the $10^{-1}/10^{-4}$ µg, $10^{-1}/10^{-3}$ µg, or $10^{-1}/10^{-2}$ µg blends (Figure 2A). All but one landed at >5 cm from the source. Interrupted orientation flights, rarely seen in tests of 10^{-1} µg Z5-10:OH alone (Figure 1), were observed with 10.6% to 28.9% males in response to the $10^{-1}/10^{-6}$ µg to $10^{-1}/10^{-3}$ µg blends. The $10^{-1}/10^{-1}$ µg blend did not elicit any upwind flights (Figure 2A).

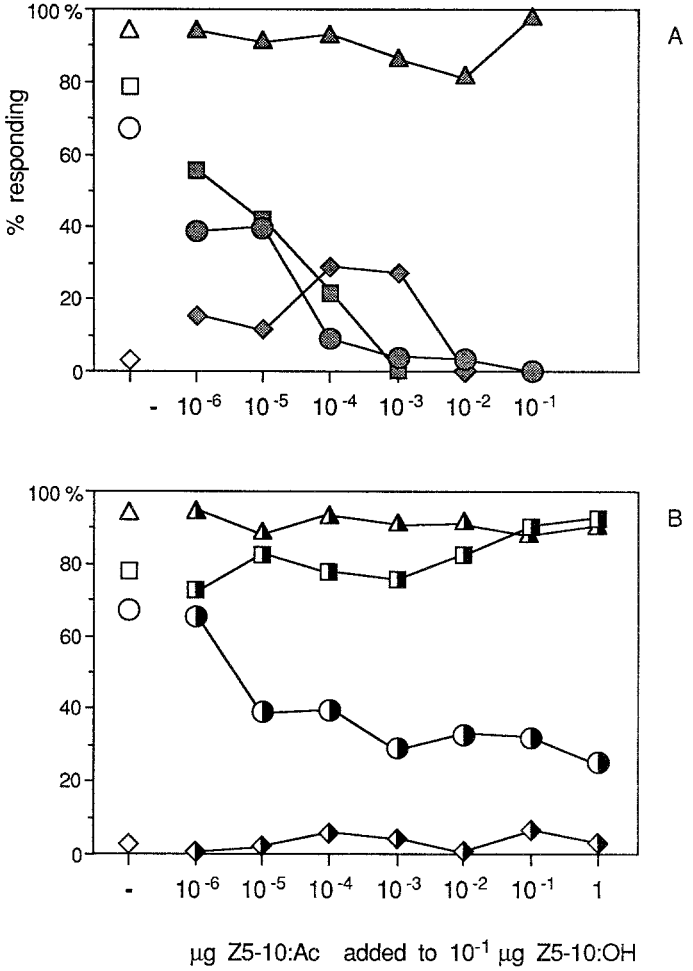


FIG. 2. Responses of *C. laricella* males to 10^{-1} µg Z5 -10:OH plus varying amounts of Z5 -10:Ac in the wind tunnel. (A) Blend sources; both compounds applied to the same filter paper. (B) Separate sources; compounds applied to two different filter papers placed 5 cm apart and perpendicular to the airstream. Plot symbols as in Figure 1.

These data show that Z5–10:Ac, when blended with the attractant alcohol Z5–10:OH, modifies the pheromonal behavior of *C. laricella* males in several respects: the rate of orientation flights to the source decreases, whereas the rate of interrupted flights increases; males land at a greater distance from the source, and they spend less time near it. The most sensitive behavioral criterion was the rate of successful orientation flights, which was significantly lower on addi-

tion of only 0.001% Z5-10:Ac (blend ratio $10^{-1}/10^{-6}$ μg). Visual inspection of video recordings showed that flights towards blends were rather erratic with wide excursions as compared to flights to alcohol alone.

Separate Sources of Z5-10:OH and Z5-10:Ac. The effects of seven different doses of Z5-10:Ac on responses to 10^{-1} μg Z5-10:OH were studied by placing the two filter papers 5 cm apart. Takeoff rates ranged between 88.9% and 95.7%. The rate of orientation flights that led to landing near the 10^{-1} μg Z5-10:OH source was not altered (65.9%) by 10^{-6} μg, but was significantly reduced (38.1%) by 10^{-5} μg Z5-10:Ac, decreasing further (25.5%) for the highest Z5-10:Ac dose of 1 μg (Figure 2B). In an additional test ($N = 49$ males), 10 μg of the acetate was placed only 1 cm from the 10^{-1} μg Z5-10:OH source. Even with this 100-fold amount of the "inhibitor," eight males still reached the Z5-10:OH source by oriented flight.

As in tests with blends, rapid changes in flight direction were observed frequently at some distance from the two sources, whereas over the last 20–30 cm males were often seen to advance to the alcohol source in rather straight flight. Accordingly, mean flight duration continually decreased with the dose of the separate Z5-10:Ac source, from 16.2 ± 9.7 sec at 10^{-6} μg to 8.4 ± 2.5 sec at 1 μg.

For all Z5-10:OH/Z5-10:Ac combinations, the majority of males that reached the Z5-10:OH source landed within 5 cm of it, such as in tests with 10^{-1} μg Z5-10:OH alone; at the high acetate doses of 10^{-1} μg and 1 μg even 90.5% and 92.7% (Figure 2B). After landing, the males generally remained longer within the 5 cm range compared to tests of Z5-10:OH alone; their mean stay within this area ranged from 38.2 ± 21.1 sec to 73.9 ± 58.3 sec for the different Z5-10:OH/Z5-10:Ac combinations. No landing responses directed to the Z5-10:Ac rather than the Z5-10:OH source were seen.

From these data, the following effects of a separate Z5-10:Ac source on responses of *C. laricella* males to Z5-10:OH are apparent: less males orient successfully towards the attractant source, flight duration is shorter, males land closer to the source and stay longer near it after landing. The separate Z5-10:Ac source did not raise the rate of uncompleted orientation attempts, thus again differing from its effects in the blend source tests.

DISCUSSION

As with "pheromone inhibitors" in other Lepidoptera, the effect of Z5-10:Ac on pheromonal attraction of *C. laricella* to Z5-10:OH was first established by field tests, using compound blends released from the same dispenser (Priesner et al., 1982). However, subsequent field observations showed quite

contrasting effects between blends and separate sources of attractant and inhibitor (Priesner and Witzgall, 1984).

The behavioral effects of the inhibitory acetate observed in this wind-tunnel study and the field (Priesner and Witzgall, 1984) appear analogous. In both tests, even small amounts of acetate in the blend reduced the rate of upwind flights to the source, induced landings further away from the source, and shortened the duration of postlanding behaviors. With separate sources of attractant and inhibitor, even high amounts of the acetate inhibitor did not suppress orientation flights, but flight duration was shorter and landings were directed closer to the alcohol source, both in the laboratory and the field.

The successful orientation of *C. laricella* males to a source of Z5–10:OH with a high dose of Z5–10:Ac placed only a few centimeters apart shows that the odor filaments emanating from the two sources were sufficiently separated to be distinguished by the flying moths, a conclusion compatible with current concepts of fine-scale characteristics of odor plumes (Murlis and Jones, 1981). Sanders (1981) expressed the view that a male moth, flying in an atmosphere permeated with attractant and inhibitor released from separate sources, would move towards the source as long as he encounters "pockets" of pure attractant.

In this context it has been proposed that the contrasting behavioral effects of the inhibitory Z5–10:Ac, on male *C. laricella*, observed in blend-source vs. separate-source experiments are primarily due to a different temporal pattern of perception of the two compounds (Priesner and Witzgall, 1984). It is, according to this view, the simultaneous (as opposed to intermittent) stimulation of the Z5–10:OH and Z5–10:Ac receptor neurons which appears to be particularly "inhibitory."

Some behaviors observed with separate attractant and inhibitor, such as landings closer to the alcohol source or certain modifications of flight tracks (Priesner and Witzgall, 1984), even suggest that males reduce perception of the acetate by active movements.

Using the same wind-tunnel setup, Preiss and Priesner (1988) studied effects of several attraction inhibitors in *Laspeyresia pomonella* as reflected by orientation flight rates and landing responses towards blend sources. Similarly, in wind-tunnel studies on *Trichoplusia ni*, *Mamestra suasa*, and *Ostrinia nubilalis*, antagonistic effects of chemicals blended with pheromone were evaluated by arrestment of upwind flight (Linn et al., 1984; Frérot et al., 1989; Glover et al., 1989). In male *Lymantria dispar*, the inhibitory (−)-enantiomer of disparlure lowered the percentage of males initiating upwind flight and reduced stabilization of altitude and speed during tethered flight but had little effect on preflight or postlanding male responses (Cardé and Hagaman, 1979; Cardé, 1981; Preiss and Kramer, 1983; Preiss, 1985). As shown here for *C. laricella*, an "attraction inhibitor" may affect not only upwind orientation flight but also

other pheromonal behaviors and may have quite contrasting modifying effects depending on its presentation with the pheromone stimulus.

Acknowledgments—We thank Dr. R. Preiss, Hamburg, for the use of his wind tunnel and Dr. J.A. Byers, Lund, for comments on the manuscript.

REFERENCES

CARDÉ, R.T. 1981. Precopulatory sexual behavior of the adult gypsy moth, pp. 572–587, *in* C.C. Doane, and M.L. McManus (eds.). The Gypsy Moth: Research towards Integrated Pest Management. USDA For. Serv. Tech. Bull. 1584, Washington D.C.

CARDÉ, R.T., and HAGAMAN, T.E. 1979. Behavioral responses of the gypsy moth in a wind tunnel to air-borne enantiomers of disparlure. *Environ. Entomol.* 8:475–484.

FRÉROT, B., LUCAS, P., and ROCHAT, D. 1989. Responses of *Mamestra suasa* male moths to synthetic pheromone compounds in a wind tunnel. *Entomol. Exp. Appl.* 53:81–87.

GLOVER, T.J., PEREZ, N., and ROELOFS, W.L. 1989. Comparative analysis of sex-pheromone-response antagonists in three races of European corn borer. *J. Chem. Ecol.* 15:863–873.

LINN, C.E., JR., BJOSTAD, L.B., DU, J.W., and ROELOFS, W.L. 1984. Redundancy in a chemical signal: Behavioral responses of male *Trichoplusia ni* to a 6-component sex pheromone blend. *J. Chem. Ecol.* 10:1635–1658.

MURLIS, J., and JONES, C.D. 1981. Fine-scale structure of odour plumes in relation to insect orientation to distant pheromone and other attractant sources. *Physiol. Entomol.* 6:71–86.

PREISS, R. 1985. Lack of effect of (−)-disparlure on orientation towards (+)-disparlure source in walking and flying gypsy moth males. *J. Chem. Ecol.* 11:885–894.

PREISS, R., and KRAMER, E. 1983. Stabilization of altitude and speed in tethered flying gypsy moth males: Influence of (+)- and (−)-disparlure. *Physiol. Entomol.* 8:55–68.

PREISS, R., and PRIESNER, E. 1988. Responses of male codling moths (*Laspeyresia pomonella*) to codlemone and other alcohols in a wind tunnel. *J. Chem. Ecol.* 14:797–813.

PRIESNER, E. 1987. (Z) -5-Dodecen-1-ol, another inhibitor of pheromonal attraction in *Coleophora laricella*. *Z. Naturforsch.* 42c:1349–1351.

PRIESNER, E., and WITZGALL, P. 1984. Modification of pheromonal behaviour in wild *Coleophora laricella* male moths by (Z)-5-decenyl acetate, an attraction-inhibitor. *Z. Angew. Entomol.* 98:118–135.

PRIESNER, E., ALTENKIRCH, W., BALTENSWEILER, W., and BOGENSCHÜTZ, H. 1982. Evaluation of (Z)-5-decen-1-ol as an attractant for male larch casebearer moths, *Coleophora laricella*. *Z. Naturforsch.* 37c:953–966.

SANDERS, C.J. 1981. Disruption of spruce budworm mating—state of the art, pp. 339–349, *in* E.R. Mitchell (ed.). Management of Insect Pests with Semiochemicals. Plenum Press, New York.

WITZGALL, P. 1985. Diurnal and seasonal flight activity of male *Coleophora laricella* as determined by sex-attractant traps. *Z. Angew. Entomol.* 100:225–233.

WITZGALL, P., and PRIESNER, E. 1984. Behavioural responses of *Coleophora laricella* male moths to synthetic sex attractant, Z-5-decen-1-ol, in the field. *Z. Angew. Entomol.* 98:15–30.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

INHIBITION OF SUNFISH FEEDING BY DEFENSIVE STEROIDS FROM AQUATIC BEETLES: STRUCTURE–ACTIVITY RELATIONSHIPS

DONALD J. GERHART,[1,*] MARIA E. BONDURA,[2] and JOHN A. COMMITO[2]

[1]*Duke University Marine Laboratory*
Beaufort, North Carolina 28516
[2]*Department of Biology*
Hood College
Frederick, Maryland 21701

(Received January 14, 1991; accepted March 7, 1991)

Abstract—The vertebrate hormone deoxycorticosterone is the most commonly occurring component of defensive secretions from aquatic beetles in the family Dytiscidae. Deoxycorticosterone and the structurally related steroids pregn-4-en-20α-ol-3-one and pregn-4-en-20β-ol-3-one were tested for their ability to inhibit feeding by bluegill sunfish, *Lepomis macrochirus*, in laboratory assays. Deoxycorticosterone at oral doses of 660 μg (2×10^{-6} mol) per pellet caused 94% inhibition in the acceptance of artificial food pellets. At the same molar dosage, pregn-4-en-20α-ol-3-one inhibited food consumption by 58%, while its epimer, pregn-4-en-20β-ol-3-one, did not significantly inhibit feeding. These results indicate that specific stereochemical conditions must be satisfied for the pregnenes to be noxious to *L. macrochirus* and suggest the existence of a receptor–ligand interaction. The potency of the three steroids in assays of feeding inhibition contradicts earlier results based on toxicity and anesthetic assays in which fish were immersed in solutions of steroids.

Key Words—Deoxycorticosterone, pregnene, Dytiscidae, chemical defense, structure–activity relationships, bluegill sunfish, *Lepomis macrochirus*.

INTRODUCTION

Aquatic beetles in the family Dytiscidae secrete from the prothoracic glands a complex mixture of steroids. The prothoracic gland discharges to the outer sur-

*To whom correspondence should be addressed.

0098-0331/91/0700-1363$06.50/0

face of dytiscid beetles and secretions of the gland provide a chemical defense against potential predators (reviewed by Scrimshaw and Kerfoot, 1987). A major component of the prothoracic secretions is the "vertebrate" hormone deoxycorticosterone (Schildknecht et al., 1966), an adrenocortical steroid that is involved in the regulation of salt and water balance. Pregnene derivatives structurally related to deoxycorticosterone also are present in the prothoracic secretions of dytiscid beetles.

Predatory fish reject dytiscid beetles, and fish immersed in aqueous solutions of dytiscid steroids become anesthetized and eventually die (Selye and Heard, 1943; Miller and Mumma, 1976a,b). The relative potencies of steroidal components of dytiscid secretions have been quantified using these toxicity assays (Miller and Mumma, 1976a). In toxicity assays, aqueous solutions of test agents are in contact for extended periods of time with the gill membranes and other external surfaces of fish. Miller and Mumma (1976b) suggested that the defensive steroids acted through the destabilization and lysis of fish membranes. Alternatively, the defensive steroids may be sensed by olfactory and/or gustatory receptors of fish and perceived as noxious (Clayton, 1964; Miller and Mumma, 1976b). Fish that attack dytiscid beetles frequently do not swallow the insects but rather spit them out, apparently after sensing the noxiousness of the beetle (Scrimshaw and Kerfoot, 1987).

The goals of this study were to: (1) determine whether deoxycorticosterone and two related pregnenes from dytiscid beetles (Figure 1) were distasteful to

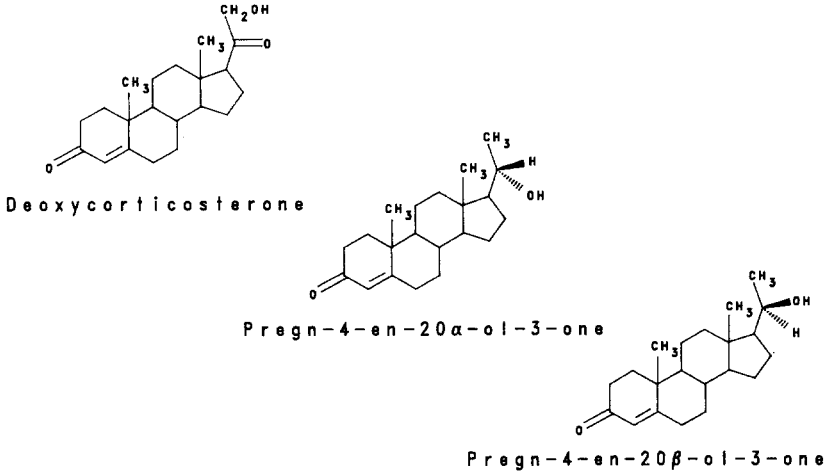


FIG. 1. Structures of deoxycorticosterone, pregn-4-en-20α-ol-3-one, and pregn-4-en-20β-ol-3-one. All three compounds are present in the prothoracic secretions of dytiscid beetles.

the sympatric, predatory fish *Lepomis macrochirus* (bluegill sunfish); (2) determine whether these three pregnenes varied significantly in their noxiousness to *L. macrochirus*; and (3) identify structural features of steroids associated with noxiousness to fish.

METHODS AND MATERIALS

Food pellets were produced by dissolving agarose in hot water to yield a 3% solution of agarose. Commercial fish food at a concentration of 5% (w/w) was added to the agarose as a flavoring agent. Deoxycorticosterone, pregn-4-en-20α-ol-3-one [= pregn-4-en-20(*S*)-ol-3-one], and pregn-4-en-20β-ol-3-one [= pregn-4-en-20(*R*)-ol-3-one, Figure 1] were dissolved in methanol to produce stock solutions of 1.8×10^{-1} M. Sufficient steroid solution then was dispersed through the agarose to yield 2×10^{-6} mol of steroid in a 50-μl aliquot of agarose mixture. Control pellets contained an equal amount of methanol but no steroids. Pellets were produced by cooling 50-μl aliquots of agarose mixture to room temperature. Steroid-treated pellets contained equimolar amounts of either deoxycorticosterone (660 μg), pregn-4-en-20α-ol-3-one (633 μg), or pregn-4-en-20β-ol-3-one (633 μg). Schildknecht (1971) noted that quantities of deoxycorticosterone can be as high as 1 m per beetle; therefore, the level of this steroid in the treated pellets was similar to levels that can occur in dytiscid beetles. The tested dosages of pregn-4-en-20α-ol-3-one and pregn-4-en-20β-ol-3-one were higher than those that occur in dytiscids and were employed to permit an assessment of potency relative to deoxycorticosterone.

Individual bluegill sunfish (*Lepomis macrochirus*) were placed in glass aquaria (25 × 15 × 18 cm) and randomly assigned to treatments. Following 24 hr of starvation, control and steroid-treated pellets were presented singly to the isolated fish in the following sequence: control pellet, steroid pellet, steroid pellet, control pellet. The initial action of each fish after pellet presentation was scored as acceptance (pellet swallowed) or rejection (pellet ignored or pellet mouthed and rejected). Only initial responses of the fish were used, since repeated mouthings and extended immersion can alter concentrations of test agents in the agarose pellets. The use of behavior scoring and sequential offering of single pellets allowed detection of facilitation or inhibition of feeding during the course of the experiment, such as satiation of the test fish or formation of a learned aversion to treated foods (Gerhart, 1984; Gerhart et al., 1988).

Rates of pellet acceptance for the different treatments were compared statistically using *G* tests for independence and employing Yates' correction (Sokal and Rohlf, 1981). Transition matrices (Legendre and Legendre, 1983) were constructed and analyzed to determine whether consumption or rejection of the

first treatment pellet significantly influenced the probability of consumption of the second. The percent inhibition of feeding caused by addition of each of the steroids relative to controls was calculated from the combined data. Inhibition was calculated as the difference between control and treatment acceptance rates, divided by the control rate.

RESULTS

Deoxycorticosterone significantly lowered rates of pellet acceptance relative to the rate of acceptance of controls (Figure 2A, Yates' corrected $G = 9.5$ for both the first and second treatment pellets, $df = 1$, $P < 0.005$). Pregn-4-en-20α-ol-3-one also significantly lowered rates of pellet acceptance relative to controls (Figure 2B; Yates' corrected $G = 8.6$, $P < 0.005$ for the first treatment pellet; Yates' corrected $G = 6.0$, $P < 0.025$ for the second treatment pellet; $df = 1$ for both comparisons). Addition of pregn-4-en-20β-ol-3-one did not significantly alter the rate of pellet acceptance relative to the rate of acceptance of control foods (Figure 2C; Yates' corrected $G = 0.04$; $df = 1$, $P > 0.5$).

The rate of acceptance of control pellets (Table 1) did not differ significantly between beginning and end of experiments for any steroid treatment (Yates' corrected $G < 0.6$, $df = 1$, $P > 0.1$ for each of the three treatments), or for the combined data for all experiments (Yates' corrected $G = 0.5$; $df = 1$, $P > 0.1$). Thus, there was no indication that fish became satiated through the course of the experiment. Furthermore, analysis of the transition matrices showed no sequence-dependent facilitation or inhibition (chi-square test for goodness of fit; $\chi^2 < 1.5$, $df = 3$, $P > 0.5$ for each treatment). Since there was no evidence that responses to the two treatment pellets were statistically dependent (Legendre and Legendre, 1983), the results for each of the three treatments were combined to calculate the relative potency of the steroids at the level employed in the feeding inhibition assays.

Analysis of the combined data (Table 2) showed that the degree of feeding inhibition was dependent upon the structure of the steroid added to the agarose pellets (Yates' corrected $G = 17.4$; $df = 2$, $P < 0.001$). Deoxycorticosterone was the most potent of the compounds, inhibiting pellet consumption by 94% relative to controls. Pregn-4-en-20α-ol-3-one was intermediate in potency, causing a 58% inhibition of feeding. Pregn-4-en-20β-ol-3-one, which did not significantly reduce pellet acceptance, inhibited consumption by 11%.

DISCUSSION

Deoxycorticosterone was the most active of the three steroids tested in this study. Pregn-4-en-20α-ol-3-one, which differs from deoxycorticosterone only in the structure of the C-17 side-chain (Figure 1), possessed decreased potency

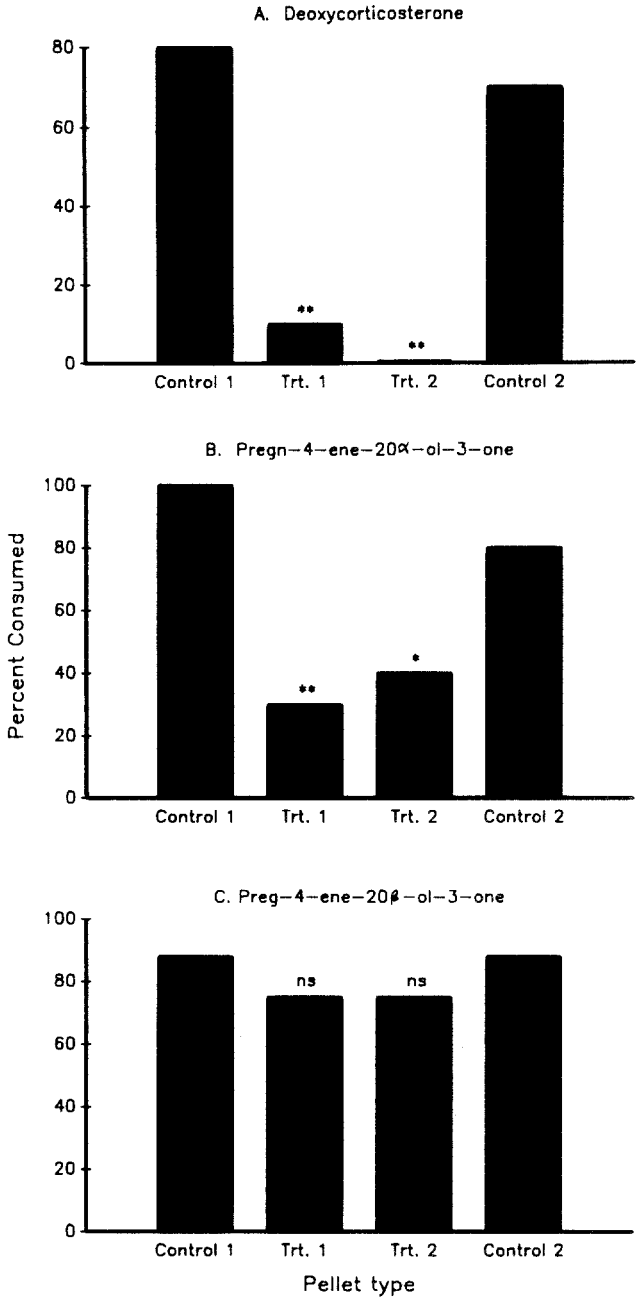


FIG. 2. Acceptance of steroid-containing food pellets and control pellets by bluegill sunfish (*Lepomis macrochirus*). Experiments utilized sequential offering of foods to fish. Key to symbols: ns = not significantly different from controls; * = significantly different from controls with $P < 0.025$; ** = significantly different from controls with $P < 0.005$.

TABLE 1. COMPARISON OF RATES OF ACCEPTANCE OF INITIAL AND FINAL CONTROL PELLETS BY *Lepomis macrochirus* FOR EACH EXPERIMENT AND COMBINED DATA SET[a]

	Eaten (*N*)	Not eaten (*N*)	Yates' corrected *G*
Deoxycorticosterone			
First control pellet	8	2	0.0
Last control pellet	7	3	
Pregn-4-en-20α-ol-3-one			
First control pellet	10	0	0.579
Last control pellet	8	2	
Pregn-4-en-20β-ol-3-one			
First control pellet	7	1	0.0
Last control pellet	7	1	
Combined data			
First control pellet	25	3	0.533
Last control pellet	22	6	

[a]Rates of acceptance did not change significantly during the trials, indicating that satiation of the fish did not occur.

TABLE 2. COMBINED ACCEPTANCE DATA AND RELATIVE POTENCIES OF DEOXYCORTICOSTERONE, PREGN-4-EN-20α-OL-3-ONE, AND PREGN-4-EN-20β-OL-3-ONE

Treatment	Pellets eaten (%)	Inhibition of feeding (%)	Relative potency
Deoxycorticosterone	5 (*N* = 20)	94	1.000
Pregn-4-en-20α-ol-3-one	35 (*N* = 20)	58	0.620
Pregn-4-en-20β-ol-3-one	75 (*N* = 16)	11	0.113
Controls	84 (*N* = 56)		

in the bioassay. This result indicates that structure of the C-17 side-chain is important for inhibition of fish feeding.

Transformation of pregn-4-en-20α-ol-3-one to pregn-4-en-20β-ol-3-one by epimerization at carbon 20 is accompanied by a fivefold reduction in potency in the sunfish feeding assay. This high degree of stereospecificity suggests that, in order to be perceived as noxious by *Lepomis macrochirus*, dytiscid steroids must interact with gustatory receptors in the buccal or pharyngeal region of the fish. Detection of dytiscid steroids by the fish is followed by rejection of the food item.

The potency relationships of the three steroids differ from earlier relationships based on assays in which steroids were externally applied to fish. Miller

and Mumma (1976a) concluded that the steric positions of hydroxyl groups were of only slight importance with respect to potency of steroids in anesthetic and toxicity assays. Both Clayton (1964) and Miller and Mumma (1976b), however, suggested that the defensive properties of steroids could result from noxiousness and not from anesthetic or toxic properties. Assays of feeding inhibition mimic the way in which defensive steroids are delivered to potential predators more closely than do anesthetic or toxicity assays. Thus, the potency relationships found in this study should reflect more closely the defensive capability of dytiscid steroids in nature.

The potency of the three steroids as feeding inhibitors parallels the taxonomic breadth of distribution of the chemicals among families of aquatic beetles. Deoxycorticosterone, the most potent of the three tested steroids, is also the most broadly distributed prothoracic gland steroid in the family Dytiscidae, occurring in 15 species. Pregn-4-en-20α-ol-3-one, which was intermediate in effectiveness and 62% as potent as deoxycorticosterone at the tested levels, occurs in three beetle species. The least active of the three steroids, pregn-4-en-20β-ol-3-one, has been reported from two beetle species (Scrimshaw and Kerfoot, 1987). The two steroid epimers do not appear to co-occur in any single species of beetle (Scrimshaw and Kerfoot, 1987). Elegant studies have been performed to measure the chemical defensive titer of dytiscids and its rate of regeneration (Fescmeyer and Mumma, 1983). The results of the present study, however, suggest that the prothoracic gland steroids of dytiscids may not be equally potent predator deterrents. Future studies of the defensive titer of dytiscids may gain further insight by focusing on the steroids that are most effective at deterring predation.

Why do some species of dytiscid beetles contain significant quantities of pregn-4-en-20β-ol-3-one, as well as other pregnenes with a 20β-hydroxy functionality (Scrimshaw and Kerfoot, 1987), when these compounds appear to have little effectiveness against *Lepomis macrochirus?* One possible explanation is that the 20β-hydroxy steroids are effective against other predator species that have different steric requirements for noxiousness in their olfactory receptors. Alternatively, the presence of palatable steroids in dytiscids may reflect the use of prothoracic secretions for both antipredator and nondefensive roles. Eisner (1970) and Blum (1981) noted that earlier researchers believed the prothoracic steroids of dytiscids played an antimicrobial or hydrophobic role, and Scrimshaw and Kerfoot (1987) suggested that the prothoracic secretions could serve multiple functions. Additional experiments are required to elucidate further the ecological roles of dytiscid prothoracic secretions.

Acknowledgments—We thank Dan Rittschof, Paul Scheuer, and Charles Kerfoot for their help in shaping the ideas contained in this manuscript. Dan Rittschof critically reviewed the manuscript. Helen Nearing supplied expert secretarial support. M.E.B. and J.A.C. acknowledge support from a Whitaker Foundation grant to Hood College. D.J.G. acknowledges support from the Office of Naval Research and the Duke University Research Council.

REFERENCES

BLUM, M.S. 1981. Chemical Defenses of Arthropods. Academic Press, New York.

CLAYTON, R.B. 1964. The utilization of sterols by insects. *J. Lipid Res.* 5:3–18.

EISNER, T. 1970. Chemical defense against predation in arthropods, pp. 235–280, *in* E. Sondheimer and J.B. Simeone (eds.). Chemical Ecology. Academic Press, New York.

FESCMEYER, H.W., and MUMMA, R.O. 1983. Regeneration and biosynthesis of dytiscid defensive agents (Coleoptera: Dytiscidae). *J. Chem. Ecol.* 9:1449–1463.

GERHART, D.J. 1984. Prostaglandin A_2: An agent of chemical defense in the Caribbean gorgonian *Plexaura homomalla. Mar. Ecol. Progr. Ser.* 19:181–187.

GERHART, D.J., RITTSCHOF, D., and MAYO, S.J. 1988. Chemical ecology and the search for natural antifoulants: Studies of a predator–prey symbiosis. *J. Chem. Ecol.* 14:1905–1917.

KERFOOT, W.C. 1982. A question of taste: Crypsis and warning coloration in freshwater zooplankton communities. *Ecology* 63:538–554.

LEGENDRE, L., and LEGENDRE, P. 1983. Numerical Ecology. Elsevier Scientific, Amsterdam.

MILLER, J.R., and MUMMA, R.O. 1976a. Physiological activity of water beetle defensive agents. I. Toxicity and anesthetic activity of steroids and norsesquiterpenes administered in solution to the minnow *Pimephales promelas* Raf. *J. Chem. Ecol.* 2:115–130.

MILLER, J.R., and MUMMA, R.O. 1976b. Physiological activity of water beetle defensive agents. II. Absorption of selected anesthetic steroids and norsesquiterpenes across the gill membranes of the minnow *Pimephales promelas* Raf. *J. Chem. Ecol.* 2:131–146.

SCHILDKNECHT, H. 1971. Evolutionary peaks in the defensive chemistry of insects. *Endeavour* 30:136–141.

SCHILDKNECHT, H., SIEWERDT, R., and MASCHWITZ, U. 1966. A vertebrate hormone as defensive substance of the water beetle (*Dytiscus marqinalis*). *Angew. Chem. Int. Ed. Eng.* 5:421–422.

SCRIMSHAW, S., and KERFOOT, W.C. 1987. Chemical defenses of aquatic organisms: Beetles and bugs, pp. 240–262, *in* W.C. Kerfoot and A. Sih (eds.). Predation: Direct and Indirect Impacts on Aquatic Communities. University of New Hampshire Press, New Hampshire.

SELYE, H., and HEARD, R.D.H. 1943. The fish assay for the anesthetic effect of the steroids. *Anesthesiology* 4:36–47.

SOKAL, R., and ROHLF, F.J. 1981. Biometry: Principles and Practice of Statistics in Biological Research. Freeman Press, New York.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

POTENT NATURAL EGG-LAYING STIMULANT FOR CABBAGE BUTTERFLY *Pieris rapae*

ROGER M.M. TRAYNIER [1,*] and ROGER J.W. TRUSCOTT[2]

[1]*Division of Entomology, CSIRO*
GPO Box 1700
Canberra ACT 2601, Australia
[2]*Department of Chemistry*
University of Wollongong
Wollongong NSW 2500, Australia

(Received February 9, 1990; accepted March 11, 1991)

Abstract—Solutions of glucobrassicin (3-indolylmethyl glucosinolate) purified from foliage and sinigrin (allyl glucosinolate) elicited oviposition by the cabbage butterfly, *Pieris rapae*, at threshold concentrations as low as 10^{-6} M. At higher concentrations, glucobrassicin elicited a faster oviposition rate and a stronger visual response to the substrate through associative learning. Solutions of 10^{-5} M glucobrassicin and 10^{-2} M sinigrin stimulated equally. Their enzymic hydrolysis products failed to influence oviposition. The markedly greater potency of glucobrassicin is consistent with known glucosinolate profiles of crucifers which show indolyl glucosinolates predominant in foliage.

Key Words—Plant–insect interaction, *Brassica campestris*, oviposition, 3-indolylmethyl glucosinolate, learning, reflective spectra, *Pieris rapae*, Lepidoptera, Pieridae.

INTRODUCTION

The botanical specificity of insects restricted to crucifers has long been linked with the presence in host plants of a class of involatile sulfur compounds, the glucosinolates, whose toxicity has been overcome and which now induce feeding and oviposition by the specialized insects (Feeny et al., 1970; Blau et al., 1978; Städler, 1978; Koritsas et al., 1989). The chemical ecology of insects of crucifers has the potential to be understood thoroughly, although impediments

*To whom correspondence should be addressed.

0098-0331/91/0700-1371$06.50/0

have been insufficient chemical analysis of glucosinolates (GSLs), and a lack of behaviorally interpretable bioassays. More than 70 GSLs are known, with 50 in the Brassicaceae, differing in a side chain derived from one of a range of amino acids (Kjaer, 1976; Fenwick, 1983). A major advance in GSL chemistry was the combination of a desulfonating enzyme with HPLC (Minchinton et al., 1982) to identify GSLs directly and more reliably than previously by inference from hydrolysis products. The new method confirmed the predominance of indolyl GSLs in brassica foliage (Sang et al., 1984). The present report seems to be the first concerning the influence of an indolyl GSL on the behavior of an insect. Nonindolyl GSLs have been compared as oviposition stimulants (Ma and Schoonhoven, 1973; Nair and McEwen, 1976) and feeding stimulants (Hicks, 1974; Nault and Styer, 1972), but no ecologically interpretable pattern emerged for different GSLs. Green cards treated with aqueous cabbage extracts elicited more oviposition by *P. rapae* than those treated with allyl GSL (sinigrin) solution (Renwick and Radke, 1983), and after further experiments with plant extracts, it was suggested that unknown substances that were not GSLs were involved (Renwick and Radke, 1988).

In field experiments, Jones (1987) recorded that a mated female *P. rapae* distributed several hundred eggs during several days by repeated, single ovipositions on host plants, in bouts of oviposition in which landings on potential host plants alternated with short flights. In the laboratory, also, flights alternated with oviposition on paper wetted with aqueous GSL solution, provided the paper was green, yellow, or pale blue. *P. rapae* learned to respond to the appearance of a chemically favorable substrate (Traynier, 1984, 1986) but did not learn to associate the presence of a chemical deterrent with the appearance of its substrate (Traynier, 1987). Interpretable bioassays of GSLs therefore required a consideration of visual responses including the influences of learning.

METHODS AND MATERIALS

In earlier experiments on oviposition by *P. rapae*, a traditional bioassay with different solutions offered on substrates of identical appearance revealed little preference for higher concentrations of sinigrin (Traynier, 1986). This lack of chemical discrimination probably resulted from butterflies learning to associate the presence of sinigrin with the appearance of the test substrate, and then responding sufficiently to visual stimuli to override chemical influences. In contrast, when butterflies were offered different solutions on disks of different but equally attractive appearance, they learned to land and oviposit on disks wetted with a 100-ppm rather than a 75-ppm solution of sinigrin. This assay method, with differently colored but equally attractive disks, was employed in the present experiments to measure oviposition by *P. rapae* elicited by different GSLs

offered at the same concentration. The insects were reared on Chinese cabbage, *Brassica campestris*.

Mated female *P. rapae*, 5–8 days old, without ovipositional experience and carrying about 100 oocytes, were tested in 60 × 60 × 60-cm cages under artificial light at 6000 lux from "daylight" tubes. Artificial blue flowers provided sucrose solution. A tray of damp peat moss in the floor of the cage humidified it to 70% relative humidity at 27°C. Experiments were made on a black bench surrounded by pink organza drapes (Sandoz dye, Nylosan Red N2RBL) within either one or a pair of cages made from a tubular frame covered by nylon mesh of the same color. Against this pink background, the green blotting papers (1) Ford's Great Green and (2) Australian Paper Manufacturers' Shoalhaven Green were accepted equally for oviposition when wetted with the same GSL solution. Butterflies oviposited on horizontal 58-mm-diam. disks made from a double layer of 0.15-mm thick celluloid supported horizontally by a central pin and covered on top with a disk of blotting paper of the same diameter. The paper was wetted with 0.8 ml of water or solution, and two disks were offered with centers 20 cm apart in tests of 20 min duration, to butterflies in groups of three, which were numbered on the hindwings for individual recognition. To start the tests, butterflies were taken in turn with their wings between the thumb and forefinger and made to walk on the oviposition sites for 10 sec to contact the test solution with their tarsal chemoreceptors and initiate oviposition behavior.

The landings and ovipositions of the butterflies were tape recorded by spoken commentary. After 10 min, 20% of the water had evaporated from the disks and was replaced around their edges with drops from a pipet. Air in the laboratory was stirred by a fan to encourage flight by the butterflies.

RESULTS

Reflectance spectra were obtained to define the oviposition substrates and cage fabric (Figure 1). Although the cage fabric was pink, it reflected a range of wavelengths including even more green and yellow (500–600 mm) than the wet green oviposition substrates. As *P. rapae* showed no ovipositional response to the pink fabric by tarsal drumming, we concluded that wavelengths additional to the green–yellow range might actively deter oviposition rather than being merely neutral.

Wet versus Dry Substrates. The most common GSL in experimental analyses of the chemical ecology of crucifers has been sinigrin (allyl glucosinolate) obtained from mustard seed. Since sinigrin and water were needed together to elicit oviposition by *P. rapae* (Traynier, 1984), we tested glucobrassicin with and without water. A green paper disk was wetted with 10^{-4} M glucobrassicin

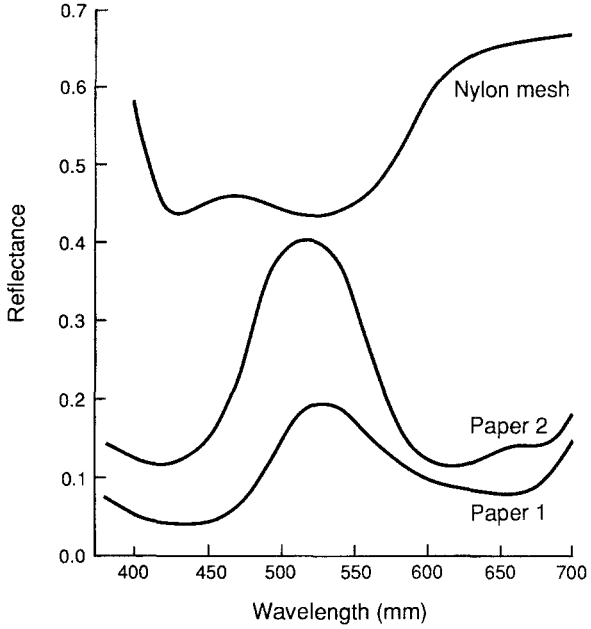


FIG. 1. Diffuse spectral reflectance from pink nylon mesh (Nylosan Red N2RBL Sandoz dye) and wetted blotting papers: (1) Ford's Great Green; (2) Australian Paper Manufacturers' Shoalhaven Green.

solution and allowed to dry. Ten naive, gravid female butterflies were made to walk on the dry paper to establish tarsal contact, then released. They all flew off, only one made a return flight, and none oviposited. In similar tests made with the same butterflies, and the same paper rewetted with water, nine of the 10 butterflies flew back to the disk to attempt oviposition and eight succeeded. We concluded that glucobrassicin, like sinigrin, elicited oviposition only in the presence of water. Our subsequent experiments used these GSLs in aqueous solution.

Threshold Concentrations. Previous results (Traynier, 1984) suggested a threshold concentration for sinigrin at about 10^{-6} M, and we tested sinigrin and glucobrassicin at this concentration. Gravid butterflies were made to walk on a green paper disk with a GSL solution for 10 sec to establish tarsal contact, then released. Of 30 butterflies placed on sinigrin solution, 14 made one or more return flights and nine of these laid a mean of five eggs (SE $\pm$ 2.2). Of 30 butterflies that contacted glucobrassicin, 17 returned and 16 laid a mean of nine eggs (SE $\pm$ 2.1). These differences were not significant, either between the number of butterflies landing or ovipositing (2×2 χ^2) or between mean oviposition per butterfly (Mann-Whitney). We concluded that both compounds had

closely similar threshold concentrations at about 10^{-6} M for ovipositional behaviors. Confirmatory evidence came from a comparison of 10^{-6} M and 10^{-8} M glucobrassicin, conducted as before, in which 10^{-8} M failed to elicit oviposition from any of 30 butterflies that contacted it, whereas 13 of 30 oviposited on disks with 10^{-6} M solution.

Comparisons above Threshold. To test concentrations above threshold, 10^{-4} M solutions of sinigrin and glucobrassicin were offered on different shades of green paper for 15 min. Each butterfly preferred to land and to oviposit on glucobrassicin disks ($P < 0.001$, sign test) irrespective of their shade of green (Figure 2). There were 78 ± 4% and 79 ± 4% of ovipositions by each butterfly on glucobrassicin disks of green shades 1 and 2, respectively, with respective landings of 65 ± 3% and 71 ± 3%. In contrast, when the solutions were offered in a separate experiment on disks of identical appearance (green 2), the butterflies showed no preference, with 7 ± 2 and 8 ± 2 ovipositions on sinigrin and glucobrassicin disks, respectively. The landing choice of butterflies paralleled the ovipositional choice, as in previous experiments in which butterflies learned to recognize the appearance of acceptable substrates (Traynier, 1984, 1986). We concluded that glucobrassicin was more stimulating than sinigrin at 10^{-4} M, but this influence could be overridden by visual learning with disks of the same color, as butterflies associated the presence of the most stimulating GSL with the appearance of its substrate and thereafter visual responses obscured differences between GSL solutions.

Glucobrassicin versus Water. The potency of the indolyl compound was

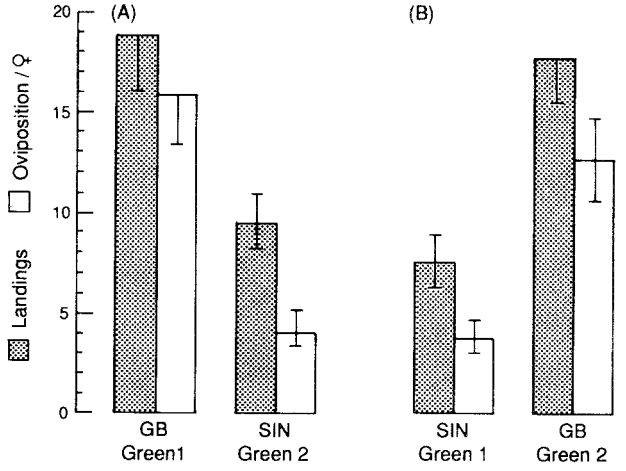


FIG. 2. Mean landings (shaded) and ovipositions (unshaded) (±SE) by 15 ovipositing *P. rapae* on a binary choice of green disks wetted with either sinigrin or glucobrassicin at 10^{-4} M, offered simultaneously in paired cages (A, B) on alternative green papers.

confirmed by a comparison of water and 3×10^{-4} M glucobrassicin applied to different shades of green. Glucobrassicin was markedly preferred with mean egg counts from 15 butterflies of 18 ± 3 vs. 2 ± 0.07 (respective solutions on green shades 1 and 2) and 16 ± 3 vs. 2 ± 0.05 (on shades 2 and 1). Oviposition on the water disks was 15% of the egg count in the first half of the test period, but only 3% in the second. The counts from individual butterflies showed an increase in oviposition preference between the first and second halves ($P < 0.001$, sign test). As *P. rapae* are known to learn, it seems likely they had learned to be more discriminating in the course of the test period.

Influence of Experience. In a comparison at even higher concentration (10^{-3} M) on different shades of green, glucobrassicin was again preferred to sinigrin (Figure 3). Two hours after these tests ended, the same butterflies were offered a choice of the two kinds of green disks, but wetted only with water. The butterflies tended to land initially on the same shade of green on which they had contacted glucobrassicin previously. Of 18 butterflies tested with GSLs, 17 landed later on water disks with a first landing choice of 15:2 in favor of the shade of green that mimicked the previously experienced glucobrassicin disk. Moreover, they oviposited with a preference for appearance corresponding to their earlier chemical preference ($P < 0.001$, Wilcoxon matched-pairs). Thus, these butterflies had learned a stronger response to the appearance of disks with glucobrassicin as opposed to sinigrin. In addition, a learned response to water

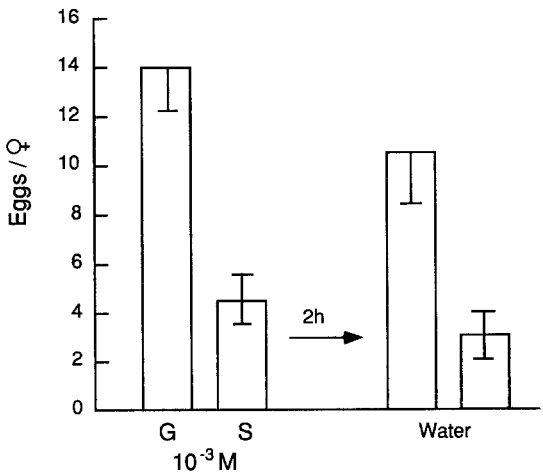


FIG. 3. *P. rapae* oviposition on alternative shades of green disks with either sinigrin or glucobrassicin at 10^{-3} M in an initial 15-min test, followed 2 hr later by oviposition on water disks of the same shade of green.

from the initial test might have contributed to the later oviposition on disks with water alone.

Equipotent Concentrations of Sinigrin and Glucobrassicin. As the responses to the two GSLs had been not significantly different at 10^{-6} M, the differences in experiments with 10^{-4}–10^{-3} M indicated a steeper dose–response curve for glucobrassicin. Oviposition equivalent to 10^{-5} M glucobrassicin was elicited by 10^{-2} M sinigrin; each induced a mean of 6 $\pm$ 1 eggs per butterfly offered these solutions on different shades of green disk.

Influence of Allyl Isothiocyanate. The question remained as to whether hydrolysis products were implicated, either singly or jointly, with GSLs. It was known that allyl isothiocyanate, derived from sinigrin, fails to elicit oviposition from *P. rapae* in the presence of water (Terofal, 1965; Traynier, 1984). As glucobrassicin and sinigrin hydrolyze to different chemical species at neutral pH, we tested the natural enzymic hydrolysis products of each in combination with the alternative GSL. We tested allyl isothiocyanate with potassium sulfate, the coproduct of enzymic hydrolysis at neutral pH, together with glucobrassicin, all three substances at 10^{-4} M in water on the one disk, against a solution of 10^{-4} M glucobrassicin on another disk of the alternative shade of green, to ensure good chemical discrimination by the butterflies. Mean oviposition by 12 butterflies given this choice yielded 7 $\pm$ 1.3 eggs on the mixture and 7 $\pm$ 1.4 eggs on the GSL with no significant preference. Clearly all oviposition had been elicited by the GSL alone.

Influence of Indolyl-3-carbinol. Enzymic hydrolysis of glucobrassicin at neutral pH, as can occur in leaves, yields indolyl-3-carbinol and the thiocyanate ion (Kjaer, 1976). Accordingly, a sinigrin solution with indolyl-3-carbinol in suspension and with potassium thiocyanate, all at 10^{-3} M, was compared with a 10^{-3} M sinigrin solution on the alternative shade of green paper. There was no significant difference. In another experiment, an aqueous suspension of indolyl-3-carbinol corresponding to 10^{-3} M was offered to *P. rapae*, together with a separate water disk, and a sinigrin solution disk, all three of green shade 2. Following contact with sinigrin, butterflies learned to oviposit on disks of the same appearance but treated with water only or with the aqueous carbinol suspension. This allowed a comparison between water with carbinol suspension for their effects on oviposition, knowing that oviposition deterrents do not contribute to associative learning. Ten butterflies oviposited 8 $\pm$ 1.2 eggs on sinigrin disks and 3 $\pm$ 0.8 eggs on both the carbinol and the water disks. Again the carbinol suspension showed no significant influence on oviposition as compared with water.

Glucobrassicin at acid pH can form indolyl-3-acetonitrile (Kjaer, 1976). This was tested like the carbinol and also showed no significant influence on oviposition.

DISCUSSION

It is known that crucifers vary in susceptibility to oviposition by *P. rapae* even after the butterfly has landed on them (Ives, 1978), and in previous attempts to analyze these effects, either total GSL content or GSL profiles without indole compounds were obtained (Nair et al., 1976; Rodman and Chew, 1980; Ahman, 1982). Although indolyl GSLs generally predominate in *Brassica* foliage, they occur in different amounts, and the oil-seed variety Zem 2 of *Brassica juncea* is unusual with sinigrin predominant in its foliage and indolyl GSLs present at low levels (Fenwick et al., 1983). From the present findings, such a plant might be expected to show a low chemical susceptibility to *P. rapae*. Another consideration, however, is that the leaf surface with which *P. rapae* makes only superficial contact (Traynier and Hines, 1987) might have a different GSL profile from the entire leaf. Taking visual responses into account, the least susceptible of all crucifers might not only lack indolyl GSLs but also be less stimulating visually and less distinguishable by *P. rapae* from other host plants by its appearance.

The finding that hydrolysis products of GSLs were ineffective oviposition stimulants for *P. rapae* differs from findings for cabbage root fly, *Delia radicum*, which oviposits in soil adjacent to the host following responses to both isothiocyanates and to GSLs (Finch, 1980; Schöni et al., 1987). The lack of responses of *P. rapae* to isothiocyanates might reflect a requirement for healthy, insect-free foliage as optimal larval food, whereas undamaged foliage might be less important to the root feeding larvae of *D. radicum*. Despite differences in responses to hydrolysis products, other insects that respond to crucifer foliage might, like *P. rapae*, be highly responsive to indolyl GSLs.

The suggestion arising from the spectral reflectance measurements that "red" wavelengths from foliage might actively deter oviposition may be of more general interest since some "red" brassicas are less preferred by other insects of crucifers as well as by ovipositing *P. rapae* (Dunn and Kempton, 1976; Rothschild and Schoonhoven, 1977; Latheef and Irwin, 1979; Myers, 1985), and it is known that additional wavelengths reflected from the sky by plastic or metal deter aphids from green foliage (Kring, 1972; Wyman et al., 1979).

Acknowledgments—We thank N. Michajlovskij, Slovak Academy of Sciences, Bratislava Kramare, Czechoslovakia, for glucobrassicin TMA salt, and E. van Papenrecht for technical assistance. Reflectance spectra were provided through the Unisearch Scheme by Dr. S. Dain and C. Stamp of the Optometry Department, University of New South Wales, Sydney, Australia.

REFERENCES

AHMAN, I. 1982. A comparison between high and low glucosinolate cultivars of summer oilseed rape (*Brassica napus* L.) with regard to their level of infestation by the brassica pod midge (*Dasineura brassicae* Winn.) *Z. Angew. Entomol.* 94:103–109.

BLAU, P.A., FEENY, P., CONTARDO, L., and ROBSON, D.S. 1978. Allylglucosinolate and herbivorous caterpillars: A contrast in toxicity and tolerance. *Science* 200:1296–1298.

DUNN, J.A., and KEMPTON, D.P.H. 1976. Varietal differences in the susceptibility of Brussels sprouts to lepidopterous pests. *Ann. Appl. Biol.* 82:11–19.

FEENY, P., PAAWE, K.L., and DEMONG, N.J. 1970. Flea beetles and mustard oils: host plant specificity of *Phyllotreta cruciferae* and *P. striolata* adults (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 63:832–841.

FENWICK, G.R., HEANEY, R.K., and MULLIN, W.J. 1983. Glucosinolates and their breakdown products in food and food plants. *C.R.C. Crit. Rev. Food Sci. Nutr.* 18:123–201.

FINCH, S. 1980. Chemical attraction of plant-feeding insects to plants. *Appl. Biol.* 5:67–143.

HICKS, K.L. 1974. Mustard oil glucosides: Feeding stimulants for adult cabbage flea beetles, *Phyllotreta cruciferae* (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 67:261–264.

IVES, P.M. 1978. How discriminating are cabbage butterflies? *Aust. J. Ecol.* 3:261–276.

JONES, R.E. 1987. Behavioural evolution in the cabbage butterfly (*Pieris rapae*). *Oecologia* 72:69–76.

KJAER, A. 1976. Glucosinolates in the Cruciferae, pp. 207–219, *in* J.G. Vaughan, A.J. MacLeod, and B.M.G. Jones (eds.). *The Biology and Chemistry of the Cruciferae*. Academic Press, London.

KORITSAS, V.M., LEWIS, J.A., and FENWICK, G.R. 1989. Accumulation of indole glucosinolates in *Psylloides chysocephala* L.-infested, or -damaged tissues of oilseed rape (*Brassica napus* L.). *Experientia* 45:493–495.

KRING, J.B. 1972. Flight behavior of aphids. *Annu. Rev. Entomol.* 17:461–492.

LATHEEF, M.A., and IRWIN , R.D. 1979. Factors affecting oviposition of *Pieris rapae* on cabbage. *Environ. Entomol.* 8:606–609.

MA, W.C., and SCHOONHOVEN, L.M. 1973. Tarsal contact chemosensory hairs of the large cabbage white butterfly *Pieris brassicae* and their possible role in oviposition behavior. *Entomol. Exp. Appl.* 16:342–357.

MINCHINTON, I., SANG, J., BURKE, D., and TRUSCOTT, R.J.W. 1982. Separation of desulphoglucosinolates by reversed-phase high performance liquid chromatography. *J. Chromatogr.* 247:141–148.

MYERS, J.H. 1985. Effect of physiological condition of the host plant on the ovipositional choice of the cabbage white butterfly *Pieris rapae*. *J. Anim. Ecol.* 54:193–204.

NAIR, K.S.S., and MCEWEN, F.L. 1976. Host selection by the adult cabbage maggot, *Hylemya brassicae* (Diptera: Anthomyiidae): Effect of glucosinolates and common nutrients on oviposition. *Can. Entomol.* 108:1021–1030.

NAIR, K.S.S., MCEWEN, F.L., and SNIECKUS, V. 1976. The relationship between glucosinolate content of cruciferous plants and oviposition preferences of *Hylemya brassicae* (Diptera: Anthomylidae). *Can. Entomol.* 108:1031–1036.

NAULT, L.R., and STYER, W.E. 1972. Effects of sinigrin on host selection by aphids. *Entomol. Exp. Appl.* 15:423–437.

RENWICK, J.A.A., and RADKE, C.D. 1983. Chemical recognition of host plants for oviposition by the cabbage butterfly, *Pieris rapae* (Lepidoptera: Pieridae). *Environ. Entomol.* 12:446–450.

RENWICK, J.A.A., and RADKE, C.D. 1988. Sensory cues in host selection for oviposition by the cabbage butterfly, *Pieris rapae*. *J. Insect Physiol.* 34:251–257.

RODMAN, J.E., and CHEW, F.S. 1980. Phytochemical correlates of herbivory in a community of native and naturalized Cruciferae. *Biochem. Syst. Ecol.* 8:43–50.

ROTHSCHILD, M., and SCHOONHOVEN, L.M. 1977. Assessment of egg load by *Pieris brassicae* (Lepidoptera: Pieridae). *Nature* 266:352–355.

SANG, J., MINCHINTON, I.R., JOHNSTONE, P.K., and TRUSCOTT, R.J.W. 1984. Glucosinolate profiles in the seed, root and leaf tissue of cabbage, mustard, rapeseed, radish and swede. *Can. J. Plant Sci.* 64:77–93.

SCHÖNI, R., STÄDLER, E., RENWICK, J.A.A., and RADKE, C.D., 1987. Host and non-host chemicals influencing the oviposition behaviour of several herbivorous insects, pp. 31–36, *in* V. Labeyrie, G. Fabres, and D. Lachaise (eds.). *Insects–Plants*, Dr. W. Junk, Dordrecht.

STÄDLER, E. 1978. Chemoreception of host plant chemicals by ovipositing females of *Delia (Hylemya) brassicae*. *Entomol. Exp. Appl.* 24:511–520.

TEROFAL, F. 1965. Zum Problem der Wirtsspezifität bei Pieriden (Lep.) unter besonderer Berücksicktigung der einheimischen Arten *Pieris brassicae* L., *P. napi* L. und *P. rapae* L. *Mitt. Münchener Entomol. Ges.* 55:1–76.

TRAYNIER, R.M.M. 1984. Associative learning in the oviposition behaviour of the cabbage butterfly, *Pieris rapae*, induced by contact with plants. *Physiol. Entomol.* 4:87–96.

TRAYNIER, R.M.M. 1986. Visual learning in assays of sinigrin solution as an oviposition releaser for the cabbage butterfly, *Pieris rapae*. *Entomol. Exp. Appl.* 40:25–33.

TRAYNIER, R.M.M. 1987. Learning without neurosis in host finding and oviposition by the cabbage butterfly, *Pieris rapae*, pp. 243–247, *in* V. Labeyrie, G. Fabres, and D. Lachaise (eds.). *Insects–Plants*. Dr. W. Junk, Dordrecht.

TRAYNIER, R.M.M., and HINES, E.R. 1987. Probes by aphids indicated by stain induced fluorescence in leaves. *Entomol. Exp. Appl.* 45:198–201.

WYMAN, J.A., TOSCANO, N.C., KIDO, K., JOHNSON, H., and MAYBERRY, K.S. 1979. Effects of mulching on the spread of aphid-transmitted watermelon mosaic virus to summer squash. *J. Econ. Entomol.* 72:139–143.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

REPLACEMENT OF THE TERMINAL METHYL GROUP IN A MOTH SEX PHEROMONE COMPONENT BY A HALOGEN ATOM: HYDROPHOBICITY AND SIZE EFFECTS ON ELECTROPHYSIOLOGICAL SINGLE-CELL ACTIVITIES

STIG JÖNSSON,[1] TOMMY LILJEFORS,[1,*] and BILL S. HANSSON[2]

[1]*Department of Organic Chemistry 3*
Chemical Center, University of Lund
P.O. Box 124, S-221 00 Lund, Sweden
[2]*Department of Animal Ecology*
Ecology Building, University of Lund
S-223 62 Lund, Sweden

(Received November 28, 1990; accepted March 14, 1991)

Abstract—Analogs of (*Z*)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*, with a chloro, bromo, or iodo substituent in place of the terminal methyl group have been synthesized and tested by electrophysiological single-sensillum recordings. The electrophysiological results have been interpreted in terms of substituent size and hydrophobicity. Interaction energies based on receptor interaction models have been calculated using the molecular mechanics [MM2(85)] method. The results support our previously suggested receptor interaction model in which the terminal alkyl chain interact with a complementary hydrophobic receptor "pocket" with very limited flexibility.

Key Words—Lepidoptera, noctuidae, Agrotis segetum, (*Z*)-5-decenyl acetate, pheromone analogue, halides, structure-activity, single-sensillum recordings, receptor-interaction.

INTRODUCTION

Our previous structure–activity studies on chain-elongated (Liljefors et al., 1985, 1987), chain-shortened (Bengtsson et al., 1990), and alkyl-substituted (Jönsson et al., 1991) analogs of (*Z*)-5-decenyl acetate (**1**, Figure 1) a phero-

*To whom correspondence should be addressed.

0098-0331/91/0700-1381$06.50/0

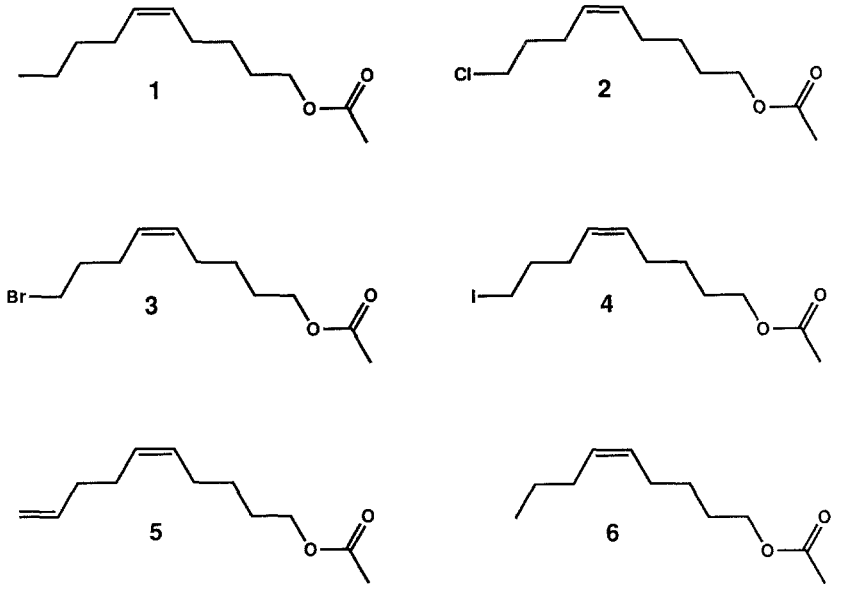


FIG. 1. Compounds studied.

mone component of the turnip moth, *Agrotis segetum*, have revealed a close fit between the receptor and the terminal methyl group of the pheromone component. The electrophysiological single-cell activities of chain-elongated and alkyl-branched analogs of **1** were found to be significantly reduced compared to that of **1**. This was explained by steric repulsive interactions between the substrate and the receptor, which in the chain-elongated case made it necessary for the substrate to undergo conformational changes. Chain-shortening of **1** by one methylene unit resulted in a drop of the electrophysiological activity by a factor of 16, indicating that the region of the receptor cavity which is interacting with the terminal part of the substrate must closely correspond to the natural substrate.

In addition to the *Z*-double bond and the acetate group, the importance of the terminal methyl group for the full activity of a pheromone component seems to be a general feature in structure–activity studies on moth sex pheromone components (Priesner et al., 1975; Priesner, 1979a,b). Recently, a few studies on structural modifications of the terminal methyl group in moth sex pheromone components have been reported. Schwarz et al. (1990) and McLean et al. (1989) have studied the replacement of a terminal CH_3 group by a CH_2F group. This small perturbation of the terminal methyl group resulted in retained or only slightly lowered biological activity (EAG, wind tunnel, field trapping) for the

species studied. However, Sun and Prestwich (1989) report that a 12,12,12-trifluoromethyl analog of (Z)-9-dodecenyl acetate analog is inactive in vivo.

To further characterize the important interaction between the terminal part of the pheromone component and its receptor, we have in the present work studied the replacement of the terminal methyl group in **1** by chlorine (**2**), bromine (**3**), and iodine (**4**) (Figure 1). These analogs provide a variation of the size and the hydrophobicity of the terminal group in a controlled way. We report the synthesis of compounds **2–4** and their biological activities determined by the single-cell technique. By using simple receptor–substrate interaction models, the steric and hydrophobic contributions to the receptor interaction and the ability of the receptor to adapt to terminal substituents of different size are investigated, employing molecular mechanics calculations. Two previously studied analogs, **5** (Bengtsson et al., 1990) and **6** (Bengtsson et al., 1987), are included in the discussions for comparison.

METHODS AND MATERIALS

Synthesis

All reactions were performed under a nitrogen atmosphere in oven-dried glassware. Anhydrous diethyl ether and tetrahydrofuran (THF) were distilled from solutions of sodium benzophenone. The final products were purified by high-performance liquid chromatography (HPLC) on a semipreparative straight-phase column (Kromasil, particle size 10 μm, 250 × 10 mm ID) and monitored with a differential refractometer. Analytical GC was performed with a Varian 3400 capillary GC fitted with a DB-Wax 30-m × 0.25-mm-ID column.

[^{1}H]-and [^{13}C]NMR spectra were determined in $CDCl_3$ solution on a Varian XL-300 spectrometer; chemical shifts (δ) are expressed downfield relative to TMS. The $CHCl_3$ signal was used as an internal reference (7.26 ppm). High-resolution mass spectra were obtained using a Jeol JMS-SX 102 spectrometer with GC inlet.

The synthetic scheme for the preparation of **2–4** is shown in Figure 2.

(Z)-5-Decenyl Acetate **(1)**. This was prepared as previously described (Olsson et al., 1983).

9-Chloro-(Z)-5-nonenyl Acetate **(2)**. To a stirred solution of lithium bis[(Z)-6-(1-ethoxyethoxy)-hex-1-enyl] cuprate (6 mmol) in 50 ml ether (Jönsson et al., 1991), was added at −30°C 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (DMPU) (10 ml) in THF (25 ml) and then 1-bromo-3-chloropropane (5.0 ml, 50 mmol). After 6 hr of stirring at −30°C to −10°C, the mixture was hydrolyzed with 3 M HCl, worked up, and acetylated by acetic acid–acetyl chloride (10:1) at 35°C. The crude acetate was purified by flash chromatography with TLC-silica gel 60H (Merck) with petroleum ether–ethyl acetate

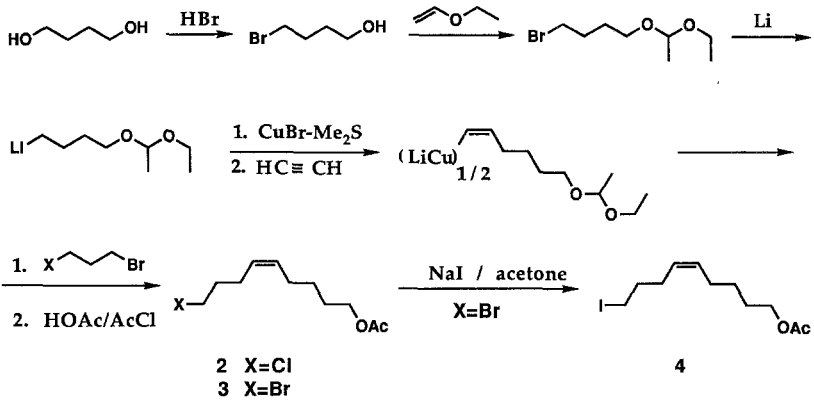


FIG. 2. Synthetic route to compounds **2–4.**

(20 : 1) as eluent, yielding 1.4 g of product. Part of this was then further purified by HPLC with petroleum ether–ethyl acetate (500 : 4). The purity of the isolated product was >99.5% (GC). δ_H (300 MHz) 1.36–1.46 (m, 2H, $=CCCH_2$), 1.59–1.68 (m, 2H, CH_2-C-O), 1.77–1.86 (m, 2H, $ClCCH_2$), 2.04–2.25 (m, 4H, $=CCH_2$), 2.04 (s, 3H, $COCH_3$), 3.53 (t, 2H, J = 6.5 Hz, $ClCH_2$), 4.06 (t, 2H, CH_2O), 5.28–5.46 (m, 2H, J = 10.8 Hz, CH=CH); δ_C (75.4 MHz) 21.0, 24.4, 26.0, 26.8, 28.2, 32.4, 44.5, 64.4, 128.2, 130.9, 171.2; MS (high-resolution), *m/e* calc. for ($C_{11}H_{19}ClO_2-C_2H_4O_2$) 158.0863, obs. 158.0829, ($C_{11}H_{19}ClO_2-C_4H_8O_2$) calc. 130.0549, obs. 130.0543.

9-Bromo-(Z)-5-nonenyl Acetate **(3)**. This compound was prepared in analogy with **2** from 1,3-dibromopropane to give 1.4 g of product. Part of this was further purified by HPLC, with petroleum ether–ethyl ether (10 : 1) as eluent, which gave a final product >98.5% pure (GC). δ_H (300 MHz) 1.36–1.47 (m, 2H, $=CCCH_2$), 1.59–1.69 (m, 2H, CH_2-C-O), 1.82–1.95 (m, 2H, $BrCCH_2$), 2.03–2.23 (m, 4H, $=CCH_2$), 2.04 (s, 3H, $COCH_3$), 3.41(t, 2H, J = 6.6 Hz, $BrCH_2$), 4.06 (t, 2H, CH_2O), 5.28–5.47 (m, 2H, J = 10.8 Hz, CH=CH). δ_C (75.4 MHz) 21.0, 25.6, 26.0, 26.9, 28.2, 32.6, 33.4, 64.4, 128.1, 131.0, 171.2; MS (high-resolution), *m/e* calc. ($C_{11}H_{19}BrO_2-C_2H_4O_2$) 202.0357, obs. 202.0353.

9-Iodo-(Z)-5-nonenyl Acetate **(4)**. Purified compound **3** (0.15 g, 0.7 mmol) was added to a solution of NaI (3.0 g, 20 mmol) in anhydrous acetone (15 ml). The solution immediately turned cloudy and was left stirring for 3 hr. Pentane (15 ml) was added, and the mixture was filtrated. The solvent was removed and the residue taken up in a new portion of pentane (10 ml). Repeated filtration and evaporation of the solvent gave 0.15 g of a yellow oil. Purification by HPLC using petroleum ether–ethyl ether (500 : 15) yielded a colorless oil of very high purity as judged by analytical HPLC (analysis by GC was not suc-

cessful due to thermal instability of the product). δ_H (300 MHz) 1.36–1.47 (m, 2H, $=CCCH_2$), 1.59–1.69 (m, 2H, CH_2-C-O), 1.82–1.92 (m, 2H, $ICCH_2$), 2.04–2.18 (m, 4H, $=CCH_2$), 2.04 (s, 3H, $COCH_3$), 3.19 (t, 2H, J = 6.8 Hz, ICH_2), 4.06 (t, 2H, CH_2O), 5.27–5.46 (m, 2H, J = 10.8 Hz, CH=C). δ_C (75.4 MHz) 6.6, 21.0, 26.0, 27.0, 27.9, 28.2, 33.3, 64.4, 127.9, 131.0, 171.2.

Electrophysiology

Electrophysiological activities were determined by the single-sensillum technique (Kaissling, 1974). Olfactory receptor cells specifically tuned to (*Z*)-5-decenyl acetate (**1**) are present in antennal sensilla type SW1 of *Agrotis segetum* and are readily accessible for single-cell recordings (Hallberg, 1981; Löfstedt et al., 1982, van der Pers and Löfstedt, 1983). The method used was modified according to van der Pers and Den Otter (1978) and has previously been described (Liljefors et al., 1987; Bengtsson et al., 1990). Dose–response curves were constructed using five different stimulus amounts from 10^{-4} μg to 1 μg for **1** and 10^{-3} μg to 100 μg for **2–4** in decadic steps. For each stimulus loading, 10 replicates were recorded, and the mean value of the number of action potentials generated during 1 sec from the onset of stimulation was used in the construction of the dose–response curves. Errors are expressed as standard errors of the mean (SEM). The relative activity of each compound is expressed as the reciprocal of the relative quantities required to elicit the same response from the receptor cell, as the natural pheromone component.

Corrections for differences in volatility have been made using relative vapor pressures, as previously described (Liljefors et al., 1985; Bengtsson et al., 1990). The correction factors used are 5.2 for compound **2**, 11.0 for compound **3**, and 29.5 for compound **4**. These factors are based on vapor pressure data for decane and its halogen-substituted analogs (Dykyj and Repás, 1979).

Molecular Mechanics Calculations

Energy-minimized geometries and intermolecular interactions were calculated using the molecular mechanics program MM2(85) (Burkert and Allinger, 1982). Starting structures for the energy-minimization program were obtained from the molecular modeling system MIMIC (Liljefors, 1983; von der Lieth et al., 1984).

RESULTS

Chemicals. Compounds **2–4** were prepared by carbocupration of acetylene followed by alkylation of the intermediate (*Z*)-dialkyl cuprate according to Alexakis et al. (1979). The synthetic scheme is shown in Figure 2. This syn-

thetic method is known to afford products of very high stereoisomeric purity without using argentation chromatography. The complete conversion of bromide to iodide was performed with a Finkelstein reaction in the conventional manner.

Electrophysiological Recordings. The electrophysiological single-cell activities for compounds **1–4** are shown as dose–response curves in Figure 3. Compounds **2** and **3** exhibit virtually identical receptor responses. The recordings show that the amount loaded on filter paper of these two compounds had to be about 150 times higher than that of **1** in order to elicit the same number of spikes/sec as the natural component **1**. The corresponding dose–response curve for compound **4** is significantly displaced to higher stimulus loadings. In this case the stimulus amount has to be about 1800 times higher than that of **1** in order to give the same receptor response as **1**. The relative amounts of the analogs delivered to the antenna, however, are not same as the relative amounts loaded on filter paper. For a final analysis, corrections for differences in volatility have to be included as described in the Materials and Methods section. The relative single-cell activities, including volatility corrections, for compounds **1–4** are shown in Figure 4 along with the activities for two previously studied related compounds (**5** and **6**) (Bengtsson et al., 1987, 1990) for comparison.

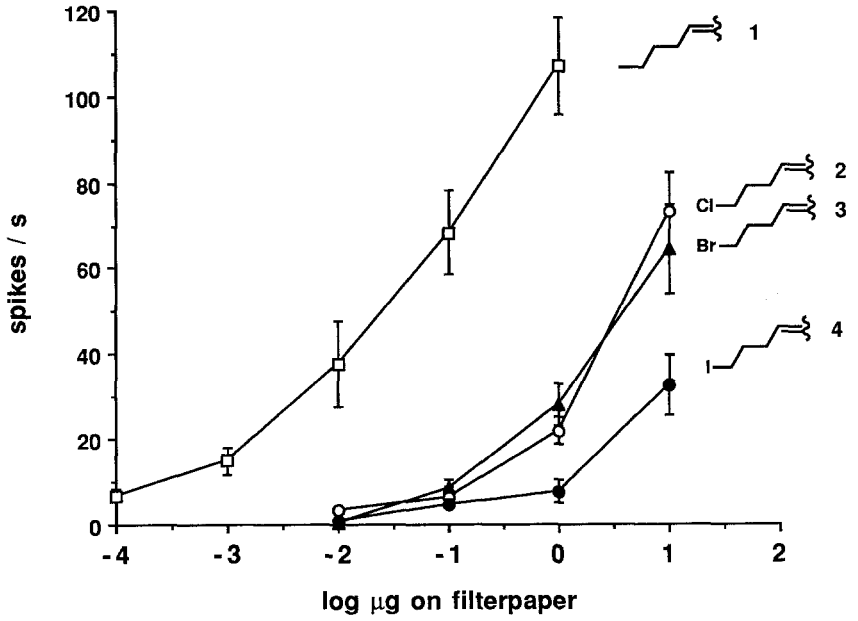


FIG. 3. Single-cell dose–response curves for compounds **1–4.**

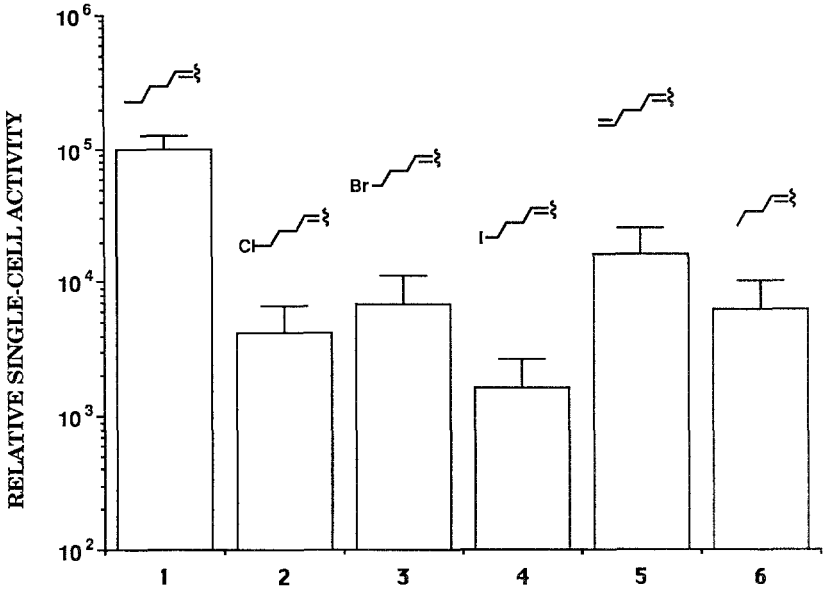


FIG. 4. Experimental single-cell activities (+SEM) for compounds **1–6.** The data are corrected for differences in volatility. The data for compounds **5** and **6** are taken from Bengtsson et al. (1987, 1990).

All analogs display a reduced activity compared to the natural pheromone component **1**. The chloro analog **2** displays an activity drop by a factor of 24, while the bromo analog **3** is less active than **1** by a factor of 14. The iodo analog **4** is the least active of the compounds studied. Its activity is lower than **1** by a factor of 60. In comparison to **5**, the halogen analogs **2–4** display significantly less activity and a comparison with the chain-shortened analog **6** shows that **2** and **3** have activities close to this analog. Thus, replacing the terminal methyl group in **1** by a chlorine or bromine substituent has about the same effect on the receptor cell response as chain-shortening by one methylene unit. Replacing the methyl group in **1** by iodine is in this respect approximately equivalent to chain-shortening by two methylene units (Bengtsson et al., 1990).

DISCUSSION

The replacement of the terminal methyl group in compound **1** by a halogen atom results in changes of the hydrophobic character and the size of the terminal substituent. Thus, hydrophobicity as well as substituent size must be taken into account in the interpretation of the experimental data.

The conformational properties of compounds **2–4** are virtually identical to those of compound **1** (Jönsson and Liljefors, unpublished results). Thus, conformational differences cannot rationalize the observed activity differences.

Hydrophobicity. The hydrophobic character of a compound is commonly expressed in terms of the logarithm of the partition coefficient (log P) describing the partition of the compound between an aqueous phase and *n*-octanol (Leo et al., 1971). Experimental log P data for some aliphatic *n*-alkanes and haloalkanes are given in Table 1. From these data it is clear that the methyl group has a higher hydrophobicity than the halogens and that the iodides are more hydrophobic than the chlorides and bromides. This is also reflected in the fragment constants developed by Hansch and Leo (1979) shown in Table 1. As log P may be predicted from addition of fragment constants (Hansch and Leo, 1979) and since the compounds studied are closely related, the differences between the fragment constants in Table 1 are identical to differences in predicted log P for compounds **1–6**.

If hydrophobic effects alone determine the affinity of a compound for a nonpolar site, a more hydrophobic compound should have a higher affinity. Assuming that the electrophysiological activity of a pheromone component or an analog is directly related to the affinity for the receptor, examination of Table 1 shows that the activities of compounds **1–4** are expected to decrease in the order **1** > **4** > **3** > **2**. The single-cell activities in Figure 4 show that **1** is indeed more active than its halogen analogs, but the order of the observed activities for **2, 3,** and **4** does not follow their relative hydrophobicities. Further-

TABLE 1. EXPERIMENTAL LOG P VALUES FOR SOME HYDROCARBONS AND HALOALKANES AND FRAGMENT CONSTANTS FOR TERMINAL SUBSTITUENTS IN COMPOUNDS **1–6**

	Log P_{obs}[a]			
Compound R–X or R′–X	R=C_2H_5 R′=C_2H_4	R=C_3H_7 R′=C_3H_6	R=C_4H_9	Fragment constant f_X[b]
R–CH_3	2.36	2.89	3.39	0.89
R–Cl	1.43	2.04	2.64	0.06
R–Br	1.61	2.10		0.20
R–I	2.00			0.59
R–H	1.81	2.36	2.89	0.35[c]
R′=CH_2	1.77	2.40		0.34[d]

[a] Suzuki and Kudo (1990).
[b] Hansch and Leo (1979).
[c] Derived from the f_H constant = +0.23 and the bond factor F_b = −0.12 (Hansch and Leo, 1979).
[d] Derived from the $F_=$ factor (Hansch and Leo, 1979).

more, on the basis of hydrophobicity arguments alone, compounds **5** and **6** should display essentially identical activities, in disagreement with experimental data (Figure 4). Consequently, hydrophobicity alone cannot rationalize the observed electrophysiological activities; the size of the substituent should also be of importance for the activity.

Substituent Size. The values of the van der Waals (vdW) radii for the terminal substituents in compounds **1–6**, employed by the MM2(85) molecular mechanics program, are tabulated in Table 2 along with the corresponding distances to a bonded carbon atom. The sum of these parameters [$D(X)$, Table 2] represents the size of a substituent X in an intermolecular interaction in the direction of the C—X bond. A particular problem is how to define the size of nonspherical substituents like the methyl group. An approach to this problem for symmetric top substituents is to calculate the effective length of the group in the C—X bond direction by simple geometry and trigonometry, using vdW radii and bond lengths (Charton, 1979). The value for the methyl group calculated in this way, using radii and bond lengths according to MM2(85) is 3.36 Å (Table 2). This shows, as expected, that the methyl group is significantly smaller in the C—X bond direction than the halogens. Verloop et al. (1976) have developed parameters (STERIMOL) that give a measure of the dimensions of a substituent in different directions. The STERIMOL-parameter L representing the length of a substituent is included in Table 2 for comparison. The L parameters and the D parameters give a very similar description of differences of substituent size. Thus, we can safely conclude that the relative size of the substituents in the C—X direction are H < CH_3 ≈ CH_2= < Cl < Br < I. Figure 5 illustrates the variation in the vdW size of the terminal substituents of the compounds studied in this work.

TABLE 2. PARAMETERS FOR DESCRIPTION OF SIZE IN C–X BOND DIRECTION OF TERMINAL SUBSTITUENTS IN COMPOUNDS **1–6**

Substituent X	vdW radius (Å)	Bond length C–X (Å)	$D(X)^a$ (Å)	STERIMOL parameter L (Å)
CH_3		1.53	3.36[b]	3.00
Cl	2.03	1.79	3.82	3.52
Br	2.18	1.95	4.13	3.82
I	2.32	2.15	4.47	4.23
H	1.50	1.11	2.61	2.06
$=CH_2$		1.34	3.41[b]	

[a] $D(X)$ = Σ [vdW radius (X) + C–X bond length].
[b] Calculated maximum length in C–X direction (see text).

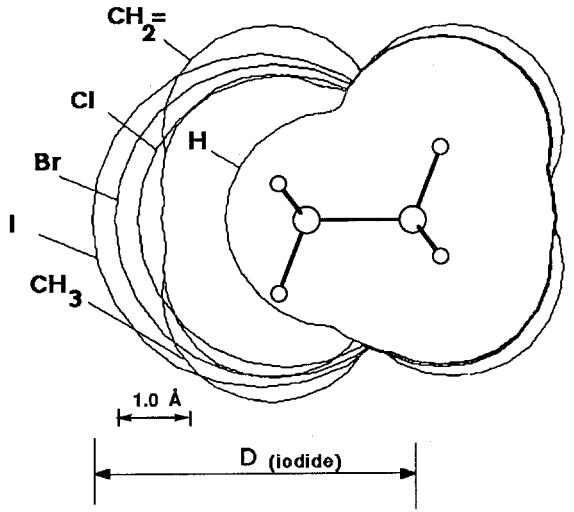


FIG. 5. Superimposed van der Waals contours of the terminal part of compounds **1–6**. The superimposed ethane molecule (corresponding to the contour marked CH_3) indicates the orientation of the contours. The parameter D is the sum of the C—X bond length and the van der Waals radius of atom X (see Table 2). The parameter D is exemplified for X = iodine.

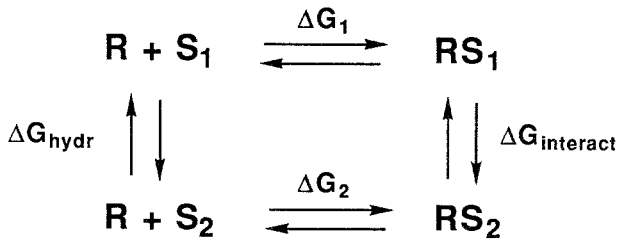
In a geometrically well-defined receptor "pocket" that is highly complementary to a methyl group, the halogen substituents will cause repulsive steric interactions due to their larger size in the C—X bond direction. The observed low activity of the iodo analog **4** is in line with this expectation, but the relative activities of compounds **2** and **3** cannot be rationalized in this way. Compound **6** is also less active and compound **5** more active than may be expected on the basis of size alone. Apparently, to fully rationalize the experimental data, hydrophobicity and size must simultaneously be taken into account. This requires a computational approach.

Molecular Mechanics Calculations. In order to simulate hydrophobic and repulsive steric interactions simultaneously, we have performed molecular mechanics calculations on the interaction between models of the terminal alkyl chain in compounds **1–6** and a lipophilic environment.

On comparing biological activities of molecules with different polarities, differences in free energies of hydration (ΔG_{hydr}) must be taken into account. Using the thermodynamic cycle (Scheme 1) it follows that

$$\Delta\Delta G = \Delta G_2 - \Delta G_1 = \Delta G_{interact} - \Delta G_{hydr} \quad (1)$$

$\Delta\Delta G$ corresponds to the difference in affinity of substrates S_1 and S_2 for



SCHEME 1.

the receptor R, and $\Delta G_{\text{interact}}$ is the difference in the interaction energies of the two substrate–receptor complexes RS_1 and RS_2 (Tembe and McCammon, 1984; McCammon, 1987). The equation above shows that if the interaction energies of the substrate–receptor complexes of the two substrates are identical, the substrate that is less soluble in water (most positive ΔG_{hydr}) will have the highest affinity for the receptor. If the substrates have identical free energies of hydration, the interaction energies of the substrate–receptor complex completely determine their relative affinities.

Differences of the free energies of hydration for alkyl chlorides, bromides, and iodides are very small, at most a few tenths of a kilocalorie per mole (Cabani et al., 1981). This is reflected in the additive group contributions to ΔG_{hydr} developed by Cabani et al. (1981). For Cl, Br, and I these contributions are -1.74, -1.85 and -1.90 kcal/mol, respectively. Thus, the order of the electrophysiolological activities for **2–4** should largely be determined by their interactions (repulsive and dispersive) with the receptor. ΔG_{hydr} for *n*-alkanes is ca. 2.5 kcal/mol more positive than that for the corresponding haloalkanes (the group contribution of CH_3 to ΔG_{hydr} is $+0.76$ kcal/mol). This must be taken into account in a comparison of the activities for **1** and its halogen analogs.

Since the structure of the receptor site is unknown, we have employed simple models for the interaction between the terminal substituents in compounds **1–6** and a lipophilic environment with defined geometrical properties. As a first model, two ethane molecules, simulating a fragment of the receptor site and the terminal part of the substrate molecule, were positioned as shown in Figure 6 and energy-minimized with molecular mechanics calculations [MM2(85)] in order to find the energetically most favorable distance between the molecules. In the calculation, the two C—C bonds were restricted to a common axis. The methyl group in one of the ethanes was in turn replaced by chlorine, bromine, iodine, and hydrogen to simulate the analogs **2–4** and **6**. Ethylene was used as a model for analog **5**. These complexes were then energy-minimized keeping the distance d in Figure 6 fixed to the value (5.18 Å) obtained from the minimization of two ethane molecules. This simple model simulates the interactions in the C—X bond direction due to repulsion and dis-

persion energies of the different substituents in a complex with a fixed geometry optimal for a methyl group. The energy of interaction is the difference in energy between the complex and the energy of the two separate parts of the complex. The calculated interaction energies are given in Table 3, model A. A negative energy indicates net attraction between the components. As interaction entropies should be very similar for the different cases, interaction energy differences should be close to free energy differences. As expected, the methyl substituent gives the most stable complex, while bromine and, in particular, iodine show strong repulsive interactions due to their larger size in the C—X bond direction.

To investigate the validity of the simple model above, a more elaborate model was constructed, simulating a three-dimensional lipophilic cavity optimally complementary to a methyl group. Five isobutane molecules were positioned around the methyl group in the terminal alkyl chain of **1** (simulated by a butane molecule), creating a three-dimensional cavity. The complex was energy-minimized by MM2(85), and the interaction energy between butane and the constructed lipophilic "receptor" was calculated as the energy difference between the complex and the two separate components. The energy-minimized

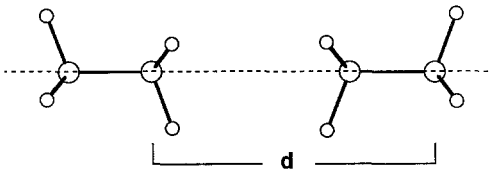


FIG. 6. Interaction complex describing model A. The figure shows the case in which the substituent is CH_3. The distance d is the optimal distance between the two ethane molecules. This distance is kept fixed in the calculations for other substituents.

TABLE 3. MOLECULAR MECHANICS [MM2(85)] CALCULATED INTERACTION ENERGIES (kcal/mol)

	Model A		Model B	
X	Interaction energy	$\Delta(X-CH_3)$	Interaction energy	$\Delta(X-CH_3)$
CH_3-	−1.0	0.0	−10.6	0.0
Cl—	−0.3	0.7	−9.4	1.2
Br—	1.2	2.2	−7.9	2.7
I—	6.4	7.4	−3.9	6.7
$CH_2=$	−0.8	0.2	−8.9	1.7
H—	−0.2	0.2	−7.8	2.8

complex is shown as a stereo-pair in Figure 7. The calculations then were repeated for models of the terminal alkyl chains of compounds **2–6** by replacing the interacting methyl group in butane by an appropriate substituent. The size of the "receptor" was kept fixed by completely restricting the motion of the central carbon in each isobutane molecule during energy-minimization. In addition, the position of the noninteracting methyl group in butane and its halogen-substituted analogs was kept fixed to assure that the substrate molecules have the same positions in the lipophilic pocket. This corresponds to a fixed distance *d* in the calculations described above (model A).

The results of these calculations are shown in Table 3, model B. Although the interaction energies are much larger in these calculations compared to the model A calculations, the differences in interaction energies [$\Delta(X-CH_3)$, Table 3] with CH_3 as reference substituent are similar for Cl, Br, and I in the two models, supporting the validity of the simple model A for these substituents. For the substituents $CH_2=$ and H, it is obvious that only the full treatment of model B gives a realistic description of the interactions between these substituents and a lipophilic cavity. Interestingly, the calculated interaction energy difference between **6** and **1**, 2.8 kcal/mol (model B, Table 3), which, after correction for differences in the loss of conformational entropy on binding becomes about 1.8 kcal/mol (Bengtsson et al., 1990), is almost identical to the energy value, 1.9 kcal/mol, which we previously deduced from the observed relative activities of **1** and **6** (Bengtsson et al., 1990). Furthermore, this energy value is in good agreement with the maximum hydrophobic binding energy for a methyl group in a perfectly complementary receptor pocket, determined from

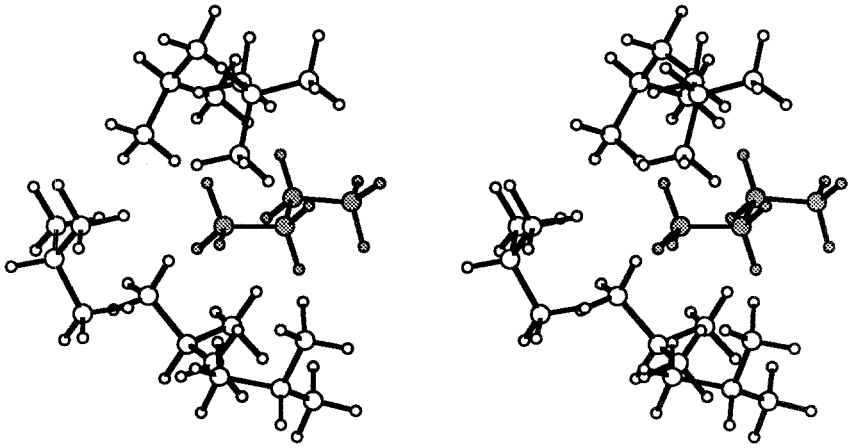


FIG. 7. Stereo-view of the energy-minimized structure of the hypothetical receptor "pocket" (model B) binding a butane molecule (filled atoms).

binding experiments with enzymes (Fersht, 1985; Bengtsson et al., 1990). This agreement indicates that the calculated interaction energies are realistic. Taking differences in free energies of hydration into account by subtracting 0.70 and 0.12 kcal/mol (Cabani et al., 1981) from the interaction energies for $CH_2=$ and H, respectively, in Table 3, the total differences in interaction energies between CH_3 and $CH_2=$, and CH_3 and H becomes 1.0 and 1.7 kcal/mol, respectively. These energies correspond to $\Delta\Delta G$ in equation 1 for the affinity of compounds **5** and **6**, in relation to compound **1**, for the constructed lipophilic "receptor" in Figure 7. The calculated energies predict the order of activities to be **1** > **5** > **6** in complete agreement with experiments (Figure 3).

From the calculated data in Table 3, it is clear that the interaction energies for the bromine and iodine substituents are too repulsive in a rigid receptor pocket that is "perfectly" complementary to a methyl group. For instance, the interaction energies for chlorine and bromine should be essentially the same in order to rationalize the experimental data in Figure 4. Thus, in order to rationalize these data, it is necessary to invoke some ability of the receptor site to respond to an increase in the size of the ligand through small changes of its geometry.

Interaction energies involving the halogen-substituted compounds, calculated as a function of the intermolecular separation, are shown in Figure 8. Since model A was shown above to be valid for the halogen substituents, we have used this simple model for these calculation. It is clear from Figure 8 that the interaction energies for the halides are strongly dependent on the intermolecular distance. The distance employed in the model A calculations in Table 3, 5.18 Å, is indicated by the left arrow in Figure 8. At this distance, the interaction energy of the methyl substituent is optimal, while the bromine and iodine substituents show large repulsive interactions. At somewhat larger intermolecular distances, the stability of the complexes with halogen substituents rapidly increases due to a decrease in the steric repulsions between the halogen substituent and the methyl group. At 5.7 Å (indicated by the right arrow in Figure 8), the interaction energies for the chlorine- and bromine-substituted compounds are identical, corresponding to a predicted equal affinity for the receptor. A further small increase of the distance d leads to stronger interaction for the bromine substituent than for the chlorine substituent, corresponding to a somewhat higher affinity for the bromo compound than for the chloro compound. Thus, if we assume a limited degree of flexibility of the receptor site corresponding to an increase of the distance d by ca. 0.5 Å, approximately one third of a C—C bond length, the calculated binding energy differences for the halogen-substituted compounds correspond very well to the order of the biological activities of these compounds. As may be inferred from Figure 8, a significantly larger degree of receptor flexibility leads to the prediction that the iodo

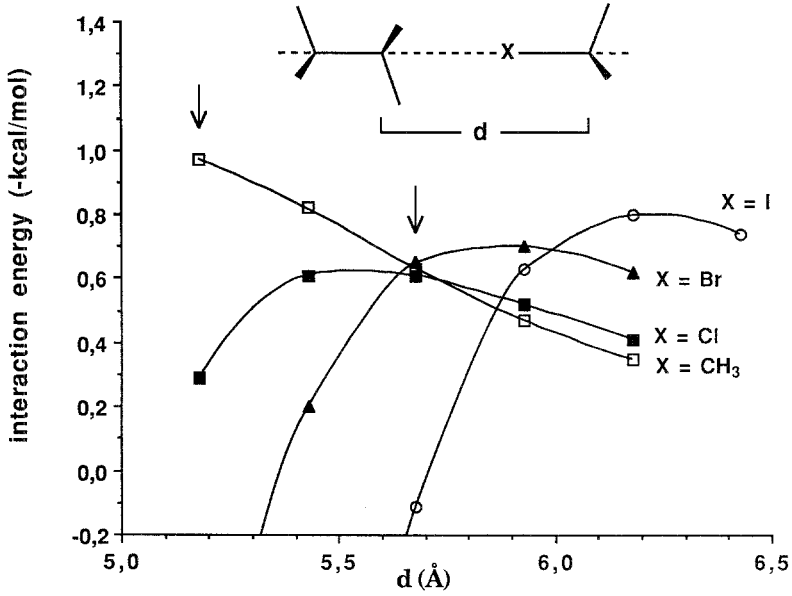


FIG. 8. Interaction energy as a function of the intermolecular distance d for the interaction between ethane and CH_3-X with various substituents X corresponding to the terminal substituent in compounds **1–4**.

compound should have a higher affinity for the receptor than at least the chloro compound. This is in disagreement with experimental data in Figure 4.

An alternative rationalization of the experimental data is that the receptor pocket binding the terminal methyl group of the natural component **1** is not perfectly complementary to a methyl group but slightly larger, approximately corresponding to the size (in the C—X bond direction) of a bromo substituent. Although the calculated interaction energies in Figure 8 then become very similar for CH_3, Cl, and Br, the more positive ΔG_{hydr} for the methyl group, as discussed above, implicates that compound **1** should still be significantly more active than the halogen substituted analogs **2–4**.

CONCLUSIONS

The replacement of the terminal methyl group in **1** by a chloro, bromo, or iodo group leads to a significant reduction of the electrophysiological single-cell activity. The observed decrease in activity is concluded to be determined by a combination of hydrophobic and sterically repulsive contributions to the

substrate–receptor interaction. The results support our previously suggested receptor interaction model in which the terminal alkyl chain interacts with a complementary hydrophobic receptor "pocket" with very limited flexibility. It is concluded that the receptor may have a small degree of flexibility approximately corresponding to the difference in vdW volumes between a methyl and a bromine substituent and/or that the steric repulsions in the receptor pocket may be relieved by a correspondingly small adjustment of the position of the terminal alkyl chain.

Acknowledgments—This work was financially supported by the Swedish Natural Science Research Council, which is gratefully acknowledged.

REFERENCES

ALEXAKIS, A., CAHIEZ, G., and NORMANT, J.F. 1979. Vinyl-copper derivatives. X. Alkylation of (*Z*)-dialkenylcuprates. *Synthesis* 10:826–830.

BENGTSSON, M., LILJEFORS, T., and HANSSON, B.S. 1987. Dienic analogues of (*Z*)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*: Synthesis, conformational analysis and structure-activity relationships. *Bioorg. Chem.* 15:409–422.

BENGTSSON, M., LILJEFORS, T., HANSSON, B.S., LÖFSTEDT, C., and COPAJA, S.V. 1990. Structure-activity relationships for chain-shortened analogs of (*Z*)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*. *J. Chem. Ecol.* 16:667–684.

BURKERT, U., and ALLINGER, N.L. 1982. Molecular Mechanics. American Chemical Society, Washington D.C.

CABANI, S., GIANNI, P., MOLLICA, V., and LEPORI, L. 1981. Group contributions to the thermodynamic properties of non-ionic organic solutes in dilute aqueous solution. *J. Solut. Chem.* 10:563–593.

CHARTON, M. 1979. Dependence of bond angles upon the steric effect. 1. XMX bond angles. *J. Am. Chem. Soc.* 101:7356–7362.

DYKYJ, J., and REPÁS, M. 1979. The Vapour Pressure of Organic Compounds. Veda, Bratislava (in Slovak).

FERSHT, A. 1985. Enzyme Structure and Mechanism. W.H. Freeman and Company, New York.

HALLBERG, E. 1981. Fine-structural characteristics of the antennal sensilla of *Agrotis segetum* (Insecta: Lepidoptera). *Cell Tissue Res.* 218:209–218.

HANSCH, C., and LEO, A. 1979. Substituent Constants for Correlation Analysis in Chemistry and Biology. John Wiley & Sons, New York.

JÖNSSON, S., LILJEFORS, T., and HANSSON. 1991. Alkylsubstitution in the terminal chain of (*Z*)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*. Synthesis, single-sensillum recordings and structure-activity relationships. *J. Chem. Ecol.* 17:103–122.

KAISSLING, K.-E. 1974. Sensory transduction in insect olfactory receptors, pp. 243–273, *in* L. Jaenicke (ed.). Biochemistry of Sensory Functions. Springer-Verlag, Berlin.

LEO, A., HANSCH, C., and ELKINS, D. 1971. Partition coefficients and their uses. *Chem. Rev.* 71:525–616.

LILJEFORS, T. 1983. MOLBUILD—an interactive computer graphics interface to molecular mechanics. *J. Mol. Graph* 1:111–117.

LILJEFORS, T., BENGTSSON, M., and HANSSON, B.S. 1987. Effects of double-bond configuration on interaction between a moth sex pheromone component and its receptor: A receptor–interaction model based on molecular mechanics. *J. Chem. Ecol.* 13:2023–2040.

LILJEFORS, T., THELIN, B., VAN DER PERS, J.N.C., and LÖFSTEDT, C. 1985. Chain-elongated analogs of a pheromone component of the turnip moth, *Agrotis segetum*. A structure–activity study using molecular mechanics. *J. Chem. Soc. Perkin Trans.* 2:1957–1962.

LÖFSTEDT, C., VAN DER PERS, J.N.C., LÖFQUIST, J., LANNE, B.S., APPELGREN, M., BERGSTRÖM, G., and THELIN, B. 1982. Sex pheromone components of the turnip moth, *Agrotis segetum*: Chemical identification, electrophysiological evaluation and behavioral activity. *J. Chem. Ecol.* 8:1305–1321.

MCCAMMON, J.A. 1987. Computer-aided molecular design. *Science* 238:486–491.

MCLEAN, J.A. MORGAN, B., SWEENEY, J.D., and WEILER, L. 1989. Behavior and survival of western spruce budworm, *Choristoneura occidentalis* Freeman, exposed to an ω-fluorinated pheromone analogue. *J. Chem. Ecol.* 15:91–103.

OLSSON, A.-M., JÖNSSON, J.-Å., THELIN, B., and LILJEFORS, T. 1983. Determination of the vapor pressures of moth sex pheromone components by a gas chromatographic method. *J. Chem. Ecol.* 9:375–385.

PRIESNER, E. 1979a. Progress in the analysis of pheromone receptor systems. *Ann. Zool. Ecol. Anim.* 11:533–546.

PRIESNER, E. 1979b. Specificity studies on pheromone receptors of noctuid and tortricid lepidoptera, pp. 57–71, *in* F.J. Ritter (ed.). Chemical Ecology: Odour Communication in Animals. Elsevier/North-Holland, Amsterdam.

PRIESNER, E., JACOBSON, M., and BESTMANN, H.J. 1975. Structure–response relationships in noctuid pheromone perception. An introductory report. *Z. Naturforsch.* 30c:283–293.

SCHWARZ, M., KLUN, J.A., and UEBEL, E.C. 1990. European cornborer sex pheromone. Inhibition and elicitation of behavioral response by analogs. *J. Chem. Ecol.* 16:1591–1604.

SUN, W.-C., and PRESTWICH, G.D. 1989. Partially fluorinated analogs of (*Z*)-9-dodecenyl acetate: Probes for pheromone hydrophobicity requirements. *Tetrahedron Lett.* 31:801–804.

SUZUKI, T., and KUDO, Y. 1990. Automatic log *P* estimation based on combined additive modeling methods. *J. Comput.-Aided. Mol. Design* 4:155–198.

TEMBE, B.L., and MCCAMMON, J.A. 1984. Ligand–receptor interactions. *Comput. Chem.* 8:281–283.

VAN DER PERS, J.N.C., and DEN OTTER, C.J. 1978. Single cell responses from olfactory receptor of small ermine moths (Lepidoptera: Yponomeutidae) to sex attractants. *J. Insect Physiol.* 24:337–343.

VAN DER PERS, J.N.C., and LÖFSTEDT, C. 1983. Continuous single sensillum recording as a detection method for moth pheromone components in the effluent of a gas chromatograph. *Physiol. Entomol.* 8:203–211.

VERLOOP, A., HOOGENSTRAATEN, W., and TIPKER, J. 1976. Development and application of new steric substituent parameters in drug design, pp. 165–207, *in* E.J. Ariëns (ed.). *Drug Design*, Vol. VII. Academic Press, New York.

VON DER LIETH, C.W., CARTER, R.E., DOLATA, D.P., and LILJEFORS, T. 1984. RINGS—a general program to build ring systems. *J. Mol. Graph.* 2:117–123.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

SEX PHEROMONE OF THE EUROPEAN SUNFLOWER MOTH, *Homoeosoma nebulellum* (DEN. & SCHIFF.) (LEPIDOPTERA: PYRALIDAE)

P. ZAGATTI,[1,*] M. RENOU,[1] C. MALOSSE,[1] B. FRÉROT,[1] C. PAVIS,[1] M. LETTERE,[1] C. DESCOINS,[1] A. PERMANA,[2] Y. PIVOT,[2] and F. LECLANT[2]

[1]*INRA Laboratoire des Médiateurs Chimiques Domaine de Brouessy, 78114 Magny-les-Hameaux France*

[2]*U.F.R. d'Ecologie animale et de Zoologie agricole ENSA-INRA, Place Pierre Viala, 34000 Montpellier France*

(Received December 18, 1990; accepted March 15, 1991)

Abstract—Four components, (*Z*)-9-tetradecenal (8.6%), (*Z,E*)-9,12-tetradecadienal (4.8%), (*Z*)-11-hexadecenal (49.5%), and (*Z*)-13-octadecenal (37.1%), were identified in extracts of female pheromone glands of the European sunflower moth, *Homoeosoma nebulellum* (Lepidoptera: Pyralidae) using GC and GC-MS analyses. EAG and single-cell recordings of male antennal receptors gave strong evidence for (*Z,E*)-9,12-tetradecadienal as the antennal key compound of sex pheromone detection in this species. This result was confirmed by field trapping; removal of (*Z,E*)-9,12-tetradecadienal from quaternary blends completely suppressed the male catches. The synthetic blends with this compound as a major component caught five times less males than the blends reproducing the ratio found in the female extracts [5% of (*Z,E*)-9,12-tetradecadienal only]. The occurrence of a minor component perceived as the most biologically relevant compound is discussed.

Key Words—Lepidoptera, Pyralidae, *Homoeosoma nebulellum*, European sunflower moth, sex pheromone, identification, multicomponent blend, (*Z,E*)-9,12-tetradecadienal.

INTRODUCTION

The European sunflower moth (ESM), *Homoeosoma nebulellum* (Den. & Schiff.) is a palearctic phycitid reported as a pest of sunflowers (*Helianthus*

*To whom correspondence should be addressed.

0098-0331/91/0700-1399$06.50/0

annuus L.) in eastern Europe (Balachowsky, 1972). In western Europe, the larvae usually develop in the inflorescences of wild Compositae, such as *Silybum marianum* L. or *Senecio jacobea* L. (ragwort) in the southern and northern parts of Europe, respectively (Goater, 1986).

The first damage on cultivated sunflowers in southern France was reported in 1983 in late crops (Permana et al., 1991). *H. nebulellum* has not yet been found on sunflower in temperate Europe, but with the recent development of cold hardy sunflower cultivars, the importance of this pest species may increase. We therefore decided to develop an effective monitoring technique to be used in control programs for this potential pest.

In this paper, we report on the identification of the components of the female sex pheromone in the ESM, and we propose an efficient attractant for field trapping.

METHODS AND MATERIALS

Insects. A laboratory colony was established on artificial diet for noctuids (Poitout and Bues, 1970), which was modified by addition of sunflower akene powder (100 g/5 liters) and sunflower oil (5 ml/5 liters). The colony was restocked annually using larvae collected near Montpellier (Hérault) and in the Camargue near St. Gilles (Gard) on *Silybum marianum* or sunflower. All rearing and testing were carried out at 23°C, 70% relative humidity under a 16:8 light–dark photoperiod.

Individuals were sexed as pupae and the two sexes held separately.

Females. Calling behavior was observed at 15-min intervals throughout the entire 24-hr period on 30 virgin 2- or 3-day-old females. Observations during the scotophase were made with a portable, incandescent red lamp.

The structure of the pheromone gland was examined histologically. The abdominal tips of 10 two-day-old virgin females were fixed for 22 hr in alcoholic Bouin's and subsequently stained with Masson-Goldner trichrome (Martoja and Martoja-Pierson, 1967). Sections were made of both invaginated glands (noncalling position) or with the ovipositor extended by injecting the fixing agent into the abdomen before removing the last few abdominal segments.

Pheromone Extraction and Analysis. The VIII–IX abdominal segments of 323 two- or three-day old females were clipped 1 hr prior to the onset of photophase and soaked for 2 hr in 1 ml hexane at room temperature. The extract then was filtered through glass wool, reduced to ca. 100 μl under a N_2 flow and kept at −30°C until used.

The sex pheromone extract was analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS). GC analyses were performed on either a Carlo Erba Fractovap 2900 with flame ionization detector

(FID), equipped with a nonpolar column (50 m WCOT SE-52, 0.2 mm ID; Girdel, Suresnes, France) heated from 45° to 160°C at 25°/min and from 160° to 210°C at 5°/min, or with a Hewlett-Packard 5890 chromatograph (FID) equipped with a polar column (34 m WCOT Carbowax 20 M, 0.3 mm ID; Girdel), operated isothermally at 180°C. Separation of the isomers of dienic aldehydes was performed on the Hewlett-Packard 5890 chromatograph equipped with a polar column (50 m WCOT CPSil 88, 0.32 mm ID, Chrompack, Middelburg, The Netherlands), operated isothermally at 100°C. GC-MS was performed on a Girdel 32 gas chromatograph linked to a Nermag R 10-10C quadrupole mass spectrometer (electron impact at 70 eV). The chromatograph was fitted with a fused silica capillary column (25 m WCOT CPSil 5 CB, 0.22 mm ID; Chrompack) and operated isothermally at 180°C.

Electrophysiology. Electroantennograms (EAGs) were recorded on male ESMs secured in a styrofoam block. After a few antennal segments had been snipped off, the distal end of one antenna was inserted in the tip of the recording electrode. The indifferent electrode was implanted in the neck of the insect. Both electrodes were glass capillaries filled with Roeder's saline (NaCl, 9.0 g/liter; KCl, 0.2 g/liter; glucose, 4.36 g/liter). Electrodes were connected to a WPI preamplifier through chloridized silver wires. After filtration (DC to 300 Hz) the signal was fed into an oscilloscope and monitored directly on the storage display screen.

To stimulate the antenna, air was puffed (0.5 sec; 1.5 liters/min) on the antenna through a glass cartridge containing a piece of filter paper loaded with 0.5 μg of the test compound. Thirty-one pure compounds were screened, including those reported as pheromone components in the Phycitinae subfamily (Arn et al., 1986), those found in gland extracts of female ESMs, and a number of analogs.

EAG data, in millivolts, were corrected in order to eliminate differences of absolute sensitivity between individuals (Renou et al., 1988). The response of one individual to a given compound was divided by the mean of the responses of this individual to the 31 compounds. Measurements were replicated on one antenna of eight different males, and the means of the corrected EAG values were calculated to produce the EAG response profile.

Single-cell recordings were carried out on excised antennae, using the tip recording technique (Kaissling and Thorson, 1980; Lucas and Renou, 1989). The base of the antenna was inserted in a glass microelectrode filled with hemolymph saline (Kaissling and Thorson, 1980). The tip of a few sensilla trichodea were cut off with sharpened forceps, and the recording electrode, filled with receptor lymph saline, was slipped over the cut end of a single hair. The action potentials were filtered (150–5000 Hz), amplified ($\times$1000), and recorded on a electrostatic fast-recording chart system (Gould ES1000). Some of the recordings were digitized, with a 10-kHz conversion rate, by a A/D converter

board (Dash 16 of Metrabyte) integrated in a PC microcomputer. Then, the responses were analyzed using a specific program written in Asyst (Macmillan Software Company) language (Lucas and Renou, 1989).

The outlet of a Y-shaped glass tube was positioned 2 cm from the antenna and a permanent flow of humidified pure air (1.5 liters/min) was continuously blown out over the antenna through the lateral branch. A stimulation cartridge was introduced in the main branch of the Y tube. Stimulation was achieved by blowing a puff of air (1.0 sec; 0.5 liters/min) through the cartridge.

All the tested chemicals were synthesized at the Laboratoire des Médiateurs Chimiques, with a purity greater than 95%.

Field Trapping. Experiments were conducted in 1988 and 1989 in southern France at St. Gilles in the Camargue and Melgueil near Montpellier using chemicals synthesized at the Laboratoire des Médiateurs Chimiques, with an isomeric purity greater than 98%. In the first experiment (from July 8, 1988, to October 21, 1988), sticky traps (INRA-Carol, Bordeaux, France) were baited with one of six treatments, which included the candidate compounds from female extract analysis or male EAG studies. In all blends, the major component was the dienic aldehyde (*Z*,*E*)-9,12-tetradecadienal (*Z*9,*E*12–14:Ald), which gave the highest responses in electrophysiology (Table 1).

All tested blends were diluted in 1 ml hexane and loaded on methanol extracted rubber septa (SNL, Paris, France). Traps were hung 15 m apart in sunflower fields (var. Viki) on a metallic support in such a way that the base of the traps was 1.5 m above the ground. The catches were recorded every three days during three months, and dispensers replaced every three weeks. Two sets of six traps were tested at the same time, one at St. Gilles and one at Melgueil. Each three-day collection (sum of the St. Gilles and Melgueil identical treatments) was considered a replicate. The results (six treatments with 35 replicates) were submitted to Kruskal-Wallis test.

In a second experiment, nine blends were compared during 20 days (May 9–29, 1989), with three replicates at St. Gilles and three at Melgueil. This experiment was designed to compare the efficiency of blends with *Z*9,*E*12–14:Ald as the major component to blends containing components in the ratio found in gland extracts. Some of these treatments included (*Z*)-13-octadecenal, a compound not identified in the first experiment (Table 2).

Blend dilution and trap design were the same as in first experiment. The catches were recorded every three days, and the results were expressed as the total number of males caught in one trap location. The data were transformed to $\sqrt{(x + 1)}$ and the treatments with a variance significantly different from zero submitted to ANOVA, i.e. three treatments with six replicates, then the means were ranked using Newman-Keuls test (Statitcf software, ITCF, Paris, France).

A third experiment was performed in the autumn of 1989: seven synthetic

TABLE 1. TOTAL CATCHES OF MALE *H. nebulellum* AT TRAPS BAITED WITH DIFFERENT SYNTHETIC BLENDS FROM JULY 8 TO OCTOBER 21, 1988 (2 REPLICATES)

	Compounds (μg)				Total catches	Mean catch/day/trap[a]
	*Z*9,*E*12-14:Ald (%)	*Z*9-14:Ald (%)	*Z*11-16:Ald (%)	*Z*9,*E*12-14:Ac (%)		
HN1	600 (67)		300 (33)		106	0.5a
HN2	600 (91)			60 (9)	2	0.0c
HN3	600 (63)		300 (31)	60 (6)	6	0.0c
HN4	600 (95)	30 (5)			7	0.0bc
HN5	600 (65)	30 (3)	300 (32)		52	0.3ab
HN6	600 (61)	30 (3)	300 (30)	60 (6)	7	0.0bc

[a]Catches followed by the same letter are not significantly different at $P = 0.05$ (Kruskal-Wallis test).

TABLE 2. TOTAL CATCHES OF MALE *H. nebulellum* AT TRAPS BAITED WITH DIFFERENT SYNTHETIC BLENDS FROM MAY 9 TO MAY 29, 1989 (6 REPLICATES)

	Compounds (μg)				Total catches	Mean catch/day/trap[a]
	Z9,E12-14:Ald (%)	*Z9*-14:Ald (%)	*Z11*-16:Ald (%)	*Z13*-18:Ald (%)		
HN1	600 (67)		300 (33)		4	0.0[b]
HN4	600 (95)	30 (5)			2	0.0[b]
HN5	600 (65)	30 (3)	300 (32)		1	0.0[b]
HN7	600 (100)				1	0.0[b]
HN8	600 (82)	30 (4)		100 (14)	5	0.0[b]
HN9	600 (58)	30 (3)	300 (29)	100 (10)	74	0.6b
HN10	48 (5)	86 (9)	495 (50)	371 (37)	371	3.1a
HN11	60 (67)		30 (33)		4	0.0[b]
HN12	60 (65)	3 (3)	30 (32)		11	0.1c

[a]Catches followed by the same letter are not significantly different at $P = 0.05$ (single way ANOVA after trasnsformation of data to $\sqrt{(x + 1)}$ and Newman-Keuls test).

[b]Treatments with null variance, not included in the ANOVA (rejection of the χ^2 test of homoscedasticity). No significant differences found with Kruskal-Wallis test.

blends and a pair of virgin females were compared during 37 days (August 28 to October 4), with three replicates at St. Gilles and three replicates at Melgueil.

In this experiment, the effects of dosage of the quaternary blend were tested, using 1000 μg and 100 μg as the sum of the four compounds, in the ratio found in the gland extract. These quaternary blends were also compared to the four possible ternary blends (Table 3).

The female-baited traps consisted of two 1-day-old virgin females in a screened cage, replaced every three days. Blend dilution, trap design, and statistical analysis (seven treatments with six replicates) were the same as in second experiment.

RESULTS

Calling Behavior and Pheromone Gland Morphology. The calling behavior of *H. nebulellum* females occurred within 30 min of lights on and lasted for about 1 hr. Female moths exhibited a characteristic posture, with the abdomen bent anterodorsally to a 90° angle and the ovipositor fully elongated, similar to that shown by *Homoeosoma ellectellum* (Arthur, 1978).

A cross section of the female abdominal tip revealed a ventrolateral glandular layer in the membrane between segments VIII and IX (Figure 1). The cells are mid-sized (10 μm) and rather cubic, contrary to many other moth species, which show tall, hypertrophic cells (Percy and Wheatherston, 1974). The gland is situated into a simple fold and does not appear as an eversible sac as in many noctuid moths (Jefferson et al., 1968). Thus a complete evagination of the ovipositor is necessary for the pheromone to evaporate in the air.

Pheromone Analysis. The GC analysis of a 323-female gland extract showed four principal peaks. GC-MS data (in electron impact mode) identified two 14-carbon aldehydes, a monoene [*m/z* 41, 55 (100%), 67, 81, 95, 98, 192 (M−18)] and a diene [*m/z* 41, 55 (100%), 67, 68, 79, 81, 95, 208 ($M^{+}\cdot$)], one 16-carbon monoenic aldehyde [*m/z* 41, 55 (100%), 69, 81, 95, 98, 220 (M−18)], and one 18-carbon monoenic aldehyde [*m/z* 41, 55 (100%), 69, 81, 95, 98, 248 (M−18)]. Comparisons of the GC retention times with standards have shown the following structures: (*Z*)-9-tetradecenal (*Z*9–14 : Ald: 8.6%), (*Z*,*E*)-9,12-tetradecadienal (*Z*9,*E*12–14 : Ald: 4.8%; 500 pg per female in the extract), (*Z*)-11-hexadecenal (*Z*11–16 : Ald: 49.5%), and (*Z*)-13-octadecenal (*Z*13–18 : Ald: 37.1%). A complete GC resolution of the isomers of 9,12-tetradecadienal was achieved by means of a CPSil 88 column operated at 100°C.

To confirm the low amount of *Z*9,*E*12–14 : Ald found in the extract, washes of whole ovipositors during 10 sec in pentane were performed on three sets of 10, 20, and 30 virgin females, but the GC analyses gave similar results; the *Z*9, *E*12–14 : Ald was always found in minor proportions, never exceeding 10% of the whole blend.

TABLE 3. TOTAL CATCHES OF MALE *H. nebulellum* AT TRAPS BAITED WITH DIFFERENT SYNTHETIC BLENDS FROM AUGUST 28 TO OCTOBER 4, 1989 (6 REPLICATES)

	Compounds (μg)				Total catches	Mean catch/day/trap[a]
	Z9,E12-14:Ald (%)	Z9-14:Ald (%)	Z11-16:Ald (%)	Z13-18:Ald (%)		
HN9	600 (58)	30 (3)	300 (29)	100 (10)	382	1.7b
HN10	48 (5)	86 (9)	495 (50)	371 (37)	1801	8.1a
HN13	5 (5)	9 (9)	50 (50)	37 (37)	355	1.6b
HN14	48 (5)		495 (54)	371 (41)	1918	8.6a
HN15		86 (9)	495 (52)	371 (39)	0	0[b]
HN16	48 (10)	86 (17)		371 (73)	42	0.2b
HN17	48 (8)	86 (14)	495 (79)		36	0.2b
2 virgin females					310	1.4b

[a]Catches followed by the same letter are not significantly different at $P = 0.05$ (single way ANOVA after transformation of data to $\sqrt{(x + 1)}$ and Newman-Keuls test).

[b]Treatment with null variance, not included in the ANOVA (rejection of the χ^2 test of homoscedasticity). Significant differences found with Kruskal-Wallis test between HN15 vs. HN16 and HN17.

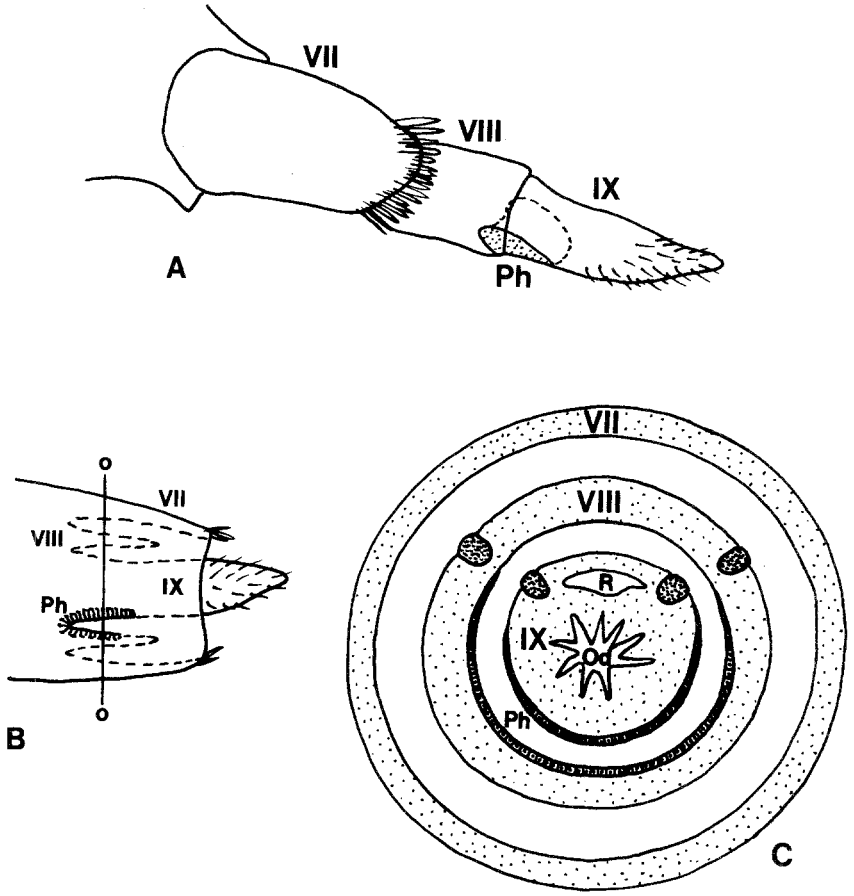


FIG. 1. Schematic view of the ovipositor of the female of *H. nebulellum:* (A) fully elongated; (B) invaginated; (C) cross section along the o-o axis. Ph: sex pheromone gland area; VII, VIII, IX: abdominal segments VII, VIII, and IX; R: rectum; Od: oviduct.

Electrophysiology. The EAG response profile of male ESMs (Figure 2) revealed that the active compounds were the C-14 aldehydes. The most active compound (EAG key compound) was *Z*9,*E*12–14:Ald followed by the monoene, *Z*9–14:Ald. The alcohols and acetate analogs of *Z*9,*E*12–14:Ald also elicited high amplitude EAGs, while *Z*11–16:Ald and *Z*13–18:Ald, two other components found in female glands, did not.

Recordings from single olfactory hairs revealed two classes of action potentials (Figure 3), indicating the presence of at least two olfactory neurons,

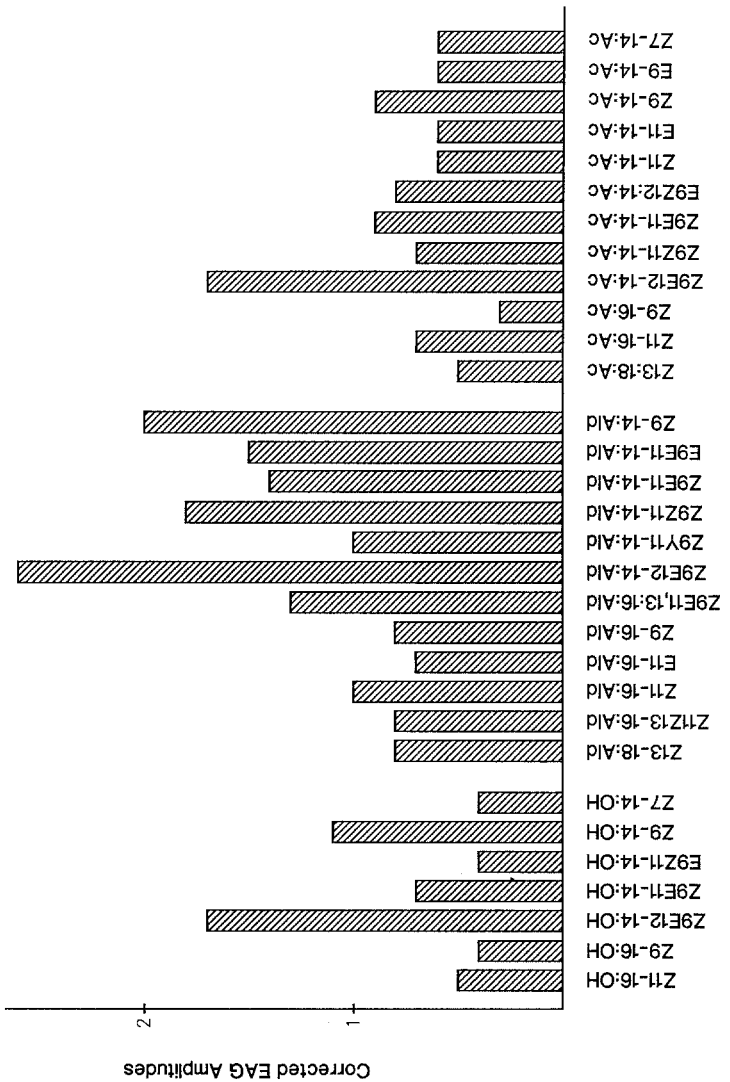


FIG. 2. Electroantennogram screening performed on male antenna with 31 compounds known as attractants or analogs in the Phycitinae or identified in the pheromone gland extracts of *H. nebulellum* females. Name of compounds were abridged as follows: Z9- geometry and position of the double bond; Y11- triple bond; -14: chain length; : OH, : Ald, : Ac, chemical function = alcohol, aldehyde, acetate.

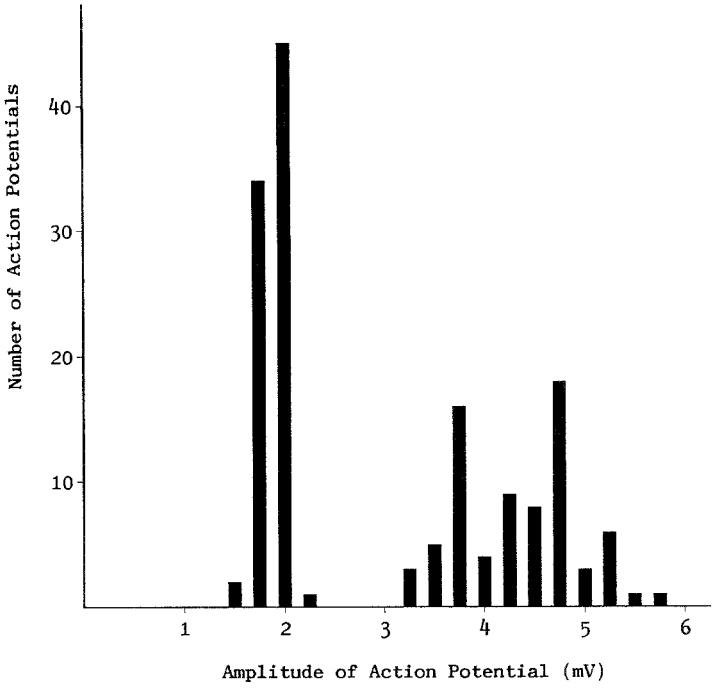


FIG. 3. Typical action potential histogram showing two classes of amplitude, recorded during spontaneous activity and stimulations with *Z*9,*E*12–14 : Ald and *Z*9,*E*12–14 : Ac from a single sensillum trichodeum.

housed within the sensilla trichodea of male ESMs. Type A neurons, with the highest amplitude action potentials, were stimulated almost exclusively by *Z*9,*E*12–14 : Ald, the EAG key compound, and slightly by *Z*9–14 : Ald (Figure 4A). Type B neurons with small amplitude action potentials were principally excited by *Z*9,*E*12–14 : Ac (Figure 4B). One female-equivalent of ESM gland extract elicited a strong firing of A but not B cells. Olfactory neurons sensitive to other components of the female pheromone secretion may exist, but none was detected within the sample of olfactory hairs from which we recorded.

Field Trapping. The first field trials with synthetic blends were carried out in 1988, when *Z*13–18 : Ald was not yet identified in the female secretion. The results (Table 1) reveal that the blends containing two compounds, *Z*9,*E*12–14 : Ald and *Z*11–16 : Ald, were attractive to the males. In contrast, *Z*9,*E*12–14 : Ac, which excited B cells in sensilla recordings, appeared to reduce the catches when added at the 10% ratio.

Further field tests were conducted in the spring of 1989 in order to determine the effects of the addition of *Z*13–18 : Ald to the mixtures that gave the

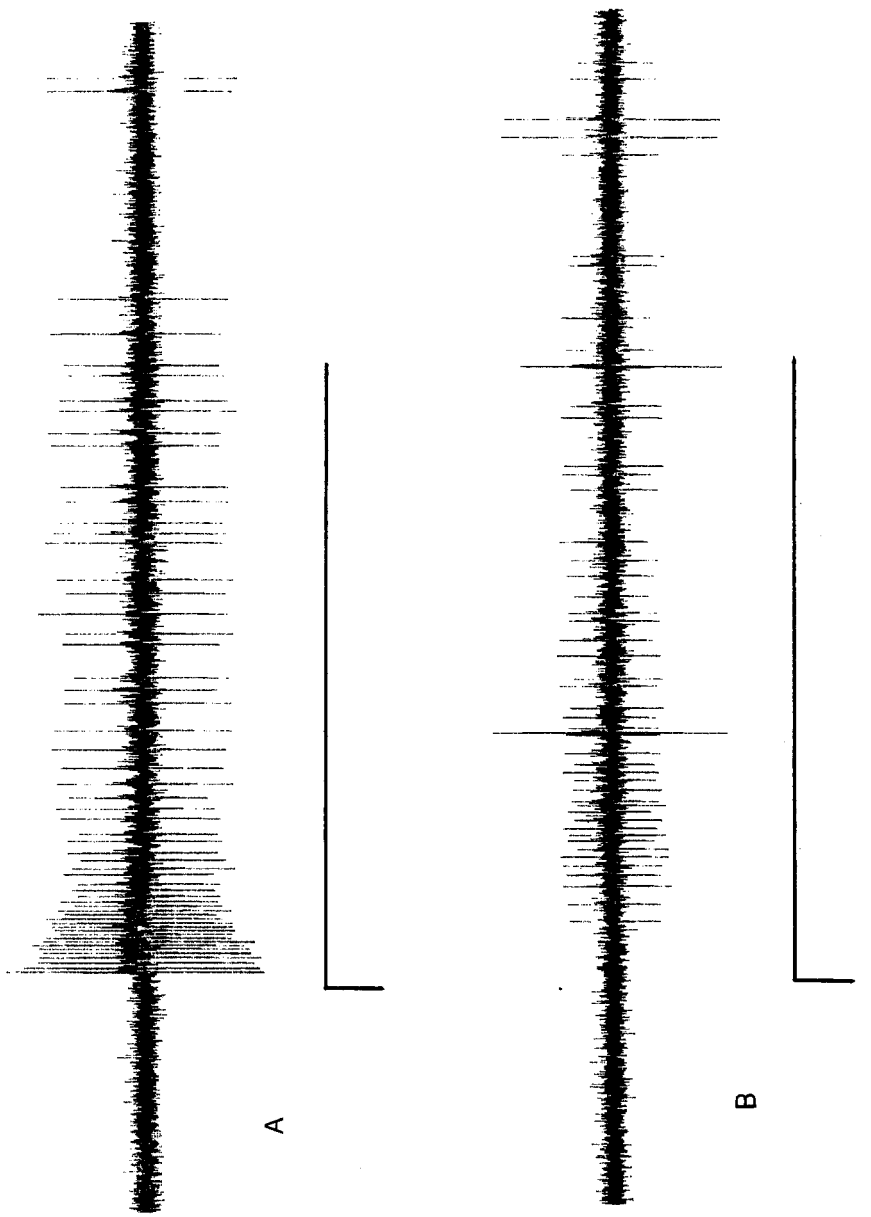


FIG. 4. Responses recorded from a single sensillum trichodeum in male *H. nebulellum*: (A) stimulation by 0.1 μg of Z9,*E*12–14:Ald during 1 sec; (B) stimulation by 0.1 μg of Z9,*E*12–14:Ac during 1 sec. Vertical scale: 1 mV; horizontal scale: 1 sec.

best results in 1988 (Table 2). This compound was proven to increase the attractivity of a ternary blend (HN5 vs. HN9), but the most important catches were obtained with the four-component blend in the same ratio as in the female extract, i.e., 4.8% of *Z*9,*E*12–14 : Ald. In contrast, a blend including this dienic aldehyde as the major constituent caught five times fewer males.

In another experiment in the summer of 1989, the role of single components was investigated by comparing the attractivity of three- component versus four-component blends (Table 3). The removal of *Z*9,*E*12–14 : Ald (HN15) completely suppressed the captures. The importance of *Z*11–16 : Ald (HN16) and *Z*13–18 : Ald (HN17) for attraction also was demonstrated, whereas *Z*9–14 : Ald did not seem to be involved in sexual attraction in our experimental conditions (HN14).

DISCUSSION AND CONCLUSION

The terminology of pheromone components used in moth communication systems is quite ambiguous for *H. nebulellum*. In many species, the major component of the female secretion is easily detected by EAG screenings (Roelofs, 1984), and sometimes it is sufficient to attract males when used alone or it dramatically suppresses catches when removed from a complex blend mimicking the natural pheromone (Linn et al., 1984). Most behavioral studies have shown the importance of minor compounds added to the major component to evoke complete mating sequences in olfactometers or higher capture levels in field tests.

The results obtained with *H. nebulellum* lead us to choose slightly different terms: (1) The analyses of gland extracts have shown that *Z*11–16 : Ald is the major component of female secretion (50%). (2) The EAG and single-cell recordings clearly demonstrated *Z*9,*E*12–14 : Ald to be the "antennal key compound." (3) From the field trapping results it appeared that *Z*9,*E*12–14 : Ald was not attractive when used alone but was essential to attraction in ternary blends, thus *Z*9,*E*12–14 : Ald is the behavioral key compound.

If *Z*9,*E*12–14 : Ald is the antennal or behavioral key compound, one would expect it to be the major component in the female secretion, as seen in nearly all previous identification studies that include GC analyses, EAG studies and bioassays (see Tamaki, 1985; Arn et al., 1986, for the most recent reviews). Nevertheless, extracts obtained by various methods in *H. nebulellum* have shown that the percentages of *Z*9,*E*12–14 : Ald never exceeded 10%. This low amount does not seem to be an artifact due to an inadequate extraction method, because the blends containing 5% of this compound are more attractive in the field than those containing 60% (HN9 vs. HN10, Tables 2 and 3). Furthermore, the most attractive blends in 1988, HN1 and HN5, with 65% of *Z*9,*E*12–14 : Ald

attracted five males only in 1989 when in competition with other blends containing 5% of this compound (Table 2). The role of Z9–14:Ald cannot be deduced from these experiments; in some cases, it appears as a slight but not significant inhibitor (Table 3). An evaluation of the blend really emitted by the female is needed before including it as a component of the sex pheromone.

A complete pheromone identification is generally achieved by the collection of airborne effluvia emitted by the calling females, but the heterogeneity of chain lengths of *H. nebullellum* pheromone compounds did not provide a suitable model for a collection of volatiles. The occurrence of 14, 16, and 18 carbon chain lengths could prevent a reliable quantification of relative percentages (and all subsequent EAG or behavioral studies) without previous studies on adsorbance and elution of these compounds on either glass or resin.

By examining the previous pheromone identification studies in Lepidoptera, we found that the antennal and behavioral key compound was always the major component of the female secretion (in many cases, a blend of two geometric isomers acts as a unit in the same way). Thus the chemical communication system used by *H. nebulellum* seems to be rather unusual. In another Phycitinae, *Elasmopalpus lignosellus*, the major component of the female secretion, (*Z*)-9-hexadecenol, is not essential to the male attraction (Lynch et al., 1984), but EAG data are not available for this species and the key compound does not appear clearly from this study.

The American sunflower moth, *Homoeosoma electellum*, is a species that seems to occupy the same ecological niche as *H. nebulellum* in Europe. In both species kairomonal relationships were reported between the female moths and a substance released by the pollen of sunflowers (Delisle et al., 1989,for *H. ellectellum*; Le Metayer, to be published, for *H. nebulellum*). Thus these species appear to be very closely related. By contrast, the sex pheromone system of *H. ellectellum* is quite different from the European species. The major component, (*Z,E*)-9,12-tetradecadienol (*Z*9,*E*12–14:OH), is able to attract more males than virgin females, even at low dosage (1 μg), and other compounds identified in the female secretion, (*Z*)-9-tetradecenol and tetradecanol, do not enhance the efficiency of the attractant tested (Underhill et al., 1979, 1982).

The occurrence of a key compound in the ESM that is not the major constituent of the female blend raises many questions about the perception of multicomponent sex pheromones. The importance of minor compounds essential to attraction was pointed out nearly 20 years ago by Klun et al. (1973). Since then, two different systems were proposed to explain the perception of complex blends. In the first one, the major component acts as a long-range releaser to evoke taking off and initial orientation. The minor compounds may act at short-range in eliciting subsequent behaviors in the mating sequence, like landing or courtship initiation. Then, each component, or a partial combination of components, would play a different role during the mating sequence (Bradshaw et

al., 1983). More recently, it appeared that, instead of different steps of behavior released by different components, the whole blend acts as a unit to evoke a behavioral sequence. This problem was reviewed by Linn and Roelofs (1989), who proposed the terms "component paradigm" and "blend paradigm" to define the two possible processes. According to the most recent works, the blend paradigm tends to be recognized as a general phenomenon (Linn and Roelofs, 1989).

At this time, the precise blend emitted in the air by the calling female remains unknown. If *Z*9,*E*12–14 : Ald really acts at very low percentages, the ESM could represent a new model to understand the perception of multicomponent pheromones in Lepidoptera. Work to investigate this further is in progress.

Acknowledgments—We are grateful to Dr. Jeremy N. McNeil for his helpful comments on the manuscript and reviewing the English text.

REFERENCES

ARN, H., TOTH, M., and PRIESNER, E. 1986. List of sex pheromones of Lepidoptera and related attractants. OILB-SROP, Paris. 123 pp.

ARTHUR, A.P. 1978. The occurrence, life history, courtship, and mating behaviour of the sunflower moth, *Homoeosoma ellectellum* (Lepidoptera: Phycitidae), in the Canadian prairie provinces. *Can. Entomol.* 110:913–916.

BALACHOWSKY, A.S. 1972. Les *Homoeosoma* Curtis, pp. 1184–1185, *in* A.S. Balachowsky (ed.). Entomologie appliquée à l'agriculture, T. 2, Lépidoptères, Vol. 2. Masson, Paris.

BRADSHAW, J.W.S, BAKER, R., and LISK, J.C. 1983. Separate orientation and releaser components in a sex pheromone. *Nature* 304:265–267.

DELISLE, J., MCNEIL, J.N., UNDERHILL, E.W., and BARTON, D. 1989. *Helianthus annuus* pollen, an oviposition stimulant for the sunflower moth, *Homoeosoma ellectellum*. *Entomol. Exp. Appl.* 50:53–60.

GOATER, B. 1986. British Pyralid Moths. A Guide to their Identification. Harley Books, Colchester, England. 175 pp.

JEFFERSON, R.N., SHOREY, H.H., and RUBIN, R.E. 1968. Sex pheromones of noctuid moths. XVI. The morphology of the female sex pheromone glands of eight species. *Ann. Entomol. Soc. Am.* 61:861–865.

KAISSLING, K.E., and THORSON, J. 1980. Insect olfactory sensilla: Structural, chemical and electrical aspects of the functional organization, pp. 261–282, *in* D.B. Satelle, L.M. Hall, and J.G. Hildebrand (eds.). Receptors for Neurotransmitters, Hormones and Pheromones in Insects. Elsevier, Amsterdam.

KLUN, J.A., CHAPMAN, O.L., MATTES, K.C., WOJTKOWSKI, P.W., BEROZA, M., and SONNET, P. 1973. Insect sex pheromones: Minor amount of opposite geometrical isomer critical to attraction. *Science* 181:661–663.

LINN, C.E., JR., and ROELOFS, W.L. 1989. Response specificity of male moths to multicomponent pheromones. *Chem. Senses* 14:421–437.

LINN, C.E., JR., BJOSTAD, L.B., DU, J.W., and ROELOFS, W.L. 1984. Redundancy in a chemical signal: Behavioral responses of male *Trichoplusia ni* to a 6-component sex pheromone blend. *J. Chem. Ecol.* 10:1635–1658.

LUCAS, P., and RENOU, M. 1989. Responses to pheromone compounds in *Mamestra suasa* (Lepidoptera: Noctuidae) olfactory neurones. *J. Insect Physiol.* 35:837–845.

LYNCH, R.E., KLUN, J.A., LEONHARDT, B.A., SCHWARZ, M., and GARNER, J.W. 1984. Female sex pheromone of the lesser cornstalk borer, *Elasmopalpus lignosellus* (Lepidoptera: Pyralidae). *Environ. Entomol.* 13:121–126.

MARTOJA, R., and MARTOJA-PIERSON, M. 1967. Initiation aux techniques de l'histologie animale. Masson, Paris, 322 pp.

PERCY, J.E., and WHEATHERSTON, J. 1974. Gland structure and pheromone production in insects, pp. 11–34, *in* M.C. Birch (ed.). Pheromones, Frontiers of Biology, Vol. 32. North-Holland Publishing, Amsterdam.

PERMANA, A., PIVOT, Y., and LECLANT, F. 1991. La Pyrale du Tournesol: Curiosité ou menace? *Phytoma* In press.

POITOUT, S., and BUES, R. 1970. Elevage de plusieurs espèces de Lépidoptères Noctuidae sur milieu artificiel riche et sur milieu simplifié. *Ann. Zool.-Ecol. Anim.* 2:79–91.

RENOU, M., LALANNE-CASSOU, B., DORE, J.-C., and MILAT, M.-L. 1988. Electroantennographic analysis of sex pheromone specificity in neotropical Catocalinae (Lepidoptera: Noctuidae): A multivariate approach. *J. Insect Physiol.* 34:481–488.

ROELOFS, W.L. 1984. Electroantennogram assays: Rapid and convenient screening procedures for pheromones, pp. 131–159, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, New York.

TAMAKI, Y. 1985. Sex pheromones, pp. 145–191, *in* G.A. Kerkut and L.I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 9, Behaviour. Pergamon Press, Oxford.

UNDERHILL, E.W., ARTHUR, A.P., CHISHOLM, M.D., and STECK, W.F. 1979. Sex pheromone components of the sunflower moth, *Homoeosoma electellum*: Z9,*E*12-tetradecadienol and Z9-tetradecenol. *Environ. Entomol.* 8:740–743.

UNDERHILL, E.W., ROGERS, C.E., CHISHOLM, M.D., and STECK, W.F. 1982. Monitoring field populations of the sunflower moth, *Homoeosoma electellum* (Lepidoptera: Pyralidae), with its sex pheromone. *Environ. Entomol.* 11:681–684.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

VOLATILE COMPONENTS IN DEFENSIVE SPRAY OF THE SPOTTED SKUNK, *Spilogale putorius*

WILLIAM F. WOOD,* CHRISTOPHER G. MORGAN, and ALISON MILLER

Department of Chemistry
Humboldt State University
Arcata, California 95521

(Received December 17, 1990; accepted March 18, 1991)

Abstract—GC-MS analysis of the anal sac secretion from the spotted skunk, *Spilogale putorius*, showed three major volatile components: (*E*)-2-butene-1-thiol, 3-methyl-1-butanethiol, and 2-phenylethanethiol. Minor volatile components identified from this secretion were: phenylmethanethiol, 2-methylquinoline, 2-quinolinemethanethiol, bis[(*E*)-2-butenyl] disulfide, (*E*)-2-butenyl 3-methylbutyl disulfide, bis(3-methylbutyl) disulfide. All of these compounds except 2-phenylethanethiol have been identified previously from the striped skunk, *Mephitis mephitis*. The thioacetate derivatives *S*-(*E*)-2-butenyl thioacetate, *S*-3-methylbutanyl thioacetate, and *S*-2-quinolinemethyl thioacetate found in the striped skunk were not seen in this species.

Key Words—Mustelidae, *Spilogale putorius*, spotted skunk, anal sac secretion, (*E*)-2-butene-1-thiol, 3-methyl-1-butanethiol, 2-phenylethanethiol, phenylmethanethiol, 2-methylquinoline, 2-quinolinemethanethiol, bis[(*E*)-2-butenyl] disulfide, (*E*)-2-butenyl 3-methylbutyl disulfide, bis(3methylbutyl) disulfide.

INTRODUCTION

The four species of skunks (Carnivora, Mustelidae, subfamily Mephitinae) found in North America are the striped skunk, *Mephitis mephitis*; the spotted skunk, *Spilogale putorius*; the hog-nosed skunk, *Conepatus leuconotus*; and the hooded skunk, *M. macroura*. All are known for their potent means of chemical defense: the spraying of a repulsive-smelling liquid from their anal glands. Only

*To whom correspondence should be addressed.

0098-0331/91/0700-1415$06.50/0

the defensive secretion of the most common member of this group, the striped skunk, *M. mephitis*, has been reported and was the subject of many investigations (Swarts, 1862; Aldrich, 1896; Aldrich and Jones, 1897; Stevens, 1945; Andersen and Bernstein, 1975; Andersen et al., 1982; Wood, 1990). In this study, we report the volatile components of this malodorous secretion from the spotted skunk, *S. putorius*.

METHODS AND MATERIALS

Two spotted skunks, a male and a female, were obtained on removal from areas where they were a nuisance in Humboldt County, California. They were sacrificed with diethyl ether, and within an hour a sample of the defensive secretion was obtained by inserting a needle into the anal sac and withdrawing the contents. The secretion was immediately placed in a freezer at −20°C for several weeks until it could be analyzed by gas chromatography–mass spectrometry (GC-MS).

GC-MS was done on a Hewlett Packard gas chromatograph (model 5890) fitted with a mass selective detector (model 5970) using a 12-m cross-linked methyl silicone capillary column. The gas chromatograph was programmed so the oven temperature was kept at 40°C for 4 min, then increased to a final temperature of 250°C at a rate of 30°C/min, and kept at this temperature for 4 min. Mass spectral fragments below $m/z = 35$ were not recorded. An isotope peak ($M^+ + 2$) was observed for all sulfur-containing compounds that had a molecular ion. The relative amount of each component is reported as the percent of the total ion current.

Reference compounds were obtained as follows: (*E*)-2-Butene-1-thiol was obtained from the anal sac of a striped skunk (Andersen and Bernstein, 1975). 3-Methyl-1-butanethiol, phenylmethanethiol (benzyl mercaptan), 2-methylquinoline, and benzyl methyl sulfide were purchased from Aldrich Chemical Co. 1-Phenylethanethiol and 2-phenylethanethiol were prepared by the method of Urquhart et al. (1955) from (1-bromoethyl)benzene and (2-bromoethyl)benzene, respectively. 2-Quinolinemethanethiol was prepared as described by Wood (1990). Bis[(*E*)-2-butenyl] disulfide, (*E*)-2-butenyl 3-methylbutyl disulfide, and bis(3-methylbutyl) disulfide were prepared by treatment of the defensive secretion with iodine as described by Wood (1990).

Since the thioacetates detected from the striped skunk (Wood, 1990) were not found in this study, an experiment was done to see if they were lost on storage. A sample of the striped skunk secretion that had been kept in a freezer for six months was examined. GC-MS analysis showed the thioacetates had not been destroyed during storage.

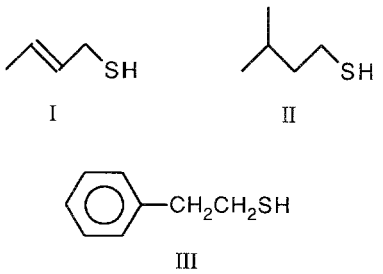
RESULTS

GC-MS analysis of a diethyl ether solution of the anal sac secretion from the spotted skunks showed three major components (Figure 1). These compounds and their amounts (percentage male, percentage female) are (*E*)-2-butene-1-thiol (I; 36%, 30%), 3-methyl-1-butanethiol (II; 48%, 66%) and 2-phenylethanethiol (III; 5%, 2%). (*E*)-2-Butene-1-thiol was identified by comparison of the GC retention time and mass spectrum with a sample of this compound from the striped skunk. 3-Methyl-1-butanethiol had identical retention time and mass spectra as an authentic sample.

The mass spectrum of the third major component was: *m/z* 140 (M^++2, 0.9), 138 (M^+, 21), 105 (5), 104 (14), 103 (5), 92 (14), 91 (100), 78 (6), 77 (10), 65 (12), and 51 (12). Since an isotope peak at *m/z* 140 (M^++2) was 4% of the molecular ion (*m/z* 138), it indicates the compound contains one sulfur atom and suggests it has the molecular formula, $C_8H_{10}S$. The base peak at *m/z* 91 is likely a tropylium ion ($C_7H_7^+$) and indicates the sulfur to be on the side chain of a monosubstituted benzene. The three possible candidates that fit these criteria are benzyl methyl sulfide, 1-phenylethanethiol, and 2-phenylethanethiol. When these compounds were checked by GC-MS, only 2-phenylethanethiol had the same retention time and mass spectrum as the compound from the skunk.

A number of minor components (Figure 2) were identified from this secretion by comparison of GC retention times and mass spectra with reference compounds. These compounds and their amounts (percentage male, percentage female) are phenylmethanethiol (IV; 0.8%, 0.2%), 2-methylquinoline (V; 0.9%, 0.3%), 2-quinolinemethanethiol (VI; 0.3%, 0.2%), bis[(*E*)-2-butenyl] disulfide (VII; trace, trace), (*E*)-2-butenyl 3-methylbutyl disulfide (VIII; 0.6%, 0.2%), and bis(3-methylbutyl) disulfide (IX; 0.1%, 0.2%).

The thioacetate derivatives of thiols found by Wood (1990) in the striped skunk could not be detected in the spotted skunk. These compounds (Figure 3)



I II III

FIG. 1. The major volatile components of spotted skunk spray: (*E*)-2-butene-1-thiol (I), 3-methyl-1-butanethiol (II), and 2-phenylethanethiol (III).

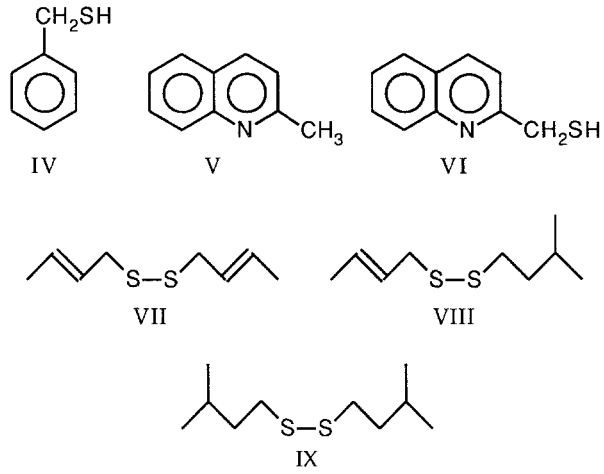


FIG. 2. The minor volatile components in spotted skunk spray: phenylmethanethiol (IV), 2-methylquinoline (V), bis[(*E*)-2-butenyl] disulfide (VII), (*E*)-2-butenyl 3-methylbutyl disulfide (VIII), and bis(3-methylbutyl) disulfide (IX).

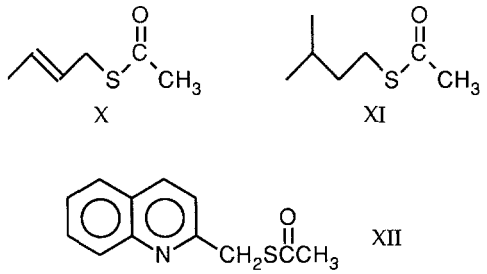


FIG. 3. The thioacetates found in striped skunk spray that are not found in the spotted skunk: *S*-(*E*)-2-butenyl thioacetate (X), (*S*)-3-methylbutanyl thioacetate (XI), and *S*-2-quinolinemethyl thioacetate (XII).

are *S*-(*E*)-2-butenyl thioacetate (X), *S*-3-methylbutanyl thioacetate (XI), and *S*-2-quinolinemethyl thioacetate (XII). To see if these compounds had been lost during the several weeks of storage, a six-month-old sample of striped skunk secretion stored at −20°C was analyzed by GC-MS. The thioacetates were still present.

DISCUSSION

The two major thiols in the spotted skunk secretion, (*E*)-2-butene-1-thiol and 3-methyl-1-butanethiol, are also the major components in the defensive spray of the striped skunk (Andersen and Bernstein, 1975). The ratio of these

compounds is the reverse of the striped skunk. (*E*)-2-Butene-1-thiol is the major volatile component of the striped skunk (Wood, 1990), while 3-methyl-1-butanethiol is the major volatile component of the spotted skunk. The third most abundant compound, 2-phenylethanethiol, has not been reported from the defensive secretion of the striped skunk. Andersen and coworkers (1982) did report an isomer of this compound, 1-phenylethanethiol, as a minor component from the striped skunk. It is clear that the compound from the spotted skunk is 2-phenylethanethiol as the 1-phenyl isomer had a different mass spectrum.

The minor components found in the spotted skunk defensive secretion have been identified previously in striped skunk defensive secretion. Andersen and coworkers (1982) identified phenylmethanethiol at less than 1%. They also found the three disulfides that form on oxidation of the two major thiols, bis[(*E*)-2-butenyl] disulfide, (*E*)-2-butenyl 3-methylbutyl disulfide, bis(3-methylbutyl) disulfide, as minor components (less than 1%). 2-Methylquinoline was first identified by Aldrich and Jones (1897), and 2-quinolinemethanethiol was found by Wood (1990) in the striped skunk.

A major difference in the secretions from the striped and spotted skunks is the lack of thioacetates in the spotted skunk secretion. Wood (1990) found a thioacetate derivative for each of the free thiols found in the striped skunk. It is clear that these thioacetates were not present in the spotted skunk secretion and were not lost on storage. The fact that the thioacetates are not present in the spotted skunk secretion may give a clue to the biosynthesis of these compounds and their corresponding thiols. The thioacetates also may play a part in the difference in effectiveness of the two secretions. The thioacetate derivatives will slowly hydrolyze and continue to release the offensive-smelling thiols over a period of time. Anecdotal reports from an individual whose pets have been sprayed by both species indicates the defensive secretion of the striped skunk is more persistent and harder to remove (W.C. Lancaster, Humboldt State University, personal communication). This individual also reports "skunk odor" to return to sprayed animals long after an encounter with a striped skunk when their hair is wet.

Acknowledgments—William Stanley, Assistant Curator, Vertebrate Museum, Humboldt State University, is thanked for obtaining one skunk and for confirming the identification of the species. Tom Borgers, Department of Chemistry, Humboldt State University, is thanked for helping collect one of the skunks. This study was partially supported by a Research and Creative Activity Award No. 1-11482 from the California State University.

REFERENCES

ALDRICH, T.B. 1896. A chemical study of the secretion of the anal glands of *Mephitis mephitica* (common skunk), with remarks on the physiological properties of this secretion. *J. Exp. Med.* 1:323-340.

ALDRICH, T.B., and JONES, W. 1897. α-Methyl-quinoline as a constituent of the secretion of the anal glands of *Mephitis mephitica*. *J. Exp. Med.* 2:439–452.

ANDERSEN, K.K., and BERNSTEIN, D.T. 1975. Some chemical constituents of the scent of the striped skunk (*Mephitis mephitis*). *J. Chem. Ecol.* 1:493–499.

ANDERSEN, K.K., BERNSTEIN, D.T., CARET, R.L., and ROMANCZYK, L.J., JR., 1982. Chemical constituents of the defensive secretion of the striped skunk (*Mephitis mephitis*). *Tetrahedron* 38:1965–1970.

STEVENS, P.G. 1945. American musk III. The scent of the common skunk. *J. Am. Chem. Soc.* 67:407–408.

SWARTS. 1862. Über das Öl des Stinkthiers. *Justus Liebigs Ann. Chem.* 123:266–270.

URQUHART, G.G., GATES, J.W., JR., and CONNOR, R. 1955. *n*-Dodecyl (lauryl) mercaptan, pp. 363–365, *in* E.C. Horning (ed.). Organic Syntheses Collective, Vol. 3. John Wiley & Sons, New York.

WOOD, W.F. 1990. New components in defensive secretion of the striped skunk, *Mephitis mephitis*. *J. Chem. Ecol.* 16:2057–2065.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

INTERSPECIFIC PAIRING BETWEEN TWO SIBLING *Ips* SPECIES (COLEOPTERA: SCOLYTIDAE)

J.W. FOX,[1,*] D.L. WOOD,[1] and J.H. CANE[2]

[1]*Department of Entomological Sciences*
University of California
Berkeley, California 94720

[2]*Department of Entomology*
Auburn University
Auburn, Alabama 36849-5413

(Received September 24, 1990; accepted March 18, 1991)

Abstract—Host and conspecific discrimination were tested as reproductive isolating mechanisms between *Ips paraconfusus* Lanier infesting *Pinus coulteri* (Torrey) and *I. confusus* (Le Conte) infesting *P. monophylla* (Fremont). In two areas (one area largely Coulter pine and the other largely pinyon pine) where these bark beetles and hosts cooccur in southern California, we induced pheromone production in host and nonhost logs using males of each species. *Ips paraconfusus* females joined both heterospecific and conspecific males tunneling in both tree species in each area. *Ips confusus* females failed to join *I. paraconfusus* males in Coulter pine, but joined *I. paraconfusus* in pinyon pine. Sympatry was demonstrated when females of both sibling species joined conspecific males in their respective hosts. Males attacked all four beetle–host treatment combinations in both areas. Laboratory tests confirmed these results. Males did not displace heterospecific males from nuptial chambers in hosts, and they did not occupy a gallery in which heterospecific males produced frass. Females that left conspecific males in a host were readily accepted by heterospecific males and oviposited in a nonhost.

Key Words—Interspecific mating, *Ips confusus*, *Ips paraconfusus*, Coleoptera, Scolytidae, reproduction, reproductive isolation.

INTRODUCTION

The engraver bark beetle genus *Ips* contains about 60 recognized species in North and Central America, Europe, and Asia (Hopping, 1963, 1965; S. L.

*To whom correspondence should be addressed.

Wood, 1982). Three sibling species in species group *grandicollis* Wood (1982), *I. confusus* (Le Conte), *I. hoppingi* Lanier, and *I. paraconfusus* Lanier are virtually identical morphologically and electrophoretically (Cane et al., 1990b), which suggests a recent origin from a common ancestor. Their species status is based primarily upon their host association, geographic distribution, failure to hybridize, karyology, and differences in the striation density on the *pars stridens*, a cephalic stridulatory apparatus (Lanier, 1966, 1970, 1971, 1972). In the San Bernardino National Forest there are areas of nearly pure stands of Coulter pine, *P. coulteri* (Torrey), a host of *I. paraconfusus*, and single-leaf pinyon, *P. monophylla* (Fremont), a host of *I. confusus*, as well as narrow zones of host sympatry (Critchfield and Little, 1966). One potential postmating barrier, failure of heterospecific larvae to successfully develop in a nonhost, does not occur (Cane and Wood, unpublished). Another postmating barrier, failure to hybridize, demonstrated by Lanier (1971), was recently confirmed by Merrill (1991) when she reared beetles in the laboratory and paired them with virgin females of known identity. The study reported here continues our investigation (Cane et al., 1990a, b) of possible premating barriers to hybridization between *I. paraconfusus* and *I. confusus*.

Premating barriers likely involve discrimination of host from nonhost, conspecific from heterospecific pheromones, and mate recognition or proximal behavioral cues. Host discrimination by *Ips* species occurs prior to or after landing, or after penetration of the bark. Host discrimination by *I. paraconfusus* may be influenced by the presence of feeding stimulants and the absence of feeding deterrents in the host phloem (Elkinton and Wood, 1980; Elkinton et al., 1980). However, Cane et al. (1990a) concluded that host discrimination was not absolute for these populations because they demonstrated that: (1) both male and female *I. confusus* and *I. paraconfusus* colonize host and nonhost pine bolts (pinyon and Coulter pine) presented to populations adjacent to a zone of sympatry; and (2) pioneering *I. paraconfusus* and *I. confusus* males, under the influence of pheromones, are trapped in large numbers at uninfested pinyon and Coulter pine bolts in both habitats. Stridulation can be important for mate recognition in *Ips* species and other scolytids (Barr, 1969; Alcock, 1982). *Ips paraconfusus* and *I. confusus* have different densities of striae on the pars stridens of the stridulatory apparatus (Lanier, 1970; Cane et al., 1990a) but produce nearly identical fundamental frequencies of vibration. However, their bisyllabic chirps are temporally distinctive (Lewis and Cane, unpublished).

Pheromones may serve as premating barriers for bark beetles. *Ips* species produce pheromones that elicit behavior, resulting in aggregation of beetles on the new host. This aggregation must be both timely and of sufficient magnitude to exploit the new resource. Pheromones are produced in the insect gut and frass (fecal pellets with phloem and xylem fragments) after prolonged feeding on phloem and have been identified for *I. paraconfusus* and *I. confusus* (sum-

marized in D. L. Wood, 1982). Differences in response to and production of pheromones has been documented in *I. pini* and may account for some species differentiation (Lanier et al., 1972, 1980; Birch et al., 1980). Lanier and Wood (1975) tested the pheromone specificity of 17 species of *Ips* and discovered that species within the same species groups are cross-responsive, unlike species in different species groups. Species with broadly sympatric distributions in the same host are from different species groups, while members in the same species group are largely allopatric. *Ips paraconfusus* and *I. confusus* females were attracted to frass produced by males of the other species when presented individually in the laboratory olfactometer and in simultaneous, paired comparisons with conspecific frass. Cross-responsiveness and interspecific pairing (i.e., females joining male galleries) between *I. confusus* and *I. paraconfusus* has been demonstrated in the field, but intermating was not established (Lanier and Wood, 1975). Cane et al. (1990a) reported that for beetles in flight, discrimination of host volatiles and conspecific pheromones were incomplete and asymmetrical because female *I. paraconfusus* did not have the same degree of discrimination as female *I. confusus*.

In the San Bernardino National Forest, heterospecific pairing and natural colonization of nonhosts were not located in zones of sympatry despite persistent search in this and earlier studies (Cane et al., 1990a). Therefore, we further investigated whether this incomplete discrimination of host volatiles and pheromones, coupled with proximal behavioral cues subsequently available to beetles freely walking on the bark, may be sufficient to elicit associations with the native host and conspecific mates. First, we tested the hypothesis that beetles in the field can pair with heterospecifics and colonize nonhosts. Next, this hypothesis was partitioned into specific behavioral elements. In the laboratory, experiments were designed to determine: (1) the likelihood of beetles encountering a heterospecific at a nuptial chamber excavated by a conspecific male; (2) the ability of beetles to discriminate hosts and conspecifics in an atmosphere saturated with pheromones of both species; (3) the influence of host on conspecific discrimination; (4) if heterospecific pairing will occur when populations are limited; and (5) if conspecific-inseminated females will join heterospecific males.

METHODS AND MATERIALS

Field Tests

Two test sites were selected during June 1–15, 1986, in the San Bernardino Mountains of southern California. One test site was located near Lake Arrowhead, California, at approximately 1500 m elevation on U.S. Forest Service property along Hook Creek Road 2N26Y, Range 2W, and Township 2N. Here-

after, this site is referred to as Lake Arrowhead. The dominant canopy vegetation was mixed conifer, consisting of mainly Coulter and ponderosa pines with some incense cedar, white fir, and oaks. The nearest pinyon pines were estimated to be 5–7 km distant. An intensive Coulter pine thinning operation provided logging debris heavily infested with *I. paraconfusus*.

The second site was approximately 67 km east of Lake Arrowhead near Baldwin Lake at approximately 2000 m elevation on Highway 38, Range 2E, and Township 3N. Hereafter, this site is referred to as Baldwin Lake. Here the dominant vegetation was primarily pinyon pines, oaks, Joshua trees, and cacti. Pinyon pines weakened by blackstain root disease [*Leptographium wageneri* (Harrington and Cobb)] (Cobb et al., 1974) were attacked by *I. confusus*. The nearest ponderosa and Jeffrey pines, potential hosts to *I. paraconfusus*, were estimated to be 2–4 km distant. Coulter pines are not present at this locality. Both areas were monitored for bark beetle activity using multiple-funnel traps (Lindgren, 1983) baited with *I. paraconfusus* males tunneling in ponderosa pine. These traps consistently caught approximately 16 beetles/day for three days.

Ips paraconfusus in Coulter pine were collected 5 km north of Lake Arrowhead, and *I. confusus* in pinyon pine were collected at Little Pine Flat, 17 km east of Baldwin Lake. Four living pinyon pines and two Coulter pines, 20–25 cm in diameter, were felled, and 24 hr later, cut into sections each providing 2000 cm^2 surface area. Males of each species were paint-marked on the elytral declivity (which had no apparent effect on beetle behavior) and were singly confined using 2 × 2-cm aluminum screen stapled over artificial entrance tunnels formed in host and nonhost pines. There were 20 logs in both areas, five replicates of each treatment, each containing 25 males: (1) *I. confusus* on pinyon, (2) *I. confusus* on Coulter, (3) *I. paraconfusus* on pinyon, and (4) *I. paraconfusus* on Coulter pine. After 24 hr, each log had 20–25 beetles producing frass. Logs were assigned randomly over a grid pattern in a fairly uniform area of forest with each position separated from another by at least 25 m. Following placement, the small aluminum screens were removed and the logs were monitored every other day for fresh attacks, termed "volunteers."

After five days, the bark and phloem were cut and removed near the nuptial chamber. All adults were identified and galleries were recorded by tracing them onto clear acetate sheets. Test logs not dissected in the field were wrapped in aluminum screen, returned to Berkeley within 48 hr, and placed in cold storage. One hundred ninety-eight heads were examined by SEM (method described in Cane et al., 1990a), approximately 20 from each treatment at each site and 20 beetles captured as emergent adults from infested host trees at each site. Because a randomized block design was used where each block contains four treatments each with five logs at each of the two sites, the data from each treatment were analyzed using an ANOVA for randomized complete blocks (SAS, 1982). When the test indicated significant differences, all pairs of treatments were compared

by using the Studentized range test with Bonferroni's inequality (Bedard et al., 1980). Laboratory tests were analyzed using Bonferroni's inequality. All tests were made at the 5% level of significance.

Laboratory Behavior Tests

Experimental logs were placed in exclusion chambers measuring 1 m in each direction, producing an atmosphere which was assumed to be saturated with pheromones. Pinyon and Coulter pine hosts, stored at 5°C for one month, were cut into sections each providing 2000 cm^2 surface area. Factitious entrance tunnels were made in log sections using a drill, and beetles were confined in tunnels using 2 × 2-cm aluminum screen cages. All beetles used were uniquely paint-marked. At the terminus of the experiments, logs were quick-frozen at −30°C and dissected. Beetle identity, presence of an egg gallery, number of eggs and larvae, gallery length, and female and male position in the gallery were recorded on clear acetate sheets.

Displacement of Males in Nuptial Chambers. If female discrimination is important and if females are to reliably recognize a male's species status solely by the pheromone in his frass, which is produced during his excavation of the nuptial chamber, then males should remain associated with their nuptial chambers. If a heterospecific male usurps a nuptial chamber excavated by another male, unproductive pairings can occur. To test the hypothesis that males can displace resident males in nuptial chambers, 25 males of one beetle species were placed in a nonhost and 50 males of another beetle species that normally colonizes that host were released in the same chamber. After six days, these logs were removed from the boxes and external beetles were retrieved. The identity of the beetle occupying the nuptial chamber was observed.

Specific Mate Recognition with Host Discrimination. Conspecific pheromones emanating adjacent to heterospecific entrance tunnels may confound discrimination. To test this hypothesis, we examined the ability of beetles to discriminate conspecifics from heterospecifics when they were placed together in a host within an atmosphere saturated with pheromones of both species. Twenty each of *I. confusus* and *I. paraconfusus* were confined in one pinyon pine log and 20 each of both species were placed in one Coulter pine log, so that each male's nearest neighbor was a heterospecific. After 36 hr aluminum screen cages were removed from male entrance tunnels and both logs were placed in a box with 45 female *I. confusus* and 45 female *I. paraconfusus*. After six more days, the logs were removed and dissected.

Specific Mate Recognition with No Host Choice. Our field tests indicated that *I. confusus* females had a preference for their host. By eliminating host choice, we hoped to test conspecific discrimination only. We used one pinyon log only and 20 *I. confusus* and 20 *I. paraconfusus* males introduced into arti-

ficial entrance tunnels for 36 hr so that each male's nearest neighbor was a heterospecific. Next, the small aluminum screen cages were removed and the log was placed in a chamber with 45 *I. confusus* females. After six days, the log was frozen and dissected.

Heterospecific Females Mixed in Galleries. Conspecifics and heterospecifics may segregate when both species are plentiful, but join heterospecific males when populations are limited, because there may be an optimum number of beetles that improves the success of the family gallery (Kirkendall, 1983). To test this hypothesis, 20 *I. paraconfusus* males were introduced into artificial entrance tunnels in a Coulter pine log and placed in a separate chamber. Twenty *I. confusus* males were similarly placed in a pinyon pine log. After 36 hr the confining cages were removed and 15 females of each species were released into each chamber. After nine days the logs were frozen and dissected.

Females Leaving Conspecific Males and Pairing with Heterospecific Males. In the laboratory, interspecific-matings between these two species produce inviable eggs (Lanier, 1970). In the field we observed several males with heterospecific females producing larvae. One explanation for these observations may be that conspecific-inseminated females had joined the heterospecific male. To test this hypothesis, 20 male *I. paraconfusus* and 20 male *I. confusus* were confined in entrance tunnels on their hosts and placed in separate chambers for 36 hr. Conspecific females were introduced, one per day, into each male gallery until each male had accepted three females. Two days after this, a log containing only heterospecific males in their host was placed into each box. Therefore, females recovered from these nonhost logs containing heterospecific males could only come from the host logs containing conspecific males. To bracket the 14-day period that females may leave their egg galleries and possibly join heterospecifics, two other similar nonhost logs containing heterospecific males were added after 10 more days had elapsed. All logs were frozen and dissected.

RESULTS

Field Tests

Measurements of 10 striae of the pars stridens of 20 females that emerged from Coulter pine collected at Lake Arrowhead ranged from 0.42 to 0.49 μm ($\bar{X} = 0.46$). For 19 females emerged from pinyon pine collected at Baldwin Lake, the measurements ranged from 0.56 to 0.69 μm ($\bar{X} = 0.63$). These data generated a size range of the striae for both species (Table 1). Lake Arrowhead appears to be almost exclusively an *I. paraconfusus* population, with only 2% *I. confusus* present. Here only two of 79 females removed from the treatment logs were found with striae widths that did not fit the size ranges of either *I. confusus* or *I. paraconfusus* that had emerged from their respective hosts. We

TABLE 1. IDENTITIES OF FEMALE *Ips* COLLECTED IN THE SAN BERNARDINO MOUNTAINS[a]

Treatment	SEM Striae Measurements		
	I. paraconfusus (range 0.42–0.49 μm)	Unidentified (range 0.51–0.54 μm)	*I. Confusus* (range 0.56–0.69 μm)
Lake Arrowhead			
I. paraconfusus in Coulter pine	20	0	0
I. confusus in Coulter pine	19	1	0
I. paraconfusus in pinyon pine	20	0	0
I. confusus in pinyon pine	17	1	2
I. paraconfusus in Coulter pine[b]	20	0	0
Baldwin Lake			
I. paraconfusus in Coulter pine	14	6	0
I. confusus in Coulter pine	2	3	14
I. paraconfusus in pinyon pine	3	3	13
I. confusus in pinyon pine	4	0	14
I. confusus in pinyon pine[b]	0	0	19

[a] Identification is based upon the width of 10 striations of the supracephalic stridulatory organ (*pars stridens*) of the female. Beetles were removed from experimental logs placed in the field or emerged from logging debris collected on site. Beetles within the range 0.51–0.54 μm could not be confidently identified and were designated as unidentified.
[b] Collected from naturally infested logging debris on site.

classed these females as "unidentified." A mixed species population was present at Baldwin Lake with a ratio of about two *I. confusus* to one *I. paraconfusus* and 15% of the female *Ips* population at Baldwin Lake were in the "unidentified" size range.

The mean number of males that formed nuptial chambers ranged from 21.4 to 24.4 for the 25 artificial entrance tunnels at both test sites. A nuptial chamber was formed if the engraving on the surface of the sapwood was similar in size and shape to other nuptial chambers that yielded a successfully joined male. At Lake Arrowhead, fewer *I. confusus* in pinyon pine produced frass than the other treatments and at Baldwin Lake fewer *I. paraconfusus* produced frass in Coulter

pine than the other treatments, but these differences were not significant. At Baldwin Lake fewer *I. paraconfusus* and *I. confusus* tunneling in pinyon produced frass than these species tunneling in Coulter pine. These differences were significant. Interestingly, more nuptial chambers were abandoned in pinyon pine than in Coulter pine at both sites.

At Lake Arrowhead, *I. paraconfusus* females joined all treatments. However, treatments with Coulter pine were significantly different from treatments with pinyon pine (Table 2). Thus *I. paraconfusus* females appear to be able to discriminate their host. At Baldwin Lake, no *I. confusus* females joined male

TABLE 2. SUMMARY OF OBSERVATIONS[a] ON FEMALES FLYING TO AND PAIRING WITH 25 MALES OF *I. paraconfusus* AND *I. confusus* FACTITIOUSLY INTRODUCED INTO COULTER AND PINYON PINE, SAN BERNARDINO NATIONAL FOREST, JUNE 1986

	Species of male factitiously introduced into logs			
	Coulter pine		Pinyon pine	
Observations	*I. paraconfusus*	*I. confusus*	*I. confusus*	*I. paraconfusus*
Lake Arrowhead				
Nuptial chambers produced	24.4 ± 1.3a	24.0 ± 2.2a	21.8 ± 2.9a	23.2 ± 2.1a
Nuptial chambers abandoned	2.0 ± 4.5a	1.4 ± 3.1a	5.8 ± 4.0a	5.0 ± 4.8a
Frass production after 2 days	22.4 ± 1.1a	22.2 ± 2.1a	19.2 ± 3.1a	20.4 ± 2.1a
Available males unjoined	3.0 ± 2.6a	2.0 ± 1.6a	4.2 ± 3.6a	4.0 ± 2.9a
Available males paired with females	19.6 ± 6.4a	19.8 ± 5.5a	11.2 ± 4.1b	13.4 ± 7.2b
Volunteers	18.4 ± 3.7a	13.4 ± 8.5a	5.0 ± 3.5b	4.6 ± 3.1b
Baldwin Lake				
Nuptial chambers produced	23.4 ± 1.3a	23.4 ± 1.1a	24.0 ± 0.7a	21.4 ± 2.7a
Nuptial chambers abandoned	3.6 ± 2.1a	3.6 ± 2.3a	8.4 ± 2.8b	8.2 ± 1.3b
Frass production after 2 days	16.6 ± 6.8a	17.6 ± 4.4a	21.4 ± 2.6b	19.8 ± 2.2b
Available males unjoined	15.6 ± 3.7a	15.2 ± 3.8a	9.8 ± 2.2b	9.0 ± 6.0b
Available males paired with females	3.6 ± 2.3a	3.8 ± 3.8a	6.0 ± 2.2a	4.0 ± 3.4a
Volunteers	2.4 ± 2.7a	2.2 ± 2.3a	2.6 ± 1.7a	3.6 ± 2.7a

[a] Means followed by different letters in rows are significantly different ($P < 0.05$) using ANOVA for randomized blocks.

I. paraconfusus in Coulter pine (Table 1). However, *I. paraconfusus* females clearly preferred conspecific males in their host Coulter pine. The remainder of *I. paraconfusus* and a small number of "unidentified" females joining males were distributed almost equally among the other three treatments. At Baldwin Lake, *Ips confusus* females appeared to be capable of discrimination of the combination of *I. paraconfusus* in Coulter pine from all other host–species combinations.

There were no significant differences in mean number of females recovered per male (Table 3). At Lake Arrowhead, the mean number of egg galleries in pinyon pine was different (i.e., lower) from that in Coulter pine, due to the reduced female joining observed in pinyon pine (Table 2). Egg gallery length in pinyon pine with male *I. confusus* and *I. paraconfusus* also was reduced compared to other treatments in Coulter pine. At Baldwin Lake, mean egg gallery length in the treatment with Coulter pine and *I. paraconfusus* females was reduced. There were significant differences between abandoned nuptial chambers in both pine species at Baldwin Lake. More than twice as many nuptial

TABLE 3. SUMMARY OF OBSERVATIONS[a] ON EGG GALLERIES FORMED BY FEMALES OF *I. paraconfusus* AND *I. confusus* IN COULTER PINE AND PINYON PINE, SAN BERNARDINO NATIONAL FOREST, JUNE 1986

Observations	Coulter pine		Pinyon pine	
	I. paraconfusus	*I. confusus*	*I. confusus*	*I. paraconfusus*
Lake Arrowhead				
Males paired with females	19.6 ± 6.4a	19.8 ± 5.5a	11.2 ± 4.1b	13.4 ± 7.2b
Eggs	17.6 ± 5.2a	17.4 ± 6.5a	10.8 ± 3.7a	11.0 ± 7.7a
Larvae	8.6 ± 8.3a	3.80 ± 5.0ab	1.6 ± 1.5b	3.8 ± 4.2ab
Females	2.1 ± 1.0a	1.2 ± 0.9a	1.8 ± 1.5a	1.2 ± 0.9a
Egg galleries	2.5 ± 0.7a	2.4 ± 0.8a	1.4 ± 0.8b	2.2 ± 0.7b
Gallery length (cm)	5.8 ± 2.8a	4.6 ± 1.5a	3.6 ± 1.5b	3.6 ± 1.6b
Volunteers	18.4 ± 3.7a	13.4 ± 8.5a	5.0 ± 3.5b	4.6 ± 3.1b
Eggs	2.2 ± 0.9a	2.3 ± 0.7a	1.6 ± 1.2b	1.3 ± 0.9b
Baldwin Lake				
Males paired with females	3.6 ± 2.3a	3.8 ± 3.8a	6.0 ± 2.2a	4.0 ± 3.4a
Eggs	1.4 ± 1.5a	2.8 ± 3.0a	4.2 ± 3.1a	3.0 ± 4.2a
Larvae	0a	1.0 ± 1.0ab	1.8 ± 2.5b	0.20 ± 0.5a
Females	1.4 ± 0.7a	1.3 ± 0.5a	1.2 ± 0.7a	1.2 ± 0.7a
Egg galleries	1.5 ± 0.8a	1.3 ± 0.5a	1.5 ± 0.7a	1.3 ± 0.7a
Gallery length (cm)	2.3 ± 0.9a	5.0 ± 4.0b	5.3 ± 2.7b	4.7 ± 2.6b
Volunteers	2.4 ± 2.7a	2.2 ± 2.3a	2.6 ± 1.7a	3.6 ± 2.7a
Eggs	0a	0.6 ± 0.7a	0.8 ± 1.1a	0.3 ± 0.7a

[a] Means followed by different letters in rows are significantly different ($P < 0.05$) using ANOVA for randomized blocks.

chambers were abandoned in pinyon pine than in Coulter pine. These differences were not observed at Lake Arrowhead.

Male "volunteer" attacks were significantly different between sites (Table 2). There were no significant differences between volunteers on Coulter and pinyon pine at Baldwin Lake. At Lake Arrowhead, there were significant differences between Coulter and pinyon pine, where Coulter pine was more frequently attacked and more eggs were produced (Table 3).

Laboratory Tests

Displacement of Males in Nuptial Chambers. Heterospecific displacement was observed in only two nuptial chambers. These were in artificial entrance tunnels that displayed no frass after 24 hr. Generally males of one species do not displace males of another species from nuptial chambers.

Specific Mate Recognition with Host Discrimination. The mean number of nuptial chambers for each species was 16.25 (range 14–18). An average of 11.25 females (range 7–20) were distributed among these nuptial chambers. There were no significant differences between species of females joining males and pines in each treatment. However, there was a significant difference for females joining host, i.e., more females joined males in Coulter than males in pinyon pine. In pinyon pine, *I. confusus* males were not joined by *I. paraconfusus* females. Five *I. paraconfusus* males in pinyon were joined by *I. confusus* females but they produced no larvae. In Coulter pine, two males of each species were joined by heterospecific females, but these crosses yielded no larvae.

Specific Mate Recognition with No Host Choice. Six female *I. confusus* joined male *I. confusus* and six female *I. confusus* joined male *I. paraconfusus* when both male species were placed in pinyon pine. Four egg galleries with eggs and larvae were formed by female *I. confusus* paired with *I. confusus* males. Two egg galleries were formed, one with larvae, when *I. confusus* females joined *I. paraconfusus* males in pinyon pine.

Heterospecific Females in Mixed Galleries. Two of 11 galleries formed by *I. confusus* males in pinyon pine contained only *I. paraconfusus* females but no galleries contained *I. confusus* and *I. paraconfusus* females together. The remaining male galleries contained conspecific females. For male *I. paraconfusus* in Coulter pine, there were no families with *I. confusus* females only. Four male galleries contained *I. confusus* and *I. paraconfusus* females together. One male gallery had only conspecific females. There were no differences in numbers of single versus multiple gallery systems found in pinyon pine treatments; five galleries had single females and six galleries had two or more females. In Coulter pine eight male galleries contained multiple females and two contained single females. Galleries more often contained heterospecifics than conspecifics.

Females Leaving Conspecific Males and Pairing with Heterospecific Males. In all situations, we found that females left conspecifics in their host and readily paired with or were admitted by heterospecific males in the nonhosts. Seven *I. confusus* females joined *I. paraconfusus* males in Coulter pine and 10 *I. paraconfusus* females joined *I. confusus* males in pinyon pine. These females produced eggs and larvae in gallery systems with the conspecific males, but only *I. paraconfusus* females produced eggs and larvae in heterospecific galleries in pinyon pine.

DISCUSSION

Speciation in engraver bark beetles must be accompanied by barriers to gene flow. For synchronistic and sympatric populations of two or more species, such barriers can exist as premating mate recognition systems or reproductive isolation mechanisms. One premating mate recognition system may involve the density of striae on the pars stridens of the female stridulatory apparatus, which are different between *I. paraconfusus* and *I. confusus*. The female of either species stridulates at the entrance tunnel to the male nuptial chamber, and this may provide an opportunity to discriminate conspecifics. However, Lewis and Cane (unpublished) recently demonstrated that for these species: (1) fundamental stridulatory frequencies are not different, (2) significant differences appeared in their stridulatory tempos, but (3) males in freshly established nuptial chambers accepted stridulating heterospecific females walking on the bark surface.

In 1986, SEM examinations revealed that the test area at Baldwin Lake contained a mixed population of these sibling species together with a sizable number of "unidentified" individuals. Species were indiscriminately joining with heterospecifics at both Baldwin Lake and Lake Arrowhead, except that *I. confusus* females did not join *I. paraconfusus* males in Coulter pine at Baldwin Lake.

The preferences of joining females in the field follow the asymmetrical prelanding patterns of beetles found by Cane et al. (1990a). Heterospecific females joined males in all of our laboratory tests, which supports our field observations; however, few gallery systems contained females of both species during these laboratory tests. Finally, larval production for heterospecific crosses can be explained by prior mating of the females (Anderson et al., 1979). From these results we concluded that there were incomplete premating reproductive barriers between these populations of *I. paraconfusus* and *I. confusus*.

In the laboratory, heterospecific males do not displace other males in nuptial chambers and do not use a nuptial chamber that has been excavated by a male of another species. In the field, males likely abandon a nuptial chamber if not joined by a female. At Baldwin Lake, where pinyon pine is the only

conifer present, *I. confusus* males abandoned their host more frequently than the nonhost, Coulter pine. At both sites, a number of nuptial chambers initiated by experimentally introduced males were occupied by females only, or by no beetles; however, larvae or eggs were present. Adults in some bark beetle species are known to leave after oviposition is completed (Coulson et al., 1978; Schultz and Bedard, 1987).

There were some beetles that exhibited strial density measurements between the size ranges of both sibling species taken from their respective hosts (Table 1). If these "unidentified" individuals represent errors in the SEM measurements, we would expect to find them distributed in all treatments. Instead, we find them mainly clustered in treatments at Baldwin Lake. At either site "unidentified" individuals did not join endemic, conspecific males in their hosts, i.e. *I. paraconfusus* in Coulter pine at Lake Arrowhead and *I. confusus* in pinyon at Baldwin Lake. The unusual combinations of beetles in pines provided by our experiments may favor beetles that have intermediate strial density measurements. These beetles may be of limited number in the population. Possibly, they may be hybrids of *I. paraconfusus* and *I. confusus*. A zone of sympatry for beetles but not for hosts has formed at Baldwin Lake. Blackstain root disease has weakened pinyon pines in this area, making them available for bark beetle colonization for decades (Ernest Del Rio, San Bernardino National Forest, personal communication). Surprisingly, we have not found these "unidentified" individuals or heterospecifics emerging from infested logs collected in (Table 1) or near (Cane et al., 1990a) either locations.

Ancestors common to both *I. confusus* and *I. paraconfusus* may have been allopatric and different in degree of polyphagy than these species are today. These ancestors may have had their diet restricted due to the reduction in the distribution of the more mesic pines or altered due to the invasion of these areas by the xeric pinyons. Isozyme studies suggest that these beetle species diverged hundreds of thousands of years ago (Cane et al., 1990b). Why haven't effective premating barriers arisen and spread from the regions of host sympatry, given the reproductive costs incurred by heterospecific parents in "fatal matings" (Kirkendall, 1983)? Perhaps the ecotones shared by the beetles' hosts have always been geographically limited (Wells and Berger, 1967). If discriminating premating behaviors were of some selective disadvantage outside of sympatry, such as mistaken rejection of conspecific mates, and there was limited gene flow into areas of sympatry (likely for these vagile beetles), then premating barriers may not be manifest. Relative to mortality caused by other factors during a generation (e.g., predators, parasites, disease, host defenses, and poor food quality), reduced fecundity due to a "fatal mating" may be rare, and of little reproductive consequence, especially if the female mates more than once (Lanier and Oliver 1966), or mates at least once with a conspecific male during her lifetime.

The preference for the host and conspecific pheromone displayed by *I. confusus* may further reduce the chance of a mating mistake (Cane et al., 1990a). *Ips paraconfusus* females displayed little olfactory preference among conspecific and heterospecific pheromones at Lake Arrowhead; however, this species exhibited considerable preference for conspecific males in Coulter pine at Baldwin Lake. Females can avoid a nonproductive mating by rejecting the heterospecific pheromone. Yet females may encounter a conspecific mate at a resource where pheromones are produced by heterospecifics, as our tests demonstrate. When mated females join heterospecific males, they procure resources for their progeny. Although these males have not contributed genes to these offspring, they may gain a female, which contributes to the overall success of the gallery system by diminishing individual risks due to predation and parasitism (Kirkendall, 1983). If this is so, then this could be why ancestral semiochemical recognition has persisted. Genes leading to the cytoplasmic incompatibility accounting for the postmating reproductive barriers may be more flexible than genes for premating barriers and set by genetic drift (Paterson, 1981; Wiley, 1978). For engraver beetles in southern California, reinforcement of postmating isolation by premating isolation has not played a significant role in the production of separate species (Sanderson, 1989).

Acknowledgments—We wish to dedicate this manuscript to the memory of Gerry N. Lanier who pioneered this research. We are appreciative of the dedicated field and laboratory work of Dr. Margaret E. Silliker, Dr. Stephanie Meyers, Lester Casher, and Linda Kervin. We thank Laura A. Merrill for her review of the manuscript. This research was made possible through NSF grant BSR-8415910 (D.L.W., J.H.C., Isao Kubo, University of California Berkeley; Molly Stock, University of Idaho) and USDA/SEA Regional Research Project W-110.

REFERENCES

ALCOCK, J. 1982. Natural selection and communication among bark beetles (Scolytidae). *Fl. Entomol. Soc.* 65(1):17–32.

ANDERSON, W.W., BERISFORD, C.W., and KIMMICH, R.H. 1979. Genetic differences among five populations of the southern pine beetle. *Ann. Entomol. Soc. Am.* 72:323–327.

BARR, B.A. 1969. Sound production in Scolytidae (Coleoptera) with emphasis on the genus *Ips*. *Can. Entomol.* 101:636–672.

BEDARD, W.D., WOOD, D.L., TILDEN, P.E., LINDAHL, K.Q., JR., SILVERSTEIN, R.M., and RODIN, J.O. 1980. Field response of the western pine beetle and one of its predators to host- and beetle-produced compounds. *J. Chem. Ecol.* 6:625–641.

BIRCH, M.C., LIGHT, D.M., WOOD, D.L., BROWNE, L.E., SILVERSTEIN, R.M., BERGOT, B.J., OHLOFF, G., WEST, J.R., and YOUNG, J.C. 1980. Pheromonal attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. *J. Chem. Ecol.* 6(3):703–717.

CANE, J.H., WOOD, D.L., and FOX, J.W. 1990a. Ancestral semiochemical attraction persists for adjoining populations of sibling *Ips* bark beetles (Coleoptera: Scolytidae). *J. Chem. Ecol.* 16(4):993–1013.

CANE, J.H., STOCK, M.W., WOOD, D.L., and GAST, S.J. 1990b. Phylogenetic relationships of bark beetles: Electrophoretic and morphometric analyses of the *grandicollis* group. *Biochem. Syst. Ecol.* 18(5):359–368.

COBB, F.W., JR., PARMETER, J.R., JR., WOOD, D.L., and STARK, R.W. 1974. Root pathogens as agents predisposing ponderosa pine and white fir to bark beetles. Proceedings 4th International Conference on *Fomes annosus*, Athens, Ga. International Union, Forest Research Organization Section 24: Forest Protection, pp. 8–15.

COULSON, R.N., FARGO, W.S., PULLEY, P.E., POPE, D.N., RICHERSON, J.V., and PAYNE, T.L. 1978. Evaluation of the reemergence of parent adult *Dendroctonus frontalis* (Coleoptera: Scolytidae). *Can. Entomol.* 110:475–486.

CRITCHFIELD, W.B., and LITTLE, E.L. JR. 1966. Geographic distribution of the pines of the world. USDA Forest Service Miscellaneous Publication 991.

ELKINTON, J.S., and WOOD, D.L. 1980. Feeding and boring behavior of *Ips paraconfusus* (Coleoptera: Scolytidae) on the bark of a host and non-host tree species. *Can. Entomol.* 112:797–809.

ELKINTON, J.S., and WOOD, D.L., and HENDRY, L.B. 1980. Pheromone production by the bark beetle *Ips paraconfusus*, in the non-host, white fir. *J. Chem. Ecol.* 6:979–987.

HOPPING, G.R. 1963. The natural groups of species in the genus *Ips* De Geer (Coleoptera: Scolytidae). *Can. Entomol.* 95:508–516.

HOPPING, G.R. 1965. North American species in Group IX of *Ips* De Geer (Coleoptera: Scolytidae). *Can. Entomol.* 97:422–434.

KIRKENDALL, L.R. 1983. The evolution of mating systems in bark and ambrosia beetles (Coleoptera: Scolytidae and Platypodidae). *Zool. J. Linn. Soc.* 77:293–352.

LANIER, G.N. 1966. Interspecific mating and cytological studies of closely related species of *Ips* De Geer and *Orthotomicus* Ferrari (Coleoptera: Scolytidae). *Can. Entomol.* 98:175–188.

LANIER, G.N. 1970. Biosystematics of North American *Ips* (Coleoptera: Scolytidae). Hopping's group IX. *Can. Entomol.* 102:1139–1163.

LANIER, G.N. 1971. Cytoplasmic incompatibility and breeding isolation in bark beetles (Coleoptera: Scolytidae). *Can. J. Genet. Cytol.* 13:160–163.

LANIER, G.N. 1972. Biosystematics of the genus *Ips* (Coleoptera: Scolytidae) in North America. Hopping's groups IV and X. *Can. Entomol.* 104:361–388.

LANIER, G.N., and WOOD, D.L. 1975. Specificity of response to pheromones in the genus *Ips*. *J. Chem. Ecol.* 1:9–23.

LANIER, G.N., and OLIVER, J.H., JR. 1966. Sex-ratio condition: Unusual mechanisms in bark beetles. *Science* 153:208–209.

LANIER, G.N., BIRCH, M.C., SCHMITZ, R.F., and FURNISS, M.M. 1972. Pheromones of *Ips pini* (Coleoptera: Scolytidae): Variation in response among three populations. *Can. Entomol.* 104:1917–1923.

LANIER, G.N., CLASSON, A., STEWART, T., PISTON, J.J., and SILVERSTEIN, R.M. 1980. The basis for interpopulational differences in pheromone biology. *J. Chem. Ecol.* 6(3):677–687.

LINDGREN, B.S. 1983. A multiple tunnel trap for scolytid beetles (Coleoptera). *Can. Entomol.* 115:299–302.

MERRILL, L.A. 1991. Biological barriers to hybridization in closely related species of *Ips* (Coleoptera: Scolytidae). Ph.D. dissertation. University of California, Berkeley, 121 pp.

PATERSON, H.E. 1981. The continuing search for the unknown and the unknowable: A critique of contemporary ideas on speciation. *S. Afr. J. Sci.* 77:113–119.

SANDERSON, N. 1989. Can gene flow prevent reinforcement? *Evolution* 43(6):1223–1235.

SAS User's Guide: Statistics, 1982 Edition. Cary, North Carolina.

SCHULTZ, D.E., and BEDARD, W.D. 1987. California five-spined *Ips*. USDA Forest Service Insect and Disease Leaflet 102, 8 PP.

WELLS, P.V., and BERGER, R. 1967. Late Pleistocene history of coniferous woodland in the Mojave Desert. *Science* 155:1640–1647.

WILEY, E.O. 1978. The evolutionary species concept reconsidered. *Syst. Zool.* 27:17–26.

WOOD, D.L. 1982. The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles. *Annu. Rev. Entomol.* 27:411–446.

WOOD, S.L. 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. Great Basin Naturalist Memoirs, Vol. 6. Brigham Young University, Provo, Utah.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

SEX PHEROMONE OF THE MULLEIN BUG, *Campylomma verbasci* (MEYER) (HETEROPTERA: MIRIDAE)

ROBERT F. SMITH,[1,3,*] HAROLD D. PIERCE, JR.,[2] and JOHN H. BORDEN[1]

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

(Received December 10, 1990; accepted March 20, 1991)

Abstract—The first known sex pheromone for the family Miridae is reported for the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). Extracts of and Porapak Q-trapped volatiles from females were attractive to males in field bioassays. Butyl butyrate and hexyl butyrate were the predominant compounds in the females' volatiles, but these compounds previously had been found to be inactive when released alone or in combination. Butyl butyrate in combination with an isolated trace chemical, (*E*)-crotyl butyrate (which was inactive alone), rivaled the attraction elicited by five live females. When synthetic butyl butyrate and (*E*)-crotyl butyrate in a 16:1 ratio were released at 91 or 183 μg/day, they comprised an effective attractant for male bugs.

Key Words—*Campylomma verbasci*, mullein bug, Heteroptera, Miridae, sex pheromone, apple pest, butyl butyrate, (*E*)-crotyl butyrate, hexyl butyrate.

INTRODUCTION

The mullein bug, *Campylomma verbasci* (Meyer), is a sporadic pest of apples in several regions of North America (Thistlewood, 1986). Current management of *C. verbasci* is based on economic thresholds that correlate nymphal density

*To whom correspondence should be addressed.
[3]Present Address: Agriculture Canada Research Station, Kentville, Nova Scotia, Canada B4N 1J5.

0098-0331/91/0700-1437$06.50/0

in the spring with subsequent fruit damage (Whalon and Croft, 1984; Thistlewood et al., 1989a). *C. verbasci* in apple trees is sampled by jarring a branch with several sharp blows from a stick and counting the fallen nymphs on a tray (B.C. Ministry of Agriculture and Fisheries, 1989, Hardman et al., 1984, Washington State University, 1988). Among other drawbacks (Smith, 1989), this time-consuming method requires many limb-tap samples per hectare for reliable prediction. In the fall, catches of adult male *C. verbasci* in traps baited with live females can be correlated with subsequent densities of nymphs the following spring (Smith and Borden, 1990). However, most monitoring programs cannot rely on such a variable and uncertain stimulus as live females.

Thistlewood et al. (1989b) found that female mullein bugs emit a sex pheromone that attracts males to female-baited traps in a manner similar to that found in the mirids *Lygus lineolaris* (P. de Beauvois) (Scales, 1968), *Lygocoris communis* (Knight) (Boivin and Stewart, 1982), *Distantiella theobroma* (Dist.) (King, 1973) and *Helopeltis clavifer* (Walker) (Smith, 1977; Staddon, 1986). Staddon (1986) also found that butyl butyrate and hexyl butyrate were female-produced compounds, the former sex-specific, but alone or in combination these compounds failed to attract male *C. verbasci*. The exact identity of the pheromones for any mirid is unknown (Aldrich, 1988).

Our objectives were: (1) to identify the sex pheromone components of *C. verbasci*, and (2) to determine the bioactivity of synthetic compounds in the field.

METHODS AND MATERIALS

Chemicals. Butyl and hexyl butyrate, butyryl chloride, (*E*)-crotonaldehyde, and 1-hexanol were purchased. Hexyl acetate was prepared by reaction of 1-hexanol with acetic anhydride in pyridine. Reduction of (*E*)-crotonaldehyde with sodium borohydride in 50% aqueous ethanol gave a (*E*)-crotyl alcohol containing 4.3% 1-butanol and 1.7% *Z* isomer. (*E*)--Crotyl butyrate was prepared by reaction of (*E*)-crotyl alcohol with butyryl chloride in ether in the presence of triethylamine. After work-up and distillation at reduced pressure, the ester was 90.6% pure and contained 4.5% butyl butyrate.

Analytical Instrumentation. Hewlett-Packard 5830, 5880, and 5890 gas chromatographs equipped with capillary inlet systems and flame-ionization detectors were employed for analyses by gas–liquid chromatography (GLC). Glass columns (30–40 m × 0.5 mm ID) coated with SP-1000 (Supelco, Bellafonte, Pennsylvania) or fused silica columns (15 m × 0.25 mm ID) coated with DB-1 (J & W Scientific, Inc., Folsom, California) were used. The injection port and detector temperatures were 260°C and 270°C, respectively. A Hewlett-Packard 5895B GC-MS-DS was employed for coupled gas chromatog-

raphy–mass spectroscopy (GC-MS). Fused silica columns (0.32 mm ID) coated with DB-1 (30 or 60 m) or DB-WAX (60 m) (J & W Scientific Inc.) were inserted directly into the ion source. The injection port, transfer line, and ion source temperatures were 260°C, 250°C, and 200°C, respectively. Helium was the carrier gas for GLC and GC-MS.

Isolation and Separation of Volatiles. The isolation and identification of the sex pheromone followed the protocol in Figure 1. Mullein bugs were collected from natural stands of common mullein, *V. thapsus* L. Females were crushed in double-distilled pentane at −78°C, and the extracts were stored at −20°C in screw-cap vials with Teflon-lined lids. The concentration of extracts was expressed as bug equivalents (beq) (one crushed female bug or any undiluted fraction thereof = 1 beq). A micro-steam distillation–continuous extrac-

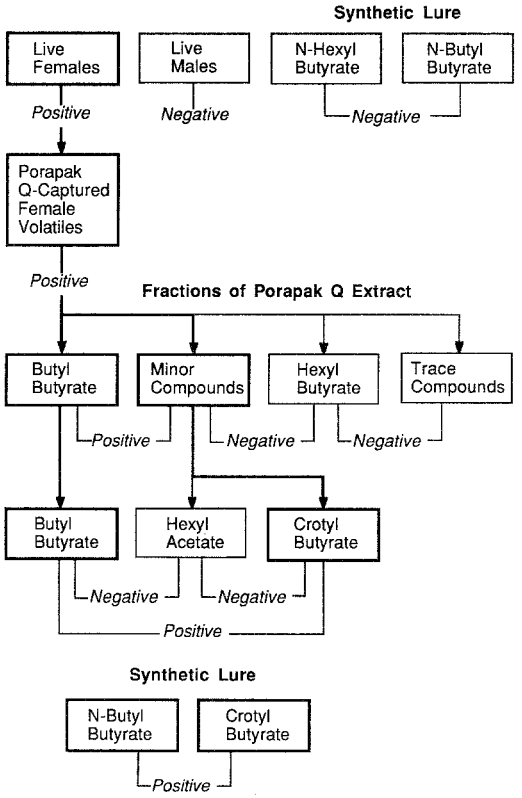


FIG. 1. Flow chart depicting sequence of experiments culminating in identification of the sex pheromone of *C. verbasci*. Live males as well as hexyl butyrate and butyl butyrate were tested by Thistlewood et al. (1989b).

tion apparatus (Godefroot et al., 1981) was employed for the isolation of volatiles from the pentane extracts of crushed bugs.

Volatiles from live *C. verbasci* females were collected in Porapak Q (50–80 mesh, Applied Science Laboratories Inc.) using methods described by Pierce et al. (1984). Air was drawn at 1.9 liters/min through a glass aeration chamber (15 cm OD × 30 cm) containing females on mullein stalks. At approx. 48-hr intervals, the aeration chamber was cleaned and fresh plant material and female bugs were added. Captured volatiles were expressed as bug-hours (bh) (1 bh = volatiles produced by 1 female for 1 hr). Volatiles from female bugs feeding on mullein were recovered by overnight extraction of the Porapak Q in a Soxhlet extractor with pentane. The solution was concentrated to approx. 10 ml by distilling off the pentane through a 30-cm Dufton column.

A Varian 1200 gas chromatograph fitted with a stainless-steel column (3.05 m × 3.18 mm OD) packed with 10% SP-1000 on Supelcoport (100/120), 10:1 effluent splitter, and thermal gradient collector (Brownlee and Silverstein, 1968) was used for micropreparative separation of steam-distilled or Porapak Q-trapped female volatiles. Before separation, a portion of the solution containing the volatiles was set aside for field tests. The remainder of the sample was concentrated under a stream of nitrogen at −10 to 0°C to approx. 30 μl before injection into the chromatograph. Fractions were rinsed from the collection tubes into 1 ml volumetric tubes containing ca. 200 μl of hexane or pentane, which were made up to volume with hexane. Aliquots from these were transferred to screw-cap vials for later release in field traps.

Field Bioassays. Bioassay experiments were used to monitor the isolation and identification of the sex pheromone (Figure 1). Five experiments were done at the Agriculture Canada Research Station, Summerland, British Columbia. Unless stated otherwise, all treatments were replicated four times in a completely randomized design. A 2-liter ice-cream carton trap with a sticky liner insert (Thistlewood et al., 1989b) was used in all experiments and set at a height of 1.5 m in both orchard and mullein field test sites. Those in an orchard were placed 0.5 m from the outer canopy edge. All bait receptacles were suspended inside the center of the trap. Data were transformed to $\sqrt{(n + 0.5)}$ prior to statistical analysis (ANOVA), and Tukey's pairwise comparison of the means (SAS Institute, 1985).

In experiment 1, fractions of the steam-distilled volatiles from crushed bugs (250 beq) or unfractionated extract (100 beq) were dissolved in 2 ml of pentane and held in a 5-ml glass vial. The screw cap for each vial was drilled to give a 2-mm vent. Traps were deployed in a mixed planting of Red Delicious and Golden Delicious apples for four days commencing August 29, 1986. Treatments were separated by at least 6 m, and replicates by 8 m.

Porapak Q-collected female volatiles or fractions thereof were tested in experiment 2 to establish a dose–response relationship. Concentrated extract in

a diethyl ether–pentane solvent was transferred by syringe into a 2-ml centrifuge tube and released through a 2-mm hole in the cap. Treatments were doses of 29,400, 9800, and 2900 bh, solvent control (ether–pentane), and a caged-female control (five per trap). The caged females were replicated three times; all other treatments had four replicates. This experiment was conducted over a six-day period commencing September 6, 1987.

In experiment 3, fractions of Porapak Q-trapped volatiles were placed into 2-ml centrifuge tubes through a 2-mm diam. hole in the cap. Fractions were tested at a strength of 8000 bh. Control stimuli were five caged females and solvent. Tests ran for 11 days commencing September 21, 1987. The objective was to verify the activity initially defined by the fractions from the crude insect crushes. This experiment was set up in an apple orchard; live-caged females had three replicates, all others had four. A fourth experiment using 10,000 bh was undertaken in the same orchard to resolve whether butyl butyrate alone had any attractiveness.

The fifth and final experiment involving 7000 bh was conducted to determine the identity of the minor component that interacts in combination with butyl butyrate. It was set up August 1988, in a field of mullein planted a 2 × 2-m grid. There were 4 m between treatments and 8 m between replicates.

Experiments with Synthetic Pheromone. Synthetic *n*-butyl butyrate and (*E*)-crotyl butyrate (16:1, w/w) were tested in a three-replicate dosage–response experiment in a Bartlett pear orchard in September 1988. Persistence of the lure was enhanced by mixing the butyrates with light paraffin oil. Treatments were 4 ml oil in an open 6-ml glass vial containing a mixture of butyl and crotyl butyrate at 2.0, 1.0, 0.1, and 0.01%, compared with paraffin oil and caged-female controls. Release rate of the butyrates was determined by the Porapak Q-collection method (Pierce et al., 1984). Numbers of males captured were transformed to $\sqrt{(n + 0.5)}$, prior to ANOVA and pairwise comparison of the means using the least significant difference test (SAS Institute, 1985).

Synthetic butyl butyrate and (*E*)-crotyl butyrate (16:1, w/w) also were tested in three release devices and compared as baits to five live-caged females. Dispensers were: (1) capillary tubes 1.18 ± 0.03 mm ID × 40 mm, (2) coagulation tubes 0.91 ± 0.03 mm ID × 40 mm, and (3) 20-ml glass vials. Four microliters of the butyrate mixture was transferred by syringe into the tubes, while 0.04 ml of the 16:1 synthetic pheromone blend was mixed in 4 ml light paraffin oil in the 20-ml vials.

Release rates of 5 μl synthetic lure from coagulation and capillary tubes ($N = 7$) were determined in the laboratory at 25 ± 1°C by measurement of miniscus regression.

All dispensers were suspended from the center of a 2-liter carton trap, hung at 1.5 m in an orchard block of Red Delicious in September 1988. Traps were set in a completely randomized design with each treatment having three repli-

cates. Data were transformed to $\sqrt{(n + 0.5)}$, prior to ANOVA and least significant difference test, $P < 0.05$ (SAS Institute, 1985).

RESULTS AND DISCUSSION

Pheromone Identification. Pheromone activity was centered in fraction 2 of the steam-distilled bug volatiles (Figure 2A), which contained butyl butyrate and minor compounds eluting after it and before hexyl butyrate (Table 1, experiment 1).

Porapak Q-captured female volatiles were as active at 29,400 bh as five females (Table 1, experiment 2). Attraction was less at 9800 or 2900 bh, but still higher than the solvent control. The capture rates in comparison to that of live females may be misleading, because the longevity of the extracts was <4 days (unpublished data) and attraction must have declined rapidly once they were placed in the traps.

The Porapak Q-trapped volatiles and a combination of fractions 1 and 2 thereof (Figure 1B) achieved captures approaching those of caged females (Table 1, experiments 3 and 4), verifying the response to fraction 2 of the female steam-distilled extract (Table 1, experiment 1). Hexyl butyrate (fraction 3) did not influence male captures alone, in combination with butyl butyrate (Smith, 1989; Thistlewood et al., 1989b), or in combination with fraction 2 (Table 1, experiment 3) and was eliminated as a candidate phermone. Butyl butyrate alone (Figure 1B, fraction 1) was inactive (Table 1, experiment 4), yet attraction was achieved when it was combined with the minor compounds in fraction 2, which were inert when tested alone (Table 1, experiment 3).

The minor components were separated into three fractions. Of these, only the combination of butyl butyrate and the fraction containing peak CB (Figure 2B) was as active as the unfractionated female volatiles (Table 1, experiment 5). Thus, the sex pheromone of the mullein bug was identified as the synergistic combination of butyl butyrate and compound CB.

Structures of Minor Compounds. The mass spectrum of peak CB (Figure 1A and B) exhibited a base peak at *m/z* 71, a parent ion at *m/z* 142, and a weak peak at *m/z* 89 ($C_4H_9O_2^+$), a strong peak in the spectrum of butyl butyrate (mol wt = 144). Since females of several *Lygus* spp. contain and emit hexyl and (*E*)-hexenyl butyrate (Aldrich, 1988), peak CB was hypothesized to be an unsaturated analog of butyl butyrate, i.e., (*E*)-crotyl butyrate [(*E*)-2-butenyl butanoate]. Comparison of the mass spectrum and retention time of the unknown to those of the synthetic sample confirmed this hypothesis. Present in both male and female volatiles were hexyl acetate and 1-hexanol (Figure 1A and C). These compounds were identified by GC-MS and search of reference spectra. The assignments were confirmed by comparison of their mass spectra and retention times to authentic samples.

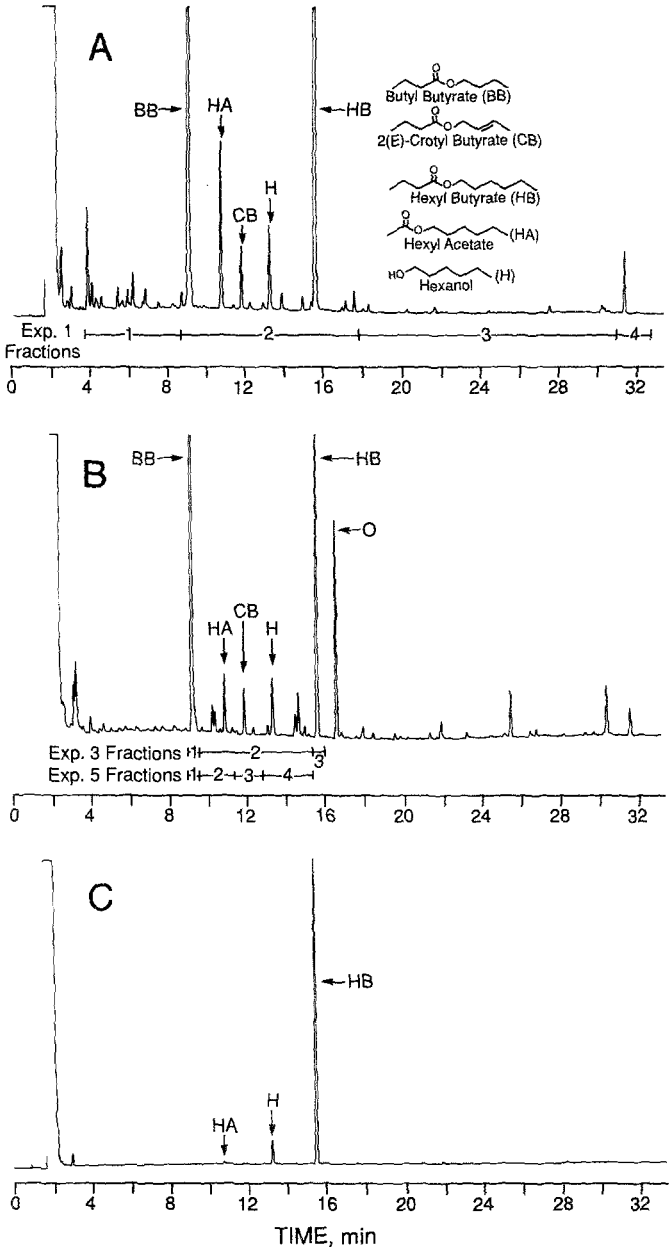


FIG. 2. Gas chromatograms of *C. verbasci* volatiles: (A) steam-distilled volatiles from females; (B) Porapak Q-trapped volatiles from females feeding on mullein; and (C) steam-distilled volatiles from males. BB = butyl butyrate, HA = hexyl acetate, CB = (*E*)-crotyl butyrate, H = 1-hexanol, HB = hexyl butyrate, and O = 1-octen-3 ol (from mold growing on mullein).

TABLE 1. CAPTURES OF MALE *C. verbasci* IN TRAPS BAITED WITH FRACTIONS OF CRUSHED FEMALE EXTRACT OR EXTRACT OF PORAPAK Q-CAPTURED FEMALE VOLATILES, 1986–1988

Experiment and description	Treatment[a]	Stimulus strength	Replicates (*N*)	Males caught ($X \pm SE$)[b]
Experiment 1	Female extract	100 beq	3	17.3 ± 1.8b
Crushed bug fractions	Fraction 1	250 beq	4	0.3 ± 0.3c
	Fraction 2	250 beq	4	35.8 ± 5.4a
	Fraction 3	250 beq	4	0.5 ± 0.3c
	Fraction 4	250 beq	3	2.0 ± 0.6c
	Pentane	5 ml	4	0.0 ± 0.0[c]
			3	8.3a
Experiment 2	5 live females			
Dose–response	Captured volatiles	29,000 bh	4	5.8 ± 1.8ab
Porapak		9,800 bh	4	3.3 ± 0.8bc
Q-collected		2,900 bh	4	2.0 ± 0.7c
volatiles	Pentane	5 ml	4	0.0[c]
Experiment 3	5 live females		4	17.0 ± 3.8a
Porapak Q extract	Porapak Q extract	8,000 bh	4	8.3 ± 0.6b
fractions	Fractions 1 and 2	8,000 bh	4	11.5 ± 3.6ab
	Fraction 2	8,000 bh	4	0.5 ± 0.5c
	Fractions 2 and 3	8,000 bh	4	0.0[c]
Experiment 4	5 live females		3	7.7 ± 1.2a
Porapak Q extract,	Porapak Q extract	10,000 bh	4	12.1 ± 1.1a
test of fraction	Fractions 1 and 2	10,000 bh	4	9.8 ± 2.9a
1 (butyl butyrate)	from Exp. 3	10,000 bh	4	
alone	Fraction 1 from Exp. 3		4	0.0[c]
Experiment 5	5 live-caged females		4	5.0 ± 0.6a
Porapak Q extract,	Porapak Q extract	7,000 bh		4.0 ± 0.4ab
identity of minor	Fraction 1 +		4	3.3 ± 0.6b
component	crotyl butyrate		4	1.0 ± 0.5c
	Fraction 1 + hexyl acetate		4	0.6 ± 0.3c
	Fraction 1 + hexanol		4	
			4	
	Hexane	5ml	4	1.1 ± 0.5c

[a] Fraction numbers correspond to those on gas chromatograms of female extracts or Porapak Q-captured female volatiles (Figure 2).

[b] Means within an experiment followed by a common letter are not significantly different, $P < 0.05$, Tukey's pairwise comparison test (SAS Institute, 1985).

[c] Zero means excluded from analysis.

Response to Synthetic Pheromone. More male *C. verbasci* were trapped using synthetic lure at 1.0% and 2.0% concentration in paraffin oil than at lower dosages (Table 2). A concentration as low as 0.1% induced a significant response. Attraction to the synthetic pheromone released from two devices equalled that of live females (Table 3).

On the basis of these results, we conclude that the two-component sex pheromone in *C. verbasci* is comprised of butyl butyrate and (*E*)-crotyl butyrate acting synergistically, and that these components occur naturally in a ratio of

TABLE 2. MEAN NUMBER OF MALE *C. verbasci* CAPTURED IN CONCENTRATION RESPONSE EXPERIMENT TO BUTYL AND CROTYL BUTYRATE (16:1) IN 4 ML PARAFFIN OIL[a]

Synthetic lure concentration	Males caught ($X \pm$ SE)[b]
2.0%	11.3 ± 2.3a
1.0%	8.7 ± 1.3a
0.1%	4.3 ± 1.8b
0.01%	2.0 ± 0.6bc
5 live females	1.0 ± 0.6c
Unbaited control	0.0 ± 0.0[c]

[a] Each treatment was replicated three times in a completely randomized design in an apple orchard, September 1988.
[b] Means followed by a common letter are not significantly different, $P < 0.05$, least significant difference test (SAS Institute, 1985).
[c] Zero means excluded from analysis.

TABLE 3. MEAN NUMBER OF MALE *C. verbasci* CAPTURED IN TRAPS USING THREE LURE RELEASE DEVICES CONTAINING 4 μL OF SYNTHETIC LURE[a]

Release device	Release rate (μg/day)	Males caught ($X \pm$ SE)[b]
Coagulation tube	91	11.7 + 1.5a
Capillary tube	183	7.7 + 1.9ab
10-ml vial (1% pheromone in mineral oil)	25	5.7 ± 1.8b
Unbaited control		0.0[c]
5 live females		11.0 ± 2.9ab

[a] Each treatment was replicated three times in a completely randomized design in an apple orchard, September 1988.
[b] Means followed by a common letter are not significantly different, $P < 0.1$, LSD test (SAS Institute, 1985).

16:1. This is the first successful identification of a sex pheromone in the family Miridae.

Acknowledgments—We thank the Agriculture Canada Research Station, Kentville, Nova Scotia, for granting educational leave to R.F. Smith, F.L. Fenton-Smith, and K. Calla for assistance in field and laboratory projects; the Agriculture Canada Research Station, Summerland, British Columbia, for use of research facilities; and the Natural Sciences and Engineering Research Council of Canada, grants A3881, G1697, and 000434 for financial support.

REFERENCES

ALDRICH, J.R. 1988. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol.* 33:211–238.

B.C. MINISTRY OF AGRICULTURE AND FISHERIES. 1989. Province of British Columbia. Tree fruit production guide for interior districts. Victoria, B.C.

BOIVIN, G., and STEWART, R.K. 1982. Attraction of male green apple bugs, *Lygocoris communis* (Hemiptera: Miridae) to caged females. *Can. Entomol.* 114:765–766.

BROWNLEE, R.G., and SILVERSTEIN, R.M. 1968. A micropreparative gas chromatograph and a modified carbon skeleton determinator. *Anal. Chem.* 40:2077–2079.

GODEFROOT, M., SANDRA, P., and VERZELE, M. 1981. New method for quantitative essential oil analysis. *J. Chromatogr.* 203:325–335.

HARDMAN, J.M., ROGERS, R.E.L., and MACLELLAN, C.R. 1984. Pest abundance, pesticide usage and levels of fruit damage in a pest management program in Nova Scotia apple orchards. XVII International Congress on Entomology, Hamburg, Germany, 1985.

KING, A.B.S. 1973. Studies of sex attraction in the cocoa capsid *Distantiella theobroma* (Heteroptera: Miridae). *Entomol. Exp. Appl.* 16:243–254.

PIERCE, H.D., PIERCE, A.M., MILLAR, J.G., WONG, J.W., VERIGIN, V.G., OEHLSCHLAGER, A.C., and BORDEN, J.H. 1984. Methodology for isolation and analysis of aggregation pheromones in the genera *Cryptolestes* and *Oryzaephilus* (Coleoptera: Cucujidae), pp. 121–137, *in* Proceedings, 3rd International Working Conference on Stored Products Entomology, Manhattan, Kansas.

SAS INSTITUTE. 1985. SAS user's guide: statistics, 5th ed. SAS Institute, Cary, North Carolina.

SCALES, A.L. 1968. Female tarnished plant bugs attract males. *J. Econ. Entomol.* 61:1466–1467.

SMITH, E.S.C. 1977. Presence of a sex attractant pheromone in *Helopeltis clavifer* (Walker) (Heteroptera: Miridae). *J. Aust. Entomol. Soc.* 16:113–116.

SMITH, R.F. 1989. Exploitation of seasonal development and semiochemicals for the refinement of pest management programs involving the mullein bug, *Campylomma verbasci* (Meyer) and pear psylla, *Psylla pyricola* Foerster. PhD thesis. Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia.

SMITH, R.F., and BORDEN, J.H. 1990. Relationship between catches of *Campylomma verbasci* (Meyer) (Heteroptera: Miridae) in traps baited with females in the fall and density of nymphs in the spring. *J. Econ. Entomol.* 83:1506–1509.

STADDON, B.W. 1986. The scent glands of Heteroptera. *Adv. Insect. Physiol.* 14:351–418.

THISTLEWOOD, H.M.A. 1986. The bionomics and monitoring of *Campylomma verbasci* (Meyer) on apple in the Okanagan Valley, British Columbia. PhD thesis. Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia.

THISTLEWOOD, H.M.A., MCMULLEN, R.D., and BORDEN, J.H. 1989a. Damage and economic injury levels of the mullein bug *Campylomma verbasci* (Meyer) (Heteroptera: Miridae), on apple in the Okanagan Valley. *Can. Entomol.* 121:1–9.

THISTLEWOOD, H.M.A., BORDEN, J.H., SMITH, R.F., PIERCE, H.D., and MCMULLEN, R.D. 1989b.

Evidence of a sex pheromone in the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). *Can. Entomol.* 121:737–744.

WHALON, M.E., and CROFT, B.A. 1984. Apple I.P.M. implementation in North America. *Annu. Rev. Entomol.* 29:435–470.

WASHINGTON STATE UNIVERSITY. 1988. Spray guide for tree fruit in eastern Washington. Cooperative Extension. Publication EB0419. Pullman, Washington.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

BEHAVIORAL RESPONSES OF MALE *Epiphyas postvittana* (WALKER) TO SEX PHEROMONE-BAITED DELTA TRAP IN A WIND TUNNEL

S.P. FOSTER,[1,*] S.J. MUGGLESTON,[1] and R.D. BALL[2]

[1]*D.S.I.R. Plant Protection*

[2]*D.S.I.R. Physical Sciences*
Mt Albert Research Centre
Private Bag, Auckland, New Zealand

(Received December 17, 1990; accepted March 20, 1991)

Abstract—The effects of parameters associated with a Delta sticky trap on the sex pheromone-mediated responses of male *Epiphyas postvittana* (Walker) were tested in a wind tunnel. Males flying to a pheromone source landed closer to the source when other males were stuck on the base, suggesting the importance of visual cues in the landing behavior of males of this species. With an increase in time (numerical order of the male in the experiment), males became stuck on the base farther from the source whether or not other males were stuck on the base. The alignment of the trap to the wind or the location of the pheromone source within the trap did not significantly affect the percentages of males that entered the trap, but both significantly affected the position at which males entered the trap. When these data were corrected for the probability of catching males, a treatment with the source to the side of the trap was predicted to catch more moths than the other treatments tested, although this difference was not significant. However, in a field-trapping experiment the treatment with the source to the side caught significantly more moths than treatments with the source either in the middle or near the top of the trap. This latter result is probably due to the greater efficiency of the trap when the source is in this position, as indicated in the wind-tunnel experiment, rather than an increase in the number of males entering the trap. Finally, the pheromone-mediated responses of male *Planotortrix octo* were tested to the various trap alignments. As the angle of the trap to the wind increased, significantly fewer *P. octo* males entered the trap, due principally to both fewer males orienting to the source and proceeding to enter the trap after having landed on it. This difference between the responses of *P. octo* and *E. postvittana* males is, we believe, due to the breakdown in plume structure

*To whom correspondence should be addressed.

0098-0331/91/0700-1449$06.50/0

from the source as the trap angle to the wind increases and to a greater sensitivity of *P. octo* males to a more structured pheromone plume. This accounts, at least to some extent, for the consistently greater field catches of *E. postvittana* over *P. octo* (and possibly other New Zealand leafroller moths) in many locations throughout New Zealand.

Key Words—Sex pheromone, traps, wind tunnel, behavior, *Epiphyas postvittana*, *Planotortrix octo*, Lepidoptera, Tortricidae.

INTRODUCTION

Field-trapping is one of the most commonly used techniques in sex pheromone studies. Generally, it is used to bioassay chemicals in order to develop a newly identified pheromone blend or to improve the efficacy of an already identified pheromone blend of a particular species. In addition to its use in identification studies, field-trapping using sex pheromones is extensively used for monitoring pest populations. The empirical determination of a threshold catch allows decisions to be made concerning the application of insecticide (or other) treatments for the control of a pest. By this technique, the number of insecticide treatments applied to a crop can be reduced or the efficacy of the treatments improved.

Although the catches of insects in traps are used by researchers to infer behavioral effects mediated by chemicals, these catches are the result of a sequence of behavioral events exhibited by a number of individuals (see Kennedy, 1977). In its natural environment, a male moth exhibits a number of behavioral responses when locating a female releasing sex pheromone (Baker, 1989). However, the surrounding of a pheromone source by a trap alters the environment that the male experiences and probably results in the modification of his behavioral responses, particularly at close range, used in locating the source. Consequently, the probability that the male may find the pheromone source may differ from the natural situation.

The effects of parameters associated with a trap on the ability of a male to locate the pheromone source inside the trap has long been recognized. In particular, studies examining the location (particularly height), design, and color of traps with respect to the number of insects caught in the trap are common (see, for example, Robacker et al., 1990). The actual effects of these factors on male behavior are usually inferred from the number of insects caught in the trap and presumed to relate to how the insect normally responds in its natural environment (see for example, Bakke and Riege, 1982). Despite the probable importance of a number of parameters associated with traps in modifying behavioral responses of insects, and hence influencing trap catches, there is little understanding of which behavioral responses these parameters actually modify.

In New Zealand, a group of endemic and introduced tortricid species forms a serious pest complex of many fruit crops (Wearing et al., 1991). The intro-

duced Australian species, the light-brown apple moth, *Epiphyas postvittana* (Walker), is one of the most economically important of these pests. Its sex pheromone has been identified as a mixture of (*E*)-11-tetradecenyl acetate (*E*11–14:OAc) and (*E,E*)-9,11-tetradecadienyl acetate (*E,E*-9,11–14:OAc) (Bellas et al., 1983), and, when used in sticky traps, is highly efficacious for catching male *E. postvittana* (Suckling et al., 1988). Compared to the large numbers of male *E. postvittana* caught in most parts of New Zealand, the numbers of the endemic New Zealand leafroller moth species caught are generally smaller in a given location (Suckling et al., 1988). While this may relate to the respective distributions and population sizes of the species, the consistency of the difference in trap catch suggests that there may be an innate behavioral or ecological difference between these species that contributes to the different trap catches. Under standard wind-tunnel conditions, similar percentages of males of *E. postvittana* (Muggleston and Foster, 1989) and various New Zealand species (Foster et al., 1990; J.R. Clearwater and S.P. Foster, unpublished) appear to respond to their respective synthetic sex pheromone blends, suggesting that the efficacy of the synthetic blend, under controlled conditions, does not account for this difference.

In order to investigate how some of the parameters associated with a sticky trap influence male behavior, we have studied the sex pheromone-mediated responses of male *E. postvittana* to changes in these parameters. To ascertain whether differences in behavioral responses to these parameters could account for the lower trap catches of the endemic New Zealand leafroller moth species, the responses of male *Planotortrix octo* (Dugdale) to changes in one of these parameters also have been studied.

METHODS AND MATERIALS

Insects. *E. postvittana* and *P. octo* are maintained as laboratory cultures at D.S.I.R. Plant Protection. For the experiments reported here, insects of both species were handled similarly. Larvae of both species were reared on synthetic diet (Singh, 1974). Male pupae were sorted and placed in an incubator at 18°C and a 16:8 light–dark photoperiod. Male moths were collected within 24 hr of emergence and placed in a 2-liter plastic container with moist vermiculite on the bottom, along with a 10% honey solution for food.

Chemicals. *E*11–14:OAc was purchased from Sigma Chemical Company (St. Louis, Missouri) and was greater than 99% isomerically pure as determined by capillary gas chromatography (GC). Tetradecyl acetate (14:OAc) was purchased from Bedoukian Fine Chemicals (Danbury, Connecticut); GC analysis showed no tetradecenyl acetates as impurities in this chemical. The (*Z*)-8-tetradecenyl acetate (*Z*8–14:OAc) and *E,E*-9,11–14:OAc used in this work were

gifts from Shin Etsu Chemical Company (Tokyo, Japan); they were greater than 97% and 94% isomerically pure, respectively. The chemicals were dissolved in pentane and applied to rubber septa (A.H. Thomas Co., Philadelphia, Pennsylvania) for use in the wind-tunnel and field-trapping trials. For *E. postvittana*, the blend used in the wind-tunnel and field trials was *E*11–14:OAc (100 μg) and *E,E*-9,11–14:OAc (10 μg) (Muggleston and Foster, 1989). A septum loaded with *Z*8–14:OAc (100 μg) and 14:OAc (100 μg) (Galbreath et al., 1985) was used in the wind-tunnel experiment involving *P. octo*. When not in use, the septa were stored in 4-ml glass vials at −15°C.

Wind-Tunnel Protocol. A 1.4-m-long wind tunnel based on the design of Miller and Roelofs (1978) was used for the experiments. Individual male moths, 3–6 days old, were placed individually in wire mesh cylinders (with a plastic lid at one end) and allowed to acclimate to the temperature (19 ± 1°C) and light conditions (0.5 lux) of the tunnel at least 1.5 hr before the start of an experiment. Individual males were introduced into the tunnel between the second and fourth hours of the scotophase as described (Muggleston and Foster, 1989).

The following experiments were conducted in the wind tunnel:

1. A corrugated plastic sheet (18 × 18 cm), coated with a thin layer of sticky glue (Product No. 633, Davis Gelatin Ltd., Christchurch), was rested (sticky side up) on a small, 12.5-cm-high wire table in the center of the tunnel, with one edge 5 cm from the farthest point upwind in the tunnel. The plastic sheet was divided into a grid of 9 × 9 (2-cm) squares. The pheromone source was placed in the middle of the grid. Individual *E. postvittana* males were released into the tunnel, and their initial point of contact on the grid and final position on the grid (i.e., where they stuck) were recorded. Males were allowed 2 min from the time of release within which to become stuck to the base, otherwise they were removed from the tunnel and not included in the data set for initial and final positions on the grid. Three hundred twenty males were flown over four days.

2. This was essentially a repeat of experiment 1 except that after males had become stuck to the sheet, they were removed from the base. Fifty males were tested.

3. A white Delta-shaped trap (see Figure 1) was used in this and other experiments. The trap, without a sticky base, was placed on the wire table in the tunnel. The trap was placed with the long axis parallel, 45°, or 90° to the direction of the wind. *E. postvittana* males were released into the tunnel and scored for the following four behavioral categories: (1) activation and ability to fly, (2) orientation upwind and initiation of upwind pheromone-mediated flight, (3) flight to the midpoint of the tunnel, and (4) position of landing on and entry into the trap. Four different landing and entry positions were distinguished (Figure 1): males landed directly on the base (base, "a" in Figure 1); males landed

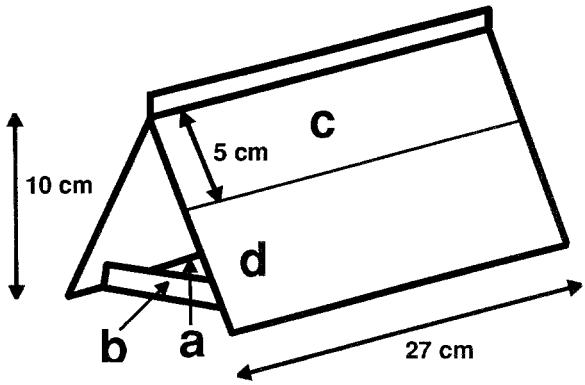


FIG. 1. The Delta trap used in the experiments. The letters indicate the various defined positions that insects landed on and subsequently entered the trap from (refer also to the text): a = base, b = flap, c = top, d = side.

on the outside of the flap ("b" in Figure 1), and walked over the flap to enter the trap (flap); males landed on and entered the trap within 5 cm of the top ("c" in Figure 1) of the trap (top); males landed on and entered the trap below 5 cm from the top ("d" in Figure 1) of the trap (side). Included in this experiment was a treatment consisting only of a base (without glue) and source. The behavioral responses of 60 males to each of the treatments were recorded.

4. This experiment, testing the effect of the location of the pheromone source within the trap on male *E. postvittana* behavioral responses, was conducted similarly to that for the effect of trap alignment. For the different treatments, the trap was aligned with the wind and the source placed inside the trap at four different locations: in the center of the base, with the large cavity of the septum pointing downwind (0°); in the center of the base with the large cavity upwind (180°); pinned to the middle of the trap, approximately 2 cm from the apex, with the large cavity pointing downwind (top); and placed on the base halfway along the long axis of the trap and 2 cm from one side, with the large cavity of the septum pointing downwind (side). The responses of 56 males to each of these treatments was recorded using the same design as for experiment 3.

5. In order to determine the efficacy of the various trap and source alignments used in the two preceding experiments for catching male *E. postvittana*, the percentages of males that entered the trap at each of the four defined positions (i.e., base, top, side, and flap) and stuck on the base were recorded. For each of the positions, 25 males were observed entering the trap and scored for (1) sticking on or (2) escaping from the trap.

6. Thirty-nine *P. octo* males were flown to each of the four trap align-

ments tested for *E. postvittana*, and their responses determined as in experiment 3.

Field-Trapping. The field trial for *E. postvittana* was run at Kumeu Research Orchard (near Auckland). The Delta traps were placed on various shelter (poplar and pine) trees, approximately 1.5 m from the ground. Traps within a replicate series were placed at least 10 m apart; replicates were placed at least 50 m apart. The numbers of moths caught in the traps were counted, the moths removed, and the traps rerandomized, weekly. The trial tested the effect of source location within the trap on catches of males. The different locations of the source within the trap were as in wind-tunnel experiment 5, except the 0° and 180° positions were equivalent in the field due to changing wind directions.

Statistical Analyses. The initial landing and final resting positions of the moths in experiments 1 and 2 were compared using regression analysis. The initial landings were analyzed using the computer program PLUM, a method for analysis of ordered categorical data (McCullagh, 1980). The model fitted was: $Y = C + a + bt$, where Y is the distance from the source, C is a constant, and b is the rate of increase in distance from the source with time t (i.e., the number of moths that have already landed). Distributions of the moths on the surface with regard to upwind or downwind location were compared using chi-squared tests.

For wind-tunnel experiments 3, 4, and 5, distributions were compared using chi-squared tests. A chi-squared test for each species was used to test for the difference in numbers entering the trap in the comparison of experiments 6 and 3. Additionally, in the comparison of these two experiments, the individual behaviors were classified as none; orient only; orient and land but did not enter; and orient, land, and enter, and were analyzed as ordered categorical data using the PLUM program (McCullagh, 1980).

Data from the field trial were transformed to $\sqrt{(X + 0.5)}$ values and analyzed by a two-way ANOVA followed by a comparison of means using Fisher's protected least significant differences test.

Observation of Plume Structure. The structure of the plumes emerging from the Delta trap, aligned at different positions to the wind, were visualized using a small incense stick, emitting smoke, placed at the center of the base.

RESULTS

Landing and Sticking Positions (Wind-Tunnel Experiments 1 and 2). After initially contacting the base, *E. postvittana* males moved a great deal over the surface of the base, either by walking or flying, often towards the pheromone source. Males were also frequently observed to fly away from the base and

return. Of the 320 male *E. postvittana* (28.4%) tested in experiment 1, 91 stuck on the base (Figure 2). A reasonably constant catch, of approximately 35%, was recorded for the first 240 males flown. However, subsequent to this only 7.5% (6) of the next 80 males flown stuck to the base. A higher proportion (50.0%) of males tested was caught on the base when males previously flown to the source were removed from the base (experiment 2).

Statistical comparison of the initial landing positions of male *E. postvittana* between experiments 1 (previous males not removed from the base) and 2 (previous males removed from the base) gave a significant landing parameter (Figure 3A and B). Thus with an increase in time (i.e., numerical sequence), males landed significantly closer to the pheromone source in experiment 1 than in experiment 2. This effect was due predominantly to the significantly (chi-squared test, $P = 0.99$) higher proportion of males that first landed in the grid position containing the pheromone source in experiment 1 (34.1%) compared to experiment 2 (4.0%). In experiment 1, landing at the grid position containing the pheromone source occurred relatively infrequently (one in seven males that landed) over the first 28 males that landed on the base; however, subsequent to this it occurred at a frequency of just under one in 2.5 males. In both experiments, a high proportion of males landed at the downwind edge of the base (53.8% and 72.0% of males landed at grid positions on this edge in experiments 1 and 2, respectively).

The final positions of the males (i.e., where they stuck) in both experiments (Figure 3 C and D) bore little or no apparent relationship to the initial

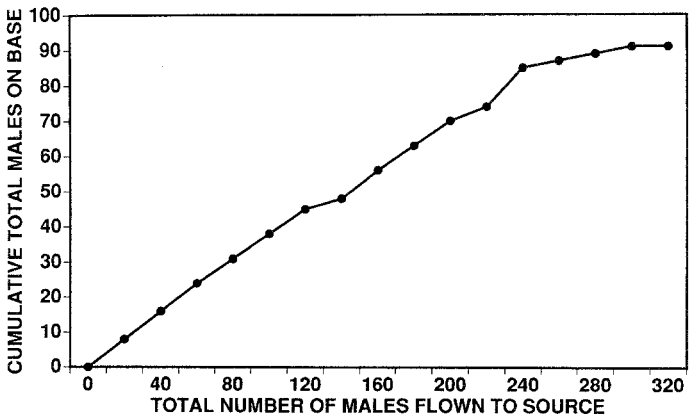


FIG. 2. Number of male *Epiphyas postvittana* caught on a sticky base, out of the total number of males flown to the pheromone source; the data are divided into sequential groups of 20 males flown.

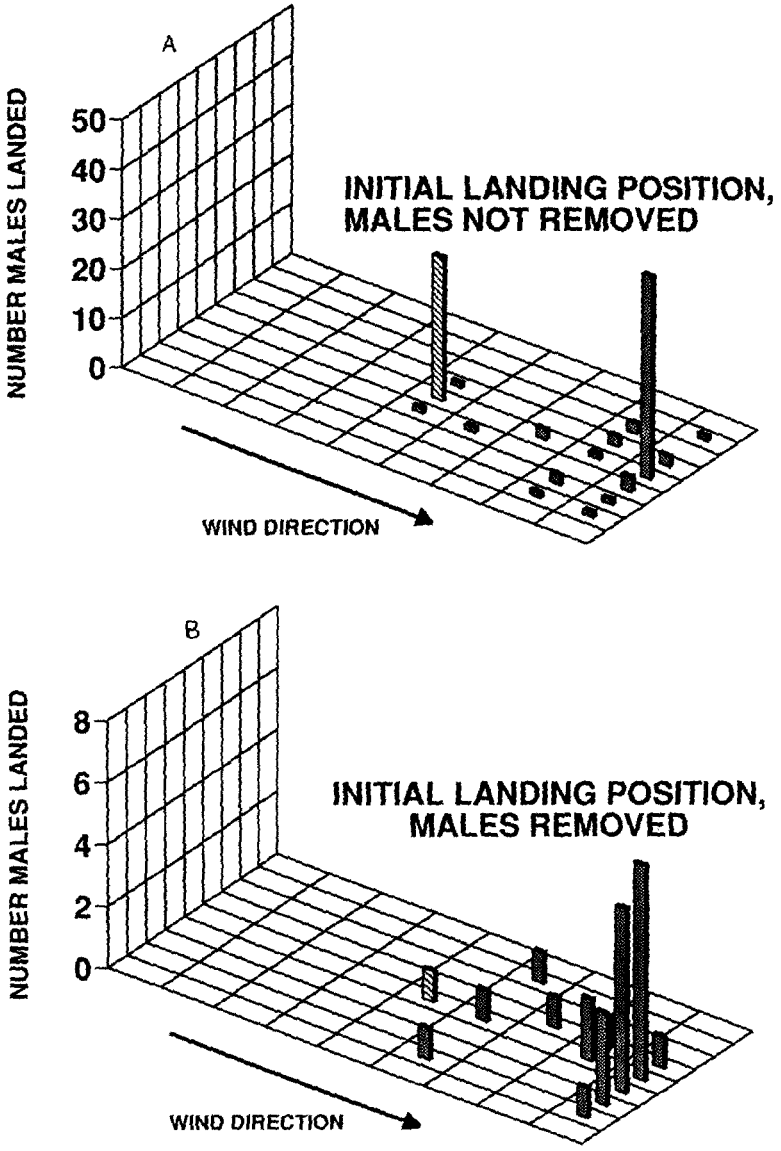
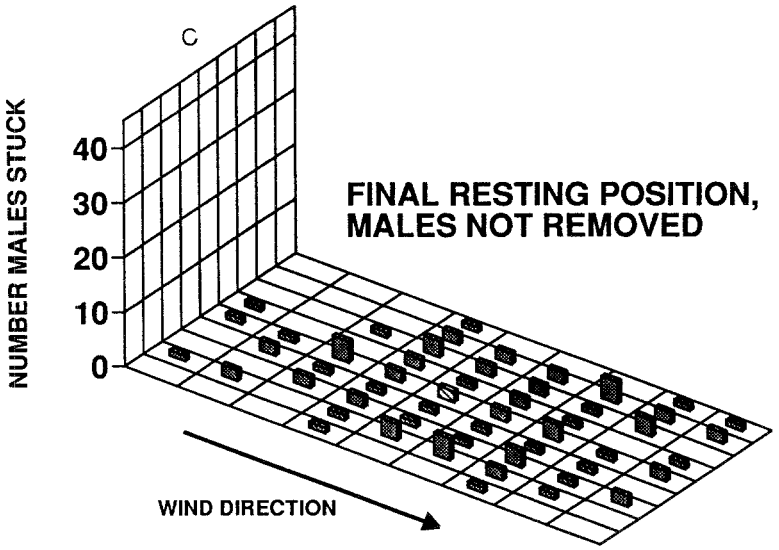


FIG. 3. Position and frequency of male *Epiphyas postvittana* landing or sticking on a base containing a pheromone source (from experiments 1 and 2); (A) initial landing position when other males were left on the base; (B) initial landing position, when other males were removed from the base; (C) final resting position (i.e., where males stuck), when other males were left on the base; (D) final resting position when other males were removed from the base. The location of the pheromone source on the base is shown by the bar with the diagonal stripe.



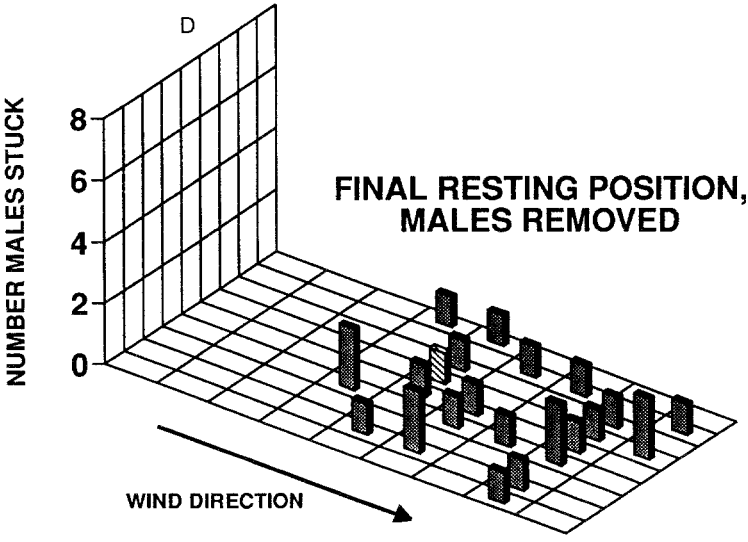


FIG 3. Continued

landing position. There was no significant difference in the distance that males became stuck from the source between experiments 1 and 2. However, there was a significant ($P < 0.05$) difference between the two experiments in the distributions of males stuck on the base. Overall, a significantly higher proportion of males became stuck downwind of the source in experiment 2 (males removed) than in experiment 1. However, there was no significant difference in the distributions of the first 25 males stuck on the base between these experiments, indicating that males became stuck upwind of the source mainly after the downwind positions largely were occupied. The final position of males in both experiments tended to increase in distance from the source with time.

Effect of Trap Alignment on Response and Catch (Wind-Tunnel Experiments 3 and 5). In experiment 3, 82% of the male *E. postvittana* tested to the base and source (without trap), contacted the base (Figure 4). Very high proportions of males entered the trap, whether it was aligned at 0°, 45°, or 90° to the wind. Although there was a slight tendency for the proportion of males

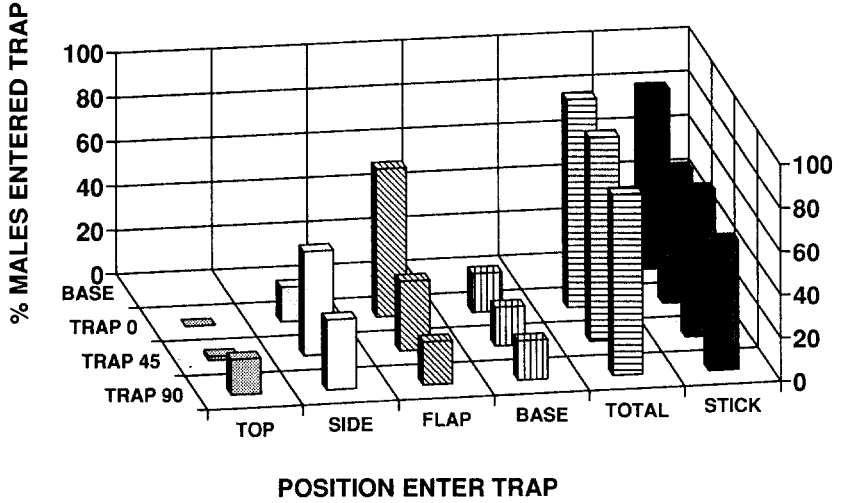


FIG. 4. The proportions of male *E. postvittana* that landed on and entered the trap at different positions for different alignments of the trap to the wind. TOP = males landed on and entered the trap within 5 cm of the top of the trap; SIDE = males landed on and entered the trap below 5 cm from the top of the trap; FLAP = males landed on and entered the trap through the entrance flap of the trap; BASE = males landed on and entered the trap directly on the base; TOTAL = sum of the individual percentages. These total percentages were corrected for probability of capture of males in the trap and these data are shown as STICK. Trap 0, 45 and 90 are the three trap alignments (in degrees), while BASE is a sticky base only.

entering the trap to decrease as the angle of the trap to the wind direction increased, there were no significant differences ($P > 0.05$) between these three treatments. However, there were differences in the proportions of males that entered the trap at the different positions, between the treatments (Figure 4). Thus, with a trap alignment of 0°, a significantly ($P < 0.01$) higher proportion of males landed and entered the trap at the flap position (66.7%) of all males entering the trap) than was observed for the other treatments. The remaining males that entered the trap aligned 0° to the wind landed and entered at either the bottom or side positions in approximately equal proportions. However, with the trap aligned 45° to the wind, the greatest single percentage of males that landed and entered (48.2%) did so at the side. The proportion of the males entering the trap at this position was significantly ($P < 0.05$) greater for this treatment than for the other two trap alignments. Quite a large proportion (32.1%) of the males flown to this treatment (45°) entered the trap at the flap position. When the trap was aligned 90° to the wind direction, males landed and entered the trap in more similar percentages for all four positions. The most significant ($P < 0.01$) difference in male response between this alignment and the other alignments was the relatively high proportion (18.0%) of males that landed and entered the trap near the top (0.0% and 1.8% of the males entered at this position when the trap was aligned 0° and 45°, respectively).

In experiment 5, the percentages of males that stuck to the base after entering the trap at the four different positions were determined as 100.0%, 52.0%, 76.0%, and 52.0% for the base, flap, side, and top positions, respectively (not significantly different, $P > 0.05$). If the data from experiment 3 are corrected for capture of the male (by sticking) by multiplying the percentages of males that landed and entered the trap at the different positions by the probability of a male sticking to the base after entering the trap at these positions (i.e., the proportions that were experimentally observed to stick), then the percentages of trap capture can be calculated for the different trap alignments; these corrected values are shown in Figure 4 along with the uncorrected (percentage of males entering trap) data. No significant differences ($P > 0.05$) in trap capture between the different alignments were detected.

Effect of Source Location on Response and Catch (Wind-Tunnel Experiment 4). There were no significant differences ($P > 0.05$) in the percentages of males that landed on and entered the trap between treatments that tested each of the four locations of the source (Figure 5).However, as in experiment 3, there were significant differences in the proportions of males that landed on and entered the trap at the different positions for the four treatments. As observed in experiment 3, a very high proportion of the males that landed on and entered the trap with the source at 0° did so at the flap position (87.8%). The pattern with the source at 180° was similar to this, although overall slightly fewer males entered the trap. Significantly greater ($P < 0.01$) percentages of males entered

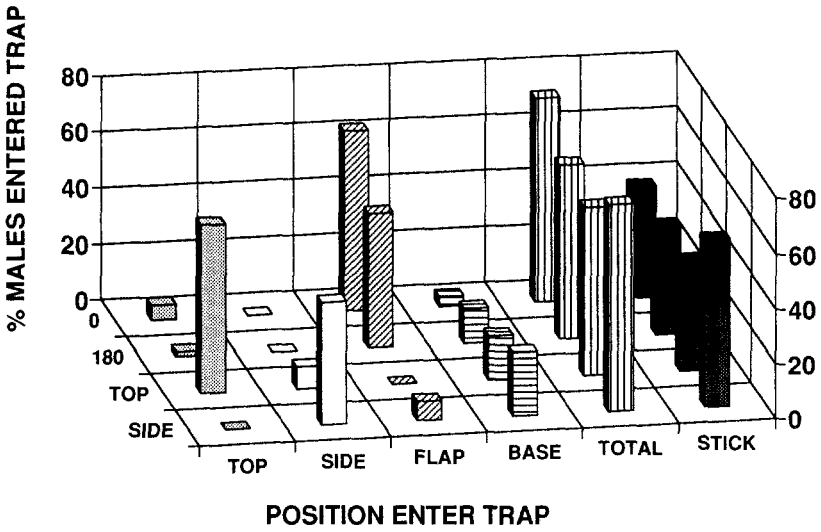


FIG. 5. The proportions of male *E. postvittana* that landed on and entered the trap at different positions for the different source locations. 0 = source placed in center of base with the large cavity facing downwind; 180 = source placed in center of base with the large cavity facing upwind; TOP = source placed in middle, 2.5 cm from the apex, of the trap; SIDE = source placed on base, halfway along the long axis and 2 cm from the side of the trap. Data corrected for probability of capture of males in the trap (STICK) are also shown. Acronyms are as in Figure 4.

the trap at this position (flap) for these two source locations, than for the other two source locations tested. In contrast to this, a large proportion (67.6%) of males landed on and entered the trap near the top for the treatment with the source near the top of the trap, significantly greater ($P < 0.01$) than for all the other treatments. For the treatment with the source located near the side of the trap, a significantly greater ($P < 0.01$) percentage of males (59.5%) entered the trap at the side position than for all the other treatments tested.

If these data are corrected for catch, as for experiment 3, then the treatment with the source placed to the side would be expected to give the highest percentage of males caught (61.0% of males tested), compared to the other treatments, which would be expected to catch approximately 40% of the males tested (Figure 5). Although suggestive, this difference was not significant at $P = 0.05$.

Comparative Responses of Male P. octo *and* E. postvittana *to Different Trap Alignments (Wind-Tunnel Experiments 6 and 3).* Male *P. octo* were flown to the three trap alignments (plus a base) as for *E. postvittana* in experiment 3. The data are shown in Figure 6A along with the comparative data for *E. postvittana* (Figure 6B). Virtually the same percentages of *P. octo* and *E. postvit-*

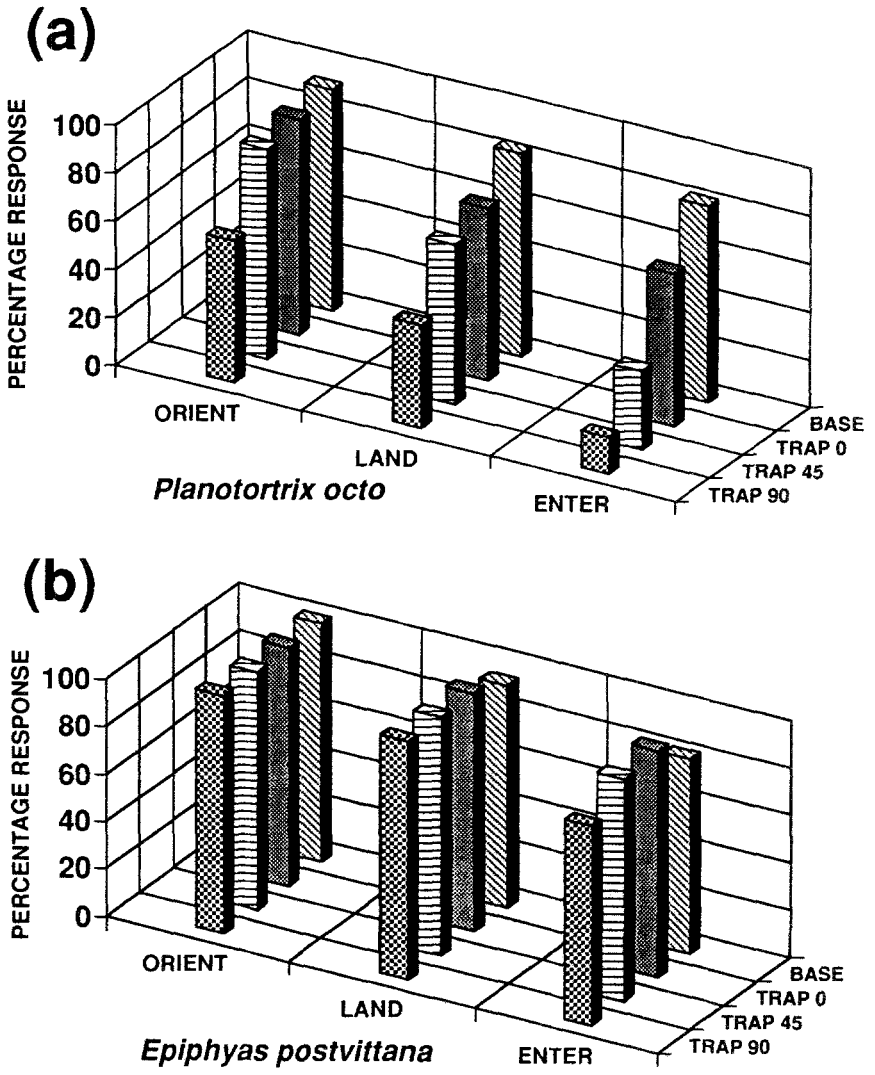


FIG. 6. The percentages of males that oriented to the source and flew upwind (ORIENT), landed on the trap (LAND), and entered a trap (ENTER) aligned at different directions to the wind (other acronyms as in Figure 4) of (A) *Planotortrix octo*, and (B) *Epiphyas postvittana*.

tana males touched the pheromone source (82.1% and 81.7%, respectively) when it was presented on the base alone. However, with the inclusion of the trap, significant differences in the percentages of the two species entering the trap were apparent. With the trap aligned 0° to the wind, a significantly smaller ($P < 0.01$) percentage of *P. octo* males (64.1%) entered the trap than did *E. postvittana* males (95.0%). This difference was due to the compounding effects of a slightly smaller percentage of *P. octo* males (89.7%) orienting upwind than did *E. postvittana* males (100.0%), and a greater proportion of *P. octo* males arresting upwind flight prior to landing on the trap. The differences between the responses of the two species were more apparent when the trap was aligned 45° to the wind. Thus, in contrast to the high percentage of *E. postvittana* males that entered the trap (93.3%), only 33.3% (significantly less, $P < 0.01$) of the *P. octo* males tested actually entered the trap. This decrease in males entering the trap over that observed when the trap was aligned at 0° was due in very large part to the high proportion of the *P. octo* males that landed on, but did not enter, the trap. Only 50% of the males that landed on the trap proceeded to enter it, compared with 89% when the trap was aligned at 0°; in comparison, 93.3% of the *E. postvittana* males entered the trap when it was aligned 45° to the wind. The difference in response between the two species was emphasized further when the trap was aligned 90° to the wind; only 15.4% of *P. octo* males entered the trap compared to 83.3% of *E. postvittana* males. Again this difference was due largely to the relative proportions of males that landed on, but did not enter, the trap (35.3% of *P. octo* males compared with 83.3% of *E. postvittana* males), although another important factor was the smaller percentage of *P. octo* males that oriented upwind relative to that of *E. postvittana* (59.0% compared to 100.0%). Overall, *P. octo* males were less likely to exhibit the more advanced behaviors as the trap alignment with respect to the wind increased, than were *E. postvittana* males.

Field-Trapping. Results from the field trial testing the effect of source location within the trap on the number of *E. postvittana* males caught in the trap are shown in Table 1. Of the three treatments tested, the greatest number of males ($P < 0.05$) were caught in the trap with the source located to the side. The other two treatments (i.e., top and 0°) caught approximately equal numbers of moths.

Observations of Smoke Plume. Compared to the relatively tight smoke plume released from the base alone, the plume from the trap aligned 0° to the wind was more turbulent and diffuse. With the trap aligned 45° to the wind direction, the turbulent and diffuse structure of the plume was even more enhanced. The plume exiting the trap aligned 90° to the wind was highly diffuse, with the diameter at the trap mouth exceeding the width of the trap (Figure 7); the filamentous structure of the plume was barely discernible.

TABLE 1. CATCHES OF *Epiphyas postvittana* MALES IN TRAPS BAITED WITH PHEROMONE SOURCES[a] AT DIFFERENT POSITIONS[b]

Position of source[c]	No. of males/trap[d]
Top	15.0b
Side	29.8a
Center	16.2b
Blank	0.0c

[a] Traps baited with 100 μg (*E*)-11-tetradecenyl acetate and 10 μg (*E*, *E*)-9,11-tetradecadienyl acetate on a rubber septum.

[b] Trial conducted at Kumeu Research Orchard between November 30, 1989, and February 1, 1990. Five replicates were used for each treatment.

[c] Top = source placed in middle, 2.5 cm from the ceiling, of the trap; side = source placed on base, halfway between the entrances, and 5 cm from the side, of the trap; center = source placed in the middle of the base.

[d] Means followed by the same letter are not significantly different at $P < 0.05$ (ANOVA followed by Fisher's protected least significant difference test).

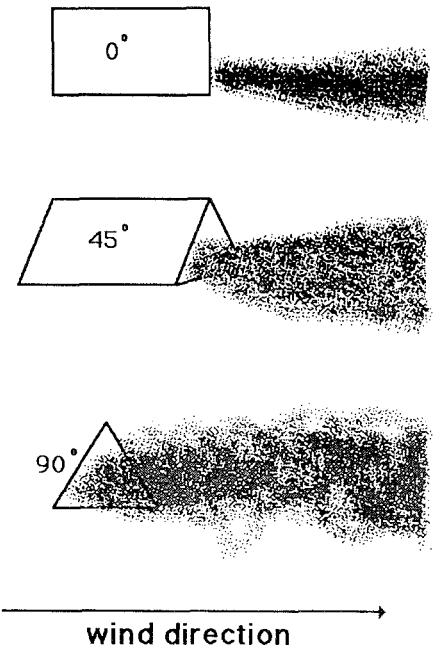


FIG. 7. Sketches showing density of the smoke plume emerging from a trap aligned at different angles (in degrees) to the wind.

DISCUSSION

In New Zealand, the greatest use of synthetic sex pheromones is in the monitoring of phenologies of leafroller moth pests using sticky traps (Suckling et al., 1988). We have investigated several parameters associated with a particular type of trap (Delta) and have found that they influence aspects of the pheromone-mediated behavior of male *E. postvittana*.

A comparison of the initial landing positions on a sticky base when males previously flown to the pheromone source were left on the base and when these males were removed from the base showed a significant difference between the two treatments; incoming males tended to land closer to the pheromone source on the base in the former treatment. Overall, on the base containing males, the initial landing positions were much more focused than they were on the base with no males, with some 79% of the males landing at just two positions on the grid (see Figure 3). The most notable difference in landing position between the two treatments was the much greater proportion of males that landed at the grid position containing the source, on the base containing males.

In addition to the female sex pheromone, there are conceivably three other sensory inputs that could influence the landing position of the males: chemical, tactile, or visual. Male *E. postvittana* are not known to have any scent organs that mediate behavior in other males or females, such as those reported in other species of moths (see for example *Grapholita molesta*; Baker and Cardé, 1979a). Therefore, it appears unlikely that the males already on the trap are releasing chemicals that influence the landing position of the incoming male. Another possibility is the presence of an increased number of male scales in the air, around the base containing the males, influencing male landing behavior. Moth scales, in combination with visual stimuli, have been shown to influence copulatory attempts (and hence close-range behavior) by male *Adoxophyes sp.* (Shimizu and Tamaki, 1980).

Perhaps the cues most likely to influence the landing behavior of males, however, are visual ones. After alighting, Oriental fruit moth males, in the presence of female sex pheromone, are influenced by visual inputs at close range to the pheromone source (Baker and Cardé, 1979b). In the presence of pheromone, males of this species exhibit a complex series of courtship behaviors. These behaviors are exhibited more frequently when a visual stimulus is associated with the pheromone source than when the two factors are separated (Baker and Cardé, 1979b). Although this postalighting visual aspect has been studied, as have the optomotor responses that male moths use in the control of their groundspeed during flight (Baker, 1989), little is known about the importance of visual cues that trigger landing responses of male moths under pheromone-mediated flight. Visual inputs, such as front-to-back motion over the eye

and size and contrast of the visual input, are thought to trigger the landing behavior of the housefly, *Musca domestica* (Borst, 1989).

E. postvittana males do not exhibit such a complex sequence of courtship behaviors as do male Oriental fruit moths. Rather, males tend to land on the surface a few centimeters away from the pheromone source and rapidly approach the source fanning their wings before attempting copulation (Muggleston and Foster, unpublished). The data presented in these experiments show that the presence of other males on the trap base influenced incoming males to bypass landing at the edge of the base more frequently and land on, or very close to, the pheromone source. This effect could be the result of either or both of two visual inputs. First, the males on the base presented a lesser degree of contrast between the downwind edge of the base and the floor of the tunnel, compared to that of a white base without males; hence males were not induced to land until they were extremely close to the pheromone source. Second, the contrast of the pheromone source (a red septum) against the white base was not as great when other males were on the base, and therefore males were unable to visually detect the pheromone source, and consequently land, until they were extremely close to it. Whether males in this situation were ultimately induced to land by visual or olfactory cues, these data suggest that *E. postvittana* males are able to distinguish the spatial position of an olfactory source in a field of similar visual inputs.

The differences in the final positions of the males between the two experiments (i.e., a greater proportion of males sticking downwind of the source in experiment 1 over experiment 2) was probably a consequence of the surface of the base becoming less sticky downwind of the source, due to the deposition of male bodies and/or scales on the surface of the glue and hence males eventually become stuck on a sticky (upwind) surface. This explanation is further supported by the observation of the final position of the male tending to increase in distance from the source with time. These data also show that the sticky surface of the base becomes less efficient at catching male *E. postvittana* after approximately 86 males are caught on the base. Although this figure would probably vary according to the sizes of males caught on the base, as well as factors such as the effect of the trap to constrain movement of a male on the base, this figure probably represents a useful guideline for the saturation point of the trap for this species.

The alignment of the trap, with respect to the wind direction, appears to have little or no effect on the number of male *E. postvittana* entering, or even being caught in, the trap. However, it does have a significant effect on the responses of the males; males land on and enter the trap at different positions, in different proportions, according to the alignment of the trap. In contrast to these results for *E. postvittana*, the alignment of the trap to the wind does drastically affect the number of *P. octo* males that land on and enter the trap. Thus,

as the alignment of the trap to the wind was changed from 0° to 45° to 90°, the percentages of males that entered the trap decreased from 64.1% to 33.3% to 15.4%, respectively. This decrease was predominantly due to the larger proportions of the *P. octo* males that landed on but did not enter the trap, as the alignment of the trap to the wind direction increased. A further contributing factor was the decrease in the percentages of males that oriented to the wind and exhibited upwind flight as the angle between the trap and the wind direction increased. This latter factor became more significant at an angle of 90°.

This decrease in the number of *P. octo* males that actually entered the trap correlates with the change in the plume structure of the pheromone released from the trap (see Figure 7). The structure of the chemical plume is an important factor in pheromone-mediated flight (Baker, 1989). Lewis and Macaulay (1976) demonstrated a correlation between the shape of the chemical plume given off by traps and the number of male *Cydia nigricana* caught in the traps; trap designs releasing broader and less structured plumes caught fewer moths. Likewise as the angle of the Delta trap increased with respect to the wind direction, the plume released from the trap became broader and less structured, and, in the case of *P. octo*, fewer males oriented and ultimately entered the trap. As noted, this effect was not observed for *E. postvittana* males, suggesting that the two species have different responses to the changes in plume structure, i.e., *P. octo* males are more sensitive to loss of plume structure.

The different responses of males of the two species to the alignment of the trap is probably a significant factor in the relatively smaller catches of *P. octo* (and possibly other species in this genus and the genus *Ctenopseustis*), compared to *E. postvittana* males, that are observed in field-trapping experiments (Suckling et al., 1988). Under controlled conditions in the wind tunnel, similar percentages of *P. octo* and *E. postvittana* males responded to their respective synthetic blends, suggesting that the differences in field catches are probably not due to the relative efficacies of the isolated synthetic blends to elicit upwind flight of the respective males. In the field, traps for these species are usually placed with little or no regard for the wind direction, which is assumed to be a random effect on replicated trap catches. However, a trap will probably spend only a relatively small proportion of its time aligned to the wind. Given equal population sizes of *P. octo* and *E. postvittana* in a particular location, then catches of *P. octo* should be lower, as is observed. It should be noted, however, that although this effect is probably a major contributor to the lower relative trap catches of *P. octo*, the relative sizes of the populations of the two species in particular locations may also be important.

The location of the pheromone source within the trap also can have a significant effect on the behavioral responses of males. Although the location of the source within the trap had only a small effect on the numbers of males entering the trap, it significantly affected where males landed and entered the

trap. This wind-tunnel experiment indicated that placing the pheromone source to the side of the trap (as opposed to the other source locations) resulted in more efficient capture of the males entering the trap. The data from the field trial testing the trap captures resulting from the various source locations were consistent with the wind-tunnel data. These experiments show that the effects of certain parameters associated with field-trapping can be tested under controlled conditions, and, if promising, can be validated or invalidated in the field. In practical terms, these particular experiments have shown that the catch by the Delta trap can be improved for trapping *E. postvittana* (and possibly other species) by placing the pheromone source at the side rather than in the normal center position. This improvement probably is due to an increased probability that males are caught in the trap after entering it, rather than more males actually entering the trap.

This approach of studying pheromone trapping in a wind tunnel has allowed us to improve trap efficiency for *E. postvittana* as well as to understand how some of the parameters associated with pheromone trapping affect the behavior of male *E. postvittana* and *P. octo* in response to a pheromone source within the trap. Such controlled studies may allow researchers to open the "black box" of field trapping for different species and allow field-trapping techniques and data to be improved.

Acknowledgments—We thank Mr. D. Rogers and Ms. A. Barrington for supplying the insects, and Dr. M.O. Harris for comments on an earlier version of the manuscript.

REFERENCES

BAKER, T.C. 1989. Pheromones and flight behaviour, pp. 231–255, *in* G.G. Goldsworthy and C. Wheeler (eds.). Insect Flight. CRC Press, Boca Raton, Florida.

BAKER, T.C., and CARDÉ, R.T. 1979a. Analysis of pheromone-mediated behaviors in male *Grapholitha molesta*, the Oriental fruit moth (Lepidoptera: Tortricidae). *Environ. Entomol.* 8:956–968.

BAKER, T.C., and CARDÉ, R.T. 1979b. Courtship behavior of the Oriental fruit moth (*Grapholitha molesta*): Experimental analysis and consideration of the role of sexual selection in the evolution of courtship pheromones in the Lepidoptera. *Ann. Entomol. Soc. Am.* 72:173–188.

BAKKE, A., and RIEGE, L. 1982. The pheromone of the spruce bark beetle *Ips typographus* and its potential use in the suppression of bark beetle populations, pp. 3–15, *in* A.F. Kydonieus and M. Beroza (eds.). Insect Suppression with Controlled release Pheromone Systems, Vol. 2. CRC Press, Boca Raton, Florida.

BELLAS, T.E., BARTELL, R.J., and HILL, A. 1983. Identification of two components of the sex pheromone of the moth, *Epiphyas postvittana* (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 9:503–512.

BORST, A. 1989. Temporal processing of excitatory and inhibitory motion stimuli in the fly's landing system. *Naturwissenschaften* 76:531–534.

FOSTER, S.P., CLEARWATER, J.R., MUGGLESTON, S.J., and SHAW, P.W. 1990. Sex pheromone of

a *Planotortrix excessana* sibling species and reinvestigation of related species. *J. Chem. Ecol.* 16:2461–2474.

GALBREATH, R.A., BENN, M.H., YOUNG, H., and HOLT, V.A. 1985. Sex pheromone components in the New Zealand greenheaded leafroller, *Planotortrix excessana* (Lepidoptera: Tortricidae). *Z. Naturforsch.* 40c:266–271.

KENNEDY, J.S. 1977. Behaviorally discriminating assays of attractants and repellents, pp. 215–229, *in* H.H. Shorey and J.J. McKelvey, Jr. (eds.). Chemical Control of Insect Behavior: Theory and Application. Wiley, New York.

LEWIS, T., and MACAULAY, E.D.M. 1976. Design and elevation of sex-attractant traps for pea moth, *Cydia nigricana* (Steph.) and the effect of plume shape on catches. *Ecol. Entomol.* 1:175–187.

MCCULLAGH, P. 1980. Regression models for ordinal data. *J. R. Stat. Soc. B* 42:109–142.

MILLER, J.R., and ROELOFS, W.L. 1978. Sustained-flight tunnel for measuring insect responses to wind-borne sex pheromones. *J. Chem. Ecol.* 4:187–198.

MUGGLESTON, S.J., and FOSTER, S.P. 1989. Sustained-flight tunnel responses of male lightbrown apple moth to synthetic sex pheromone. *Physiol. Entomol.* 14:443–449.

ROBACKER, D.C., MORENO, D.S., and WOLFENBARGER, D.A. 1990. Effects of trap color, height, and placement around trees on capture of Mexican fruit flies (Diptera: Tephritidae). *J. Econ. Entomol.* 83:412–419.

SHIMIZU, K., and TAMAKI, Y. 1980. Releasers of male copulatory attempt in the smaller tea tortrix moth (Lepidoptera: Tortricidae). *Appl. Entomol. Zool.* 15:140–150.

SINGH, P. 1974. A chemically defined medium for rearing *Epiphyas postvittana* (Lepidoptera: Tortricidae). *N.Z. J. Zool.* 1:241–243.

SUCKLING, D.M., WALKER, J.T.S., SHAW, P.W., and WHITE, V. 1988. Spray less for leafroller control. *Orchardist N.Z.* 61:21–24.

WEARING, C.H., THOMAS, W.P., DUGDALE, J.S., and DANTHANARAYANA, W. 1991. Australian and New Zealand species, *in* L. Van der Geest (ed.). Tortricoid Pests, Their Biology, Natural Enemies and Control. Elsevier, Amsterdam. In press.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

ANALYSIS OF CHARACTERISTIC ODORS FROM HUMAN MALE AXILLAE

XIAO-NONG ZENG,[1] JAMES J. LEYDEN,[2] HENRY J. LAWLEY,[1] KIYOHITO SAWANO,[3] ISAO NOHARA,[3] and GEORGE PRETI[1,2,*]

[1]*Monell Chemical Senses Center*
3500 Market Street
Philadelphia, Pennsylvania 19104

[2]*Department of Dermatology, School of Medicine*
Hospital of the University of Pennsylvania
3400 Spruce Street
Philadelphia, Pennsylvania 19104

[3]*Takasago International Corporation*
3-19-22 Takanawa Minato-ku
Tokyo, Japan

(Received January 7, 1991; accepted March 22, 1991)

Abstract—A number of studies concerning the analysis of axillary odors have assumed that the characteristic odor produced in the axillae is due to volatile steroids and isovaleric acid. Organoleptic evaluation of chromatographic eluants from axillary extracts was employed to isolate the region in the chromatogram where the characteristic odor eluted. The odor of the dissolved eluant was eliminated when it was treated with base, suggesting that acids make up the characteristic axillary odor. Subsequent extraction of the pH-adjusted axillary extract in conjunction with organoleptic evaluation of the chromatographic eluant, preparative gas chromatography, and analysis by GC-MS as well as GC-FTIR showed the presence of a number of C_6 to C_{11} straight-chain, branched, and unsaturated acids as important contributors to the axillary odor. The major odor component is (*E*)-3-methyl-2-hexenoic acid. Three homologous series of minor components are also important odor contributors; these consist of the terminally unsaturated acids, the 2-methyl-C_6 to -C_{10} acids and the 4-ethyl-C_5 to -C_{11} acids. These types of acids have not been reported previously as components of the human axillary secretions and have not been proposed previously as part of the principal odor components in this area.

*To whom correspondence should be addressed.

0098-0331/91/0700-1469$06.50/0

Key Words—Axillary odors, (*E*)-3-methyl-2-hexenoic acid, androstenone, human axillary secretions, malodors, 7-octenoic acid.

INTRODUCTION

The human axillary region is a body area with unique odor-producing characteristics. In the axillae, apocrine, sebaceous, eccrine, as well as the recently described apoeccrine, glands (Sato et al., 1987), provide an excellent environment for a large permanent population of microorganisms (Leyden et al., 1981). These microorganisms are believed to generate a variety of odoriferous compounds that characterize our underarm region (Labows et al., 1982). Previous analyses of both the apocrine secretion and the total axillary sweat have shown the presence of a variety of both volatile and nonvolatile steroids (Brooksbank, 1970; Brooksbank et al., 1974; Claus and Alsing, 1976; Labows et al., 1979b; Bird and Gower, 1980).

The apocrine secretion when freshly collected at the skin surface is odorless. (Hurley and Shelly, 1960; Shehadeh and Kligman, 1963). However, incubation with the resident bacteria results in the production of a characteristic odor, which appears to be unique to the organism used (Leyden et al., 1981). The micrococci bacteria present in the axillae impart an acidic odor to the secretion. The headspace above apocrine secretion incubated with micrococci has been reported to contain isovaleric acid (Labows, 1979; Labows et al., 1982). The diphtheroid bacteria give similar chromatographic headspace profiles with isovaleric acid being present. However, the odor is more distinct and pungent, suggesting the presence of other unidentified volatiles (Labows et al., 1982).

Analysis of freshly collected apocrine secretions in our laboratory have demonstrated the presence of two androgen steroid sulfates: 17-oxo-5α-androstan-3α-yl sulfate (androsterone sulfate) and 17-oxo-5α-androsten-3β-yl sulfate (dehydroepiandrosterone sulfate) (Labows et al., 1979b). Both dehydroepiandrosterone sulfate and androsterone sulfate are present in high concentrations (Labows et al., 1979b; Preti et al., 1987) and may serve as precursors for more volatile, odoriferous steroids.

Studies recently completed in our laboratories employed axillary extracts obtained from men and women to influence the menstrual cycle length and timing (Preti et al., 1986; Cutler et al., 1986). Another report from our laboratory discussed the analysis of the extracts and the levels of several steroids present in them. The extracts also contained a series of aliphatic acids ranging from two to 18 carbons (Preti et al., 1987).

Investigators have collected the volatiles in the axillary area for chromatographic analysis and evaluation of individual peaks for malodor (Dravnieks et al., 1975). However, no structure elucidation was performed in these studies.

Two recent reviews (Labows, 1988; Gower, 1989), have suggested, but not proven, that the characteristic odor in the axillae is due to the presence of volatile steroids and isovaleric acid. The volatile steroids suggested as contributing to the axillary odor are 5α-androst-16-en-3α-ol (androstenol), 5α-androst-16-en-3-one (androstenone), and 4,16-androstadien-3-one (androstadienone). These steroids are formed, in part, by the action of cutaneous microorganisms (Labows, 1988) and have low olfactory threshold (Amoore et al., 1977); however, there is approximately a 40–50% anosmia to androstenone in the adult United States population (Labows and Wysocki, 1984), which implies that this odor is not recognized by half the population.

Since axillary secretion extracts contain a complex mixture of compounds that are not well characterized, it is possible that secretion components (other than those noted above) are important for both mediating the odor character and physiological activity. We report here the isolation and identification of compounds from a combined male axillary secretion extract that contains the characteristic odors present in the axillae.

METHODS AND MATERIALS

General. [^{1}H]NMR spectra for the (*E*)-3-methyl-2-hexenoic acid collected by preparative gas chromatography from the axillary extract were recorded on a Bruker AM-500 spectrometer, employing $CDCl_3$ as solvent with $CHCl_3$ as reference; 21,000 scans were used to obtain sufficient data for structural determination. For the synthetic samples, [^{1}H]NMR spectra were recorded on a Bruker AM-400 spectrometer, employing $CDCl_3$ as solvent with TMS as reference.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. A Finnigan 4510 GC-MS data system equipped with a split–splitless injector, a fused silica capillary column, and capabilities for operation in both electron impact and chemical ionization modes was used for analysis. The columns employed were a 30-m × 0.32-mm-ID fused silica column with a 0.25-μm coating of Stabilwax (cross-bonded polyethylene glycol), or a 30-m × 0.32-mm-ID fused silica RTX-1 column with a 0.25-μm coating of cross-bonded methyl silicon (Restek, Port Matilda, Pennsylvania). Analysis conditions for each column were as follows: Stabilwax, 60°C (4 min hold) to 220°C, at 3°C/min, hold for 45 min; RTX-1, 60°C (4 min hold) to 300°C, at 4°C/min, hold for 15 min. The mass spectrometer is interfaced to a Nova 4X computer, which utilizes the Super Incos software for data acquisition, analysis, and quantitation. The mass range employed during these analyses was *m/z* 40–450. This mass range was scanned once each second and a typical run included 4000 scans. The data system also included the NBS library of 42,000 compounds.

Identifications were based on interpretation of unknown spectra and their

comparison with both the NBS library and mass spectra generated from synthetic and/or commercially available standard compounds. In addition, the relative chromatographic retention times of unknowns and known standards were compared. A mixture of fatty acid ethyl esters was used to determine the relative retention times (van den Dool and Kratz, 1963) and generate an ethyl ester unit for each compound.

Gas Chromatography–High-Resolution Mass Spectrometry (GC-HRMS) This was performed using a Carlo Erba Fractovap 4160 series gas chromatograph interfaced to a Kratos MS-50. High-resolution spectra of the eluting components were obtained using a scan time of 5 sec for the decade 20–200 daltons, an ionizing voltage of 70 eV, and an ionizing chamber temperature of 150°C. Data were acquired, reduced, and exact masses calculated using the Kratos DS-55 software.

Gas Chromatography–Fourier Transform Infrared Spectra. These were obtained using a Hewlett-Packard 5890 gas chromatograph interfaced to a Hewlett-Packard 5965A infrared detector. Samples were analyzed using either a 30-m × 0.32-mm-ID fused silica column with a bonded 0.5-μm coating of Supelco Wax (60°C for 4 min then programmed to 220°C at 3°C/min) or a Hewlett-Packard 50-m × 0.32-mm-ID column with 0.5-μm bonded coating of methyl silicone. Spectra were obtained at 4 wave number resolution with 1.6 scan/sec or 8 wave number with 3 scans/sec.

Collection of Axillary Secretions. The axillary secretions used in this investigation were collected from six healthy male volunteer donors (ages 25–40). Each donor had large numbers of lipophilic diphtheroids in his axillary region. These microorganisms have been associated with production of the strongest axillary odors (Labows et al., 1982; Leyden et al., 1981). The protocol followed by donors and the methods used for the collection were described previously (Cutler et al., 1986; Preti et al., 1987).

Preparation of Axillary Secretion Extract. Extracts were prepared by extracting 12 pads at a time with doubly distilled ethanol, as previously described (Cutler et al., 1986; Preti et al., 1987). The ethanol-soaked pads were subsequently soaked with a mixture of 85 : 15 chloroform–methanol for 1 hr and then squeezed (Nanograde Solvents from Mallinkrodt, St. Louis, Missouri; these solvents contain little or no impurities by GC-MS analysis when 100–200 ml are concentrated to ≤ 50 μl) (15 ml/pad). Approximately 95% of the chloroform–methanol was recovered. The two extracts were combined, then concentrated to a volume of 50–100 μl by a rotary evaporator, and subsequently stored at −60°C until analysis. Through the use of standards in model experiments, we determined that 59% of the 3-methyl-2-hexenoic acids and 85% of the C_7–C_9 acids were recovered from the pads.

Preparative Gas Chromatographic (PGC) Fractionation and Collection of Axillary Secretion Extracts. PGC fractionation of axillary secretion extracts was

performed on a Perkin-Elmer 990 gas chromatograph equipped with hydrogen flame-ionization detector, linear temperature programmer, and modified with a capillary effluent splitter with a 10:1 split ratio as well as a thermal gradient collector (Brownlee and Silverstein, 1968). Fractions were collected in 40-cm × 1.8-mm-OD glass capillary tubes. Retrieval of the collected fraction was accomplished by breaking the end of the tube, drawing one end to a point, and rinsing the tube with small aliquots (about 20–30 μl) of desired solvent. The column used for separation was 30 m × 0.52 mm ID coated with Stabilwax; the analysis conditions used for the separation were as follows: 60°C for 4 min and then 3°C/min to 220°C with a hold for 48 min at the final temperature.

Isolation of Acidic Components From Axillary Secretion Extract. The concentrated axillary secretion extract (100 μl) was put into a centrifuge tube and then cooled to 0°C. Saturated aqueous sodium bicarbonate (2 ml) was added into the tube. After shaking, the mixture was extracted with $CHCl_3$ (1 ml × 2). The resulting two layers were separated by centrifugation for 5 min. The bottom organic layer, containing the basic and neutral components, was transferred to a 5-ml pear-shaped flask by using a thinly drawn pipet. This was stored at −10°C until analysis.

The aqueous layer remaining in the centrifuge tube was cooled to 0°C, and acidified with 0.5 M HCl to a pH of 2. The mixture was extracted with $CHCl_3$ (1 ml × 2). The organic layer was again separated as described above and concentrated to 20 μl.

A second procedure was performed for the isolation of acidic components. This involved treatment of the cooled (0°C), concentrated (without adding $NaHCO_3$, as above) axillary secretion extract with 0.5 M HCl, and extraction with $CHCl_3$. The two layers formed were separated by the same procedures as above. The organic layer was treated with saturated aqueous sodium bicarbonate and separated. The basic aqueous layer produced from the last step was acidified and extracted with $CHCl_3$. After separation of the two layers, the organic layer that contains the acidic components was concentrated and analyzed. The procedure yielded the same array of compounds as was found when the extract was first exposed to bicarbonate.

Synthesis of 7-Octenoic Acid. To a stirred mixture of 1,6-heptadiene (3.57 g, 0.039 mol) and toluene (5 ml), 8.2 ml (1/3 mol eq) of $Al(i\text{-butyl})_2H$ was added while maintaining the temperature below 40°C. This mixture was then stirred for 3 hr at 50–60°C. After cooling this mixture to room temperature, 7.7 ml (1/3 mol eq) of *n*-butyl lithium was added. We then bubbled CO_2 gas into the mixture (at room temperature) and followed the formation of 7-octenoic acid by gas chromatography. The addition of CO_2 was ceased when no further acid was found. The mixture was subsequently poured into a 10% HCl solution and extracted with 3 × 30 ml of ether. The ether layer then was extracted with 3 × 3 ml of 10% NaOH solution and the NaOH solution subsequently acidified

with 2 M HCl and extracted with 3 × 30 ml of diethylether. The ether layer was dried and concentrated to give 2.5 g (45%) of an oil which contained 77% of 7-octenoic acid. Pure quantities of the acid for subsequent experiments were obtained by preparative gas chromatography as described above.

Preparation of Trimethylsilyl Derivatives. In order to form trimethylsilyl (TMS) esters and/or ethers, 5 μl of the concentrated acid fraction was reacted at 70°C for 30 min with 10 μl *N,O*-bistrimethylsilyltrifluoroacetamide (BSTFA) and 2 μl trimethylsilyl chloride. The resulting reaction mixture was analyzed by GC-MS using the non-polar RTX-1 column described above.

Organoleptic Evaluation of Chromatographically Separated Components (Smell Chromatography). To determine which of the chromatographic eluants had odors resembling the axillary extract, a panel of two to four judges were assembled to perform the smell chromatography. Each panel member was able to perceive the "urinous" odor of androstenone and the "musky" odor of androstenol (Amoore et al., 1977; Labows et al., 1982). Prior to smell chromatography, the entire mixture to be analyzed was evaluated after placing a small (1–5 μl) aliquot on a filter paper strip and allowing the solvent to dry. The odor of the entire mixture then was evaluated by the panel. The sample was left in a fumehood within easy access should it be needed for reference. Olfactory sampling began approximately 3 min after injection. Each judge sampled the chromatographic eluate every 30–40 sec on a rotating basis. They were asked to record which portions of the eluate most resembled the original "bouquet" in terms of quality and intensity. Panelists were instructed not to communicate with each other during judging and to make sure that other panelists could not see their records. Smell chromatography was performed with the flame ionization detector off and olfactory sampling of the chromatographic eluent occurring at each 30-sec interval by two or more observers. The analyses were performed using the following conditions: Sil-8, 60°C (4 min) to 300°C at 4°C/min; Stabilwax, 60°C (4-min hold) to 220°C at 3°C/min. The carrier gas (He) flow rate in each analysis was 2 ml/min.

Hydrogenation of Collected Peak K. Into a 0.4 ml V-shaped reaction vial were placed 10 mg of 10% palladium on charcoal catalyst, 50 μl nanograde methanol, and collected compound [K] (≤ 0.2 μg in 10 μl of $CHCl_3$). A small stream of hydrogen was introduced into the mixture at room temperature. After 5 min, the mixture was transferred to a 1-ml centrifuge tube and centrifuged at top speed for 5 min. The clear reaction mixture was withdrawn into a small pear-shaped vial and concentrated to about 5 μl using a Savant Speed Vac Concentrator (model SVC-100H) prior to analysis by GC-MS.

Structural Data for Synthetic (E)- *and* (Z)-*Methyl-2-Hexenoic Acids.* The 3-methyl-2-hexenoic acids were synthesized using the procedure of Wadsworth and Emmons (1961) to condense triethylphosphonoacetate with 2-pentanone. This procedure gives a 1:3 mixture of the *Z* and *E* isomers.

The NMR spectrum for the *E* isomer has been previously reported using a 100-MHz instrument (Smith et al., 1969). However, FT-IR data for the *E* as well as 500-MHz NMR, FT-IR, and MS data for the *Z* compound are given below:

(E)-*3-Methyl-2-Hexenoic Acid.* IR max (vapor) cm^{-1} 3581, 2971, 1751, 1651, 1391, 1270, 1198, 1106, 858. Ethyl ester unit = 13.49.

(Z)-*3-Methyl-2-Hexenoic Acid.* [^{1}H]NMR ($CDCl_3$) 0.95 (3H, triplet, J = 7.3, C—6H), 1.15 (2H, sextuplet, J = 7.5, C—5H), 1.91 (3H, doublet, J = 1.3, C—7H), 2.62 (2H, triplet, J = 7.7, C—4H), 5.69 (1H, singlet, C—2H); IR max (vapor) cm^{-1} 3580, 2973, 1750, 1649, 1385, 1271, 1186, 1112, 1009, 860. Mass Spectrum: *m/z* (rel. int.) M^+ 128 (71), 113 (81), 111 (21), 100 (50), 95 (72), 87 (32), 83 (25), 82 (59), 72 (13), 71 (42), 69 (100), 67 (85), 57 (24), 56 (13), 55 (73), 53 (37), 45 (25), 43 (88), 42 (27), 41 (93); ethyl ester unit = 13.05.

RESULTS

The suggestion that volatile steroids might be involved in the characteristic axillary malodors initially prompted us to perform organoleptic evaluation of the chromatographic eluant (henceforth, smell chromatography) with the concentrated axillary extracts using a nonpolar, methyl silicon column. We previously had established reliable retention times for the volatile steroids using this type of column (Preti et al., 1987). The results of this experiment are illustrated in Figure 1 (top). This shows that within the first 15 min of the analysis, a strong, characteristic axillary odor eluted. The urinous, musky odors characteristic of the volatile steroids (such as androstenol, androstenone, 3,5-androstadienone, and the pyrolysis products of dehydroepiandrosterone sulfate and androsterone sulfate) elute in the area shown at about 40–45 min. The identical extract injected onto the more polar, Carbowax-type phase (Figure 1, bottom), shows the characteristic axillary odor eluting at a retention time of 38–53 min. This is preceded by the sharp, acidic smells of short-chain aliphatic acids (such as butyric and isovaleric acids). On this column, the volatile steroids eluted after 65–70 min. These results suggested that a yet unidentified group of compounds was responsible for the characteristic axillary odor.

Collection of the area where the characteristic axillary odors elute was performed using preparative gas chromatography. The collected eluant was dissolved in methylene chloride, divided in two, and treated with two aqueous base solutions: (1) $NaHCO_3$ (pH 9.9); and (2) $Na_2B_4O_7$ (pH 10.8). Both treatments resulted in an elimination of the characteristic axillary odor. This experiment suggested to us that acidic components with C_6 or greater carbons (based on chromatographic retention times) carried the characteristic underarm odor.

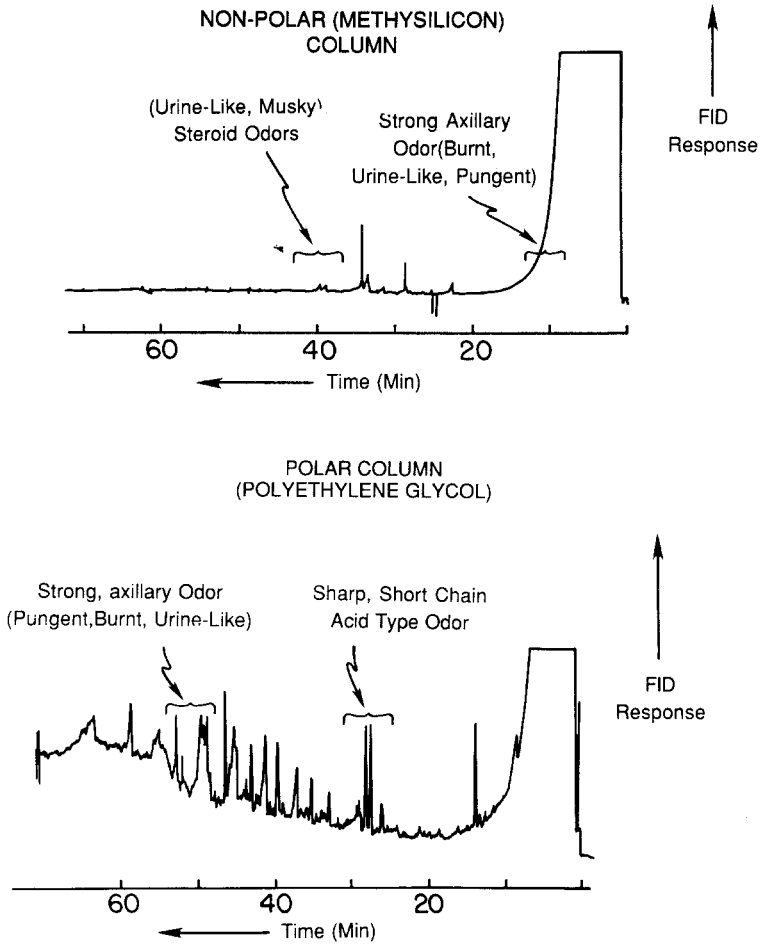


FIG. 1. Top: Flame ionization detector (FID) response and organoleptic evaluation of compounds in the axillary secretion extract eluting from the bonded methyl silicone column. Strong, characteristic axillary type odors elute with the first 10–18 min. The odor of the volatile steroids (such as androstenone and androstenol) as well as the pyrolysis products of the steroid sulfates are found in the time frame shown. Bottom: Results of the organoleptic evaluation of compounds in the axillary secretion extract eluting from the bonded Carbowax-like (Stabilwax) column. The characteristic axillary odors elute in the time frame shown. Steroids such as androstenone and androstenol have long retention times on this column (>65 min).

We subsequently isolated the acidic components from a combined (six male donors) concentrated male axillary extract. The extract containing the acid components was concentrated to 20 μl by rotary evaporation. This acidic concentrate had a characteristic axillary malodor—identical to that of the entire extract. The neutral and basic part of the extract had little or no odor. A second procedure employing initial acidification of the extract gave identical results (see Methods and Materials).

Smell chromatography of the acidic fraction using the polar (Stabilwax column), showed a series of components with burnt, urinous, and axillary-like qualities eluting from times 37–54 min. Therefore, a number of the compounds eluting in this time frame carried a significant portion of the axillary odor. However, the major component, found at ethyl ester unit (EEU) of approximately 13.50 (peak K in Figure 3) appeared to be a major contributor to the axillary odor.

The reconstructed ion chromatogram (RIC) obtained from separation and analysis of the combined acid fraction from the male donors is shown in Figure 2. The area outlined in Figure 2 is expanded in Figure 3 (bottom). The shaded areas indicate where important odors elute. Correlation of the data from smell chromatography with the GC-MS data is carried out by matching the relative retention times (based on ethyl ester indices; van den Dool and Kratz, 1963) on both the chromatograph where the smell chromatography was performed and on the GC-MS system.

Figure 3 also shows a plot (mass chromatogram, top) of m/z 60. This "McLafferty rearrangement" ion is characteristic of aliphatic acids (with an available γ-hydrogen and an unsubstituted α-carbon) (Budzikiewicz et al., 1967). In this figure, m/z 60 is particularly large for the normal, straight-chain acids.

In addition to the mass spectral and relative retention time data, further analysis of the acidic extract was performed by combined gas chromatography–Fourier transform-infrared spectroscopy (GC-FTIR). All of the compounds identified in this mixture are listed with their corresponding ethyl ester units in Table 1. Those compounds thought to be important contributors to the odor are marked with an asterisk.

Characterization of (E)- *and* (Z)-*3-Methyl-2-Hexenoic Acid.* Mass spectra obtained by combined GC-MS using both polar and nonpolar columns showed that the major component (peak K) had the mass spectrum shown in Figure 4. A molecular ion at m/z 128, as well as losses of 15, 17, and 28 from the molecular ion are seen. Also noted was a small component eluting at approximately 13.00 EEU (peak G in Figure 3), which had an identical mass spectrum, suggesting the presence of a stereoisomer. A high-resolution mass spectrum of peak K showed its molecular ion to have an elemental composition of $C_7H_{12}O_2$ (exact mass = 128.0837; found = 128.0820).

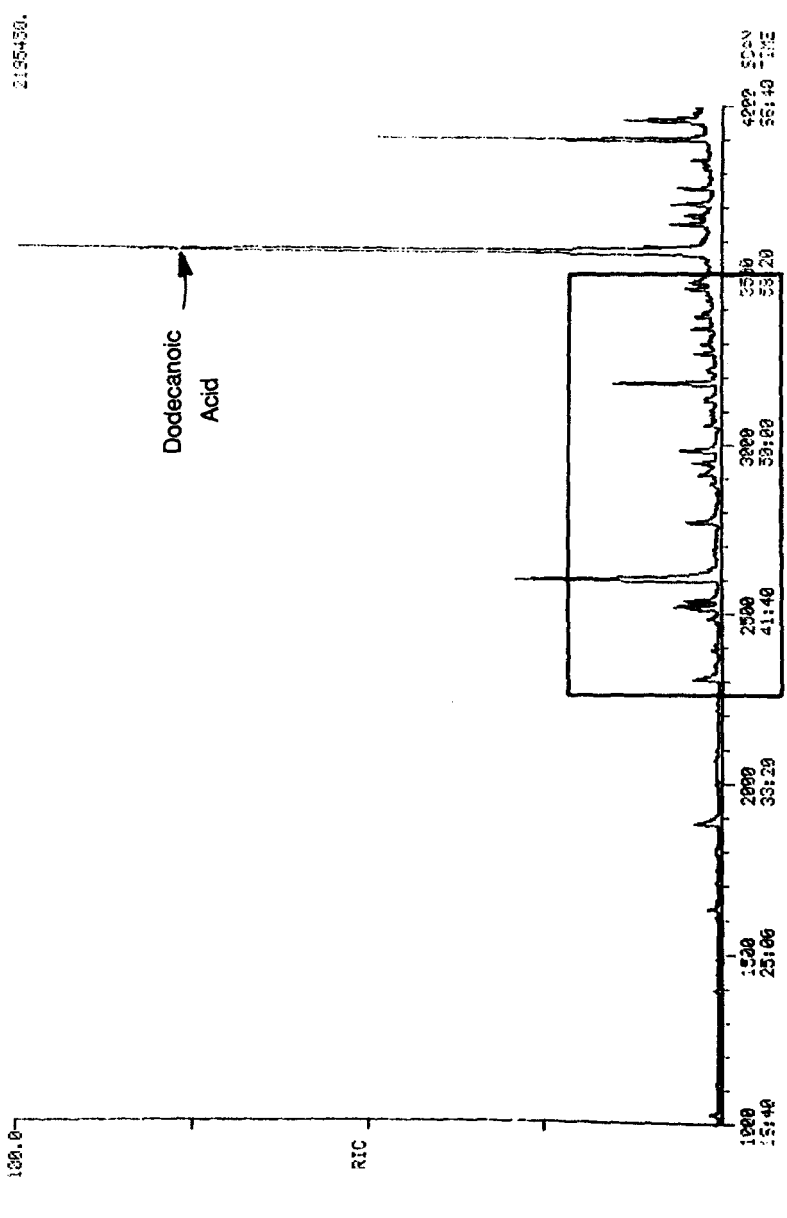


FIG. 2. The computer reconstructed chromatogram generated by the analysis of the total acid portion of the axillary extract. The outlined area is the part of the chromatogram where the characteristic axillary odors are found, as shown in Figure 3. The major component seen at approximately 3590 scans is nonodorous normal dodecanoic acid.

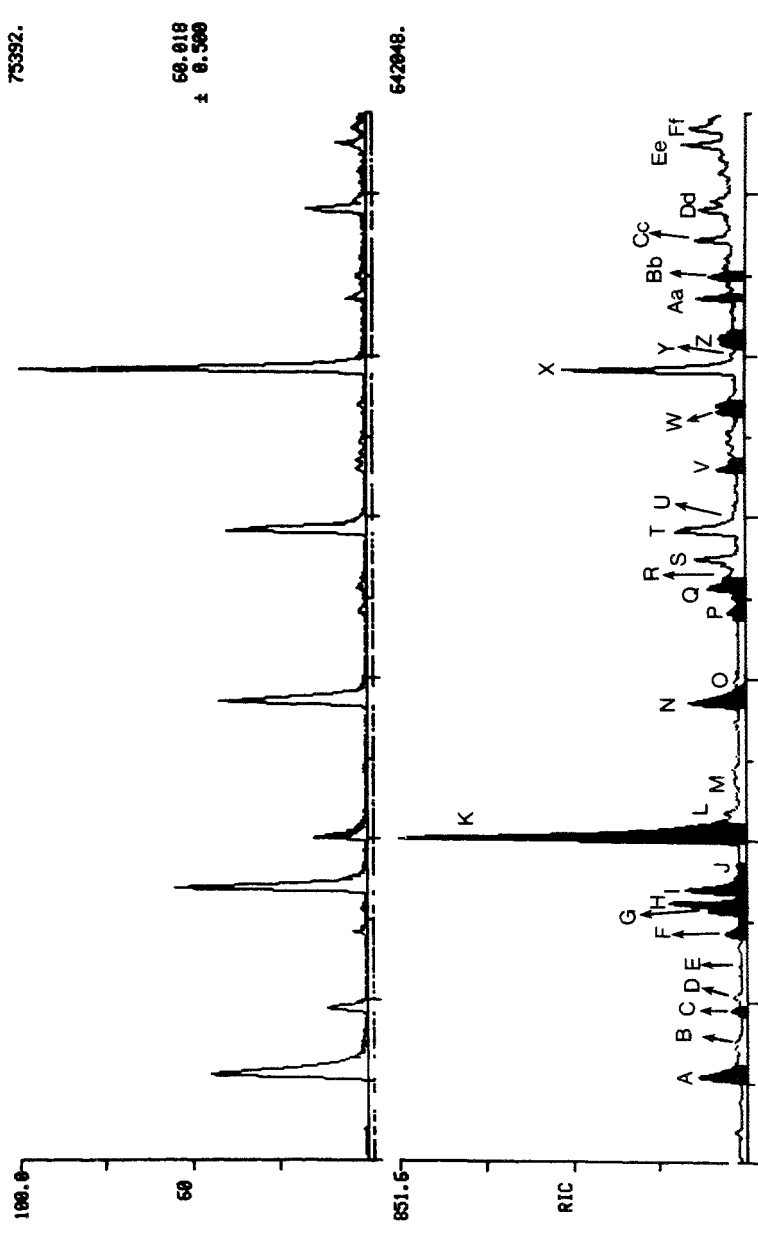


FIG. 3. The outlined portion of the chromatogram from Figure 2 is expanded here. In addition the top part of the figure shows a plot of *m/z* 60 ("mass chromatogram") in this region. This ion is characteristic of aliphatic acids with an available α-carbon as discussed in the text. This ion is particularly large for the normal, straight-chain acids. The shaded areas represent portions of the chromatogram where characteristic axillary-type odors eluted. The identity of all the labeled peaks in the chromatogram may be found in Table 1. Those compounds in the shaded areas are marked with an asterisk in Table 1.

TABLE 1.[a]

Peak		Molecular weight	Retention time (ethyl ester unit)
A	*n*-hexanoic acid[C,G]	116	12.20*
B	2-methylhexanoic acid[G]	130	12.39
C	3-methylhexanoic acid[G]	130	12.55*
D	dimethylsulfone[G] ($C_2H_6SO_2$)	94	12.63
E	γ-C_8-lactone[G^]	142	12.79
F	4-ethylpentanoic acid[G]	130	12.97*
G	(*Z*)-3-methyl-2-hexenoic acid[G]	128	13.10*
H	2-ethylhexanoic acid[G]	144	13.13
I	*n*-heptanoic acid[C,G]	130	13.22*
J	2-methylheptanoic acid[T,G]	144	13.36*
K	(*E*)-3-methyl-2-hexenoic acid[C,G]	128	13.50*
L	phenol	94	13.65
M	γ-C_9-lactone[G]	156	13.91
N	*n*-octanoic acid[C,G]	144	14.28*
O	2-methyloctanoic acid[G]	158	14.41
P	4-ethylheptanoic acid[G]	158	14.81*
Q	7-octenoic acid[G]	142	14.95*
R	γ-C_{10}-lactone	70	15.01
S	*n*-tetradecanol	214	15.21
T	*n*-nananoic acid[C,G]	158	15.28
U	2-methylnonanoic acid[T,G]	172	15.38
V	4-ethyloctanoic acid[G] ("goat acid")	172	15.64*
W	unsaturated C_9 acid[T]	172	16.04*
X	*n*-decanoic acid[C,G]	156	16.28
Y	2-methyldecanoic acid[T,G]	186	16.36
Z	unsaturated C_{10} acid[T]	170	16.46*
Aa	4-ethylnonanoic acid[T]	186	16.69*
Bb	9-decenoic acid[G]	170	16.90*
Cc	*n*-hexadecanol	242	17.24
Dd	*n*-undecanoic acid[C,G]	186	17.29
Ee	4-ethyldecanoic acid[T,G]	200	17.66
Ff	unsaturated C_{11} acid[T] (10-undecenoic acid?)	184	17.76

[a]The following symbols are used in this table: T = tentatively assigned by mass spectral data. C = FTIR spectrum corresponds to assigned structure; G = correspondence of mass spectrum and relative chromatographic retention times with commercially available or synthetic sample. *Indicates compound thought to be a contributor to axillary odor. ^ C_5–C_7 γ-lactones also present,

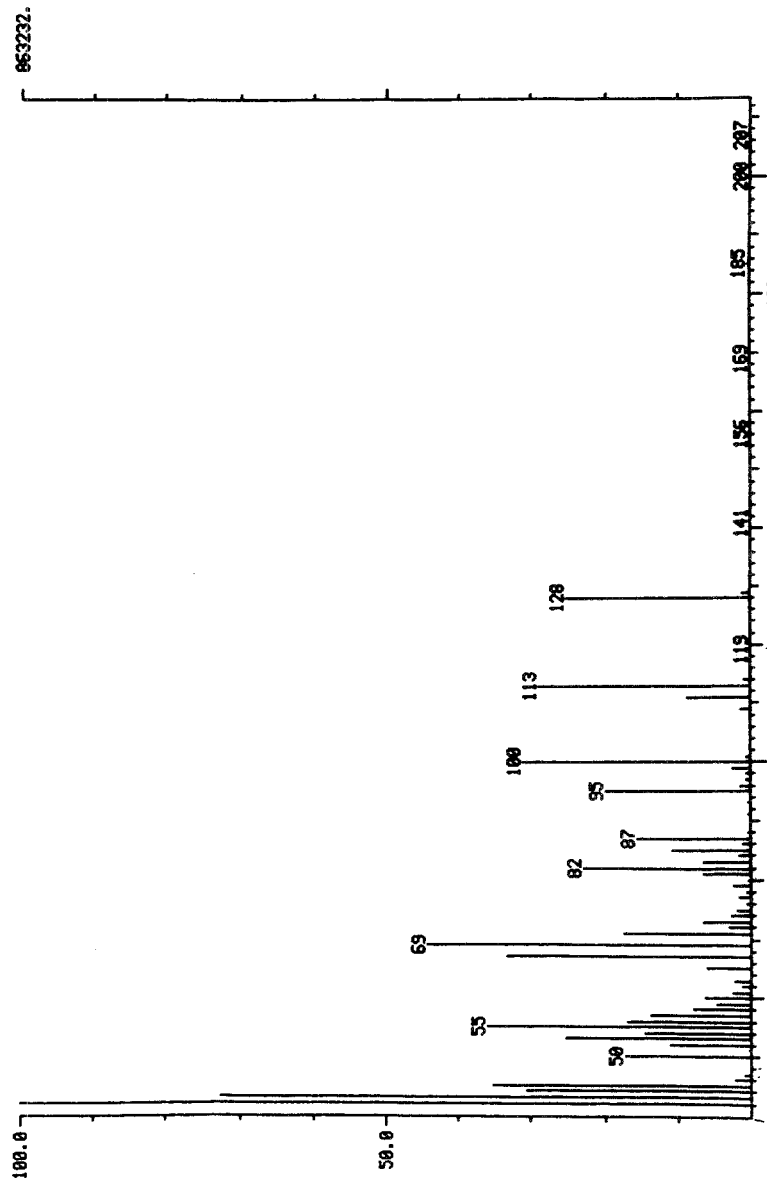


FIG. 4. Mass spectrum obtained from peak K in Figure 3, subsequently characterized as (*E*)-3-methyl-2-hexenoic acid.

Two analyses of the acid mixture were performed using GC-FTIR. The compound of interest showed identical vapor phase IR spectra on both polar and nonpolar columns (Figure 5). A search of a compilation of IR vapor-phase spectra suggested an α,β-unsaturated acid (personal communication from Mr. Michael Boruta, Sadtler Research Laboratories, after searching unknown spectra against Sadtler's IR-Vapor Phase Data Base). In addition, the EEU of peak K and the MS data also suggested a branched, unsaturated C_7 acid. We isolated a small amount (≤ 0.2 μg) of the unknown acid and performed a microhydrogenation. GC-MS analysis of the resultant reaction mixture showed a mass spectrum that was consistent with the structure of 3-methylhexanoic acid.

From the above data, the most probable structure was postulated to be 3-methyl-2-hexenoic acid. This compound was synthesized using the procedure of Wadsworth and Emmons (1961) to condense triethyl phosphonoacetate with 2-pentanone. This procedure gave a 1:3 mixture of the *Z*- and *E* isomers; each was isolated using PGC. The MS, EEU, FTIR and [^{1}H]NMR spectra for each isomer were obtained, allowing assignment of the *E* structure to peak K; the *Z* isomer was the minor component, peak G.

As further confirmation of our structural assignments, PGC using a wide-bore (0.53 mm) bonded capillary column was employed to collect sufficient amounts of the proposed *E* isomer from a concentrated extract. This extract was obtained from a collection of 20 pads worn by one donor (with the strongest axillary extract odor) across a 20-day period. Approximately 4.6 μg of the proposed *E* isomer were collected and used to obtain the FT-[^{1}H]NMR spectrum. This quantity yielded sufficient data to confirm that the collected compound was the (*E*)-3-methyl-2-hexenoic acid.

Characterization of Other Axillary Odor Compounds. Figure 6 shows the FTIR spectra of peaks A, N, T, and X, which correspond to the C_6, C_8, C_9, and C_{10} straight-chain acids. In contrast to solution-phase IR spectra, the vapor-phase spectra show very sharp absorption at 3577 cm^{-1} for the carboxyl OH stretching.

In addition to the (*Z*)- and (*E*)-3-methyl-2-hexenoic acids, several other unsaturated compounds appear to be present, including compounds with terminal double bonds. The C_8 unsaturated acid, 7-octenoic acid, is a minor component of the mixture but appears to have a high odor impact. The mass spectrum of 7-octenoic acid as well as that of a synthetic sample are shown in Figure 7. In addition to the correspondence of the mass spectra, the relative retention times of the naturally occurring and synthetic 7-octenoic acids are identical. Several other terminally unsaturated compounds are also present (Table 1). Since retention times and mass spectra suggested these compounds might be homologous to 7-octenoic acid, we obtained 8-nonenoic acid from Takasago International Corp. (Tokyo, Japan), 9-decenoic acid from Tokyo Kasei Kogyo Co., Ltd. and 10-undecenoic acid from Sigma Chemical Co. Although both the EEU

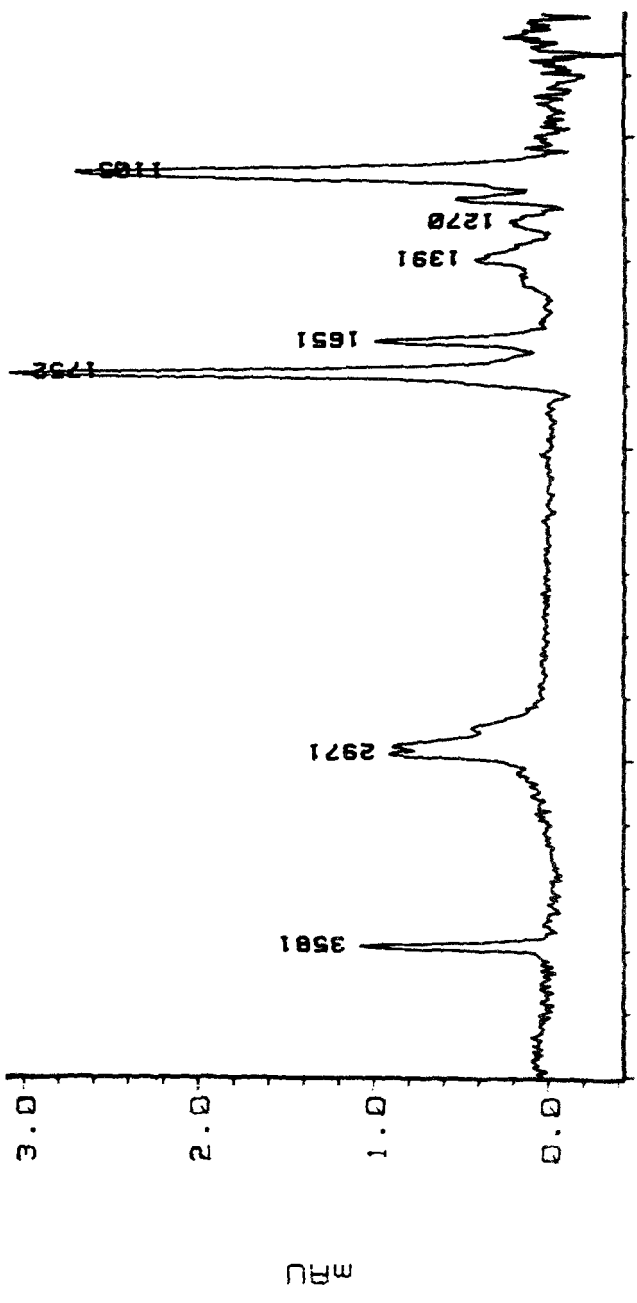


FIG. 5. Vapor-phase IR spectrum obtained from peak K in Figure 3. Note the sharp absorption at 3581 cm^{-1} due to the carboxyl-OH function, in contrast to the broad absorption seen in solution-phase IR spectra of acids.

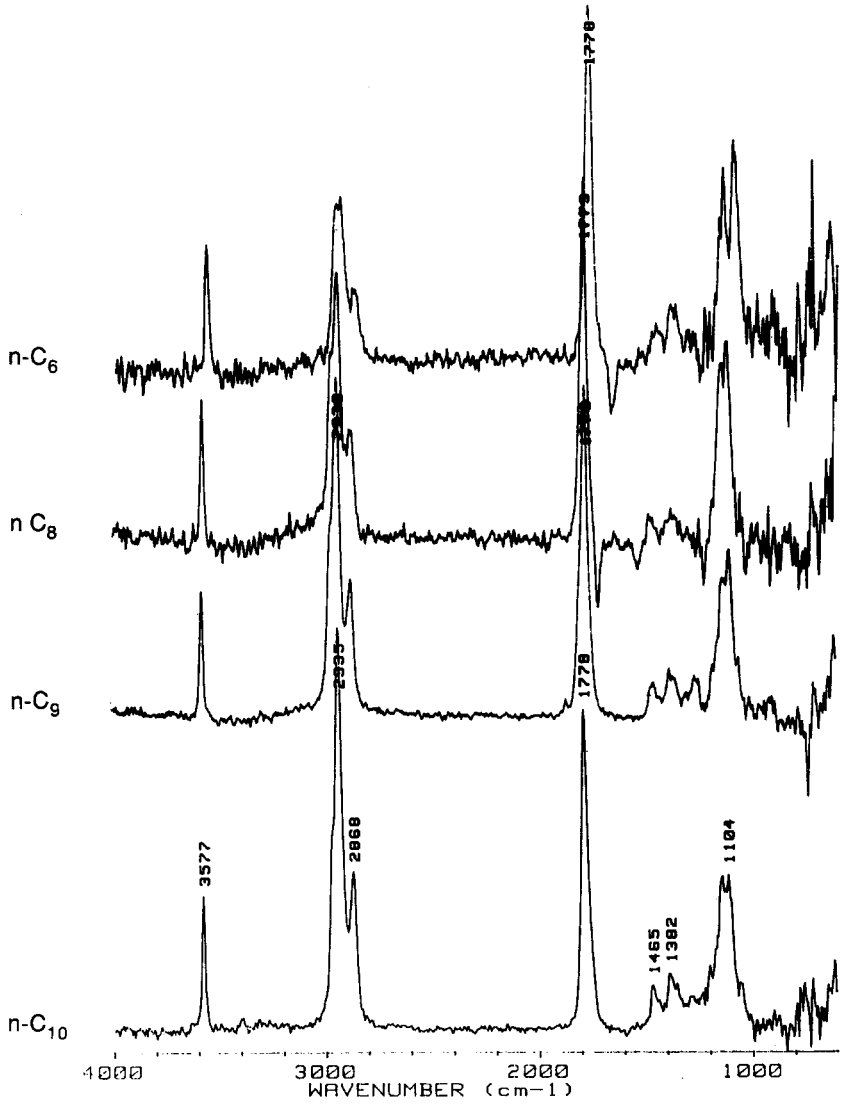


FIG. 6. Shows four overlapping FTIR spectra from normal hexanoic, octanoic, nonanoic, and decanoic acids. Note the sharp adsorption at 3577 cm^{-1} for the carboxyl OH stretching.

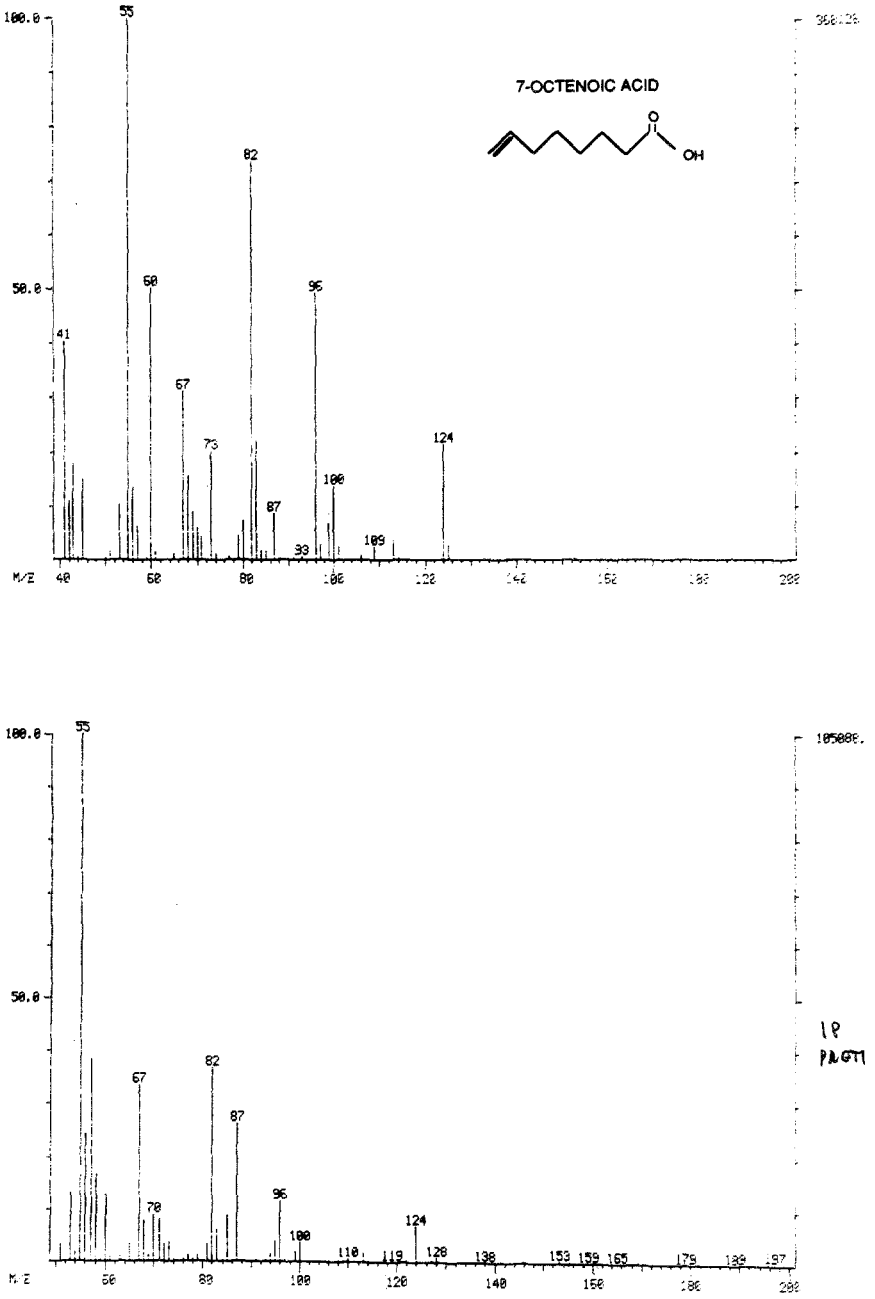


FIG. 7. Mass spectrum of synthetic 7-octenoic acid (top) compared with that of the proposed 7-octenoic acid found in the axillary extract centered at scan 2915 (bottom).

and mass spectra of 9-decenoic acid were identical to that of the unknown in the extract, the 8-nonenoic had an EEU of 15.86 (unknown = 16.04, Table 1). In addition, the mass spectrum of the unknown showed a larger proposed molecular ion and a different overall fragmentation pattern than that of the standard sample. The EEU of standard 10-undecenoic acid was 17.84 (unknown = 17.76); however, the mass spectrum of the unknown appeared to be "mixed," since it appears to elute with another compound. Consequently, it is difficult to conclude if 10-undecenoic acid is present since the mass spectra could not be compared directly in this sample.

Branched-Chain, Saturated Acids. The GC-MS data also showed two series of branched-chain acids. One homologous series consisted of 2-methyl acids beginning with 2-methyl hexanoic acid (peaks B, J, O, U, and Y). The mass spectra of the 2-methyl acids are characterized by ions at both *m/z* 74 ("McLafferty rearrangement" ion in α-methyl substituted acids), and *m/z* 87. Although these are the same ions that are present in the low mass end of methyl ester mass spectra, neither their relative retention times nor the molecular ion regions of their mass spectra correspond to methyl esters.

A second homologous series of acids possessed an ethyl group on the 4-position in the chain (peaks F, P, V, Aa, and Ee in Figure 3). The 4-ethyl-octanoic acid in the series (peak V) has a "goatlike" smell and has been previously isolated from sebaceous gland secretions of mature male goats (Sugiyama et al., 1981). In addition, this compound has been studied because of its low odor threshold (Boelens et al., 1983). This series of acids appears to be characterized by ions at M^+-29 and M^+-59, which in 4-ethyloctanoic acid ("goat acid") are seen at *m/z* 143 and 113, respectively (Figure 8).

γ-Lactones. Trace quantities of a series of C_5—C_{10} γ-lactones were found from scan numbers 1500–3000 by examining the mass chromatogram of *m/z* 85 (Labows et al., 1979a). Except for C_5 γ-lactone, all the other γ-lactones show a base peak at *m/z* 85, which is the characteristic peak of γ-lactone. This series of compounds has been reported previously to be formed by *Pityospirum ovale* isolated from the scalp (Labows et al., 1979a).

Analysis Using a Nonpolar Column. To further examine the axillary odor components, we chromatographed the mixture on a nonpolar column with and without derivatization. The analysis without derivatization did not reveal any new components but did show that (*Z*)- and (*E*)-3-methyl-2-hexenoic acids as well as many of the other C_6—C_{11} acids shown in Table 1 elute within the first 15–18 min. This would explain the presence of the strong axillary odors shown eluting in the smell chromatogram of the nonpolar column in Figure 1 (top). In addition, this analysis also showed the presence of both the volatile steroids (androstenone and androstenol) and the pyrolysis products of the two steroid sulfates (androsterone sulfate and dehydroepiandrosterone sulfate) described in

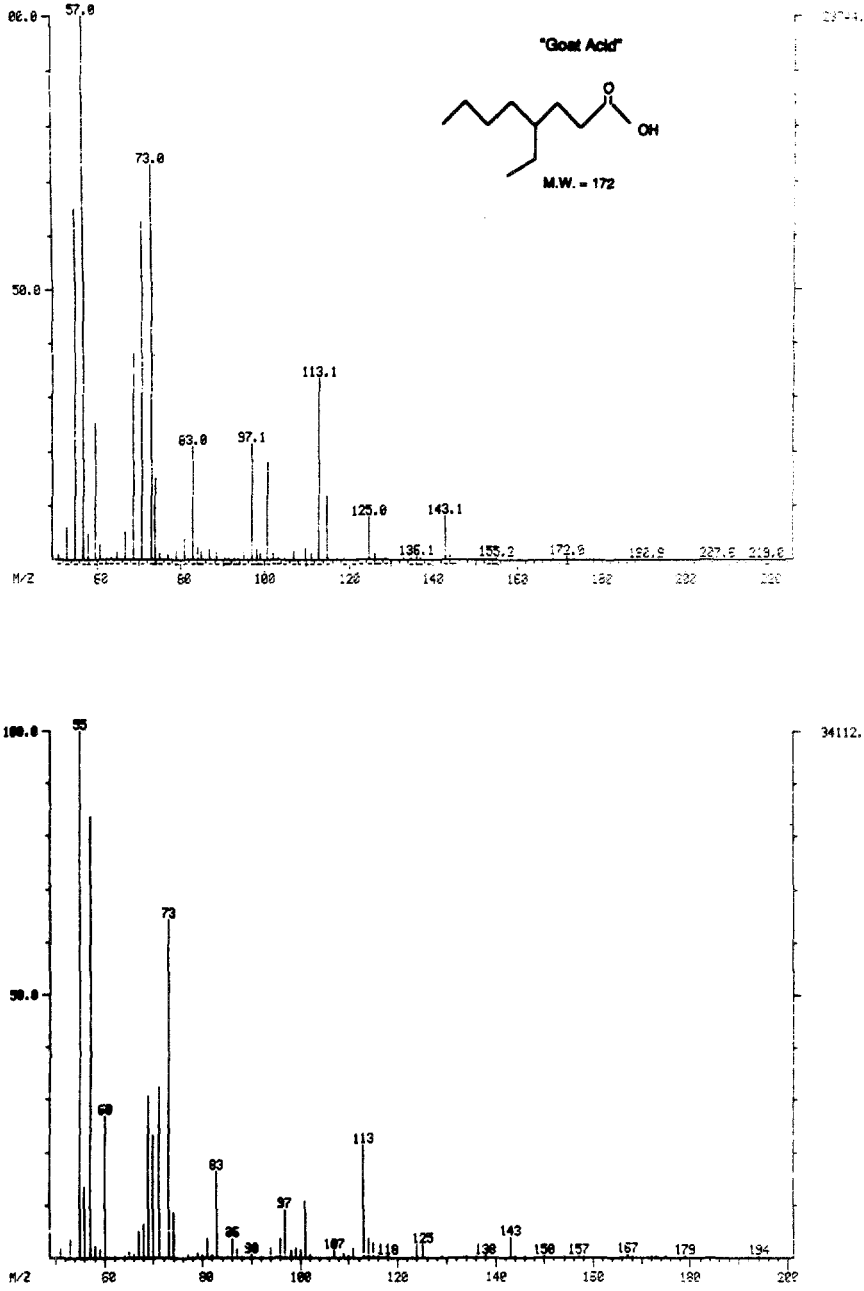


FIG. 8. Mass spectrum of commercially available 4-ethyloctanoic acid ("goat acid"; top) compared with that of the proposed 4-ethyloctanoic acid found in the axillary extract centered at scan 3062 (bottom).

previous studies from our laboratory (Labows et al., 1979b). These eluted at the predicted retention times, as shown in Figure 1.

Analysis of the trimethylsilyl derivatives revealed a variety of components not seen in the earlier analysis; however, a majority of the compounds are not contributors to the odors since they are higher-molecular-weight (C_{12}—C_{18}), straight-chain saturated, branched, and unsaturated acids. Several aromatic acidic compounds were identified including benzoic, phenylacetic, *o*-methylbenzoic and phenylpropanoic acids, and *p*-hydroxybenzaldehyde. The largest of these components appears to be *p*-hydroxybenzaldehyde; however, it is relatively odorless and only phenylacetic acid appears to lend some odor quality to the axillary odor.

DISCUSSION

The results obtained from this study suggest that volatile C_6—C_{11} straight-chain, branched, and unsaturated acids are the major contributors to underarm odor. Previous investigations of axillary secretions have not reported such a complex mixture of acids nor have these compounds been suggested as important axillary odorants (Labows, 1988; Gower, 1989). Those compounds with branching or unsaturation seem to have a high odor impact. A major contributor to the axillary odor is (*E*)-3-methyl-2-hexenoic acid; it is both unsaturated and branched. The *Z* isomer, which is present at one-tenth the concentration of the *E* isomer, also has a high odor impact. However, its odor quality is more like aliphatic acid.

(*E*)-3-Methyl-2-hexenoic acid was once thought to be the odor that characterized patients suffering from schizophrenia (Smith et al., 1969). Further studies later showed the compound to be present in normal patients also (Gordon et al., 1973). These previous studies employed whole-body sweat collected from patients placed in plastic bags between two electric blankets; consequently, the specific body site(s) for production of this malodorous compound could not have been correctly determined. Further, the *Z* isomer was not reported in these past studies of whole body sweat.

Mass spectrometric evidence for the presence of the volatile steroids in the acidic fraction was seen using the nonpolar methyl silicone column. However, in view of the relatively large odor impact of the acids (smell chromatography experiment), the high prevalence of a specific anosmia to one of the steroids (androstenone) and the relatively (to the steroids) high concentration of the acid compounds, we do not feel that the steroids are important malodor contributors. Further, their retention times, as noted above, do not suggest that they elute at the time when the strongest axillary odors elute.

Labows (1988) recently suggested that organoleptic descriptions of the

axillary odors implied the presence of not only isovaleric acid but also medium-chain acids, such as 4-ethyloctanoic acid. The latter is present (see Table 1) in the acidic axillary extract and has a very low olfactory threshold (Boelens et al., 1983). No data are available concerning the olfactory threshold for (*E*)-3-methyl-2-hexenoic acid or many of the other acids identified in our study.

As noted in the results, the neutral and basic fractions possessed very little odor. An organoleptic panel (four persons) that evaluated the concentrated extract as well as its acid, base, and neutral fractions found that all the characteristic axillary odor was in the acid fraction.

Recent applications of the technique of smell chromatography to malodor problems in the food industry (Sevenants and Sanders, 1984; McGorrin et al., 1987) and odor threshold measurements (Marin et al., 1988) suggested it would be an ideal way to pursue the chromatographic retention times of the characteristic axillary odors. This proved to be correct. Its application here showed that several of the compounds present at relatively low concentrations (such as the terminally unsaturated acids) were important contributors to the odor. Since the smell chromatography experiment suggested that the region where 7-octenoic acid eluted contained an important odor, we carefully investigated each of the minor chromatographic components in the area to determine which might possess an axillary-like odor. The result was the identification of 7-octenoic acid and subsequently the other terminally unsaturated homologs noted in Table 1.

We noted that one of us (H.J.L.) could not perceive either of the 3-methyl-2-hexenoic acids. Consequently, we are currently examining how extensive this specific anosmia might be. When asked to describe the odor, people tested who can smell these acids gave as their most frequent replies: urinous, sweaty, burnt, woody, acrid, musty, and musky. Many of these descriptors also have been attached to the odor of volatile steroids (Ohloff et al., 1983).

In the study reported here, the axillary secretion extracts were combined from six male donors. We are currently collecting and evaluating data from individuals. It is believed that there will be quantitative differences in the composition of axillary from each donor. Therefore, a different group of the combined male donor axillary secretion extracts may give a different ratio of the characteristic odor components.

The low levels of γ-lactones seen in the analyses (Table 1) may be due to the metabolism of *Pityrosporum ovale*. These yeast organisms are found on human skin areas that contain large numbers of sebaceous glands and may be part of the normal resident axillary flora. These yeasts previously have been shown to produce γ-lactones in the presence of human sebum. The lactones have a pleasant odor (Labows et al., 1979a).

The research of Hurley and Shelly (1960) as well as Shehadeh and Kligman (1963) demonstrated that freshly produced apocrine secretion is odorless and

that the axillary odor arises from the action of cutaneous microorganisms upon apocrine secretion. Consequently, the precursors for the odor are contained within the apocrine secretion. Previous analyses of the secretion have shown that it contains steroid sulfates, cholesterol, lipids, and about 10% protein (Labows et al., 1979b, 1982). In addition, considerable speculation has focused on the formation of the volatile steroids from apocrine secretion precursors (Labows, 1988; Gower, 1989). However, since the compounds reported here were not known to be important malodor constituents, no speculation has appeared concerning their precursors. The structure of the characteristic odors suggests that the precursors for these compounds would almost certainly not be amino acid-like in composition due to the chain length and branching of the acids involved. In addition, since the odor is formed very quickly in both in vivo and in vitro experiments, it would suggest a simple bond cleavage to form the odor and not a complex bacterial catabolism from higher molecular components. Consequently, the apocrine secretion may contain the characteristic odor compounds esterified to higher-molecular-weight lipid components or perhaps even bound to the proteins in some fashion.

Acknowledgments—The authors wish to acknowledge the help of Mr. Steve Huhn of Nabisco Brands, Inc. (Schaeberle Technology Center, East Hanover, New Jersey) and Mr. Walter O. Ledig of International Flavors and Fragrances (Union Beach, New Jersey) for obtaining the FTIR spectra. We also thank: Mr. Michael Boruta of Sadtler Research Laboratories (a division of Bio-Rad Laboratories) for employing their PC-IR Search Software to compare our FTIR data to their IR-Vapor Phase Data Base; Mr. Ken McGinley of the Duhring Laboratories, Department of Dermatology, University of Pennsylvania, for screening axillary secretion donors for the correct microflora and odor production; and Mrs. Janice Blescia for typing this manuscript. NMR spectra of the chromatographically collected compounds were obtained with the help of Dr. George Furst, Director of the Analytical NMR Facility in the Department of Chemistry, University of Pennsylvania. This work was supported in part by a postdoctoral fellowship awarded to the Monell Chemical Senses Center by Takasago International Corporation.

REFERENCES

AMOORE, J., PELOSI, P., and FORRESTER, J. 1977. Specific anosmias to 5α-androst-16-en-3-one and pentadecalactone: The urinous and musky primary odors. *Chem. Senses Flavor* 2:401–425.

BIRD, S., and GOWER, D.B. 1980. Measurement of 5α-androst-16-en-3-one in human axillary secretions by radioimmunoassay. *J. Endocrinol.* 85:8–9.

BOELENS, H., HARING, H.G., and DE RIJKE, D. 1983. Threshold values of and human preferences for 4-ethyl octanoic and 3-methyl butanoic acid. *Perfum. Flavorist* 8(1):71–74.

BROOKSBANK, B.W.L. 1970. Labeling of steroids in axillary sweat after administration of ^{3}H-5-pregnenolone and ^{14}C-progesterone to a healthy male. *Experientia* 26:1012–1016.

BROOKSBANK, B.W.L., BROWN, R., and GUSTAFSSON, J.A. 1974. The detection of 5-α-androst-16-en-3α-ol in human male axillary sweat. *Experientia* 30:864–865.

BROWNLEE, R.G., and SILVERSTEIN, R.M. 1968. A micro-preparative gas chromatograph and a modified carbon skeleton determinator. *Anal. Chem.* 40:2077–2079.

BUDZIKIEWICZ, H., DJERASSI, C. and WILLIAM, D.H. 1967. Mass Spectrometry of Organic Compounds. Holden-Day, San Francisco. pp. 155–162; 214–218.

CLAUS, R., and ALSING, W. 1976. Occurrence of 5α-androst-16-en-one, a boar pheromone, in man and its relationship to testosterone. *J. Endocrinol.* 68:483–484.

CUTLER, W.B., PRETI, G., KRIEGER, A., HUGGINS, G.R., GARCIA, C.R., and LAWLEY, H.J. 1986. Human axillary secretions influence women's menstrual cycles: The role of donor extract from men. *Horm. Behav.* 20:463–473.

DRAVNIEKS, A. 1975. Evaluation of human body odors: Methods and interpretations. *J. Soc. Cosmet. Chem.* 26:551–571.

GORDON, S.G., SMITH, K., RABINOWITZ, J.L., and VAGELOS, P.R. 1973. Studies of *trans*-3-methyl-2-hexenoic acid in normal and schizophrenic humans. *J. Lipid Res.* 14:495–503.

GOWER, D. 1989. The significance of odorous steroids in axillary odour, pp. 47–75, *in* S. Van Toller and G.H. Dodd (eds.). Perfumery: The Psychology and Biology of Fragrance. Chapman and Hall, London.

HURLEY, J., and SHELLY, W. 1960. The Human Apocrine Gland in Health and Disease. Charles C Thomas, Springfield, Illinois.

KLIGMAN, A.M., LEYDEN, J.J., and MCGINLEY, K.J. 1976. Bacteriology of skin. *J. Invest. Dermatol.* 67:160–168.

LABOWS, J.N. 1979. Human odors: What can they tell us? *Perfum. Flavorist* 4:12–17.

LABOWS, J.N. 1988. Odor detection, generation, and etiology in the axilla, pp. 321–343, *in* C. Felgen, and K. Laden (eds.). Antiperspirants and Deodorants. Marcel-Dekker, New York.

LABOWS, J.N., and WYSOCKI, C.J. 1984. Individual differences in odor perception. *Perfum. Flavorist* 9:21–26.

LABOWS, J.N., MCGINLEY, K.J., LEYDEN, J.J., and WEBSTER, G.F. 1979a. Characteristic γ-lactone odor production of the genus *Pityrosporum*. *Appl. Environ. Microbiol.* 38(3):412–415.

LABOWS, J.N., PRETI, G., HOELZLE, E., LEYDEN, J., and KLIGMAN, A.M. 1979b. Steroid analysis of human apocrine secretion. *Steroids* 34:249–258.

LABOWS, J.N., MCGINLEY, K.J., and KLIGMAN, A.M. 1982. Perspectives on axillary odor. *J. Soc. Cosmet. Chem.* 34:193–202.

LEYDEN, J.J., MCGINLEY, K.J., HOELZLE, E., LABOWS, J.N., and KLIGMAN, A.M. 1981. The microbiology of the human axillae and its relation to axillary odors. *J. Invest. Dermatol.* 77:413–416.

MARIN, A.B., ACREE, T.E., and BARNARD, J. 1988. Variation in odor detection thresholds determined by charm analysis. *Chem. Senses* 13(3):435–444.

MCGORRIN, R.J., POFABL, T.R., and CROASMUN, W.R. 1987. Identification of the musty component from an off-odor packaging film. *Anal. Chem.* 59:1109A–1111A.

OHLOFF, G., MAURER, B., WINTER, B., and GIERSCH, W. 1983. Structural and configurational dependence of the sensory process in steroids. *Helv. Chim. Acta* 66(20):192–201.

PRETI, G., CUTLER, W.B., GARCIA, C.R., HUGGINS, G.R., and LAWLEY, H.J. 1986. Human axillary secretions influence women's menstrual cycles: The role of donor extracts of females. *Horm. Behav.* 20:474–482.

PRETI, G., CUTLER, W.B., CHRISTENSEN, C.M., LAWLEY, H., HUGGINS, G.R., and GARCIA, C.R. 1987. Human axillary extracts: Analysis of compounds from samples which influence menstrual timing. *J. Chem. Ecol.* 13(4):717–731.

SATO, K., LEIDAL, R., and SATO, F. 1987. Morphology and development of an apoeccrine sweat gland in human axillae. *Am. J. Physiol.* 252:166–180.

SEVENANTS, M.R., and SANDERS, R.A. 1984. Anatomy of an off-flavor investigation: The "medicinal" cake mix. *Anal. Chem.* 56:293A–295A.

SHEHADEH, N., and KLIGMAN, A.M. 1963. The bacteria responsible for apocrine odor, Part II. *J. Invest. Dermatol.* 41:1–5.

SMITH, K., THOMPSON, G.F., and KOSTER, H.D. 1969. Sweat in schizophrenic patients: Identification of the odorous substances. *Science* 166:398–399.

SUGIYAMA, T., SASADA, H., MASAKI, J. and YAMASHITA, K. 1981. Unusual fatty acids with specific odor from mature male goats. *J. Agric. Biol. Chem.* 45:2655-2658.

VAN DEN DOOL, H., and KRATZ, P.D. 1963. A generalization of the retention index system including linear programmed gas–liquid partition chromatography. *J. Chromatogr.* 11:463–471.

WADSWORTH, W.S., and EMMONS, W.D. 1961. The utility of phosphate carbanions in olefin synthesis. *J. Am. Chem. Soc.* 83:1733–1738.

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

Book Review

Phytochemical Ecology: Allelochemicals, Mycotoxins, and Insect Pheromones and Allomones. Chang-Hung Chou and George R. Waller (eds.). Taipei: Institute of Botany, Academia Sinica, 1989. 504 pp.

This book is the proceedings of the meeting on Phytochemical Ecology: Allelochemicals, Mycotoxins and Insect Pheromones and Allomones held in Taipei and Kenting, Taiwan, December 13–20, 1988. The order of the papers follows the sequence at the meeting, and all papers are included. The topics covered were allelopathy, plant–animal interactions, mycotoxins, and insect pheromones and allomones. This was an extremely broad range of subjects to be covered by 35 papers. The papers supposedly were arranged under eight major headings according to the contents. These included Frontiers of Phytochemical Ecology, Allelopathy in Natural and Agricultural Ecosystems, Biochemical and Physiological Basis of Allelochemicals, Allelochemicals in Soils and Rhizosphere, Allelochemical Actions of Fungal Metabolites, Allelochemicals in Weed Control, Mycotoxins, and Pheromones. Three to six papers were listed under each of the headings and the relationships between several of the papers and the headings were not clear. The inclusion of mycotoxins with the other topics seemed rather illogical, even though the subject is a very important one.

The wide range of topics does appear reasonable when one considers the overall goal of the symposium. That goal was to stimulate cooperative research projects in phytochemical ecology between scientists in Taiwan and other participating countries. Unfortunately, this resulted in a proceedings that has a very narrow treatment of most topics and decreased its value as a reference for scientists in specialized areas. In spite of these limitations, most of the papers are of excellent quality and many of them were presented by some of the top scientists in the world in the areas of presentation.

This reviewer finds it very difficult to summarize the papers presented in this book, because it cannot be done accurately by the major headings listed in the contents. Five chapters dealt primarily with the allelopathic potentials of selected plant species, chiefly in Taiwan, Korea, Australia, Mexico, and Florida in the United States. One chapter concerned interactive effects of allelochemicals and environmental stress, one involved allelopathy and agricultural sustainability, five covered the topic of allelochemicals in weed control, three dealt with the fate of allelochemicals in soils, one chapter concerned the use of *Pseudomonas cepacia* (K413) as a potential biofungicide, and four chapters

0098-0331/91/0700-1493$06.50/0

dealt primarily with the identifications of new and old allelochemicals. The 20 chapters listed to this point were obviously concerned primarily with allelopathy.

One chapter dealt in part with allelopathic interactions of algae and in part with the production by algae of chemicals toxic to fish, humans, and other animals. Six chapters were concerned with mycotoxins, six chapters with pheromones, one chapter with the control of insects by allelochemicals, and one with the abiotic synthesis of humic polymers in soil.

In addition to the technical chapters, the proceedings includes a preface, four opening addresses, an introduction, conclusions and recommendations, summary remarks, an author index and a subject index.

Elroy L. Rice
David Ross Boyd Professor Emeritus of Botany
Department of Botany and Microbiology
University of Oklahoma
Norman, Oklahoma 73019

Journal of Chemical Ecology, Vol. 17, No. 7, 1991

Book Review

CRS Handbook of Insect Pheromones and Sex Attractants. M.S. Mayer and J.R. McLaughlin. Boca Raton, Florida: CRC Press, 1991. 1083 pp. $295 (outside the U.S., $345).

The arrival of the *Handbook of Insect Pheromones and Sex Attractants* in our laboratory was akin to a mail delivery in Antarctica. Our minds were already fully occupied, but when the book arrived, we were immediately tempted to explore this informative compendium. With enthusiasm, and in considerable awe of this work, we set about to evaluate it. It soon became evident that this handbook is an invaluable research tool.

The handbook is divided into two parts. Part I, the major section, is entitled Insect Sex Pheromones, and begins with a list of Genera by Order and Family followed by a List of Species. The bulk of the informative text is comprised of many Entries in Order of Genera. Part I ends with three indexes, one to Footnoted Species, for which taxonomic clarification is given, the second to Common Names, and the third to Chemicals .

The smaller Part II deals with pheromone chemistry. It begins with a Pheromone Synthesis Table of Contents in which pertinent references are listed alphabetically under the IUPAC name of each compound. The book ends with two independent lists, in which alphabetically ordered references are grouped under two headings, General Analytical Methods and General Synthetic Methods.

Because most of the text of part I is arranged alphabetically by genera, readers who know the scientific names of the species on which they seek information will easily thumb through the pages until they find them. If one were interested, for example, in the genus *Phyllonorycter*, he or she would find on pages 705–708 entries for nine species, from *P. blancardella* to *P. watanabei*. Exploring *P. blancardella* further, one would find the generic synonym (*Lithocolletis*), the species author [(Fabricius)], the common name (spotted tentiform leafminer), and the order and family (Lepidoptera: Gracillariidae). Three headings follow the above taxonomic information: Field Attractant, indicating biological activity, but no proven function as a pheromone component for the one compound listed by IUPAC name below the heading; Biological Assay, under which there is a concise and useful description of field and electrophysiological assays done on this species; and References, under which are listed five pertinent publications.

0098-0331/91/0700-1495$06.50/0

For other species, the headings are justifiably different, yet descriptive, and the information is greater in extent. Thus, the headings for *Heliothis zea* are: Female Sex Pheromone Components, Isolation and Identification, Biological Assay, and References, and the text and references cover two full pages.

Despite the authors' obvious concern for accuracy, there are several errors or omissions with regard to taxonomy, spelling, and chemical accuracy. More seriously, many readers will be confused about the content of the book. While the title reads in part, Insect Pheromones, the preface indicates that the scope of the book is restricted to chemicals that have "sex pheromonal" or "mating" activity, including aggregants linked to reproduction. Yet the text includes questionable assignments, e.g., as Female Sex Pheromone Components for two multifunctional compounds produced by honeybee queens. The book would be even more useful if it had included all pheromones, avoiding difficult and arbitrary decisions as to whether a given compound was truly involved in reproduction. As it stands now, there are huge omissions; e.g., there is a single entry for the Formicidae, an obscure reference to one species being attracted to citronellol. Expansion to embrace a wider spectrum of behavioral activity is recommended for future editions, which it is hoped are being contemplated even now. It would also be helpful if there were an index to species, giving the pages on which they appeared. Finally, and unavoidably, the book refers only to literature published before 1988 and is thus already out of date.

Nevertheless, this volume will be an essential resident in all laboratories that conduct serious research on insect pheromones and should be acquired by all university and other libraries that have a scientific clientele. At a somewhat daunting price of $295 (U.S.) or $348 (U.S.) outside of the United States, one cannot expect too many individuals to purchase it. However, if I were about to graduate in insect chemical ecology, or if I had not received a review copy, I might already be strongly hinting that it would make a nice graduation or Christmas present.

John H. Borden
Centre for Pest Management
Department of Biological Sciences
Simon Fraser University
Burnaby, British Columbia V5A 1S6 Canada

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

ANNOUNCEMENT

SYMPOSIUM ON INSECT–PLANT RELATIONSHIPS

The 8th International symposium on Insect–Plant Relationships will be held on March 9–13, 1992, at Wageningen, The Netherlands.

Topics to be discussed will include host-plant selection, insect–plant communities, plant defenses, multitrophic interactions, genetics and evolution, and plant resistance.

In addition to invited papers, the program will include submitted oral and poster presentations. Proceedings of the meeting will be published before the end of the year.

First circular and organizational matters:

IAC, Section OCC
P.O. Box 88
6700 AB Wageningen, The Netherlands
Telephone +31-8370-90111, FAX +31-8370-18552

Further inquiries:

Dr. J.H. Visser
Research Institute for Plant Protection
P.O. Box 9060
6700 GW Wageningen, The Netherlands
Telephone +31-8370-76002, FAX +31-8370-10113

0098-0331/91/0800-1497$06.50/0

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

WHAT DEFENSE DOES *Schouwia purpurea* (CRUCIFERAE) HAVE AGAINST THE DESERT LOCUST?
Secondary compounds and nutritive value

SAID GHAOUT,[1] ALAIN LOUVEAUX,[2,*]
ANNE MARIE MAINGUET,[2] MAX DESCHAMPS,[3]
and YOUSSEF RAHAL[2]

[1]*Direction de la Protection des Végétaux*
B.P. 1308 Rabat, Morocco
[2]*Laboratoire de Biologie évolutive*
et Dynamique des Populations
Bât. 446 Université Paris-Sud
91405 Orsay Cedex, France
[3]*Station d'Amélioration des plantes*
Laboratoire du Colza INRA
Domaine de la Motte Vicomte 35650
Le Rheu, France

(Received November 26, 1990; accepted March 25, 1991)

Abstract—A field study of the food eaten by solitary desert locusts was carried out in a winter breeding area in Mauritania. The food eaten, determined by the plant epidermis found in fecal pellets, was compared to the plant's availability in the habitat. *Schouwia purpurea*, well represented in the diets, was dominant at the study site. Adults had a preference for *Tribulus terrester*. Growth and feeding on these two plants were compared. The high water content of *S. purpurea* leaves limited the dry matter eaten and slowed down growth. Glucosinolates were separated and quantified by gas chromatography. There are 132 μmol/g dry matter in green leaves. In multiple choice tests, with paper disks, glucosinolate extracts were phagostimulant at a low concentration (21 μmol/g dry matter) and repulsive at a higher one (214 μmol/g dry matter). Biting behavior on *S. purpurea* was recorded and analyzed on video. The importance of *Schouwia purpurea* in desert locust habitats and its defenses is discussed.

Key Words—Sahara, Mauritania, ecology, *Schistocerca gregaria*, Orthop-

*To whom correspondence should be addressed.

tera, Acrididae, *Schouwia purpurea*, Cruciferae, glucosinolates, defense, nutrition, *Tribulus terrester*.

INTRODUCTION

Schouwia purpurea is a typical Saharian crucifer that grows in few places, although it is sometimes found with a dense and impressive vegetative development in large areas. Most of the surveys on the desert locust report the presence of this crucifer in its habitats (Roffey and Popov, 1968). In spite of its importance, very few studies have been devoted to *S. purpurea* (Boulet, 1966; Hemming and Symmons, 1969). It is found mainly in Talak, Aïr, and Tibesti. It is also found in Egypt and Arabia and has been introduced recently in India (Bhaumik, 1983). In Mauritania *S. purpurea* grows only in Aftout Faïe and the southern part of Adrar.

Cruciferae contain secondary plant compounds such as glucosinolates. Since some are toxic for nonhost insects, they could be defensive substances (Erickson and Feeny, 1974). Glucosinolates are found in all species of the Cruciferae family and are present in seeds, roots, and leaves. Their types and concentrations are taxon specific (Josefsson, 1972; Cole, 1976). Glucosinolates biosynthesis depends on the environment and can be enhanced by physiological stress such as low temperatures (MacLeod, 1976).

Selective feeders on Cruciferae have evolved detoxifying ability (Slansky and Feeny, 1977). The desert locust, known to be a generalist, does not refuse *S. purpurea* and is currently reared on cabbage. Therefore, is *Schistocerca gregaria* adapted to *S. purpurea?*

During its migrations throughout the Sahara, the desert locust, although very mobile, has to find patches of vegetation to feed on. At the same time, successful reproduction depends on locating the right food plants in the right place for oviposition. While there are many data on desert locust flight and swarm displacements, studies on solitary populations are rare. Information is still needed in the field on what makes reproduction a success in Saharian habitats.

Food plant quality is an important feature, and the following studies analyze what occurs with the most conspicuous annual forb encountered by the desert locust in its winter breeding area in Mauritania.

Study Site. The investigations lasted two successive years (October–December 1987 and August–December 1988) in Aftout Faïe, 250 km from Nouakchott (18°50N, 13°55W) (Ghaout, 1990). The El Gourarya site is a wadi, 3 km long, which is flooded during the rainy season. The bottom part of the valley is obstructed by live sand dunes, where water accumulates. Humidity persists for many months. Wells 0.5–3 m deep contained water for three months during the 1987 winter. The soil is an alluvial clay with gravel on the surface.

This wadi was a pasture of annual forbs of about 100 hectares in 1987 and was reduced to 3 hectares in 1988. The extent of the pasture depends on seasonal rains, but vegetation is always scanty with a sparse ground cover. *Schouwia purpurea*, *Tribulus terrester*, *Boerhavia repens*, and *Colocynthis vulgaris* form a plant community typical of desert locust habitat (Table 1) (Ozenda, 1983; Popov et al., 1984).

Schouwia purpurea–Desert Locust Association. In West Africa, *Schistocerca gregaria* populations move north from the Sahelian zone to the Sahara at the same time Inter-Tropical Convergence Zone (ITCZ) is retreating south. Breeding in *Schouwia* and *Tribulus* biotopes occurs in September, and the larvae develop in these habitats during the winter season. They are known to be selectively chosen by locusts to breed and are monitored by locust-control organizations (Popov et al., 1984).

S. purpurea is the only annual in the Sahara that remains green for more than four months and even up to seven months during a rainy winter. *S. purpurea* requires large quantities of water and needs a clay soil. This explains the

TABLE 1. VEGETATION AT EL GOURARYA SITE ON NOVEMBER 1, 1987[a]

		Frequency	Cover (%)	Availability (%)
Recorded on strip transect				
Cruciferae	*Schouwia purpurea* (Forsk.)	63	6.0	73.2
Zygophyllaceae	*Tribulus terrester* L.	2	+	
	Fagonia olivieri Boiss.	3	+	
Nyctaginaceae	*Boerhavia repens* L.	28	2.1	25.6
Cucurbitaceae	*Colocynthis vulgaris* (L.)	2	0.1	1.2
Cyperaceae	*Cyperus conglomeratus* Rottb.	1	+	
Recorded on study site				
Poaceae	*Aristida* sp. L.		+	
Zygophyllaceae	*Seetzenia africana* R.Br.		+	
Boraginaceae	*Heliotropium undulatum* Vahl.		+	
Papilionaceae	*Indigofera* sp. L.		+	
Trees				
Capparidaceae	*Capparis decidua* (Forsk.)		+	
	Maerua crassifolia Forsk.		+	
Mimosaceae	*Acacia raddiana* Savi.		+	
Asclepiadaceae	*Calotropis procera* Ait.		+	

[a] Strip transect was 1 m wide and 100 m long. On each square meter plant species occurrence was recorded and each plant surface was measured. +, rare plants, only present, were searched for on all the study sites.

very limited areas where *S. purpurea* can grow (Hemming and Symmons, 1969). It is worth noting that the eggs of the desert locust, which are also very demanding of water, develop well in such a soil.

T. terrester grows more quickly than *S. purpurea* and requires sandy soil. It flowers one month after rain and provides food and shelter to young larvae early in the breeding season. This annual plant does not last long, however.

These two plants are often associated, each colonizing a different part of the soil. Typically, *T. terrester* is found on live sand at the surface of the clay, and *S. purpurea* has its roots growing in cracks. We studied a site where *S. purpurea* was dominant, but *T. terrester* was dominant in other places in the Aftout Faïe area.

METHODS AND MATERIALS

Field Work

Feeding preferences of solitary hoppers and adults were determined from the leaf epidermis remaining in the feces. In November and December, once a week about 10 hoppers and adults were caught at the study site, between 11 AM and noon. Each individual was kept without food in a clear plastic box until the next morning to collect feces, and returned to the study site. Fecal pellets were dried and stored in a bag until they could be brought back to the laboratory for examination. The method used gave the locusts enough time to complete their meal after an overnight rest; therefore the probability of obtaining individuals with a full alimentary tract was higher. The feces collected were what the insects had eaten in the morning. Determining diet from epidermis remaining in feces is not destructive, and a small number of solitary locusts could be followed in the same place from hatching to adulthood. The feces of 52 hoppers and 74 adults were individually examined between October and December 1987.

The remaining fragments found in the whole feces (10–20 pellets) of each individual were hydrated for 24 hr in water with a few drops of a detergent (Teepol). The plant material was shredded and the epidermis separated and treated for 30 sec in hypochlorite (15%). The epidermis was rinsed and dehydrated in ethanol. Seven mounted slides were made for each individual and examined with a microscope. A collection of epidermis was prepared for identification purposes by giving locusts known plants to feed on. Rejected plants and tree leaves were prepared from collection samples. In this particular case, the epidermis had to be loosened in boiling water. *Colocynthis vulgaris* is one of the plants that the desert locust refused to eat.

Diet determination and feeding preferences were studied where the hoppers hatched: that is, in a limited part of the site (1 hectare) narrowed by sand dunes. On November 1, the plant cover was estimated in this part of the study site

from a strip transect 1 m wide and 100 m long. Rare plants were sought for throughout the site (Table 1). Since plants were sparse and covered less than 10% of the ground, the length and width of each plant had to be measured to calculate the approximate area. Availability percentage give some indication of the probability for a locust to be in contact with a particular plant species. During November, the diet of the 24 hoppers and 36 adults caught was compared to the availability of various plant species.

The nutritive value of food plants was determined from rearings conducted in the field, under a tent roof protecting the insects from direct insolation. The air temperature and humidity conditions were measured in the shade. The mean temperature varied from 33.6°C in October to 25°C in December. First-instar hoppers caught at the study site had the typical green color of the solitary phase. Contacts were prevented by rearing the hoppers in individual transparent plastic boxes 21 × 15 × 8 cm. Experiments were limited to five males and five females, due to the scarcity of solitary hoppers at the study site. They received a monospecific diet of *S. purpurea* or *T. terrester* leaves renewed every day. All rearing began on the same day in order to compare development. The length of the hopper period was calculated from the day of second instar molt to imaginal molt. Food consumption and growth were calculated, starting on the day following the molt, for seven days during the fifth instar and 10 days during adulthood. Food consumption and ECI Waldbauer's indices were used in order to compare the nutritional value of *S. purpurea* and *T. terrester*.

$$\text{Efficiency of conversion of ingested food (ECI)} = \frac{\text{weight gained}}{\text{weight ingested}} \times 100$$

In these field experiments, the dry weights were obtained by an infrared thermocontrol balance which desiccated the plant material at 90°C until its weight was stable.

Preparation of Plants for Chemical Analysis. S. purpurea and *T. terrester* were harvested by hand on October 10, 1988. Senescent *S. purpurea* leaves were harvested specifically in early December to establish a comparison with the green leaves collected in October. Plant material was air dried in a shady place, at a maximum air temperature of 43°C, and stored in plastic bags. Brought back to the laboratory in January 1989, they were freeze-dried, milled, and stored in plastic bags at −40°C until analyzed. Feces collected from experimental locusts fed with one food plant were dried, milled, and stored in the same way.

Laboratory Work

Extraction and Chemical Analysis of Nutrients. Kjeldahl nitrogen determination was used for the nitrogen content of leaves and feces (six replicates each). It was measured on a photocolorimeter with a Nessler reagent according

to Strauch's protocol (1965). Cellulose was extracted from plant material (two replicates) with NaOH, HCl, sodium hypochlorite, and hydrogen peroxide as an oxidizing agent according to Jenkins (1930). In the feces, uric acid and allantoic acid were determined apart (four replicates) in order to quantify excreted nitrogen (Mainguet and Le Berre, 1973). The assimilated part of nitrogen was measured as the difference between nitrogen ingested and nitrogen rejected minus nitrogen excreted. The enzymatic breakdown of uric acid by uricase was determined on a photocolorimeter and that of allantoic acid according to the Schryver-Fosse phenylhydrazine method (Fosse and Brunel, 1929; Razet, 1961).

Extraction and Analysis of Mustard Oil Glucosides. Glucosinolates tested for their antifeedant properties were isolated from 100 g of dried green *S. purpurea* leaves, following Nayar and Thorsteinson's (1963) method. Mustard oil glucosides were extracted with 70% ethanol in a Soxhlet apparatus for 24 hr. The brownish syrupy concentrate was chromatographed on a neutral alumina column, then on a cellulose column to purify the extract.

Glucosinolate analyses were performed on the leaves and seeds of *S. purpurea* and also on feces. Paper filter disks prepared for multiple choice tests, with the purified extract, were analyzed in order to determine the quantity of glucosinolates deposited. All these analyses were performed at the INRA laboratory of Le Rheu. Individual glucosinolates were separated and quantified by gas chromatography. Sinigrin (allyl glucosinolate) was used as an internal standard. Derivatization with silylating reagents [N-methyl-N-trimethylsilylheptafluorobutyramide (MSHFBA) and trimethylchlorosilane (TMCS)] was carried out according to Thies (1976, 1977, 1979) and Heaney and Fenwick (1980). The volatilized glycosides were separated using a capillary column (25 m × 0.32 mm ID), packed with 1% OV-1 on 100% methyl. The oven temperature was increased by 10°C/min from 185°C to 280°C. The injection port temperature was 280°C and the detector temperature was 290°C. Hydrogen was used as a carrier gas.

Behavioral Tests. Some mustard oil glucoside extracts from *S. purpurea* leaves were tested in the laboratory for their antifeedant effects on 3-day-old fifth-instar nymphs. Paper filter disks impregnated or not with glucosinolates were given in a multiple choice test, to six females starved for 3–5 hr. The test was run in a clear plastic box (11 liters) kept at 33°C, heated and lighted by electric bulbs. Disks (5.5 cm diameter) impregnated or not with the extract were presented pinned on a cork. The test ended when half a disk had been eaten. The remaining area of each disk was measured with an electronic area measurer and the amount eaten calculated. Three concentrations of mustard oil glucosides were tested and replicated three times each.

Biting behavior on fresh *S. purpurea* leaves was recorded in the laboratory with a Hitachi video camera fixed on a stereomicroscope. Three-day-old, fifth-

instar nymphs, ready to feed if given suitable food (a fresh cabbage leaf), were tested and their biting recorded for 1 min, then *S. purpurea* was given and biting recorded for 1 min. Finally a cabbage leaf was given again to confirm readiness. The biting sequences of 16 individuals were analyzed on a videotape recorder.

Statistics

The effect of the food plant treatment on the locusts' growth was determined by the statistical analysis ANOVA for three-factor designs and complete blocks. We used the statistical computer package SX STATISTIX.

RESULTS

Feeding Preferences of the Solitary Phase. Gut content analysis, from the feces collected, showed that the hoppers had rarely eaten more than one plant (12.5%), but 31.1% of the adults had eaten two plants and 8.1% had eaten three. The adults' diet is more diversified (8 plants eaten to 10 recorded) than that of hoppers (4/10) but it decreased in diversity from October to December (Table 2).

The frequency of plant species found in the feces was compared to their frequency on the strip transect. Since plant cover was determined on November 1, the comparison was limited to locusts caught during November. The hoppers' diet was not different from what was available on the strip transect. *S. purpurea* and *Boerhavia repens* (dominant) were found in feces in the same proportions (Figure 1).

On examination of adult feces, *T. terrester* was found more frequently than expected. This plant was recorded twice on the strip transect (Table 1) and 12 times in feces. Chi-square test comparing *T. terrester* frequencies on a 2×2 contingency table is highly significant $\chi_1^2 = 28.4$. *Colocynthis vulgaris*, although a little more abundant than *T. terrester*, was never present in feces (Table 1, Figure 1). In fact *C. vulgaris* was definitely rejected by desert locusts when given to feed in rearings.

Nutritional Value. The water content (88% fresh weight), and nitrogen were higher in *S. purpurea* than in *T. terrester*, but the cellulose content was lower (15% dry weight) (Table 3). Senescent, fallen leaves were conserved to be eaten by locusts. They contained less secondary substances than green leaves but also less nitrogen (Tables 3 and 4).

At the end of larval development the hoppers, reared since their second instar on fresh *S. purpurea* leaves, were about three days late compared to those reared on *T. terrester*. The mean length of each instar, male and female, was regularly a little bit longer on *S. purpurea* than on *T. terrester;* the Wilcoxon

TABLE 2. NUMBER OF INDIVIDUALS THAT ATE A PLANT SPECIES[a]

Plant species	Instar I 11/3 (N = 8)	II 11/10 (N = 9)	III 11/21–24 (N = 11)	IV 12/5–11 (N = 11)	12/17–27 (N = 13)	10/20–30 (N = 20)	11/3–20 (N = 36)	12/1–20 (N = 18)
Schouwia purpurea (Forsk.)	5	6	7	11	12	16	29	18
Boerhavia repens L.	3	3	1	1	1	8	10	1
Tribulus terrester L.	0	1	0	0	1	9	12	0
Fagonia olivieri Boiss.	0	0	0	0	1	1	0	1
Cyperus conglomeratus Rottb.	0	0	0	0	0	2	0	0
Seetzenia africana R. Br.	0	0	0	0	0	0	1	0
Indigofera sp. L.	0	0	0	0	0	1	0	0
Heliotropium undulatum Vahl.	0	0	0	0	0	1	0	0
Aristida sp. L.	0	0	0	0	0	0	0	0
Colocynthis vulgaris (L.)	0	0	0	0	0	0	0	0

[a]Hoppers and adults caught between October 20 and December 20, 1987, at the El Gourarya site.

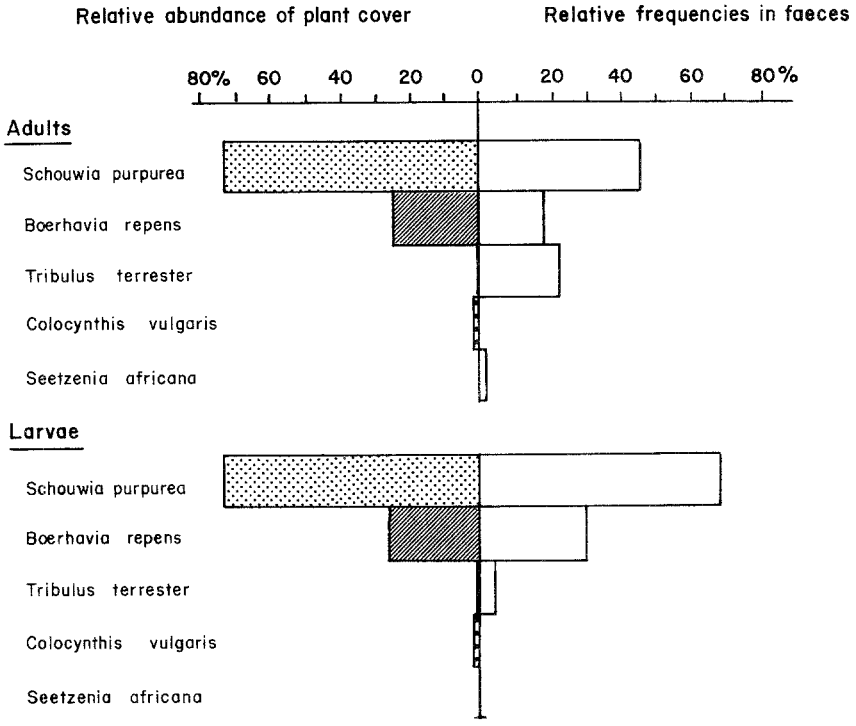


FIG. 1. Relative availability of plant species at the El Gouraraya site and their frequencies in feces. Plant availability is calculated according to the surface covered (Table 1). The frequency of plants eaten is based on examination of 24 hoppers and 36 adults caught in November 1987 (Table 2).

TABLE 3. WATER, NITROGEN, AND CELLULOSE CONTENT OF FOOD PLANTS; GREEN LEAVES COLLECTED IN OCTOBER AND SENESCENT LEAVES IN DECEMBER; MEAN PERCENTAGES GIVEN

	Water (% fresh weight)	Nitrogen (% dry weight)	Cellulose (% dry weight)
Schouwia purpurea			
Green leaves	88	4.3	14.8
Senescent leaves	< 10	2.2	17.3
Tribulus terrester	71	2.5	37.6

nonparametric test on paired mean lengths was significant ($P < 0.024$, Table 5).

During rearing, fresh food was not limited, and the daily quantity given was about the same for the two diets (Table 6). The effect of the food plant on consumption and growth, tested by ANOVA analysis (Table 6), showed that

TABLE 4. GLUCONISOLATES IN LEAVES AND SEEDS OF *Schouwia purpurea* (μmol/g dry matter)[a]

		Peaks						
	Replicate	GNA	GBN	PRO	A	B	40H GBS	Total
Retention time		1.79	1.98	2.18	4.64	4.72	4.83	
Green leaves	1	57.0	0.6	13.2	37.8		3.7	112.3
(Oct. 1988)	2	63.0	0.6	13.2	52.4		3.2	132.4
	3	72.4	0.2	13.8	64.9		0.6	151.9
Senescent leaves	1	5.3	0.2	3.0	15.7	9.2	4.0	37.4
(Dec. 1988)	2	5.7	0.2	6.8	13.5	6.3	2.9	35.4
	3	5.9		2.6	23.3	0.3	1.3	33.4
Seeds	1	3.1		86.5			0.8	90.4
Green leaf extract	1	14.0	0.1	3.2	4.7		0.03	22.03
on paper filter	2	12.4	0.1	3.6	4.4		0.07	20.57

[a]GNA = gluconapin, GBN = glucobrassicanapin, PRO = progoitrin, 40H GBS = 4-OH-Glucobrassicin.

TABLE 5. MEAN GROWTH LENGTH (DAYS $\pm$ SE) OF HOPPERS REARED ON MONOSPECIFIC DIET[a]

		Male				Female		
Instar	*N*	S.p.	*N*	T.t.	*N*	S.p.	*N*	T.t.
II	5	7.7 $\pm$ 0.3	5	7.2 $\pm$ 0.2	5	7.6 $\pm$ 0.5	5	7.5 $\pm$ 0.3
III	4	9.7 $\pm$ 0.8	4	8.6 $\pm$ 0.4	5	11.2 $\pm$ 0.9	5	8.2 $\pm$ 0.5
IV	4	9.7 $\pm$ 0.5	4	9.4 $\pm$ 0.2	5	9.6 $\pm$ 0.5	5	9.7 $\pm$ 1.0
V	4	14.7 $\pm$ 0.5	4	14.4 $\pm$ 0.2	5	15.2 $\pm$ 0.5	5	14.0 $\pm$ 0.4
Total length		42.0 $\pm$ 1.2		39.6 $\pm$ .2		43.6 $\pm$ 1.2		39.4 $\pm$ 1.1

[a]S.p. = *Schouwia purpurea*; T.t. = *Tribulus terrester*; *N* = number of individuals surviving.

TABLE 6. MEAN DAILY FOOD CONSUMPTION AND GROWTH RATE OF NYMPHS AND ADULTS RAISED ON TWO DIETS[a]

	Sex (*N*)	Fresh wt given	Dry wt		FWE	DWE	DWG	ECI
			Uneaten	Feces				
Nymphs								
S. purpurea	F(5)	5350.8	436.2	102.3	1716.0	205.9	22.2	10.8
	M(4)	4381.6	355.2	67.4	1421.0	170.5	18.8	11.0
T. terrester	F(5)	5977.2	1266.6	239.9	1609.6	466.8	33.6	7.2
	M(4)	5797.7	1388.5	150.9	1010.5	292.8	21.8	7.4
Adults								
S. purpurea	F(3)	4338.3	375.3	88.0	1210.3	145.2	7.6	5.2
	M(5)	4520.0	420.8	71.6	1013.4	121.6	5.1	4.2
T. terrester	F(4)	6003.7	1147.5	324.4	2046.8	593.5	19.4	3.3
	M(4)	4626.6	1098.9	128.0	837.8	242.8	6.2	2.6
				F	0.03	50.21	17.01	14.73
				P	0.86	0.006	0.05	0.002

[a]Mean weights (mg/day). F( ) = number of females, M( ) = number of males. FWE = mean fresh weight eaten. DWE = mean dry weight eaten. DWG = mean dry weight gain. ECI = efficiency of conversion of food ingested. ANOVA for plant, age, sex, and complete blocks.

the dry matter eaten on *S. purpurea* was about half that eaten on a *T. terrester* diet. This difference is highly significant ($F = 50.21$, $P < 0.01$). Daily weight increase was different for the two diets; hoppers and adults grew more on *T. terrester* than on *S. purpurea* ($F = 17.01$, $P = 0.05$), but the dry weight gain/dry weight consumed ratio (ECI) was better on *S. purpurea* ($F = 14.73$, $P < 0.01$). The effect of age was highly significant in ECI analysis ($F = 37.78$, $P < 0.01$). This supports earlier results (Hoekstra and Beenakkers, 1976).

These results indicate that *S. purpurea* food was digestible due to a low cellulose content, but it is difficult to discuss food assimilation; AD and ECD indices are more subject to unacceptable errors than ECI (Schmidt and Reese, 1986). There was no difference concerning the fresh weight of *S. purpurea* or *T. terrester* consumed ($F = 0.03$, $P > 0.80$, Table 6). This result, which is different from that of dry weight consumption, can be explained by the high water content of *S. purpurea* leaves.

Secondary Substances in S. purpurea. Gas chromatography indicated that six glucosinolates were present in *S. purpurea* leaves (Table 4). Four components were identified: gluconapin, glucobrassicanapin, progoitrin, and 4-OH-glucobrassicin. Two peaks (A and B) had nearly the same gas chromatographic retention times as indole glucosinolates. Analysis, without the internal standard, confirmed that sinigrin was present as traces in leaves. Senescent leaves have less glucosinolates, especially gluconapine, than green leaves. Seasonal variation of the glucosinolate contents in the different crucifer parts have been described by Feeny and Rosenberry (1982); there was always a marked decrease with age, sometimes associated with flowering (Rodman and Louda, 1984). The total glucosinolate content in green leaves (132 μmol/g dry matter) (Table 4) was very high compared to cultivated crucifers. In *Brassica napus*, a food well accepted by *S. gregaria* in laboratory experiments, it varied from 18 μmol/g dry matter in the leaf to 40 μmol/g dry matter in the stem (Bradshaw et al., 1984). In *S. purpurea* seeds, glucosinolates were not very important (90 μmol/g dry matter) compared to what was found in *B. napus* seeds, the highest content being 160–180 μmol/g for the Jet Neuf cultivar and the least about 10 μmol/g in the selected cultivar (Tapis d'or cv.). The presence of only trace amounts of glucosinolates in the feces could indicate that they were being either absorbed or extensively degraded. Derivatives such as isothiocyanates were not investigated.

Behavioral Tests. Locusts feeding on a plant cut off pieces of the leaf blade and chewed them with regular biting movements. On accepted food, this rhythm sometimes lasted for over 1 min without interruption. If there were any unacceptable portions, the mandibular movements stopped and another biting site was tried. A shortening of the biting sequences showed that an antifeedant had been detected. Tests on fifth-instar nymphs, ready to feed on a cabbage leaf

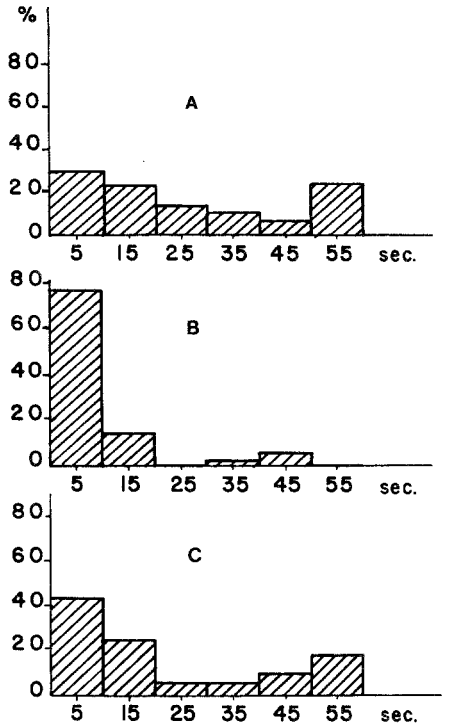


FIG. 2. Histogram of the mean durations of continuous biting on a 1-min feeding video record. Fifth-instar nymphs were successively given (A) cabbage leaf; (B) *Schouwia purpurea* leaf; (C) cabbage leaf.

showed that the biting sequences had various lengths; 50% were over 20 sec (Figure 2). If the same individual was given a *S. purpurea* leaf immediately after, biting sequences were then shortened, 80% being 10 sec or less.

In order to test if the glucosinolates contained in the leaves should be considered as antifeedants, a multiple-choice test on paper filter disks impregnated with a glucosinolate extract was compared to sucrose and paper controls (Table 7). The extract was obtained from *S. purpurea* green leaves collected in the field. The concentration of glucosinolates had an effect on the percentage of disks eaten. ANOVA analysis was significant on paper disks impregnated with the extract and on paper disks impregnated with the extract plus sucrose. At a low concentration (2 μmol/g dry matter), glucosinolates were phagostimulants, but at a higher level (214 μmol/g dry matter) they were dissuadants and consumption shifted to the control disks. This concentration was only twice that found in *S. purpurea* leaves.

TABLE 7. MEAN PERCENTAGES OF PAPER FILTER DISKS EATEN IN MULTIPLE-CHOICE TEST[a]

Disks impregnated	Doses			F	P
	A	B	C		
Extract alone	31.9	10.8	5.8	7.90	0.021
Extract + sucrose 10%	30.6	61.9	7.7	11.52	0.009
Sucrose 10%	23.7	18.0	66.3	6.38	0.033
Paper filter alone	13.9	9.2	20.2	0.81	0.49

[a]Three replicates for each concentration of glucosinolates. B = initial extract, 21.4 μmol glucosinolates/g paper filter. A = initial extract diluted ten times. C = initial extract concentrated ten times. One-way ANOVA analysis on arcsin transformation of percentages.

DISCUSSION

Desert locusts have no true preference for *Schouwia purpurea*. Since solitary hoppers are less active than adults, they usually feed mainly on plants used for shelter. Field observations indicate that young hoppers were effectively standing on *S. purpurea* leaves. Solitary hoppers are less active than gregarious ones, especially in dense cover (Uvarov, 1977). They rest for long periods on vegetation, basking or sheltering, and are more likely to feed where they stay. In the diet observed, *S. purpurea* represents no more than 70% of the plants eaten, and adults complete their diet with various dicotyledons. Adults fly easily, and it is impossible to determine that they have eaten their meal where they were captured; this is shown by the rare plants eaten and not recorded on the transect. *T. terrester* was rare all over the study site, and this plant was consumed more than it should be. There is certainly a preference for it.

For locusts reared on one plant with no choice, *S. purpurea* is not the best food, compared to *T. terrester*. Positive nutritional factors, such as a high nitrogen content and not too much cellulose are counterbalanced by factors that reduce consumption such as a high water content. Too much water is known to be unfavorable to the desert locust (Lewis and Bernays, 1985), and the amount of water ingested with fresh *S. purpurea* leaves probably limits the volume of food intake in the foregut by proprioceptive feedback control.

In the field, during the 1988 campaign, gregarious adults were observed eating dried *S. purpurea* leaves, although green ones were plentiful. This could be a way of regulating water intake and enhancing growth. Laboratory observations on the feeding behavior of the desert locust (Lewis and Bernays, 1985) indicate a preference for food with various water contents. Growth is better if the locust can regulate the proportion of water and dry matter intake.

A preference for food with a low concentration of glucosinolates, especially gluconapine and progoitrine, that decrease in senescent leaves could be the reason why adults were seen eating dead fallen *S. purpurea* leaves. *Melanoplus sanguinipes* reduces feeding when glucosinolate extracts, which contain progoitrine and gluconapine, are added to its diet (Pawlowski et al., 1968). Our tests showed that glucosinolates were phagostimulant at low concentrations but deterrent at high ones, even given with sucrose in multiple-choice tests. In our laboratory experiments, the desert locust had short biting sequences on green *S. purpurea* leaves. The enzymatic hydrolysis of glucosinolates when leaves are crushed yields glucose, sulfate, and mustard oils, which are volatile allyl and butenyl isothiocyanates, responsible for the strong-smelling odors; this could induce a chemical barrier when present in high concentrations, as in *S. purpurea*.

The four glucosinolates found are known to be toxic constituents for vertebrates since they are degraded by myrosinase. They do not seem to be toxic for locusts since they can be fed throughout their development.

A commercially available cabbage glucoside, sinigrin, is a phagostimulant for the desert locust (Le Berre and Tira, 1977) but sinigrin has been found to be nearly absent in *Schouwia purpurea*, the native crucifer, and therefore probably does not play a significant part in the insect–plant relationship.

Our results indicate that *S. gregaria* is relatively well adapted to its host plant but that *S. purpurea* is mainly interesting for its water content and the shelter it provides when the locust is able to diversify its diet with various other food plants. Under desert conditions, the persistence of vegetation is important to maintain the locust for several months in its habitat long enough to allow reproduction. Popov et al. (1984), describing *S. gregaria* biotopes in Tamesna, indicated that the mixed vegetation *S. purpurea–T. terrester* is more likely to provide food and shelter, in the long run, than a monospecific vegetation. The complementarity of these two plants is also suggested in our results and should be more specifically investigated.

Acknowledgments—We would like to thank Y. Gillon of ORSTOM Paris for his helpful comments and J.F. Briane of the Lab. Biologie végétale B Orsay for his statistical analysis. Video recordings were realized in Pr Jallon's laboratory, CNRS Gif. S. Ghaout was supported by a FAO doctoral fellowship.

REFERENCES

BHAUMIK, G.H. 1983. On the genus *Schouwia* DC. (Brassicaceae) in India. *J. Econ. Tax. Bot.* 4:291–294.

BOULET, R. 1966. Observations pédologiques dans le Tamesna oriental (Niger). Relations sol-végétation. ORSTOM, Dakar. 67 pp.

BRADSHAW, J.E., HEANEY, R.K., MACFARLANE SMITH, W.H., GOWERS, S., GEMMELL, D.L., and

FENWICK, G.R. 1984. The glucosinolate content of some fodder Brassicas. *J. Sci. Food Agric.* 35:977-981.

COLE, R.A. 1976. Isothiocyanates, nitriles and thiocyanates as products of autolysis of glucosinolates in cruciferae. *Phytochemistry* 15:759-762.

ERICKSON, J.M., and FEENY, P.P. 1974. Sinigrin a chemical barrier to the black swallowtail butterfly, *Papilio polyxenes*. *Ecology* 55:103-111.

FEENY, P., and ROSENBERRY, L. 1982. Seasonal variation in the glucosinolate content of North American *Brassica nigra* and *Dentaria* species. *Biochem. Syst. Ecol.* 10:23-32.

FOSSE, R., and BRUNEL, A. 1929. Sur le ferment producteur d'acide allantoïque par hydratation de l'allantoïne. Sa présence dans le règne animal. *C.R. Acad. Sci. Paris* 188:1067.

GHAOUT, S. 1990. Contribution à l'étude des ressources trophiques de *Schistocerca gregaria* (Forsk.) (Orthoptera, Acrididae) solitaire en Mauritanie occidentale et télédétection de ses biotopes par satellite. *Doctoral thesis*. Paris XI, Orsay. 203 pp.

HEANEY, R.K., and FENWICK, G.R. 1980. The analysis of glucosinolates in *Brassica* species using gas chromatography. Direct determination of the thiocyanate on precursors, glucobrassicin and neoglucobrassicin. *J. Sci. Food Agric.* 31:593-599.

HEMMING, C.F., and SYMMONS, P.M. 1969. The germination and growth of *Schouwia purpurea* (Forsk) and its role as habitat of the Desert Locust. Anti-Locust Bulletin 46. 38 pp.

HOEKSTRA, A., and BEENAKKERS, A.M.T. 1976. Consumption, digestion, and utilization of various grasses by fifth-larvae and adults of the migratory locust. *Entomol. Exp. Appl.* 19:130-138.

JENKINS, S.H. 1930. The determination of cellulose in straws. *Biochem. J.* 24:1428-1432.

JOSEFSSON, E. 1972. Variation of pattern and content of glucosinolates in seed of some cultivated cruciferae. *Z. Pflanzenzucht.* 68:113-123.

LE BERRE, J.R., and TIRA, R. 1977. Stimulation, par la Sinigrine, de la prise de nourriture du Criquet pélerin *Schistocerca gregaria* Forsk. (Orthoptera Acrididae). *C.R. Acad. Sci. Paris (Ser. D)* 284:573-576.

LEWIS, A.C., and BERNAYS, E.A. 1985. Feeding behavior: selection of both wet and dry food for increased growth in *Schistocerca gregaria* nymphs. *Entomol. Exp. Appl.* 37:105-112.

MACLEOD, A.J. 1976. Volatile flavour compounds of the Cruciferae, pp. 307-330, *in* J.G. Vaughan, A.J. MacLeod, and B.M.G. Jones (Editors) The Biology and Chemistry of the Cruciferae. Academic Press, New York.

MAINGUET, A.M., and LE BERRE, J.R. 1973. Nutrition du Criquet migrateur *Locusta migratoria* L. (Orthoptera Acrididae). II Excrétion azotée en fonction de divers aliments. *Arch. Sci. Physiol.* 27:91-113.

NAYAR, J.K., and THORSTEINSON, A.J. 1963. Further investigations into the chemical basis of insect-host plant relationships in an oligophagous insect, *Plutella maculipennis* (Curtis) (Lepidoptera: Plutellidae). *Can. J. Zool.* 4:923-929.

OZENDA, P. 1983. Flore du Sahara. Ed. CNRS Paris 622 pp.

POPOV, G.B., WOOD, T.G., and HARRIS, M.J. 1984. Insect pests of the Sahara, pp. 145-174, *in* J.L. Cloudsley-Thompson (ed.). Sahara Desert. Pergamon Press, New York.

PAWLOWSKI, S.H., RIEGERT, P.W., and KRZYMANSKI, J. 1968. Use of grasshoppers in bioassay of thioglucosides in rapeseed (*Brassica napus*). *Nature* 220:174-175.

RAZET, P. 1961. Recherches sur l'Uricolyse chez les insectes. Doctoral thesis. Rennes. 206 pp.

RODMAN, J.E., and LOUDA, S.M. 1984. Phenology of glucosinolate concentrations in roots, stems and leaves of cardamine cordifolia. *Biochem. Syst. Ecol.* 12:37-46.

ROFFEY, J., and POPOV, G.B. 1968. Environmental and behavioural processes in a desert locust outbreak. *Nature* 219:446-450.

SLANSKY, F., and FEENY, P. 1977. Stabilization of the rate of nitrogen accumulation by larvae of the cabbage butterfly on wild and cultivated food plants. *Ecol. Monographs* 47:209-228.

SCHMIDT, D.J., and REESE, J.C. 1986. Sources of error in nutritional index studies of insects on artificial diet. *J. Insect Physiol.* 32:193–198.

STRAUCH, L. 1965. Ultramicro-méthode pour le dosage de l'azote daus les substances biolopiques (translation). *Z. Klin. Chem.* 5:165–167.

THIES, W. 1976. Quantitative gas liquid chromatography of glucosinolates on a microliter scale. *Fette Seifen Anstrichm.* 78:231–234.

THIES, W. 1977. Analysis of glucosinolates in seeds of rapeseed (*Brassica napus* L.): Concentration of glucosinolates by ion exchange. *Z. Pflanzenzucht.* 79:331–339.

THIES, W. 1979. Detection and utilization of a glucosinolate sulphohydrolase in the edible snail, *Helix pomatia*. *Naturwissenschaften* 66:364–365.

UVAROV, B.P. 1977. Grasshoppers and Locusts. A Handbook of General Acridology, Vol. II C.O.P.R. Publ., London. 613 pp.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

IPSENOL: AN AGGREGATION PHEROMONE FOR *Ips latidens* (LECONTE) (COLEOPTERA: SCOLYTIDAE)

DANIEL R. MILLER,[1,*] JOHN H. BORDEN,[1] G.G.S. KING,[2] and KEITH N. SLESSOR[2]

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

(Received January 9, 1991; accepted March 25, 1991)

Abstract—Ipsenol was identified from the frass of male, but not female, *Ips latidens* from British Columbia, feeding in phloem tissue of lodgepole pine, *Pinus contorta* var. *latifolia*. The responses of *I. latidens* to sources of ipsenol and *cis*-verbenol were determined with multiple-funnel traps in stands of lodgepole pine in British Columbia. Ipsenol attracted both male and female *I. latidens*, verifying that it is a pheromone for this species. Male *I. latidens* showed a slight preference for (*S*)-(−)-ipsenol. *cis*-Verbenol was not produced by beetles of either sex and, in contrast to an earlier report, both enantiomers inhibited attraction to ipsenol-baited traps. The predators, *Enoclerus sphegeus* and *Thanasimus undatulus* (Cleridae), were attracted to traps baited with *cis*-verbenol and ipsenol.

Key Words—Pheromone, ipsenol, *cis*-verbenol, chirality, *Ips latidens*, Coleoptera, Scolytidae, predator, kairomone, *Enoclerus sphegeus*, *Thanasimus undatulus*, Cleridae.

INTRODUCTION

In British Columbia, the bark beetle, *Ips latidens* (LeConte) (Coleoptera: Scolytidae), feeds and breeds in the phloem tissue of lodgepole and ponderosa pines, *Pinus contorta* var. *latifolia* Engelmann and *P. ponderosa* Douglas ex Lawson

*To whom correspondence should be addressed at: Phero Tech Inc., 7572 Progress Way, RR #5, Delta, British Columbia, Canada V4G 1E9.

0098-0331/91/0800-1517$06.50/0

and Lawson, respectively (Bright, 1976; Furniss and Carolin, 1980; Wood, 1982). Like many other bark beetles, *I. latidens* has the potential to be a significant pest in stands of lodgepole pine, particularly in association with the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Scolytidae), or during periods of chronic drought (Furniss and Carolin, 1980; Miller and Borden, 1985).

Ipsenol (2-methyl-6-methylene-7-octen-4-ol) has been implicated as a pheromone for *I. latidens* in California. *Ips latidens* were caught, albeit in low numbers, on traps baited with either ipsenol or a mixture of ipsenol and *cis*-verbenol (*cis*-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol) (Wood et al., 1967). In Idaho, *I. latidens* were attracted to sources of racemic ipsenol; alone and in combination with bolts of ponderosa pine (Furniss and Livingston, 1979). However, the question of whether ipsenol is a pheromone for *I. latidens* is still unresolved since the production of ipsenol by *I. latidens* has not yet been determined.

Our objective was to determine the identity of pheromone(s) used by *I. latidens* in stands of lodgepole pine in British Columbia. Various scolytid species show behavioral responses to different enantiomers of ipsenol and the related chiral alcohol, ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) (Borden, 1982). Therefore, we tested the three following hypotheses: (1) one or both sexes of *I. latidens* would produce one or both enantiomers of ipsenol and/or one or both enantiomers of *cis*-verbenol; (2) *I. latidens* would be attracted to chiral ipsenol; and (3) *cis*-verbenol would act synergistically in increasing attraction of *I. latidens* to chiral ipsenol. Concurrent research by Seybold et al. (1991) tested similar hypotheses for *I. latidens* in California, breeding in ponderosa pine.

METHODS AND MATERIALS

In 1984, adult *I. latidens* were obtained from a 2-year-old colony that originated near the east gate of Manning Park, British Columbia. Using the gelatin-pill-capsule technique (Borden, 1967), 16 adult males and five adult females were restrained, individually, on noninfested bolts of lodgepole pine, collected near Princeton, British Columbia. Beetles were allowed to bore into the bark and feed for 24 hr. The frass of each individual was crushed in 150 μl of pentane. These extracts were analyzed by splitless capillary gas chromatography (Hewlett Packard HP 5890 using a 30-m $\times$ 0.25-mm-ID fused silica column). The identities and integrities of ipsenol and *cis*-verbenol were verified by mass spectrometry using splitless capillary gas chromatography (Hewlett Packard HP 5985B).

Racemic ipsenol (chemical purity, 98%) was obtained from Bedoukian

Research Inc. (Danbury, Connecticut). B.J. Johnston (Department of Chemistry, Simon Fraser University, Burnaby, British Columbia) supplied chiral ipsenols (optical purities, 96% (*S*)-(−) and 94% (*R*)-(+), respectively; chemical purities, 98%). (−)-β-Phellandrene was obtained from the SCM Corporation (Jacksonville, Florida).

Phero Tech Inc. (Vancouver, British Columbia) supplied polyethylene, bubble-cap devices containing the following chemicals: (1) racemic ipsenol (chemical purity, 98%) in solution with 1,3-butanediol; (2) 1,3-butanediol (chemical purity, >98%); (3) ethanol (chemical purity, 99%); and (4) chiral *cis*-verbenols (optical purities, 84% (*S*)-(−) and 94% (*R*)-(+), respectively; chemical purities, 98%).

β-Phellandrene was released from closed, polyethylene, microcentrifuge tubes (1.8 ml) (Evergreen Scientific, Los Angeles, California). The release rate was approximately 8 mg/day at 27–30°C (determined by weight loss). Ipsenol release devices consisted of either 10-cm lengths of C-flex tubing (ID = 1.6 mm; OD = 2.4 mm) (Concept Inc., Clearwater, Florida) filled with a solution of ipsenol in ethanol, or polyethylene, bubble-cap devices filled with a solution of ipsenol in 1,3-butanediol, and heat-pressure sealed. The release rates of ipsenol from these devices were approximately 0.6 and 0.2–0.3 mg/day, respectively, at 24°C (determined by collection of volatiles on Porapak-Q). 1,3-Butanediol was not released from either C-flex or bubble-cap lures. Ethanol release devices consisted of either 10-cm lengths of C-flex tubing or polyethylene bubble-caps, each filled with ethanol and heat-pressure sealed. The release rates of ethanol from these devices were approximately 10 and 6 mg/day, respectively, at 24°C (determined by weight loss). *cis*-Verbenol was released from polyethylene, bubble-cap devices at a rate of 3–6 mg/day at 27–30°C (determined by weight loss).

In all experiments, eight-unit, multiple-funnel traps (Lindgren, 1983) (Phero Tech) were set in mature stands of lodgepole pine near Princeton, British Columbia. Each trap was suspended such that the top funnel of each trap was 1.3–1.5 m above ground. No trap was within 2 m of any tree. Treatments were assigned randomly within replicates. Sexes of *I. latidens* captured in experiment 1 were determined by dissection and examination of genitalia. Sexes of *I. latidens* captured in other experiments were not determined due to insufficient numbers for most of the treatments. Sexes of other beetles caught in traps were not determined.

In experiments 1–3, replicate grids were placed at least 100 m apart, and traps were spaced 10–15 m apart within each replicate. The effect of chiral ipsenol was tested in experiment 1. Eleven replicates of six traps per replicate, were set in grids of 2 × 3, from May 23 to July 2, 1987. The treatments, using C-flex devices, were as follows: (1) blank control; (2) ethanol control; (3) racemic ipsenol (0.6 mg/day); (4) racemic ipsenol (1.2 mg/day); (5) (*S*)-(−)-

ipsenol (0.6 mg/day); and (6) (*R*)-(+)-ipsenol (0.6 mg/day). All ipsenol devices contained ethanol.

Experiment 2 tested the effects of ethanol and the interaction between ipsenol and ethanol. Seven replicates of four traps per replicate were set in grids of 2 × 2, from August 6 to 31, 1989. The treatments, using bubble-cap devices, were as follows: (1) 1,3-butanediol; (2) ethanol and 1,3-butanediol (as two separate devices); (3) racemic ipsenol; and (4) racemic ipsenol and ethanol (as two separate devices). All ipsenol devices contained 1,3-butanediol.

Experiment 3 tested for interaction between (*S*)-(−)-*cis*-verbenol and the combination of ipsenol and β-phellandrene. β-Phellandrene is used as a kairomone by *I. latidens* (Miller and Borden, 1990). Nine replicates of four traps per replicate were set in grids of 2 × 2, from May 21 to June 23, 1988. The treatments, using C-flex devices, were as follows: (1) ethanol control; (2) racemic ipsenol and β-phellandrene (as two separate devices); (3) (*S*)-(−)-*cis*-verbenol; and (4) the combination of racemic ipsenol, β-phellandrene, and (*S*)-(−)-*cis*-verbenol (as three separate devices). All ipsenol devices contained ethanol.

Experiment 4 tested for interaction between (*R*)-(+)-*cis*-verbenol and ipsenol. β-Phellandrene was not used due to lack of availability. Traps were placed 50 m apart in a single, large grid pattern measuring 200 × 400 m. Ten replicate blocks of four linearly consecutive traps per block were set along parallel trap lines, spaced 50 m apart, from June 21 to July 10, 1989. The treatments, using bubble-cap lures, were as follows: (1) 1,3-butanediol; (2) racemic ipsenol; (3) (*R*)-(+)-*cis*-verbenol; and (4) racemic ipsenol and (*R*)-(+)-*cis*-verbenol (as two separate devices). All ipsenol devices contained 1,3-butanediol.

The data were analyzed using the SAS statistical package version 5.0 (SAS Institute Inc., Cary, North Carolina). Trap catches of all species were transformed by $\ln(Y + 1)$ to remove heteroscedasticity. Sex ratio data for *I. latidens* were normalized by an arcsin transformation. Homoscedastic data were subjected to either one-, two-, or three-way analysis of variance (ANOVA). Evidence of synergy in the attraction of beetles, due to the interaction of multiple components, was determined by the interaction term in ANOVA. Two multiple contrasts were performed in experiment 1. Ryan-Einot-Gabriel-Welsch (REGW) multiple-range tests were used in experiments 2–4 when $P < 0.05$.

RESULTS AND DISCUSSION

Ipsenol is a pheromone for *Ips latidens*. It was found in the frass of 10 of 16 male *I. latidens* (estimated range, 10 ng to 1 mg), but not in the frass of any female. The chirality of ipsenol was not determined because we were unable to separate the acetyl lactate diastereomers (Slessor et al., 1985) of synthetic

racemic ipsenol by gas chromatography. *cis*-Verbenol was not found in any samples. Similar results were found by Seybold et al. (1991) for Californian *I. latidens*. The major monoterpene in the frass was β-phellandrene. β-Phellandrene is the major monoterpene in the phloem tissue of lodgepole pine (Mirov, 1961; Shrimpton, 1972, 1973) and is a kairomone for *I. latidens* (Miller and Borden, 1990).

In experiments 1 and 2, *I. latidens* were significantly attracted to ipsenol, with a slight preference for (*S*)-(−)-ipsenol (Figures 1A and 2). The results in

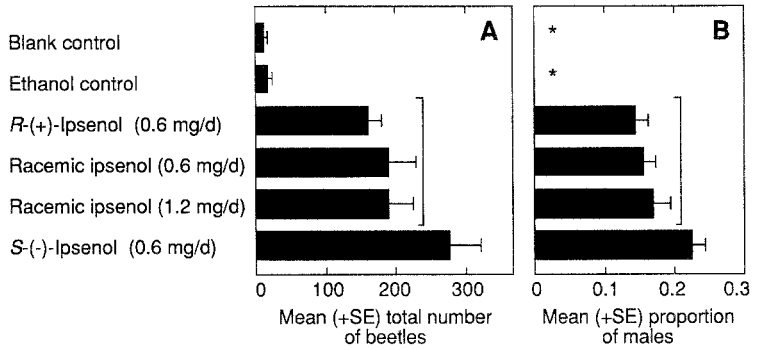


FIG. 1. The effect of chiral ipsenol on the number (A) and sex ratio (B) of *Ips latidens* responding to multiple-funnel traps near Princeton, British Columbia, in experiment 1 from May 23 to July 2, 1987 ($N = 11$). Mean numbers grouped by a line are significantly different from the blank and ethanol controls as well as (*S*)-(−)-ipsenol [multiple contrasts, $F(1,49)$, $P < 0.001$ and $P = 0.025$, respectively, on data transformed by $\ln(Y + 1)$]. Mean proportions of males grouped by a line are significantly different from (*S*)-(−)-ipsenol [multiple contrast, $F(1,30)$, $P = 0.008$, on data transformed by $\arcsin(Y)$]. Some treatments (*) had insufficient numbers for determinations of sex ratios.

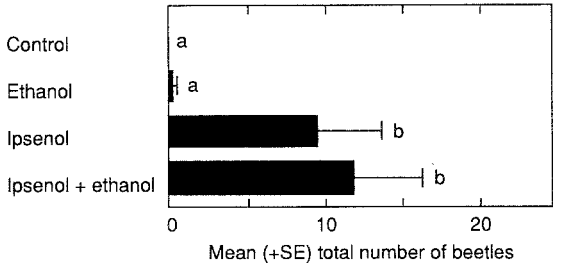


FIG. 2. The effect of ethanol and ipsenol on the attraction of *Ips latidens* to multiple-funnel traps near Princeton, British Columbia, in experiment 2 from August 6 to September 2, 1989 ($N = 7$). Means followed by the same letter are not significantly different at $P = 0.05$ [REGW multiple range test on data transformed by $\ln(Y + 1)$].

experiment 1 can be attributed solely to ipsenol, since ethanol alone was not attractive and there was no significant interaction between ethanol and ipsenol in experiment 2 [ANOVA, $F(1,24)$, $P = 0.441$ and $P = 0.989$, respectively]. The sex ratios of *I. latidens* captured in experiment 1 were affected by chirality [ANOVA, $F(3,30)$, $P = 0.048$]. Proportionally more males responded to (*S*)-(−)-ipsenol than to either racemic or (*R*)-(+)-ipsenol (Figure 1B). Our results agree with the field data of Wood et al. (1967) and Furniss and Livingston (1979) and recent laboratory data of Seybold et al. (1991).

In contrast to results from California (Wood et al., 1967), both enantiomers of *cis*-verbenol inhibited the response of *I. latidens* to sources of ipsenol (Figures 3 and 4). *cis*-Verbenol was not produced by male *I. latidens*. *cis*-Verbenol is produced by sympatric species of bark beetles such as *D. ponderosae* (Pierce et al., 1987; Libbey et al., 1985) and may act as a synomone

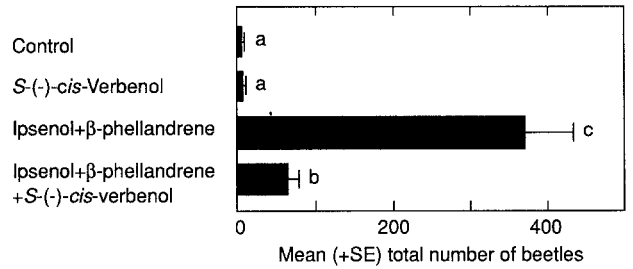


FIG. 3. The effect of (*S*)-(−)-*cis*-verbenol and the combination of ipsenol and β-phellandrene on the attraction of *Ips latidens* to multiple-funnel traps near Princeton, British Columbia, in experiment 3 from June 8 to 23, 1988 ($N = 9$). All treatments contained ethanol. Means followed by the same letter are not significantly different at $P = 0.05$ [REGW multiple range test on data transformed by $\ln(Y + 1)$].

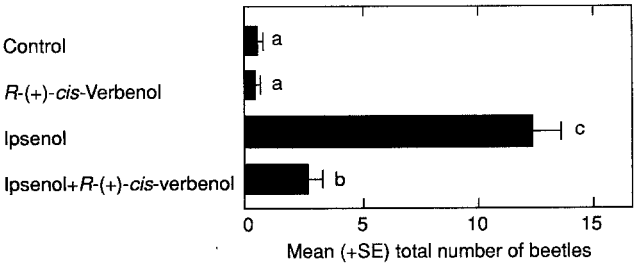


FIG. 4. The effect of (*R*)-(+)-*cis*-verbenol and ipsenol on the attraction of *Ips latidens* to multiple-funnel traps near Princeton, British Columbia, in experiment 4 from June 21 to July 10, 1989 ($N = 10$). Means followed by the same letter are not significantly different at $P = 0.05$ [REGW multiple range test on data transformed by $\ln(Y + 1)$].

(Nordlund and Lewis, 1976), facilitating resource partitioning and minimizing interspecific competition for phloem tissue. The role of synomones in cross-attraction and host partitioning has been demonstrated in bark beetle communities such as the loblolly pine, *P. taeda* L., community of southern pine beetles in the southern United States (Hedden et al., 1976; Vité et al., 1978; Dixon and Payne, 1979; Birch et al., 1980; Svihra et al., 1980; Paine et al., 1981; Watterson et al., 1982).

Sources of ipsenol and/or *cis*-verbenol and/or ethanol also were attractive to other species of bark beetles. In experiment 1, *Ips mexicanus* (Hopkins) preferred traps baited with ipsenol, regardless of chirality or release rate (Table 1). The treatment with the highest release rates of ipsenol and ethanol were preferred by *Hylurgops porosus* (LeConte) and a *Pityophthorus* Eichhoff species. Unlike the *Pityophthorus* species, *H. porosus* was attracted to ipsenol alone. *Hylastes longicollis* Swaine did not exhibit any preferences in experiment 1. However, in experiment 3, ipsenol with β-phellandrene was the preferred treatment for *H. longicollis* as well as for *I. mexicanus* and a *Pityophthorus* species (Table 2). *Hylurgops porosus* showed equal preference for the combinations of ipsenol and β-phellandrene, and ipsenol with β-phellandrene and (*S*)-(−)-*cis*-verbenol.

TABLE 1. EFFECTS OF CHIRAL IPSENOL ON ATTRACTION OF *Ips mexicanus, Hylastes longicollis, Hylurgops porosus*, AND A *Pityophthorus* sp. (SCOLYTIDAE) TO MULTIPLE-FUNNEL TRAPS NEAR PRINCETON, BRITISH COLUMBIA, IN EXPERIMENT 1, MAY 23 TO JULY 2, 1987 ($N = 11$)

Treatment	Mean (±SE) total number of beetles[a]			
	Ips mexicanus	*Hylastes longicollis*[b]	*Hylurgops porosus*	*Pityophthorus* species
Blank control	0.1 ± 0.1 a	6.3 ± 1.4	12.9 ± 4.2 a	5.7 ± 3.1 a
Ethanol	0.1 ± 0.1 a	8.7 ± 1.8	23.0 ± 5.3 bc	4.0 ± 1.9 a
Ethanol + (*R*)-(+)-ipsenol (0.6 mg/day)	2.8 ± 1.1 b	7.9 ± 1.2	25.3 ± 4.6 bc	7.4 ± 2.1 ab
Ethanol + racemic ipsenol (0.6 mg/day)	2.5 ± 0.7 b	6.3 ± 1.3	22.8 ± 5.1 bc	7.8 ± 1.8 ab
Ethanol + racemic ipsenol (1.2 mg/day)	2.4 ± 0.9 b	9.0 ± 1.7	31.7 ± 4.9 c	13.3 ± 3.7 b
Ethanol + (*S*)-(−)-ipsenol (0.6 mg/day)	2.5 ± 0.9 b	5.8 ± 1.4	19.2 ± 4.0 b	3.3 ± 0.8 a

[a] Means within a column followed by different letters are significantly different at $P = 0.05$ [REGW multiple range test on data transformed by $\ln(Y + 1)$].
[b] No significant differences among means [ANOVA, $F(5, 49)$, $P = 0.232$].

TABLE 2. EFFECTS OF (*S*)-(−)-*cis*-VERBENOL AND COMBINATION OF RACEMIC IPSENOL AND β-PHELLANDRENE ON ATTRACTION OF *Ips mexicanus, I. perturbatus, Hylastes longicolis, Hylurgops porosus,* A *Pityphthorus* SPECIES (SCOLYTIDAE), AND *Enoclerus sphegeus* (CLERIDAE), TO MULTIPLE-FUNNEL TRAPS NEAR PRINCETON, BRITISH COLUMBIA, IN EXPERIMENT 3, JUNE 8–23, 1988 ($N = 7$)

Teatment	Mean (± SE) total number of beetles[a]					
	Ips mexicanus	*Ips perturbatus*	*Hylastes longicollis*	*Hylurgops porosus*	*Pityophthorus* species	*Enoclerus sphegeus*
Ethanol	0.2 ± 0.2 a	0.8 ± 0.5 a	3.8 ± 0.6 a	9.0 ± 3.5 a	0.8 ± 0.3 a	0.7 ± 0.7 a
Ethanol + (*S*)-(−)-*cis*-verbenol	2.0 ± 0.6 b	1.4 ± 0.9 a	5.1 ± 1.8 a	8.6 ± 1.7 a	1.3 ± 0.4 a	5.3 ± 1.6 bc
Ethanol + ipsenol + β-phellandrene	18.9 ± 3.5 c	1.4 ± 0.8 a	11.4 ± 2.5 b	21.3 ± 3.0 b	11.1 ± 3.0 b	1.8 ± 0.7 ab
Ethanol + ipsenol + β-phellandrene + (*S*)-(−)-*cis*-verbenol	3.3 ± 0.5 b	1.9 ± 3.7 b	3.9 ± 1.2 a	14.6 ± 3.9 ab	2.1 ± 1.2 a	15.0 ± 5.1 c

[a]Means within a column followed by different letter are significantly different at $P = 0.05$ [REGW multiple-range tests on data transformed by $\ln(Y + 1)$].

Explanations for significant treatment differences in these species must be viewed as speculative. Responses by bark beetles suggest that ipsenol and/or *cis*-verbenol are used as either pheromones or synomones (Tables 1 and 2). However, there are no data on the phenology of pheromone production by any of these species. The low numbers caught in traps, relative to *I. latidens*, may be a consequence of four factors: (1) low population numbers; (2) missing pheromones; (3) other semiochemical functions; or (4) random chance.

The experimental areas were selected for abundance of *I. latidens*. We have no data on the abundance of the other species. However, *I. pini* (Say) and *Pityogenes knechteli* Swaine were very abundant in both years but neither was trapped. Ipsenol inhibits the response of *I. pini* to suitable hosts and to its own pheromone, ipsdienol (Birch and Light, 1977; Birch et al., 1977; Furniss and Livingston, 1979). It seems reasonable to hypothesize that ipsenol and/or *cis*-verbenol are also inhibitory to *P. knechteli*, or have no effect.

The preferred treatment for the clerid (Coleoptera), *Enoclerus sphegeus* F., in experiment 3 was the combination of ipsenol with ethanol, β-phellandrene and (*S*)-(−)-*cis*-verbenol (Table 2). The interaction between treatments had an additive, not synergistic, effect on the attraction of *E. sphegeus* [ANOVA, $F(1,24)$, $P = 0.822$]. In experiment 4, both *E. sphegeus* and *Thanasimus undatulus* Say (Cleridae) preferred the combination of ipsenol and (*R*)-(+)-*cis*-verbenol over all other treatments (Table 3). The interaction between ipsenol and (*R*)-(+)-*cis*-verbenol had a synergistic effect on the attraction of *E. sphegeus* [ANOVA, $F(1,36)$, $P = 0.001$] but an additive effect on the attraction of *T. undatulus* [ANOVA, $F(1,36)$, $P = 0.560$].

TABLE 3. EFFECTS OF (*R*)-(+)-*cis*-VERBENOL AND RACEMIC IPSENOL ON ATTRACTION OF *Enoclerus sphegeus* AND *Thanasimus undatulus* (CLERIDAE) TO MULTIPLE-FUNNEL TRAPS NEAR PRINCETON, BRITISH COLUMBIA, IN EXPERIMENT 4, JUNE 21 TO JULY 10, 1989 ($N = 10$)

Treatment	Mean (± SE) total number of beetles[a]	
	Enoclerus sphegeus	*Thanasimus undatulus*
Blank control	0.1 ± 0.1 a	0.1 ± 0.1 a
(*R*)-(+)-*cis*-Verbenol	0.2 ± 0.1 a	2.5 ± 1.0 b
Ipsenol	1.2 ± 0.3 b	2.3 ± 0.5 b
Ipsenol + (*R*)-(+)-*cis*-verbenol	5.9 ± 0.9 c	6.0 ± 1.1 c

[a]Means within a column followed by different letters are significantly different at $P = 0.05$ [REGW multiple range tests on data transformed by $\ln(Y + 1)$].

Responses by the clerids, *E. sphegeus* and *T. undatulus*, to sources of *cis*-verbenol and the combination of ipsenol and *cis*-verbenol (Tables 2 and 3) are consistent with other studies demonstrating the use of bark beetle pheromones as kairomones by predators (Borden, 1982; Dahlsten, 1982). The lack of specificity in prey of *E. sphegeus* is exemplified by its capacity to respond to pheromones of other species such as *exo*-brevicomin produced by *Dendroctonus* and *Dryocoetes* species (Borden et al., 1987).

Acknowledgments—We thank D.L. Wood and S.J. Seybold for their encouragement, collaboration, and reviews of the manuscript. Additional comments were received from two anonymous reviewers. D.E. Bright and E. Rickey kindly verified the identifications of Scolytidae and Cleridae, respectively. Voucher specimens have been deposited with the Entomology Museum at Simon Fraser University. G. Owen provided assistance with gas chromatography and spectral analyses, and B.J. Johnston resolved both enantiomers of ipsenol. L. Wheeler, T. Richerson, and C. Matteau assisted in processing captured beetles. Assistance in the manufacture of ipsenol release devices was provided by Phero Tech Inc. This research was supported in part by an H.R. MacMillan Family Fund Fellowship and a Simon Fraser University Graduate Research Fellowship to D.R.M., the Natural Sciences and Engineering Research Council of Canada (Operating Grant A3881 and Strategic Grant G1611),and the Science Council of British Columbia [Grant 1 (RC 14-16)].

REFERENCES

BIRCH, M.C., and LIGHT, D.M. 1977. Inhibition of the attractant pheromone response in *Ips pini* and *I. paraconfusus* (Coleoptera: Scolytidae): Field evaluation of ipsenol and linalool. *J. Chem. Ecol.* 3:257-267.

BIRCH, M.C., LIGHT, D.M., and MORI, K. 1977. Selective inhibition of response of *Ips pini* to its pheromone by the (*S*)-(−)-enantiomer of ipsenol. *Nature* 170:738-739.

BIRCH, M.C., SVIHRA, P., PAINE, T.D., and MILLER, J.C. 1980. Influence of chemically mediated behavior on host tree colonization by four cohabiting species of bark beetles. *J. Chem. Ecol.* 6:395-414.

BORDEN, J.H. 1967. Factors influencing the response of *Ips confusus* (Coleoptera: Scolytidae) to male attractant. *Can. Entomol.* 99:1164-1193.

BORDEN, J.H. 1982. Aggregation pheromones, pp. 74-139, *in* J.B. Mitton and K.B. Sturgeon (eds.). Bark Beetles in North American Conifers. University of Texas Press, Austin, Texas.

BORDEN, J.H., PIERCE, A.M., PIERCE, H.D., JR., CHONG, L.J., STOCK, A.J., and OEHLSCHLAGER, A.C. 1987. Semiochemicals produced by western balsam bark beetle, *Dryocoetes confusus* Swaine (Coleoptera: Scolytidae). *J. Chem. Ecol.* 13:823-836.

BRIGHT, D.E., JR. 1976. The bark beetles of Canada and Alaska (Coleoptera: Scolytidae). Agriculture Canada Publication 1576.

DAHLSTEN, D.L. 1982. Relationships between bark beetles and their natural enemies, pp. 140-182, *in* J.B. Mitton and K.B. Sturgeon (eds.). Bark Beetles in North American Conifers. University of Texas Press, Austin, Texas.

DIXON, W.N., and PAYNE, T.L. 1979. Sequence of arrival and spatial distribution of entomophagous and associate insects on southern pine beetle-infested trees. Texas Agric. Exp. Stn. Misc Publ. 1432.

FURNISS, R.L., and CAROLIN, V.M. 1980. Western forest insects. U.S. For. Serv. Misc. Publ. 1339.

FURNISS, M.M., and LIVINGSTON, R.L. 1979. Inhibition by ipsenol of pine engraver attraction in northern Idaho. *Environ. Entomol.* 8:369–372.

HEDDEN,R., VITÉ, J.P., and MORI, K. 1976. Synergistic effect of a pheromone and a kairomone on host selection and colonisation by *Ips avulsus*. *Nature* 261:696–697.

LIBBEY, L.M., RYKER, L.C., and YANDELL, K.L. 1985. Laboratory and field studies of volatiles released by *Dendroctonus ponderosae* Hopkins (Coleoptera, Scolytidae). *Z. Angew. Entomol.* 100:381–392.

LINDGREN, B.S. 1983. A multiple-funnel trap for scolytid beetles. *Can. Entomol.* 115:299–302.

MILLER, D.R., and BORDEN, J.H. 1985. Life history and biology of *Ips latidens* (LeConte) (Coleoptera: Scolytidae). *Can. Entomol.* 117:859–871.

MILLER, D.R., and BORDEN, J.H. 1990. The use of monoterpenes as kairomones by *Ips latidens* (LeConte) (Coleoptera: Scolytidae). *Can. Entomol.* 122:301–307.

MIROV, N.T. 1961. Composition of gum turpentines of pines. U.S. For. Serv. Tech. Bull. No. 1239.

NORDLUND, D.A., and LEWIS, W.J. 1976. Terminology of chemical releasing stimuli in intraspecific and interspecific interactions. *J. Chem. Ecol.* 2:211–220.

PAINE, T.D., BIRCH, M.C., and SVIHRA, P. 1981. Niche breadth and resource partitioning by four sympatric species of bark beetles (Coleoptera: Scolytidae). *Oecologia* 48:1–6.

PIERCE, H.D., JR., CONN, J.E., OEHLSCHLAGER, A.C., and BORDEN, J.H. 1987. Monoterpene metabolism in female mountain pine beetles, *Dendroctonus ponderosae* Hopkins, attacking ponderosa pine. *J. Chem. Ecol.* 13:1455–1480.

SEYBOLD, S.J., WOOD, D.L., WEST, J.R., SILVERSTEIN, R.M., OHTSUKA, T., and KUBO, I. 1991. Laboratory investigations of the aggregation pheromone of *Ips latidens* (LeConte) (Coleoptera: Scolytidae). *J. Chem. Ecol.* In press.

SHRIMPTON, D.M. 1972. Variation in the extractives from lodgepole pine sapwood and heartwood. Forestry Canada Information Report NOR-X-18.

SHRIMPTON, D.M. 1973. Extractives associated with wound response of lodgepole pine attacked by the mountain pine beetle and associated microorganisms. *Can. J. Bot.* 51:527–534.

SLESSOR, K.N., KING, G.G.S., MILLER, D.R., WINSTON, M.L., and CUTFORTH, T.L. 1985. Determination of chirality of alcohol or latent alcohol semiochemicals in individual insects. *J. Chem. Ecol.* 11:1659–1667.

SVIHRA, P., PAINE, T.D., and BIRCH, M.C. 1980. Interspecific olfactory communications in southern pine beetles. *Naturwissenschaften* 67:518–520.

VITÉ, J.P., OHLOFF, G., and BILLINGS, R.F. 1978. Pheromonal chirality and integrity of aggregation response in southern species of the bark beetle *Ips* sp. *Nature* 272:817–818.

WATTERSON, G.P., PAYNE, T.L., and RICHERSON, J.V. 1982. The effects of verbenone and brevicomin on the within-tree populations of *Dendroctonus frontalis*. *J. Ga. Entomol. Soc.* 17:118–126.

WOOD, D.L., STARK, R.W., SILVERSTEIN, R.M., and RODIN, J.O. 1967. Unique synergistic effects produced by the principal sex attractant compounds of *Ips confusus* (LeConte) (Coleoptera: Scolytidae). *Nature* 215:206.

WOOD, S.L. 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. Great Basin Natur. Mem. 6.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

ENANTIOSPECIFIC SYNTHESIS OF (*R*)-1,7-DIOXASPIRO [5.5]UNDECANE [MAJOR COMPONENT OF OLIVE FRUIT FLY (*Dacus Oleae*) SEX PHEROMONE] FROM D-FRUCTOSE[1]

ISIDORO IZQUIERDO CUBERO,* MARIA T. PLAZA LÓPEZ-ESPINOSA, and RAFAEL ACUÑA CASTILLO

Department of Organic Chemistry, Faculty of Pharmacy, University of Granada, 18071 Granada (Spain).

(Received November 28, 1990; accepted March 26, 1991)

Abstract—The synthesis of the title compound **13** has been carried out through the preparation of its precursor, (3*R*,4*R*,5*S*,6*R*)-3,4,5-trihydroxy-1,7-dioxaspiro[5.5]undecane (**6**), obtained from D-fructose using Wittig's methodology, reduction, and spiroketalation. Compound **6** was transformed into **13** by a Barton deoxygenation at C-5 followed by a Corey dideoxygenation at C-3,4 of the appropriately protected derivatives.

Key Words—Enantiospecific, synthesis, spiroacetal, pheromone, olive fruit fly, D-Fructose, *Dacus oleae*, Diptera, Tephritidae.

INTRODUCTION

Since Baker et al. (1980) isolated and identified the major component of the sex pheromone of the olive fruit fly as 1,7-dioxaspiro [5.5]undecane (**13**), several racemic (Ley and Lygo, 1984; Brinker et al., 1985; DeShong et al., 1988) and three enantiospecific (Redlich and Francke, 1984; Mori et al., 1985; Iwata et al., 1985) syntheses of this compound have been reported. Retrosynthetic analysis of **13** (Scheme 1) indicated that it could be synthesized via a double cyclization of a δ, δ'-dihydroxyketone. Since the δ-hydroxyketone moiety is present in a hexulose, synthesis from this source would only necessitate an elongation

*To whom correspondence should be addressed.

[1]Enantiospecific synthesis of spiroacetals. Part II. For Part I, see Izquierdo and Plaza (1990).

0098-0331/91/0800-1529$06.50/0

of the sugar chain at C-1 (Wittig methodology) with the appropriately functionalized synthon.

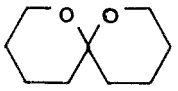
We report here an enantiospecific synthesis of (*R*)-1,7-dioxaspiro[5.5]undecane (**13**) using D-fructose as the chiral precursor, with the chirality of the spiranic center assured by a double anomeric effect (Deslongchamps et al., 1981; Chan et al., 1985; Aamlid et al., 1987).

METHODS AND MATERIALS

General Methods. Melting points were determined with an electrothermal melting point apparatus and are uncorrected. Solutions were dried over $MgSO_4$ before concentration under diminished pressure. [^{13}C]-(75.4 MHz) and [^{1}H]NMR spectra (300 MHz, internal Me_4Si) were recorded with a Bruker AM-300 spectrometer of solutions in $CDCl_3$. The assignment of resonances for **13** was assisted by ^{13}C–^{1}H COSY spectra (Figure 1). Infrared spectra were recorded with a Perkin-Elmer 782 spectrophotometer and mass spectra with a Hewlett-Packard HP-5988-A spectrometer. Optical rotations were measured on solutions in chloroform (1-dm tube) with a Perkin-Elmer 141 polarimeter. Gas–liquid chromatography (GLC) was performed on a Perkin-Elmer 8410 gas chromatograph equipped with a flame-ionization detector and a steel column (2 × 0.125 in. id) packed with 5% OV-17 on Chromosorb W (100–120 mesh) at 250° (A) and 115° (B). The N_2 carrier gas flow rate was 30 ml/min, the injection port temperature was 290° (A) and 200° (B), and the detector zone temperature was 290° (A) and 200° (B). Thin-layer chromatography (TLC) was performed on silica gel G (Merck) (0.25 mm) with detection by charring with sulfuric acid. Column chromatography was done with silica gel Merck (7734). Tetrahydrofuran (THF) was distilled from sodium-benzophenone ketyl and stored over 4-Å molecular sieves prior to use.

The noncrystalline compounds for which elemental analyses were not obtained were shown to be homogeneus by chromatography and characterized by NMR and mass spectrometry.

(3-Benzoxypropyl)triphenylphosphonium Bromide (1). A solution of 25.8 g (98.6 mmol) of triphenyl phosphine and 22.6 g (98.6 mmol) of 3-benzoxypropane bromide (Smith and Sprung, 1943) in 220 ml of dry toluene was heated under reflux for three days. During this time **1** (49 g , quantitative) precipitated as a white crystalline solid, which was collected by filtration, washed with ether–hexane (1 : 1), and dried; mp 158–160°; ν^{KBr} 3049, 3006 (C—H, aromatic), 2991, 2893, 2865 (C—H), 1438 (benzyl), 1184, 1115, 1101, 1064, 1017, 995, and 888 (C—O—C), 753 and 736 cm^{-1} (aromatic). [^{1}H]NMR (80 MHz): δ 8.00–7.50 (m, 15H, 3 Ph), 7.11 (s, 5H, $\underline{Ph}CH_2$), 4.45 (s, 2H, $PhC\underline{H}_2$), 4.10–3.60 (m, 4H, H-1,1′,3,3′), 2.20–1.62 (m, 2H, H-2,2′).



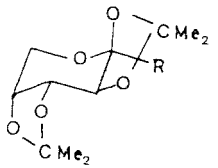
$\Rightarrow$ $HOCH_2-(CHOH)_3-CO-(CH_2)_4OH$

$\Downarrow$

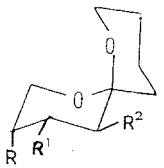
D-FRUCTOSE + $X-(CH_2)_2-CH_2OH$

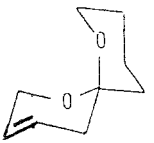
$Ph_3P^+ - CH_2 - CH_2 - CH_2 - OBn \quad Br^-$

1



2 R = CHO
3 R = CH = CH - $(CH_2)_2OBn$
4 R = $(CH_2)_4OBn$
5 R = $(CH_2)_4OH$





12

6 R = R^1 = R^2 = OH
7 R = R^1 = $O_O^{\sim}CMe_2$, R^2 = OH
8 R = R^1 = $O_O^{\sim}CMe_2$, R^2 = OCSSMe
9 R = R^1 = $O_O^{\sim}CMe_2$, R^2 = H
10 R = R^1 = OH, R^2 = H
11 R = R^1 = $O_O^{\sim}C{=}S$, R^2 = H
13 R = R^1 = R^2 = H

SCHEME 1.

TABLE 1. [^{13}C]NMR DATA FOR **3–5**[a]

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	$\underline{C}Me_2$	$C\underline{Me}_2$	$\underline{CH}_2Ph$	Ph
3	70.06	29.04	130.29	130.51	102.81	74.68	70.58 a	70.47 a	60.84	109.03	26.27	72.78	138.69 i
										108.17	25.95		128.36 m
											24.77		127.68 p
											24.22		127.50 o
4	70.48	30.10	20.03	40.69	104.19	73.70	70.89 b	70.68 b	60.93	108.88	26.47	72.99	138.71 i
										107.37	26.47		128.34 m
											25.20		127.67 p
											24.12		127.46 o
5	62.63	32.89	19.39	40.45	104.12	73.71	70.81 c	70.59 c	60.88	108.89	26.41		
										107.43	25.77		
											25.15		
											24.05		

[a]Assignment uncertain within each pair marked with the same letter.

Analysis: Calc. for $C_{28}H_{28}BrOP$: C, 68.43; H, 5.74. Found: C, 68.28; H, 5.74.

(Z)-1-O-Benzyl-2,3,4-trideoxy-5,6:7,8-di-O-isopropylidene-β-D-arabino-non-3-ene-5-ulo-5,9-pyranose (3). To a stirred solution of potassium *tert*-butylate (0.6 g, 5 mmol) in anhydrous tetrahydrofuran (THF) (10 ml) under N_2, **1** (2.45 g, 5 mmol) was added at room temperature. The mixture was stirred for 30 min to yield an orange solution of the ylide. A solution of 2,3:4,5-di-*O*-isopropylidene-β-D-*arabino*-hexos-2-ulo-2,6-pyranose **2** (Izquierdo and Plaza, 1990) (1.29 g, 5 mmol) in dry THF (5 ml) was added dropwise. The reaction was left at room temperature for 1 hr. TLC (ether–hexane, 3:2) revealed the presence of a new compound and the absence of **2**. The mixture was poured into ice-water, extracted with ether (3 × 100 ml), and the combined extracts washed with brine. Concentration of the solvent gave a residue that was chromatographed on silica gel (ether–hexane, 1:3) to yield **3** as a syrup (2.91 g, 64%), retention time 12.01 min (A), $[\alpha]_D = -11.2°$ (*c* 1); ν^{film} 3067 and 3032 (C—H, aromatic), 2991, 2938 and 2906 (C—H), 1664 (C=C), 1455 (benzyl), 1383 and 1373 (CMe_2), 1253, 1212, 1171, 1102, 1071, 987 and 898 (C—O—C and 1,3-dioxolane ring), 738, and 698 cm^{-1} (aromatic).[^{1}H]NMR: δ 7.35–7.20 (m, 5H, $CH_2\underline{Ph}$), 5.63 (dt, 1H, $J_{2,3}$ 7, $J_{3,4}$ 11.7 Hz, H-3), 5.53 (dd, 1H, $J_{2,4}$ 1.4 Hz, H-4), 4.58 (dd, 1H, $J_{6,7}$ 2.4, $J_{7,8}$ 8 Hz, H-7), 4.50 (s, 2H, $\underline{CH_2}Ph$), 4.20 (dm, 1H, H-8), 4.18 (d, 1H, H-6), 3.84 (dd, 1H, $J_{8,9a}$ 2, $J_{9a,9e}$ 13 Hz, H-9*a*), 3.72 (d, 1H, H-9*e*), 3.58–3.46 (m, 2H, H-1,1′), 2.88–2.68 (m, 2H, H-2,2′), 1.52, 1.46, 1.33, and 1.32 (4 s, 12H, 2 CMe_2). For the [^{13}C]NMR, see Table 1. Mass spectrum: *m/z* 375 (1.9, M^+-Me), 267 (2.1), 229 [1.8, $M^+-CH=CH-(CH_2)_2OBn$], 209 (1.8), 184 (5.2), 171 (6.9), 170 (6.5), 160 (7.9), 143 (40.1), 126 (10.4), 105 (44.2), 101 (15.3), 99 (13.4), 91 (100, $C_7H_7^+$), 83 (33.7), and 43 (30.8, Ac^+).

2,3,4-Trideoxy-5,6:7,8-di-O-Isopropylidene-β-D-arabino-5-ulo-5,9-pyranose (5) and its 1-O-benzyl derivative (4). Compound **3** (8.07 g, 20.7 mmol) in methanol (100 ml) was hydrogenated at 4 atm over Raney nickel (7 g). GLC after 6 hr, revealed that **3** had disappeared and that two new compounds [retention times 11.94 and 1.47 min (A)] were present. The catalyst was removed by filtration, washed with methanol, and the filtrate concentrated to a residue that was chromatographed (ether–hexane, 1:2 to 2:1) to yield first syrup **4** (2.18 g, 27%), $[\alpha]_D = -8.6°$, $[\alpha]_{365} = -21.7°$ (*c* 1.1); ν^{film} 3091 and 3068 (C—H, aromatic), 2991, 2939 and 2871 (C—H), 1455 (benzyl) 1383 and 1373 (CMe_2), 1253, 1213, 1107, 1066 and 899 (C—O—C and 1,3-dioxolane ring), 736, and 698 cm^{-1} (aromatic). [^{1}H]NMR (80 MHz): δ 7.30 (s, 5H, $CH_2\underline{Ph}$), 4.54 (dd, 1H, $J_{6,7}$ 2.5, $J_{7,8}$ 8 Hz, H-7), 4.48 (s, 2H, $\underline{CH_2}Ph$), 4.18 (dm, 1H, H-8), 4.06 (d, 1H, H-6), 3.88 (dd, 1H, $J_{8,9a}$ 1.5, $J_{9a,9e}$ 12.5 Hz, H-9*a*), 3.65 (dd, 1H, $J_{8,9e}$ 1 Hz, H-9*e*), 3.46 (bt, 2H, H-1,1), 1.88–1.50 (m, 6H, H-2,2′,3,3′,4,4′), 1.48, 1.43, and 1.30 (3 s, 12H, relative intensity 1:1:2, 2 CMe_2). For the

TABLE 2. [^{13}C]NMR DATA FOR **6–13**[a]

Compound	C-2	C-3	C-4	C-5	C-6	C-8	C-9	C-10	C-11	$\underline{C}Me_2$	$C\underline{Me}_2$	C = S	S$\underline{Me}$
6	61.33	69.64	70.81	73.12	99.52	62.61	24.78	18.10	29.96				
7	59.01	73.85	74.62	77.35	97.67	61.57	24.66	18.01	29.84	109.09	28.24 26.26		
8	59.10	74.25 a	74.36 a	82.94	97.74	61.73	24.51	17.88	30.21	109.54	27.78 26.50	217.36	19.41
9	59.93	71.70 b	69.96 b	38.61	96.46	61.06	24.96	18.39	35.52	108.39	28.00 26.13		
10	63.10	67.89	65.34	38.68	97.28	60.83	24.91	18.33	34.83				
11	58.30	78.24	76.60	36.02	95.79	61.68	24.59	18.22	35.50			191.61	
12	61.61	124.87	121.51	35.86	94.49	59.95	25.21	18.76	34.95				
13	60.17	25.78	19.04	36.21	94.87								

[a]Assignment uncertain within each pair marked with the same letter.

[^{13}C]NMR, see Table 1. Mass spectrum: *m/z* 394 (0.3, M^++2), 393 (1.9, M^++1), 392 (7.7, M^+), 377 (5.3, M^+−Me), 334 (1.3, M^+−Me_2CO), 319 (0.4, M^+−Me−Me_2CO), 317 (0.2, M^+−Me−AcOH), 301 (1.3, M^+−C_7H_7), 262 (1.5), 259 (1.1, M^+−Me−AcOH−Me_2CO), 241 (2.4), 229 [1.4, M^+−$(CH_2)_4$OBn], 191 (2.1), 185 (4.4), 171 [3.9, M^+−$(CH_2)_4$OBn−Me_2CO], 143 (13.9), 101 (10.8), 91 (100, $C_7H_7^+$), and 43 (67.5, Ac^+).

Eluted second was syrup **5** (4.21 g, 67.4%), $[\alpha]_D = -15°$ (*c* 1.13); ν^{film} 3450 (OH), 2992, 2939 and 2877 (C−H), 1383 and 1374 (CMe_2), 1253, 1212, 1189, 1085, and 1067 cm^{-1} (C−O−C and 1,3-dioxolane ring). [^{1}H]NMR: δ 4.51 (dd, 1H, $J_{6,7}$ 2.5, $J_{7,8}$ 8 Hz, H-7), 4.16 (dd, 1H, H-8), 4.04 (d, 1H, H-6), 3.80 (dd, 1H, $J_{9a,9e}$ 13, $J_{8,9a}$ 1.9 Hz, H-9*a*), 3.66 (d, 1H, H-9*e*), 3.58 (bt, 2H, H-1,1), 1.92 (bs, 1H, OH-1), 1.89–1.49 (m, 6H, H-2,2′,3,3′,4,4′), 1.46, 1.43, and 1.29 (3 s, 12H, relative intensity 1:1:2, 2 CMe_2). For the [^{13}C]NMR, see Table 1. Mass spectrum: *m/z* 302 (0.3, M^+), 287 (74.8, M^+−Me), 244 (2.1, M^+−Me_2CO), 229 [12.9, M^+−Me−Me_2CO or M^+−$(CH_2)_4$OH], 227 (16.6, M^+−Me−AcOH), 211 (21.3), 184 (17.9), 171 [15.7, M^+−Me-$(CH_2)_4$OH −Me_2CO], 169 (35.4, M^+−Me −AcOH−Me_2CO), 157 (24.0), 151 (53.0), 143 (46.5), 126 (23.2), 101 (100, $C_5H_9O_2^+$), 86 (22.6), and 43 (60.3, Ac^+).

When **3** (12.4 g, 31.8 mmol) was hydrogenated over Raney nickel (13 g) in methanol (150 ml) at 1.5 atm for 48 hr, only **5** (8.86 g, 92.3%) was obtained.

(3R,4R,5S,6R)-3,4,5-Trihydroxy-1,7-dioxaspiro[5.5]undecane (6). A solution of **5** (8.86 g, 29.3 mmol) in 70% aqueous trifluoroacetic acid (45 ml) was kept at room temperature for 20 hr. TLC (chloroform–methanol, 5:1) revealed the absence of **5** and the presence of a new compound of lower mobility. Concentration of the mixture and repeated codistillation with water and then with dichloromethane gave a residue that was purified by column chromatography (chloroform–methanol, 15:1) to yield **6** (6.47 g, quantitative), as a colorless syrup, $[\alpha]_D = -116.5°$ (*c* 1); ν^{film} 3416 (OH), 2948 and 2876 (C−H), 1265, 1223, 1122, 1072, 989, and 919 cm^{-1} (C−O−C). [^{1}H]NMR (80 MHz): δ 4.40–3.40 (bm, 10H) and 2.10–1.30 (bm, 6H). For the [^{13}C]NMR, see Table 2. Mass spectrum: *m/z* 205 (0.1, M^++1), 204 (0.4, M^+), 178 (0.9), 143 (0.5), 132 (1.2), 114 (9.4), 101 (100, $C_5H_9O_2^+$), 83 (23.2), 60 (27.9), 55 (25.1), and 43 (12.7, Ac^+).

(3R,4R,5S,6R)-5-Hydroxy-3,4-isopropyldioxy-1,7-dioxaspiro[5.5]undecane (7). To a stirred solution of **6** (2.9 g, 14.2 mmol) in dry acetone (25 ml) was added anhydrous copper sulfate (5 g) and *p*-toluene-sulfonic acid (0.5 g). The reaction mixture was kept at room temperature for 24 hr. TLC (ether) revealed the presence of a new compound of higher mobility. The reaction mixture was neutralized (K_2CO_3), filtered, and concentrated. Column chromatography (ether–hexane, 1:2 to 2:1) of the residue, yielded crystalline **7** (2.95 g, 85.3%), mp 136–138° (from hexane), $[\alpha]_D = -173°$ (*c* 1);

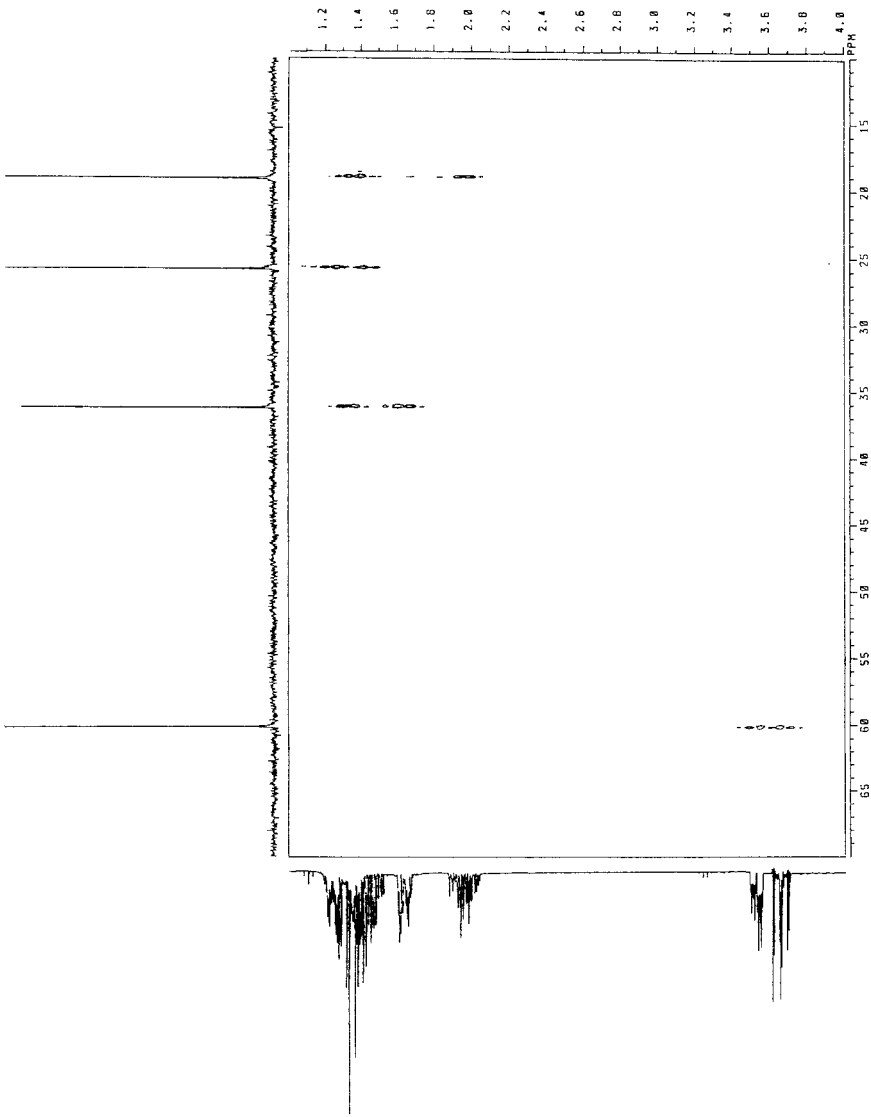


FIG. 1. Two-dimensional $^{13}C-^{1}H$ heteronuclear shift-correlation spectrum of **13**, at a ^{1}H frequency of 300 MHz. To clarify the spectrum, [^{1}H]- and [^{13}C]NMR spectra have been included along the axes of the two-dimensional spectrum.

ν^{KBr} 3476 (OH), 2970, 2947 and 2904 (C—H), 1383 and 1374 (CMe_2), 1249, 1226, 1216, 1174, 1113, 1071, and 991 cm^{-1} (C—O—C and 1,3-dioxolane ring). [^{1}H]NMR: δ 4.17 (dd, 1H, H-3), 4.07 (dd, 1H, $J_{3,4}$ 5.7, $J_{4,5}$ 7.3 Hz, H-4), 3.96 (d, 1H, $J_{2a,2e}$ 13.4 Hz, H-2*a*), 3.81 (dd, 1H, $J_{2e,3}$ 2.7 Hz, H-2*e*), 3.67 (m, 1H, H-8*e*), 3.56 (dt, 1H, $J_{8a,8e} = J_{8a,9a} = 11$, $J_{8a,9e}$ 3.3 Hz, H-8*a*), 3.34 (bt, 1H, H-5), 2.32 (d, 1H, $J_{HO,5}$ 8.1 Hz, HO-5), 1.92 (dt, 1H, $J_{10a,11a} = J_{11a,11e} = 13$, $J_{10e,11a}$ 4.6 Hz, H-11*a*), 1.79 (tq, 1H, $J_{10a,10e} = J_{9a,10a} = 13$, $J_{9e,10a} = J_{10a,11e} = 4$ Hz, H-10*a*), 1.64 – 1.43 (m, 4H, H-9*a*,9*e*,10*e*,11*e*), 1.49, and 1.33 (2 s, 6H, CMe_2). For the [^{13}C]NMR, see Table 2. Mass spectrum: *m/z* 244 (0.8, M^+), 229 (3.4, M^+-Me), 169 (0.4, $M^+-Me-AcOH$), 159 (2.1), 157 (2.1), 130 (2.6), 114 (10.0), 101 (100, $C_5H_8O_2^+$), 85 (58.0, $C_5H_8O_2^+-Me$), 83 (29.9), 59 (25.5, Me_2COH^+), and 43 (41.8, Ac^+).

Analysis: Calc. For $C_{12}H_{20}O_5$: 58.99; H, 8.25. Found: C, 58.56; H, 8.37.

(3R,4R,5S,6R)-3,4-Isopropyldioxy-5-[(thiomethyl)thiocarbonyloxy]-1,7-dioxaspiro[5.5]undecane (8). A solution of **7** (5.06 g, 21 mmol) in anhydrous THF (60 ml) was added to a stirred solution of sodium hydride (2.3 g, 96 mmol) (80% oil dispersion) in the same solvent (40 ml) and imidazole (100 mg) under N_2 at room temperature. The mixture was refluxed for 30 min, cooled, and carbon disulfide (2.9 ml, 47.4 mmol) was added dropwise. The reaction was refluxed again for 30 min, cooled, and methyl iodide (2.4 ml, 38 mmol) was added slowly, and the mixture was heated under reflux for 1 hr. TLC (ether–hexane, 3:2) revealed the absence of **7** and the presence of a new more mobile product. The excess hydride was destroyed by cautious addition of ether saturated with water (50 ml) and water (15 ml). The organic phase was separated and the aqueous phase extracted with ether (2 × 15 ml). The combined extracts were washed with brine and water, concentrated, and the residue chromatographed (ether–hexane, 1:2) to yield crystalline **8** (6.8 g, quantitative), mp 143–146° (from hexane), $[\alpha]_D = -154°$ (*c* 1); ν^{KBr} 2984, 2964, 2950 and 2875 (C—H), 1384 and 1375 (CMe_2), 1264, 1241, 1223, 1206 (C=S), 1172, 1117, 1084, 1069, 1030 and 985 cm^{-1} (C—O—C, C—S, and 1,3-dioxolane ring). [^{1}H]NMR: δ 5.83 (d, 1H, $J_{4,5}$ 8 Hz, H-5), 4.42 (dd, 1H, $J_{3,4}$ 5.4 Hz, H-4), 4.28 (dd, 1H, H-3), 4.05 (d, 1H, $J_{2a,2e}$ 13.4 Hz, H-2*a*), 3.88 (dd, 1H, $J_{2e,3}$ 2.8 Hz, H-2*e*), 3.75–3.65 (dm, 1H, H-8*e*), 3.59 (dt, 1H, $J_{8a,8e} = J_{8a,9a} = 11$, $J_{8a,9e}$ 3.5 Hz, H-8*a*), 2.59 (s, 3H, S—Me), 1.83–1.70 (m, 1H), 1.63–1.42 (m, 5H), 1.55 and 1.33 (2 s, 6H, CMe_2). For the [^{13}C]NMR, see Table 2. Mass spectrum: *m/z* 319 (1.2, M^+-Me), 259 (3.9, $M^+-Me-AcOH$), 227 (18.9, $M^+-OCS-MeSH$), 196 (46.3), 168 (68.1, $M^+-OCS-MeSH-Me_2CO$), 151 (17.6), 143 (23.0), 125 (27.7), 91 (78.7, $MeSCS^+$), 85 (50.6, $C_4H_5O_2^+$), 54 (82.5) and 43 (100, Ac^+).

Analysis: Calc. for $C_{14}H_{22}O_5S_2$: C, 50.27; H, 6.63. Found: C, 48.67; H, 6.28.

(3R,4S,6R)-3,4-Isopropyldioxy-1,7-dioxaspiro[5.5]undecane (9). A solu-

tion of **8** (3.7 g, 10.7 mmol) and azobisisobutyronitrile (50 mg) in dry toluene (40 ml) was added dropwise to a stirred solution of refluxing tri-*n*-butyltin hydride (4 ml, 14 mmol) in the same solvent (20 ml) under N_2. Refluxing was continued overnight. TLC (ether–hexane, 1:2) revealed that **8** had disappeared and that a new compound of lower mobility was present. Concentration of the solvent, followed by column chromatography (ether–hexane, 1:4) of the residue afforded crystalline **9** (2.65 g, quantitative), mp 58–60° (from hexane), $[\alpha]_D = -151.3°$ (*c* 1); ν^{KBr} 2992, 2960, and 2945 (C—H), 1373 (CMe_2), 1275, 1246, 1220, 1156, 1080, 1067, 1034, and 989 cm^{-1} (C—O—C and 1,3-dioxolane ring). [^{1}H]NMR: δ 4.35 (dt, 1H, $J_{3,4} = J_{4,5e} = 6.1$, $J_{4,5a}$ 8.3 Hz, H-4), 4.02 (bdd, 1H, H-3), 3.91 (d, 1H, $J_{2a,2e}$ 13.2 Hz, H-2*a*), 3.77 (dd, 1H, $J_{2e,3}$ 2.6 Hz, H-2*e*), 3.59 (dt, 1H, $J_{8a,8e} = J_{8a,9a} = 11.2$, $J_{8a,9e}$ 3.4 Hz, H-8*a*), 3.51 (dm, 1H, H-8*e*), 1.84 (dd, 1H, $J_{5a,5e}$ 13.7 Hz, H-5*e*), 1.86–1.69 (m, 2H), 1.61 (dd, 1H, H-5*a*), 1.56–1.45 (m, 3H), 1.39 (dt, 1H, $J_{10a,11a} = J_{11a,11e} = 13.2$, $J_{10e,11a}$ 4.2 Hz, H-11*a*), 1.45, and 1.29 (2 s, 6H, CMe_2). For the [^{13}C]NMR, see Table 2. Mass spectrum: *m/z* 228 (2, M^+), 213 (88, M^+-Me), 173 (11), 157 (25), 153 (32, $M^+-Me-AcOH$), 113 (56), 100 (80), 85 (72, $C_4H_5O_2^+$), 59 (28, Me_2COH^+), 55 (51) and 43 (100, Ac^+).

Analysis: Calc. for $C_{12}H_{20}O_4$: C, 63.13; H, 8.83. Found: C, 63.47; H, 9.09.

*(3R,4S,6R)-3,4-Dihydroxy-1,7-dioxaspiro[5.5]undecane (**10**).* A solution of **9** (2.5 g, 11 mmol) in 60% aqueous acetic acid (20 ml) was heated at 50° for 30 min. TLC (ethyl acetate) revealed the presence of a new compound of lower mobility. The reaction mixture was concentrated and the residue coevaporated repeatly with toluene in order to remove the remaining acetic acid. Column chromatography (ethyl acetate) of the residue afforded **10** (2.36 g, quantitative) as a colorless syrup, $[\alpha]_D = -94°$ (*c* 1.4); ν^{film} 3414 (OH), 2945 and 2876 (C—H), 1224, 1094, 1072, 1002, and 986 cm^{-1} (C—O—C). [^{1}H]NMR (80 MHz): δ 4.25–3.40 (m, 6H), 3.00 (bs, 2H, HO-3,4), and 2.00–1.25 (m, 8H). For the [^{13}C]NMR, see Table 2. Mass spectrum: *m/z* 188 (5, M^+), 171 (3, M^+-OH), 157 (19), 145 (27), 133 (33), 112 (23), 101 (79, $C_5H_9O_2^+$), 99 (60), 83 (35) and 43 (100, Ac^+).

*(3R,4S,6R)-3,4-Thiocarbonyldioxy-1,7-dioxaspiro[5.5]undecane (**11**).* To a stirred solution of **10** (1 g, 5.32 mmol) and 4-dimethylaminopyridine (1.6 g, 13 mmol) in dry dichloromethane (25 ml) at 0° under N_2 was added 90% thiophosgene (0.6 ml) in the same solvent (5 ml). The reaction mixture was stirred for 45 min at 0° and then 12 hr at room temperature. TLC (ethyl acetate) showed the presence of a new faster running compound. The mixture was filtered, the filtrate concentrated, and the residue chromatographed (ether–hexane, 1:1) to yield crystalline **11** (0.73 g, 63%), mp 136–139° (from hexane), $[\alpha]_D = -77.5°$ (*c* 1); ν^{KBr} 2964, 2948, and 2880 (C—H), 1364, 1354, 1344, 1272, 1211 (C=S), 1181, 1098, 1068, and 996 cm^{-1} (C—O—C). [^{1}H]NMR: δ 5.14 (dt,

1H, $J_{3,4} = J_{4,5e} = 5.9$, $J_{4,5a}$ 7 Hz, H-4), 4.82 (bdd, 1H, H-3), 4.06 (d, 1H, $J_{2a,2e}$ 14 Hz, H-2*a*), 3.84 (dd, 1H, $J_{2e,3}$ 2.4 Hz, H-2*e*), 3.62 (dt, 1H, $J_{8a,9e}$ 3.6, $J_{8a,8e} = J_{8a,9a} = 11$ Hz, H-8*a*), 3.60–3.52 (m, 1H, H-8*e*), 2.03 (dd, 1H, $J_{5a,5e}$ 14.7 Hz, H-5*e*), 1.89 (dd, 1H, H-5*a*), 1.85–1.72, and 1.62–1.40 (2 m, 6H, H-9,9′,10,10′,11,11′). For the [^{13}C]NMR, see Table 2. Mass spectrum: *m/z* 232 (4, $M^+ + 2$), 231 (9, $M^+ + 1$), 230 (70, M^+), 174 (20), 152 (15), 127 (15), 101 (63, $C_5H_9O_2^+$), 99 (56), 86 (30), 84 (51), 83 (44), and 55 (100, $C_4H_7^+$ or $C_3H_4O^+$).

Analysis: Calc. for $C_{10}H_{14}O_4S$: C, 52.15; H, 6.13. Found: C, 52.11; H, 6.20.

(R)-*1,7-Dioxaspiro[5.5]undec-3-ene (12).* A solution of **11** (0.46 g, 2 mmol) in trimethylphosphite (1.5 ml) was heated under reflux for 48 hr. TLC (ether–hexane, 2:1) revealed the presence of a new faster running compound. Aqueous 20% sodium hydroxide (5 ml) was added with stirring and the mixture kept at room temperature for 4 hr, then extracted with ether (3 × 15 ml), and the combined extracts washed with water and concentrated to give a residue that was chromatographed (ether–hexane, 1:2) to afford **12** (0.26 g, 83%), retention time 6.1 min (B), $[\alpha]_D = -51°$ (*c* 2). [^{1}H]NMR: δ 5.74 (dm, 1H, H-3), 5.66 (ddt, 1H, $J_{3,4}$ 10.4, $J_{4,5}$ 6.7, $J_{2,4} = J_{4,5'} = 1.8$ Hz, H-4), 4.16 (dddd, 1H, $J_{2,2'}$ 16, $J_{2,3}$ 5.5, $J_{2,4}$ 3.5, $J_{2,5}$ 2 Hz, H-2), 4.03 (dddd, 1H, $J_{2',3}$ 4.5, $J_{2',4}$ 3, $J_{2',5}$ 1.5 Hz, H-2′), 3.75 (dt, 1H, $J_{8a,8e} = J_{8a,9a} = 11.3$, $J_{8a,9e}$ 3.1 Hz, H-8*a*), 3.66 (dm, 1H, H-8*e*), 2.16 (ddt, 1H, $J_{5,5'}$ 17.8, $J_{2,5} = J_{3,5} =$ 2.5 Hz, H-5), 2.03 (dm, 1H, H-5′), 1.87 (tq, 1H, $J_{9a,10a} = J_{10a,10e} = J_{10a,11a} = 13.4$, $J_{9e,10a} = J_{10a,11e} = 4.2$ Hz, H-10*a*), 1.73–1.45 (m, 5H, H-9*a*,9*e*,10*e*,11*a*,11*e*). For the [^{13}C]NMR, see Table 2.

(R)-*1,7-Dioxaspiro[5.5]undecane (13).* A solution of **12** (100 mg, 0.65 mmol) in anhydrous ether (8 ml) was hydrogenated over Raney Nickel (60 mg) at 1.5 atm for five days. Monitoring the reaction by GLC indicated the transformation of the peak with retention time 6.1 min into a faster running compound (retention time 4.41 min) (B). Removal of the catalyst by filtration and concentration of the filtrate, gave a residue that was chromatographed (ether–*n*-pentane, 1:4) to afford **13** (70 mg, 70%) as a colorless volatile oil, $[\alpha]_D = -119°$ (*c* 1.5, pentane), [lit (Redlich and Francke, 1984) $[\alpha]_D = -122.8°$ (*c* 3.2, pentane); lit. (Mori et al., 1985) $[\alpha]_D = -121°$ (*c* 1.84, pentane)]; ν^{film} 2944 and 2874 (C—H), 1386, 1231, 1208, 1180, 1096, 1069, 1041, 991 and 877 cm^{-1} (C—O—C). [^{1}H]NMR (C_6D_6): δ 3.66 (ddd, 2H, $J_{2a,2e} = J_{8a,8e} =$ 11, $J_{2a,3a} = J_{8a,9a} = 12.3$, $J_{2a,3e} = J_{8a,9e} = 2.5$ Hz, H-2*a*,8*a*), 3.53 (ddt, 2H, $J_{2e,3e} = J_{2e,3a} = J_{8e,9e} = J_{8e,9a} = 4.7$, $J_{2e,4e} = J_{8e,10e} = 1.8$ Hz, H-2*e*,8*e*), 1.95 (tq, 2H, $J_{4a,4e} = J_{3a,4a} = J_{4a,5a} = J_{10a,10e} = J_{9a,10a} = J_{10a,11a} = 13.1$ $J_{3e,4a} = J_{4a,5e} = J_{9e,10a} = J_{10a,11e} = 4$ Hz, H-4*a*,10*a*), 1.63 (ddt, 2H, $J_{4e,5e} = J_{4a,5e} = J_{10e,11e} = J_{10a,11e} = 4$, $J_{3e,5e} = J_{9e,11e} = 1.5$ Hz, H-5*e*,11*e*), 1.44 (ddq, 2H, $J_{3a,3e} = J_{9a,9e} = 12.5$, $J_{3a,4e} = J_{9a,10e} = 3.7$ Hz, H-3*a*,9*a*), 1.36

(dt, 2H, $J_{5a,5e} = J_{11a,11e} = 13.4$, $J_{4e,5a} = J_{10e,11a} = 4.4$ Hz, H-5*a*,11*a*), 1.36 (dm, 2H, H-4*e*,10*e*), and 1.23 (dm, 2H, H-3*e*,9*e*). For the [^{13}C]NMR, see Table 2. Mass spectrum: *m/z* 156 (19.4, M^+), 128 (12), 111 (17.7), 101 (100, $C_5H_9O_2^+$), 98 (92.7), 83 (39), 56 (14.4), 55 (33, $M^+ - C_5H_9O_2$), and 43 (12.3, Ac^+).

RESULTS AND DISCUSSION

Reaction of the ylid from (3-benzoxypropyl)triphenylphosphonium bromide (**1**) with 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose (**2**) (Izquierdo and Plaza, 1990) in dry tetrahydrofuran gave only (*Z*)-1-*O*-benzyl-2,3,4-trideoxy-5,6:7,8-di-*O*-isopropylidene-β-D-*arabino*-non-3-en-5-ulo-5,9-pyranose (**3**). The *Z* configuration at C-3 was assigned on the basis of the $J_{3,4}$ value (11.7 Hz). The high stereoselectivity found was attributed to the use of a non-lithium-containing base to generate the ylide.

Hydrogenation of **3** over Raney nickel for short or long periods produced the saturated benzyl derivative (**4**) or the corresponding de-*O*-benzylated compound **5**, respectively.

Treatment of **5** with 70% aqueous trifluoroacetic acid for 20 hr resulted in deacetonation and cyclization to afford (3*R*,4*R*,5*S*,6*R*) -3,4,5-trihydroxy-1,7-dioxaspiro[5.5]undecane (**6**).[2] The structure of **6** was established from its [^{13}C]NMR spectrum where the chemical shift value of the spiranic carbon (δ 99.52) was in good agreement with those previously reported.

Conventional acetonation of **6** gave the corresponding 3,4-isopropyldioxy derivative **7**, which was deoxygenated at C-5, via its 5-xanthate derivative (**8**), by reduction (Barton and McCombie, 1975) to yield **9**.

Controlled hydrolysis of **9** in acid medium gave its deacetonated compound **10**, which was dideoxygenated by Corey and Winter's (1963) method via its 3,4-thiocarbonyldioxy derivative **11**, to produce (*R*)-1,7-dioxaspiro[5.5]undec-3-ene (**12**), which was subsequently hydrogenated to yield (*R*)-1,7-dioxaspiro[5.5]undecane (**13**), whose physical and spectroscopic data were in good accordance with those found in the literature (Redlich and Francke, 1984; Mori et al., 1985; Iwata et al., 1985). We have included the assignment of the [^{1}H]- and [^{13}C]NMR data.

REFERENCES

AAMLID, K.H., HOUGH, L., RICHARDSON, A.C., and HENDRY, D. 1987. An enantiospecific synthesis of (*R*)-1,4,7-trioxaspiro[5.5]undecane from D-fructose. *Carbohydr. Res.* 164:373–390.

BAKER, R., HERBERT, R., HOWSE, P.E., JONES, O.T., FRANCKE, W., and REITH, W. 1980. Iden-

[2]The [^{13}C]NMR data of compounds **6–13** are included in Table 2 with the numbering according to the nomenclature for spirocompounds.

tification and synthesis of the major sex pheromone of the olive fly (*Dacus oleae*). *J. Chem. Soc. Chem. Commun.* 52–53.

BARTON, D.H.R., and MCCOMBIE, S.W. 1975. A new method for the deoxygenation of secondary alcohols. *J. Chem. Soc. Perkin Trans. I.* 1574–1585.

BRINKER, U.H., HAGHANI, A., and GOMANN, K. 1985. Spiroacetal formation by carbene (carbenoid) insertion reaction: Synthesis of the major constituent of the sex attractant of the olive fly (*Dacus oleae*). *Angew. Chem. Int. Ed. Engl.* 24:230–231.

CHAN, J.Y.C., HOUGH, L., and RICHARDSON, A.C. 1985. The synthesis of (*R*)-and (*S*)-spirobi-1,4-dioxane and related spirobicycles from D-fructose. *J. Chem. Soc. Perkin Trans. I.* 1457–1462.

COREY, E.J., and WINTER, R.A.E. 1963. A new stereospecific olefin synthesis from 1,2-diols. *J. Am. Chem. Soc.* 85:2677–2678.

DESHONG, P., WALTERMIRE, R.E., and AMMON, H.L. 1988. A general approach to the stereoselective synthesis of spiroketals. A total synthesis of the pheromones of the olive fruit fly and related compounds. *J. Am. Chem. Soc.* 110:1901–1910.

DESLONGCHAMPS, P., ROWAN, D.D., POTHIER, N., SAUVÉ, T., and SAUNDERS, J.K. 1981. 1,7-Dioxaspiro[5.5]undecanes. An excellent system for the study of stereoelectronic effects (anomeric and *exo*-anomeric effects) in acetals. *Can. J. Chem.* 59:1105–1121.

IWATA, C., FUJITA, M., HATTORI, K., UCHIDA, S., and IMANISHI, T. 1985. Intramolecular Michael addition reaction to chiral vinylic sulfoxides. An enantioselective synthesis of (*R*)- and (*S*)-1,7-dioxaspiro[5.5]undecane. *Tetrahedron Lett.* 26:2221–2224.

IZQUIERDO CUBERO, I., and PLAZA LOPÉZ-ESPINOSA, M.T. 1990. An enantiospecific synthesis of (−)-talaromycins A and B from D-fructose. *Carbohydr. Res.* 205:293–304.

LEY, V., and LYGO, B. 1984. A new route to spiroketals using the Horner-Wittig reaction of 2-diphenylphosphinoxy cyclic ethers. *Tetrahedron Lett.* 25:113–116.

MORI, K., WATANABE, H., YANAGI, K., and MINOBE, M. 1985. Synthesis of the enantiomers of 1,7-dioxaspiro[5.5]undecane, 4-hydroxy-1,7-dioxaspiro[5.5]undecane and 3-hydroxy-1,7-dioxaspiro[5.5]undecane, the components of the olive fruit fly pheromone. *Tetrahedron* 41:3663–3672.

REDLICH, H., and FRANCKE, W. 1984. Synthesis of enantiomerically pure 1,7-dioxaspiro[5.5]undecanes, pheromone components of the olive fly (*Dacus oleae*). *Angew. Chem. Int. Ed. Engl.* 23:519–520.

SMITH, L.I., and SPRUNG, J.A. 1943. Vitamin E. XLI. Synthesis of 1-chloro-3,7,11,15-tetramethylhexadecanol-3, and its condensation with trimethylhydroquinone to form α-tocopherol. *J. Am. Chem. Soc.* 65:1276–1283.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

DEVELOPMENTAL PROFILE OF SINALBIN (*p*-HYDROXYBENZYL GLUCOSINOLATE) IN MUSTARD SEEDLINGS, *Sinapis alba* L., AND ITS RELATIONSHIP TO INSECT RESISTANCE

ROBERT P. BODNARYK

Section on Germplasm Development and Biotechnology
Agriculture Canada, Research Station
195 Dafoe Road
Winnipeg, Manitoba, Canada R3T 2M9

(Received December 18, 1990; accepted March 1, 1991)

Abstract—Sinalbin was identified as a chemical component of insect antixenosis and antibiosis resistance mechanisms in seedlings of *Sinapis alba* by DEAE-Sephadex chromatography, HPLC, treatment with sulfatase and myrosinase, various feeding tests using artificial and natural substrates, and by measuring sinalbin concentrations in cotyledons and leaves during seedling development. The effects of sinalbin on feeding were dependent upon the insect species and upon the rapidly changing profile of sinalbin concentrations in the developing seedling. The high concentrations of sinalbin found in young cotyledons (up to 20 mM) and leaves (up to 10 mM) deterred the feeding of the flea beetle, *Phyllotreta cruciferae* Goeze and larvae of the bertha armyworm, *Mamestra configurata* Walker. The protection that sinalbin confers upon the vulnerable, newly emerged seedling (and upon tiny, young leaves) appears critical for the first few days of survival of *S. alba* under feeding pressure from flea beetles in the field. The lower concentrations of sinalbin found in older cotyledons and leaves (2–3 mM) offer little or no protection against *P. cruciferae* and may actually stimulate the feeding of this crucifer specialist. These concentrations of sinalbin, however, are still effective in reducing the level of feeding by larvae of the more generalist feeder *M. configurata*.

Key Words—Sinalbin, glucosinolate, resistance, *Sinapis alba*, *Brassica napus*, *Phyllotreta cruciferae*, Coleoptera, Chrysomelidae, *Mamestra configurata*, Lepidoptera, Noctuidae.

0098-0331/91/0800-1543$06.50/0

INTRODUCTION

The mustard, *Sinapis alba* L., shows a high level of resistance in the field to damage from adults of the crucifer flea beetle, *Phyllotreta* spp. (Putnam, 1977; Lamb, 1984). Antixenosis (nonpreference) and tolerance have been identified as two mechanisms in seedlings of *S. alba* that likely account for flea beetle resistance in this species (Bodnaryk and Lamb, 1991). Although flea beetle resistance is inherently valuable in growing *S. alba*, the question of utilizing its resistant traits in other more extensively grown crucifer crops, such as the oilseed rapes *Brassica napus* L., and *B. campestris* L., has not been addressed. Recent hybridization of *S. alba* and *B. napus* via embryo rescue (Ripley and Arnison, 1990) indicates that transfer of useful traits of *S. alba* to *B. napus* may not be as formidable a task as once imagined.

Chemically based resistance must be compatible with the end use of the crop for which the resistance is intended. The chemical basis of resistance in *S. alba* to pest insects is currently unknown and the potential that this resistance may have for transfer to other crops is therefore also unknown. The purpose of this research is to identify chemical factors that contribute to resistance in *S. alba* using two insect pests, namely the flea beetle *P. cruciferae* (a crucifer specialist) and the bertha armyworm, *M. configurata*, a more generalist feeder (Beirne, 1971). The research also examines the developmental profile of an identified resistance factor in *S. alba* and its relationship to insect resistance during early seedling growth.

METHODS AND MATERIALS

Insects. Flea beetles, *Phyllotreta* sp., were collected in May from allyl isothiocyanate baited traps in the field (Burgess and Wiens, 1980) and maintained in the laboratory in screened cages 0.5 × 0.5 × 0.5 m on cabbage leaves, *B. oleracea* L. obtained from a supermarket. Water was provided through a dental wick held in a flask. Prior to tests, *P. cruciferae* were selected and transferred by aspiration to a holding cage for one day with water only. The bertha armyworm, *M. configurata*, was reared on an artificial diet at 20°C and 60–70% relative humidity under an 18-hr light:6-hr dark cycle (Bucher and Bracken, 1976).

Plants. Uniform, large seeds from registered cultivars of mustard, *S. alba* cv. Ochre, and canola, *B. napus* cv. Westar, were selected by eye for individual planting in vermiculite in 175-ml styrofoam coffee cups having a 0.5-cm-diam. hole punched in the bottom. The cups were subirrigated with Hoagland's nutrient solution and held under wide-spectrum (Gro-Lux) artificial lighting in the laboratory at 20 ± 1°C, 50–60% relative humidity, and 18-hr light:6-hr dark

cycle. Plants were kept 8–10 cm from the lights and received about 200 μE/sec/m^2 of photosynthetically active radiation as determined using a LI-COR LI-190SB quantum sensor (LI-COR Inc., Lincoln, Nebraska).

Chemicals. Sinalbin was isolated from 200 g of seeds of *S. alba* as described by Hanley et al. (1983).

Extraction and Quantitation of Sinalbin. Cotyledons and first true leaves of *S. alba* were extracted in boiling 80% methanol as described by Bodnaryk and Palaniswamy (1990). Sinalbin was estimated as its desulfo derivative by HPLC as described by Minchinton et al. (1982).

Preparation of a Glucosinolate Fraction from Cotyledons. Cotyledons (5 g wet weight) from 4-day-old *S. alba* or 5-day-old *B. napus* seedlings were held for 5 min in 80 ml of boiling 80% methanol contained in the stainless steel cup of a Sorval homogenizer. The methanol–cotyledon mixture was then homogenized for 1 min at maximum speed and the homogenate centrifuged at 12,000*g*. The pellet was rehomogenized and further extracted with 80 ml of hot 80% methanol, centrifuged as above,and the extracts combined. Methanol was removed from the combined extract on a rotary evaporator at 40°C and the concentrated aqueous extract was adjusted to contain 0.05 M barium–lead acetate in a final volume of 80 ml. The mixture was stirred for 1 hr at ambient temperature and then centrifuged at 12,000*g* for 10 min to remove precipitated material. The clear supernatant was applied to a column of DEAE–Sephadex A-25 (0.2 g dry weight) that had been treated with 0.5 M pyridine–acetate and equilibrated with water. The column was washed with 20 ml of distilled water (discarded) and then with 3 ml of 0.1 M pyridine–acetate, 10 ml of 0.5 M pyridine–acetate and 10 ml of 1.0 M pyridine–acetate. The pyridine–acetate fractions were dried thoroughly on a rotary evaporator and reconstituted in 1.0 ml of distilled water for incorporation into feeding assays and for glucosinolate analysis.

Enzymatic Treatments: Sulfatase. Cotyledons (5 g wet wt) from 4-day-old *S. alba* were extracted as above and the extract bound to the top of a DEAE–Sephadex A-25 column (0.2 g dry wt). The extract was exposed "on column" to sulfatase (EC 3.1.6.1, Sigma Chemical Co., St. Louis, Missouri) for 24 hr as described by Minchinton et al. (1982). Desulfo glucosinolates were eluted with 2 ml of water, lyophilized, and reconstituted in 1 ml of distilled water for incorporation into feeding assays and for HPLC. The column was further eluted to produce 0.1, 0.5, and 1.0 M pyridine–acetate fractions for feeding assays and HPLC as described above.

Enzymatic Treatments: Myrosinase. Cotyledons (5 g wet wt) from 4-day-old *S. alba* or 5-day-old *B. napus* seedlings were homogenized in 60 ml of distilled water and held at 20 to 22°C for 3 hr to expose glucosinolates to endogenous myrosinase (β-thioglucosidase, E.C.3.2.3.1) (Bones, 1990). After

3 hr the mixture was adjusted to contain 80% methanol, boiled for 5 min, and processed as above to produce 0.1, 0.5, and 1.0 M pyridine–acetate fractions for feeding assays and HPLC.

Feeding Assays Using a Gelatin Substrate. Plant glucosinolate fractions or sinalbin were incorporated into gelatin for analysis of feeding inhibition or stimulation. The gelatin medium was prepared by adding 5 g of gelatin (type B from bovine skin, approx. 60 bloom) slowly to 50 ml of vigorously stirred distilled water at 20–22°C. The suspension was then heated at 100°C for 10 min with occasional swirling to dissolve the gelatin and then kept at 37°C for use throughout the day. Glucosinolate fractions or sinalbin contained in 0.5 ml distilled water were added to 2.0 ml of gelatin solution in 15 × 85-mm test tubes at 37°C. The tubes were capped with Parafilm and the contents mixed by inversion. Controls received 0.5 ml distilled water. An aliquot (100 μl) of the test mixture was pipetted onto the bottom of each of 20 polystyrene Petri dishes using an Eppendorf pipetter fitted with a disposable tip whose end had been enlarged to about 2 mm internal diameter for easy pipetting. An aliquot (100 μl) of the control mixture was pipetted adjacent to the test mixture leaving a space of about 2 cm. The tops of the Petri dishes were put in place, using care not to disturb the gelatin, and metal trays containing ice were placed over the dishes for about 20 min for gelling. "Seal tight" Petri dishes (9 × 50 mm, Fisher Scientific) were most convenient to use, but disposable Petri dishes (15 × 100 mm) with loose-fitting lids were also suitable if kept at high relative humidity throughout the feeding assay.

The feeding assay was begun by adding five adult flea beetles or one bertha armyworm larva to the Petri dishes and allowed to run for 24 hr. After 24 hr, lids and insects were removed and the gelatin allowed to dry at ambient temperature for 48 hr. The amount of feeding on the control and test disks was estimated gravimetrically in the case of bertha armyworm larvae, which consumed a significant proportion of the disks. The amount of gelatin consumed by flea beetles in the feeding assays was only a tiny proportion of the total, so that weighing did not give an accurate estimate of the amount eaten. Therefore, flea beetle feeding was estimated as surface area consumed. Feeding "spots" on the thin, transparent gelatin disks were visualized as their shadows cast on the white stage of a Wild M8 dissecting microscope by oblique (45°) lighting. The area of the discrete, well-defined dark "spots" was obtained using an optical grid fitted to an eyepiece of the microscope.

Feeding Assays Using Uptake by Excised Seedlings. Sinalbin was assayed for feeding inhibition or stimulation after uptake through the cut stem of seedlings of *B. napus*. This species contains no sinalbin (Bodnaryk and Palaniswamy, 1990) and is palatable to flea beetles and bertha armyworm larvae. The stems of 40 13-day-old seedlings were cut with a pair of fine, sharp scissors under distilled water and placed in 10-ml glass vials containing either distilled

water (controls, 20 plants) or 2 mM sinalbin (20 plants). The plants were held 8 cm under wide spectrum artificial lighting for 18 hr. Ten pairs of plants held in distilled water or 2 mM sinalbin were offered to flea beetles or bertha armyworm larvae in individual screened cages measuring 30 × 30 × 30 cm. In the case of the bertha armyworm feeding tests, each pair of vials containing the seedlings was put in an aluminum pan, which was then filled with vermiculite to reach the top of the vials. The vermiculite was moistened with distilled water and provided a convenient substrate for the bertha armyworm larvae which were introduced into the feeding test by placing them on the vermiculite in the middle of the space separating the two vials. After 6–24 hr the insects were removed from the plants, and the area eaten from cotyledons and first true leaves was estimated using an optical grid. The remaining plants not used in the feeding tests were analyzed for sinalbin levels in their cotyledons and first true leaves.

Paired Feeding Tests with S. alba *and* B. napus. Styrofoam coffee cups containing vermiculite were planted with *S. alba* and *B. napus* seeds in alternating order. Two experiments were conducted. In the first, 4-day-old seedlings of *S. alba* and 5-day-old seedlings of *B. napus* (two of each per cup; six cups per test; three replicates) were offered to *P. cruciferae* (five beetles per seedling) or *M. configurata* (one larva per seedling) for 24 hr. In the second, 13-day-old seedlings of *S. alba* and 14-day-old seedlings of *B. napus* (one of each per cup; six cups per test; three replicates) were offered to *P. cruciferae* (10 beetles per seedling or *M. configurata* (two larvae per seedling) for 24 hr. For the experiments with older seedlings, cotyledons were removed with sharp scissors 1 hr before the start of the experiment to permit a paired feeding test of first true leaves only. At the end of all feeding tests, the insects were removed and the amount eaten was determined by weighing cotyledons or leaves in the case of experiments with *M. configurata* or by estimating the area consumed with an optical grid in the case of *P. cruciferae*.

Growth and Development of M. configurata *Fed* S. alba *or* B. napus. Two experiments were conducted. In the first, 10 first-instar larvae were placed in each of six cages as above containing 72 4-day-old seedlings of *S. alba* or 72 5-day-old seedlings of *B. napus*. Seedlings were replaced daily. Since growing larvae of *M. configurata* are voracious feeders, it became necessary to add larger and larger quantities of seedlings as the larvae grew, effectively limiting the number of larvae that could be reared to maturity with the available facilities.

In the second experiment, egg masses containing 80–100 eggs from a single female were divided in half. The number of chorionated (fertile) eggs in each half was counted one or two days before hatch. Each half then was placed on an 18- to 25-day-old plant of *S. alba* or *B. napus* from which cotyledons had been removed by scissors. A minimum of four replicates were obtained for each plant species.

For both experiments, records of survival and body weight were obtained

on day 10, day 21, and at pupation. Survival to eclosion and the diapause-nondiapause state of the pupae also were recorded. Nondiapausing pupae were identified by the appearance of adult structures seen through the pupal cuticle. Diapausing pupae were dissected at the end of the study to confirm that no adult development had taken place.

RESULTS

Effect of DEAE-Sephadex A-25 Fractions of Extracted Cotyledons on Feeding. Incorporation into gelatin of the thoroughly dried 0.5 M pyridine-acetate DEAE-Sephadex A-25 fraction of cotyledons of *S. alba* that had been extracted in boiling 80% methanol deterred the feeding of flea beetles, *P. cruciferae*, and the bertha armyworm, *M. configurata* (Table 1, experiment 1). The deterrent effect was strongest for *M. configurata*, where consumption of the test gelatin containing the 0.5 M pyridine-acetate fraction was less than 5% of total feeding (Table 1, experiment 1). The 0.1 and 1.0 M pyridine-acetate fractions from *S. alba* (Table 1, experiment 1) and the 0.1, 0.5,and 1.0 M fractions from *B. napus* cotyledons had relatively little or no effect on feeding (Table 1, experiment 2).

Incorporation into gelatin of the 0.5 M pyridine-acetate fraction of cotyledons of *S. alba* that were extracted in water at 20–22°C and allowed to stand for 3 hr before boiling in 80% methanol had little effect on feeding (Table 1, experiment 3).

Investigation by HPLC of the 0.5 M pyridine-acetate fraction of cotyledons of *S. alba* extracted in boiling 80% methanol revealed a high concentration of *p*-hydroxybenzyl glucosinolate (sinalbin), the predominant glucosinolate of this mustard. Other glucosinolates were not detected in this fraction. When cotyledons of *S. alba* were extracted in water at 20–22°C and allowed to stand for 3 hr before boiling in 80% methanol, the 0.5 M pyridine-acetate fraction contained only a trace (0.24%) of sinalbin, an observation consistent with the destruction of this glucosinolate by endogenous myrosinase (Bones, 1990).

Incorporation into gelatin of the water and the 0.1, 0.5, and 1.0 M pyridine-acetate fractions of cotyledons of *S. alba* that were extracted in boiling 80% methanol, then treated on-column with sulfatase had little or no effect on feeding by *P. cruciferae* or *M. configurata* (Table 1, experiment 4). Investigation of the water fraction by HPLC revealed a high concentration of desulfosinalbin (not shown), which the feeding experiments in Table 1, experiment 4, demonstrated to be without deterrent effect. Investigation of the 0.1, 0.5, and 1.0 M pyridine-acetate fractions by HPLC revealed only a trace of sinalbin in the 0.5 M fraction, attesting to the efficient action of the sulfatase (not shown).

Effect of Sinalbin on Feeding. Sinalbin, when incorporated into gelatin,

TABLE 1. EFFECT OF VARIOUS DEAE-SEPHADEX FRACTIONS OF SEEDLINGS OF *S. alba* OR *B. napus* INCORPORATED INTO GELATIN ON FEEDING RESPONSE OF FLEA BEETLES, *P. cruciferae* AND BERTHA ARMYWORM, *M. configurata*

Pyridine-acetate fractions eluted from DEAE-Sephadex A-25 column	Normalized test feeding (control = 1)	
	P. cruciferae	*M. configurata*
Experiment 1:		
Cotyledons of *S. alba* extracted in boiling 80% methanol		
0.1 M	0.90	0.83
0.5 M	0.28[a]	0.049[a]
1.0 M	0.84	0.88
Experiment 2:		
Cotyledons of *B. napus* extracted in boiling 80% methanol		
0.1 M	0.98	0.87
0.5 M	0.83	0.81
1.0 M	0.93	0.97
Experiment 3:		
Cotyledons of *S. alba* extracted in water at 20–22°C		
0.5 M	0.95	0.91
Experiment 4:		
Cotyledons of *S. alba* extracted in boiling 80% methanol, then treated on column with sulfatase		
Water		
0.1 M	0.88	0.96
0.5 M	0.82	0.86
1.0 M	0.89	0.83
	0.98	0.95

[a] Normalized test feeding responses significantly less than 1 indicate feeding inhibition by the test fraction. $P < 0.05$, t test.

altered the feeding response of *P. cruciferae* (Figure 1). Sinalbin concentrations of 0.5 to 2 mM appeared to stimulate feeding slightly while higher concentrations deterred feeding. Sinalbin strongly deterred feeding of *M. configurata* in a dose-dependent manner (Figure 1). At 20 mM, the highest concentration tested, feeding was partially deterred in *P. cruciferae* and completely deterred in *M. configurata* (Figure 1).

Uptake of Sinalbin by Excised B. napus *Seedlings and its Effect on Feeding.* Two-week-old seedlings of *B. napus* cv. Westar took up sinalbin through their cut stems and concentrated it in the cotyledons and leaves (Table 2, A). The concentration of sinalbin in excised seedlings held in a 2 mM sinalbin

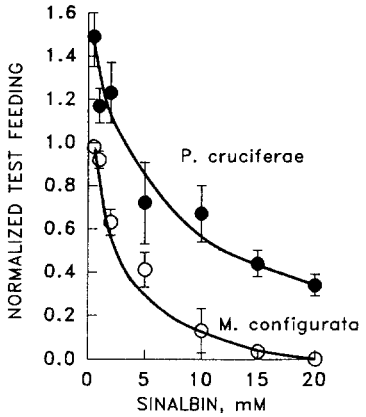


FIG. 1. Effect of sinalbin (*p*-hydroxybenzyl glucosinolate) on the feeding of flea beetles, *P. cruciferae*, and the bertha armyworm, *M. configurata*. Sinalbin was incorporated into gelatin for paired feeding tests of equal amounts of a test gelatin containing sinalbin and a control gelatin containing only water. Twenty paired tests each having five flea beetles or one armyworm were done for each concentration of sinalbin. Values for the amount eaten by the insects were normalized (control = 1). Normalized test feeding values > 1 indicate feeding stimulation by sinalbin; values < 1 indicate feeding inhibition.

solution of 18 hr was about 7 mM in cotyledons and 15 mM in leaves (Table 2, A).

P. cruciferae and *M. configurata* readily discriminated between excised seedlings of *B. napus* incubated in water or 2 mM sinalbin and fed less on the sinalbin-containing cotyledons and leaves (Table 2, A). The deterrent effect was greater in the leaves that had sinalbin concentrations approximately double those found in the cotyledons.

Paired Feeding Tests with Seedlings of B. napus *and* S. alba. There were remarkable between-plant and between-insect differences in the paired feeding tests described in Table 2, B. When the cotyledons of young seedlings were compared, both insects preferred to eat *B. napus*, by about 2 to 1 for *P. cruciferae* and by almost 7 to 1 for *M. configurata*. The concentration of sinalbin in the cotyledons of *S. alba* was about 10 mM and undetected in *B. napus* (Table 2, B, experiment 1). When the first true leaves of older plants were compared, *P. cruciferae* now preferred *S. alba* by nearly 9 to 1, a complete reversal in the feeding preference observed at the cotyledon stage. *M. configurata* continued to prefer *B. napus*, but now by only about 3 to 1. The concentration of sinalbin in the leaves of *S. alba* was 2.8 mM and undetected in *B. napus*.

Growth and Survival of M. configurata *fed* S. alba *or* B. napus. Larvae of *M. configurata* fed young seedlings of *S. alba* performed poorly compared to

TABLE 2. UPTAKE OF SINALBIN BY EXCISED SEEDLINGS OF *B. napus* CV. WESTAR AND ITS EFFECT ON FEEDING BY FLEA BEETLES, *P. cruciferae*, AND BERTHA ARMYWORM, *M. configurata* (A), AND PAIRED FEEDING TESTS OF SEEDLINGS OF *B. napus* CV. WESTAR AND *S. alba* CV. OCHRE (B)

Paired feeding test	Sinalbin concentration (μmol/g wet wt)		Normalized feeding			
			P. cruciferae		*M. configurata*	
	Cotyledons	Leaves	Cotyledons	Leaves	Cotyledons	Leaves
A						
14-day-old excised seedlings of *B. napus* cut stems held in water for 18 hr	0	0	1	1	1	1
14-day-old excised seedlings of *B. napus* cut stems held in 2 mM sinalbin for 18 hr	7.1 ± 1.4	15.3 ± 1.9	0.68[a]	0.33[a]	0.63[a]	0.25[a]
B						
Experiment 1						
5-day-old seedlings of *B. napus*	0		1		1	
4-day-old seedlings of *S. alba*	10.1 ± 1.7		0.42[a]		0.15[a]	
Experiment 2						
14-day-old seedlings of *B. napus*	N.D.[c]	0	N.D.	1	N.D.	1
13-day-old seedlings of *S. alba*	N.D.	2.8 ± 0.4	N.D.	8.80[a]	N.D.	0.31[a]

[a] $P < 0.05$, paired t test.
[b] In experiment 2, the cotyledons were removed from plants with scissors 1 hr prior to the beginning of the experiment to permit a paired feeding test of first true leaves only.
[c] N.D. = not determined.

cohorts fed young seedlings of *B. napus* or older plants of both species (Table 3). Survival of larvae was lower, survival to the pupal stage and to eclosion were lower, body weights were usually less, and the percentage of nondiapause pupae was abnormally low (Table 3).

M. configurata grew best on the cotyledons of *B. napus* (Table 3). Survival and body weights on older plants of *B. napus* cv. Westar (Table 3) were similar to those reported for larvae fed the detached leaves of another cultivar, *B. napus* cv. Regent (Turnock, 1985).

Developmental Profile of Sinalbin in S. alba *Seedlings.* Newly emerged seedlings of *S. alba* had high concentrations (>20 mM, Figure 2A) of sinalbin in their cotyledons. The concentration fell rapidly during the first few days of growth and tended to level off at about 2 mM. The amount of sinalbin in the cotyledons, however, remained almost unchanged during the same period (Figure 2B), indicating that the precipitous decline in the concentration of sinalbin

TABLE 3. EFFECT OF MAINTAINING BERTHA ARMYWORM, *M. configurata*, ON SEEDLINGS OR OLDER PLANTS OF MUSTARD, *S. alba* CV. OCHRE, OR OILSEED RAPE, *B. napus* CV. WESTAR, ON LARVAL AND PUPAL SURVIVAL, BODY WEIGHT, AND DIAPAUSE (MEANS ± SD)[a]

	Food plant			
	4- to 5-day-old seedlings		18- to 25-day-old plant	
Stage and parameter	*S. alba*	*B. napus*	*S. alba*	*B. napus*
Larvae				
First instar	$N = 60$	$N = 60$	$N = 197$	$N = 169$
Survival (%), day 10	60 a	96 b	88 b	91 b
Weight (mg) day 10	7.3 ± 1.0 a	26.6 ± 5.0 b	9.0 ± 2.0 a	13.0 ± 2.5 c
Survival (%), day 21	50 a	90 b	88 b	91 b
Weight (mg) day 21	294 ± 179 a	725 ± 311 c	272 ± 121 a	354 ± 159 d
Survival (%) to pupal stage	20 a	82 b	85 b	88 b
Pupae				
Weight (mg)				
Male	273 ± 61 a	381 ± 40 c	316 ± 32 b	328 ± 26 b
Female	298 ± 79 a	435 ± 58 c	355 ± 50 b	353 ± 32 b
Survival (%) to ecolsion	10 a	78 b	82 b	84 b
Nondiapause (%)	50 a	93 b	73 a	83 b

[a] Values in any row followed by different letters differ significantly ($P < 0.05$, ANOVA). Values for day 21 and beyond are based on four replicates each having 20 insects.

was due simply to "dilution" by the rapidly increasing biomass of the cotyledons.

Young leaves of *S. alba* also had a high concentration of sinalbin, which in 8-day-old seedlings exceeded 10 mM. The concentration fell to about 3 mM during the next six days of growth (Figure 2A). During the same period, the amount of sinalbin per leaf increased by more than threefold (Figure 2B), but whether this increased amount was derived from de novo synthesis or translocation was not investigated. Undoubtedly, the fall in the concentration of sinalbin in growing leaves would have been much greater if the amount of sinalbin per leaf had remained unchanged. In spite of apparently different mechanisms, the concentration of sinalbin in older cotyledons and leaves was maintained at about 2 mM, a value close to that reported earlier for 4- to 6-week-old leaves of *S. alba* (1.68 mol/g fresh weight) (Reed et al., 1989).

DISCUSSION

This research has established that the high concentrations of *p*-hydroxybenzyl glucosinolate (sinalbin) found in the cotyledons of young seedlings and

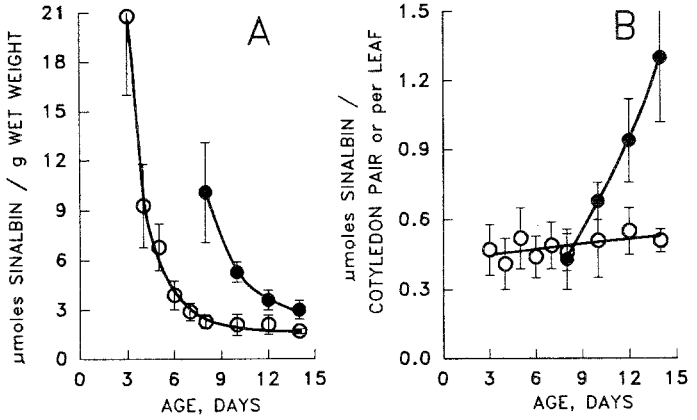


FIG. 2. Changes in the level of sinalbin in *S. alba* cv. Ochre during seedling growth. (A) changes in the concentration of sinalbin; (B) changes in the amount of sinalbin per cotyledon pair or per leaf. Open circles, cotyledons; closed circles, first true leaf. Each point represents the mean of the sinalbin level in four to eight samples that each contained 10 cotyledon pairs or 4–10 first true leaves. Vertical bars, standard deviation.

in young leaves of *S. alba* deter the feeding of flea beetles, *P. cruciferae*, and the bertha armyworm, *M. configurata*. The evidence in support of this finding was obtained from several diverse experiments. First, a glucosinolate fraction from cotyledons of *S. alba* obtained by DEAE–Sephadex A-25 chromatography deterred the feeding of *P. cruciferae* and *M. configurata* when incorporated into gelatin. A similar fraction prepared from the cotyledons of *B. napus*, which contains no sinalbin, did not deter feeding. Treatment of the glucosinolate fraction from cotyledons of *S. alba* with myrosinase and sulfatase, enzymes that are known to destroy glucosinolates, abolished the feeding deterrent activity. HPLC chromatography of the deterrent glucosinolate fraction revealed the presence of a high concentration of sinalbin and no other glucosinolates. Secondly, incorporation of known concentrations of sinalbin into gelatin altered the feeding response of both insects in a dose-dependent manner. Thirdly, uptake of sinalbin through the cut stems of seedlings of *B. napus*, a species normally palatable to *P. cruciferae* and *M. configurata*, reduced the feeding response of both insects. Finally, a study of sinalbin concentrations in developing seedlings of *S. alba* verified that the concentration of sinalbin shown to deter feeding in the artificial studies actually occurs in young cotyledons and leaves. Sinalbin, therefore, is a chemical component of the antixenosis (nonpreference) resistance mechanism described earlier for the cotyledons of *S. alba* (Bodnaryk and Lamb, 1991). The possibility that there are other as yet unidentified factors that contribute to the resistance of *S. alba* has not been ruled out by this study.

The effectiveness of sinalbin in deterring insect feeding needs careful evaluation in terms of both insect species and the rapidly changing profile of sinalbin concentration in the developing seedling. The high concentration of sinalbin found in young cotyledons (up to 20 mM) and young leaves (up to 10 mM) deterred the feeding of both *P. cruciferae* and *M. configurata*. The protection that sinalbin confers upon the vulnerable, newly emerged seedling appears to be critical for the first few days of survival under feeding pressure from flea beetles. Young seedlings of *B. napus*, which contain no sinalbin and no other feeding deterrent, are most susceptible to flea beetle feeding and are heavily damaged or killed in the field unless protected for two to three weeks by an insecticide (Lamb, 1984; Bodnaryk and Lamb, 1991). Tiny young leaves of *S. alba* may also be protected by their high concentration of sinalbin. These deterrent levels, however, are transitory, and the concentration of sinalbin in the cotyledons and leaves rapidly declines in the developing plant, largely due to simple "dilution" by the increasing tissue biomass. The lower concentrations of sinalbin found in the older *S. alba* plant (2–3 mM) offer little or no protection against *P. cruciferae* and may actually stimulate the feeding of this crucifer specialist. The less severe consequences of flea beetle feeding on the old, large leaves of a well-established plant may make the economics of maintaining a high concentration of sinalbin throughout a large tissue mass not worthwhile. The concentrations of sinalbin in the leaves of older plants are still effective in reducing the level of feeding by larvae of the more generalist feeder *M. configurata*, perhaps indicating an interesting compromise in *S. alba* between the costs of insect herbivory and the metabolic cost of protection.

In addition to its role in antixenosis, sinalbin also contributed to a second resistance mechanism identified in plants by Painter (1951), namely antibiosis. Like antixenosis, antibiosis caused by sinalbin is concentration dependent. Larvae of *M. configurata* fed young seedlings of *S. alba* in which the concentration of sinalbin in the cotyledons exceeded 20 mM had reduced levels of fitness as measured by their low survival, low body weights, and abnormal diapause compared to cohorts fed older plants of *S. alba* or *B. napus*. Although some of these effects may be due to partial starvation, it seems likely that sinalbin at low concentrations is antixenotic and at high concentrations antixenotic and antibiotic for larvae of *M. configurata*.

Glucosinolates in Brassicaceae comprise a group of some 80 thioglucosides with side chains of varying structure (reviewed in Larsen, 1981; Rodman, 1981) and having a variety of effects upon organisms that come in contact with them or their breakdown products (reviewed in Chew, 1988). There is a need for caution in interpreting the putative effects of glucosinolates on insect feeding. In some modern brassicas, glucosinolates do not determine the feeding rate of the flea beetle, *P. cruciferae* (Bodnaryk and Palaniswamy, 1990). As shown in this study, the effect of the glucosinolate sinalbin on insect feeding depends on

the stage of host plant development, the concentration of sinalbin, and the insect species being considered. The significance of glucosinolates as determinants of host-plant interaction must therefore be assessed on a case-by-case basis (Chew, 1988).

Sinalbin is a unique product of the mustard plant that has been highly selected for a specialized commercial use. Sinalbin also deters the feeding of important insect pests at critical times during the development of *S. alba*, making insect resistance in this plant the envy of crop protection entomologists. It seems clear, however, that the components of insect resistance in *S. alba* (antixenosis, antibiosis) that have sinalbin as their chemical basis would not be useful for transfer to the oilseed rapes or to other commercial crucifers because the presence of this glucosinolate usually would be incompatible with their end uses. It is possible to increase the level of insect resistance in the cotyledons and leaves of oilseed rape as shown by the experiments in which sinalbin was artificially introduced into *B. napus*. Incorporation of sinalbin into *B. napus* might be achieved by transfer of genes for sinalbin biosynthesis but would be acceptable only if the genes were not expressed to any significant degree in the seed pod. Unlike many other glucosinolates, trace amounts of sinalbin would be unlikely to pose significant human or animal toxicological problems since this product has been an accepted part of the human diet for centuries.

S. alba has other insect-resistance mechanisms apparently absent from the oilseed rapes, notably tolerance (Bodnaryk and Lamb, 1991) and wound-induced resistance (Palaniswamy and Bodnaryk, unpublished) that may not involve sinalbin. The utility of these traits for the oilseed rapes awaits laboratory and field evaluation of *S. alba* × *B. napus* hybrids (Ripley and Arnison, 1990) for insect resistance and canola quality.

Acknowledgments—I thank Lubomir Kudryk for skilled technical assistance and Drs. R.J. Lamb and P. Palaniswamy for helpful advice and discussion. This paper is dedicated to Dr. Tom Atkinson on the occasion of his retirement as Director of the Winnipeg Research Station.

REFERENCES

BEIRNE, B.P. 1971. Pest insects of annual crop plants in Canada. *Mem. Entomol. Soc. Can.* 78.

BODNARYK, R.P., and LAMB, R.J. 1991. Mechanisms of resistance to the flea beetle, *Phyllotreta cruciferae* (Goeze) in mustard seedlings, *Sinapis alba* L. *Can. J. Plant Sci.* 71:13–20.

BODNARYK, R.P., and PALANISWAMY, P. 1990. Glucosinolate levels in the cotyledons of mustard, *Brassica juncea* L. and rape, *B. napus* L. do not determine feeding rates of the flea beetle, *Phyllotreta cruciferae* (Goeze). *J. Chem. Ecol.* 16:2735–2746.

BONES, A.M. 1990. Distribution of beta-thioglucosidase activity in intact plants, cell and tissue cultures and regenerate plants of *Brassica napus* L. *J. Exp. Bot.* 41:737–744.

BUCHER, G.E., and BRACKEN, G.K. 1976. The bertha armyworm, *Mamestra configurata* (Lepidoptera: Noctuidae): Artificial diet and rearing technique. *Can. Entomol.* 108:1327–1338.

BURGESS, L., and WIENS, J.E. 1980. Dispensing allyl isothiocyanate as an attractant for trapping crucifer-feeding flea beetles. *Can. Entomol.* 112:93–97.

CHEW, F.S. 1988. Searching for defensive chemistry in the cruciferae, or, do glucosinolates always control interactions of cruciferae with their potential herbivores and symbionts? No. 1, pp. 81–112, *in* K.C. Spencer (ed.). Chemical Mediation of Coevolution. Academic Press, Toronto.

HANLEY, A.B., HEANEY, R.K., and FENWICK, G.R. 1983. Improved isolation of glucobrassicin and other glucosinolates. *J. Sci. Food Agric.* 34:869–873.

LAMB, R.J. 1984. Effects of flea beetle, *Phyllotreta* spp. (Chrysomelidae: Coleoptera), on the survival, growth, seed yield and quality of canola, rape and yellow mustard. *Can. Entomol.* 116:269–280.

LARSEN, P.O. 1981. Glucosinolates, pp. 501–525, *in* E.E. Conn (ed.). The Biochemistry of Plants, Vol. 7. Academic Press, Toronto.

MINCHINTON, I., SANG, J., BURKE, D., and TRUSCOTT, R.J.W. 1982. Separation of desulphoglucosinolates by reversed-phase high performance liquid chromatography. *J. Chromatogr.* 247:141–148.

PAINTER, R.H. 1951. Insect Resistance in Crop Plants. University of Kansas Press, Lawrence, Kansas.

PUTNAM, L.G. 1977. Response of four Brassica seed crop species to attack by the crucifer flea beetle, *Phyllotreta cruciferae*. *Can. J. Plant Sci.* 57:987–989.

REED, D.W., PIVNICK, K.A., and UNDERHILL, E.W. 1989. Identification of chemical oviposition stimulants for the diamondback moth, *Putella xylostella*, present in three species of Brassicaceae. *Entomol. Exp. Appl.* 53:277–286.

RIPLEY, V.L., and ARNISON, P.G. 1990. Hybridization of *Sinapis alba* L. and *Brassica napus* L. via embryo rescue. *Plant Breed.* 104:26–33.

RODMAN, J.E. 1981. Divergence, convergence, and parallelism in phytochemical characters: The glucosinolate–myrosinase system, pp. 43–79, *in* D.A. Young and D.A. Seigler (eds.). Phytochemistry and Angiosperm Phylogeny. Praeger, New York.

TURNOCK, W.J. 1985. Developmental, survival, and reproductive parameters of bertha armyworm, *Mamestra configurata* (Lepidoptera: Noctuidae) on four plant species. *Can. Entomol.* 117:1267–1271.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

FATE OF QUINOLIZIDINE ALKALOIDS THROUGH THREE TROPHIC LEVELS: *Laburnum anagyroides* (Leguminosae) AND ASSOCIATED ORGANISMS

Á. SZENTESI[1,*] and M. WINK[2]

[1]*Plant Protection Institute*
Hungarian Academy of Sciences
Budapest, POB. 102, H-1525, Hungary
[2]*Ruprecht-Karls-Universität Heidelberg*
Institut für Pharmazeutische Biologie
Im Neuenheimer Feld 364, D-6900 Heidelberg, Germany

(Received December 26, 1990; accepted March 1, 1991)

Abstract—The quinolizidine alkaloids (QA) of golden rain, *Laburnum anagyroides*, and those of phytophagous insects associated with the plant, as well as of parasitoids of the latter, were analyzed by capillary GLC and GLC-MS. The alkaloid content in samples of vegetative plant parts was high at the beginning of the season, then decreased, while that of reproductive organs was high throughout flowering, pod formation, and maturation. The analyses showed that the QA of the plant passed through two higher trophic levels (herbivorous insects and their parasitoids) and that the alkaloid pattern changed little during the passage. The alkaloids were present in two phytophagous insect species associated with golden rain: the predispersal seed predator, *Bruchidius villosus* [5–13 μg/g fresh weight (fw)], and *Aphis cytisorum* (182–1012 μg/g fw), an aphid that feeds on shoots, leaves, and inflorescences. Braconid and chalcidoid parasitoids emerging from the bruchid host also contained alkaloids (1.3–3 μg/g fw), as did three foraging ant species, *Lasius niger*, *Formica rufibarbis*, and *F. cunicularia* (45 μg/g fw), that visited the aphid colonies or honeydew-covered leaves of aphid-infested plants. The hypothesis that developing bruchid larvae and/or the plant "manipulate" QA supply to infested seeds was not supported, because QA content of leftover endosperm in seeds after bruchid development was similar to that of uninfested seeds. The frass of developing bruchid larvae was rich in QA (31 mg/g dry weight). While aphids sequestered, the bruchid larvae took up and eliminated QA with the frass without chemical transformation.

Key Words—*Laburnum anagyroides*, *Robinia pseudacacia*, *Aphis cytiso-*

*To whom correspondence should be addressed.

rum, Homoptera, Aphididae, *Bruchidius villosus*, Coleoptera, Bruchidae, *Lasius niger*, *Formica* species, Hymenoptera, Formicidae, *Triaspis thoracicus*, Braconidae, Chalcidoidea, quinolizidine alkaloids.

INTRODUCTION

Evidence is accumulating that interactions at three trophic levels can be mediated and/or influenced by chemicals originating from only one member of the food web, the plant (Price et al., 1980). For example, the development time of the parasitoid *Hyposoter exiguae* was prolonged in its host, *Heliothis zea*, and the percentage of adult survivorship decreased, if α-tomatine, the naturally occurring steroid glycoside in tomatoes, was present in the host's diet to the extent of 0.3–0.5% (Campbell and Duffey 1979, 1981). Similarly, the percentage of emerging *Apanteles congregatus* parasitoids from the host *Manduca sexta* was significantly lower in comparison with the control when they developed in a host reared on a diet containing a higher level of nicotine (Thurston and Fox, 1972). Benn et al. (1979) found that pyrrolizidine alkaloids were present in the parasitoid *Microplitis* emerging from the insect host *Nyctemera* living on *Senecio spathulatus* plants. However, the insect hosts were not affected by the chemicals in the above studies.

Quinolizidine alkaloids (QA) are characteristic secondary metabolites of the phylogenetically primitive tribes of the family Leguminosae (Kinghorn and Balandrin, 1984) and are especially abundant in the members of the tribe Genisteae (Waller and Nowacki, 1978). They are assumed to play an important role in plant defense against nonadapted phytophages (Waller and Nowacki, 1978; Wink, 1984c, 1985, 1988), but also constitute a nitrogen source for the plant seedlings (Nowotnowna, 1928, cited in Waller and Nowacki, 1978; Wink, 1985; Wink and Witte, 1985a). Phytophagous insects, such as aphids, acquire defense against generalist predators by sequestering QA (Wink and Römer, 1986). QA also have bacterio-and fungistatic properties (Wink, 1984a; Tyski et al., 1988) besides being highly toxic to most vertebrates and inhibitory for the development of other plant species (Pöhm, 1966; Waller and Nowacki, 1978; Wink, 1983).

The primary aim of the present paper wa⁻ to follow the fate of QA, both qualitatively and quantitatively, through a complex multispecies food web from the primary producer (*Laburnum anagyroides*) to the first (herbivorous) and second (carnivorous) consumer levels, as well as to scavengers (aphid-visiting ant species). For this purpose, we sampled and analyzed plant parts, and insect herbivores and organisms associated with them (parasitoids and ant species). We also were interested in the question of whether QA taken up by the bruchid seed predator would be found changed in a quantitative and compositional fashion when eliminated.

For some time it also has been known that some legume species are able to selectively abort seeds and/or pods containing seed predator larvae. Such and other "manipulations" by the plant can affect transport of nutrients and non-nutrients into the seeds (Stephenson, 1981). As, in the present plant seed predator system, egg-laying by bruchid females occurs on green pods and larvae develop in the ovules, the plant, by means of active defense, may alter the chemical environment within the seeds. Therefore, we also examined whether infested seeds contained a higher level of QA than uninfested ones.

METHODS AND MATERIALS

Host Plant. Laburnum anagyroides Medik (Leguminosae) is an endemic plant species in central and southern Europe (Soó, 1966). The tree is also widely grown as an ornamental in parks and gardens throughout Europe. Yellow flowers bloom within inflorescences through May and June. Pods containing one to nine seeds appear in June, grow to 2–8 cm in length, and harden by the end of August. Pods are dehiscent and remain on the plant. Many pods open in late September; however, most of them open only during the next spring. Some seeds fall out of the pod during the following spring, but many remain in the pod for a year or longer. Seeds are dark brown and 2–4 mm long.

Insect Herbivores. Aphis cytisorum Hart. (Homoptera: Aphididae) is a dark grey aphid with a waxy coating covering the body. Aphids hatch from overwintering eggs placed on the plant's trunk close to the soil surface. They move up the plant and form colonies on distal parts of new shoots, inflorescence axes, and leaves' lower surfaces. Individual aphid colonies are distinct; however, if the level of infestation is high, they later coalesce. Honeydew is produced in large amounts and is collected by several ant species.

The adults of the predispersal seed predator, *Bruchidius villosus* Fabr. (Coleoptera: Bruchidae), emerge from seeds of *L. anagyroides* and probably other overwintering sites such as under tree bark, although this is not well established. Females lay eggs on the surface of immature pods. Larvae enter through the pod wall and feed on developing seeds. By mid-August, larvae complete development and pupate, and adults emerge from open pods at the end of August or overwinter within seeds of closed pods on the plant.

Parasitoids. B. villosus is parasitized by chalcidoid and braconid species that emerge either in August–September or overwinter as larvae or adults inside the seeds and emerge the following May–June. (See more details in the Results section.)

Sampling Sites and Preparation of Plant and Insect Material for Chemical Analysis. Because *L. anagyroides* generally occurs as sporadically scattered individual trees and only rarely forms larger, homogeneous stands, both insect

and plant materials came from several collection sites in Hungary. Individual trees of (1) 2–3 m height and max. 10-cm trunk diameter, for which the local climatic and soil conditions varied greatly; and (2) one homogeneous stand (ca. 20 trees approx. 10–15 years old, growing on a long-abandoned damp area, on clay soil) were sampled for green plant parts, aphids, and ant species during 1987 (see Table 1 for dates of sampling).

TABLE 1. QUALITATIVE QA COMPOSITION OF *L. anagyroides* PARTS

Date (1987)/sample	Composition (% of total QA)			
	1[a]	2	3	4
May 27				
Leaf petioles and shoots	13.7	75.9	5.7	1.0
Leaf laminas	56.2	38.4	0.2	0.6
Inflorescence axis and flower pedicels	16.3	82	1.1	0.4
Flowers (sine pedicels)	14.9	85.1		
Very young pods[b]	30.6	67.6	0.7	0.3
June 8				
Inflorescence axis and flower pedicels	9.7	85	2.4	1.7
Half-grown pods I[c]	27.3	69.8	2.6	0.2
Half-grown pods II[d]	26.1	73.6		
July 22				
Leaf petioles and laminas	4.6	95		
Inflorescence axis and pod pedicels	1.0	89.9	2.3	3.8
Fully grown pods	1.3	97.7	0.7	
September 2–4				
Leaf petioles	6.3	88	5.2	1.0
Leaf laminas	11.8	88.2		
Mature dry pod wall (sine seeds)	4.1	12.7	74.4	8.1
1987 from 4 localities	6.9	15.1	76.7	1.8
1989	3.1[e]	95.5		
	(1.3)	(1.3)		
Leftover endosperm after bruchid development	4.5	94		
Endosperm (control)	3.4	95		
Testa of bruchid-infested seeds	1.5	97		
Testa (control)	5.4	93		

[a] 1 = *N*-Methylcytisine; 2 = cytisine; 3 = 5,6-dehydrolupanine; 4 = anagyrine.
[b] 0.5–1.5 cm long.
[c] "Small": 2–5 cm long.
[d] "Big": 4–6 cm long.
[e] Mean (SD).

For alkaloid analyses of green plant parts, lyophilized samples were used. One 6- to 60-g fresh weight sample of living plant parts, pooled from one to two trees for each sampling date and plant part (as indicated in Table 1) were removed and immediately put into a cool box, transferred to the laboratory, and stored at −60°C until further processing. The deep-frozen samples were crushed in a mortar filled with liquid N_2, put into a lyophilizer (Edwards, Great Britain, model EF 03), freeze-dried, ground, placed in vials, and finally placed back into the lyophilizer for sealing under vacuum. The samples were 2–9 g dry weight (dw) after lyophilization. In addition to the above, dry pods (without seeds), and whole mature seeds (4–5 g each sample; one from 1987 and several from 1989), testa (516 mg), and endosperm (1 g) of mature, dry intact seeds, as well as testa (184 mg) and leftover endosperm of seeds (447 mg) in which the predispersal seed predator bruchid had completed development, were collected and analyzed.

Aphids were collected from infested trees by brushing them into a container, and ants from aphid colonies and honeydew-covered leaves were collected with an aspirator. There were three samples of *A. cytisorum* collected at three points of time in 1987 (see Table 2), each containing approximately 4900, 7500 and 14,000 aphids, and two samples of ants, one a mixed sample of *Formica rufibarbis* F. and *F. cunicularia* Latr. and the other a homogeneous sample of 444 *Lasius niger* L. (Hymenoptera: Formicidae).

On September 2, 1987, a large sample of pods, with even mature pods still closed, were collected for obtaining bruchid adults and parasitoids for chemical analysis. The seeds from one batch of pods were removed and placed, at 25–26°C, under a photoperiod of 20:4 hr (light–dark). The containers were covered with linen cloth and humidified with wet cotton until both bruchids and parasitoids emerged from the seeds.

Emerging insects were collected daily and stored at −20°C in 70% EtOH. For alkaloid analysis, the following were used: (1) two samples of *B. villosus* containing 357 and 520 beetles, respectively; (2) three samples of parasitoids, one of 43 braconids, one of 64 pteromalids, and a third of 82 specimens of about three species of parasitoids. Parasitoids emerged from the same seed samples from which the bruchids originated.

Following the emergence of bruchids and parasitoids, one sample of empty cocoons (94 mg) of the braconid parasitoid, and one sample of bruchid larval frass together with pupation chambers of larvae lined with cemented fecal material (134 mg) were collected from infested seeds and used for analysis.

Chemical Analysis of Plant and Insect Material. Plant samples (500 mg) were homogenized in 15 ml 0.5 M HCl and left standing for 1 hr. The homogenates were made alkaline by adding 6 N NaOH and applied to standard Chem-Elute columns for solid-phase extraction. After elution with methylene chloride, the solvent was evaporated in vacuo.

TABLE 2. QUANTITATIVE AND QUALITATIVE QA COMPOSITION OF INSECTS ASSOCIATED WITH *L. anagyroides*

Sample/date	Alkaloid content (μg/g)[a]	Alkaloids (% of total QA)			
		1[b]	2	3	4
A. cytisorum					
June 16, 1987	182	5.6	92.5	1.7	
July 5, 1987	450	4.6	91	4.2	
July 29, 1987	1,012	3.2	94	2.7	0.4
B. Villosus					
Sept. 9–15, 1987	13		100		
Sept. 18, 1987	5		100		
Larval frass	31,276[c]	0.8	98		
Lasius niger					
June 16, 1987	45	2.5	95	1.2	1.2
Formica rufibarbis and *F. cunicularia*					
June 16–July 5, 1987	[d]	1.9	91	6.5	0.3
Triaspis thoracicus					
Sept. 8–17, 1987	3		100		
Triaspis cocoons	1,617[c]		99.5		
Parasitoids I[e]					
Sept. 10, 1987–Jan. 25, 1988	1.3		100		
Parasitoids II[f]					
Sept. 18, 1987–Jan. 11, 1988	[d]	40	60		

[a] Measured on a fresh weight basis.
[b] 1 = *N*-Methylcytisine; 2 = cytisine; 3 = 5,6-dehydrolupanine; 4 = anagyrine.
[c] Measured on a dry weight basis.
[d] Due to the lack of data on body weights, alkaloid contents per gram body weight could not be calculated; however, the samples contained 76 and 5 μg alkaloids, respectively.
[e] Pure sample of one pteromalid species.
[f] Mixed sample of a minimum of three eulophid and pteromalid species.

Alkaloid extracts were analyzed by TLC (cyclohexane–diethylamine, 7 : 3) and capillary GLC: Perkin Elmer GLC 8500 or Chrompack CP438A; DB-1 column (30 m × 0.3 mm); oven: 150°C, 2 min isothermal, to 320°C with 20°C/min; injector: 250°C; detector: 320°C; carrier gas: helium, 92 kPa. Quantification was carried out with cytisine as an external standard using a nitrogen-specific detector. GLC-MS measurements were performed as described in Wink et al. (1983). Alkaloids were identified according to their specific

Kovats retention indices and mass spectra and by using alkaloid standards (for details see Wink et al., 1983).

The QA contents in plant materials were determined on a dry weight (dw) basis, those of insects on a fresh weight (fw) basis. The QA contents of bruchid adults, measured on a fresh weight basis, were converted into dry weight to compare with QA content of larval feces that was measured on a dry weight basis. For the conversion we assumed that QA content was 10 times as high in dry materials.

RESULTS

Parasitoids. There was one braconid species, *Triaspis thoracicus* Curt. (Hymenoptera), present in the samples. Among the chalcidoid species, pteromalids, eulophids, and eupelmids were represented, and the presence of a *Tetrastichus* sp. (Hymenoptera: Eulophidae) was confirmed. In case of a *L. anagyroides* seed sample (containing approximately 5000 seeds), pteromalids and braconids emerged from ca. 18% of the bruchid-infested seeds.

Ants. At least three ant species (*Lasius niger*, *Formica rufibarbis*, and *F. cunicularia*, roughly in a 15 : 1 : 2 ratio) regularly visited the aphid colonies or, in the case of high aphid infestation, only the twigs and the leaves that were covered with honeydew.

Alkaloid Analysis: Qualitative Aspects. QA were detected in all components of food chain examined. Both the plant and insect samples showed similar alkaloid patterns (Tables 1 and 2). There were four quinolizidine alkaloids identified by TLC, capillary GLC, and GLC-MS: cytisine, *N*-methylcytisine, 5,6-dehydrolupanine, and anagyrine (Figure 1).

As for seasonal change in alkaloid composition, cytisine remained the major component in almost all plant samples. There was not much change in minor (5,6-dehydrolupanine and anagyrine) alkaloids either. The only substantial variation was shown in *N*-methylcytisine. An unusual pattern was detected in mature seeds and pod walls (without seeds) collected in 1987. Here, the cytisine content was very low, while the level of 5,6-dehydrolupanine was high. In the 1989 seed samples, cytisine was the main alkaloid (95.5 $\pm$ 1.3%, mean $\pm$ SD, $N = 4$), and *N*-methylcytisine was present in 3.1 $\pm$ 1.3% ($N = 4$). In comparison with alkaloids in the endosperm of seeds, *N*-methylcytisine made up a smaller percentage of two alkaloids found in the frass of *B. villosus* (Tables 1 and 2).

Bruchidius villosus beetles, *T. thoracicus*, and one pteromalid parasitoid sample contained predominantly cytisine; the other alkaloids were not present or occurred at levels too low to detect. In parasitoid sample II (Table 2), however, in addition to cytisine, a substantial proportion of *N*-methylcytisine was

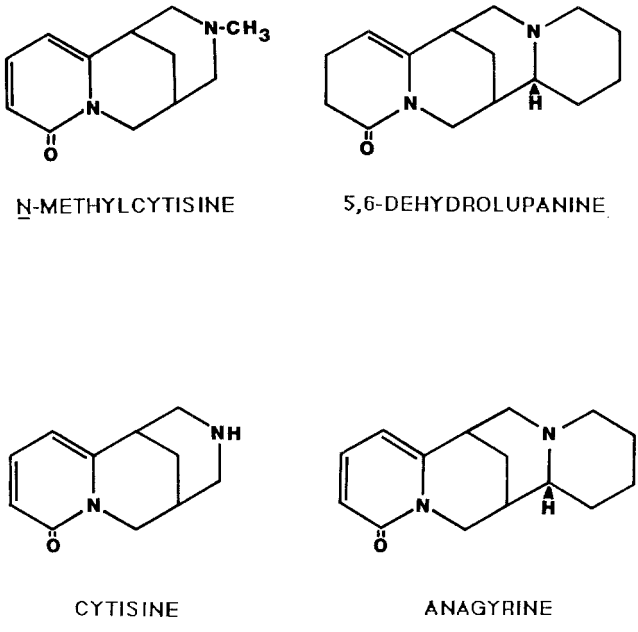


FIG. 1. Structural formulas of the quinolizidine alkaloids found in the samples.

present also. Larval feces of the bruchid and the cocoons of the braconid parasitoid were low in *N*-methylcytisine and rich in cytisine (Table 2).

Quantitative Aspects. Individual plant parts (on a microgram per gram dry weight basis) showed a seasonal trend in total alkaloid contents (Figure 2). In May, much of the alkaloid content was found in the reproductive organs, specifically in inflorescence axes and individual flower pedicels. Since flowering and pod formation is continuous within a given inflorescence and within a time period, the very young pods already present at peak flowering also contained a relatively very high amount of QA. Gradually, stems, leaves, and inflorescence axis and/or flower pedicels lost their QA contents by September, while developing pods maintained some (ca. 1 mg total QA/g dry plant material) until ripening. Mature seeds were especially rich in QA (Figure 2). In four samples of such seeds (collected at four different localities in 1989), QA content ranged from 9.075 to 23.161 mg/g dw. Of the seed parts (on a microgram per gram dry weight basis), endosperm often contained four to five times as much QA as seed testa (Figure 3).

Alkaloid contents of bruchids (determined on a fresh weight basis) were much lower than either those in fully grown green pods, mature dry seeds, or endosperm (Table 2, Figure 3). On the other hand, in the aphids, the alkaloid levels were higher than in the plant (Table 2) and were in accordance with the

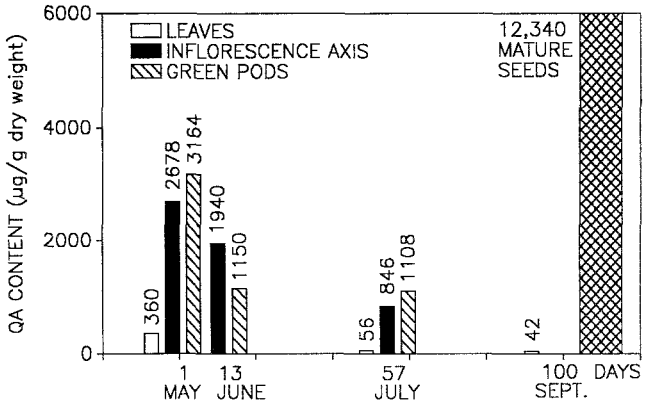


FIG. 2. QA content of various parts of *L. anagyroides* between May 27 and September 2.

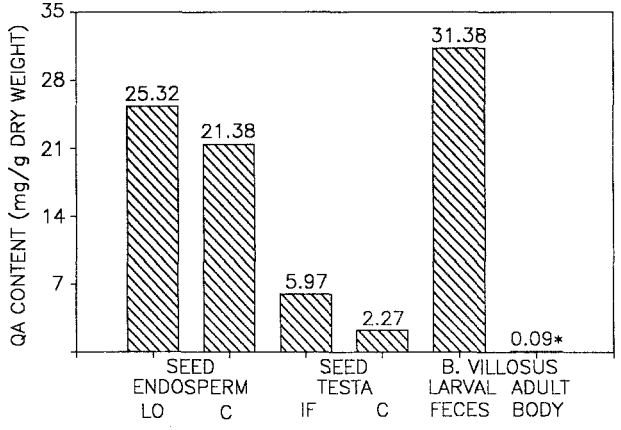


FIG. 3. QA contents of control and seed predator-infested *L. anagyroides* seed parts, as well as larval feces and bruchid adults. LO = leftover (i.e., bruchid-infested) endosperms, IF = infested, C = control. Asterisk denotes that the QA contents of adult bruchids were measured on a fresh weight basis, but were converted into dry weight values (see Methods and Materials for details).

seasonal change in the plant's QA content. Although the contents of QA in aphids were measured on a fresh weight basis, a cautious estimate on a dry weight basis (see Methods and Materials) would yield values 10 times as high as those found in the corresponding plant tissues. The most frequently visiting ant species, *L. niger*, weighed ca. 2 mg fw/individual and contained ca. 0.1

μg/ant of alkaloids. Alkaloid contents of parasitoids were much lower than those either in plant parts or in the host, but still detectable (Table 2).

QA content of endosperm of bruchid-infested and uninfested seeds were in the same range. The larval frass had a very high QA content (Figure 3 and Table 2). The peak oviposition period of bruchids (July; Szentesi, unpublished observation) coincided with a relatively low alkaloid content in the pod (a third to half of that present at early pod development).

DISCUSSION

Analysis of the quinolizidine alkaloid content of *L. anagyroides* and of associated insects showed that this major group of secondary plant metabolites passed through several trophic levels almost unchanged in terms of composition. One or more of the plant alkaloids appeared in two herbivores (an aphid and a bruchid), in various parasitoids, and in three ant species. This is remarkable if one considers the diversity and host specificity of participating organisms (see a more detailed discussion of this later).

Typical components of the QA group are cytisine (found in more than 79 plant species), *N*-methylcytisine, and anagyrine (Figure 1), constituting from trace quantities to 98% of total alkaloid content of a plant species (Mears and Mabry, 1971). Main sites of accumulation are the epidermal layers (Wink et al., 1984) and the reproductive organs, e.g., fruits and/or seeds (Wink, 1987; and present study).

Host Plant. In *L. anagyroides*, fresh leaves and stems contain about 150 μg/g fw cytisine (Wink, 1984c), and in both seeds and pods cytisine is the major alkaloid (Wink, 1984d). The QA level changed in different plant parts during the season. First young shoots, then inflorescences, and finally developing and mature pods and/or seeds contained the highest amount of QA (Figure 2). As reported also for other leguminous species (Waller and Nowacki, 1978; Wink and Hartmann, 1981), we found that *L. anagyroides* mobilized QA out of leaves and into seeds by the end of the season, showing that such compounds were not simply waste products. These findings are in agreement with the results of Greinwald et al. (1990) on *L. watereri* (Kirchn.) Dipp., a hybrid of *L. alpinum* and *L. anagyroides*. However, there were unusually high 5,6-dehydrolupanine and low cytisine levels in the 1987 pod wall and mature seed samples. The cause of these lower levels is unclear.

Insect Herbivores. Both *A. cytisorum* and *B. villosus* took up QA from *L. anagyroides*. The quantity present in the body, however, differed considerably between the two species (Table 2). *A. cytisorum* accumulated alkaloids against a concentration gradient. On two other legume species, *Petteria ramentacea* (Wink and Witte, 1985b) and *Cytisus scoparius* (Wink et al., 1982), *A. cyti-*

sorum also showed a higher level of QA content in the body than found in particular plant parts. The quantities of QA determined in aphid samples taken at three different points of time (Table 2) showed a seasonal change similar to that found in the plant. One of the reasons for this is that the aphids followed fresh tissue growth from shoot to flower axis to pod. Like many aphid species, *A. cytisorum* also taps the phloem sap, where the QA are reported to be transported in *Cytisus* and *Lupinus* plant species (Wink and Witte, 1984).

In the case of the bruchid species, the only stage feeding on the plant is the larva. Therefore, the QA uptake and quantity refer to this stage, although these were determined in the adults. The alkaloids present in the larvae eventually pass through the pupal stage to reach the adults. We think that the amount of QA taken up by a bruchid larva at any time during its development must have been in the range (ca. 1 mg/g dw) found in the plant tissue in which it developed in July. (In contrast, by the time the larvae completed development and adults emerged, there was a 10-fold increase of QA content in mature seeds in comparison with that in green pods.)

There was a high amount of QA continuously excreted by the bruchid larval feces: ca. 30 times as high as the quantity found in pods, and 200–600 times as high as the amount found in beetles (the latter was measured by a fresh weight basis and converted to dry weight). This reveals a very effective QA-eliminating process in the larvae. We assumed that they either continuously and quickly excreted the QA taken up while feeding, or the QA were degraded metabolically. The first mechanism seemed to be operating, although some changes in the qualitative QA pattern did occur. The fact that the feces were enriched in QA was the result of nutrients removal, and not the consequence of increasing QA content in the plant. We assume that, through *N*-demethylation reaction in the gut, some metabolic transformations also occur in the bruchid larva.

In connection with the described quantitative relationships, we believe that the possibility of "manipulating" QA content in the bruchid-infested seeds of *L. anagyroides* could exist both on the plant's and the seed predator's side. Janzen (1976) and Stephenson (1981) listed cases when fruits with bruchid larvae inside were subsequently aborted, although no proximal causes of this were known. In the case of *Laburnum/Bruchidius*, we considered the following possibilities: (1) The plant responded to the presence of bruchid larvae by delaying or hindering the maturation of seeds by not transporting all the nutrients, but increasing transport of the QA into the seeds. (2) Similarly, bruchid larvae could have avoided much of the QA transported to the seeds by either (a) influencing the transport itself or (b) completing development by the time of peak QA build-up (early to mid-August). The first hypothesis could be rejected with the present bruchid–plant relationship because infested and control seed endosperms contained the same quantities of QA (Figure 3); that is, the plant did not "manip-

ulate'' seed development. If abortion of bruchid-infested seeds occurred at all with *Laburnum*, it must have taken place at an earlier phase of seed development. As for the second hypothesis, assumption ''a'' is a possibility; however, we do not have evidence for it. Assumption ''b'' seems to be a realistic one, although it also implies that a high QA content is not preferred by the bruchid larvae.

As for the qualitative composition: the composition found in the aphids was similar to that demonstrated by the chemical analyses in the hostplant *L. anagyroides* (Tables 1 and 2). In other studies, the similarity was only partial. For example, although the most abundant alkaloid in *C. scoparius* is sparteine, 17-oxosparteine (derived from sparteine) was the most abundant alkaloid in aphids feeding on this plant (Wink et al., 1982). In *A. cytisorum* feeding on *Petteria ramentacea*, there was only one alkaloid, cytisine, found in the aphid, out of six occurring in the plant (Wink and Witte, 1985b).

The green *Laburnum* fruit varies more in QA composition during the time of bruchid larval development than does the mature seed or the bruchid adult. Although three components of the QA assemblage were present in fully grown pods, only two of them were found in larval frass, and one (cytisine) in the adult bruchid (Tables 1 and 2). Obviously, the plant gives the base for changes in the proportion of chemicals found in the herbivore feeding on it. For instance, more recently, Greinwald et al. (1990) identified 17 different QA from pods of *L. watereri*, of which 15 were not present in seeds.

We assume that both the aphid and the bruchid are likely to face much inter- and intraspecific chemical diversity in plant hosts. Besides the host plant, *L. anagyroides*, known to us, both insect species are reported from six other QA-containing plant species, and from a species, *Robinia pseudacacia*, devoid of QA (Krécsy, 1886; Kiss, 1895; Vadas, 1911; Escherich, 1923; Wahl, 1925; Zacher, 1936; Fischer, 1938; Hoffmann, 1945; Börner, 1952; F.P. Müller, personal communication). The main constituents of the latter are flavonoids and the nonprotein amino acid, canavanine (Bell, 1971). With some exceptions (see Wink, 1984b; Wink and Witte, 1984; Greinwald et al., 1990), few quantitative data are available on the seasonal, diurnal, or within-plant distribution of QA.

Herbivore infestation level and QA content of the host plant may be convergent. For example, the feeding sites of the legume-specialist aphid, *Acyrthosiphon spartii*, on the plant *Sarothamnus scoparius* were correlated with the highest concentration of the major alkaloid, sparteine (Smith, 1966). In contrast, the population density of a generalist aphid, *Macrosiphum euphorbiae*, decreased with increasing alkaloid level in *L. angustifolius* and *Cytisus scoparius*, respectively (Brusse, 1962; Wink et al., 1982). Possibly the within-plant density of a generalist insect species is inversely proportional, while that of an adapted specialist is directly proportional, to the alkaloid content of the plant.

Parasitoids. There was a decrease in QA content at the third trophic level

(Table 2), suggesting a limited, but still significant, passage of alkaloids. Similar passage of plant allelochemicals to parasitoids through an insect herbivore have been demonstrated under experimental conditions by Thurston and Fox (1972), Campbell and Duffey (1979, 1981), Thorpe and Barbosa (1986), and Barbosa et al. (1986). Benn et al. (1979) found about 300 μg alkaloid/larva in the lepidopterous host *Nyctemera* collected on *Senecio* plants in the field and stated that the alkaloids were present in the braconid parasitoids too, although no data were given. Despite the low level of QA detected in parasitoids in the present study, it is noteworthy that a substantial amount of cytisine was passed also to the pupal chamber wall, i.e., the cocoon, spun by the braconid parasitoid larvae (Table 2). The composition of QA found in the parasitoid samples corresponded to that present in adult bruchids with one exception (parasitoid sample II, Table 2) where *N*-methylcytisine occurred in large amounts.

Among the species found, *Tetrastichus* is reported both as a primary parasitoid (on the herbivore, *B. villosus*) and a secondary one (on the braconid, *T. thoracicus*), that is a (hyper)parasitoid (Medvedeva, 1978), or "metaparasite" sensu Steffan (1981), which may further complicate the possible routes of passage of alkaloids throughout the foodweb.

The parasitoid species we studied live in various bruchid species; e.g., *T. thoracicus* parasitizes a number of other unrelated bruchid species (*Bruchus atomarius*, *B. lentis*, and *B. rufimanus*) (Luca, 1970, 1977). These species, in turn, inhabit phytochemically diverse host plants.

Ants. The alkaloid analyses demonstrated (Table 2) that ants gather a substantial amount of QA from honeydew, both directly from tending aphids and from leaf surfaces. We think that in the latter case, UV radiation may decrease the compounds' stability. QA and/or the breakdown products are probably taken back to the nests where their fate is not known.

QA Along the Food Web. It is believed that peak QA quantities in plant parts throughout the season serve plant defense purposes (Waller and Nowacki, 1978; Wink, 1984c, 1985; Wink and Hartmann, 1981). Indeed, QA have important effects on the behavior and physiology of nonadapted herbivores. Their feeding-deterrent activity was demonstrated on the tortricid *Choristoneura fumiferana* (Bentley et al., 1984), on the pea aphid *Acyrthosiphon pisum* (Dreyer et al., 1985), and even on mollusks (Wink, 1984c).

The QA found in *L. anagyroides* have been sequestered by the aphids and excreted without much change by other members of the food web investigated. This suggests that QA might not play a primary role in the lives of the latter species. We do not know whether *A. cytisorum* on *L. anagyroides* enjoys any protection from specialized parasitoids or predators. However, evidence indicates that QA-containing aphids can gain protection from generalist predators. Wink and Römer (1986) and Gruppe and Römer (1988) demonstrated that two predators, *Carabus problematicus* and first-instar larvae of *Coccinella septem-*

punctata, were narcotized (temporarily immobilized) and killed, respectively, after feeding on *Macrosiphum albifrons* colonies grown (and containing 1.3 mg/g fw alkaloids) on a "bitter" *Lupinus* variety, whereas they preferentially consumed the aphids developing on a "sweet," i.e., alkaloid-free, lupine without exhibiting adverse symptoms.

In summary, all trophic levels studied tolerated the presence or absence of QA. This ability may prove to be a good strategy in a varying environment, variability meaning that both within- and among-plant species show chemical diversity.

Acknowledgments—An earlier version of the manuscript profited much from the careful reading and suggestions of Dr. Marion O. Harris (U.S.A.). We are also grateful to Drs. C.B. Montllor (U.S.A.), T. Jermy (Hungary), and E.A. Vogt (U.S.A.) for critical remarks; to Drs. L. Gallé, L. Szalay-Marzsó, C. Erdélyi, J. Papp (Hungary), L. Borowiec (Poland), Prof. F.P. Müller, Dr. H. Wendt (Germany), and Mr. A. Podlussány (Hungary) for species identification and comments; and to Mr. L. Tibai and Miss K. Ferencz (Hungary) for technical assistance. Special thanks are due to Dr. L. Witte (Germany) for carrying out the GLC-MS measurements.

REFERENCES

BARBOSA, P., SAUNDERS, J.A., KEMPER, J., TRUMBULE, R., OLECHNO, J., and MARTINAT, P. 1986. Plant allelochemicals and insect parasitoids: Effects of nicotine on *Cotesia congregata* (Say) (Hymenoptera: Braconidae) and *Hyposoter annulipes* (Cresson) (Hymenoptera: Ichneumonidae). *J. Chem. Ecol.* 12:1319–1328.

BELL, E.A. 1971. Comparative biochemistry of non-protein amino acids, pp. 179–206, *in* J.B. Harborne, D. Boulter and B.L. Turner (eds.). Chemotaxonomy of the Leguminosae. Academic Press, London.

BENN, M., DE GRAVE, J., GNANASUNDERAM, C., and HUTCHINS, R. 1979. Host-plant pyrrolizidine alkaloids in *Nyctemera annulata* Boisduval: Their persistence through the life-cycle and transfer to a parasite. *Experientia* 35:731–732.

BENTLEY, M.D., LEONARD, D.E., REYNOLDS, E.K., LEACH, S., BECK, A.B., and MURAKOSHI, I. 1984. Lupine alkaloids as larval feeding deterrents for spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). *Ann. Entomol. Soc. Am.* 77:398–400.

BÖRNER, C. 1952. Europe centralis Aphides—Die Blattläuse Mitteleuropas. *Mitt. Thüring. Bot. Ges.* Heft 4. Bheft 3. Weimar. 484 pp.

BRUSSE, M.J. 1962. Alkaloid content and aphid infestation in *Lupinus angustifolius* L. *N.Z. J. Agric. Res.* 5:188–189.

CAMPBELL, B.C., and DUFFEY, S.S. 1979. Tomatine and parasitic wasps: Potential incompatibility of plant antibiosis with biological control. *Science* 205:700–702.

CAMPBELL, B. and DUFFEY, S.S. 1981. Alleviation of α-tomatine-induced toxicity to the parasitoid, *Hyposoter exiguae*, by phytosterols in the diet of the host, *Heliothis zea*. *J. Chem. Ecol.* 7:927–946.

DREYER, D.L., JONES, K.C., and MOLYNEUX, R.J. 1985. Feeding deterrency of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphid (*Acyrthosiphon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. *J. Chem. Ecol.* 11:1045–1051.

ESCHERICH, K. 1923. Die Forstinsekten Mitteleuropas. B.2. Paul Parey Verlag, Berlin. 662 pp.

FISCHER, A. 1938. *Spermophagus cisti* F. (syn. *Bruchus cisti* F.) als Schädling der Wildformen von *Lupinus angustifolius* L. und *Lupinus luteus* L. *Z. Pflanzenkrankh. Pflanzenschutz* 48:592–597.

GREINWALD, R., SCHULTZE, W., and CZYGAN, F.-C. 1990. Über der Alkaloidzusammensetzung der oberirdischen Teile von *Laburnum watereri* (Kirchn.) Dipp. *Biochem. Physiol. Pflanzen* 186:1–10.

GRUPPE, A., and RÖMER, P. 1988. The lupin aphid (*Macrosiphum albifrons* Essig, 1911) (Hom., Aphididae) in West Germany: Its occurrence, host plants and natural enemies. *J. Appl. Entomol.* 106:135–143.

HOFFMANN, A. 1945. Faune de France. 44. Coléoptères Bruchides et Anthribides. Lechevalier, Paris. 184 pp.

JANZEN, D.H. 1976. Two patterns of predispersal seed predation by insects on Central American deciduous forest trees, pp. 179–188, *in* J. Burley and B.T. Styles (eds.). Tropical Trees, Variation, Breeding, and Conservation. Linnaean Society Symposium Series 2, London.

KINGHORN, A.D., and BALANDRIN, M.F. 1984. Quinolizidine alkaloids of the Leguminosae: structural types, analysis, chemotaxonomy, and biological activities, pp. 105–149, *in* W.S. Pelletier (ed.). Alkaloids: Chemical and Biological Perspectives. John Wiley & Sons, New York.

KISS, F. 1895. Rovarkárositások a szegedi m. kir. erdögondnokság kerületében [Damages caused by insect pests in the Royal Hungarian Forest at Szeged]. *Erdészeti Lapok* 34:1025–1030 (in Hungarian).

KRÉCSY, B. 1886. Az akáczfa levél-tetü tömeges megjelenéséröl [An outbreak of black locust aphid]. *Rovartani Lapok* 3:148 (in Hungarian).

LUCA, Y. DE. 1970. Catalogue des metazoaires parasites et predateurs de bruchides (Coléoptères). *Ann. Soc. Hortic. Hist. Nat. Hérault* 110:1–23.

LUCA, Y. DE. 1977. Catalogue des metazoaires parasites et predateurs des Bruchides (Col.) (troisième note). *Bull. Soc. Etud. Sci. Nat. Nimes* 55:5–22.

MEARS, J.A., and MABRY, T.J. 1971. Alkaloids in the Leguminosae, pp. 73–178, *in* J.B. Harborne, D. Boulter, and B.L. Turner (eds.). Chemotaxonomy of the Leguminosae. Academic Press, London.

MEDVEDEVA, G.S. (ed.). 1978. Opredelitel nasekomikh Europeiskoi tschasti SSSR, Vol. III. Perepontschatokrilie. 2 [A guide for the identification of insects of the European part of the Soviet Union, Vol. III. Hymenoptera. 2]. Nauka, Leningrad. 758 pp. (in Russian).

NOWOTNOWNA, A. 1928. *Pamietniki PINGW* 9:5–15. (No title was available. Cited in Waller and Nowacki, 1978.)

PÖHM, M. 1966. Cytisin und *N*-Methylcytisin in Keimpflanzen. *Abh. Dtsch. Akad. Kl. Chem.* 3:251–254.

PRICE, P.W., BOUTON, C.E., GROSS, P., MCPHERON, P.A., THOMPSON, J.N., and WEIS, A.E. 1980. Interactions among three trophic levels: Influence of plants on interactions between insect herbivores and natural enemies. *Annu. Rev. Ecol. Syst.* 11:41–65.

SOÓ, R. 1966. A magyar flóra és vegetáció rendszertani-növényföldrajzi kézikönyve. II [Plant taxonomy and biogeography of the Hungarian flora and vegetation]. Akad. Kiadó, Budapest. 655 pp. (in Hungarian).

SMITH, B.D. 1966. Effect of the plant alkaloid sparteine on the distribution of the aphid *Acyrthosiphon spartii* (Koch). *Nature* 212:213–214.

STEFFAN, J.R. 1981. The parasites of bruchids, pp. 223–229, *in* V. Labeyrie (ed.). The Ecology of Bruchids Attacking Legumes (Pulses). Series on Entomology, Vol. 19. Dr. W. Junk Publ., The Hague.

STEPHENSON, A.G. 1981. Flower and fruit abortion: Proximate causes and ultimate factors. *Annu. Rev. Ecol. Syst.* 12:253–279.

THORPE, K.W., and BARBOSA, P. 1986. Effects of consumption of high and low nicotine tobacco

by *Manduca sexta* (Lepidoptera: Sphingidae) on survival of gregarious endoparasitoid *Cotesia congregata* (Hymenoptera: Braconidae). *J. Chem. Ecol.* 12:1329–1337.

THURSTON, R., and FOX, P.M. 1972. Inhibition by nicotine of emergence of *Apanteles congregatus* from its host, the tobacco hornworm. *Ann. Entomol. Soc. Am.* 65:547–550.

TYSKI, S., MARKIEWICZ, M., GULEWICZ, K., and TWARDOWSKI, T. 1988. The effect of lupin alkaloids and ethanol extracts from seeds of *Lupinus angustifolius* on selected bacterial strains. *J. Plant Physiol.* 133:240–242.

VADAS, J. 1911. Az akácfa monográfiája, különös tekintettel erdögazdasági jelentöségére [A monograph of the black locust with special regard to its forestry significance]. Pátria Irod. Váll. Nyomd. Rt., Budapest. 236 pp. (in Hungarian).

WAHL, B. 1925. Bericht über die Tätigkeit der Bundesanstalt für Pflanzenschutz in Wien im Jahre 1924. *Z. Landw. Versuchsw. Deutschösst.* 28:21–48 (only an abstract from *Rev. Appl. Entomol.* 14:189, 1926).

WALLER, G.R., and NOWACKI, E.K. 1978. Alkaloid Biology and Metabolism in Plants. Plenum Press, New York. 294 pp.

WINK, M. 1983. Inhibition of seed germination by quinolizidine alkaloids. Aspects of allelopathy in *Lupinus albus* L. *Planta* 158:365–368.

WINK, M. 1984a. Chemical defense of Leguminosae. Are quinolizidine alkaloids part of the antimicrobial defense system of lupins? *Z. Naturforsch.* 39c:548–552.

WINK, M. 1984b. Biochemistry and chemical ecology of lupin alkaloids, pp. 326–343, *in* Proceedings, 3rd International Lupin Congress, La Rochelle, France, 1984.

WINK, M. 1984c. Chemical defense of lupins. Mollusc-repellent properties of quinolizidine alkaloids. *Z. Naturforsch.* 39c:553–558.

WINK, M. 1984d. *N*-Methylation of quinolizidine alkaloids: An *S*-adenosyl-*L*-methionine : cytisine *N*-methyltransferase from *Laburnum anagyroides* plants and cell cultures of *L. alpinum* and *Cytisus canariensis*. *Planta* 161:339–344.

WINK, M. 1985. Chemische Verteidigung der Lupinen: Zur biologischen Bedeutung der Chinolizidinalkaloide. *Plant Syst. Evol.* 150:65–81.

WINK, M. 1987. Chemical ecology of quinolizidine alkaloids, pp. 524–533, *in* G.R. Waller (ed.). Allelochemicals. Role in Agriculture and Forestry. ACS Symposium Series 330, American Chemical Society, Washington, D.C.

WINK, M. 1988. Plant breeding: importance of plant secondary metabolism for plant breeding. *Theor. Appl. Genet.* 75:225–233.

WINK, M., and HARTMANN, T. 1981. Sites of enzymatic synthesis of quinolizidine alkaloids and their accumulation in *Lupinus polyphyllus*. *Z. Pflanzenphysiol.* 102:337–344.

WINK, M., and RÖMER, P. 1986. Acquired toxicity—the advantages of specializing on alkaloid-rich lupins to *Macrosiphum albifrons* (Aphidae). *Naturwissenschaften* 73:210–212.

WINK, M., and WITTE, L. 1984. Turnover and transport of quinolizidine alkaloids. Diurnal fluctuations of lupanine in the phloem sap, leaves and fruits of *Lupinus albus* L. *Planta* 161:519–524.

WINK, M., and WITTE, L. 1985a. Quinolizidine alkaloids as nitrogen source for lupin seedlings and cell cultures. *Z. Naturforsch.* 40c:767–775.

WINK, M., and WITTE, L. 1985b. Quinolizidine alkaloids in *Petteria ramentacea* and the infesting aphids, *Aphis cytisorum*. *Phytochemistry* 24:2567–2568.

WINK, M., HARTMANN, T., WITTE, L., and RHEINHEIMER, J. 1982. Interrelationship between quinolizidine alkaloid producing legumes and infesting insects: Exploitation of the alkaloid-containing phloem sap of *Cytisus scoparius* by the broom aphid *Aphis cytisorum*. *Z. Naturforsch.* 37c:1081–1086.

WINK, M., WITTE, L., HARTMANN, T., THEURING, C., and VOLZ, V. 1983. Accumulation of

quinolizidine alkaloids in plants and cell suspension cultures: Genera *Lupinus*, *Cytisus*, *Baptisia*, *Genista*, *Laburnum*, and *Sophora*. *Planta Med.* 48:253–257.

WINK, M., HEINEN, H.J., VOGT, H., and SCHIEBEL, H.M. 1984. Cellular localization of quinolizidine alkaloids by laser desorption mass spectrometry (LAMMA 1000). *Plant Cell Reports* 3:230–233.

ZACHER, F. 1936. Beitrag zur Nährpflanzenkenntniss der Samenkäfer (Col. Bruch.-Lariidae). *Mitt. Dtsch. Entomol. Ges.* 7:10–13.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

2,2′-OXO-1,1′-AZOBENZENE: MICROBIAL TRANSFORMATION OF RYE (*Secale cereale* L.) ALLELOCHEMICAL IN FIELD SOILS BY *Acinetobacter calcoaceticus:* III

WILLIAM R. CHASE, MURALEEDHARAN G. NAIR,* ALAN R. PUTNAM,[1] and SAROJ K. MISHRA[2]

Bioactive Natural Products Laboratory
Department of Horticulture
Michigan State University
East Lansing, Michigan 48824

(Received January 24, 1991; accepted March 1, 1991)

Abstract—*Acinetobacter calcoaceticus*, a gram-negative bacterium isolated from field soil, was found to be responsible for the biotransformation of 2(3H)-benzoxazolinone (BOA) to 2,2′-oxo-1,1′-azobenzene (AZOB). Experiments were conducted to evaluate the transformation of BOA to AZOB by this microbe in sterile and nonsterile soil. Transformation studies with soils inoculated with *A. calcoaceticus* indicated that the production of AZOB increased linearly with the concentration of BOA in sterile soil and showed a quadratic trend in nonsterile soils. This also indicated that all soil types studied for the transformation experiments might contain *A. calcoaceticus* capable of the conversion of benzoxazolinones.

Key Words—Allelopathy, 2,4-dihydroxy-1,4(2H)-benzoxazine-3-one, 2(3H)-benzoxazolinone, 2,2′-oxo-1,1′-azobenzene, *Acinetobacter calcoaceticus*.

INTRODUCTION

Rye is considered an important cover crop in Michigan, other parts of the United States, and around the world (Smeda and Weller, 1986; Smeda and Putnam,

*To whom correspondence should be addressed.
[1] Present address: 49823 Gallatin Road, Gallatin Gateway, Montana 59730.
[2] Present address: Microbiology Laboratory, Krug International, 1290 Hercules Drive, Houston, Texas 77058.

0098-0331/91/0800-1575$06.50/0

1988). Numerous allelochemicals with apparent weed control activity have been isolated and identified from rye (*Secale cereale* L.) herbage and found to inhibit lettuce (*Lactuca sativa* L.), redroot pigweed (*Amaranthus retroflexus* L.), and common lambsquarters (*Chenopodium album* L.) seed germination and root growth (Chou and Patrick, 1976; Patrick, 1955; Shilling et al., 1985). Barnes and Putnam (1986, 1987) and Barnes et al. (1987) isolated two hydroxamic acids, 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) and 2(3H)-benzoxazoline (BOA), from rye residues, compared their toxicity to phenyllactic acid and hydroxybutyric acid, the allelochemicals reported earlier from rye (Patrick, 1955; Shilling et al., 1985), and found that the hydroxamic acids, DIBOA and BOA, were the most toxic to both monocots and dicots. The benzoxazinones are thought to occur in plants as glucosides. Upon injury, they are released as DIBOA by glucosidase enzymes (Hofman and Hofmanova, 1968; Zungica et al., 1983). The quantities of these compounds in plants vary with the species, age, and the plant part used (Almeida, 1985; Barnes and Putnam, 1986; Tang et al., 1975; Virtanen et al., 1957). Recently, Nair et al. (1990) reported the production of an azoperoxide, 2,2′-oxo-1,1′-azobenzene (AZOB) in field soils incubated with BOA. The benzoxazinones produced by rye, DIBOA and BOA, were rapidly converted to AZOB in nonsterile bins over heat-sterilized soil, suggesting a microbial basis for the reaction. This new transformed allelochemical was more toxic to plants than its precursors, BOA or DIBOA (Hietala and Virtanen, 1960; Nair et al., 1990).

Allelochemicals can be released into the environment by processes such as volatilization, root exudation, leaching, microbial transformation, and release of toxins from decomposition of plant residues (Chapman and Lynch, 1983; Rice, 1984). The microorganisms either release or produce phytotoxins directly from residues or use the residues as substrate in the production of bioactive compounds (Atlas and Bartha, 1987; Patrick, 1955; Proebsting and Gilmore, 1941). For example, the contribution of microorganisms in the production of toxins from peach root bark was investigated, and several soil-born microorganisms were found to degrade amygdalin, an allelochemical from the peach tree, to HCN and benzaldehyde (Patrick, 1955; Proebsting and Gilmore, 1941). These degradation products were shown to be the toxic allelochemicals present in the soil containing decayed peach tree roots. This paper describes the isolation and identification of a soil microbe capable of transforming benzoxazinone to the more toxic AZOB and the rate of transformation of BOA to AZOB by this microbe in some Michigan field soils.

METHODS AND MATERIALS

General Experimental. Ultraviolet (UV) and visible absorption spectral analysis were performed on a Gilford Response II spectrophotometer. BOA was obtained commercially from the Aldrich Chemical company, Milwaukee, Wis-

consin, and was uniformly mixed with soil using a mortar and pestle, before addition of water and vortexing (1 min). Column chromatography was performed using a J.T. Baker Flash Column (20 × 2.7 cm, 250 ml). The silica column used was Analtech (35–75 μm particle size, 60 Å pore size), and the flow rate was 3 ml/min, unless otherwise specified. All analytical TLC (250 μm) and preparative TLC (1500 μm) were done on Uniplates (Analtech, Inc., Newark, Delaware). Homogenization of microbial colonies was done in a Brinkman Polytron (CH-6010) blender for 5 min. Soil was sterilized by autoclaving (1 hr, 15 atm, 120°C) for four successive days, and distilled water and media used were autoclaved for 20 min.

Soil Collection. All soil collection sites were in the State of Michigan. Spinks loamy sand soil (Psammentic, Hapludalfs, sandy, mixed, mesic) was collected (November, 1987) from the Horticulture Teaching and Research Center field, located on Jolly Road in East Lansing, Michigan. Soil was passed through an aluminum sieve (2 × 12 mm opening) and stored in a cubic yard bin at room temperature (15–22°C) for one year prior to use. Three other soils, Kalamazoo sandy loam (Typic Hapludalfs, fine-loamy, mixed, mesic), Metea loam (Arenic Hapludalfs, loamy, mixed, mesic) and Oshtemo sandy loam (Typic Hapludalfs, coarse-loamy, mixed, mesic) were collected in October and November of 1988 and stored under the same conditions. The Kalamazoo Sandy loam soil was collected at the Clarksville Research Station located in Clarksville, the Oshtemo sandy loam was from the Sodus Horticulture Research Farm located in Sodus, and the Metea loam was collected from the Moore Seed Farm located in Elsie.

Preparation of Media. Yeast maltose glucose (YMG) agar (yeast extract, 4 g; maltose, 10 g; glucose, 4 g; bacto-agar 18 g/liter), potato dextrose agar (PDA, Difco) (39 g/liter), and Nz-Amine agar (Nz-Amine, ICN Biomedicals Inc., 3 g/liter, bacto-agar 18 g/liter) were used for growth and identification of soil microbes. Antibacterial plates were prepared by adding chloramphenicol to a final concentration of 100 μg/ml. Antifungal Nz-Amine agar plates were prepared by adding nystatin and cycloheximide at concentrations of 30 μg and 500 μg/ml, respectively.

Isolation of Soil Microbes. Spinks loamy sand soil (1 g) was suspended in sterile physiological saline (9 ml) (0.85% NaCl in distilled water, w/v), vortexed (15 min), and allowed to settle (1 hr). This soil extract was tested for in vitro transformation of BOA to AZOB by incubating BOA (10 mg) with sterile soil (100 g), according to the procedure published earlier (Nair et al., 1990). Cultures were allowed to incubate in the dark at 26°C (96 hr), and extracted with MeOH (50 ml × 3). Biotransformation of BOA to AZOB by the soil extract containing microbes (1 ml) was verified by TLC comparison with the standard AZOB and by UV–visible spectroscopy.

Separation of Total Soil Microbes into Bacteria and Fungi. Serial 10-fold dilutions of the supernatant–soil extract was prepared in sterile distilled water.

Soil extracts (1 ml each) were pipetted into 10 different autoclaved test tubes, diluted to 10 ml each by sterile distilled water, and mixed well. Solutions from each tube (10 ul) were spread onto individual YMG plates. Plates at dilution of 10^{-6} showed the best separation of fungal and bacterial colonies. A total of nine individual colonies was further transferred onto YMG plates separately and assayed with BOA for the production of AZOB. Colonies found to be positive for transformation of BOA to AZOB were transferred to Nz-Amine agar and PDA plates enriched with antibacterial or antifungal compounds, respectively, and incubated (72 hr). Two fungi and one bacterium thus obtained were assayed for the transformation of BOA to AZOB.

Identification of Microbe. The bacterium that converted BOA to AZOB was further purified on antifungal Nz-Amine plates. Morphological and biochemical properties of the bacterium were determined with a Vitek Auto Microbic System using Vitek GNT gram-negative identification cards.

Spray Reagents for TLC Detection. DIBOA was detected on thin-layer plates with ferric chloride ($FeCl_3$) spray reagent consisting of 5% $FeCl_3$ in 95% ethanol, acidified with concentrated HCl. A spray reagent consisting of 1% ceric sulfate ($CeSO_4$) in concentrated H_2SO_4 was used to detect BOA.

Production and Extraction of AZOB from Soil. A commercial sample of BOA (4 g) was mixed thoroughly with Spinks loamy sand (4 kg) and distilled water (400 ml) and incubated (26°C, 96 hr) in four 2-liter Erlenmeyer flasks, as published earlier (Long et al., 1974). The soil was extracted with methanol (8 × 500 ml) and filtered through a Buchner funnel, using filter paper (Whatman No. 1). The extract was further filtered through a sintered glass filter (fine, 4–5 μm), and the solvent was removed by rotary evaporation. A preliminary TLC (toluene–ethylacetate, 5:4) of the crude extract indicated the presence of AZOB and unreacted BOA. Separation of BOA from AZOB was achieved by flash column chromatography (260 g, toluene–ethylacetate, 5:4). The orange band eluted was collected and dried in vacuo to produce dark brown crystals of AZOB (1.18 g).

Quantification of BOA and AZOB in Soil Extract. The samples were analyzed spectrophotometrically for BOA and AZOB. Standard curves for BOA and AZOB were prepared using known quantities of BOA (from 0.748 to 286.0 μg/100 ml in MeOH) and AZOB (from 1.51 to 96.402 μg/100 ml in MeOH) and by recording the absorbance at 273 and 432 nm, respectively. Standard solutions of each of the dried soil extracts stored at −20°C were prepared by dissolving the extract in MeOH in a 100-ml volumetric flask. The absorbance was recorded for each solution. Concentrations of BOA and AZOB in a given sample were obtained from the standard curve.

In Vitro Transformation Studies with Pure Cultures of A. calcoaceticus. Cultures of *A. calcoaceticus* were used from the stock cultures grown on YMG plates, and transformation of BOA to AZOB was carried out as reported earlier

(Nair et al., 1990). All treatments were incubated (26°C) in the dark for 1, 2, 4, 6, 12, and 24 days and extracted with MeOH (3 × 150 ml) and quantified for BOA and AZOB by UV spectroscopic procedure. Microbial populations were estimated by monitoring the colony-forming units (CFU) of the stock solution and were calculated to be 5.3×10^{10}.

Production of AZOB in Liquid Media. Baffle-bottomed Erlenmeyer flasks (500 ml) containing distilled water (100 ml) were autoclaved and cooled to room temperature. BOA (80 mg) and freshly homogenized colonies of *A. calcoaceticus* (5.3×10^{10} CFU, 1 ml) were added to the water and incubated (26°C) under shake conditions. The fermentation broth (96 hr old) was extracted with EtOAc (3 × 75 ml). Shake and nonshake flasks were extracted with EtOAc separately. The EtOAc layer from shake flasks was dried over anhydrous $MgSO_4$ and evaporated *in vacuo* (four days). Purification of AZOB from BOA by TLC (toluene–ethylacetate, 5:4) gave an orange band, free from BOA, and was eluted with $CHCl_3$–MeOH (1:1) and dried *in vacuo* affording pure crystals of AZOB (46 mg).

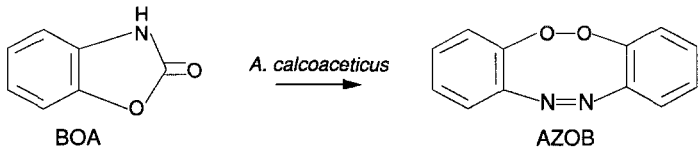
Microbial Transformation of BOA to AZOB. Experiments were conducted to study the rate of BOA transformation by the naturally occurring microbial population of *A. calcoaceticus* in field soil. BOA (30 mg each) was mixed with Spinks loamy sand (10 g each) containing distilled water (3.5 ml) in culture tubes (2.5 × 15.0 cm). Sterile foam plugs were used to cap the tubes, which were then wrapped with aluminum foil for the duration of the experiment. Culture tubes were placed in the dark and incubated (26°C) for periods of 1, 2, 4, 6, 12, and 24 days. At the end of the incubation period, the soil was extracted with MeOH (3 × 150 ml). The extracts were dried over anhydrous $MgSO_4$ and the solvent was removed *in vacuo*. All dried samples were stored at −20°C.

Statistical Methods. Data obtained in duplicated transformation studies were subjected to analysis of variance and means were compared with the least significant difference (LSD) test.

RESULTS AND DISCUSSION

Initial purification of soil extracts containing total microbial isolates were positive for the conversion of BOA to AZOB. Partial purification of the total isolates into bacteria and fungi afforded one bacterium and two fungi positive for the transformation of BOA to AZOB (Scheme 1). However, final purification of the fungi on bactericidal plates did not show transformation of BOA, and hence they were not investigated further.

The microbe capable of transforming BOA to AZOB in field soil was identified as a gram-negative rod-shaped bacterium with a tendency to form coccoid bodies. The organism grew well in a medium containing 0.4% peptone and



SCHEME 1. Transformation of BOA to AZOB in field soils using *A. calcoaceticus*.

0.02% tryptophan, under aerobic conditions, and was capable of using malonate as the sole source of carbon. Decarboxylase tests of the bacterium were negative with lysine, ornithine,and arginine. Glucose was not fermented in the presence of specific inhibitors, DP300 and *p*-coumaric, and the microbe did not produce acid with raffinose, sorbitol, sucrose, inositol, adonitol, rhamnose, arabinose, or glucose. Moreover, the microbe did not hydrolyze esculin or *o*-nitrophenyl-β-D-galactopyranoside. Under aerobic conditions, this organism did not produce acid with glucose, lactose, maltose, mannitol, or xylose. Tests for the production of H_2S, urease, and plant indican were negative. The aforementioned properties are in conformity with those of *A. calcoaceticus* described in *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt, 1984).

Although analytical methods for benzoxazolines were reported earlier (Long et al., 1974; Nair et al., 1990; Virtanen et al., 1957), these methods are not convenient or quantitative for the determination of BOA and AZOB in soil extracts. Since BOA and AZOB gave characteristic UV–visible absorption maxima (Barnes et al., 1987; Nair et al., 1990), it is easy to calculate the concentrations of these compounds from their mixtures by monitoring the optical density (OD). This was confirmed from the concentration curves obtained for BOA and AZOB where OD was 273 and 432 nm, respectively. Standard curves were prepared separately by plotting each compound's concentration against its OD. Concentrations of AZOB and unreacted BOA in the various soil extracts were obtained directly from the standard curves.

The percentages of AZOB isolated from *in vitro* transformation experiments was greater than previously reported (Nair et al., 1990). The modified extraction procedure of AZOB from soil gave yields three times greater (29.8% vs. 9.1%) than previously reported values (Nair et al., 1990). This could be attributed to the new solvent system employed for the separation of AZOB from BOA. In the reported procedure (Nair et al., 1990), HCOOH and NH_4OH were used as part of the developing solvent system, and these may have degraded a portion of the AZOB isolated. The rate of production of AZOB in field soil was investigated under environmentally controlled conditions over a period of 24 days using sterile and nonsterile soils. In both cases, *A. calcoaceticus* cultures were added (5.3×10^{10}) to the soils previously incorporated with BOA. The quantities of AZOB production was proportional to the quantity of BOA avail-

able in the soil (Table 1). At the end of day 12, only 7% AZOB was detected with a loss of 12% BOA. However, the 0.1 mg of BOA/g soil (nonsterile) experiment showed the highest production of AZOB at day 3 (Table 1). The maximum yield of AZOB (9.7%) was observed during the incubation period of days 2–4 with an 85% decrease in BOA concentration. This yield is similar to the 9.1% reported by Nair et al. (1990).

The recovery of unreacted BOA from the treated soil was 33% of the total amount incubated. The remainder of the BOA already could have converted to AZOB or a portion could have decomposed to products that are not yet characterized. Furthermore, BOA might have decomposed to other products under mechanisms that are not yet known. However, a possible mechanism for the production of AZOB from BOA, monomethoxy AZOB (MAZOB) from MBOA and dimethoxy AZOB (DIMAZOB) from MBOA was described earlier by Nair et al. (1990). When the concentration of BOA in field soil was raised to 1.0 mg/g soil, the AZOB production increased linearly to the concentration of BOA. This indicated that the excess BOA might have functioned as an antimicrobial agent on the total microflora in the soil and prevented the soil microbes from utilizing BOA as a carbon or nitrogen source. Usually, microbial populations present in the soil are low in numbers but explode quickly when a suitable substrate becomes available (Atlas and Bartha, 1987). Therefore, having observed different trends for the BOA transformation in nonsterile field soil, it would seem logical that, given the appropriate time interval, a quadratic trend could be expected at both rates of BOA (early lag phase followed by linear phase). Rapid AZOB production detected in the different Michigan soils studied

TABLE 1. QUANTITIES OF BOA AND AZOB ISOLATED FROM STERILE SOIL INOCULATED WITH *A. calcoaceticus* AND NONSTERILE SOIL, BOTH INCUBATED WITH 0.1 mg OF BOA/g SOIL

	Days					
Compounds	1	2	4	6	12	24
Sterile soil						
BOA (μg)	331.9	279.2	229.9	253.5	193.7	191.7
AZOB (μg)	10.4	28.9	51.1	89.3	72.5	99.3
Nonsterile soil						
BOA (μg)	118.6	97.6	47.0	33.0	18.2	[a]
AZOB (μg)	11.2	32.6	33.8	13.5	3.1	[a]

[a] Not detectable levels.

suggested the presence of *A. calcoaceticus* capable of the conversion of BOA to AZOB (Figure 1 and 2).

AZOB was produced in large quantities in aqueous shake cultures for bioassay experiments. This method was convenient, cost effective, and less time-consuming. Extraction of AZOB and BOA by EtOAc from aqueous media was easily performed, and the rate of production of AZOB under shake conditions appeared greater. Since the production of AZOB occurred readily in liquid cultures, it is not necessary to use soil for the transformation of BOA.

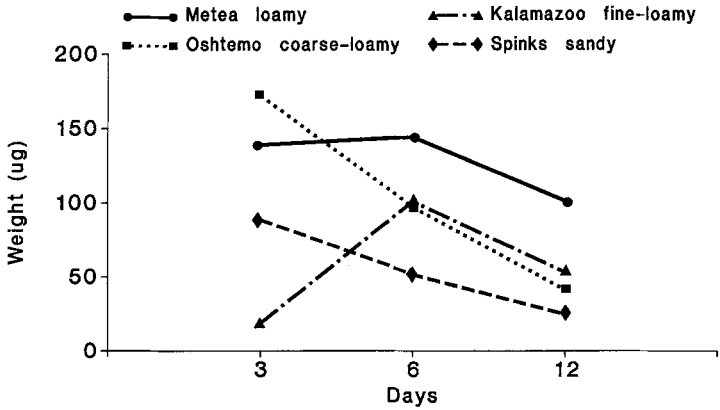


FIG. 1. Concentration of unreacted BOA present in the four Michigan soils used for transformation studies.

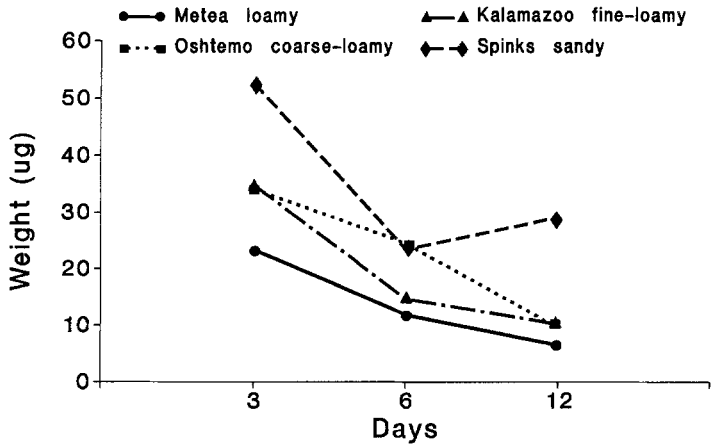


FIG. 2. Concentration of AZOB in four Michigan soils incubated with 0.1 mg of BOA per g of soil.

Even with a lower yield from the liquid medium (24%), when compared to the soil (30.8%), water was a more attractive transformation medium for large scale *in vitro* production of AZOB. The experiments with BOA in nonsterile soil also indicated that the AZOB produced might have been used by other soil microbe(s) as a substrate, thus explaining the quadratic trend observed (Table 1). These data support the difficulty encountered in the detection of AZOB from the soil incubated with rye tissue. The concentration of AZOB started to decline in the experiment with nonsterile soil (Table 1) by the end of day 4. Similar situations in the field can be envisioned where BOA leached from the rye residues is undergoing rapid transformation to AZOB. This experiment also suggested that the accumulation of AZOB will be difficult to detect in field soils with or without rye residues.

REFERENCES

ALMEIDA, F.S. 1985. Effect of some winter crop mulches on the soil weed infestation. Proceedings 1985 British Crop Protection Conference. *Weeds* 2:651–695.

ATLAS, R.M., and BARTHA, R. 1987. Microbial Ecology: Fundamentals and Applications. Benjamin/Cummings, Menlo Park, California. p. 71.

BARNES, J.P., and PUTNAM, A.R. 1986. Evidence for allelopathy by residues and aqueous extracts of rye (*Secale cereale* L.) *Weed Sci.* 34:384–390.

BARNES, J.P., and PUTNAM, A.R. 1987. Role of benzoxazinones in allelopathy by rye (*Secale cereale* L.). *J. Chem. Ecol.* 13:889–905.

BARNES, J.P., PUTNAM, A.R., BURKE, B.A., and AASEN, A.J. 1987. Isolation and Krieg characterization of allelochemicals in rye herbage. *Phytochemistry* 26:1385–190.

CHAPMAN, S.J., and LYNCH, J.M. 1983. The relative roles of micro-organisms and their metabolites in the phytotoxicity of decomposing plant residues. *Plant Soil* 74:457–459.

CHOU, C.H., and PATRICK, Z.A. 1976. Identification and phytotoxic activity of compounds produced during decomposition of rye and corn residues in soil. *J. Chem. Ecol.* 2:369–387.

HIETALA, P.K., and VIRTANEN, A.I. 1960. Precursors of benzoxazolinone in rye plants: Precursor I, the glucoside. *Acta Chem. Scand.* 14:502–504.

HOFMAN, J., and HOFMANOVA, O. 1968. 1,4-Benzoxazinone derivatives in plants: Sephadex fractionation and identification of a new glucoside. *Eur. J. Biochem.* 8:109–112.

KRIEG, N.R., and HOLT, J.G. 1984. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore. 303 pp.

LONG, B.J., DUNN, G.M., and ROUTLEY, D.G. 1974. Rapid procedure for estimating cyclic hydroxamate (DMIBOA) concentration in maize. *Crop Sci.* 14:601–603.

NAIR, M.G., WHITENACK, C.J., and PUTNAM, A.R. 1990. 2,2′-oxo-1,1′-azobenzene. A microbially transformed allelochemical from 2,3-benzoxazoline: I. *J. Chem. Ecol.* 16:353–364.

PATRICK, Z.A. 1955. The peach replant problem in Ontario. II. Toxic substances from microbial decomposition products of peach root residues. *Can. J. Bot.* 33:461–485.

PROEBSTING, E.L., and GILMORE, A.E. 1941. The relation of peach root toxicity to the re-establishing of peach orchards. *Proc. Am. Soc. Hortic. Sci.* 38:21–24.

RICE, E.L. 1984. Allelopathy, 2nd ed. Academic Press, Orlando, Florida. 266 pp.

SHILLING, D.G., LIEBL, R.A., and WORSHAM, A.D. 1985. Rye (*Secale cereale*) and wheat (*Triticum aestivum* L.) mulch: The suppression of certain broadleaved weeds and the isolation and identification of phytotoxins. American Chemical Society, Washington, D.C. 243 pp.

SMEDA, R.J., and PUTNAM, A.R. 1988. Cover crop suppression of weeds and influence on strawberry yields. *Hortic. Sci.* 23:132–134.

SMEDA, R.J., and WELLER, S.C. 1986. Weed suppression with rye in transplanted tomatoes. North Central Weed Control Conference Proceedings, Abstract 41:27–28.

TANG, C.S., CHANG, S.H., HOO, D., and YANAGIHARA, K.H. 1975. Gas chromatographic determination of 2(3)-benzoxazolinones from cereal plants. *Phytochemistry* 4:2077–2079.

VIRTANEN, A.I., HIETALA, P.K., and WAHLROOS, O. 1957. Antimicrobial substances in cereals and fodder plants. *Arch. Biochem. Biophys.* 69:486–500.

ZUNGICA, G.E., AGRANDONA, V.H., NIEMEYER, H.M., and CORCUERA, L.J. 1983. Hydroxamic acid content in wild and cultivated gramineae. *Phytochemistry* 22:2665–2668.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

HESPERETIN 7-RUTINOSIDE (HESPERIDIN) AND TAXIFOLIN 3-ARABINOSIDE AS GERMINATION AND GROWTH INHIBITORS IN SOILS ASSOCIATED WITH THE WEED, *Pluchea lanceolata* (DC) C.B. CLARKE (ASTERACEAE)

INDERJIT and K.M.M. DAKSHINI*

Department of Botany
University of Delhi
Delhi-110007, India

(Received January 28, 1991; accepted March 3, 1991)

Abstract—Hesperetin 7-rutinoside (Hesperidin) and taxifolin 3-arabinoside were detected in the soils associated with the rapidly spreading perennial weed, *Pluchea lanceolata*. In the present investigations, inhibitory potential of the aqueous extracts of the two compounds was established and confirmed through growth experiments pertaining to seed germination and seedling growth of radish, mustard, and tomato, with 10^{-4} M solutions of the authentic samples. The significance of the water-soluble compounds present in the rhizosphere zones of the weed and its interference potential is commented upon.

Key Words—Allelopathy, growth experiments, hesperidin, interference, *Pluchea lanceolata*, taxifolin 3-arabinoside.

INTRODUCTION

Previously, Inderjit and Dakshini (1990) reported that *Pluchea lanceolata* (DC) C.B. Clarke (Asteraceae) achieves its interference potential by releasing and inhibiting seed germination and seedling growth of different plant species through phytochemicals synthesized by the weed. These studies necessitated the identification of phytochemical compounds in the soils associated with the weed. Since a large number of phenolic compounds have been shown to possess

*To whom correspondence should be addressed.

0098-0331/91/0800-1585$06.50/0

allelopathic potential (Rice, 1984), isolation and characterization of only this category of compounds have been undertaken. This analysis resulted in the detection of the flavonone, hesperetin 7-rutinoside (hesperidin), and a dihydroflavonol, taxifolin 3-arabinoside, from the soils associated with the weed. Since the occurrence of these compounds in the soil has not been reported earlier, investigations on their effects on germination and growth of some plant species have been undertaken and data pertaining to these are reported here.

METHODS AND MATERIALS

The soils associated with and without (as controls) the weed, *Pluchea lanceolata*, were collected from the fields around the metropolitan city of Delhi. These samples were air-dried and stored in paper bags, to be used later for extraction of compounds.

Extraction, Purification, and Identification of Phenolic Constituents. The soil extracts (5 : 1, v/w) were prepared by shaking the soil samples with double distilled water (DDW) for 1 hr at room temperature. The extracts were filtered, dried in vacuum, and the residue extracted with 10 ml of methanol. These extracts were loaded on Whatman No. 3 (46 × 57 cm) chromatographic paper and developed by descending chromatography using the butanol–acetic acid–water (BAW, 4 : 1 : 5, upper phase) (Harborne, 1973). Chromatograms were dried and scanned bands were marked under UV ($-NH_3$) and UV($+NH_3$). Six bands appeared on the chromatogram with extracts from weed-associated soil, whereas only two bands were detected on the chromatogram with control soil extracts. Each band was eluted in methanol, loaded again separately, washed repeatedly with DDW, and finally extracted with methanol. To confirm the purity of these compounds, each compound was loaded on Whatman No. 1 chromatographic paper and run in four solvent systems: BAW, DDW, 15% acetic acid, and forestal (conc. HCl–acetic acid–water, 3 : 30 : 10). After confirming the purity of these compounds, each was again loaded on Whatman No. 3 chromatographic paper and washed repeatedly with DDW through descending chromatography. The compounds from chromatograms were finally eluted with methanol, and the eluates then were taken for spectral analysis and shifts (NaOH, $AlCl_3$, $AlCl_3$/HCl, NaOAc, and NaOAc/H_3BO_3), if any, of the parent as well as the hydrolyzed fractions. These purified compounds, after loading separately on Whatman No. 1 chromatographic paper, were developed through descending chromatography and the fluorescence (UV and UV+NH_3) and R_f (×100) values were recorded in the four solvent systems mentioned above. The DDW residue of the hydrolyzed parent compound was used for the characterization of sugar moiety, if any, following Harborne (1973). Further, the glycoside and aglycone forms were cochromatographed with the authentic samples and compared with data given by Mabry et al. (1970). The two compounds

common to both (control and weed-associated soil) were not considered for further study. Of the remaining four, two were phenolic acids and the others a flavonone and a dihydroflavonol. Since the former class of compounds has been reported earlier (Rice, 1984) in many allelopathic studies, only the last two compounds were subjected to further analysis.

Seed Germination and Growth Experiments. To investigate the allelopathic potential of the identified compounds, germination and growth experiments were performed. Since the synthetic glycoside of taxifolin (taxifolin 3-arabinoside) was not available, the synthetic aglycone (taxifolin) was used for germination studies. It could be argued that with such a step the comparison of results of two compounds may not be fully justifiable. However, in view of the observations of Rice and Pancholy (1974) that sugars are easily split from the glycoside fraction by microbial action and they tested aglycones for inhibitory activity, the results obtained in the present investigations can be compared.

The compounds hesperidin and taxifolin were dissolved initially in methanol, dried in vacuum, and dissolved in DDW to a final concentration of 10^{-4} M. In general, this low concentration of phytotoxins has been shown to be inhibitory to germination and seedling growth (McCahon et al., 1973; Williams and Hoagland, 1982). The seeds of commonly grown crops in the region, e.g., radish (*Raphanus sativus* var. pusa desi), mustard (*Brassica juncea* cv. PR 45), and tomato (*Lycopersicon esculentum* var. pusa ruby) were selected to study the allelopathic potential of the compounds. For germination studies, 50 seeds of these plants were sown on filter paper moistened with DDW (served as control) or compounds, each in equal volume, and placed in 15-cm-diam. Petri plates. To maintain uniform moisture status in the Petri plates, a cotton pad soaked in DDW or compounds was placed below the filter paper. Root and shoot lengths were recorded every 24 hr for seven days. Each treatment was replicated three times. During the period of experimentation, the temperature regime of 30 $\pm$ 5°C and diurnal regime of light condition were maintained. After seven days, fresh weight of three sets of five seedlings each was recorded. To get a better evaluation of the inhibitory effect, relative germination (RG) index was calculated as follows:

$$\text{RG index} = \frac{\text{number of seeds germinated at 48 hr} \times 100}{\text{number of seeds germinated at 168 hr}}$$

Comparison of the various parameters was made using one way analysis of variance.

RESULTS AND DISCUSSION

A flavonone, hesperidin, and a dihydroflavonol, taxifolin 3-arabinoside, were identified in the aqueous extracts of the soils associated with the weed, *Pluchea lanceolata* (Tables 1 and 2). Compared to taxifolin, hesperidin was a

TABLE 1. FLUORESCENCE, R_f (×100) VALUES, AND ABSORPTION SPECTRA OF COMPOUNDS ISOLATED FROM SOILS ASSOCIATED WITH *Pluchea lanceolata*

	Fluorescence[a]		*Rf* (×100) values[b]				Absorption spectra						
Compound	UV	UV + NH_3	DDW	BAW	HOAc	For-estal	Methanol	NaOH	$AlCl_3$	$AlCl_3$/HCl	NaOAc	NaOAc/H_3BO_3	Identification
1	Y	GY	88	49	70	83	272, 326	286, 379	272, 322sh 387	271, 322sh 374	272, 306sh 329	272, 306sh 325	Hesperidin
2	BM	BM	87	48	84	78	279, 312sh[c]	285, 353	278, 306	278 304sh	276, 350	278, 350	Taxifolin 3-arabinoside

[a] Fluorescence key: Y, yellow; GY, green yellow; BM, brown mauve.
[b] Solvent key: DDW, double distilled water; BAW, butanol–acetic acid–water (4 : 1 : 5, upper phase); HOAc, 15% acetic acid; forestal, conc. HCl–acetic acid–water (3 : 30 : 10).
[c] sh-shoulder.

TABLE 2. PAPER CHROMATOGRAPHY OF SUGAR IN WATER FRACTIONS AFTER ACID HYDROLYSIS OF TAXIFOLIN GLYCOSIDE

Compound	R_f (×100) values[a]		Color with aniline hydrogen phthalate[b]
	BAW	BBPW	
Suspected Arabinose	16.5	25.5	Pink red
Known Arabinose	17.0	25.7	Pink red

[a] Solvent key: BAW, butanol–acetic acid–water (4 : 1 : 5, upper phase); BBPW, butanol–benzene–pyridine–water (5 : 1 : 3 : 3).
[b] Prepared by dissolving 9.2 ml aniline and 16 g phthalic acid in 490 ml of butanol, 490 ml diethyl ether, and 20 ml water.

stronger inhibitor of the seed germination of the three crop species (Table 3). Hesperidin also inhibited significantly ($P < 0.05$) the growth of roots and shoots of all seedlings. Similarly, taxifolin brought about reduction in roots and shoots of the seedlings ($P < 0.05$), excepting shoot growth of mustard and root growth of tomato (Table 3). However, on a relative basis, hesperidin inhibited the shoot growth more; taxifolin, on the other hand, suppressed the root growth more effectively except in tomato, where hesperidin was more inhibitory. Similarly, both compounds reduced the fresh weight of the seedlings of all species ($0.01 < P < 0.08$) except mustard seedlings grown with hesperidin. Like shoot growth, fresh weight of seedlings was affected more by hesperidin than taxifolin. Furthermore, tomato seeds were found to be more sensitive to the compounds than the other two crop species (except grown with taxifolin). It may be noted that the effect of these two compounds varied with seeds tested. Even though the reason for this variation can not be explained, it is likely that, as opined by Williams and Hoagland (1982), this may be due to differences in seed size, seed coat permeability, differential uptake, and metabolism. However, any inference in this regard requires further investigation.

Additionally, seedlings grown with either of the compounds showed browning of the root tip and root–shoot (hypocotyl) zones. Whether this resulted from increased reduction of ascorbic acid in the xylem vessels, as thought by Rice (1984), or from simple oxidation of phenolic compounds needs further study. In spite of earlier suggestions regarding the probable effects of flavanoids on different physiological parameters (Koeppe and Miller, 1974; Lang and Racker, 1974), mechanisms leading to suppression of seedling growth are not clear. However, the present study has shown that two compounds are potent allelochemics associated with the rhizosphere in soils of the weed. Furthermore, their role in interference of seed germination and seedling growth of crop spe-

TABLE 3. EFFECT OF COMPOUNDS (10^{-4} M) ON SEED GERMINATION AND SEEDLING CHARACTERISTICS OF DIFFERENT PLANT SPECIES

Plant species	Treatment[a]	Germination (%)	RG index[b]	Root length (cm) days after sowing			Shoot length (cm) days after sowing			Fresh weight (g)[c]
				1	4	7	1	4	7	
Mustard	C	92	100	0.34 ±0.42	4.89 ±1.94	5.22 ±1.74	0.14 ±0.04	1.96 ±0.80	2.54 ±0.76	0.1726 ±0.019
	H	66	66.60		2.20***[d] ±1.20	4.16* ±2.14		0.61*** ±0.42	1.56*** ±0.97	0.1479 ±0.021
	T	98	97.95	0.28 ±0.11	3.47** ±2.04	4.10* ±2.41	0.12* ±0.06	1.53* ±0.81	2.16 ±1.31	0.1520* ±0.0070
Radish	C	100	100	0.43 ±0.20	2.82 ±1.58	3.95 ±1.40	0.30 ±0.13	1.58 ±0.82	3.01 ±1.58	0.3484 ±0.046
	H	80	100	0.29** ±0.41	1.85*** ±0.87	2.68*** ±1.29		0.80*** ±0.37	1.58*** ±0.91	0.2651* ±0.005
	T	98	100	0.27* ±0.14	1.71*** ±0.82	2.14*** ±1.07	0.23 ±0.16	1.37** ±0.61	1.98*** ±0.80	0.2910* ±0.004
Tomato	C	54	7.40		1.74 ±1.24	3.95 ±1.49		0.72 ±0.04	2.67 ±1.49	0.1036 ±0.005
	H	24	[e]		0.15 ±0.05	1.32*** ±1.66		0.10 ±0.01	0.60*** ±0.57	0.0533[f]
	T	56	[e]		1.60 ±1.51	3.73 ±2.48		0.51 ±0.37	1.87* ±1.13	0.0883* ±0.005

[a] C, control; H, hesperidin; T, taxifolin.
[b] Relative germination index.
[c] Fresh weight of five seedlings on 7th day.
[d] *$0.01 < P < 0.08$, ** $0.001 < P < 0.01$, ***$P < 0.0001$.
[e] No germination on 2nd day.
[f] Only five seedlings available.

cies should be significant since this weed is perennial and thus would maintain a sufficient supply of these compounds in addition to other phenolic acids that are also inhibitory to growth and establishment of seedlings.

Acknowledgments—We are grateful to Professor M.R. Parthasarthy, Department of Chemistry, University of Delhi, for his help in identification of compounds. The financial assistance of Council of Scientific and Industrial Research (CSIR), is gratefully acknowledged. We wish to thank Professor M.A.K. Lodhi, Missouri, U.S.A. for his valuable suggestions to improve the manuscript.

REFERENCES

HARBORNE, J.B. 1973. Phytochemical Methods. Chapman and Hall, London.

INDERJIT, and DAKSHINI, K.M.M. 1990. The nature of interference potential of *Pluchea lanceolata* (DC) C.B. Clarke (Asreraceae). *Plant Soil* 122:298–302.

KOEPPE, D.E., and MILLER, R.J. 1974. Kaempferol inhibitions of corn mitochondrial phosphorylation. *Plant Physiol.* 54:374–378.

LANG, D.R., and RACKER, E. 1974. Effect of quercetin and F_1 inhibitor on mitochondrial ATPase and energy linked reaction in submitochondrial particle. *Biochim. Biophys. Acta* 333:180–186.

MABRY, T.J., MARKHAM, K.R., and THOMAS, M.B. 1970. The Systematic Identification of Flavanoids. Springer-Verlag, New York.

MCCAHON, C.B., KELSEY, R.G., SHERIDEN, R.P., and SHAFIZADEH, F. 1973. Physiological effects of compounds extracted from sagebrush. *Bull. Torrey Bot. Club* 100:23–33.

RICE, E.L. 1984. Allelopathy, 2nd ed. Academic Press, Orlando, Florida.

RICE, E.L., and PANCHOLY, S.K. 1974. Inhibition of nitrification by climax ecosystems. III. Inhibitors other than tannins. *Am. J. Bot.* 61(10):1095–1103.

WILLIAMS, R.D., and HOAGLAND, R.E. 1982. The effects of naturally occurring phenolic compounds on seed germination. *Weed Sci.* 30:206–212.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

PALATABILITY OF APOSEMATIC QUEEN BUTTERFLIES (*Danaus gilippus*) FEEDING ON *Sarcostemma clausum* (ASCLEPIADACEAE) IN FLORIDA

DAVID B. RITLAND

Department of Zoology
University of Florida
Gainesville, Florida 32611

(Received December 17, 1990; accepted March 4, 1991)

Abstract—Queen butterflies (*Danaus gilippus*) are generally considered unpalatable to predators because they sequester and store toxic cardenolides from their larval food plants. However, a major queen food plant in Florida, the asclepiadaceous vine *Sarcostemma clausum*, is shown here to be a very poor cardenolide source, and queens reared on this plant contain no detectable cardenolide. A direct evaluation of queen palatability using red-winged blackbirds indicates that *S. clausum*-reared butterflies are essentially palatable to these predators (85% of abdomens entirely eaten), indicating little protection from either cardenolides, other sequestered phytochemicals, or de novo defensive compounds. Wild-caught queens that presumably fed as larvae upon *S. clausum* and also had access to adult-obtained chemicals, such as pyrrolizidine alkaloids (PAs), were relatively palatable as well (77% of abdomens eaten); they did not differ significantly in palatability from the lab-reared butterflies. Together, these findings suggest that: (1) *S. clausum*-fed queens are poorly defended against some avian predators, and (2) for the particular queen sample examined, PAs do not contribute substantially to unpalatability. The discovery that *S. clausum*-feeding queens are essentially palatable is of additional significance because it compels a reassessment of the classic mimicry relationship between queen and viceroy butterflies. Viceroys have been shown recently to be moderately unpalatable; therefore, the established roles of model and mimic may be reversed in some cases.

Key Words—Chemical defense, mimicry, cardenolides, pyrrolizidine alkaloids, Asclepiadaceae, Lepidoptera, Danainae, *Danaus gilippus*, *Limenitis archippus*, *Sarcostemma*.

INTRODUCTION

Unpalatability and chemical defense are recurring themes in the study of arthropod antipredator strategies. Insects whose unpalatability is due solely to food-

0098-0331/91/0800-1593$06.50/0

plant-derived chemicals may possess a labile defense; species, populations, or individuals feeding on food plants that lack sufficient quantities of essential chemicals will themselves be relatively undefended (e.g., Brower et al., 1967; Brower, 1969; Bowers, 1980). Food-plant-related variation in chemical defense must significantly affect the ecology and evolution of not only the species in question, but also its predators and any mimics.

Milkweed butterflies (Nymphalidae: Danainae) are traditionally viewed as chemically defended and unpalatable, in part because their primary larval food plants (Asclepiadaceae and Apocynaceae) contain toxic and bitter cardenolides, which some danaines sequester and store for their own defense (Parsons, 1965; Reichstein et al., 1968; reviews: Ackery and Vane-Wright, 1984, and Brower, 1984). However, generalizations concerning danaine unpalatability may not be valid, because (1) species differ in their ability to sequester and store cardenolides (e.g., Brower et al., 1975; Rothschild et al., 1975), and (2) many danaine genera feed on host plants that reportedly lack cardenolides (Ackery and Vane-Wright, 1984). On the other hand, inferring that a danaine is palatable because it feeds on a cardenolide-free food plant may be spurious as well, because the butterfly could sequester other phytochemicals (as either a larva or an adult) or synthesize its own chemical deterrents. Therefore, direct investigations of palatability using relevant predators are necessary to elucidate the ecology and evolution of palatability-mediated predator–prey and model–mimic interactions.

This paper describes an investigation into the palatability of Florida queen butterflies, *Danaus gilippus berenice* (Cramer). The queen, like its better-studied congener, the monarch [*Danaus plexippus* (L.)], stores cardenolides sequestered from some of its larval food plants (e.g., Brower et al., 1975; Cohen, 1985; Ritland, 1991c). Queen larvae in Florida feed on several milkweeds (*Asclepias* spp.) with variable cardenolide contents (Roeske et al., 1976; Cohen, 1983). In addition, in central and southern Florida (where queens are most abundant), they commonly feed on asclepiadaceous vines, especially *Sarcostemma clausum* (Jacq.) Roem. & Schult. (personal observation). The cardenolide content of *S. clausum* has not been investigated, but some asclepiadaceous vines reportedly lack cardenolides (*Cynanchum*: Abisch and Reichstein, 1962; Hegnauer, 1964; *Gonolobus*: Brower et al., 1967), raising the possibility that queens feeding on *Sarcostemma* in Florida might be poorly defended.

However, as noted above, inferring the palatability of *S. clausum*-feeding queens from the cardenolide content of other asclepiadaceous vines is risky. *Sarcostemma clausum* may in fact be cardenolide-rich, or it might possess other phytochemicals (e.g., bitter polyhydroxysteroids; Hegnauer, 1964, and references within), which queens could sequester and store as distasteful deterrents. Moreover, even if *Sarcostemma*-feeding queens do not acquire defensive compounds as larvae, they may compensate via adult-obtained chemicals. Adult

queens store pyrrolizidine alkaloids (PAs) obtained from the nectar or sap of certain plants; metabolites of these PAs are crucial components of courtship pheromones (e.g., Brower et al., 1965; Pliske and Eisner, 1969; Pliske, 1975; Edgar et al., 1979). Several workers (e.g., Rothschild and Marsh, 1978; Eisner, 1980; Conner et al., 1981) have suggested an additional defensive role for these bitter-tasting and toxic (Bull et al., 1968) PAs. The effectiveness of PAs against vertebrate predators has not yet been established (Glendinning et al., 1990), but PAs do deter spider predators (e.g., Brown, 1984; Masters, 1990), and a PA-based defense in queens could potentially compensate for meager cardenolide concentrations.

Quantifying queen palatability is important from the standpoint of understanding queen–predator interactions, but it is important also because of the queen's mimicry relationship with the viceroy butterfly [*Limenitis archippus* (Cramer)] (Brower, 1958b). The viceroy has traditionally been considered a palatable mimic of the distasteful queen and an exemplar of temperate-zone Batesian mimicry (e.g., Scudder, 1870; Opler and Krizek, 1984). Recently, however, I have shown that viceroys are unpalatable to at least one avian predator (Ritland, 1991a; Ritland and Brower, 1991); therefore, if *Sarcostemma*-feeding queens possess an inadequate chemical defense, the established roles of model and mimic (in an ecological sense) may be reversed.

To clarify the palatability of queens feeding on *Sarcostemma*, I investigated (1) cardenolide content of *S. clausum*, (2) cardenolide content of queens reared on *S. clausum*, (3) palatability of *S. clausum*-reared queens to avian predators, and (4) palatability of queens from a natural population that had presumably fed as larvae upon *S. clausum* but that also had access to adult-obtained chemicals such as PAs.

METHODS AND MATERIALS

Quantitative Cardenolide Assay of Sarcostemma clausum

Sarcostemma R. Br. comprises some 22 New World species; only *S. clausum* is indigenous to Florida (see Holm, 1950, and Rosatti, 1989, for distributional data and nomenclatural history). In addition to being a primary queen food plant in southern Florida (personal observation; M.C. Minno, personal communication), *S. clausum* is a potentially important food plant for danaines in Central and South America and the Greater Antilles (Holm, 1950; Brown and Heineman, 1972).

I assayed 16 plants collected in southern Florida; four plants were sampled from each of four sites (Figure 1; voucher specimens in University of Florida Herbarium). Leaf material (~ 10 g) collected from each plant was immediately frozen in the field (on Dry Ice) to prevent enzymatic degradation of chemical

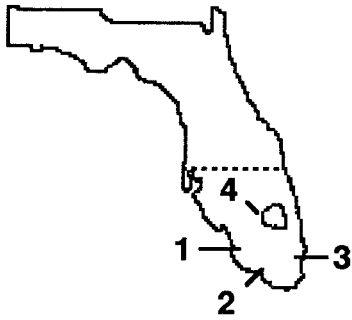


FIG. 1. Florida collection sites of *Sarcostemma clausum* for cardenolide spectroassay. Latitude and longitude data from U.S. Geological Survey maps. Dotted line represents approximate northern distributional limit of *S. clausum* (determined from University of Florida, University of South Florida Herbarium data). **1:** Lee Co., Hwy. 82, 16 km E of Fort Myers. 26° 34′ N, 81° 41′ W. **2:** Collier Co., jct. Hwy. 41 & 29. 25° 56′ N, 81° 22′ W. **3:** Dade Co., Hwy. 27 at Miami Canal. 25° 56′ N, 80° 26′ W. **4:** Glades Co., Hwy. 78, 9 km N of Hwy. 27. 26° 56′ N, 81° 7′ W.

constituents. After drying for 16 hr at 60°C, leaves from each plant were ground separately, and samples of ~0.4 g ground leaf material were extracted individually (in 10 ml 95% ethanol) in a shaker bath for 1 hr at 75–78°C. The standard spectroassay protocol (as modified by Malcolm et al., 1989) was employed to quantify cardenolide content (as digitoxin equivalents) of each sample.

Butterfly Rearing

Forty-four *S. clausum* plants were propagated from cuttings and root stock taken from ~25 plants in Collier and Glades Counties, Florida in May and October 1988 (sites 2 and 4, Figure 1). Several of these plants were propagated from the same wild plants sampled for cardenolide spectroassay. Plants were grown in pots under ambient conditions in Gainesville, Florida; I assumed that their chemical constitution approximated that of plants growing naturally. Progeny of six female queens (*ex* Collier/Glades Counties, October–November 1988) were randomly distributed among *S. clausum* plants. Plants were arranged so that larvae could move between plants and choose which leaves to eat, as they do in nature (personal observation). Thus, this study does not attempt to maintain a chemical connection between individual plants and butterflies, but rather assesses palatability of an artificial butterfly population feeding on a population of plants. Larvae were reared under ambient laboratory conditions in Gainesville, Florida, and were transferred to new plants before old ones were severely defoliated (to reduce the possibility of induced chemical defenses in the plants; e.g., Wratten et al., 1988). Butterflies were frozen one day after emergence and

stored with care to avoid desiccation (Ritland, 1991c). Red-winged blackbirds treat freshly killed and previously frozen queens in like manner, suggesting that the queen's chemical defenses are not degraded by freezing (Ritland, 1991c).

Quantitative Cardenolide Assay of S. clausum*-Reared Queens*

I assayed 12 butterflies reared on *S. clausum* to determine whether they concentrated cardenolides to higher levels than those found in the plants. Previously frozen butterflies were dried for 16 hr at 60°C, ground in petroleum ether using a tissue homogenizer, and then extracted in a shaker bath with petroleum ether for 30 min at 35–37°C to remove lipid. Lipid extraction reportedly removes negligible cardenolide (Nishio, 1980; Nelson and Brower, unpublished data). The ground butterflies were then individually extracted in 10 ml 95% ethanol in a shaker bath for 1 hr at 75–78°C, and each butterfly was individually assayed using standard spectrophotometric techniques (methods of Brower et al., 1972; modified by Brower et al., 1975, and Malcolm et al., 1991).

Palatability of S. clausum*-Reared Queens*

The role of cardenolides in danaine defense is well documented, and Brower et al. (1967, 1968, 1972), employing a combination of bioassays and spectroassays of butterflies from natural populations, demonstrated the existence of a cardenolide-based palatability spectrum in monarch butterflies. However, the possibility of de novo defensive chemicals or storage of other phytochemicals precludes reliable inference of butterfly palatability from food plant cardenolide content. Therefore, I directly assessed the palatability of *S. clausum*-reared queens (compared to palatable control butterflies) by presenting them to generalist avian predators: wild-caught male red-winged blackbirds, *Agelaius phoeniceus* (L.), Icteridae. Ritland and Brower (1991) discuss the ecological relevance of these predators.

I employed a rigorous new bioassay protocol that successfully evaluates palatability while eliminating several potential confounding factors: predators' previous experience with butterflies, aversions learned during the experiment, neophobia (fear of novel prey), and presentation order effects (Ritland, 1991c). Methodological details are presented elsewhere (Ritland, 1991c), but a key feature of my protocol was that I presented only butterfly abdomens to the birds. Birds were therefore likely to accept or reject butterflies based on postattack (gustatory) cues, rather than on visual (wing pattern) cues that they might have previously been conditioned to accept or reject in the wild. (The suitability of abdomens as estimators of whole-butterfly palatability is considered below).

Bird capture, acclimation, and training are detailed by Ritland (1991c). I conducted the experiments in specialized cages (see Coppinger, 1970) equipped with (1) a rotating "lazy-susan" device containing 12 feeding cups for butterfly

presentation and (2) one-way glass to facilitate observation. On each of two consecutive days, each bird received three *S. clausum*-reared queen abdomens randomly interspersed with three abdomens of palatable control butterflies (for a total of six queens and six controls per bird). Controls were species known to be palatable to red-wings (Ritland, 1991b): *Anartia jatrophe* (Johansson), *Phoebis sennae* (L.), *Urbanus proteus* (L.), *Papilio palamedes* Drury, and *P. glaucus* (L.). Abdomens were presented individually in the rotating feeding dishes for 12 sec at 10-min intervals.

Palatability Indices. When an abdomen was presented, I scored the bird's response as: 0 = not touched (sight-rejected), 1 = pecked once (taste-rejected), 1.5 = pecked repeatedly but not eaten, 2 = partly eaten, or 3 = entirely eaten. Thus, higher response scores indicate more vigorous attacks. If the bird ate the abdomen entirely, I recorded manipulation time (from initiation of attack to swallowing). Finally, I recorded the incidence of distress behavior (head-shaking, feather-ruffling, and vigorous bill-wiping) exhibited by each bird in response to controls and queens. Such reactions are commonly observed when birds encounter distasteful prey (e.g., Bowers, 1981; Schlee, 1986).

Each bird's responses to queens and controls were individually summarized by calculating mean response score, mean manipulation time, and percentage of trials eliciting distress behavior. I compared queens to controls via paired-sample t tests (corrected for unequal variance if necessary). In all comparisons, I tested a null hypothesis of no difference, and all probability values reported are two-tailed.

Controlling for Potential Differences in Bird Taste Sensitivity. To ensure that these particular birds were capable of tasting and rejecting defensive chemicals in butterflies, I assessed their reactions to two reference species: viceroys reared on *Salix caroliniana* Michx. and monarchs reared on *Asclepias curassavica* L. In earlier experiments, other red-wings treated viceroys as moderately unpalatable (40% eaten) and *A. curassavica*-reared monarchs as very unpalatable (6% eaten). Each bird in the current experiment received two viceroy and two monarch abdomens (in that order) at the end of the experiment, and their responses were compared to those of the red-wings used in previous feeding experiments.

Palatability of Wild-Caught Adult Queens

Adult queens were collected in Glades County, Florida (Figure 1, site 4) in November 1988. Extensive searching in the area yielded only *S. clausum* as a potential larval host plant. I therefore assumed that these butterflies fed as larvae upon *S. clausum*, and that they differed from my lab-reared queens primarily in having had local access to adult-obtained compounds such as PAs. Potential PA sources in the area at the time of queen collection included *Cro-*

talaria spectabilis Roth. and *C. retusa* L. (Fabaceae), senescent *Senecio glabellus* Poir. (Asteraceae), and *Eupatorium* spp. (Asteraceae). No queens were directly observed visiting these plants, but queens sampled from this site earlier in the year did contain variable PA concentrations (Ritland, 1991c).

I assessed the palatability of wild-caught queens in an experiment employing the same protocol outlined above; I used eight new red-wings, each of which received (over two consecutive days) six wild-caught queen abdomens randomly interspersed with six abdomens of the previously listed palatable control species. Palatability indices and statistical comparisons were as in the reared-queen experiment. These birds also received abdomens of *A. curassavica*-reared monarchs and *S. caroliniana*-reared viceroys at the end of the experiment to assess their taste sensitivity.

RESULTS

Sarcostemma clausum *Cardenolide Content*

Spectroassays revealed that seven (44%) of the 16 plants sampled contained no detectable cardenolide in their leaves (Table 1). The remaining nine plants registered peak absorbances of only 0.018–0.049, indicating very low cardenolide concentrations ranging from 2.2 to 12.5 μg (digitoxin equivalents) per 0.1 g dry leaf material. The mean cardenolide concentration of all 16 plants was only 3.8 μg/0.1 g dry weight (dw) (SD = 4.3), or 0.004%; mean concentration of the nine nonzero plants was 6.8 μg/0.1 g dw (SD = 3.4). Even these low concentrations may be overestimates, because of potential interference from coextracted phytochemicals (see also Cohen, 1983). Unless queen larvae are extremely efficient at concentrating cardenolides from their food plant, it seems unlikely that such low levels could contribute significantly to a butterfly's chemical defense (see palatability data, below). No significant difference in cardenolide concentration existed among plants from the four sites (Kruskal-Wallis one-way analysis, $H = 1.68$, $df = 3$, $P > 0.5$; but sample sizes are small). It is possible that elsewhere in its broad geographic range, *S. clausum* contains higher cardenolide levels or that cardenolide content varies seasonally as in *Asclepias* species (e.g., Nelson et al., 1981). Nonetheless, the southern Florida populations represented by these samples are evidently poor cardenolide sources for danaines.

Butterfly Cardenolide Content

Queens reared on *S. clausum* contained no detectable cardenolide; all 12 butterflies sampled registered absorbances of 0 (Table 1). They apparently were unable to concentrate the meager cardenolides of their host plant to an effective

TABLE 1. RESULTS OF CARDENOLIDE SPECTROASSAY OF 16 *Sarcostemma clausum* PLANTS COLLECTED IN SOUTHERN FLORIDA AND 12 FLORIDA QUEENS (*Danaus gilippus*) REARED IN LABORATORY ON *S. clausum*

Plants				Lab-reared butterflies		
Code	Site[a]	Absorbance[b]	Cardenolide concentration[c]	Code	Absorbance[b]	Cardenolide concentration[c]
A	1	0.018	2.2	S1	0.0	nil
B	2	0.049	12.5	S2	0.008	nil
C	3	0.031	6.4	S3	0.009	nil
D	4	0.023	3.8	S4	0.010	nil
E	1	0.019	2.6	S5	0.0	nil
F	2	0.036	8.3	S6	0.0	nil
G	3	0.006	nil	S7	0.0	nil
H	4	0.0	nil	S8	0.0	nil
I	1	0.0	nil	S9	0.001	nil
J	2	0.0	nil	S10	0.001	nil
K	3	0.037	8.4	S11	0.001	nil
L	4	0.034	7.2	S12	0.001	nil
M	1	0.0	nil			
N	2	0.0	nil			
O	3	0.0	nil			
P	4	0.042	9.9			
Mean (±SE)			3.8 ± 1.1			

[a] See Figure 1.
[b] Peak absorbance recorded for sample at 622 nm on Perkin-Elmer spectrophotometer.
[c] Micrograms of cardenolide (digitoxin equivalents) per 0.1 g. dry leaf material. Calculated from peak absorbance as in Brower et al. (1975).

level, similar to the situation with monarch butterflies reared on the cardenolide-free asclepiadaceous vine *Gonolobus rostratus* (Vahl) Roem. & Schult. (Brower et al., 1972).

Palatability of Lab-Reared Queens

Abdomens of the reared queens did not significantly differ from the palatable control abdomens in percentage eaten (paired-sample $t = 0.94$, $P = 0.62$) or response score ($t = 1.19$, $P = 0.27$) (Table 2, Figure 2). Six birds (75%) ate every queen abdomen offered, and only one bird was hesitant to eat the *S. clausum*-reared queens (perhaps due to previous experience or unusual sensitivity to low-level chemical defenses). Over 85% of the reared queens were entirely eaten, while 10% were dropped after a single peck (apparently taste-rejected).

TABLE 2. MEAN REACTIONS OF EIGHT RED-WINGED BLACKBIRDS TO ABDOMENS OF CONTROL BUTTERFLIES[a] AND QUEENS (*Danaus gilippus*) REARED IN LABORATORY ON *Sarcostemma clausum*[b]

	Butterfly type							
	Control				Reared queen			
Bird	Eaten (%)	Mean Score[c]	Mean time[d]	Distress (%)[e]	Eaten (%)	Mean score[c]	Mean Time[d]	Distress (%)[e]
S-1	100	3.00	7.3	0.0	100	3.00	10.7	0.0
S-2	83.3	2.83	3.4	0.0	100	3.00	3.6	16.7
S-3	100	3.00	4.6	16.7	16.7	1.67	12.8	50.0
S-4	83.3	2.83	4.3	0.0	67.7	2.33	11.8	16.7
S-5	100	3.00	3.5	0.0	100	3.00	18.9	0.0
S-6	100	3.00	5.8	0.0	100	3.00	4.1	0.0
S-7	100	3.00	6.8	0.0	100	3.00	19.9	0.0
S-8	100	3.00	3.9	0.0	100	3.00	21.1	16.7
Mean	95.8	2.96	5.0	2.1	85.6	2.75	12.9	12.5
SE	2.7	0.03	0.5	2.1	10.6	0.18	2.4	6.1

[a] Control species: *Papilio glaucus*, *P. palamedes*, *Urbanus proteus*, *Phoebis sennae*.
[b] $n = 6$ controls and 6 queens per bird.
[c] Mean response score calculated from scores recorded for individual presentations, ranging from 0 = not touched to 3 = entirely eaten; see Methods and Materials for details.
[d] Mean manipulation time calculated for abdomens entirely eaten.
[e] Percentage of trials that elicited "distress behavior" (head-shaking, etc.); see Methods and Materials for details.

Manipulation times of *S. clausum*-reared queen abdomens were significantly longer than those of control abdomens (mean time = 12.9 vs. 5.0 sec; $t = -3.2$, $P = 0.015$) (Table 2, Figure 2); this indicates that birds delayed between attacking and swallowing queen abdomens, perhaps due to low-level chemical defenses produced by the butterfly or sequestered from the larval host plant. An alternative hypothesis, that queens were manipulated longer due to their toughness, was explicitly eliminated by including two tough-textured control species (*Urbanus proteus*, *Phoebis sennae*); both of these were readily devoured without delay (mean times = 5.8 and 4.4 sec, respectively). The suggestion that the reared queens possess a low-level chemical deterrent gains support from the observation that 12.5% of queen presentations elicited distress behavior (compared to 2.1% of control presentations) (Figure 2). This level of distress behavior, while significantly greater than that observed with control abdomens ($t = 2.38$, $P = 0.048$), is considerably lower than that observed with

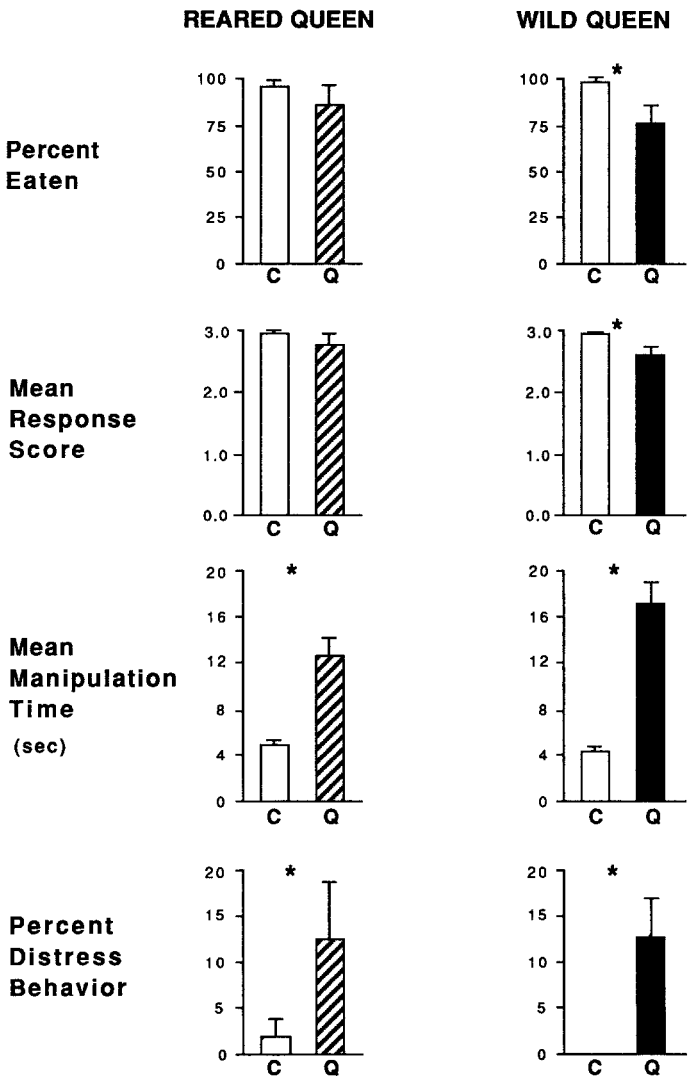


FIG. 2. Left column: Comparative palatability to eight red-winged blackbirds of control abdomens vs. abdomens of queens (*Danaus gilippus*) reared in the laboratory on *Sarcostemma clausum*. $N = 6$ controls and 6 queens per bird. See text for details on response score, manipulation time, and distress behavior. Right column: Comparative palatability to eight red-wings of control abdomens vs. abdomens of wild-caught queens that presumably fed as larvae on *S. clausum* and had access to adult-obtained defensive chemicals. $N = 6$ controls and 6 queens per bird. * indicates significant difference between queen and control (paired-sample t tests, $\alpha = 0.05$).

several other chemically defended butterflies (Brower, 1958a; Bowers, 1980; Ritland, 1991c).

Male and female queens did not differ in cardenolide content (paired-sample $t = 0.0$), percentage eaten ($t = 1.43$, $P = 0.20$), or response score ($t = 0.42$, $P = 0.68$) (Ritland, 1991c).

It is possible that *S. clausum*-reared queens were accepted because these particular red-wings were insensitive to butterfly chemical defenses in general; this explanation is eliminated by examining the treatment of *Salix caroliniana*-reared viceroys and *Asclepias curassavica*-reared monarchs offered at the conclusion of the experiment. The eight birds ate only 44% of viceroys (similar to the 40% eaten by 23 birds in an earlier study: $t = 0.23$, $P = 0.82$; Ritland, 1991b), and they ate only 12.5% of *A. curassavica*-reared monarch abdomens (similar to the 6% eaten by eight red-wings in a pilot experiment: $t = 0.61$, $P = 0.55$; Ritland, 1991c). Clearly, birds in the current experiment were capable of detecting and rejecting chemically defended butterflies.

Palatability of Wild-Caught Adult Queens

Overall, the eight red-wings ate significantly fewer wild-caught queen abdomens than control abdomens (paired-sample $t = 2.38$, $P = 0.048$), but half the birds ate every wild-collected queen abdomen offered. Seventeen percent of wild queens were taste-rejected after a single peck, and queens therefore elicited significantly lower response scores than control abdomens ($t = 2.5$, $P = 0.04$) (Table 3, Figure 2). As in the reared-queen experiment, manipulation times for wild queen abdomens were significantly longer than times for control abdomens (paired-sample $t = -4.6$, $P = 0.003$), and wild queens elicited a low but statistically significant ($t = -3.0$, $P = 0.02$) level of distress behavior (12.5%). This again suggests that a low-level chemical defense exists in the wild-caught queens. No significant difference existed between male and female wild-caught queens in percentage eaten (paired-sample $t = -1.5$, $P = 0.18$) or response score ($t = -0.8$, $P = 0.46$) (Ritland, 1991c).

As in the reared-butterfly experiment, the red-wings treated abdomens of *Salix caroliniana*-reared viceroys as moderately unpalatable; 50% were entirely eaten (similar to the 40% eaten by 23 red-wings in a previous experiment: $t = 0.67$, $P = 0.51$; Ritland, 1991b). *A. curassavica*-reared monarchs were treated as very unpalatable; 6% were entirely eaten, equaling the 6% eaten by eight red-wings in a pilot study ($t = 0.0$; $P = 1.0$; Ritland, 1991c). Therefore, the possibility that these particular birds were simply insensitive to defensive chemicals is eliminated.

TABLE 3. REACTIONS OF EIGHT RED-WINGED BLACKBIRDS TO ABDOMENS OF CONTROL BUTTERFLIES[a] AND WILD-COLLECTED QUEENS (*Danaus gilippus*) THAT PRESUMABLY FED AS LARVAE ON *Sarcostemma clausum* AND ALSO HAD ACCESS TO ADULT-OBTAINED DEFENSIVE CHEMICALS[b]

	Butterfly type							
	Control				Wild queen			
Bird	Eaten (%)	Mean score[c]	Mean Time[d]	Distress (%)[e]	Eaten (%)	Mean score[c]	Mean Time[d]	Distress (%)[e]
G-1	100	3.00	8.1	0.0	100	3.00	13.5	0.0
G-2	100	3.00	3.7	0.0	100	3.00	5.9	0.0
G-3	100	3.00	2.6	0.0	33.3	2.00	11.9	16.7
G-4	100	3.00	3.6	0.0	100	3.00	13.2	33.3
G-5	83.3	2.83	3.4	0.0	50.0	2.00	15.7	16.7
G-6	100	3.00	4.6	0.0	66.7	2.50	27.1	16.7
G-7	100	3.00	5.9	0.0	66.7	2.33	27.3	16.7
G-8	100	3.00	3.7	0.0	100	3.00	26.4	0.0
Mean	97.9	2.98	4.4	0.0	77.0	2.60	17.6	12.5
SE	2.1	0.02	0.6	0.0	9.4	0.16	2.9	4.2

[a] Control species: *Papilio glaucus*, *P. palamedes*, *Urbanus proteus*, *Phoebis sennae*.
[b] $n = 6$ controls and 6 queens per bird.
[c] Mean response score calculated from scores recorded for individual presentations, ranging from 0 = not touched to 3 = entirely eaten; see Methods and Materials for details.
[d] Mean manipulation time calculated for abdomens entirely eaten.
[e] Percentage of trials that elicited "distress behavior" (head-shaking, etc); see Methods and Materials for details.

Comparison of Reared and Wild-Caught Queens

My prediction that wild-caught queens (with access to pyrrolizidine alkaloids) would be substantially more distasteful than lab-reared queens was not upheld. While trends in this direction are apparent, no statistically significant difference existed between the two groups (Figure 2): (1) birds entirely ate 77% of wild queen abdomens (vs. 85% of reared queens eaten by the previous eight birds; $t = 0.60$, $P = 0.57$); (2) mean response score for wild queens was 2.60 (vs. 2.75 for reared; $t = 0.62$, $P = 0.55$); (3) mean manipulation time for wild queens was 17.6 sec (vs. 12.9 sec for reared; $t = 1.26$, $P = 0.22$); and (4) incidence of distress behavior was 12.5% in both cases ($t = 0.0$, $P = 1.0$).

Assessment of Experimental Procedure

The experimental protocol was designed to eliminate several factors that could confound assessment of palatability (see Ritland, 1991c), and it appears to have been successful: (1) No neophobia was evident; birds readily attacked and ate the unfamiliar and variously colored control abdomens. (2) No preexisting aversions were evident; every bird readily attacked the first queen abdomen it was offered, and abdomens were not rejected until after birds had tasted one or more of them. (3) Spearman rank correlation analysis indicates that no bird demonstrated a significant increase or decrease in response score over the course of its six queen presentations (Ritland, 1991c). This suggests that birds treated individual abdomens independently and did not learn to recognize them and accept or reject them based on visual cues. Therefore, I conclude that this experiment succeeded in measuring the birds' responses to palatability per se without the confounding influences of mimicry, previous experience, or learned aversions. (4) Extrapolating from palatability of abdomens in the laboratory to palatability of intact butterflies in the wild must be done cautiously. However, previous laboratory experiments comparing treatment of abdomens vs. intact butterflies reveal that the percentage of queen abdomens entirely eaten by birds approximates the percentage of intact queens at least partially eaten, i.e., killed (Ritland, 1991b). Therefore, in this system, measurements of abdomen palatability allow estimates of the relative palatabilities of intact butterflies and lend insight into the probability of a butterfly surviving an attack by a predator in the wild. (5) This paper considers the responses of a single predator species; since others may differ in sensitivity to the queen's defensive chemicals, future investigations must include other members of the predator community known or believed to exploit queen populations as prey (cf., Malcolm, 1990).

DISCUSSION

The spectroassays suggest that sampled *S. clausum* plants from southern Florida are very poor cardenolide sources, and the palatability trials indicate that queens reared on this food plant gain very little chemical defense against red-winged blackbird predators. (The possibility that these particular birds were simply insensitive to butterfly chemical defenses was eliminated by examining their treatment of viceroys and monarchs.) The observed palatability of *S. clausum*-reared queens parallels the findings of Brower et al. (1967), who showed that 100% of monarchs reared on a cardenolide-free vine, *Gonolobus rostratus*, were eaten by blue jay predators (*Cyanocitta cristata bromia* Oberholser).

The laboratory-reared queens contained no detectable cardenolide, and if

they stored other phytochemicals from their larval food plant, those apparently conferred little protection. I also found little evidence that queens synthesize their own defensive compounds in effective quantities (although significantly longer queen manipulation times and a low incidence of distress behavior suggest that perhaps they did possess low levels of distasteful noncardenolide compounds). I estimate that if these had been living butterflies, 90% of them would not have survived a bird attack (assuming that repeated pecks are fatal and discounting behavioral defenses). Previous workers have raised questions concerning the queen's cardenolide-based defense (Brower et al., 1975; Cohen, 1985), but the level of queen palatability revealed in the current experiment was surprising.

Wild-collected queens that presumably fed as larvae upon *S. clausum* and also had access to adult-obtained compounds such as pyrrolizidine alkaloids (PAs) were significantly less palatable than control butterflies, but the differences were not of great magnitude (Table 2, Figure 2). I estimate that had they been alive, 83% would not have survived their encounter with a bird (discounting behavioral defenses). Differences in palatability between wild-caught butterflies and reared ones were not statistically significant, but trends exist, and further investigation is required to determine whether wild-caught butterflies are less palatable. The comparison presented here suggests that at the point in time and space represented by the wild queen sample, PAs or other adult-obtained compounds were not a major component of chemical defense. The possibility remains, however, that PAs make substantial contributions to queen distastefulness in other areas or other seasons in which PA sources are more abundant or comprise a different array of species. Even within a single locale, seasonal changes in the flora (e.g., from *Senecio*-dominated in spring to *Crotalaria*- and *Eupatorium*-dominated in autumn) may result in quantitative and qualitative differences in the PAs available to queens. Studies of geographically and seasonally representative queen samples are needed to clarify the purported defensive value of PAs against vertebrate predators (cf. Glendinning et al., 1990; Ritland, 1991c).

Future investigations must also address the possibility that *S. clausum* varies geographically in cardenolide content or that queens vary geographically in ability to sequester defensive chemicals from it. Nonetheless, the finding that the sampled *S. clausum*-fed queens are poorly defended (chemically) against a generalist avian predator is of some significance, because *S. clausum* is a primary larval food plant throughout central and southern Florida and is probably utilized by queens in much of tropical America. Unless their unpalatability is bolstered by PAs or other adult-obtained substances, these conspicuously colored *S. clausum*-feeding queens are essentially "sheep in wolves' clothing" (Rothschild, 1972). It is unlikely that they are unpalatable enough to elicit con-

ditioned aversions in avian predators (see also Ritland, 1991c), and their conspicuous "warning" coloration may therefore be a liability rather than an advantage.

However, *S. clausum*-fed queens may not be completely vulnerable; they may gain some protection from predators via automimicry. An automimic is a palatable individual that is avoided by conditioned predators because of its resemblance to distasteful conspecifics (Brower et al., 1967). If palatable *S. clausum*-feeding individuals coexist with more distasteful queens (e.g., ones that fed on a high-cardenolide larval food plant such as *Asclepias curassavica*), and if predators have been conditioned previously to avoid the more distasteful queens, then *S. clausum*-fed queens may enjoy an automimetic advantage. The potential for automimicry in different natural populations and its possible consequences will be considered elsewhere.

The findings of this experiment are of additional significance because of the queen's mimicry relationship with viceroy butterflies. Viceroys are traditionally viewed as palatable (Batesian) mimics of queens, which have historically been considered unpalatable. However, as this experiment demonstrates, some queens are relatively palatable. Furthermore, recent studies (Ritland, 1991b; Ritland and Brower, 1991) reveal that Florida viceroys are themselves distasteful (40% of abdomens eaten in feeding experiments similar to this one); in fact, they are significantly more distasteful than the queens in the current experiment (viceroy vs. reared queen: $t = 3.2$, $P = 0.003$; viceroy vs. wild-caught queen: $t = 2.7$, $P = 0.01$). Hence, in areas where *S. clausum* is the primary food plant, queens essentially must be Batesian or very weak Müllerian mimics of relatively strong viceroy models. This postulated reversal of traditionally accepted roles suggests that some viceroy and queen populations are evolving in a very different selective milieu than previously believed; viceroys, rather than benefiting from their resemblance to queens, may suffer an elevated predation rate if palatable queens interfere with predators' aversive conditioning. Thus, the results of these experiments compel a comprehensive reanalysis of viceroy–queen mimicry dynamics. In addition, these findings prompt an investigation into the palatability of soldier butterflies, *Danaus eresimus* (Cramer), which also feed on *S. clausum* (personal observation) and which may be involved in a mimicry relationship with viceroys in Mexico (Chermock, 1947).

Acknowledgments—Lincoln Brower, Deane Bowers, and Ted Sargent provided very helpful comments on the manuscript; Robin Ritland assisted in obtaining plants, queens, and control butterflies; and Alfonso Alonso and Tonya Van Hook instructed me in spectroassay procedures. To all I am grateful. Spectroassays and palatability experiments were conducted in L.P. Brower's laboratory. Funding for travel and rearing equipment was provided by the National Geographic Society (grant 3417-86 to L.P. Brower) and a Sigma Xi grant-in-aid of research.

REFERENCES

ABISCH, E., and REICHSTEIN, T. 1962. Orientierende chemische Untersuchung einiger Asclepiadaceen und Periplocaceen. *Helv. Chim. Acta* 45:2090–2116.

ACKERY, P.R., and VANE-WRIGHT, R.I. 1984. Milkweed Butterflies: Their Cladistics and Biology. Cornell University Press for the British Museum (Natural History), Ithaca, New York.

BOPPRÉ, M. 1984. Chemically mediated interactions between butterflies, pp. 259–275, *in* R.I. Vane-Wright and P.R. Ackery (eds.). The Biology of Butterflies. Academic Press, London.

BOWERS, M.D. 1980. Unpalatability as a defense strategy of *Euphydryas phaeton* (Lepidoptera: Nymphalidae). *Evolution* 34:586–600.

BOWERS, M.D. 1981. Unpalatability as a defense strategy of western checkerspot butterflies (*Euphydryas* Scudder, Nymphalidae). *Evolution* 35:367–375.

BROWER, J.V.Z. 1958a. Experimental studies of mimicry in some North American butterflies. I. The Monarch, *Danaus plexippus* and Viceroy, *Limenitis archippus*. *Evolution* 12:32–47.

BROWER, J.V.Z. 1958b. Experimental studies of mimicry in some North American butterflies. III. *Danaus gilippus berenice* and *Limenitis archippus floridensis*. *Evolution* 12:273–285.

BROWER, L.P. 1969. Ecological chemistry. *Sci. Am.* 220:22–29.

BROWER, L.P. 1984. Chemical defence in butterflies, pp. 109–134, *in* R.I. Vane-Wright and P.R. Ackery (eds.). The Biology of Butterflies. Academic Press, London.

BROWER, L.P., BROWER, J.V.Z., and CRANSTON, F.P. 1965. Courtship behaviour of the queen butterfly, *Danaus gilippus berenice* (Cramer). *Zoologica* 50:1–39.

BROWER, L.P., BROWER, J.V.Z., and CORVINO, J.M. 1967. Plant poisons in a terrestrial food chain. *Proc. Natl. Acad. Sci.* 57:892–898.

BROWER, L.P., RYERSON, W.N., COPPINGER, L.L., and GLAZIER, S.C. 1968. Ecological chemistry and the palatability spectrum. *Science* 161:1349–1351.

BROWER, L.P., MCEVOY, P.B., WILLIAMSON, K.L., and FLANNERY, M.A. 1972. Variation in cardiac glycoside content of monarch butterflies from natural populations in eastern North America. *Science* 177:426–429.

BROWER, L.P., EDMUNDS, M., and MOFFITT, C.M. 1975. Cardenolide content and palatability of a population of *Danaus chrysippus* butterflies from West Africa. *J. Entomol. (A)* 49:183–196.

BROWN, F.M. and HEINEMAN, B. 1972. Jamaica and its Butterflies. E.W. Classey, Ltd., London.

BROWN, K.S. 1984. Adult-obtained pyrrolizidine alkaloids defend ithomiine butterflies against a spider predator. *Nature* 309:707–709.

BULL, L.B., CULVENOR, C.C.J., and DICK, A.T. 1968. The Pyrrolizidine Alkaloids. North-Holland Publishing Co., Amsterdam.

CHERMOCK, R.L. 1947. A new subspecies of *Limenitis archippus* (Lepidoptera, Nymphalidae). *Am. Mus. Novit.* 1365:1–2.

COHEN, J.A. 1983. Chemical interaction among milkweed plants (Asclepiadaceae) and lepidopteran herbivores. PhD dissertation. University of Florida, Gainesville.

COHEN, J.A. 1985. Differences and similarities in cardenolide content of queen and monarch butterflies in Florida and their ecological and evolutionary implications. *J. Chem. Ecol.* 11:85–103.

CONNER, W.E., EISNER, T., VANDER MEER, R.K., GUERRERO, A., and MEINWALD, J. 1981. Precopulatory sexual interaction in an arctiid moth (*Utetheisa ornatrix*): Role of a pheromone derived from dietary alkaloids. *Behav. Ecol. Sociobiol.* 9:227–235.

COPPINGER, R.P. 1970. The effect of experience and novelty on avian feeding behavior with reference to the evolution of warning coloration in butterflies. II. Reactions of naive birds to novel insects. *Am. Nat.* 104:323–335.

EDGAR, J.A., BOPPRÉ, M., and SCHNEIDER, D. 1979. Pyrrolizidine alkaloid storage in African and Australian danaid butterflies. *Experientia* 35:1447–1448.

EISNER, T. 1980. Chemistry, defence and survival: case studies and selected topics, pp. 847–878, *in* M. Locke and D.S. Smith (eds.). Insect Biology in the Future. Academic Press, New York.

GLENDINNING, J.I., BROWER, L.P., and MONTGOMERY, C.A. 1990. Taste and toxic responses of three mice species to cardiac glycosides and pyrrolizidine alkaloids. *Chemoecology*. In press.

HEGNAUER, R. 1964. *Chemotaxonomie der Pflanzen*. Band 3. Birkhauser e Verlag, Basel.

HOLM, R.W. 1950. The American species of *Sarcostemma* R. Br. (Asclepiadaceae). *Ann. Mo. Bot. Garden* 37:477–560.

MALCOLM, S.B. 1990. Mimicry: Status of a classic evolutionary paradigm. *Trends Ecol. Evol.* 5:57–62.

MALCOLM, S.B., COCKRELL, B.J., and BROWER, L.P. 1989. Cardenolide fingerprint of monarch butterflies reared on common milkweed, *Asclepias syriaca* L. *J. Chem. Ecol.* 15:819–853.

MALCOLM, S.B., COCKRELL, B.J., and BROWER, L.P. 1991. Spring migration of the monarch butterfly: cardenolide content and wing wear as labels of successive brood migration, *in* S.B. Malcolm and M.P. Zalucki (eds.). Biology and Conservation of the Monarch Butterfly. Natural History Museum of Los Angeles County, Contributions in Science. In press.

MASTERS, A.R. 1990. Pyrrolizidine alkaloids in artificial nectar protect adult ithomiine butterflies from a spider predator. *Biotropica* 22:298–304.

NELSON, C.J., SEIBER, J.N., and BROWER, L.P. 1981. Seasonal and intraplant variation of cardenolide content in the California milkweed, *Asclepias eriocarpa*, and implications for plant defense. *J. Chem. Ecol.* 7:981–1009.

NISHIO, S. 1980. The fates and adaptive significance of cardenolides sequestered by larvae of *Danaus plexippus* (L.) and *Cycnia inopinatus* (Hy. Edwards). PhD dissertation. University of Georgia, Athens.

OPLER, P.A., and KRIZEK, G.O. 1984. Butterflies East of the Great Plains. Johns Hopkins University Press, Baltimore.

PARSONS, J.A. 1965. A digitalis-like toxin in the monarch butterfly, *Danaus plexippus*. *J. Physiol.* 178:290–304.

PLISKE, T.E. 1975. Attraction of Lepidoptera to plants containing pyrrolizidine alkaloids. *Environ. Entom.* 4:455–473.

PLISKE, T.E., and EISNER, T. 1969. Sex pheromone of the queen butterfly: biology. *Science* 164:1170–1172.

REICHSTEIN, T., EUW, J. VON, PARSONS, J.A., and ROTHSCHILD, M. 1968. Heart poisons in the monarch butterfly. *Science* 161:861–866.

RITLAND, D.B. 1991a. Revising a classic butterfly mimicry scenario: Demonstration of Müllerian mimicry between Florida viceroys (*Limenitis archippus floridensis*) and queens (*Danaus gilippus berenice*). *Evolution*. In press.

RITLAND, D.B. 1991b. Unpalatability of the viceroy butterfly (*Limenitis archippus*) and its purported mimicry model, the Florida queen (*Danaus gilippus*). *Oecologia*. In press.

RITLAND, D.B. 1991c. Reassessment of viceroy butterfly (*Limenitis archippus*) mimicry and an analysis of the ecological dynamics of mimicry. PhD dissertation. University of Florida, Gainesville.

RITLAND, D.B., and BROWER, L.P. 1991. The viceroy butterfly is not a Batesian mimic. *Nature* 350:497–498.

ROESKE, C.N., SEIBER, J.N., BROWER, L.P., and MOFFITT, C.M. 1976. Milkweed cardenolides and their comparative processing by monarch butterflies (*Danaus plexippus* (L.)). *Recent Adv. Phytochem.* 10:93–167.

ROSATTI, T.J. 1989. The genera of suborder Apocynineae (Apocynaceae and Asclepiadaceae) in the southeastern United States. *J. Arnold Arbor.* 70:443–514.

ROTHSCHILD, M. 1972. Secondary plant substances and warning colouration in insects, pp. 59–83, *in* H.F. van Emden (ed.). Insect/Plant Relationships. Blackwell Scientific Publishers, Oxford, U.K.

ROTHSCHILD, M., and MARSH, N.A. 1978. Some peculiar aspects of danaid/plant relationships. *Entomol. Exp. Appl.* 24:437–450.

ROTHSCHILD, M., EUW, J. VON, REICHSTEIN, T., SMITH, D.A.S., and PIERRE, J. 1975. Cardenolide storage in *Danaus chrysippus* with additional notes on *D. plexippus*. *Proc. R. Soc.(B)* 190:1–31.

SCHLEE, M.A. 1986. Avian predation on Heteroptera: experiments on the European blackbird *Turdus m. merula* L. *Ethology* 73:1–18.

SCUDDER, S.H. 1870. Is mimicry advantageous? *Nature* 3:147.

WRATTEN, S.D., EDWARDS, P.J., and WINDER, L. 1988. Insect herbivory in relation to dynamic changes in host plant quality. *Biol. J. Linn. Soc.* 35:339–350.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

EXPOSURE TO ODORS FROM STRESSED CONSPECIFICS INCREASES PREFERENCE FOR HIGHER AMBIENT TEMPERATURES IN C57BL/6J MICE

DEL THIESSEN,* CHANA AKINS, and CARLOS ZALAQUETT

Department of Psychology
University of Texas
Austin, Texas 78712

(Received January 28, 1991; accepted March 8, 1991)

Abstract—Exposure to odors from stressed conspecifics increases preference for higher ambient temperatures in C57BL/6J mice. C57BL/6J male mice were individually allowed preferences on a thermal gradient ranging in temperature from 22°C to 42°C. Group 1 ($N = 10$) was exposed to odors from triads of foot-shocked conspecifics during the first 2-hr temperature preference trial. Group 2 ($N = 10$) was exposed to odors from triads of nonstressed conspecifics during similar testing. Body temperature (T_B) variations were measured in three animals of each group. Thermal preference was significantly higher for animals exposed to odors from stressed conspecifics than for animals exposed to odors from nonstressed animals (32.0°C vs. 29.0°C). T_B changes on the heated gradient were significantly higher for animals exposed to odors from stressed animals (+1.5°C) than for animals exposed to odors from nonstressed animals (−0.33°C). Additional animals on a nonheated thermogradient were tested for T_B when exposed to odors from stressed or from nonstressed animals ($N = 3$ per condition). There was no difference in T_B between these two groups. Increases in T_B on the heated gradient are apparently due to the higher ambient temperature choices and not due to the odor per se.

Key Words—Stress, odors, body temperature, ambient temperature, C57BL/6J mice, *Mus musculus*.

INTRODUCTION

Odors from animals under stress often signal injury, distress, or the presence of predators. The conspecific response to these signals is avoidance, dispersal,

*To whom correspondence should be addressed.

0098-0331/91/0800-1611$06.50/0

or increased activity (Muller-Velten, 1966; Carr et al., 1970; Rottman and Snowdon, 1972; Zalaquett and Thiessen, 1991). Observations of the gerbil, *Meriones unguiculatus*, and the mouse, *Mus musculus*, suggest a relationship between conspecific odors, physiological changes, and behavior (Cocke and Thiessen, 1986; Thiessen and Cocke, 1990). When gerbils are exposed to odor from stressed conspecifics, body temperatures are elevated about 0.38°C within 20 min. This effect is consistent with other findings that many forms of stress elevate body temperatures (Long et al., 1990). The gerbil becomes inactive when confined with the odor and will avoid the odor if given the opportunity. We have noted similar behavioral effects with BALB/cJ and C57BL/6J mice (Zalaquett and Thiessen, 1991; Zalaquett, unpublished observations). In addition, in mitogen assays of BALB/cJ mice, continuous exposure to odors from stressed mice for 24 hr results in immunological suppression of T-cell proliferation (Thiessen and Cocke, 1990).

These observations led us to speculate that animals may benefit from increased body temperatures. This appears true for animals exposed to pathogens and may also be the case for exposure to stressful stimuli. When a pathogen invades the body, the initial response often observed among most mammals is an increase in body temperature (Jampal et al., 1983). The febrile response is adaptive in that the spread of the infection is retarded and longevity is increased (Covert and Reynolds, 1977; Hart, 1988; Kluger et al., 1975; Kluger and Vaughn, 1978). Benefits are gained by the reduction of blood levels of iron and zinc, which are necessary for the proliferation of pathogens (Hart, 1988, 1990; Kluger, 1989). Increased body temperature also facilitates T-cell activity and interferon synthesis, and thus brings the force of the immune system into play at an early stage of infection (Downing et al., 1988; Duff and Durum, 1982).

Recent studies in this laboratory show that C57BL/6J mice seek out a higher ambient temperature when exposed to lipopolysaccharide (LPS) or to the odors from animals made ill with LPS (Akins et al., 1991; Akins and Thiessen, 1990). The similarity of reactions suggests that animals exposed to odors from stressed animals may also cause the recipients to seek higher ambient temperatures, a reaction that may facilitate immune responses.

This study tests the possibility that C57BL/6J mice subjected to body odors from stressed conspecifics will increase their temperature preferences and hence their body temperatures.

METHODS AND MATERIALS

Subjects. Fifty male C57BL/6J mice, approximately 60 days of age at the beginning of the study, were obtained from the Animal Resources Center at The University of Texas at Austin. Mice were housed in colony cages mea-

suring 16 × 16 × 13.5 cm at 26°C ambient temperature and 40% relative humidity, with ad libitum food and water under a 12:12 hr light–dark cycle. Twenty-four of these mice were assigned randomly to form four triads of stress odor (SO) donors and four triads of control odor (CO) donors. The remaining 26 animals were divided randomly into four groups housed individually and assigned one of the following conditions:

Group 1 ($N = 10$): stress odor recipients placed on the preheated temperature gradient. Body temperature was measured in three of these recipients (see Procedure below).

Group 2 ($N = 10$): control odor recipients placed on the preheated temperature gradient. Body temperature was measured in three of these recipients.

Group 3 ($N = 3$): stress odor recipients placed on the nonheated temperature gradient, measured for body temperature.

Group 4 ($N = 3$): control odor recipients placed on the nonheated temperature gradient, measured for body temperature.

Thus, animals were tested for temperature reactions to the stress and control odors and for changes in body temperature due to odor exposure or to temperature gradient exposure.

Test Apparatus. A schematic of the temperature gradient apparatus as described by Thiessen et al. (1970) is shown in Figure 1. The gradient was constructed of metal, measuring 147.3 cm in length and 11.4 cm in width. A tube attached to the bottom of the gradient carried water at room temperature one fourth the length of the gradient. Metal heating tapes were placed along the bottom adjacent to the water conduit and were spaced so that the temperature of the gradient ranged from 22°C to 42°C when heated. The heating tapes were regulated with a rheostat, and the gradient runway was enclosed with a removable Plexiglas hood.

The odor donor chambers (enclosed in a cabinet that precluded any visual or auditory contact between donors or experimental subjects) measured 50.6 × 30.8 × 30.8 cm and were sealed with weatherstripping. Colony cages, provided with wood shavings and Purina Lab Chow, were placed inside the donor chambers. The donor chambers were connected to the temperature gradient with Tygon tubing. Airflow from the donor chambers entered at the runway and was vacuumed out at a rate of 7–8 liters/min. The apparatus was housed in the test room adjacent to the colony room. The test room was maintained at room temperature (26°C).

An RCA video camera attached to an RCA video recorder monitored the behavior of animals on the temperature gradient. A Bailey type T probe needle (model BAT-12) was used to measure preferred ambient temperature along the length of the gradient.

Body Temperature Measurements. Biotelemetry devices (Mini-mitter thermistors model M) were used to measure mouse body temperature (T_B) (Thiessen

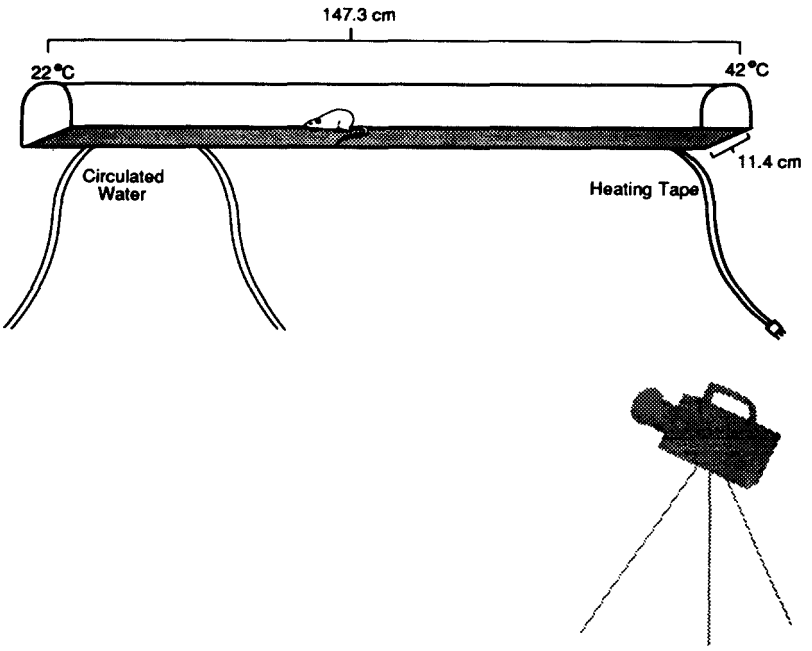


FIG. 1. Schematic diagram of the temperature gradient apparatus.

and Kittrell, 1980). Mini-mitters were reduced in size to accommodate mice by removing the plastic capping and coating the transmitter and battery with candle wax. These thermistors then were calibrated individually over a range of 11°C to 41°C in three steps. The interval between audible beats, which is temperature sensitive, was counted in milliseconds using a receiver and standard AM radio during testing and converted to body temperature using calibration data. As the termistor increases in temperature, the interval between beats decreases.

Mini-mitters were surgically implanted under anesthesia five days prior to testing. A calibrated thermistor was inserted into the interperitoneal cavity through a small incision on the right abdominal surface. The wound was sutured and mice were allowed to recover.

Shock Apparatus. The foot-shock apparatus was constructed of stainless steel with Plexiglas walls. The apparatus measured 24 × 24 × 35 cm with 0.2-cm stainless-steel grid bars spaced 0.4 cm apart forming the floor. Scrambled foot shock was supplied to the floor of the chamber by the Lafayette foot-shock apparatus (model 82404/5 SS). Shock parameters for the individual animals were three successive, 7-sec scrambled electric shocks, separated by a 60-sec rest interval (0.5 mA, 600 V).

Procedure. On the day of testing, mice were transferred from the colony

room to the test room. The temperature gradient apparatus was either preheated for 2 hr prior to testing or left at room temperature (nonheated) during the test period. Each triad of the control odor donors was habituated in the donor chamber with the active airstream 1 hr prior to testing. Each recipient mouse was placed individually on the gradient for 2 hr and received either odor from stressed donors or odor from control donors. Odors were introduced into either end of the gradient (warm or cool) in a random order to eliminate any possible position bias. Temperature preference was defined as that temperature where the mouse first rested for at least 5 min. Body temperature was recorded preceding each trial and immediately after each trial. The two groups were equated for original body temperature.

RESULTS

A *t* test for independent samples was used to test for differences in preferred ambient temperature between SO and CO recipients. These data are seen in Figure 2. Animals that received the odor from stressed conspecifics preferred

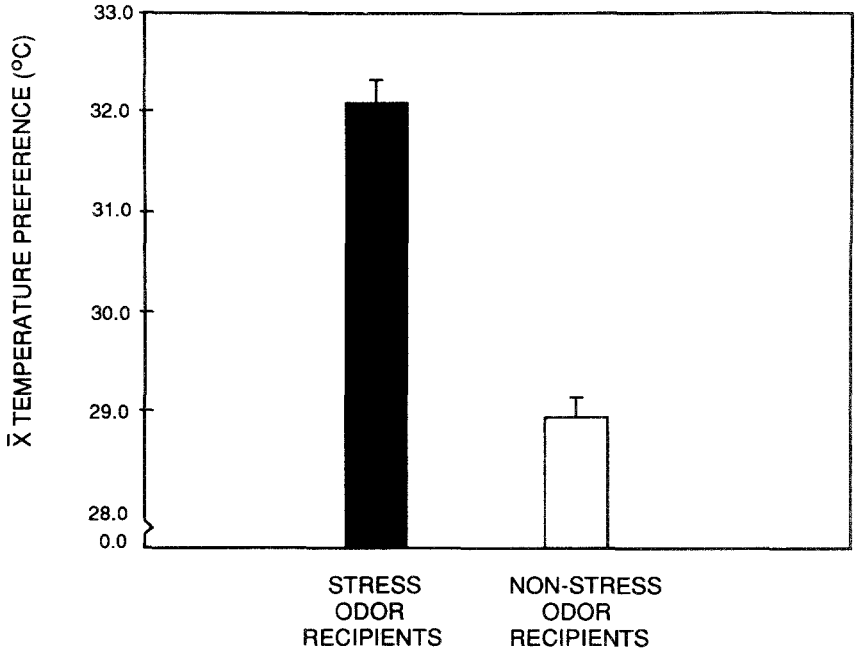


FIG. 2. Preferred ambient temperature (°C) of mice exposed to stress odors or control odors from conspecifics.

a significantly higher mean ambient temperature (32.0 ± 0.17°C) than those that received the nonstressed odors of conspecifics (29.0 ± 0.16°C), ($t = 15.53$, $df = 18$, $P < 0.005$). The difference in initial body temperature and the body temperature after each trial (2 × 2 ANOVA) was compared with SO and CO mice placed on a heated gradient and with SO and CO mice placed on a nonheated gradient. The variations in T_B between the four groups were significant ($F = 10.96$, $df = 3, 8$, $P < 0.005$). Stress odor recipients on a heated gradient had a significantly greater mean T_B increase (+1.5°C) than CO recipients on the heated gradient (−0.33°C). In addition, these SO recipients placed on the heated gradient had a greater mean T_B increase than SO recipients on the nonheated gradient (−0.33°C) and CO recipients on the nonheated gradient (−0.17°C). These data are seen in Figure 3. Among animals exposed to a heated temperature gradient (SO and CO combined), there was a significant relationship between temperature preference and T_B ($r = 0.89$, $df = 3, 8$, $P < 0.005$).

Thus, animals exposed to odors from stressed conspecifics chose a higher temperature preference on the gradient, and as a result of this preference, showed a higher body temperature.

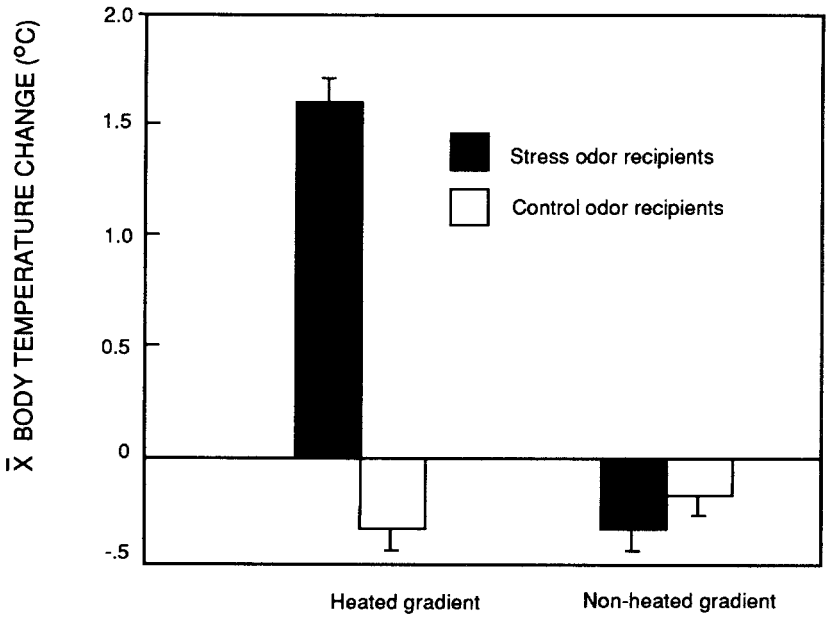


FIG. 3. Difference between initial and posttrial body temperature (°C) of stress odor and control odor recipients on a heated gradient or nonheated gradient for 2 hr.

DISCUSSION

C57BL/6J mice prefer a higher ambient temperature when subjected to the odors of stressed conspecifics. The consequence of the choice is an elevation in body temperature.

The behavioral choice of a higher ambient temperature may be adaptive. Many forms of stress are known to elevate body temperature (Long et al., 1990). In the case of odors from the stressed mice, the intensity of the stress may not be sufficient to evoke an increase in body temperature. The recipient animal may, however, gravitate toward a warmer environment, thus accomplishing the same effect.

The self-induced elevation of body temperature may indicate that the reaction to an odor from a stressed conspecific is a reflection of a more general process. Mice made ill with lipopolysaccharide, or those subjected to the odors from these individuals, also seek out a higher ambient temperature (Akins et al., 1991; Akins and Thiessen, 1990). The elevation of body temperatures that accompanies this behavior may be beneficial. Increased body temperature can, for example, limit the devastating effects of invasive organisms (Hart, 1988, 1990), and it also can facilitate T-cell activity and the synthesis of interferon (Downing et al., 1988; Duff and Durum, 1982). Hart (1988) makes the important point that animals may elevate their body temperature prophylactically when threatened with pathogen invasions.

Our observations suggest that mice react prophylactically to the possible consequences of a stress odor. Perhaps the self-induced elevation in temperature protects the mice from damage that might occur directly from the odor or from the consequences of immune depression. Animals subjected to stress are more vulnerable to disease (Hart, 1990; Khansari et al., 1990), and febrile temperatures can limit the spread of pathogens and stimulate the immune system (Hart, 1988, 1990; Downing et al., 1988; Duff and Durum, 1982).

Stress effects and consequences of pathogenic invasion often seem similar. Both cause an initial depression of T-cell and B-cell activities; both involve the activity of the pituitary–adrenal system; both elevate body temperature; and both trigger events that eventually facilitate immunoreactivity (Khansari et al., 1990; Hart, 1988).

From this perspective, what the animals appear to be doing is reacting to stress as if it were a pathogenic attack, or as if such an attack were imminent. Since disease often follows stress, it would be adaptive if the organism made no distinctions between stress and pathogenic invasion. Elevation of body temperature, either by systematic or behavioral means, could be an important initial response.

For this hypothesis to survive, it must be demonstrated that animals stressed by an odor have an advantage when they choose a higher ambient temperature.

Either their resistance to a disease must increase or their ability to cope with disease must be improved.

Acknowledgments—This study was conducted with the support of NIMH grant MH 14076-22 awarded to D. Thiessen.

REFERENCES

AKINS, C.K., and THIESSEN, D.D. 1990. Lipopolysaccharide decreases ambient temperature preference in Mongolian gerbils (*Meriones unguiculatus*). *Percept. Motor Skills*. 71:1–2.

AKINS, C.K., THIESSEN, D.D., and COCKE, R. 1991. Lipopolysaccharide ambient temperature preference in C57BL/6J adult mice. *Physiol. Behav.* In press.

CARR, W.J., MARTORANO, R.D., and KRAMES, L. 1970. Responses of mice to odors associated with stress. *J. Comp. Physiol. Psychol.*, 71(2):223–228.

COCKE, R., and THIESSEN, D.D. 1986. Chemocommunication among prey and predator species. *Anim. Learn. Behav.* 14(1):90–92.

COVERT, J.B., and REYNOLDS, W.W. 1977. Survival value of fever in fish. *Nature* 267:43–45.

DOWNING, J.F., MARTINEZ-VALDEZ, H., ELIZONDO, R.S., WALKER, E.B., and TAYLOR, M.W. 1988. Hyperthermia in humans enhances interferon-g synthesis and alters the peripheral lymphocyte population. *J. Interferon Res.* 8:143–150.

DUFF, G.W., and DURUM, S.K. 1982. T cell proliferation induced by interleukin-1 is greatly enhanced by hyperthermia. *Clin. Res.* 30(3):694A.

HART, B.L. 1988. Biological basis of the behavior of sick animals. *Neurosci. Biobehav. Rev.* 12:123–137.

HART, B.L. 1990. Behavioral adaptations to pathogens and parasites: Five strategies. *Neurosci. Biobehav. Rev.* 14:273–294.

JAMPAL, H.D., DUFF, G.W., GERSHON, R.K., ATKINS, E., and DURUM, S.K. 1983. Fever and immunoregulation III. Hyperthermia augments the primary in vitro humoral immune response. *J. Exp. Med.* 157:1229–1238.

KHANSARI, D.N., MURGO, A.J., and FAITH, R.E. 1990. Effects of stress on the immune system. *Immunol. Today* 11:170–175.

KLUGER, M.J. 1989. Body temperature changes during inflammation: Their mediation and nutritional significance. *Proc. Nutri. Soc.* 48:337–345.

KLUGER, M.J., and VAUGHN, L.K. 1978. Fever and survival in rabbits injected with *Pasteurella multicida*. *J. Physiol.* 282:243–251.

KLUGER, M.J., RINGLER, D.H., and ANVER, M.R. 1975. Fever and survival. *Science* 188:166–168.

LONG, N.C., VANDER, A.J., and KLUGER, M.J. 1990. Stress-induced rise of body temperature in rats is the same in warm and cold environments. *Physiol. Behav.* 47:773–775.

MULLER-VELTEN, H.G. 1966. Uber den angstgeruch bei der hausmaus. *Z. Vgl. Physiol.* 52:401–429.

ROTTMAN, S.J., and SNOWDON, C.T. 1972. Demonstration and analysis of an alarm pheromone in mice. *J. Comp. Physiol. Psychol.* 81:483–490.

THIESSEN, D.D., and COCKE, R. 1990. Alarm odors suppress the immune system, pp. 507–518, *in* D. Muller Schwartz (ed.). Chemical Signals in Vertebrates, Chapter 5. Plenum Press, New York.

THIESSEN, D.D., and KITTRELL, M.W. 1980. The Harderian gland and thermoregulation in the gerbil (*Meriones unguiculatus*). *Physiol. Behav.* 24:417–424.

THIESSEN, D.D., OWEN, K., and WHITSETT, M. 1970. Chromosome mapping in behavioral activities, pp. 189, *in* G. Lindzey and D.D. Thiessen (eds.). Century Psychology Series. Meredith Corp., New York.

ZALAQUETT, C., and THIESSEN, D.D. 1991. The effects of odors from stressed mice on conspecific behavior. *Physiol. Behav.* In press.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

EFFECT OF OVIPOSITION DETERRENTS FROM ELDERBERRY ON BEHAVIORAL RESPONSES BY *Heliothis virescens* TO HOST-PLANT VOLATILES IN FLIGHT TUNNEL[1]

F.C. TINGLE* and E.R. MITCHELL

Insect Attractants, Behavior, and Basic Biology Research Laboratory
Agricultural Research Service, U.S. Department of Agriculture
Gainesville, Florida 32604

(Received January 11, 1991; accepted April 15, 1991)

Abstract—In flight-tunnel assays, mated female *Heliothis virescens* (F.) moths responded by positive anemotaxis to volatiles from extracts of two host plants (cotton and tobacco), but they did not fly to an extract from elderberry (*Sambucus simpsonii* Rehd.), a nonhost that contains an oviposition deterrent for *H. virescens*. When the elderberry extract was mixed with extract from either cotton or tobacco, the flight response by moths to volatiles emanating from the extract blends was reduced significantly at most doses when compared to the positive response to extracts from either host alone. The number of landings (including brief contacts) and landings that resulted in oviposition on the substrates treated with extract blends also were reduced significantly in most tests.

Key Words—Plant attractant, plant–insect interaction, plant extracts, cotton, tobacco, elderberry, *Heliothis virescens*, Lepidoptera, Noctuidae.

INTRODUCTION

Recent studies in environmentally controlled systems have demonstrated that mated *Heliothis virescens* (F.) and *H. subflexa* (Gn.) females respond to allelochemicals from cultivated and wild host plants for host-finding and oviposition. Tingle et al. (1989) demonstrated in flight-tunnel assays that *H. subflexa*

*To whom correspondence should be addressed.

[1] This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or the recommendation of its use by USDA.

females showed positive anemotaxis (i.e., upwind flight) toward the odor source in the presence of volatiles from an extract of leaves from their only known host, *Physalis* spp. (groundcherry). Then Tingle et al. (1990) showed in dual-choice tests that *H. subflexa* females demonstrated a significant preference for volatiles from extracts of groundcherry and that *H. virescens*, a polyphagous species, responded positively by anemotaxis to extracts from susceptible tobacco, cotton, *Desmodium tortuosum* (Swartz) de Candolle (host plants), and groundcherry (nonhost). When given a choice between volatiles from extracts of leaves from a susceptible tobacco cultivar and from a resistant cultivar, the *H. virescens* females did not fly to volatiles emanating from the resistant tobacco cultivar. Furthermore, Mitchell et al. (1991) showed in no-choice tests that *H. virescens* females responded positively via upwind flight in laboratory assays to volatiles emitted from methylene chloride washes of leaves from cotton, tobacco, and *D. tortuosum*, a weed species. They found that, except for *D. tortuosum*, the response was positive and linear with dose.

Earlier, Jackson et al. (1984) reported that whole-leaf cuticular washes from green leaves of a flue-cured tobacco stimulated oviposition by *H. virescens* when sprayed onto leaves of a resistant tobacco strain. Mitchell and Heath (1987) demonstrated that methanol extracts of washes from whole leaves of groundcherry increased egg deposition by *H. subflexa* on treated tobacco plants (a nonhost) 8.5-fold over untreated controls. These results were consistent whether the extract was evaluated on plants in greenhouse-cage tests or on broadcloth as the substrate in an olfactometer that they developed for oviposition behavioral studies. Using this olfactometer, Mitchell et al. (1990) found that extracts prepared with methylene chloride washes of cotton squares (flower buds) and leaves from cotton, tobacco, *D. tortuosum* (host plants), and groundcherry (nonhost), stimulated oviposition by *H. virescens*. The *H. subflexa* moths were stimulated to oviposit by groundcherry extract, its normal host, and extract from cotton squares, a nonhost.

In contrast to the studies with the chemicals that attract *H. virescens* and *H. subflexa* to their host or stimulate oviposition upon arrival, Tingle and Mitchell (1984, 1986) reported that extracts from various indigenous plants, including elderberry (*Sambucus simpsonii* Rehd.), acted as oviposition deterrents for *H. virescens*. They found that extracts prepared from homogenized elderberry leaves with either acetone, distilled water, ethanol, hexane, methanol, or methylene chloride deterred oviposition by *H. virescens* on treated substrates in laboratory choice tests. In field-cage bioassays, moths laid significantly fewer eggs on tobacco leaves treated with elderberry leaf–water extract than on untreated leaves on the same plant.

Thiery and Visser (1986) found that blending of odors from nonhost species, namely wild tomatoes, *Lycopersicon hirsutum* f. *glabratum* C.H. Mull, or cabbage, *Brassica oleracea* L. var. *gemmifera* D.C., with the attractive plant

odor of potato plants, *Solanum tuberosum* L., blocked the release of upwind responses by female Colorado potato beetles, *Leptinotarsa decemlineata* Say. They reported that the neutralization of the beetle's orientation response was obtained without repellency. Renwick and Radke (1987) discussed how acceptance or rejection of a plant by gravid cabbage butterflies, *Pieris rapae* L., may depend on the balance of positive and negative chemical stimuli, if both are present, within the plant. The present study was undertaken to determine if blending of the volatiles from a nonhost (elderberry), which contains an oviposition deterrent (Tingle and Mitchell, 1984, 1986), with attractant volatiles from host plants (cotton or tobacco) affects the olfactory orientation or other behavioral responses by mated *H. virescens* in the flight tunnel.

METHODS AND MATERIALS

A 60 × 60 × 195-cm Plexiglas flight tunnel (Tingle et al., 1989) was used to observe the effect of an oviposition deterrent from elderberry (Tingle and Mitchell, 1984, 1986) on the flight response of mated *H. virescens* females to volatiles from crude extracts of host plants (Tingle et al., 1990; Mitchell et al., 1991). Extracts were prepared from elderberry (native), cotton (variety McNair 220), and tobacco (NC 2326) by washing ca. 400 g of fresh plant material for 30 sec in 1 liter of methylene chloride, which was filtered and stored in 1-liter glass containers at 0°C until needed (Mitchell and Heath, 1987). Collections of plant material were made from cotton and tobacco plants that were located in cultivated field plots and from natural stands of elderberry. The cotton and elderberry plants were in the flowering stage, and tobacco was in the prebloom stage. The flowering stage of host plants has been shown as the most preferred phenological state by *Helicoverpa zea* (Boddie) (Johnson et al., 1975) and *H. armigera* (Hübner) (Roome, 1975).

Methylene chloride was used as the solvent for removing components from cotton squares (flower buds) and whole leaves of tobacco and elderberry (Mitchell et al., 1990, 1991; Tingle et al., 1990). Before testing, the extracts were concentrated in a rotary evaporator to 1-g equivalent (1 gE) per 200 μl solvent.

All test insects were reared in our laboratory colony on modified pinto bean diet using the methods described by Guy et al. (1985) and Mitchell et al. (1988). This *H. virescens* colony was started in 1987 (Mitchell et al., unpublished data) from field-collected insects; new genetic material from the field has been added each year since then. For testing, the adults were confined upon eclosion for mating (21 pairs) in 5.5-liter plastic cages with screened tops and held under a reversed 14 : 10 hr light–dark cycle in a holding room that was maintained at 25–26°C and 60–70% relative humidity.

On the day of testing, the moths were sexed by gently squeezing the abdo-

men to extrude the genitalia. The females were then confined in a 25 × 25 × 25-cm Plexiglas holding cage and placed in the flight tunnel room (3 × 2.6 × 2.1 m) ca. 1 hr before scotophase. An intake vent in the wall allowed a continuous flow of fresh unfiltered air from the outside into the room, which was maintained at the same environmental conditions as the holding room. An electric timer was used to control overhead fluorescent lights (two banks of two 40-W bulbs). Three 25-W red light bulbs that were equally spaced above the tunnel remained on continuously and provided a light level of 2.4 lux inside the tunnel. Air was pulled through the tunnel at ca. 0.4 m/sec and exhausted ca. 3 m above the roof covering the flight tunnel room via a 30-cm-diam. flexible pipe.

A test was begun by introducing into the tunnel 1 gE of extract from either cotton, tobacco, or elderberry alone, or 1 gE cotton or tobacco in combination with 1, 2, or 3 gE of elderberry extract, on an evaporator substrate (white muslin) that covered the end of a glass cylinder dispenser with a 3.5-cm opening. When a combination of two extracts was applied to the substrate, they were mixed together just prior to application. After waiting 5 min for the solvent to evaporate, the dispenser was placed at the upwind end of the tunnel. Air was blown through the cylinder with an aquarium pump at the rate of 1 liter/min. to provide a continuous flow (plume) of the extract odor through the tunnel. The location and form of the plume was verified previously by introducing smoke into the dispenser system and observing the smoke trail.

Each moth was removed from the holding cage and placed into a cylindrical 4 × 6.5-cm plastic cage with screened ends. After placement of the release cage into the downwind end of the flight tunnel, the moth was released immediately and observed for 2 min. Behavioral responses, including random and oriented flight, contacts and landings, and oviposition on extract-treated substrates, were recorded. Six replicates of six or eight (mean = 7.2) *H. virescens* females per replicate were tested individually in each treatment. One replicate of the three treatments in one or more tests was completed daily. Tests with untreated controls were conducted periodically to ensure that response to the dispenser substrate (white muslin) was negligible when treated with solvent only and that contamination of the bioassay system had not occurred. A limited supply of test insects prevented daily bioassays with untreated controls.

The following treatments with the indicated doses of elderberry extract were conducted to determine the effect of oviposition deterrents from elderberry (Tingle and Mitchell, 1984, 1986) on the flight response by mated *H. virescens* females to volatiles from extracts of cotton (1 gE) in tests 1–3 and tobacco (1 gE) in tests 4–6:

Test 1. Extracts: cotton, elderberry (1 gE), and cotton–elderberry (1 gE) mixture.

Test 2. Extracts: cotton, elderberry (2 gE), and cotton–elderberry (2 gE) mixture.

Test 3. Extracts: cotton, elderberry (3 gE), and cotton–elderberry (3 gE) mixture.

Test 4. Extracts: tobacco, elderberry (1 gE), and tobacco–elderberry (1 gE) mixture.

Test 5. Extracts: tobacco, elderberry (2 gE), and tobacco–elderberry (2 gE) mixture.

Test 6. Extracts: tobacco, elderberry (3 gE), and tobacco–elderberry (3 gE) mixture.

The data from the three treatments in each test were converted to arcsine $\sqrt{x}$ and analyzed using ANOVA (Steel and Torrie, 1960). Treatment means were separated using Duncan's multiple-range test (Duncan, 1955).

After a deterrent effect by volatiles from elderberry extract on the positive response by *H. virescens* to host-plant extracts in the flight tunnel was detected, the percent reduction in flight response was calculated as follows (HPE = host plant extract; EBE = elderberry extract):

$$\frac{\%\text{ response to HPE} - \%\text{ response to HPE with EBE}}{\%\text{ response to HPE}} \times 100$$

These data were analyzed for significance between responses to substrates treated with host-plant extracts and host-plant extracts combined with elderberry extract, using Student's paired t test (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

The mated *H. virescens* females demonstrated a significant flight response by positive anemotaxis to host-plant volatiles in tests 1–6 (Figures 1 and 2) as expected. However, the moths showed no significant flight response to volatiles from elderberry at the three dosages tested. Moreover, when either 2 gE (test 2) or 3 gE (test 3) of elderberry extract was combined with 1 gE of cotton extract, significantly fewer moths flew to the extract mixture than the 47–67% that flew to the cotton extract alone (Figure 1). This resulted in reductions of 43% and 49%, respectively, in flight response to the cotton extract when 2 or 3 gE of the elderberry extract were combined with 1 gE of the extract from cotton (Table 1). No significant reduction in flight response was observed when 1 gE of the elderberry extract (test 1) was used in combination with 1 gE of cotton extract.

When extract from tobacco was used as the attractant (tests 4–6), 50–60% of the *H. virescens* females showed a positive flight response to the tobacco

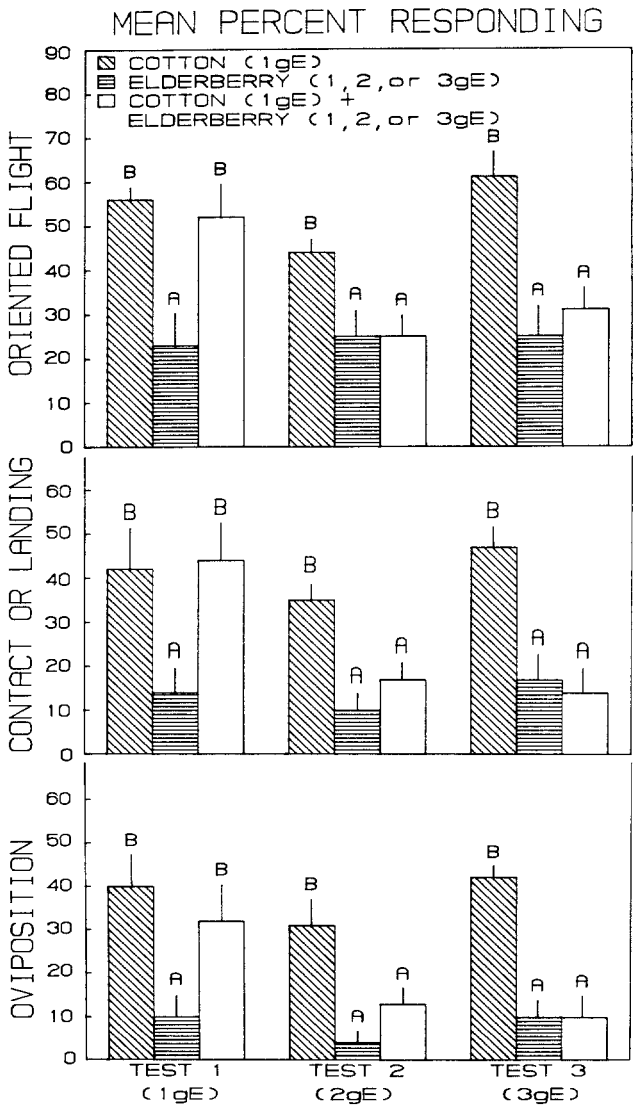


FIG. 1. Mean percentages of *Heliothis virescens* females responding to plant extracts in no-choice tests. Cotton extract (1 gE) was used as the attractant and 1, 2, or 3 gE of elderberry extract was used alone or in combination with cotton extract (1 gE) in tests 1, 2, and 3. Means within each test with the same letter are not significantly different, $P < 0.05$ [ANOVA, Steel and Torrie (1960); Duncan's multiple-range test (Duncan 1955)]. Vertical lines indicate standard error of means.

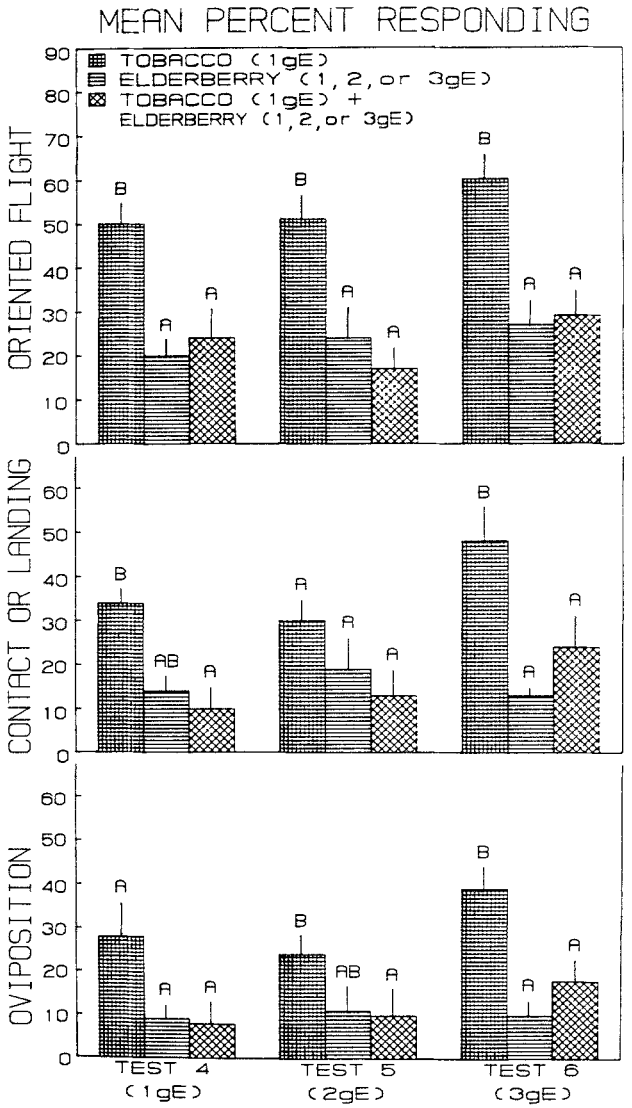


FIG. 2. Mean percentages of *Heliothis virescens* females responding to plant extracts in no-choice tests. Tobacco extract (1 gE) was used as the attractant and 1, 2, or 3 gE of elderberry extract was used alone or in combination with cotton extract (1 gE) in tests 1, 2, and 3. Means within each test with the same letter are not significantly different, $P < 0.05$ [ANOVA, Steel and Torrie (1960); Duncan's multiple-range test (Duncan 1955)]. Vertical lines indicate standard error of means.

extract alone (Figure 2). However, significant reductions of 51–66% in flight response to 1 gE of tobacco extract occurred when 1, 2, or 3 gE of the extract from elderberry were combined with the tobacco extract (Table 1). Moths that did not respond to the extracts flew in a random fashion or landed on the tunnel wall.

Reductions in the number of moths that either contacted or landed on the extract-treated substrates were similar to the flight response data when 1, 2, or 3 gE of the elderberry extract were combined with 1 gE of extract from either cotton or tobacco. More than 85% of the contacts resulted in landings on the substrates. Except when 1 gE of elderberry extract was combined with 1 gE of the cotton extract in test 1, the number of contacts and landings were significantly reduced by 53–71% (Table 1). When the substrates in tests 2 and 3 were treated with 1 gE of cotton extract combined with either 2 or 3 gE, respectively,

TABLE 1. PERCENT REDUCTION IN FLIGHT RESPONSE BY *H. virescens* FEMALES TO EXTRACTS FROM HOST PLANTS IN FLIGHT TUNNEL WHEN EXTRACT FROM ELDERBERRY LEAVES WAS ADDED TO EXTRACT FROM COTTON SQUARES (FLOWER BUDS) OR TOBACCO LEAVES

Elderberry extract dosage (gram equivalents)	Host plant extract (1 gE)			
	Cotton		Tobacco	
	Reduction (%)	*t*-value[a]	Reduction (%)	*t*-value[a]
Oriented flight to extracts[b]				
1 gE	6.3(1)	0.309NS	51.4(4)	2.571*
2 gE	42.8(2)	3.384*	66.1(5)	5.004**
3 gE	48.8(3)	3.682*	51.3(6)	3.535*
Contact or landing on extract substrates				
1 gE	0	0.194NS	69.4	3.552*
2 gE	52.8	2.572*	58.2	2.628*
3 gE	70.5	2.865*	49.2	3.001*
Oviposition on substrates				
1 gE	19.4	0.786NS	70.1	2.271NS
2 gE	60.1	2.572*	60.1	3.511*
3 gE	76.7	3.737*	53.9	3.271*

[a] Calculated by Student's unpaired *t* test from percentage of moths demonstrating a positive response to extract from either cotton or tobacco alone and in combination with extract from elderberry (six replications per dose): NS = nonsignificant, $P < 0.05$; * significant, $P < 0.05$; ** significant, $P < 0.01$.

[b] Test numbers shown in parentheses.

of elderberry extract, only 17% and 14%, respectively, of the moths contacted or landed on the substrates (Figure 1). However, 35% and 47% of the moths in test 2 and 3, respectively, contacted or landed on the substrates treated only with the extract from cotton.

When the extract from tobacco was used as the attractant in tests 4–6, significant reductions of 58–69% in the number of contacts and landing on the substrates treated with the tobacco–elderberry mixture at all three dosages were indicated when the two treatments, tobacco and tobacco–elderberry mixture, were compared by the unpaired *t* test (Table 1). However, when data from all three treatments (tobacco, elderberry, and tobacco–elderberry mixture) were analyzed by ANOVA, significant reductions in the number of contacts and landings were evident only when 1 gE or 3 gE of elderberry extract were combined with 1 gE of the tobacco extract in tests 4 and 6 (Figure 2).

There was no significant reduction in the number of landings that resulted with oviposition on the extract-treated substrates when 1 gE of elderberry extract was combined with 1 gE of extract from either cotton (test 1) or tobacco (test 4) (Table 1, Figures 1 and 2). An increase in the amount of elderberry extract from 1 gE to 2 or 3 gE significantly reduced the number of landings with oviposition on substrates treated with the elderberry–host-plant mixture.

Although untreated controls with only the solvent (methylene chloride) on the substrate were conducted periodically in the flight tunnel, six replicates were conducted on the same days that behavioral responses by *H. virescens* females to both elderberry and host-plant extracts were monitored (Table 2). Significantly more moths demonstrated a positive flight response to the substrates treated with extracts from either cotton (three replicates) or tobacco (three rep-

TABLE 2. MEAN PERCENTAGES (± SE) OF *H. virescens* FEMALES RESPONDING IN NO-CHOICE TESTS TO SUBSTRATES TREATED WITH HOST-PLANT EXTRACT, ELDERBERRY EXTRACT, OR METHYLENE CHLORIDE AS CONTROL

Extract on substrate	Mean percent response[a]		
	Oriented flight	Contact or landing	Landing with oviposition
Host Plant (1 gE)[b]	61.1 ± 3.5b	34.7 ± 5.5b	29.9 ± 5.5b
Elderberry (2 gE)	20.1 ± 8.2a	6.9 ± 4.5a	2.8 ± 2.8a
Control (200 μl)	12.5 ± 4.6a	8.3 ± 2.6a	6.3 ± 2.8a

[a] Means within each column with the same letter are not significantly different [$P < 0.05$, ANOVA, Steel and Torrie (1960), and Duncan's multiple-range test, (Duncan, 1955)].
[b] Cotton (three replicates) or tobacco (three replicates); responses to these two extracts were almost identical.

licates) than flew to either the elderberry- or methylene chloride-treated substrates. There were significantly more contacts, landings, and landings resulting in oviposition on the substrates treated with host-plant extracts. The responses by the females to the elderberry extract and to the solvent (methylene chloride) alone were statistically equal.

The effectiveness of extract from elderberry leaves began to deteriorate four to five months after the extract was prepared with methylene chloride and stored at 0°C. When 1 gE of 5- to 6-month-old elderberry extract was combined with 1 gE of tobacco extract, it did not inhibit the flight response of moths to the tobacco extract as 1 gE of <1-month-old elderberry extract did in test 4. Even when 3 gE of the aged elderberry extract were combined with 1 gE cotton extract, there was no significant effect on the positive response of *H. virescens* females to cotton extract.

Although the extract from elderberry apparently deteriorated after being stored for four to five months at 0°C, we have not detected a significant deterioration of either cotton or tobacco extract as an attractant for *H. virescens* in the flight tunnel, even when the extracts had been stored at 0°C for at least one year (unpublished data). Mitchell and Heath (1987) reported that crude extracts from whole-leaf washes of groundcherry in methanol could be stored for more than one year at 0°C without significant loss in oviposition stimulant activity for *H. subflexa*. The cotton extract used in this study was >9 months old, and the tobacco extract was >20 months old. Both extracts were still effective as attractants for *H. virescens* in the flight tunnel.

The inhibitory effect of the elderberry volatiles on the behavioral responses of *H. virescens* to attractive volatiles from host plants in our laboratory assays is evident. However, understanding the interaction of volatiles from diverse plant species, or even from various plants of a single species in varying growth stages, and how they affect insect behavior in field situations is difficult and poorly understood. Environmental conditions, including wind turbulence that blends the volatiles, must be considered. In addition to volatiles, phytophagous insects also use other plant stimuli (e.g., color, shape, size) in host selection. Elucidation of the chemistry of phytochemicals that govern plant selection and associated behaviors by insects offers opportunities to render them less attractive to specific insect species.

Acknowledgments—We gratefully acknowledge the technical assistance of P. Hall, F. Adams, A. McAshan, C. Green, and J. Leach of this laboratory.

REFERENCES

DUNCAN, D.B. 1955. Multiple range and multiple *F* tests. *Biometrics* 11:1–42.

GUY, R.H., LEPPLA, N.C., RYE, J.R., GREEN, C.W., BARRETTE, S.L., and HOLLIEN, K.A. 1985. *Trichoplusia ni*, pp. 487–494, *in* P. Singh and R.F. Moore (eds.). Handbook of Insect Rearing, Vol. 2. Elsevier Science Publishers, Amsterdam.

JACKSON, D.M., SEVERSON, R.F., JOHNSON, A.W., CHAPLIN, J.F., and STEPHENSON, M.G. 1984. Ovipositional response of tobacco budworm moths (Lepidoptera: Noctuidae) to cuticular chemical isolates from green tobacco leaves. *Environ. Entomol.* 13:1023–1030.

JOHNSON, M.W., STINNER, R.E., and RABB, R.L. 1975. Ovipositional response of *Heliothis zea* (Boddie) to its major hosts in North Carolina. *Environ. Entomol.* 4:291–297.

MITCHELL, E.R., and HEATH, R.R. 1987. *Heliothis subflexa* (Gn.) (Lepidoptera: Noctuidae): Demonstration of an oviposition stimulant from groundcherry using a novel bioassay. *J. Chem. Ecol.* 13:1849–1858.

MITCHELL, E.R., HINES, R.W., and COPELAND, W.W. 1988. *Heliothis subflexa* (Lepidoptera: Noctuidae): Establishment and maintenance of a laboratory colony. *Fla. Entomol.* 71:212–214.

MITCHELL, E.R., TINGLE, F.C., and HEATH, R.R. 1990. Oviposition response of three *Heliothis* species (Lepidoptera: Noctuidae) to allelochemicals from cultivated and wild host plants. *J. Chem. Ecol.* 16:1817–1827.

MITCHELL, E.R., TINGLE, F.C., and HEATH, R.R. 1991. Flight activity of *Heliothis virescens* (F.) females (Lepidoptera: Noctuidae) with reference to host plant volatiles. *J. Chem. Ecol.* 17:259–266.

RENWICK, J.A.A., and RADKE, C.D. 1987. Chemical stimulants and deterrents regulating acceptance or rejection of crucifers by cabbage butterflies. *J. Chem. Ecol.* 13:1771–1776.

ROOME, R.E. 1975. Activity of adult *Heliothis armigera* (Hb.) (Lepidoptera: Noctuidae) with reference to the flowering of sorghum and maize in Botswanna. *Bull. Entomol. Res.* 65:523–530.

STEEL, R.G.D., and TORRIE, J.H. 1960. Principles and Procedures of Statistics. McGraw-Hill, New York.

THIERY, D., and VISSER, J.H. 1986. Masking of host plant odour in the olfactory orientation of the Colorado potato beetle. *Entomol. Exp. Appl.* 41:165–172.

TINGLE, F.C., and MITCHELL, E.R. 1984. Aqueous extracts from indigenous plants as oviposition deterrents of *Heliothis virescens* (F.). *J. Chem. Ecol.* 10:101–113.

TINGLE, F.C., and MITCHELL, E.R. 1986. Behavior of *Heliothis virescens* (F.) in presence of oviposition deterrents from elderberry. *J. Chem. Ecol.* 12:1523–1531.

TINGLE, F.C., HEATH, R.R., and MITCHELL, E.R. 1989. Flight response of *Heliothis subflexa* (GN.) females (Lepidoptera: Noctuidae) to an attractant from groundcherry, *Physalis angulata* L. *J. Chem. Ecol.* 15:221–231.

TINGLE, F.C., MITCHELL, E.R., and HEATH, R.R. 1990. Preferences of mated *Heliothis virescens* and *H. subflexa* females for host and nonhost volatiles in a flight tunnel. *J. Chem. Ecol.* 16:2889–2898.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

VOLATILE SECRETION OF DUFOUR GLAND OF WORKERS OF AN ARMY ANT, *Dorylus (Anomma) molestus*[1]

ANNE-GENEVIEVE BAGNERES,[2,4] JOHAN BILLEN,[3] and E. DAVID MORGAN[2,*]

[2]*Department of Chemistry*
University of Keele
Staffordshire, ST5 5BG, England

[3]*Zoological Institute*
University of Leuven
Leuven, B-3000, Belgium

(Received January 10, 1991; accepted April 22, 1991)

Abstract—The Dufour glands of workers of *Dorylus (Anomma) molestus* contain chiefly linear alkenes and alkanes, with (*Z*)-9-tricosene and tricosane representing over 70%. The glands are relatively small with some indication of very small (nanogram or less) amounts of dihydrofarnesol. Minima, medium, and major workers of *Dorylus (Anomma) nigricans* contain a similar spectrum of compounds.

Key Words—Exocrine secretion, Dufour gland, *Dorylus (Anomma) molestus*, *Dorylus (Anomma) nigricans*, Dorylinae, Formicidae, Hymenoptera.

INTRODUCTION

The variation in the forms of organization of social insects is overwhelming. This can be illustrated by the variety of systems evolved within the Formicidae, which includes vegetarian seed-harvesting species and ferocious predators, strictly arboreal species and others that are entirely hypogaeic, while colony size ranges from as few as nine individuals in *Pachycondyla sublaevis* (Peeters,

*To whom correspondence should be addressed.
[1] Dedicated to Professor J.K.A. van Boven on his seventy-sixth birthday.
[4] Present address: Laboratoire de Communication Chimique, CNRS-UPR 27, 31 Chemin J. Aiguier, 13402 Marseille Cedex 9, France.

0098-0331/91/0800-1633$06.50/0

1990) up to several millions. The Old World army ants, which constitute the subfamily Dorylinae, undoubtedly represent one of the most fascinating groups among the Formicinae. They comprise the Aenictini, the majority of which are found in tropical Asia and Northeastern Australia, and the Dorylini, which are mainly distributed in Africa. The New World army ants form the separate subfamily Ecitoninae.

The African Dorylini hold the record, by a wide margin, for size of colony in a monogynous ant, with approximately 22 million individuals in a colony of *Dorylus (Anomma) wilverthi* (Raignier and van Boven, 1955). Doryline ants do not have a permanent nest, and in their nomadic progress only occupy temporary bivouacs in between massive raiding bouts. In spite of their enormous colony size, their considerable impact upon the environment, and their unusual habits, Dorylinae have not received as much attention as other more amenable subfamilies of ants. The available papers deal mainly with their general biology and taxonomic aspects (reviewed by Raignier and van Boven, 1955, and Gotwald, 1982). A few reports have been published on the morphology of the exocrine glands of dorylines (Hölldobler and Engel, 1978; Billen, 1985). Nothing, however is known as yet on the ethological function or the chemical composition of the glandular secretions. This lack of information is more understandable when considering, on the one hand, the impossibility of keeping these nestless ants alive in the laboratory, and on the other, the practical problems of having sophisticated research equipment close to their African habitat.

Using the solid sampling technique of Morgan and Wadhams (1972), however, the site and time of collection of the material and the laboratory where it is chemically examined become independant of each other (cf., Billen et al., 1987). Using this technique we have been able to carry out the first chemical examination of the Dufour glands of the workers of *Dorylus (Anomma) molestus* (Gerstaecker) as part of a comparative taxonomic survey of this gland in various subfamilies of the Formicidae (Morgan, 1990a; Attygalle and Morgan, 1984). We report here this first chemical examination of a member of the subfamily Dorylinae, together with a preliminary examination of the Dufour glands of *Dorylus (Anomma) nigricans* Illiger carried out several years earlier and not yet fully reported.

METHODS AND MATERIALS

Live ants were collected near Nairobi, Kenya, and immediately flown to Leuven, Belgium, where they were dissected and the glands sealed in glass capillaries and sent by mail to Keele. Worker ants were immobilized by cooling over liquid nitrogen. Dissection was carried out in water under a binocular

microscope as described by Morgan (1990b). The dissected glands were dried and sealed in glass capillaries and kept in a refrigerator until ready for chemical analysis by combined gas chromatography–mass spectrometry.

Gas chromatography was carried out on a Hewlett Packard model 5890 gas chromatograph using helium (1 ml/min) as carrier gas on a fused silica capillary (12 m × 0.35 mm) coated with a 0.33-μm film of immobilized dimethylsilicone fluid (equivalent to OV-1) (Cambridge Capillaries, Cambridge, U.K.). The injector was set at 150°C, and the detector at 300°C. The column oven was programmed from 30°C to 250°C at 8°C/min and then held isothermally. Injection was splitless. The analytical column was linked to the Hewlett Packard 5970B mass selective detector through a deactivated, uncoated fused silica capillary (10 × 0.35 mm). The mass spectrometer was set to scan m/z 40–350 using 70 eV ionization with a scan time of about 1 scan/1.5 sec. Data were collected and processed by a Hewlett Packard ChemStation, model HP 59970C.

Formation of dimethylthioethers from alkenes for the location of double bonds was carried out on a nanogram scale as described by Billen et al. (1986) and Attygalle and Morgan (1988). A capillary containing 3, 5, or 10 Dufour glands was crushed under hexane, and to this was added dimethyl disulfide (50 μl) and a solution of iodine in ether (2 μl, 60 mg/ml). The mixture was placed in a Keele Microreactor (Attygalle and Morgan, 1986) sealed with a screw cap and heated overnight at 100°C. The reaction was halted by adding aqueous sodium thiosulfate (50 μl). For the linked gas chromatography–mass spectrometry of the reaction products, the injector was set at 280°C and the column programmed from 200°C to 320°C at 3°C/min.

The analyses of the Dufour glands of *Dorylus (Anomma) nigricans* were carried out by the same solid sampling technique of single dissected glands, but were performed some years earlier on the technology available then, i.e., packed gas chromatography column of polyethylene glycol (PEG 20M) operated at 210°C isothermally and without the benefit of mass spectrometry. The identifications of the alkanes and tricosene were based upon identity of retention times. The technique was as described in papers of that time (e.g., Billen et al. 1986).

RESULTS

The Dufour glands of *Dorylus (Anomma) molestus* are filled with linear alkanes and alkenes with (*Z*)-9-tricosene and tricosane together providing over 70% of the total (Table 1). No terpenes or oxygenated compounds were observed among the major components. A typical gas chromatogram is shown in Figure 1. The position of the double bonds in the alkenes was determined by conversion to the α,β-dimethylthioethers with dimethyl disulfide. The position of the

TABLE 1. AVERAGE PERCENTAGE COMPOSITION OF THE DUFOUR GLANDS OF WORKERS OF *D. molestus* BASED ON ANALYSES OF SEVEN INDIVIDUALS[a]

Number in Figure 1	Compound	%	SD
1	Pentadecene	0.72	0.7
2	Pentadecane		
3	Heptadecadiene	0.58	1.5
4	Heptadecene	10.24	7.9
5	(*Z*)-Dihydrofarnesol	0.3	0.9
6	Nonadecadiene	0.55	1.5
7	Nonadecene	0.28	0.7
8	Heneicosene	0.18	0.5
9	(*Z*)-9-Tricosene	58.4	22.1
10	Tricosane	13.7	3.1
11	Pentacosadiene	6.82	9.0
12	9-Pentacosene	5.55	7.5

[a] Mean amount per gland, 200 ng.

double bonds were easily located in tricosene and pentacosene from the mass spectral fragmentation of the dimethylthioethers. The geometry of tricosene was shown by comparison of the retention time of the derivative with that of an authentic specimen of (*Z*)-9-tricosene to be *Z*. That of the *E* isomer has a different retention time. Samples containing 3, 5, and 10 glands were each used for the formation of the dimethylthioethers, but there was insufficient material in each of them to locate the position of the double bond in heptadecene or nonadecene.

The only evidence of nonlinear compounds was in one sample that showed a peak eluting immediately after heptadecene, which had the mass spectrum and retention time of an authentic sample of (*Z*)-dihydrofarnesol made by the method of Dawson et al. (1988). In the remaining samples, this was too weak to be clearly seen.

The preliminary examination of *Dorylus (Anomma) nigricans* also showed that tricosane and probably tricosene were the major compounds present and that the general pattern of compounds was similar, but lack of identification of some of the other substances makes full comparison impossible. Five individual minima workers, one medium, and two major workers were examined. The major component in all of them was that tentatively identified as tricosene (mean value of 30 ng), followed by tricosane (27 ng); the third component eluting near docosane was unidentified.

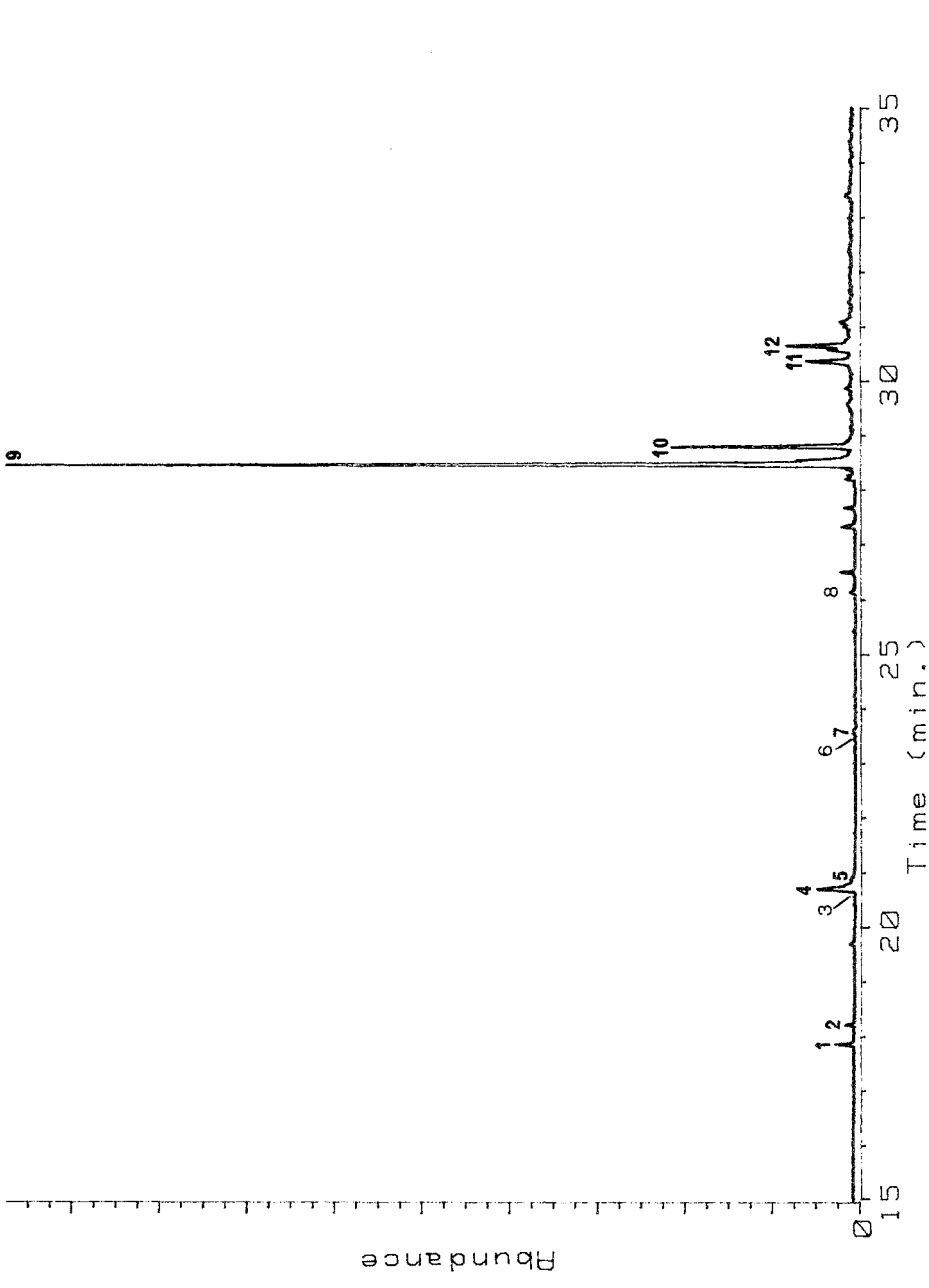


FIG. 1. Gas chromatogram of a single Dufour gland of a worker of *Dorylus (Anomma) molestus* on a capillary column. Labeled peaks are listed in Table 1.

DISCUSSION

Examination of Dufour glands of two species of *Dorylus* show that tricosene and tricosane are by far the major components in the glands of these members of the Dorylinae. Only seven single specimens of *D. molestus* were available, one of which showed a relatively high proportion of minor components, which included one terpenoid substance identified as dihydrofarnesol (Table 1) and one specimen in which the proportions of pentacosadiene and pentacosene were comparable with those of tricosene and tricosane. The result is relatively large standard deviations in the percentages in Table 1 for this small sample. The glands are comparatively small for ants, with this sample giving an average of 200 ng of secretion per gland.

The main points of interest are, first, the relatively high molecular mass (C_{23} and C_{25}) of the principal components. This we have noted previously (Attygalle et al., 1990) is characteristic of species living in a tropical climate, compared with species from a temperate climate, where C_{11}-C_{17} compounds usually predominate. Secondly, the tricosene is the same one encountered in *Atta* (Evershed and Morgan, 1981) and frequently elsewhere in insect cuticular hydrocarbons. Thirdly, although tricosane (mp 47.6°C) is a solid, tricosene is a liquid at room temperature, and the mixture of many alkenes will keep the tricosane in solution and keep the mixture liquid. Fourthly, the meager evidence for a sesquiterpene compound, dihydrofarnesol, already identified in a ecitonine ant (Morgan et al., in preparation) and now in its Old World counterpart, is interesting, although otherwise the chemistry of ecitonines and dorylines is very different.

The data on *D. nigricans* indicate that this species is not greatly different from *D. molestus*. The amount in the gland varied from 50 to 150 ng, rising with increasing size of the workers, but overall these glands are relatively small among ant Dufour glands. We may expect other species of *Dorylus* will give similar but not identical patterns of hydrocarbons in their Dufour gland.

At our present level of knowledge, there is nothing distinctive about this glandular secretion that correlates with the unusual nomadic existence of doryline ants.

Acknowledgments—We thank E.E. Martens (University of Nairobi, Kenya) for collecting *D. molestus* and J.K.A. van Boven for the *D. nigricans* and for identifying both species. J.P.J.B. and A.G.B. both thank the British Council for travel grants to visit Keele; A.G.B. thanks the Centre National de la Recherche Scientifique (CNRS/DRCI) for financial support; E.D.M. thanks the SERC for a grant for the purchase of GC-MS equipment. We thank S.J. Keegans for helpful discussion and advice.

REFERENCES

ATTYGALLE, A.B., and MORGAN, E.D. 1984. Chemicals from glands of ants. *Chem. Soc. Rev.* 13:245–278.

ATTYGALLE, A.B., and MORGAN, E.D. 1986. A versatile micro-reactor and extractor. *Anal. Chem.* 58:3054–3058.

ATTYGALLE, A.B., and MORGAN, E.D. 1988. Pheromones in nanogram quantities: Structure determination by combined microchemical and gas chromatographic methods. *Angew. Chem. Int. Ed. Engl.* 27:460–478.

ATTYGALLE, A.B., BILLEN, J.P.J., JACKSON, B.D., and MORGAN, E.D. 1990. Morphology and chemical contents of Dufour glands of *Pseudomyrmex* ants (Hymenoptera: Formicidae) *Z. Naturforsch.* 45C:691–697.

BILLEN, J.P.J. 1985. Comparative ultrastructive of the poison and Dufour glands in Old and New World army ants (Hymenoptera, Formicidae). *Actes Colloq. Insectes Soc.* 2:17–26.

BILLEN, J.P.J., EVERSHED, R.P., ATTYGALLE, A.B., MORGAN, E.D., and OLLETT, D.G. 1986. The contents of the Dufour glands of workers of three species of *Tetramorium*. *J. Chem. Ecol.* 12:669–685.

BILLEN, J.P.J., ATTYGALLE, A.B., MORGAN, E.D., and OLLETT, D.G. 1987. Gas chromatography without solvents: Pheromone studies: The Dufour gland of the ant *Pseudomyrmex occidentalis*. *Int. Anal.* 1:3–6.

DAWSON, G.W., GRIFFITHS, D.C., PICKETT, J.A., PLUMB, R.T., WOODCOCK, C.M., and ZHANG, Z.-N. 1988. Structure/activity studies on aphid alarm pheromone derivatives and their field use against transmission of barley yellow dwarf virus. *Pestic. Sci.* 22:17–30.

EVERSHED, R.P., and MORGAN, E.D. 1981. Chemical investigation of the Dufour gland contents of attine ants. *Insect Biochem.* 11:343–351.

GOTWALD, W.H. 1982. Army ants, pp. 157–254, *in* H.R. Hermann (ed.). Social Insects, Vol. IV. Academic Press, New York.

HÖLLDOBLER, B., and ENGEL, H. 1978. Tergal and sternal glands in ants. *Psyche* 85:285–330.

MORGAN, E.D. 1990a. Exocrine gland chemistry and phylogeny of ants, pp. 319–320, *in* G.K. Veeresh, B. Mallik, and C.A. Viraktamath (eds.). Social Insects and the Environment. Oxford and IBH, New Delhi.

MORGAN, E.D. 1990b. Preparation of small-scale samples from insects for chromatography. *Anal. Chim. Acta* 236:227–235.

MORGAN, E.D., and WADHAMS, L.J. 1972. Gas chromatography of volatile compounds in small samples of biological materials. *Chromatogr. Sci.* 10:528–529.

PEETERS, C. 1990. La reproduction chez les fourmis ponérines. *Actes Colloq. Insectes Soc.* 6:195–202.

RAIGNIER, A., and VAN BOVEN, J. 1955. Etude taxonomique, biologique et biométrique des *Dorylus* du sous-genre *Anomma* (Hymenoptera: Formicidae). *Ann. Mus. R. Congo Belge*, 4 (sci. zool.) (2):1–359.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

INDUCED MIMICRY OF COLONY ODORS IN ANTS

ANNE-GENEVIEVE BAGNERES,[1,*] CHRISTINE ERRARD,[2] CATHERINE MULHEIM,[3] CATHERINE JOULIE,[4] and CATHERINE LANGE[3]

[1]*C.N.R.S-L.N.B 8*
31, chemin Joseph Aiguier
13402 Marseille Cedex 9, France

[2]*Laboratoire d'Ethologie et Sociobiologie*
U.A. CNRS 667, Université Paris-Nord
93430 Villetaneuse, France

[3]*Laboratoire de chimie organique structurale*
Université P.et M. Curie, 4, place Jussieu
75005-Paris, France

[4]*Université P. et M. Curie*
4 place Jussieu
75005-Paris, France

(Received February 7, 1991; accepted April 22, 1991)

Abstract—The cuticular hydrocarbons of *Formica selysi* (Formicinae) and *Manica rubida* (Myrmicinae) reared in single species and in mixed species colonies were determined using gas chromatography (GC) and GC-mass spectrometry. In colonies containing both species, each species modified its species-specific recognition odor. This odor is composed, at least partly, of cuticular hydrocarbons. The cuticular hydrocarbons of *M. rubida* consist only of saturated alkanes (*n*-alkanes and branched alkanes). In *F. selysi* the mixture also contains unsaturated compounds (monoenes and dienes). In heterospecific colonies, a new chemical signature developed. This signature resulted from qualitative and quantitative changes in the spectrum of hydrocarbons produced by each species and permitted the two species to inhabit the same nest without displaying interspecific aggression. The readjustment seemed to be more an active synthesis or an active transfer than simply a passive transfer from one species to the other. This may imply that the ants synthesized some components of the hydrocarbon signature of the other species. These synthesizing processes may be activated under particular social environmental conditions.

Key Words—Formicinae, mimicry, artificial mixed colonies, cuticular

*To whom correspondence should be addressed.

0098-0331/91/0800-1641$06.50/0

hydrocarbons, colony recognition, ants, Hymenoptera, Formicidae, *Formica selysi*, *Manica rubida*.

INTRODUCTION

A fundamental feature of social insect colonies (bees, ants, and termites) is the fact that they are usually closed. Individuals of the same species from outside the colony are prevented from entering the nest and are repulsed, attacked, and often killed (Wilson, 1971).

There are, however, examples of mixed colonies of ants of different species coexisting in the same nest. The existence of natural associations of this kind prompted Fielde (1903) to create artificial mixed colonies of two or more species that never form such associations in nature. Using this heterospecific rearing technique, Fielde demonstrated that discrimination between nestmates and foreign individuals was based on a set of chemicals that together produced a colony-specific odor. More recently Hölldobler and Michener (1980) suggested that colony odor derives partly from chemicals produced by the colony members themselves. Analyses of these chemical recognition signals suggest that they are incorporated into the insect cuticle in termites and ants (Howard et al., 1980; Howard and Blomquist, 1982; Vander Meer et al., 1985). By transferring cuticular hydrocarbons from *Camponotus vagus* ants of one colony to those of another, Bonavita-Cougourdan et al. (1987) caused foreign ants to be treated like nestmates and so proved that hydrocarbons acted at least as one colony recognition odor. These findings have been confirmed by the results of studies on other ant species, *Camponotus floridanus* (Morel and Vander Meer, 1988) and *Cataglyphis cursor* (Nowbahari et al., 1989). Hydrocarbons also appear to act as species and colony recognition cues among termites (Howard et al., 1982; Clément et al., 1985; Bagnères et al., 1988; Haverty et al., 1988). Cuticular hydrocarbons also play an important role in the interactions between termitophilous and myrmecophilous taxa, or ant social parasites and their hosts (Howard et al., 1980; Vander Meer and Wojcik, 1982; Lemaire et al., 1986).

Larval transfers from one ant society to another have shown that the chemical signature (consisting mainly of cuticular hydrocarbons) changes in response to social environment (Bonavita-Cougourdan et al., 1989).

We have used heterospecific ant colonies as a system in which to study the mechanisms of species colonial recognition (Jaisson, 1980; Errard and Jaisson, 1985). Earlier work with heterospecific colonies concentrated on the means of producing an association of *Formica selysi* (Formicinae) and *Manica rubida* (Myrmicinae). These associations are made possible by the transient existence of a tolerance period occurring during the few days after imaginal eclosion (Errard, 1986, 1987). We also have shown that a mixture of unidentified chem-

icals, probably hydrocarbons, changed when the two species were reared in heterospecific colonies (Errard and Jallon, 1987).

Foragers from homospecific colonies were highly aggressive when forced into heterospecific confrontations in the laboratory. The resulting combats usually ended in one or both individuals being killed. Young workers of *M. rubida* and *F. selysi* put together to form heterospecific colonies showed no aggressive behavior, however. The behavior of each species rapidly adapted to the presence of allospecifics. We have shown, however, that in colonies where the two species coexist without aggression most of the individual interactions are preferentially directed towards ants of the same species. Furthermore, the two species retain their own spatial organization (Corbara and Errard, 1989, 1991).

Among the wide range of natural associations, the artificial heterospecific colonies of *F. selysi* and *M. rubida* are just an apparent myrmecobiosis (Stumper, 1950) because in these associations the ants of both species coexist in fairly close proximity but rear their own brood separately.

In the present study, we have identified the hydrocarbons extracted from the cuticle, and we also have examined the changes in composition and abundance of the cuticular hydrocarbons that occur when *F. selysi* and *M. rubida* are reared heterospecifically. We put forward hypotheses to explain the mutual tolerance that was concomitant with the chemical changes observed.

METHODS AND MATERIALS

Rearing. The species used in the present study, *Manica rubida* (Myrmicinae) and *Formica selysi* (Formicinae) were collected from the same biotope (French Alps, altitude 700 m) in July 1987, October 1988, and April 1989, at three different sites (Morillon, Fer à Cheval, Salvagny), where the two species are sympatric. Parent colonies from these three sites were reared in the laboratory (with queen) under the following conditions: natural photoperiod, temperature range 22 $\pm$ 2°C, diet consisting of honey, apple, flies, and mealworms. Two types of enclosures (open boxes containing food and water) were set up: those containing *F. selysi* cocoons and workers, and those containing *M. rubida* pupae and workers. Young workers of each species were removed within 24 hr of their emergence and used to form two kinds of colonies: control colonies (homospecific) and artificial mixed colonies (heterospecific). Each control colony contained 40–42 young workers, in addition to brood (consisting of 20 pupae in the case of *M. rubida* colonies, and 20 cocoons in the case of *F. selysi* colonies). The artificial mixed colonies consisted of 25 young workers of each species, less than 24 hr old, in addition to brood (10 pupae and 10 cocoons). None of these colonies contained any queens.

Sampling and Chemical Analysis of Cuticular Aliphatic Hydrocarbons.

Individual ants from 11 control colonies (coming from the different sites) and two heterospecific colonies were killed by freezing, and then weighed immediately, when about six months old. Each ant was then extracted with 2 ml pentane in a Wheaton tube. After 5 min, the total pentane extract was drawn off and evaporated under nitrogen. Eicosane (n-C_{20}) was added to each sample as an internal standard.

Extracts from 21 individuals of each species from homospecific colonies and 11 extracts of 11 individuals from heterospecific colonies (six *F. selysi* and five *M. rubida*) were mathematically analyzed. In our colonies, no distinction was made between individuals that emerged before or after the artificial colonies (control and mixed) had been set up. Saturated and unsaturated hydrocarbon fractions were separated on silica plates impregnated with 10% silver nitrate developed with benzene–hexane (8:92) (Howard et al., 1978). Capillary gas chromatography (GC) was carried out using a Chrompak CP Sil 5 WCOT column (25 m × 0.25 mm ID). The GC is a Delsi Girdel series 300 machine with a flame ionization detector (FID) coupled to an Enica series 10 integrator. Coefficients depending on the Delsi FID were calculated for each peak in relation to an internal standard (here n-C_{20}), using a mixture of synthetic n-alkanes (n-C_{16} to n-C_{38}) for each hydrocarbon at various concentrations, and a further coefficient of 0.8 for unsaturated alkanes was calculated also with synthetic alkenes at various concentrations. The injector was of the split-splitless type and the injection was performed for 15 sec in splitless mode. The carrying gas was helium at 1 bar and the temperature was programmed at 5°C/min from 100 to 320°C (10 min isothermal).

Mass spectra (MS) were produced with a Nermag R-1010 C linked to an IBM PC AT3 using the Spectral 30 software. Electron impact (EI) spectra were obtained at 70 eV, chemical ionization (CI) at 100 eV, and methane at 0.05 torr. The position of double bonds was determined by methyl thioether formation (Francis, 1981; Francis and Veland, 1981; Carlson et al., 1989). After determinations, a complementary GC-MS analysis (selected ion monitoring) was carried out to check whether any of the hydrocarbons' diagnostic ions, which were found to be absent from either species, might in fact be present in trace quantities. The threshold of the GC-MS was then increased by two.

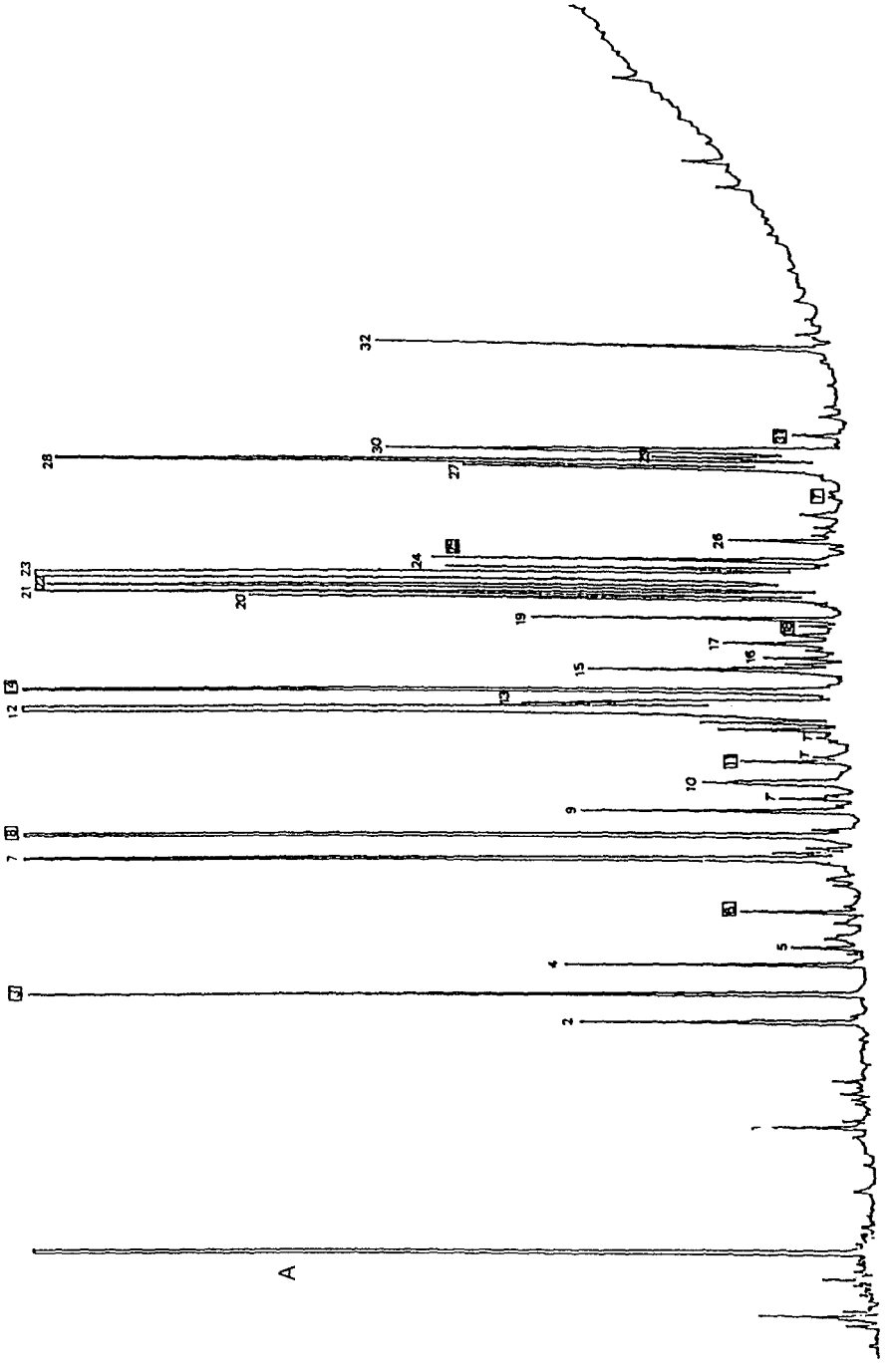
Mathematical Analysis. Hydrocarbon species present in quantities greater than 0.5 ng/insect were included in the analysis using Lotus 1-2-3 software (version 2.01). A total of 32 peaks were recorded from *F. selysi* individuals reared homospecifically, and 26 from *M. rubida* reared homospecifically, and for each peak the surface was readjusted with the coefficient. Forty-two peaks were recorded on ants of both species reared in mixed colonies. The basic statistics (mean, variance, standard deviation, etc.) were calculated on both the proportional and absolute quantities of each hydrocarbon. Mean quantities were tested using Student's t test setting the significance level at $\alpha < 0.05$. Multi-

variate principal component analysis (MANOVA) and an analysis of correlation were carried out on the data using Statgraphic Inc. software. Principal component analysis was used to separate individual ants or individual hydrocarbon species along the principal canonical axes (i.e., those capable of absorbing the greatest proportion of the sample variance). Where the quantities of hydrocarbon species were highly correlated, they were removed from the analysis. The MANOVA therefore was carried out on a restricted set of 26 of the hydrocarbons after this correlation analysis.

RESULTS

Chemistry. The hydrocarbons extracted from homospecifically reared *M. rubida* consisted entirely of alkanes. Among these, 39.2% were *n*-alkanes, 41% monomethyl-alkanes, 15.2% dimethyl-alkanes, and 4.6% were undetermined saturated molecules. The cuticular mixture from *F. selysi* control colonies was characterized by the presence of numerous unsaturated molecular species (53.3%). Among these alkenes 40% were monenes and 17.3% dienes. Numerous alkane molecules also were observed (40%), 24.3% of which were *n*-alkanes, 7.2% monomethyl-alkanes, 8.5% dimethyl-alkanes; 2.7% of molecules are unknown. Heterospecifically reared ants of both species had intermediate hydrocarbon profiles between the profiles of single species *F. selysi* and *M. rubida* colonies (Figure 1). The hydrocarbon mixture from heterospecifically reared ants contained both alkanes and alkenes. The proportions of *n*-alkanes, monomethyls, dimethyls, monoenes, and dienes were 20.8, 15.4, 15.4, 16.1, and 4.7%, respectively, in *F. selysi*, and 24.6, 35.1, 15.7, 15.7, and 7.1% in *M. rubida*. Undetermined products amounted to 6.2% and 6.6% of the totals in *F. selysi* and *M. rubida*, respectively. The hydrocarbon MS peaks were identified (Table 1) and their diagnostic ions (using EI, CI and for EI after methyl thioether formation presented together with the numerical abbreviations of the hydrocarbons). The outcome of the search for diagnostic ions, such as the unsaturated products from *F. selysi* that were not found to be present in *M. rubida* (monoenes: *m/z* 322, 350, 378, 392, 406, 434; dienes: *m/z* 404, 432, 460) was negative. The proportions and absolute quantities (in nanograms per milligram of insect) of the compounds from each category of ants were tabulated (Table 2).

Mathematical Analysis. After principal component analysis of the relative proportion of the cuticular hydrocarbons, the first and second principal components accounted for 38.4% and 17.1% of the total sample variance. A further 9.5, 6.4, and 5.9% of the variance was explained by components 3, 4, and 5. With the first and third principal components alone, it was possible to separate the four categories of ants: homospecifically reared *F. selysi* and *M. rubida*,


A
1
2
4
5
7
9
10
12
15
16
17
19
20
21
23
24
26
27
28
30
32

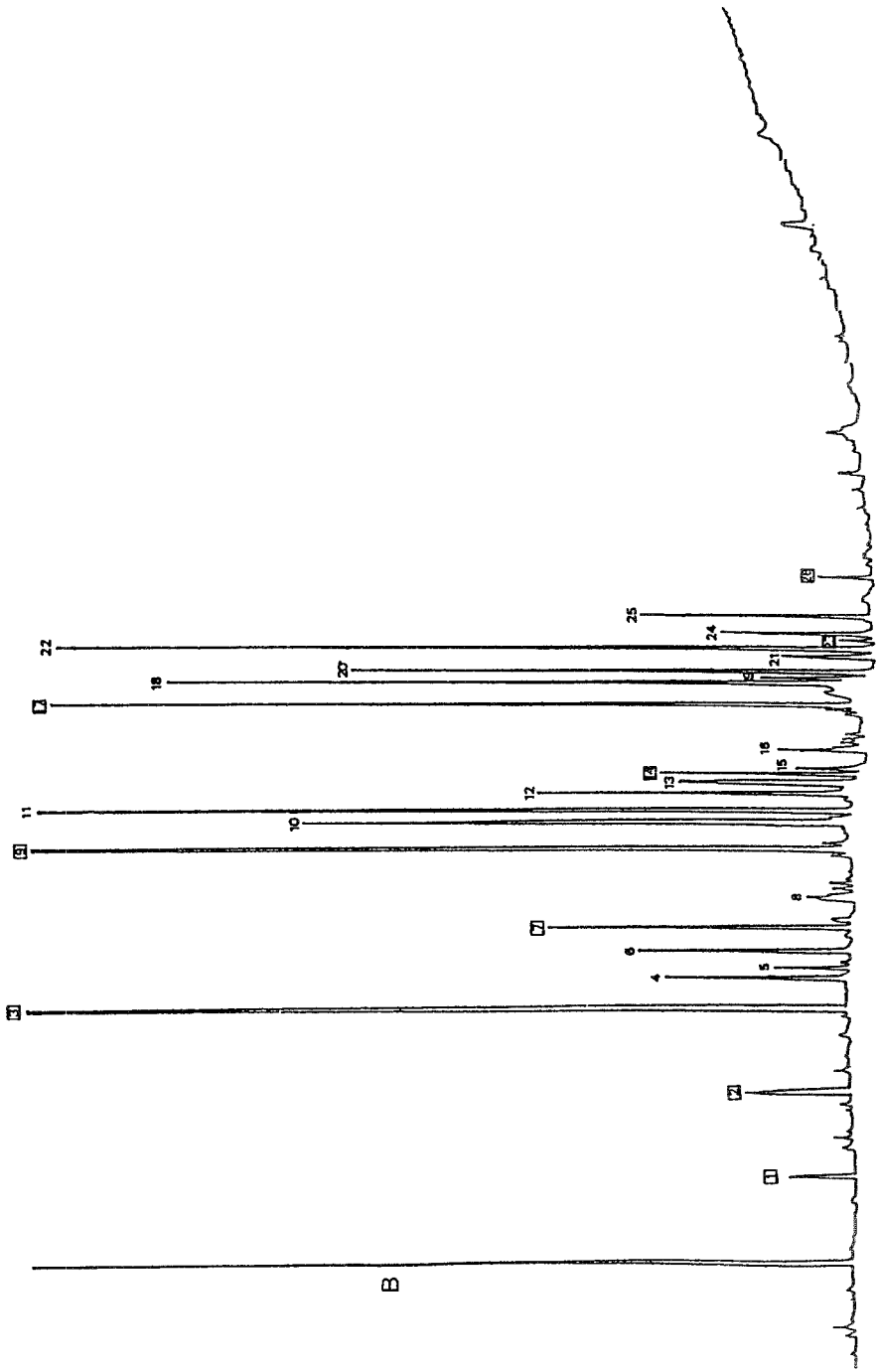


FIG. 1. Gas chromatograms of the cuticular hydrocarbons of *Formica selysi* (A) and *Manica rubida* (B) from homospecific colonies, and *F. selysi* (C) and *M. rubida* (D) from heterospecific colonies.

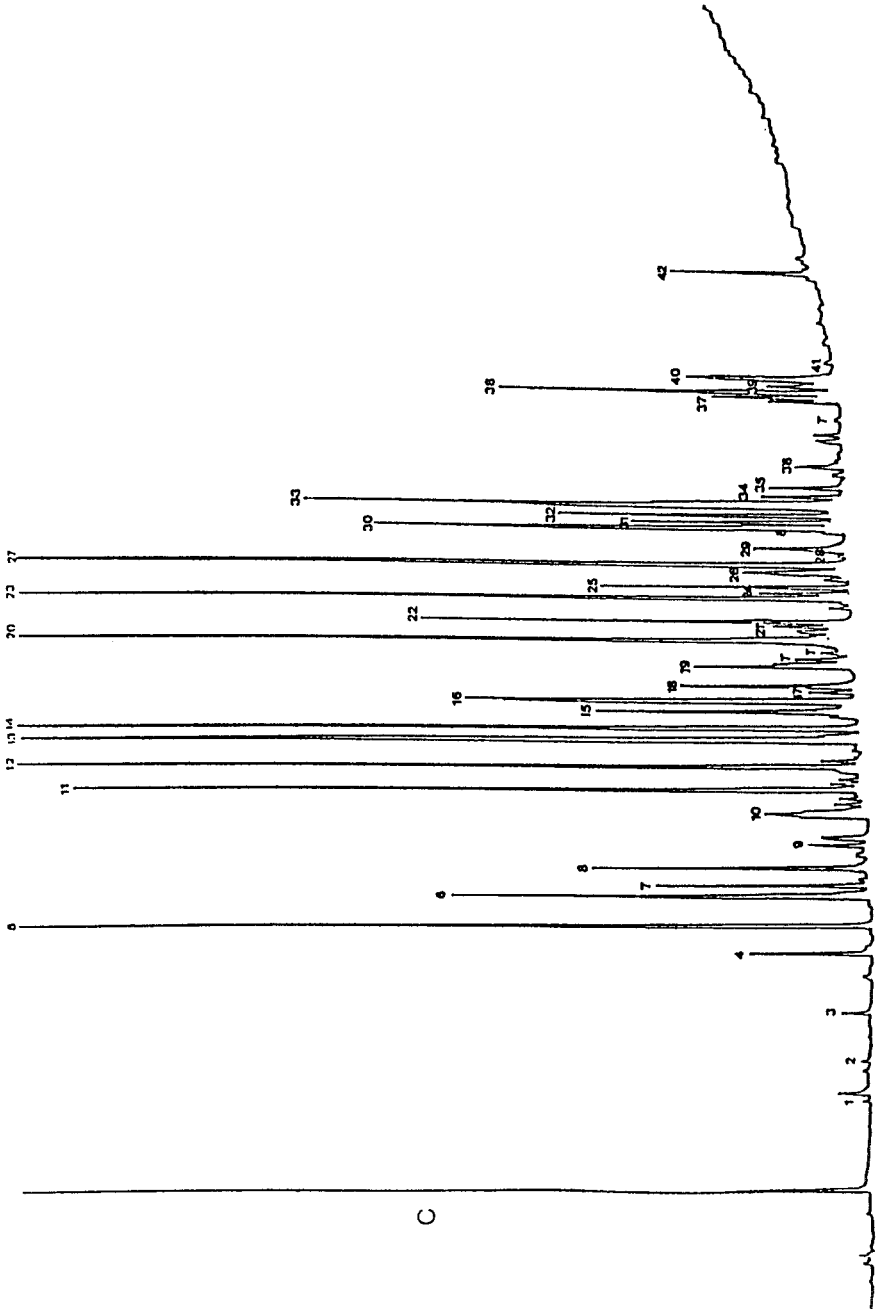


FIG. 1. Continued

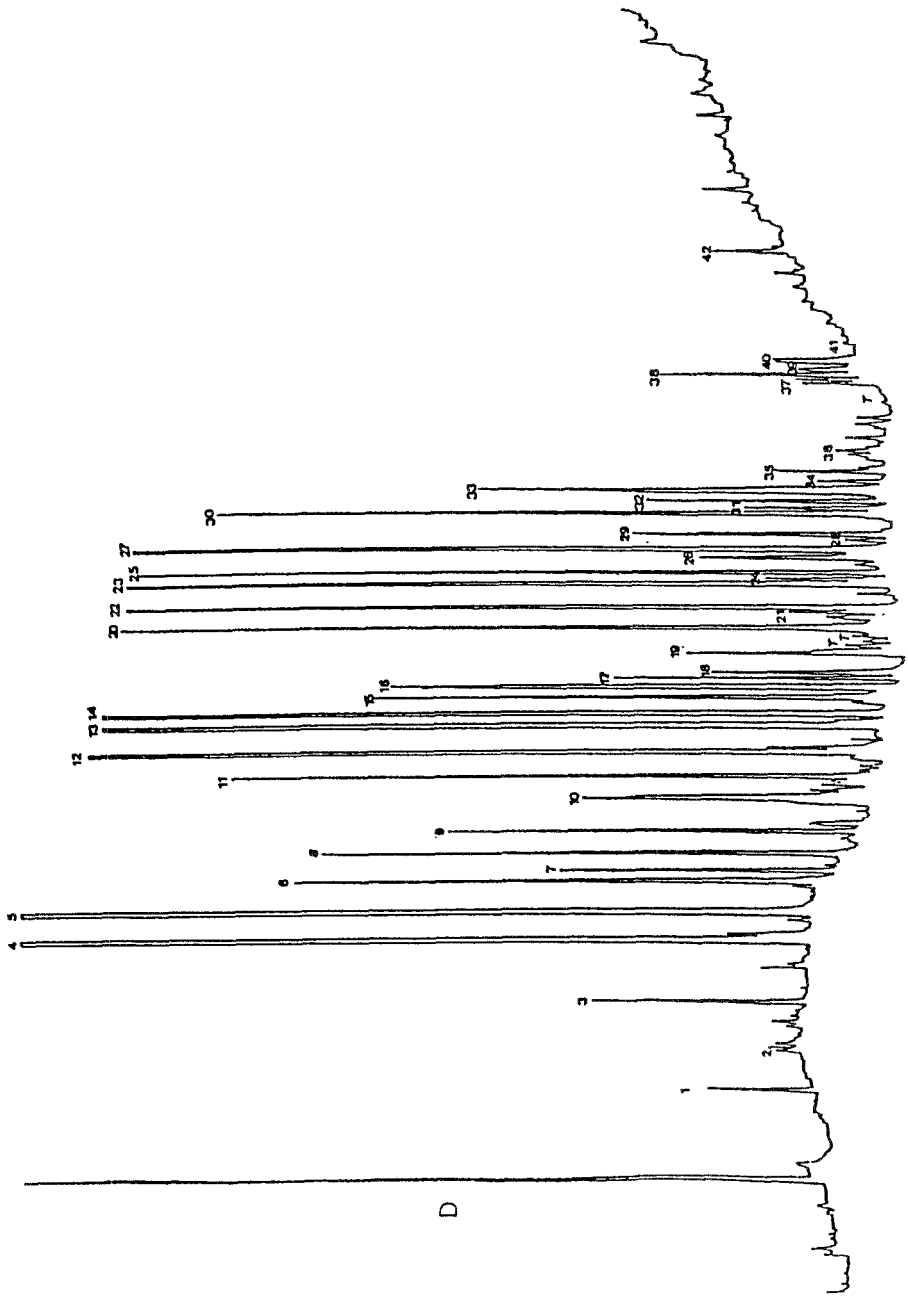


FIG. 1. Continued

TABLE 1. IDENTIFICATION OF HYDROCARBONS FROM *Formica selysi* (FORMICINAE) AND *Manica rubida* (MYRMICINAE)[a]

Compounds	CN	MW	Diagnostic EI-MS ions	CI-MS ions $(M-H)^+$	ECL	*Formica selysi*	*Manica rubida*	Mixed colonies
n-ALKANES								
n-C_{21}	21	296		295	21.00		1	1
n-C_{22}	22	310		309	22.00		2	3
n-C_{23}	23	324		323	23.00	3	3	5
n-C_{24}	24	338		337	24.00	6	7	9
n-C_{25}	25	352		351	25.00	8	9	12
n-C_{26}	26	366		365	26.00	11	14	17
n-C_{27}	27	380		379	27.00	14	17	22
n-C_{28}	28	394		393	28.00	18	23	28
n-C_{29}	29	408		409	29.00	25	26	35
n-C_{30}	30	422		422	30.00	T		T
n-C_{31}	31	436		435	31.00	31		41
Monomethyl alkanes								
11Me-C_{23}	24	338	168/169, 196/197	337	23.37	4	4	6
9Me-C_{23}	24	338	140/141, 224/225	337	23.37	4	4	6
5Me-C_{23}	24	338	84/85, 280/281	337	23.50	5	5	7
3Me-C_{23}	24	338	56/57, 308/309	337	23.70		6	8
11Me-C_{24}	25	352	168/169, 210/211	351	24.40		8	10
9Me-C_{24}	25	352	140/141, 238/239	351	24.40		8	10
13Me-C_{25}	26	366	196/197	365	25.36	9		13
11Me-C_{25}	26	366	168/169, 224/225	365	25.37	9	10	13
9Me-C_{25}	26	366	140/141, 252/253	365	25.38		10	13
5Me-C_{25}	26	366	84/85, 308/309	365	25.50	T	11	14
3Me-C_{25}	26	366	56/57, 336/337	365	25.70	10	12	15
13Me-C_{26}	27	380	196/197, 210/211	379	26.32		T	19
11Me-C_{26}	27	380	168/169, 238/239	379	26.36		16	19
9Me-C_{26}	27	380	140/141, 266/267	379	26.36		16	19

5Me-C_{26}	27	380	84/85, 322/323	379	26.52		T	T
3Me-C_{26}	27	380	84/85, 322/323	379	26.72		T	T
13Me-C_{27}	28	394	196/197, 224/225	393	27.31	15		23
11Me-C_{27}	28	394	168/169, 252/253	393	27.33	15	18	23
9Me-C_{27}	28	394	140/141, 280/281	393	27.35		18	23
7Me-C_{27}	28	394	112/113, 308/309	393	27.44		19	24
5Me-C_{27}	28	394	84/85, 336/337	393	27.50	16	20	25
3Me-C_{27}	28	394	56/57, 364/365	393	27.73	17	21	26
13Me-C_{29}	30	422	196/197, 252/253	421	29.33	26		36
11Me-C_{29}	30	422	168/169, 280/281	421	29.33	26		36
Dimethyl alkanes								
5, 17diMe-C_{27}	29	408	84, 168, 267, 351	407	27.83		22	27
x, ydiMe-C_{28}	30	422		421	28.10	19	24	29
8, 12diMe-C_{28}	30	422	126, 196, 253, 323	421	28.38	20	25	30
Monoenes								
9-$C_{23:1}$	23	322	173, 243, 416	323	22.67	2		4
9-$C_{25:1}$	25	350	173, 271, 444	351	24.70	7		11
9-$C_{27:1}$	27	378	173, 299, 472	379	26.68	12		20
9-$C_{28:1}$	28	392	173, 327, 500	393	26.73	17		26
9-$C_{29:1}$	29	406	173, 355, 548	407	26.76	23		33
9-$C_{31:1}$	31	434	173, 383, 576	435	30.74	30		40
Dienes								
9, 19-$C_{29:2}$	29	404	173/325, 187/311, 592	405	28.41	20		30
9, 21-$C_{29:2}$	29	404	173/325, 159/339, 592	405	28.48	21		31
9, 23-$C_{29:2}$	29	404	173/325, 131/367, 592	405	28.59	22		32
9, 19-$C_{31:2}$	31	432	173/353, 215/311, 620	433	30.41	27		37
9, 21-$C_{31:2}$	31	432	173/353, 187/339, 620	433	30.48	28		38
9, 23-$C_{31:2}$	31	432	173/353, 159/367, 620	433	30.59	29		39
9, 21-$C_{33:2}$	33	460	173/381, 215/339, 648	461	32.44	32		42

[a]The numbers added link up with Figure 1 (A and B different numbering, C and D common numbering) and Table 2 (''mixed'' numbering used). For monoenes and dienes, the diagnostic EI-MS ions given are those after methylthiolation.

TABLE 2. HYDROCARBONS FROM *Formica selysi* AND *Manica rubida* REARED HOMOSPECIFICALLY OR HETEROSPECIFICALLY EXPRESSED AS PERCENTAGES AND QUANTITIES

		Percentages							Quantities				
		Control colonies			Mixed colonies				Control colonies		Mixed colonies		
Peak	Identification	*F. selysi*	*M. rubida*	Stat.	*F. selysi*	Stat.	*M. rubida*	Stat.	*F. selysi*	*M. rubida*	*F. selysi*	*M. rubida*	Stat.
Alkanes													
a1	n-C_{21}		0.97	**	0.27	**	0.33	*		8.04	7.96	8.56	NS
			(0.68)		(0.07)		(0.20)			(4.18)	(2.61)	(4.80)	
a3	n-C_{22}		0.90	***	0.53	*	0.48	*		9.49	16.75	12.46	NS
			(0.27)		(0.32)		(0.14)			(5.91)	(11.29)	(4.10)	
a5	n-C_{23}	4.27	14.90	**	6.30	NS	7.26	*	37.25	187.13	190.14	174.59	NS
		(4.50)	(6.39)		(1.70)		(3.40)		(26.54)	(183.44)	(62.17)	(39.37)	
a9	n-C_{24}	0.61	1.51	**	0.87	NS	0.89	*	7.23	19.42	30.42	25.78	NS
		(0.25)	(0.50)		(0.31)		(0.28)		(7.75)	(19.35)	(21.89)	(18.02)	
a12	n-C_{25}	9.61	11.88	NS	6.96	NS	4.86	*	117.47	163.11	216.06	129.87	*
		(6.43)	(6.09)		(1.06)		(1.77)		(140.01)	(185.32)	(81.00)	(59.35)	
a17	n-C_{26}	0.90	1.36	*	0.76	NS	1.18	NS	9.10	17.61	28.41	38.07	NS
		(0.43)	(0.47)		(0.37)		(0.37)		(7.93)	(18.36)	(26.29)	(29.10)	
a22	n-C_{27}	6.41	6.13	NS	2.67	*	4.14	*	83.15	91.60	88.60	120.50	NS
		(5.88)	(3.92)		(0.27)		(1.08)		(116.46)	(116.39)	(52.44)	(63.94)	
a28	n-C_{28}	0.32	0.65	NS	0.44	NS	1.65	*	3.52	6.95	15.76	60.49	NS
		(0.53)	(0.68)		(0.36)		(1.08)		(7.42)	(7.48)	(18.14)	(68.85)	
a35	n-C_{29}	1.62	0.91	*	1.12	NS	1.35	NS	21.85	12.77	47.50	46.71	NS
		(1.13)	(0.66)		(1.01)		(0.96)		(25.32)	(18.44)	(69.91)	(42.44)	
a41	n-C_{31}	0.51	—	**	0.88	NS	2.45	*	6.25		48.73	100.32	NS
		(0.46)			(1.81)		(2.33)		(9.54)		(110.60)	(142.45)	

Monomethyls													
m6	11Me-C_{23} + 9Me-C_{23}	1.45 (1.26)	2.57 (1.46)	*	2.70 (0.46)	*	1.66 (0.73)	*	16.14 (16.23)	22.36 (13.47)	82.70 (29.02)	40.81 (11.20)	*
m7	5Me-C_{23}	0.46 (0.47)	0.68 (0.41)	NS	1.48 (0.39)	**	1.06 (0.30)	*	4.79 (5.90)	5.72 (2.34)	48.37 (24.66)	29.64 (16.50)	NS
m8	3Me-C_{23}		2.13 (1.05)	**	1.69 (0.15)	***	1.39 (0.67)	*		19.79 (11.42)	54.88 (28.06)	37.43 (24.14)	NS
m10	11Me-C_{24} + 9Me-C_{24}		1.27 (0.66)	**	1.87 (0.40)	***	1.04 (0.43)	NS		11.88 (8.05)	62.81 (37.86)	34.10 (30.06)	NS
m13	13Me + 11Me- + 9Me-C_{25}	1.40 (0.95)	7.69 (2.82)	**	7.46 (1.40)	**	4.88 (1.55)	*	15.74 (14.02)	76.19 (51.54)	228.09 (79.57)	126.91 (41.88)	*
m14	5Me-C_{25}		8.87 (1.80)	***	6.40 (1.24)	***	5.32 (1.45)	*		92.07 (53.77)	195.39 (67.66)	135.82 (31.37)	NS
m15	3Me-C_{25}	0.86 (0.40)	2.80 (1.41)	**	2.39 (0.65)	**	1.89 (0.34)	*	10.04 (8.69)	31.21 (31.91)	75.13 (36.34)	55.89 (39.23)	NS
m19	13Me + 11Me- + 9Me-C_{26}		1.68 (0.82)	**	1.99 (0.53)	**	2.06 (0.88)	NS		16.38 (10.69)	67.61 (40.13)	58.41 (31.66)	NS
m23	13Me + 11Me-C_{27} + 9Me-C_{27}	1.33 (0.71)	7.21 (2.06)	***	6.06 (0.50)	**	5.51 (1.02)	*	15.36 (12.93)	77.01 (54.80)	187.61 (68.80)	151.91 (64.86)	NS
m24	7Me-C_{27}		1.09 (0.43)	***	1.11 (0.65)	*	1.61 (0.72)	NS		12.08 (10.48)	39.16 (33.35)	53.11 (44.83)	NS
m25	5Me-C_{27}	0.41 (0.21)	3.15 (1.04)	***	1.50 (0.60)	*	3.21 (0.93)	NS	4.19 (3.31)	39.05 (33.15)	53.29 (45.49)	98.45 (67.49)	NS
me26	3Me-C_{27} + X-$C_{28:1}$	0.73 (0.38)	1.84 (0.83)	**	1.51 (1.03)	NS	2.35 (1.10)	NS	9.24 (9.10)	19.72 (17.24)	59.71 (76.47)	80.58 (82.43)	NS
m36	11Me-C_{29} + 13Me-C_{29}	0.53 (0.48)		**	0.64 (0.70)	NS	3.09 (2.34)	*	7.38 (9.06)		28.67 (47.09)	110.08 (103.64)	NS
Dimethyls													
d27	5, 17-DiMe-C_{27}		9.15 (3.56)	***	10.42 (1.62)	***	8.50 (2.42)	NS		94.44 (0.83)	324.17 (121.85)	220.62 (64.89)	NS
dx29	x, y-C_{28}	6.00 (6.47)	2.67 1.98	*	1.48 (1.07)	*	4.11 (1.57)	NS	72.10 (158.36)	29.12 (29.65)	44.55 (59.72)	117.14 (56.57)	*
dn30	8, 12-DiMe-C_{28} + 9, 19-$C_{29:2}$	2.48 (1.64)	3.29 (2.02)	NS	3.48 (0.46)	*	3.07 (0.56)	NS	22.80 (16.36)	31.33 (21.64)	114.30 (63.79)	83.99 (34.43)	NS

TABLE 2. Continued

		Percentages							Quantities				
		Control colonies			Mixed colonies				Control colonies		Mixed colonies		
Peak	Identification	*F. selysi*	*M. rubida*	Stat.	*F. selysi*	Stat.	*M. rubida*	Stat.	*F. selysi*	*M. rubida*	*F. selysi*	*M. rubida*	Stat.
Monoenes													
e4	9-$C_{23:1}$	1.52		**	0.97	*	2.89	NS	16.82		30.79	48.42	NS
		(0.92)			(0.20)		(5.27)		(13.41)		(13.01)	(70.65)	
e11	9-$C_{25:1}$	6.07		**	4.52	NS	1.47	**	70.23		142.08	38.96	*
		(3.97)			(1.05)		(0.60)		(63.52)		(65.84)	(18.01)	
e20	9-$C_{27:1}$	21.11		***	5.50	***	3.06	**	236.19		169.07	91.56	NS
		(5.13)			(1.47)		(0.73)		(200.49)		(66.26)	(68.25)	
e33	9-$C_{29:1}$	9.35		***	3.82	**	2.21	***	109.28		122.51	65.84	NS
		(3.25)			(1.15)		(0.43)		(94.67)		(63.18)	(45.51)	
e40	9-$C_{31:1}$	2.06		***	1.27	*	1.25	*	24.73		47.31	49.88	NS
		(0.70)			(0.66)		(1.06)		(21.36)		(49.21)	(67.60)	
Dienes													
n31	9, 21-$C_{29:2}$	3.67		**	0.85	**	2.10	*	34.26		29.97	69.53	NS
		(2.27)			(0.42)		(1.14)		(24.88)		(28.43)	(58.69)	
n32	9, 23-$C_{29:2}$	4.66		***	1.23	**	1.48	*	46.12		43.77	51.39	NS
		(2.04)			(0.65)		(0.72)		(36.26)		(43.67)	(51.68)	
n37	9, 19-$C_{31:2}$	1.15		*	0.62	*	0.65	NS	11.04		21.37	17.72	NS
		(1.17)			(0.18)		(0.92)		(12.64)		(14.54)	(29.36)	
n38	9, 21-$C_{31:2}$	5.54		**	0.90	**	1.28	*	56.88		31.21	44.24	NS
		(3.32)			(0.75)		(1.14)		(43.48)		(32.89)	(46.41)	
n39	9, 23-$C_{31:2}$	0.97		*	0.53	NS	1.12	*	9.54		22.25	47.24	NS
		(1.68)			(0.51)		(1.14)		(12.63)		(34.17)	(69.51)	
n42	9, 21-$C_{33:2}$	1.32		**	0.56	*	0.49	*	16.33		20.05	15.84	NS
		(0.84)			(0.43)		(0.26)		(17.04)		(23.97)	(16.04)	

Unknowns													
x2	Unknown	0.58		*	0.97	NS	0.31	**	5.83		30.24	9.09	NS
		(0.73)			(0.84)		(0.10)		(8.23)		(29.78)	(4.88)	
x16	Unknown		3.28	**	3.43	**	3.39	NS		29.63	108.67	98.27	NS
			(1.92)		(0.99)		(0.79)			(19.40)	(49.06)	(55.77)	
x18	Unknown		1.32	***	1.66	***	1.65	NS		12.90	55.76	55.92	NS
			(0.59)		(0.24)		(0.63)			(7.96)	(31.42)	(53.40)	
x21	Unknown	0.96		**		**		***	10.64				NS
		(0.48)							(8.10)				
x34	Unknown	1.07		***	0.53	***	1.30	***	12.58		20.30	54.07	NS
		(0.26)			(0.34)		(1.25)		(11.79)		(23.87)	(75.74)	
Total		100	100		100		100		1170.39	1130.18	3251.17	2910.39	NS
									(1005.19)	(809.03)	(1619.43)	(1642.80)	

[a] Standard deviation in parenthesis. Student t test: NS = not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. *F. selysi* (control $N = 21$), *M. rubida* (control $N = 22$), *F. selysi* (mixed $N = 6$), *M. rubida* (mixed $N = 5$).

and *F. selysi* and *M. rubida* from heterospecific colonies (Figure 2A). The second component accounted for the variance between individuals within species. The four groups (*F. selysi* from control colonies, heterospecifically reared ants of both species, and *M. rubida* from control colonies) separated out along the first principal component axis (Figure 2A). Control colony *F. selysi* were most clearly characterized by the alkenes 9-$C_{27:1}$ (e20) and 9-$C_{25:1}$ (e11), and the dienes *n*32 and *n*38 of 29- and 31-carbon atoms. The *M. rubida* from control

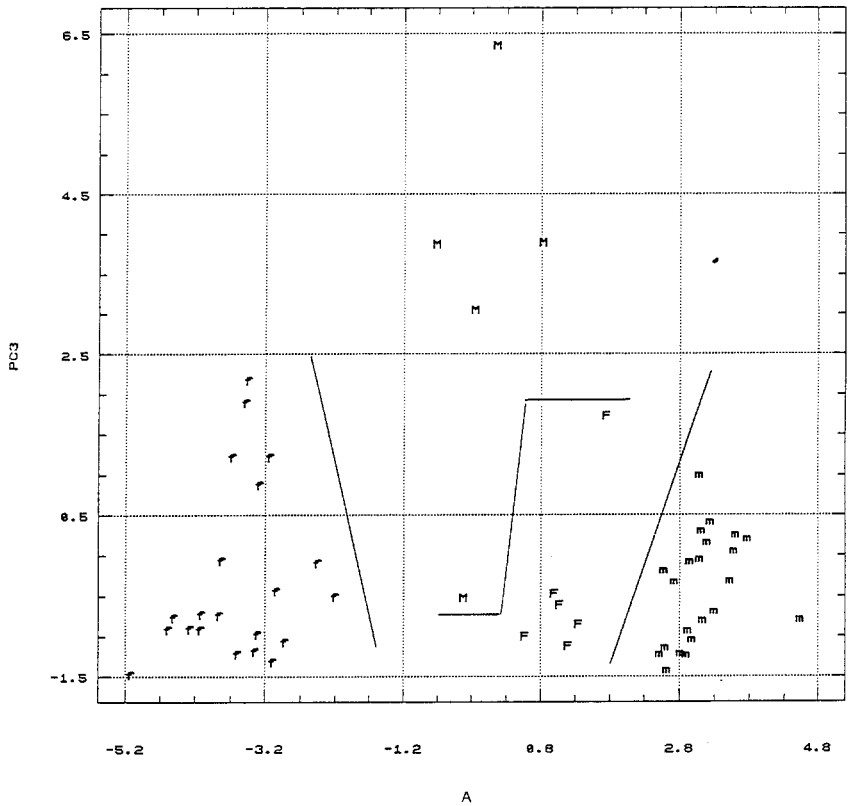


FIG. 2. Plot of the canonical axes 1 and 3 of the GC peaks obtained from the individual workers (*F. selysi* and *M. rubida* from control and mixed colonies). (A) Principal component map (the lines between groups were drawn arbitrarily to facilitate the interpretation). f: workers from control colony of *F. selysi* ($N = 21$), m: workers from control colony of *M. rubida* ($N = 22$), F: *F. selysi* workers from mixed colony ($N = 6$), M: *M. rubida* workers from mixed colony. (B) Component weight map, plotted for the 26 different hydrocarbons selected (a: *n*-alkane, m: monomethyl-alkanes, d: dimethyl-alkanes, e: monoene, n: diene).

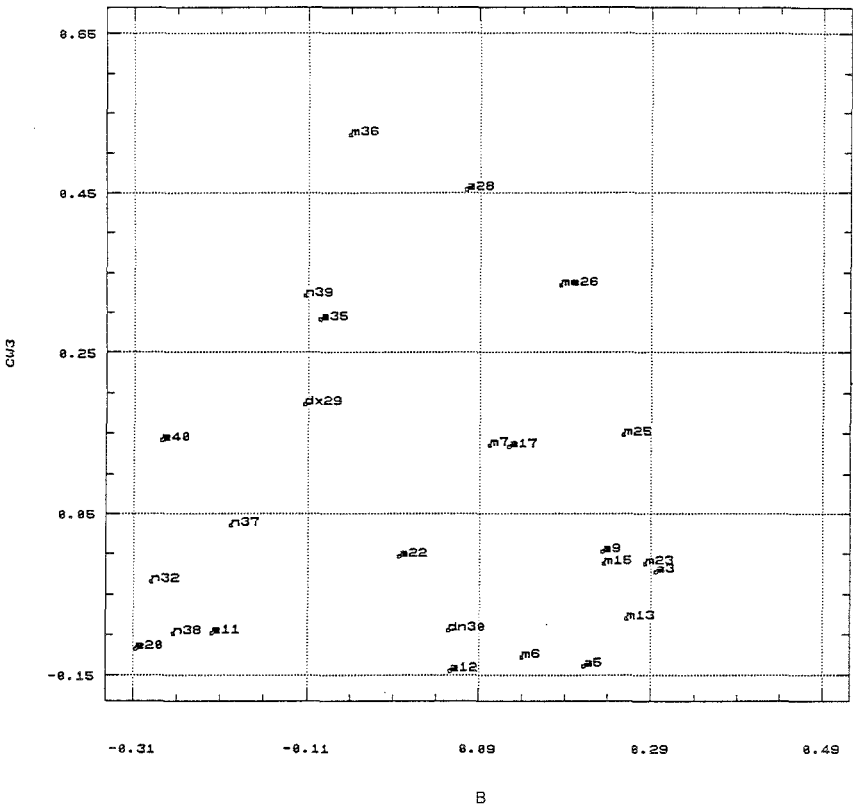


FIG. 2. Continued

colonies were characterized, however, by the alkanes n-C_{22} (a3), 13 + 11 + 9Me-C_{25} (m13) and 13 + 11 + 9Me-C_{27} (m23) (Figure 2B).

The interspecifically reared individuals clustered at the center of the first axis separated out along the third axis (Figure 2A). Four of the five heterospecifically reared *M. rubida* and a few individuals from control colonies occurred in the positive upper section of the plot. The negative lower section contained five of the six heterospecifically reared *F. selysi* and the most of individuals from control colonies.

Our analysis brought to light three categories of hydrocarbons (Table 2). One group contained cuticular hydrocarbons specific to *F. selysi* and comprised 13 peaks. A second group, with eight peaks, contained products species-specific to *M. rubida*. The third group (16 peaks) included the hydrocarbons shared by both species. The hydrocarbons that were common to both species (when reared homospecifically) formed 38.9% of those extracted from the cuticle of individ-

uals from *F. selysi* control colonies. The remainder, 58.5% were more specific to *F. selysi*; whereas the hydrocarbons common to both species amounted to 47.2% of the load from *F. selysi* reared heterospecifically. Products specific to *F. selysi* amounted to 22.3% and the remaining 24.3% were hydrocarbons specific to *M. rubida*. Control colonies of *M. rubida* carried 26.1% species-specific products and 69.3% of these were common to both species. The hydrocarbons carried by *M. rubida* from interspecific colonies, however, were 20.7% species-specific and 49.1% common to both species. The remaining 23.3% were specific to *F. selysi*. The proportion of species-specific hydrocarbons from heterospecifically reared ants of both species was conspicuously lower than in conspecific controls. Here the difference was greater (36%) in *F. selysi* than in *M. rubida* (6%). The proportion of shared hydrocarbons was 10% greater in heterospecific than in homospecific *F. selysi*. Heterospecifically reared *M. rubida*, however, had 20% less shared hydrocarbons than the conspecific controls.

As a result of these changes, the proportion of shared hydrocarbons carried by ants reared interspecifically converged towards 48% in both species. In addition, ants in heterospecific colonies possessed two or even three times as much cuticular hydrocarbons as those reared homospecifically (Table 2). The mean individual load of *F. selysi* was 1170 ng/mg of insect in control and 3251 ng/mg of insect in heterospecific colonies. Likewise in *M. rubida*, the mean individual load was 1130 ng/mg of insect among ants from homospecific colonies and was 2910 ng/mg of insect among ants from heterospecific colonies. All the categories of hydrocarbons increased by similar amounts and contributed equally to the increase in the proportions on interspecifically reared ants. The mean individual loads of some common hydrocarbons [including *n*-C_{25} (a12), *n*-C_{29} (a35), 9Me + 11Me-C_{23} (m6), 5Me-C_{23} (m7), 9Me + 11Me-C_{25} (m13) and the *x*,*y*-dimethyl-C_{28} (dx29)] were, for example, 20 ng/mg in controls and 100 ng/mg in heterospecifically reared ants (Table 2) .

With 11 of the 16 shared hydrocarbons, the increases in the amounts produced by ants from heterospecific colonies were greater in the species that produced the lower proportions of these hydrocarbons when reared in control colonies. The proportion of the mixture of 9- and 11-methyl pentacosane (m13) in *F. selysi* increased from 1.4% in control colonies to 7.5% in heterospecific ones. In *M. rubida* the proportions of this mixture decreased from 7.7% in control to 4.9% in interspecific colonies. Some *F. selysi*-specific hydrocarbons were more abundant however on *F. selysi* and *M. rubida* reared heterospecifically. These included *n*-C_{31} (a41), 11Me + 13Me-C_{29} (m36), 9-$C_{23:1}$ (e4) and nonacosadienes (*n*30, *n*31) (Figure 3). These products, which are absent in *M. rubida* reared alone, were three to four times more abundant on *M. rubida* reared heterospecifically than on *F. selysi* (which naturally synthesized these hydrocarbons) in control and mixed colonies. The loads carried by heterospecifically reared *F. selysi* were twice as much as those carried by conspecifics

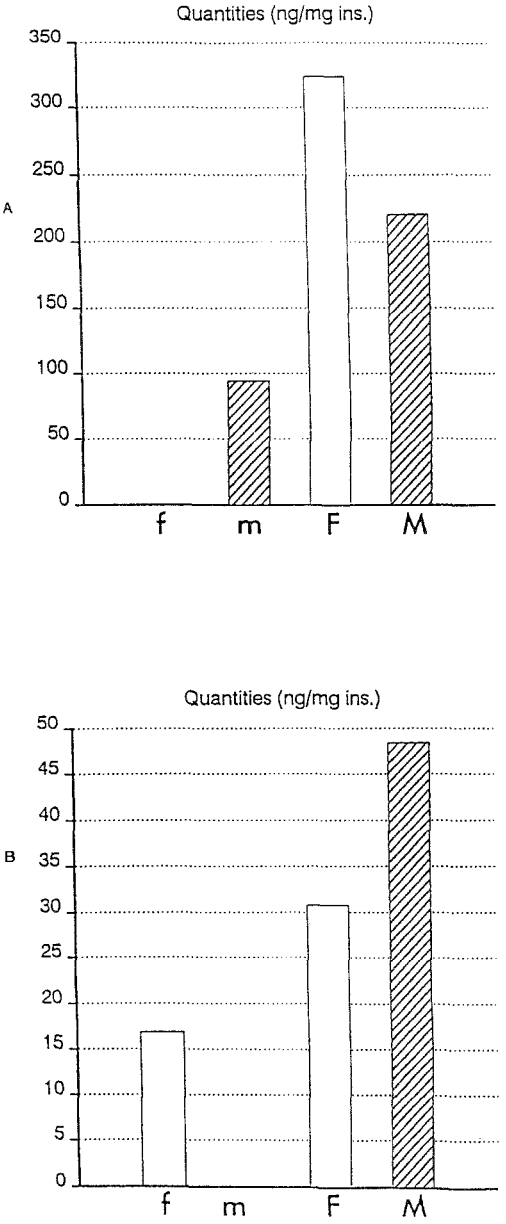


FIG. 3. Histograms of quantities in nanograms per milligram insect of two specific hydrocarbons (A: 5,17-diMe-C_{27}; B: 9-tricosene). f: homospecific reared *Formica selysi;* m: homospecific reared *Manica rubida;* F: heterospecific reared *Formica selysi;* M: heterospecific reared *Manica rubida*.

from control colonies. A similar pattern was observed in the case of 5,17-diMe-C_{27} (d27) and 3Me-C_{23} (m8) and other hydrocarbons specific to *M. rubida* (Figure 3). The proportions of *F. selysi*-specific hydrocarbons recorded from heterospecifically reared *F. selysi* were significantly different from the proportions recorded in heterospecifically reared *M. rubida* (chi-squared test, $\chi^2 = 70.01$, $df = 12$, $\alpha < 0.01$). This difference was largely attributable to six of 13 hydrocarbons *n*-C_{31} (a41), the mixture 11Me + 13Me-C_{29} (m3), 9-pentacosene (e11), 9-heptacosene (e20), 9–21 nonacosadiene (*n*31) and 9–23 hentriacontadiene (*n*39). Comparisons of *M. rubida*-specific hydrocarbons showed the existence of no significant differences between the proportions extracted from heterospecifically reared *M. rubida* and *F. selysi* (chi-squared test, $\sigma^2 = 3.47$, $df = 7$, $\alpha < 0.90$).

The two species appear to have reacted differently to heterospecific rearing. The quantities of cuticular hydrocarbons increased much more (80.6%) in *F. selysi* than in *M. rubida*, suggesting that *F. selysi* is more sensitive to the altered social environment produced by heterospecific rearing or has a different physiological development or a different nutritional behavior.

DISCUSSION

Our results demonstrate that there exists a close link between the species-specific cuticular signature—the particular spectrum of cuticular hydrocarbons—of *F. selysi* and *M. rubida*, and the tolerance between these species. This confirms similar findings in other social insects including both termites (Howard et al., 1982; Clément et al., 1985) and ants (Bonavita-Cougourdan et al., 1987; Morel and Vander Meer, 1988; Nowbahari et al., 1989). Mature adults from control colonies of the two species, when forced to occupy the same nest, are able to distinguish allospecifics on the basis of their species-specific chemical signature. Agonistic behavior occurs as soon as foreign ants are introduced into the nest and most of the foreign ants are killed in the resulting combat. By contrast, young ants reared heterospecifically show modified hydrocarbon profiles. By modifying their spectra, the two species become chemically more closely related. Individuals reared in heterospecific colonies are therefore tolerant of their allospecific nestmates and show no aggressive behavior (Corbara and Errard, 1991).

The individual hydrocarbon loads (in nanograms per milligram) carried by heterospecifically reared ants of both species were twice those of the control ants. Several processes contribute to this increase: (1) *M. rubida* "acquires" molecules species-specific to *F. selysi*, sometimes producing greater quantities of hydrocarbons than *F. selysi* from control colonies. In addition *F. selysi* reared heterospecifically greatly increase production of their own species-specific

hydrocarbons. The same phenomena occur with *F. selysi*. (2) Heterospecifically reared ants produce the hydrocarbons common to both species in much greater quantities than either species when reared alone. Despite these changes, each species retains a species-specific chemical signature. At the same time individuals of both species acquire a similar chemical signature that is specific to the colony.

What are the mechanisms mediating these phenomena that are so strongly influenced by the ants' social environment? Some authors have suggested that the queen plays an important role as a source of the chemicals that form the colony odor (Carlin and Hölldobler, 1986, 1987). In our experiments, however, there were no queens in either the heterospecific or homospecific colonies. The presence of allospecific workers alone produced all the changes observed in the hydrocarbon spectra of heterospecifically reared ants. A similar phenomenon has been observed in *Camponotus vagus* larvae (Bonavita-Cougourdan et al., 1989). The chemical and behavioral plasticity exhibited by *F. selysi* and *M. rubida* has been found to exist in many myrmecophile and termitophile species. Two hypotheses have been advanced to explain the phenomenon. Vander Meer and Wojcik (1982) suggested that cuticular hydrocarbons are merely transferred passively from the host to the myrmecophile. Howard et al. (1980, 1990), however, proposed that new chemicals are actively synthesized. In their larval transfers from one society to another, Bonavita-Cougourdan et al. (1989) gave three hypotheses to explain the changes in the chemical signature in response to social environment, in addition to its transfer from workers to larvae: (1) genetically controlled regulatory mechanisms in response to environmental stimuli; (2) workers providing larvae with entire synthesized hydrocarbons by feeding; (3) or with precursors of the hydrocarbons.

The synthesis and regulation of cuticular hydrocarbons, and likewise of venoms, appear to be controlled by Mendelian genes (Vander Meer et al., 1985; Ross et al., 1987). Passive transfer of hydrocarbons does not explain the increases in hydrocarbon loads we observed in both species reared heterospecifically. An ant receiving chemicals by passive transfer would be expected to pick up a smaller quantity of chemical than the amount carried by the donor ant. Even if the transfer of cuticular hydrocarbons was complete, the average quantities received would be equal to, but never more than, the quantities carried by the donor. This objection is particularly valid with molecules that are not normally synthesized by the receiving homospecifically reared ants. The hydrocarbon loads of heterospecifically reared *F. selysi* and *M. rubida* contained different proportions of the various species-specific and common molecular species (Figure 3). This fact implies that, even if the various hydrocarbons were transferred from one species to another, they were not exchanged all at once nor at the same rates.

It is unlikely that any "selection" of particular hydrocarbons may occur

during passive transfer because the cuticular products are chemically and physically very similar, as mathematical analysis has shown. A possible mechanism of the "transfer" type would involve the exchange of precursor molecules by trophallaxis rather than the exchange of fully synthesized hydrocarbons by cuticular contacts. This hypothesis can be rejected, however, because in our experiments there was very little trophallaxis between the two species.

Transfer of cuticular hydrocarbons also seems to be unlikely because it requires numerous interactions between the species. The molecules involved are not very volatile and would have to be deposited on the cuticle of allospecific ants during trophallaxis or mutual grooming, but interspecific behavior is uncommon in heterospecific ant colonies (Corbara and Errard, 1991). An active transfer mechanism of a different kind, however, may account for our data, one where the contents, or part of the contents, of the postpharyngeal gland are transferred during the brief interspecies contacts that occur in mixed colonies. We established previously that in both of these two species, this gland contains the same specific mixture as the cuticule (Bagnères and Morgan, 1991).

Since it is difficult to imagine a passive transfer mechanism that fits with our data, it is necessary to examine the alternative hypothesis that new hydrocarbons may be synthesized by heterospecifically reared ants of both species, quite apart from the possibility involving an active transfer of substances from the postpharyngeal gland (Bagnères and Morgan, 1991). Genetically controlled regulating mechanism may modify the synthesis of cuticular hydrocarbons and their precursor molecules in response to environmental stimuli. This mechanism also implies that there exist synthetic pathways that are activated only under certain environmental conditions or that nonspecific enzymes are involved in the synthesis of new molecules using new precursor molecules selected by unknown mechanisms.

In heterospecific colonies, therefore, a dynamic equilibrium exists. Each species synthesizes some components of the hydrocarbon signature of the other species. The differences between the species that exist when they are homospecifically reared are thus largely deleted in heterospecific colonies. This mechanism enables individual ants to escape interspecific aggression. In addition, our results show that the redistribution of hydrocarbons is such that each species retains a species-specific signature while acquiring a new odor that is specific to the colony.

We intend to carry out biochemical studies to determine whether, in fact, *F. selysi* and *M. rubida* are actually capable of synthesizing molecules that they do not normally produce.

Acknowledgments—The authors wish to thank Professor E.O. Wilson for his helpful comments on the present paper, Professor E.D. Morgan for revising the manuscript, D. Agosti for carrying out the determination of the species *Formica selysi*, and A. Davis and J. Blanc for the English translation of our manuscript.

REFERENCES

BAGNÈRES, A.G. , and MORGAN, E.D., 1991. The postpharyngeal glands and the cuticule of Formicidae contain the same characteristic hydrocarbons. *Experientia* 47:106–111.

BAGNÈRES, A.G., LANGE, C., CLEMENT, J.L., and JOULIE, C. 1988. Les hydrocarbures cuticulaires des *Reticulitermes* français: Variations specifiques et coloniales. *Actes Colloq. Insectes Soc.* 4:34–42.

BONAVITA-COUGOURDAN, A., CLÉMENT, J.L., and LANGE, C. 1987. Nestmate recognition: The role of cuticular hydrocarbons in the ant *Camponotus vagus* (scop). *J. Entomol. Sci.* 22:1–10.

BONAVITA-COUGOURDAN, A., CLÉMENT, J.L., and LANGE, C. 1989. The role of cuticular hydrocarbons in recognition of larvae by workers of the ant *Camponotus vagus*: changes in the chemical signature in response to social environment. *Sociobiology* 16:49–74.

CARLIN, N.F., and HÖLLDOBLER, B. 1986. The kin recognition system of carpenter ants (*Camponotus* spp.). I Hierarchical cues in small colonies. *Behav. Ecol. Sociobiol.* 19:123–134.

CARLIN, N.F., and HÖLLDOBLER, B. 1987. The kin recognition system of carpenter ants (*Camponotus* spp.). II Larger colonies. *Behav. Ecol. Sociobiol.* 20:209–217.

CARLSON, D.A., ROAN, C.S., YOST, R.A., and HECTOR, J. 1989. Dimethyl disulfide derivatives of long chain alkenes, alkadienes, and alkatrienes for gas chromatography/mass spectrometry. *Anal. Chem.* 61:1564–1571.

CLÉMENT, J.L., LANGE, C., BLUM, M.S., HOWARD, R.W., and LLOYD, M. 1985. Chemosystematique du genre *Reticulitermes* (Isoptères) aux U.S.A. et en Europe. *Actes Colloq. Insectes Soc.* 2:123–131.

CORBARA, B., and ERRARD, C. 1989. Organisation spatiale de *Manica rubida* et *Formica selysi* en colonies homo-et hétérospécifiques. Corrélation avec l'organisation sociale. *Actes Colloq. Insectes Soc.* 5:233–241.

CORBARA, B., and ERRARD, C. 1991. The organisation of artificial heterospecific ant colonies. The case of *Manica rubida*/*Formica selysi* (Myrmicidae, Formicidae) association: Mixed Colony or parallel colonies. *Behav. Proc.* 23:75–87.

ERRARD, C. 1986. Role of early experience in mixed colony odor-recognition in the ants *Manica rubida* and *Formica selysi*. *Ethology* 72:243–249.

ERRARD, C. 1987. Phylogenus/biotope interactions among Formicidae. *Behav. Proc.* 14:35–47.

ERRARD, C., and JAISSON, P. 1985. Etude des relations sociales dans les colonies mixtes hétérospécifiques chez les fourmis. *Folia Entomol. Mex.* 61:87–98.

ERRARD, C., and JALLON, J.M. 1987. An investigation of the development of the chemical factors in ants intra-society recognition, p. 478, *in* H. Rembold and J. Eder (eds.). Chemistry and Biology of Social Insects. Verlag J. Peperny, München.

FIELDE, A. 1903. Artificial mixed nests of ants. *Biol. Bull. Marine Biol. Lab.* 5:320–325.

FRANCIS, G.W. 1981. Alkylthiolation for the determination of double-bond position in unsaturated fatty acid esters. *Chem. Phys. Lipid J. Chromatogr.* 29:369–374.

FRANCIS, G.W., and VELAND, K. 1981. Alkylthiolation for the determination of double-bond positions in linear alkenes. *J. Chromatogr.* 219:379–384.

HAVERTY, M.I., PAGE, M., NELSON, L.J., and BLOMQUIST, G.J. 1988. Cuticular hydrocarbons of dampwood termites, *Zootermopsis*: Intra- and intercolony variation and potential as taxonomic characters. *J. Chem. Ecol.* 14:1035–1058.

HÖLLDOBLER, B., and MICHENER, C.D. 1980. Mechanisms of identification and discrimination in social Hymenoptera, pp. 35–56, *in* H. Markl (ed.). Evolution of Social Behavior: Hypotheses and Empirical Tests. Dahlem Konferenzen, Verlag Chemie, Weinheim.

HOWARD, R.W., and BLOMQUIST, G.J. 1982. Chemical ecology and biochemistry of insects hydrocarbons. *Annu. Rev. Entomol.* 27:149–170.

HOWARD, R.W., MCDANIEL, C.A., and BLOMQUIST, G.J. 1978. Cuticular hydrocarbons of the

eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae). *J. Chem. Ecol.* 4:233–245.

HOWARD, R.W., MCDANIEL, C.A., and BLOMQUIST, G.J. 1980. Chemical mimicry as an integrating mechanism: Cuticular hydrocarbons of a termitophile and its host. *Science* 210:431–433.

HOWARD, R.W., MCDANIEL, C.A., NELSON, D.R., BLOMQUIST, G.J., GELBAUM, L.T., and ZALKOW, L.H. 1982. Cuticular hydrocarbons of *Reticulitermes virginicus* (Banks) and their role as potential species and caste recognition cues. *J. Chem. Ecol.* 8:1227–1239.

HOWARD, R.W., STANLEY-SAMUELSON, D.W., and AKRE, R.D. 1990. Biosynthesis and chemical mimicry of cuticular hydrocarbons from the obligate predator, *Microdon albicomatus* Novak (Diptera: Syrphidae) and its ant prey, *Myrmica incompleta* Provancher (Hymenoptera: Formicidae). *J. Kans. Entomol. Soc.* 63:437–443.

JAISSON, P. 1980. Environmental preference induced experimentally in ants (Hymenoptera: Formicidae). *Nature* 286:388–389.

LEMAIRE, M., LANGE, C., LEFEBVRE, J., and CLÉMENT, J.L. 1986. Stratégie de camouflage du prédateur *Hypoponera eduardi* dans les sociétés de *Reticulitermes* européens. *Actes Colloq. Insectes Soc.* 3:97–101.

MOREL, L., and VANDER MEER, R.K. 1988. Ontogeny of nestmate recognition cues in the red carpenter ant (*Camponotus floridanus*). *Behav. Ecol. Sociobiol.* 22:175–183.

NOWBAHARI, E., LENOIR, A., CLÉMENT, J.L., LANGE, C., BAGNÈRES, A.G., and JOULIE, C. 1989. Individual, geographical and experimental variation of cuticular hydrocarbons of the ant *Cataglyphis cursor* (Hymenoptera, Formicidae). *Biochem. Syst. Ecol.* 18:63–74.

ROSS, K.G., VANDER MEER, R.K., FLETCHER, D.J.C., and VARGO, E.L. 1987. Biochemical phenotypic and genetic studies of two introduced fire ants and their hybrid (Hymenoptera: Formicidae). *Evolution* 41:280–293.

STUMPER, R. 1950. Etudes myrmecologiques. La myrmecobiose. *Bull. Soc. Nat. Luxembourg* 44:31–43.

VANDER MEER, R.K., and WOJCIK, D.P. 1982. Chemical mimicry in the myrmecophilous beetle *Myrmecaphodius excavaticollis*. *Science* 218:806–808.

VANDER MEER, R.K., LOFGREN, G.S., and ALVAREZ, F.M. 1985. Biochemical evidence for hybridization in fire ants. *Fla. Entomol.* 68:501–506.

WILSON, E.O. 1971. The Insect Societies. Belknap Press of Harvard University Press, Cambridge, Massachusetts.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

VOLATILES MEDIATING PLANT–HERBIVORE–NATURAL ENEMY INTERACTIONS: ELECTROANTENNOGRAM RESPONSES OF SOYBEAN LOOPER, *Pseudoplusia includens*, AND A PARASITOID, *Microplitis demolitor*, TO GREEN LEAF VOLATILES

R. RAMACHANDRAN and D.M. NORRIS*

Department of Entomology
University of Wisconsin
Madison, Wisconsin 53706

(Received February 19, 1991; accepted April 22, 1991)

Abstract—Electroantennograms were recorded from an herbivore, *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae), and a parasitoid, *Microplitis demolitor* (Wilkinson) (Hymenoptera: Braconidae), exposed to 5- through 12-carbon aliphatic compounds of several chemical classes. The response of the herbivore was higher for the 6- and/or 7-carbon hydrocarbons, alcohols, aldehydes, esters, and ketones. The response of the parasitoid was higher for the 7- and 8-carbon hydrocarbons, aldehydes, and ketones. Responses of the herbivore and the parasitoid to alcohols were similar. Both the herbivore and the parasitoid were most sensitive to aldehydes and ketones, and least sensitive to alcohols and hydrocarbons. Responses of the parasitoid to hydrocarbons, aldehydes, and ketones were numerically higher than those of the herbivore. The adaptive significance of differential olfactory sensitivity between the herbivore and the natural enemy is discussed in relation to tritrophic interactions among plants, herbivores, and natural enemies.

Key Words—Herbivores, *Pseudoplusia includens*, Lepidoptera, Noctuidae, natural enemies, *Microplitis demolitor*, Hymenoptera, Braconidae, EAG responses, green leaf volatiles.

INTRODUCTION

Volatile phytochemicals apparently serve as important messengers in the interactions between plants and other organisms. However, knowledge of the specific roles of such phytochemicals in such interactions remains limited (Buttery et al., 1984; Buttery and Ling, 1985; Visser, 1986; Khan et al., 1987; Liu et

*To whom correspondence should be addressed.

0098-0331/91/0800-1665$06.50/0

al., 1989) as compared to that for less volatile, e.g., "tastant," compounds (Schoonhoven, 1968; Staedler, 1976; Visser et al., 1979; Norris, 1986). A few earlier workers (Jermy, 1966, 1976; Gilbert et al., 1967; Gilbert and Norris, 1968) not only elucidated an importance of volatile phytochemicals in the interactions between plants and herbivores, but also demonstrated specifically that allomonic volatiles are important means by which some plants are antixenosic towards potential guests. However, most published information emphasizes the attractiveness of plant volatiles to herbivores (Visser and Thiery, 1985; Metcalf, 1986; Landolt, 1989) and natural enemies (Eller et al., 1988; Navasero and Elzen, 1989; Sheehan and Shelton, 1989; Martin et al., 1990; Turlings et al., 1990). The responses of natural enemies of herbivores to such informational volatile phytochemistry also have been discussed as dimensions of tritrophic systems (Price et al., 1980; Price, 1986). Further investigations probably will yield evidence of polytrophic communications based especially on phytochemicals.

Results of more recent studies emphasize that the odor of a plant is typically composed of tens and hundreds of chemicals. Some are unique to a plant, while others are extremely common among them (van Straten and Marse, 1983; Visser et al., 1979). As an example of the latter point, green leaf volatile chemicals contribute variously to the smell of numerous plants (Visser, 1979; Hamilton-Kemp et al., 1988, 1989; Liu et al., 1989; Hernandez et al., 1989; Lwande et al., 1989; Connick et al., 1989; Dicke et al., 1990). Electrophysiological studies indicate that chemosensory neurons of adult phytophagous insects are highly sensitive to green leaf volatiles (Visser, 1979; Guerin and Visser, 1980; Dickens, 1984; Dickens and Boldt, 1985; Light and Jang, 1987; Ramachandran et al., 1990). Such chemicals also may be important in host–habitat location by several natural enemies (Baericke et al., 1989; Whitman and Eller, 1990; Turlings et al., 1990). In spite of this apparent importance of green leaf volatiles in tritrophic communications involving plants, herbivores, and their natural enemies, specific studies focusing on how these chemicals are perceived by phytophagous insects and their natural enemies are lacking. We therefore used an electroantennogram technique (Schneider, 1957) to analyze the olfactory sensitivities of both an herbivore, *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae), and a parasitoid, *Microplitis demolitor* (Wilkinson) (Hymenoptera: Braconidae), to green leaf volatile chemicals. *Microplitis demolitor* is an introduced parasitoid (Shepard et al., 1983a) that develops successfully in *P. includens* (Shepard et al., 1983b).

METHODS AND MATERIALS

Insects. The colony of *P. includens* was maintained on a pinto bean-based diet (Shorey and Hale, 1965) at 27 ± 2°C and 50 ± 10% relative humidity

with a photoperiod of 14:10 hr light–dark. *M. demolitor* were reared on *P. includens* larvae following the methods of Herard et al. (1988). Adults of *P. includens* were maintained in cages with 10% sucrose solution, and those of *M. demolitor* were maintained with honey and water until used in experiments. Experiments utilized 4-day-old females and 2-day-old males.

Antennal Preparation. A moth was immobilized in a disposable 5-ml polypropylene pipet tip with paraffin wax. The insect's head and a part of the prothorax protruded from the narrow end of the pipet (the end had been cut to the appropriate dimension). Each parasitoid was immobilized in a similar manner in a pipet tip (1017-01-1, Robbins Scientific Corp., Mountain View, California 94043) with a small quantity of modeller's clay (Permoplast, American Art Clay Co., Inc., Indianapolis, Indiana). A wad of cotton, dipped in distilled water, was inserted in the wider end of the pipet to prevent rapid desiccation of the insect. The right antenna of each insect was removed to permit easy access for the indifferent electrode to the base of the recording left antenna. Insects were allowed to recover for 15 min before initiation of an experiment.

The distal end of the recording antenna was inserted into the tip of a glass pipet (20 μm diam) containing a chlorinated silver wire (Ag–AgCl) electrode (recording electrode) filled with a saline solution (NaCl, 3.75 g; $CaCl_2$, 0.105 g; KCl, 0.175 g; $NaHCO_3$, 0.100 g; H_2O, 500 ml) (Roelofs, 1984) containing 10% (v/v) of polyvinylpyrolidone (Aldrich Chemical Co., Milwaukee, Wisconsin). The base of the antenna was connected to a similar saline filled electrode. The Ag–AgCl electrode was connected through a Molex Soldercon IC terminal (Digi-Key part No. 1938-4) to a differential input probe (model HIP 16) of an amplifier (model P16 AC/DC microelectrode amplifier, Grass Instruments, Quincy, Massachusetts). The analog electroantennogram (EAG) signal, amplified 100 times, was converted to digital form through an analog–digital interface (Metrabyte DAS-8-PGA-analog-digital interface; Metrabyte Corporation, Taunton, Massachusetts). The digital signals were either viewed directly on a Compaq PC or imported into a spreadsheet program, Lotus 1-2-3 (Lotus Development Corporation, Massachusetts), using Labtech Notebook, Version-4, software (Laboratory Technologies Incorporation, Wilmington, Massachusetts). An EAG signal was monitored at the rate of 100 signals per second. Maximal EAG depolarizations were read from *xy* plots of time (msec) versus signal (v).

An experimental antenna was continuously bathed in a stream of calcium chloride-dried, activated charcoal-filtered air (40 ml/sec), which was rehumidified by bubbling it through an Erlenmeyer flask (2 liters) containing distilled water. The prepared air was introduced to the experimental site through the base of a modified Bunsen burner (Bjostad, 1988). The upper part of the Bunsen burner was replaced with a 10-cm-long glass delivery tube that had a 2-cm ID for the *P. includens* antenna and an 0.8-cm ID for the *M. demolitor* antenna.

The delivery tube had a 3-mm hole in its side wall to allow the tip of a disposable Pasteur pipet to be inserted into the airstream. The distal tip of the delivery tube was positioned 2 cm from the antennal preparation. Stimuli were applied into the airstream using a disposable Pasteur pipet (5.75 in. long). The tip of the pipet was introduced into the hole in the side wall of the delivery tube. Air (ca. 2 ml) was quickly puffed (ca. 30 msec) from a 5-ml syringe through the disposable pipet into the airstream. The modified Bunsen burner air delivery and the syringe were permanently mounted in front of a microscope stage that held the antennal preparation. A glass funnel (10 cm diam.), connected to a vacuum line, was positioned 10 cm behind the antennal preparation to evacuate chemicals continuously from the experimental area. The antenna was stimulated at 4-min intervals. A standardized series of stimulations with five test chemicals was preceded and followed by both the standard chemical (100 μg of *cis*-3-hexen-1-ol) and the control (1 μl of hexane) stimulations (Light and Jang, 1987). The *P. includens* antennal preparation remained in experimentally stable condition for 5–6 hr. *M. demolitor* antennal preparations were dependably utilized experimentally for 2 hr. Preparations were maintained in a room at 25 $\pm$ 2°C and 70% relative humidity.

Chemicals. The studied volatile phytochemicals, their source and purity are presented in Table 1. Each chemical was dissolved in HPLC-grade hexane (Aldrich Chemical Company) to yield 100 mg/ml solutions. Involved vials were numbered serially; during an experiment, each chemical was known and referenced only by its assigned number. Experimental hydrocarbons were used without a carrier solvent. For an experiment, the quantity of hydrocarbons was 10 μl for *P. includens* and 1 μl for *M. demolitor*.

Experimental Protocol. A hexane-washed, oven-dried, filter-paper strip (3 $\times$ 1 cm) was inserted into an otherwise prepared Pasteur pipet. One microliter of a test solution then was applied on the filter paper. Such a stimulus-containing Pasteur pipet was prepared prior to experimental stimulation in a room different from the one housing the EAG preparation. EAGs were recorded from three males and three females of *P. includens*, and four males and four females of *M. demolitor*.

Each EAG was analyzed by measuring the maximal millivolt (−mV) amplitude of depolarization elicited by a given stimulus. Each millivolt response was converted to a percentage value of the mV response to the accompanying standard (100 μg *cis*-3-hexen-1-ol). This analysis has been utilized in other EAG studies on insect olfaction (Guerin and Visser, 1980; Dickens, 1984; Dickens and Boldt, 1985; Light and Jang, 1987).

Statistical Analysis. The relative EAG responses to compounds within a chemical group (e.g., hydrocarbons, alcohols) were analyzed by ANOVA to distinguish the effects of sex and compounds. Multiple comparisons were performed with the mean responses of both sexes to individual compounds in a

TABLE 1. LIST OF CHEMICALS USED IN EAG STUDIES AND THEIR PURITY[a]

Chemicals	Purity (%)	Chemicals	Purity (%)
I Aliphatic hydrocarbons		IV Aliphatic saturated aldehydes	
Pentane	>99	Hexanal	98
Hexane	>99	Heptanal	95
Heptane	>99	Octanal	99
Octane	>99	Nonanal	95
Nonane	>99	Decanal	95
Decane	>99	Dodecyl aldehyde	95
		Tetradecyl aldehyde	80
II Aliphatic saturated alcohols		V Aliphatic unsaturated aldehydes	
1-Pentanol	99	*trans*-2-Pentanal	95
1-Hexanol	98	*trans*-2-Hexanal	99
1-Heptanol	98	*trans*-2-Heptanal	98
1-Octanol		*trans*-2-Octanal	94
1-Nonanol	99	*trans*-2-Nonanal	97
1-Decanol	>99	*trans*-2-Decanal	98
1-Dodecanol	99		
1-Tetradecanol			
III Aliphatic unsaturated alcohols		VI Aliphatic ketones	
cis-3-Hexen-1-ol	98	2-Pentenone	90
trans-3-Hexen-1-ol	98	2-Hexanone	98
1-Octen-3-ol	98	2-Heptanone	98
cis-3-Nonen-1-ol	95	3-Octanone	99
trans-5-Decen-1-ol	97	2-Nonanone	>99
cis-7-Tetradecen-1-ol	97	2-Decanone	98
cis-7-Dodecen-1-ol	99		
		VII Esters	
		Methyl caprylate	99
		Methyl caproate	99
		Methyl enanthate	99
		Methyl nonanoate	98
		Methyl decanoate	99

[a] All chemicals were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin.

group (when the effect of compounds was significant) or the mean responses of each sex to all compounds in a group (when the effect of sex was significant). Means were separated by Tukey's honestly significant difference test (Zar, 1984). When the interaction between the effect of sex and chemical was significant, differences in the responses of the two sexes to individual compounds

were evaluated by Student's t test (Zar, 1984). Responses of *P. includens* and *M. demolitor* were compared only in magnitude.

RESULTS

Electroantennogram Response

The typical electroantennogram responses of *P. includens* and *M. demolitor* were similar (Figure 1). This response was characterized by a fast depolarization followed by a slow return to the baseline. The recording technique permitted real-time analysis of the characteristics of a response. The fast depolarization phase of the *P. includens* response occurred between 0.2299 and 0.5299 sec (mean $\pm$ standard deviation = 0.41819 ± 0.078, $N = 18$). There was a significant positive linear relationship between the maximum amplitude of depolarization and the time for maximum depolarization (Figure 2, Student's t statistics = 2.69 $P < 0.05$). The recovery to baseline occurred between 1.5 and 4.3 sec (mean $\pm$ standard deviation = 2.926 ± 0.8835, $N = 18$) and also was related linearly to the amplitude of EAG response (Figure 2, Student t statistic = 2.23, $P < 0.05$).

Response to Standard

The absolute EAG amplitude elicited from male versus female *P. includens* or *M. demolitor* in response to stimulation with the standard (*cis*-3-hexen-1-ol) did not differ statistically (Table 2). EAG responses of female *M. demolitor* and female *P. includens* were similar (Mann-Whitney U test, $P < 0.4$), but male *P. includens* response was higher than that of male *M. demolitor* ($P < 0.006$).

TABLE 2. ELECTROANTENNOGRAM RESPONSES OF *P. includens* AND *M. demolitor* TO 100 μg OF *cis*-3-HEXEN-1-OL

Sex	EAG responses (mV)[a]	
	P. includens	*M. demolitor*
Female	3.67 ± 0.6ax	3.02 ± 0.3ax
Male	3.61 ± 0.2ax	2.34 ± 0.3ay

[a] Values in columns followed by the same letter are not significantly different, $P < 0.05$, Student's t test. Values in rows followed by the same letters are not significantly different, Mann-Whitney U test, $P < 0.05$.

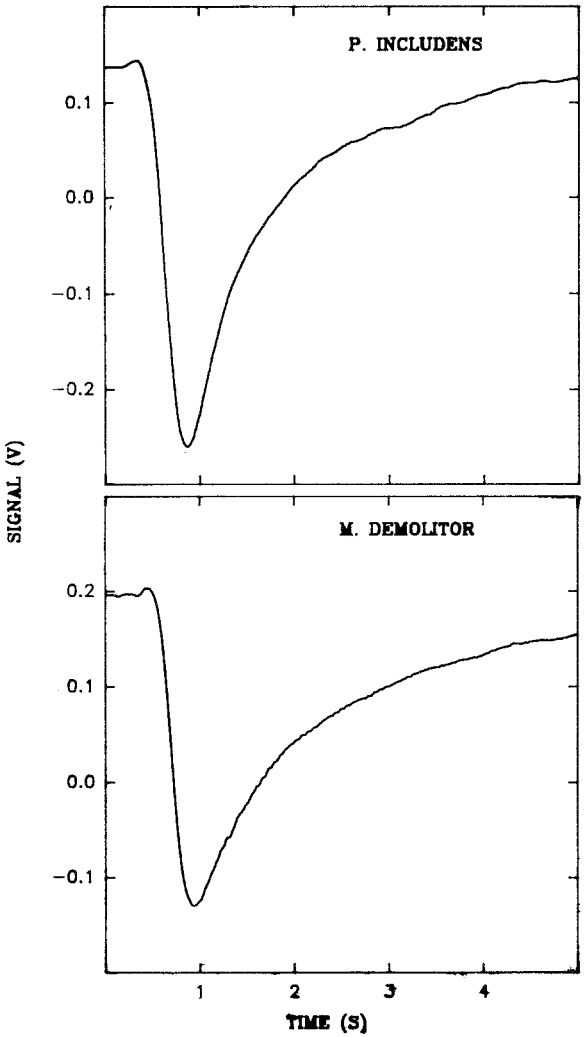


FIG. 1. Typical electroantennogram responses of *P. includens* and *M. demolitor* to stimulation with 100 μg of *cis*-3-hexen-1-ol.

Response to Hydrocarbons

Responses of male versus female *P. includens* to stimulation with hydrocarbons did not differ significantly ($F^{1,26} = 0.35$, $P < 0.350$). In both sexes, responses to all hydrocarbons differed significantly from the filter-paper control (Figure 3). Responses to compounds of different carbon-chain lengths were not

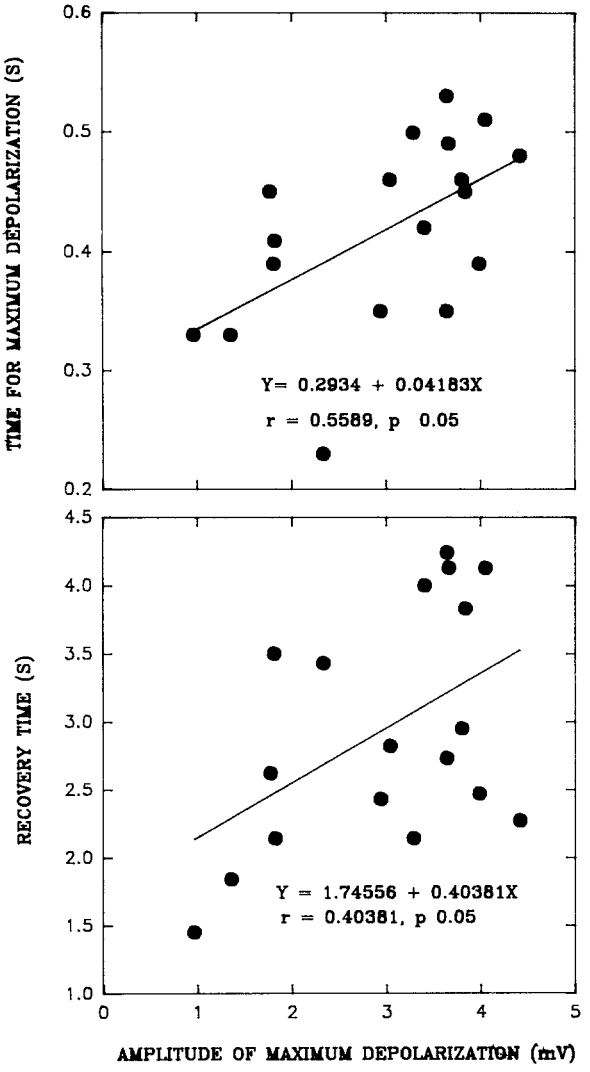


FIG. 2. Relationships between the amplitude of the EAG responses of *P. includens* and the times for depolarization and recovery.

statistically different. The recorded numerical responses, however, peaked at six carbons and decreased gradually at longer carbon-chain lengths (Figure 3).

The responses of male and female *M. demolitor* to hydrocarbons were similar ($F^{1,39} = 0.05$, $P < 0.8212$). Response to each hydrocarbon was significantly different from that to filter-paper control (Figure 3). Response to 7-, 8-,

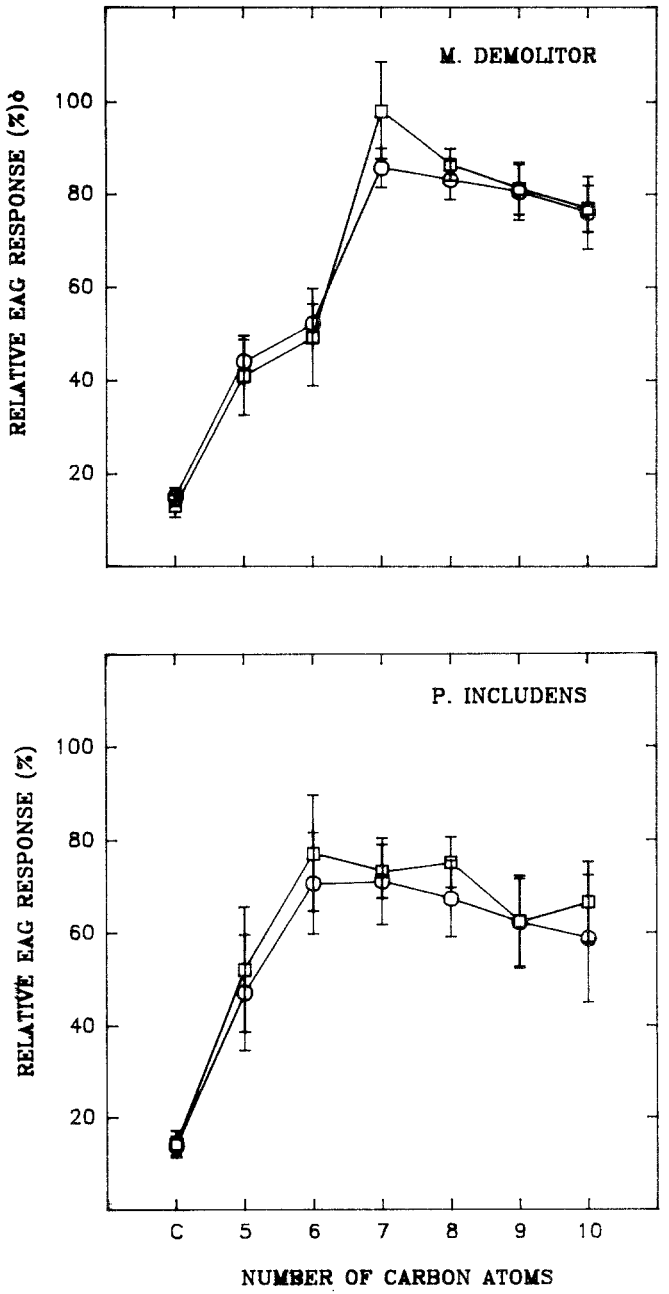


FIG. 3. EAG responses of *M. demolitor* and *P. includens* to saturated hydrocarbons. C = control (1 μl of hexane). Circles = females, squares = males.

9-, and 10-carbon compounds was significantly higher than that to 5- and 6-carbon compounds (Figure 3). More specifically the numerical response to hydrocarbons of different carbon-chain lengths peaked at 7 carbons and then decreased gradually at greater lengths (Figure 3). The response of *M. demolitor* to hydrocarbons was numerically higher than that of *P. includens*, although the quantity of stimulant was ten times lower.

Response to Aliphatic Aldehydes

Saturated Aldehydes. The magnitude (84%) of response of male *P. includens* to saturated aldehydes was higher than that (74%) of the females ($F^{1,30} = 5.71$, $P < 0.02$). Responses to 6-, 7-, 8-, and 9-carbon compounds were significantly higher than to a 10-carbon compound. Responses to 12- and 14-carbon compounds did not differ from that of the control (Figure 4). Responses peaked between the 6- and 8-carbon chain lengths.

Responses of male *M. demolitor* were higher than those of females (137.8 and 117.6% for male and female responses, respectively; $F^{1,33} = 14.51$, $P < 0.006$). Responses to 7-, 8-, and 9-carbon aldehydes were significantly higher than that to 6- and 10-carbon compounds (Figure 4). Responses of *M. demolitor* peaked at the 8-carbon chain length.

Unsaturated Aldehydes. There was a significant interaction between sex and carbon chain length regarding the responses of *P. includens* to compounds ($F^{5,22} = 3.29$, $P < 0.02$). Male responses to 6-, 7-, and 8-carbon compounds were higher than those of the females (Figure 5). Responses of both sexes to 6-, 7-, and 8-carbon compounds were significantly higher than those to 5- and 9-carbon compounds (Figure 5). Responses numerically peaked between the 6- and 7-carbon chain lengths.

Male *M. demolitor* elicited a higher EAG response (119.8%) to unsaturated aldehydes than did females (106.3%) ($F^{1,39} = 19.71$, $P < 0.05$). Responses to 7- and 8-carbon compounds were significantly higher than to all other compounds. Responses peaked between the 7- and 8-carbon chain lengths (Figure 5). Peak responses of *M. demolitor* were higher than those of *P. includens*.

Aliphatic Alcohols

Saturated Alcohols. Responses of male *P. includens* to 6- and 7-carbon alcohols were significantly higher than that of females. Highest responses were recorded for 5-, 6-, and 7-carbon compounds (Figure 6). Responses dropped ca. 40% as the carbon chain length increased beyond 7 carbons. Response to 9-, 10-, 12-, and 14-carbon compounds did not differ significantly from that to the solvent control.

Female *M. demolitor* response (79.61%) was higher than that of the males

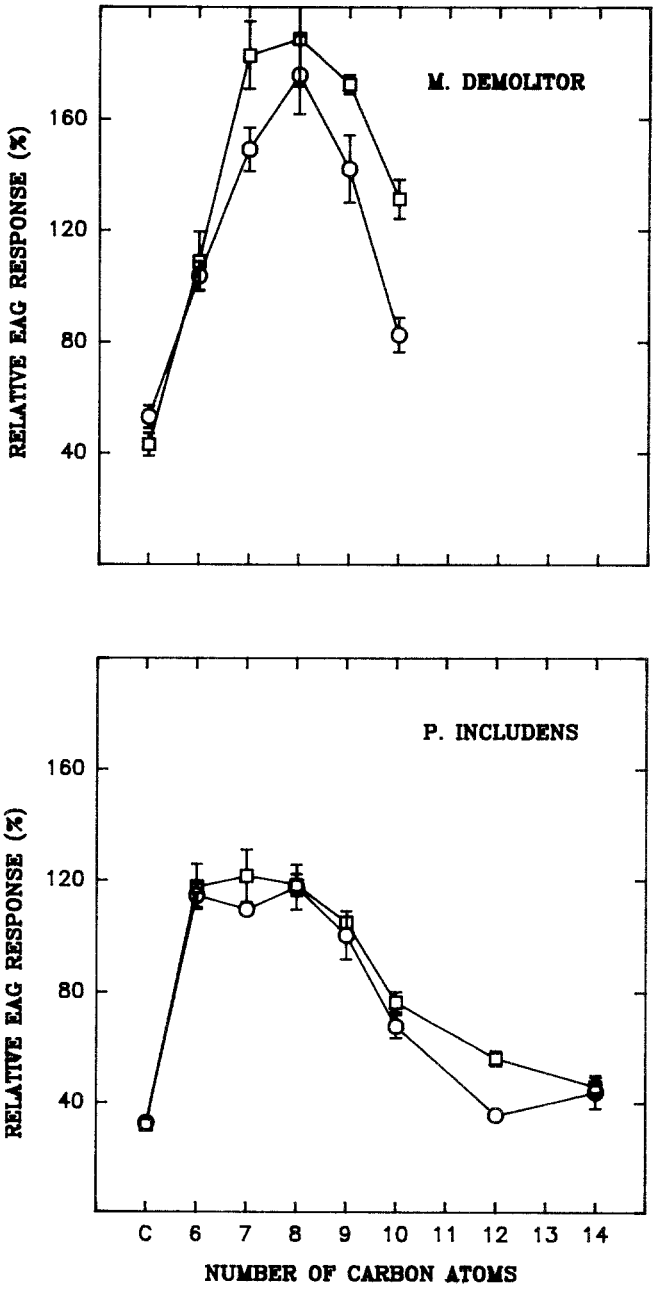


FIG. 4. EAG responses of *M. demolitor* and *P. includens* to saturated aldehydes. C = control (1 μl of hexane). Circles = females, squares = males.

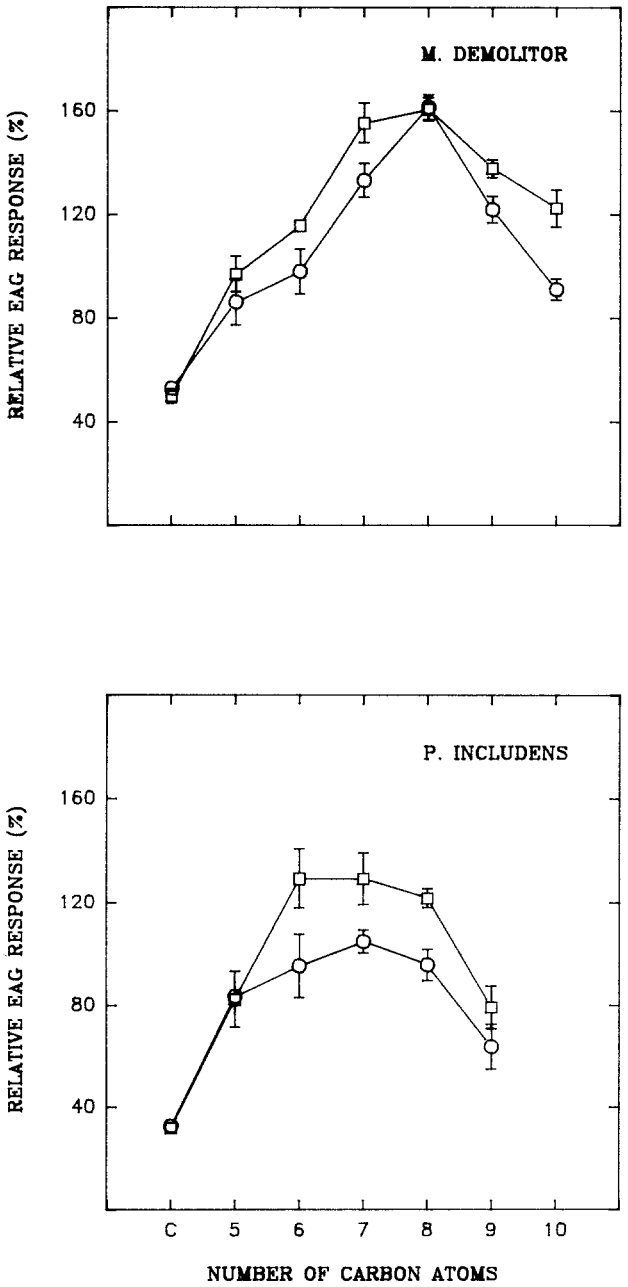


FIG. 5. EAG responses of *M. demolitor* and *P. includens* to unsaturated aldehydes. C = control (1 μl of hexane). Circles = females, squares = males.

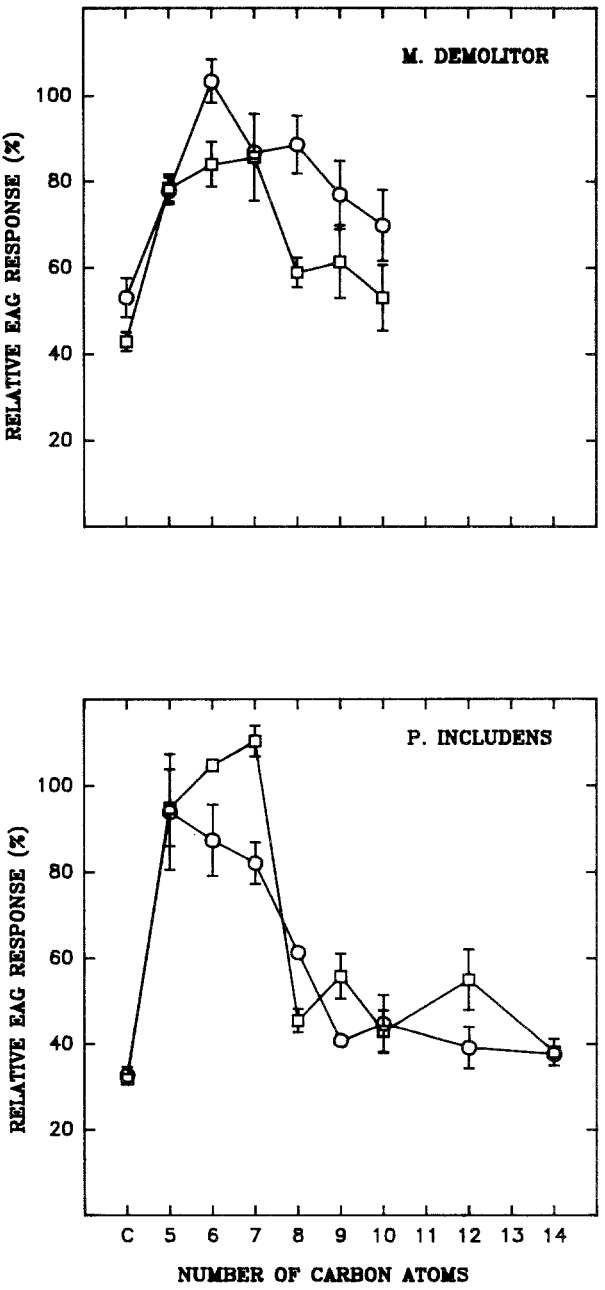


FIG. 6. EAG responses of *M. demolitor* and *P. includens* to saturated alcohols. C = control (1 μl of hexane). Circles = females, squares = males.

(67.51%) ($F^{1,37} = 15.21$, $P < 0.004$). The highest numerical response was recorded for the 6-carbon compound, although this response was not statistically different from that for 5-, 7-, and 8-carbon compounds (Figure 6). The response profiles of *M. demolitor* and *P. includens* were similar (Figure 6).

Unsaturated Alcohols. Responses of male versus female *P. includens* to unsaturated alcohols were similar ($F^{1,22} = 2.88$, $P < 0.1036$). The two optical isomers of a 6-carbon alcohol (*cis*- and *trans*-hexen-1-ol) and an 8-carbon unsaturated alcohol elicited higher responses in this chemical class (Figure 7). The responses to 9-, 10-, 12-, and 14-carbon compounds were lower than to the above alcohols but significantly higher than that to the solvent control. Responses of *M. demolitor* to these unsaturated alcohols were not studied.

Aliphatic Ketones

Male and female responses of *P. includens* were similar among the studied compounds of this chemical class ($F^{1,26} = 0.13$, $P < 0.7252$). Response to the 8-carbon ketone was statistically similar to that for the 7-carbon compound (Figure 8). Responses to the other studied ketones were lower. Aliphatic ketones elicited similar responses from male and female *M. demolitor* ($F^{1,26} = 0.81$, $P < 0.3772$). The responses could be categorized into two homogenous groups: (1) responses to 7-, 8-, and 9-carbon compounds, which were high and significantly different from that to the solvent control; and (2) responses to 5-, 6-, and 10-carbon compounds, which did not differ from those to the solvent control. Peak response was observed at the 8-carbon chain length (Figure 8). Peak response of *M. demolitor* was numerically higher than that of *P. includens*.

Esters

Responses of both sexes of *P. includens* were similar. Response to all compounds was significantly higher than that to the solvent control (Figure 9). Comparison of means indicates that the compounds could be divided into three distinct groups: the highest response, recorded with the 6-carbon compound; the intermediate, recorded with 7- and 8-carbon compounds; and the lowest responses, recorded with 9- and 10-carbon esters. Responses of *M. demolitor* to esters were not studied.

Response to Functional Groups

To determine how the chemical functional group affected the EAG response, that reaction to 6-, 7-, and 8-carbon compounds with different functional groups was analyzed by ANOVA. *P. includens* adults proved more sensitive to aldehydes (saturated or unsaturated) and ketones (Figure 10). Hydrocarbons were the least stimulatory.

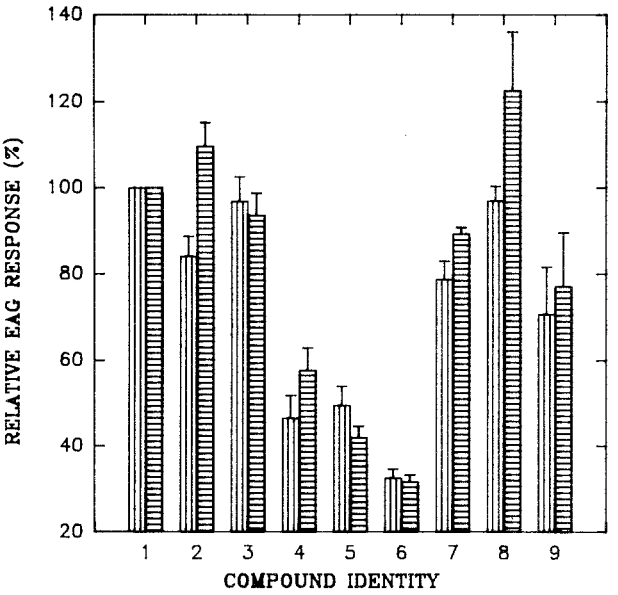


FIG. 7. EAG responses of *P. includens* to unsaturated alcohols. (1) *cis*-3-hexen-1-ol, (2) *trans*-3-hexen-1-ol, (3) 1-octen-3-ol, (4) *cis*-3-nonen-1-ol, (5) *trans*-5-decen-1-ol, (6) control (1 μl of hexane), (7) *cis*-7-tetradecen-1-ol, (8) *cis*-7-dodecen-1-ol, (9) control (10 μl of hexane). C1 = 1 μl of hexane as control, C2 = 10 μl of hexane as control. One microliter of 100 mg/ml solutions was used as stimuli for compounds 1, 2, 3, 4, and 5. Ten microliters of 10 mg/ml solutions were used for compound numbers 7 and 8. Hence these results are to be compared with their respective solvent controls (i.e., 1 or 10 μl of hexane). Columns with vertical lines = females, with horizontal lines = males.

For *M. demolitor*, saturated aliphatic aldehydes were the most stimulatory, and unsaturated aldehydes and ketones were next (Figure 10). Responses to alcohols and hydrocarbons were lower, but similar (Figure 10).

DISCUSSION

Characteristics of EAG Responses. A typical EAG response has at least three measurable parameters: (1) the amplitude of depolarization, (2) the time required for depolarization, and (3) the time required to return to the baseline after depolarization. Most studies use the amplitude of depolarization as the parameter to measure the sensitivity of insects to olfactory stimulants (Blust and Hopkins, 1987; Light and Jang, 1987; Whitehead, 1986). A few investigations have also utilized the recovery time, or half time to recovery, to the baseline

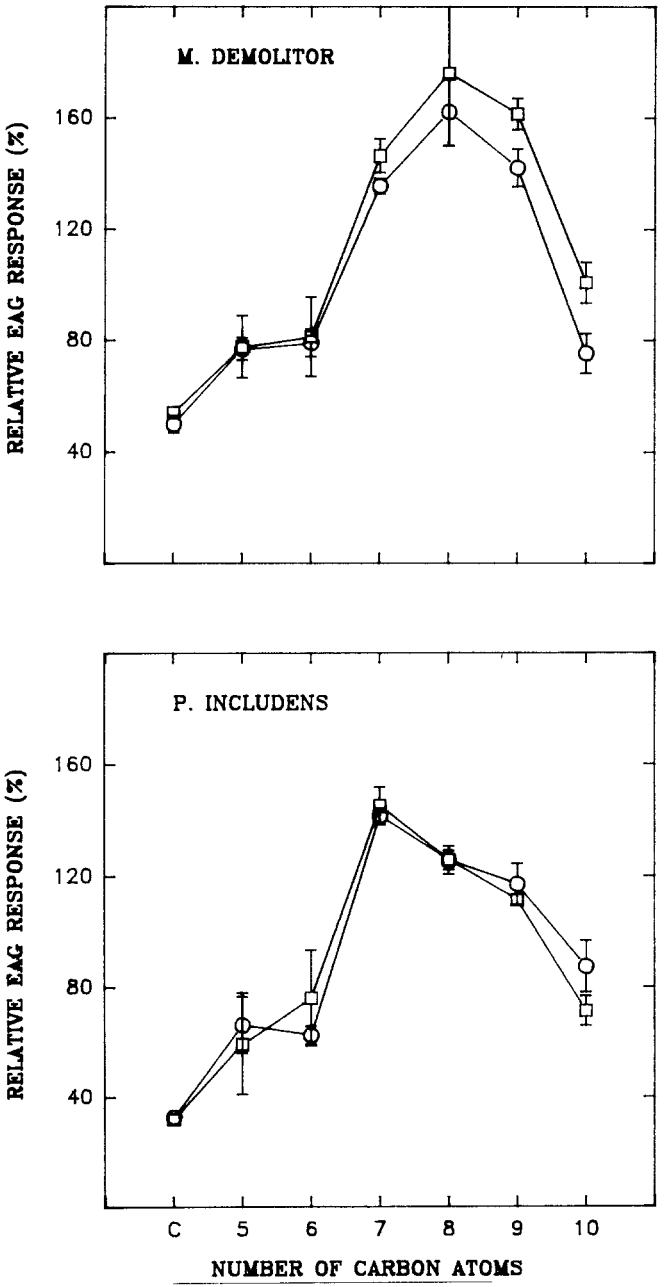


FIG. 8. EAG responses of *M. demolitor* and *P. includens* to aliphatic ketones. C = control (1 μl of hexane). Circles = females, squares = males.

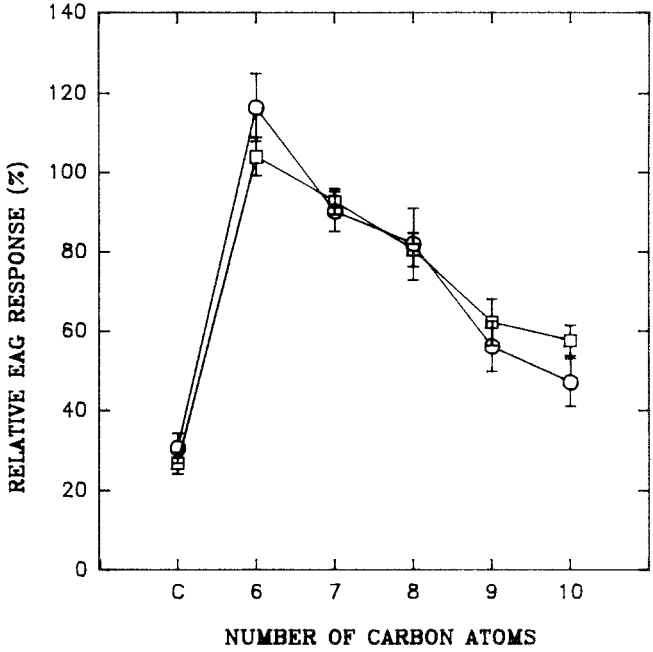


FIG. 9. EAG responses of *P. includens* to esters. C = control (1 μl of hexane). Circles = females, squares = males.

as an index of EAG response (Visser, 1979). Compounds with high biological activity are reported to have slow recovery to the baseline (Roelofs, 1986). However, relationships among the above three parameters are not well documented. Our EAGs were recorded in real time to an accuracy of 1/100th of a second. Our results indicate that both the time for depolarization and the recovery time are significantly related linearly to the amplitude of depolarization. However, only a small portion of the variability in these two parameters was accounted for (r^2 = 0.3124 and 0.1631 for depolarization and recovery time, respectively; Figure 2) by their dependence on the amplitude of depolarization.

Female adults of the herbivore, *P. includens*, and the natural enemy, *M. demolitor*, elicited similar absolute EAG responses to the standard, *cis*-3-hexen-1-ol. Since the magnitude of the EAG response to a given stimulus is directly related to the number of receptors reacting (Payne, 1975) and the length of the herbivore antenna (9.6 ± 0.5 mm) is about three times longer than that of the parasitoid (3.3 ± 0.2 mm for female antenna), then the similarity in the responses suggests a higher density of receptors per unit of antennal length on the natural enemy.

Sexual Differences. Males of both the parasitoid and the herbivore are more

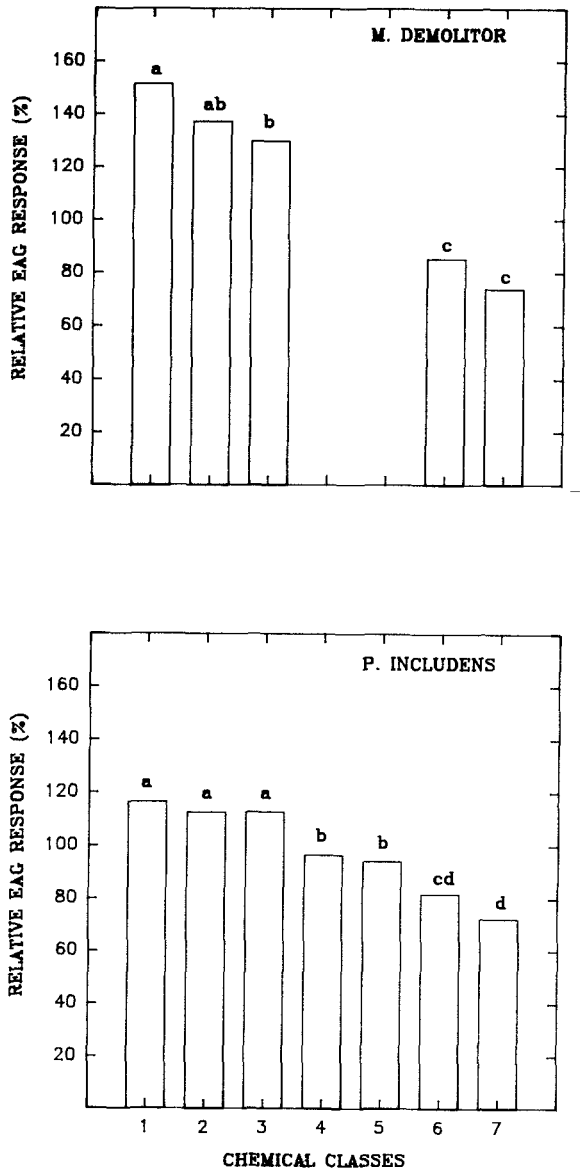


FIG. 10. Responses of *M. demolitor* and *P. includens* to compounds with different functional groups. Each bar is the mean response of male and female moths, or parasitoids, to 6-, 7-, and 8-carbon compounds; (1) saturated aldehydes, (2) unsaturated aldehydes, (3) ketones, (4) unsaturated alcohols, (5) esters, (6) alcohols, (7) hydrocarbons. Bars not bearing the same letters are statistically significant, Tukey's honestly significant difference test, $P < 0.05$.

sensitive than the female to saturated and unsaturated aldehydes. Adult males of several phytophagous insects have given a higher response than their conspecific females to some plant volatiles (Guerin and Visser, 1980; Dickens, 1984; Honda et al., 1986; Blust and Hopkins, 1987; Hansson et al., 1989; Ramachandran et al., 1990). The higher sensitivity of males to some plant volatile compounds may be explained partially on the basis that some of these compounds may also stimulate pheromone receptors and thus produce a larger EAG response through additive or synergistic effects. The observed sensitivity of male antennae to plant volatiles has led to the postulation that the perception of plant volatiles by male antennal receptors evolved before their function in pheromone detection. It has been further theorized that a primitive mate-finding system could be based on both sexes gathering around suitable food plants (Lanne et al., 1987; Hansson et al., 1989). Synergism of bark beetle and cotton boll weevil sex pheromones by plant volatiles (Bedard et al., 1969, 1980; Dickens, 1989) and the attraction of male cabbage looper moths to cabbage plants in a wind tunnel (Landolt, 1989; Landolt and Heath, 1990) support the suggestion that such resource-based mating strategies occur in insects.

Males of the parasitoids, *Diadromus pulchellus* Wesmeal and *D. collaris* Gravenhorst, are also known to perceive plant volatiles (Lecomte and Pouzat, 1985). Mate location by male *Campoletis sonorensis* (Cameron) was reported to be mediated partly by volatiles of cotton (McAuslane et al., 1990). However, other studies have reported that male parasitoids do not respond to volatiles from the host habitat (Elzen et al., 1986; Eller et al., 1988; Whitman and Eller, 1990) or that their response to such volatiles was lower than that of conspecific females (Martin et al., 1990). The EAG responses of male *M. demolitor* were comparable to those of the females for hydrocarbons and ketones, higher for saturated aldehydes, and lower for alcohols. It seems likely that both the low concentration and the unnaturalness of the stimulus source, and the physiological condition of the males, may have influenced their response in studies where male parasitoids did not orient to plant volatiles (McAuslane et al., 1990).

Carbon Chain Length Effects. Phylogenetically diverse phytophagous insects have been reported to be selectively responsive to 6- and 7-carbon alcohols and aldehydes (Visser, 1986; Light and Jang, 1987). The EAG responses of *P. includens* similarly were higher for 6- and 7-carbon hydrocarbons, saturated and unsaturated aldehydes, saturated and unsaturated alcohols, and esters. The response to ketones clearly peaked at 7 carbons. This latter significant difference may possibly be explained by the fact that the functional group in ketones is located on the second carbon, whereas it is on the first carbon in the other classes of chemical compounds tested. This difference thus might make a 7-carbon ketone the reactive equivalent of a 6-carbon compound in the other chemical classes.

With the natural enemy, *M. demolitor*, the responses were higher for the

7- and 8-carbon hydrocarbons, saturated aldehydes, unsaturated aldehydes, and ketones. In all chemical classes tested, except alcohols, there was a 1-carbon shift in the peak response by the parasitoid as compared to the herbivore. A similar higher EAG response to heptanal than hexanal was recorded for *C. sonorensis* (Baehrecke et al., 1989). Our results, along with those of Baehrecke et al. (1989), thus suggest a difference in the olfactory-response profile of a herbivore and its parasitic hymenopteran to plant volatiles. Six-carbon alcohols, aldehydes, and their derived esters are found in several plants (van Straten and Maarse, 1983; Visser et al., 1979; Visser, 1986). Consequently, higher responses of herbivores to 6-carbon compounds could be of adaptive significance. However, several plants reportedly contain a full range of ca. C_2–C_{12} aliphatic compounds (van Straten and Maarse, 1983; Connick et al., 1989; Liu et al., 1989). In view of our limited understanding of which plant volatiles parasitoids utilize to locate their host habitat (Whitman, 1988a,b), the biological significance of the observed heightened sensitivity of some parasitoids to 7-carbon compounds remains unknown. The searching behavior of the parasitic wasp, *Orgilus lepidus* (Hymenoptera: Braconidae), however, was elicited by heptanoic acid, which is present in the frass of its host, the larvae of the potato tuber moth (Hendry et al., 1973). It is well known that volatiles from the frass of host larvae play important roles in the behavior of several parasitoids (Vinson, 1984; Nordlund et al., 1988; Whitman, 1988a,b; Lewis and Tumlinson, 1988). When the above caterpillars ingest and metabolize host tissues, 7-carbon aliphatic compounds may be uniquely abundant volatile metabolites in their frass. The observed high responses of some parasitoids to 7-carbon compounds thus might be adaptive.

Several studies also have demonstrated an increase in parasitoid responses to plants damaged by herbivorous insects (Whitman and Eller, 1990; Turlings et al., 1990). Herbivore damage certainly increases the quantity of volatile compounds emanating from the plant (Whitman and Eller, 1990; Turlings et al., 1990). Whether herbivore damage produces a significant shift from 6-carbon to 7-carbon compounds in the volatile profiles of a plant is not known.

Chemical Class Effects. EAG amplitudes for phytophagous insects have been selectively greater for either aldehydes (Dickens, 1984; Dickens and Boldt, 1985; Ramachandran, 1990) or alcohols (Guerin and Staedler, 1982; Visser, 1979; Kozlowski and Visser, 1981; Van der Pers, 1981). In *P. includens*, responses to saturated and unsaturated aldehydes and ketones were higher than to other tested chemical classes. The *P. includens* response profile was similar to that of *Anthonomas grandis* (Dickens, 1984) and *Trirhabda bracharides* (Dickens and Boldt, 1985). The hierarchy of responses by the parasitoid, *M. demolitor*, and the herbivore, *P. includens*, to compounds in the tested chemical groups was remarkably similar (Figure 10). However, the response of the parasitoid to tested aldehydes, ketones, and hydrocarbons was greater than that of the herbivore. The higher responses of the parasitoid to hydrocarbons was of

particular interest because the quantity of stimulus was 10 times lower. Various studies have shown that parasitic Hymenoptera are stimulated by kairomones originating from the host's body and scales (Vinson, 1984; Nordlund et al., 1988; Whitman, 1988a,b). Since the host body and scales contain cuticular hydrocarbons, a higher response by a parasitoid to hydrocarbons may be adaptive.

Green Leaf Volatiles and Tritrophic Interactions among Plants, Herbivores, and Natural Enemies. Several authors have suggested that plant volatiles have evolved to serve tritrophic communicative roles (Whitman, 1988b, 1990; Dicke and Sabelis, 1989; Dicke et al., 1990). Based on the evidence that the release of green leaf volatiles is increased after an herbivore attack (Wallbank and Wheatley, 1976; Tollsten and Bergström, 1988; Dicke et al., 1990; Whitman and Eller, 1990) and that several parasitoids and predators are much more attracted to damaged plants than to undamaged plants (Sato, 1979; Elzen et al., 1983; Loke et al., 1983; Odell and Godwin, 1984; Nadel and van Alphen, 1987; Dicke and Sabelis, 1988; Whitman, 1988b; Whitman and Eller, 1990), it has been proposed that such release of volatiles might represent an act of "screaming for help" by the plants (Whitman and Eller, 1990). Our results, which indicate that the parasitoid antenna is much more sensitive to several plant volatiles than that of the herbivore, support the hypothesis that a higher release of such volatiles after herbivore attack is adaptive to the plant. Whether the differences in the electrophysiological responses of the herbivore and parasitoid correlate with differing behavioral responses remains to be determined. Our observed increased sensitivity of the parasitoid antenna to 7-carbon versus 6-carbon compounds suggests that the 7-carbon volatiles may be more important in parasitoid host-seeking behavior. We hypothesize that herbivore frass may be the major source of such 7-carbon volatiles. When some herbivores ingest and metabolize host tissues, 7-carbon aliphatic compounds may be uniquely abundant in their frass. Identification of heptanoic acid in the frass of the potato tuber moth as the kairomone for its parasitoid supports our contention (Hendry et al., 1973). Although emphasis currently is placed on plants as the most important source of volatile cues for parasitoid orientation (Whitman and Eller, 1990; Turlings et al., 1990), we suggest that the contribution of volatiles in herbivore frass may be more important. An alternative explanation for the observed heightened sensitivity of the parasitoid antenna to 7-carbon compounds could be that after herbivore damage there is a shift from 6- to 7-carbon volatiles being released by a plant. There is, however, no experimental evidence to support this. Our current research is examining these possibilities.

Acknowledgments—A Robert S. McNamara Fellowship from the Economic Development Institute of the World Bank to R.R. made these studies possible. This research was also supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison; and in part by funds from U.S. Hatch Projects No. 3040 and 3419, U.W. Graduate School Project No. 911427 and USDA Competitive Grant No. 88-37153-4043.

REFERENCES

BAEHRECHE, E.H., WILLIAMS, H.J., and VINSON, S.B. 1989. Electroantennogram responses of *Campoletis sonorensis* (Hymenoptera: Ichneumonidae) to chemicals in cotton (*Gossypium hirsutum* L). *J. Chem. Ecol.* 15:37–45.

BEDARD, W.D., TILDEN, P.E., WOOD, D.L., SILVERSTEIN, R.M., BROWNLEE, R.G., and RODIN, J.O. 1969. Western pine beetles: Field response to its sex pheromone and a synergistic host terpene myrcene. *Science* 164:1284–1285.

BEDARD, W.D., WOOD, D.L., TILDEN, P.E., LINDAHL, K.Q., JR., SILVERSTEIN, R.M., and RODIN, J.O. 1980. Field responses of the western pine beetle and one of its predators to host- and beetle-produced compounds. *J. Chem. Ecol.* 6:625–641.

BJOSTAD, L.B. 1988. Insect electroantennogram responses to semiochemicals recorded with an inexpensive personal computer. *Physiol. Entomol.* 13:139–145.

BLUST, M.H., and HOPKINS, L.T. 1987. Olfactory responses of a specialist and a generalist grasshopper to volatiles of *Artemisia ludoviciana* Nutt. (Asteraceae). *J. Chem. Ecol.* 13:1893–1901.

BUTTERY, R.G., and LING, L.C. 1985. Volatile components of corn roots: possible insect attractants. *J. Agric. Food Chem.* 33:772–774.

BUTTERY, R.G., KAMM, J.A., and LING, L.C. 1984. Volatile components of red clover leaves, flowers, and seed pods: Possible insect attractants. *J. Agric. Food Chem.* 32:254–256.

CONNICK, W.J., BRADOW, J.M., and LEGENDRE, M.G. 1989. Identification and bioactivity of volatile allelochemicals from amaranth residues. *J. Agric. Food Chem.* 37:792–796.

DICKE, M., and SABELIS, M.W. 1989. Does it pay plants to advertise for bodyguards? Towards a cost-benefit analysis of induced synomone production, pp. 341–358 *in* H. Lambers, M.L. Cambridge, H. Konings, and T.L. Pons (eds.). Causes and Consequences Variation in Growth Rate and Productivity of Higher Plants. SPB Academic Publishing, The Hague.

DICKE, M., VAN BEEK, T.A., POSTHUMUS, M.A., BEN DOM, N., VAN BOKHOVEN, H., and DE GROOT, A.E. 1990. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions: Involvement of host plant in its production. *J. Chem. Ecol.* 16:381–396.

DICKENS, J.C. 1984. Olfaction in the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Circulionidae): Electroantennogram studies. *J. Chem. Ecol.* 10:1759–1785.

DICKENS, J.C. 1989. Green leaf volatiles enhance aggregation pheromone of boll weevil, *Anthonomus grandis*. *Entomol. Exp. Appl.* 52:191–204.

DICKENS, J.C., and BOLDT, P.E. 1985. Electroantennogram responses of *Trirhabda bracharides* (Weber) (Coleoptera: Chrysomelidae) to plant volatiles. *J. Chem. Ecol.* 11:767–779.

ELLER, F.J., TUMLINSON, J.H., and LEWIS, W.J. 1988. Beneficial arthropod behavior mediated by airborne semiochemicals: source of volatiles mediating the host-location flight behavior of *Microplitis croceipes* (cresson) (Hymenoptera: Braconidae), a parasitoid of *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae). *Environ. Entomol.* 17:745–753.

ELZEN, G.W., WILLIAMS, H.J., and VINSON, S.B. 1983. Response by the parasitoid *Campoletis sonorensis* (Hymenoptera: Ichneumonidae) to chemicals (synomones) in plants: Implications for host habitat location. *Environ. Entomol.* 12:1872–1876.

ELZEN, G.W., WILLIAMS, H.J., and VINSON, S.B. 1986. Wind tunnel flight responses by hymenopterous parasitoid *Compoletis sonorensis* to cotton cultivars and lines. *Entomol. Exp. Appl.* 42:285–289.

GILBERT, B.L., and NORRIS, D.M. 1968. A chemical basis for bark beetle (*Scolytus*) distinction between host and non-host trees. *J. Insect Physiol.* 14: 1063–1068.

GILBERT, B.L., BAKER, J.E., and NORRIS, D.M. 1967. Juglone (5-hydroxy-1,4- naphthoquinone)

from *Carya ovata*, a deterrent to feeding by *Scolytus multistriastus*. *J. Insect Physiol.* 13:1453–1459.

GUERIN, P.M., and STAEDLER, E. 1982. Host odour perception in three phytophagous Diptera—A comparative study, pp. 95–105. *in* J.H. Visser and A.K. Minks (eds.). Proceedings, 5th International Symposium on Insect-plant Relationships. Wageningen, Pudoc, Wageningen.

GUERIN, P.M., and VISSER, J.H. 1980. Electroantennogram responses of the carrot fly, *Psila rosae* to volatile plant components. *Physiol. Entomol.* 5:111–119.

HAMILTON-KEMP, T.R., ANDERSON, R.A., RODRIGUEZ, J.G., LOUGHRIN, J.H., and PATTERSON, C.G. 1988. Strawberry foliage headspace vapor components at periods of susceptibility and resistance to *Tetranychus urticae* Koch. *J. Chem. Ecol.* 14:789–796.

HAMILTON-KEMP, T.R., RODRIQUEZ, J.G., ARCHBOLD, D.D., ANDERSEN, R.A., LOUGHRIN, J.H., PATTERSON, C.G., and LOWRY, S.R. 1989. Strawberry resistance to *Tetranychus urticae* Koch: Effects of flower, fruit and foliage removal comparisons of air-vs. nitrogen-entrained volatile compounds. *J. Chem. Ecol.* 15:1465–1473.

HANSSON, B.S., VAN DER PERS, J.N.C., and LOFQVIST, J. 1989. Comparison of male and female olfactory cell response to pheromone compounds and plant volatiles in the turnip moth, *Agrotis segetum*. *Physiol. Entomol.* 14:147–155.

HERARD, F., KELLER, M.A., and LEWIS, W.J. 1988. Rearing *Microplitis demolitor* (Wilkinson) in the laboratory for use in studies of semiochemical mediated searching behavior. *J. Entomol. Sci.* 23:105–111.

HENDRY, L.B., GREANY, P.D., and GILL, R.J. 1973. Kairomone mediated host-finding behaviour in the parasitic wasp *Orgilus lepidus*. *Entomol. Exp. Appl.* 16: 471–477.

HERNANDEZ, H.P., HSIEH, T.C-Y., SMITH, C.M., and FISCHER, N.H. 1989. Foliage volatiles of two rice cultivars. *Phytochemistry* 28:2959–2962.

HONDA, H., MARUYAMA, Y., and MATSUMOTO, Y. 1986. Comparison in EAG response to *n*-alkyl compounds between fruit- and Pinaceae-feeding type of yellow peach moth, *Conogethes punctiferalis* (Guenée) (Lepidoptera: Pyralidae). *Appl. Entomol. Zool.* 21:399–404.

JERMY, T. 1966. Feeding inhibitors and food preference in chewing phytophagous insects. *Entomol. Exp. Appl.* 9:1–12.

JERMY, T. 1976. The Host-Plant in Relation to Insect Behavior and Reproduction. Plenum Press. New York.

KHAN, Z.R., CIEPIELA, A., and NORRIS, D.M. 1987. Behavioral and physiological responses of cabbage looper, *Trichoplusia ni* (Hubner), to steam distillates from resistant versus susceptible soybean plants. *J. Chem. Ecol.* 13:1903–1915.

KOZLOWSKI, M.W., and VISSER, J.H. 1981. Host plant related properties of the antennal olfactory system in the oak flea weevil, *Rynchaenus guercus*. Electroantennogram study. *Entomol. Exp. Appl.* 30:169–175.

LANDOLT, P.J. 1989. Attraction of the cabbage looper to host plants and host plant odor in the laboratory. *Entomol. Exp. Appl.* 53:117–124.

LANDOLT, P.J., and HEATH, R.R. 1990. Sexual role reversal in mate-finding strategies of the cabbage looper moth. *Science* 249:1026–1028.

LANNE, B.S., SCHLYTER, G., BYERS, J.A., LOFQVIST, J., LEUFVEN, A., BERGSTROM, G., VAN DER PERS, J.N.C., UNELIUS, R., BAECKSTROM, P., and NORIN, T. 1987. Differences in attraction to semiochemicals present in sympatric pine shoot beetles, *Tomicus minor* and *T. piniperda*. *J. Chem. Ecol.* 13:1045–1067.

LECOMTE, C., and POUZAT, J. 1985. Responses electroantennographiques de deux parasitoides Ichneumonides, *Diadromus pulchellus* et *D. collaris*, aux odeurs de vegetaux, du phytophagehote *Acrolepiopsis assectella* et du partenaire sexuel. *Entomol. Exp. Appl.* 39:295–306.

LEWIS, W.J., and TUMLINSON, J.H. 1988. Host detection by chemically mediated associative learning in a parasitic wasp. *Nature* 331:257–259.

LIGHT, D.M., and JANG, E.B. 1987. Electroantennogram responses of the oriental fruit fly, *Dacus dorsalis*, to spectrum of alcohol and aldehyde plant volatiles. *Entomol. Exp. Appl.* 45:55–64.

LIU, S.-H., NORRIS, D.M., and MARTI, E. 1988. Behavioral responses of female adult *Trichoplusia ni* to volatiles from soybeans versus a preferred host, lima bean. *Entomol. Exp. Appl.* 49:99–109.

LIU, S.-H., NORRIS, D.M., and LYNE, P. 1989. Volatiles from the foliage of soybean, *Glycine max*, and lima bean, *Phaseolus lunatus*: Their behavioral effects on the insects *Trichoplusia ni* and *Epilachna varivestis*. *J. Agric. Food Chem.* 37:496–501.

LOKE, W.H., ASHLEY, T.R., and SAILER, R.I. 1988. Influence of fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), larvae and corn plant damage on host finding in *Apanteles marginiventris* (Hymenoptera: Braconidae). *Environ. Entomol.* 12:911–915.

LWANDE, W., MCDOWELL, P.G., AMIANI, H., and AMOKE, P. 1989. Analysis of airborne volatiles of cowpea. *Phytochemistry* 28:421–423.

MARTIN, W.R., NORDLUND, D.A., and NETTLES, W.C. 1990. Response of parasitoid *Eucelatoria bryani* to selected plant material in an olfactometer. *J. Chem. Ecol.* 16:499–508.

MCAUSLANE, H.J., VINSON, S.B., and WILLIAMS, H.J. 1990. Influence of host plant on mate location by parasitoid *Campoletis sonorensis* (Hymenoptera: Ichneumonidae). *Environ. Entomol.* 19:26–31.

METCALF, R.L. 1986. Plant volatiles as insect attractants. *Crit. Rev. Plant Sci.* 5:251–301.

NADEL, H., and VAN ALPHEN, J.J.M. 1987. The role of host-and host-plant odours in the attraction of a parasitoid, *Edidinocarsis lopezi*, to the habitat of its host, the cassava mealybug, *Phenacoccus manihoti*. *Entomol. Exp. Appl.* 45:181–186.

NAVASERO, R.C., and ELZEN, G.W. 1989. Response of *Microplitis croceipes* to host and nonhost plants of *Heliothis virescens* in a wind tunnel. *Entomol. Exp. Appl.* 53:57–63.

NORRIS, D.M. 1986. Anti-feeding compounds, pp. 97–146, *in* G. Haug and H. Hoffman (eds.). Chemistry of Plant Protection, Vol. I. Springer-Verlag, Berlin.

NORDLUND, D.A., LEWIS, W.J., and ALTIERI, M.A. 1988. Influences of plant-produced allelochemicals on the host/prey selection behavior of entomophagous insects, pp. 65–90, *in* P. Barbosa and D. Letourneau (eds.). Novel Aspects of Insect-Plant Interactions. John Wiley, New York.

ODELL, T.M., and GODWIN, P.A. 1984. Host selection by *Blepharipa pratensis* (Meigen), a tachinid parasite of the gypsy moth, *Lymantria dispar* L. *J. Chem. Ecol.* 10:311–320.

PAYNE, T.L. 1975. Bark beetle olfaction. III. Antennal olfactory responsiveness of *Dendroctonus frontalis* Zimmerman and *D. brevicomis* Le Conte (Coleoptera: Scolytidae) to aggregation pheromones and host tree terpene hydrocarbons. *J. Chem. Ecol.* 1:233–242.

PRICE, P.W. 1986. Ecological aspects of host plant resistance and biological control: Interactions among three trophics levels, pp. 11–30 *in* D.J. Boethel and R.D. Eikenbarry (eds.). Interactions of Plant Resistance and Parasitoid Predators of Insects. Ellis Horwood Limited, Chicester, England.

PRICE, P.W., BOUTON, C.E., GROSS, P., MCPHERON, B.A., THOMPSON, J.N., and WEIS, A.E. 1980. Interactions among three trophic levels: Influence of plants on interactions between insect herbivores and natural enemies. *Annu. Rev. Ecol. Syst.* 11:41–65.

RAMACHANDRAN, R., KHAN, Z.R., CABALLERO, P., and JULIANO, B.O. 1990. Olfactory sensitivity of two sympatric species of rice leaf-folders (Lepidoptera: Pyralidae) to plant volatiles. *J. Chem. Ecol.* 16:2647–2666.

REID, C.D., and LAMPAN, R.L. 1989. Olfactory responses of *Orius insidiosus* (Hemiptera: Anthocoridae) to volatiles of corn silk. *J. Chem. Ecol.* 15:1109–1115.

ROELOFS, W.L. 1984. Electroantennogram assays: Rapid and convenient screening procedures for pheromones, pp. 131–160, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, Berlin.

SAIJO, R., and TAKEO, T. 1975. Increase of *cis*-3-hexen-1-ol content in tea leaves following mechanical injury. *Phytochemistry* 14:181–182.

SATO, Y. 1979. Experimental studies on parasitization by *Apanteles glomeratus*. IV. Factors leading a female to the host. *Physiol. Entomol.* 4:63–70.

SCHNEIDER, D. 1957. Elektrophysiologische Untersuchungeu von Chemo-und Mechanorezeptoren der Antenne des Seidenspinners *Bombyx mori* L. *Z. Vergl. Physiol.* 40:8–41.

SCHOONHOVEN, L.M. 1968. Chemosensory bases of host plant selection. *Annu. Rev. Entomol.* 13:115–116.

SHEEHAN, W., and SHELTON, A.M. 1989. The role of experience in plant foraging by the aphid parasitoid *Diaeretiella rapae* (Hymenoptera: Aphidiidae). *J. Insect Behav.* 2:743–759.

SHEPPARD, M., LAWN, R.T., and SCHNEIDER, M. 1983a. Insects on grain legumes in north Australia: A survey of potential pests and their enemies. University of Queensland Press, Brisbane, Australia. 90 pp.

SHEPPARD, M., POWEL, J.E., and JONES, W.A., JR. 1983b. Biology of *Microplitis demolitor* (Hymenoptera: Braconidae) an imported parasitoid of Heliothis (Lepidoptera: Noctuidae) spp. and the soybean looper, *Pseudoplusia includens* (Lepidoptera: Noctuidae). *Environ. Entomol.* 12:641–645.

SHOREY, H.H., and HALE, R.L. 1965. Mass-rearing of the larvae of nine noctuid species on a simple artificial medium. *J. Econ. Entomol.* 58:522–524.

STAEDLER, E. 1976. Sensory aspects of insect plant interactions. Proceedings, XV International Congress on Entomology, Washington. pp. 228–248.

TOLLSTEN, L., and BERGSTRÖM, G. 1988. Headspace volatiles of whole plants and macerated plant parts of *Brassica sinapis*. *Phytochemistry* 27:4013–4018.

TURLINGS, T.C.J., TUMLINSON, J.H., and LEWIS, W.J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasp. *Science* 250:1251–1253.

VAN DER PERS, J.N.C. 1981. Comparison of electroantennogram response spectra to plant volatiles in seven species of *Fponomenta* and in the torricid *Adoxophyes Orana*. *Entomol. Exp. Appl.* 30:181–192.

VAN STRATEN, S., and MAARSE, H. 1983. Volatile compounds in food, 5th ed. Central Institute for Nutrition and food Research TNO, Zeist, The Netherlands.

VINSON, S.B. 1984. Parasite-host relationships, pp. 205–233, *in* W.J. Bell and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

VISSER, J.H. 1979. Electroantennogram responses of the Colorado beetle, *Leptinotarsa decemlineata* to plant volatiles. *Entomol. Exp. Appl.* 25:86–97.

VISSER, J.H. 1983. Differential sensory perceptions of plant compounds by insects, pp. 215–230, *in* P.A. Hedin (ed.). Plant Resistance to Insects. ACS Symposium Series 208. American Chemical Society, Washington, D.C.

VISSER, J.H. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol.* 31:121–144.

VISSER, J.H., and AVE, D.A. 1978. General green leaf volatiles in the olfactory orientation of the Colorado beetle, *Leptinotarsa decemlineata*. *Entomol. Exp. Appl.* 24:538–549.

VISSER, J.H., and THIERY, D. 1985. Behavioral responses of Colorado potato beetle to stimulation by wind and plant odors. *Bull. Agric. Exp. Stn. Univ. Mass.* 704:117–125.

VISSER, J.H., VAN STRATEN, S., and MAARSE, H. 1979. Isolation and identification of volatiles in the foliage of potato, *Solanum tuberosum*, a host plant of the Colorado beetle, *Leptinotarsa decemlineata*. *J. Chem. Ecol.* 5:13–25.

WALLBANK, B., and WHEATELY, G.A. 1976. Volatile contituents from cauliflower and other crucifers. *Phytochemistry* 15:763–766.

WHITEHEAD, A.T. 1986. Electroantennogram responses by mountain pine beetles, *Dendtroctonus ponderosae* Hopkins, exposed to selected semiochemicals. *J. Chem. Ecol.* 12:1603–1621.

WHITMAN, D.W. 1988a. Plant natural products as parasitoid cuing agents, pp. 386-396, *in* H.G. Cuttler (ed.). Biologically Active Natural Products Potential use in Agriculture. ACS Symposium Series 380. American Chemical Society, Washington, D.C.

WHITMAN, D.W. 1988b. Allelochemical interactions among plants, herbivores, and their predators, pp. 11-64, *in* P. Barbosa and D.K. Letourneau (eds.). Novel Aspects of Insect-Plant Interactions. John Wiley, New York.

WHITMAN, D.W., and ELLER, F.J. 1990. Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1:69-76.

ZAR, J.H. 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, New Jersey. 718 pp.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

BEHAVIOR OF NEONATE DIAMONDBACK MOTH LARVAE [*Plutella xylostella* (L.)] ON LEAVES AND ON EXTRACTED LEAF WAXES OF RESISTANT AND SUSCEPTIBLE CABBAGES

SANFORD D. EIGENBRODE,[1,*] KARL E. ESPELIE,[2] and ANTHONY M. SHELTON[1]

[1]*Department of Entomology*
Cornell University
New York State Agricultural Experiment Station
Geneva, New York 14456

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

(Received March 8, 1991; accepted April 22, 1991)

Abstract—Neonate *Plutella xylostella* moved more rapidly, spent more time walking, and engaged in searching behaviors more often on leaves of NY 8329, a resistant cabbage with glossy leaves, than on Round-Up, a susceptible variety with normal wax bloom. The neonates also spent significantly more time palpating and more time biting and spinning silk on the susceptible cabbage (although the latter two differences were not significant). Very similar differences in neonate behavior occurred on leaf surface wax extracts (hexane and dichloromethane) of the two cabbage genotypes. Leaf surface waxes are thus strongly implicated in eliciting reduced acceptance of the glossy cabbage by neonate *P. xylostella*. The chemical compositions of the leaf wax extracts were markedly different. Several compounds, including the triterpenols α- and β-amyrin, were found only in the glossy waxes. The percentages of some major wax constituents differed between wax extracts of the two cabbage types. These differences in wax composition may condition the plant resistance in glossy types.

Key Words—*Plutella xylostella*, Lepidoptera, Plutellidae, *Brassica oleracea*, plant epicuticular lipids, leaf surface waxes, insect movement, insect behavior, host-plant resistance.

*To whom correspondence should be addressed at: Department of Entomology, University of California, Riverside, California 92521.

0098-0331/91/0800-1691$06.50/0

INTRODUCTION

The diamondback moth, *Plutella xylostella* (L.), a serious pest of crucifer crops, is extremely difficult to control because of resistance to insecticides in many populations (Talekar and Griggs, 1986; Shelton et al., 1991). Among the alternatives to pesticides are insect-resistant crop genotypes derived from the glossy-leafed cauliflower PI 234599 (Lin et al., 1983, 1984; Eigenbrode et al., 1990; Dickson et al., 1990). These genotypes have a glossy leaf surface wax, which differs from the normal whitish wax bloom (normal bloom) of cultivated *Brassica* (Eigenbrode and Shelton, 1990).

Larval survival on some glossy genotypes of cabbage is $< 1\%$ of survival on normal bloom susceptible cabbages, and first instars are most strongly affected (Lin et al., 1983; Eigenbrode et al., 1990). Neonates move several times faster, establish fewer feeding sites, and have much higher mortality on resistant glossy-leafed plants than on susceptible plants with normal bloom. Disruption or removal of the leaf waxes of NY 8329, a resistant glossy cabbage descended from PI 234599, eliminated differences in (Dickson et al., 1984) *P. xylostella* neonate movement rates between this line and Round-Up, a susceptible cabbage variety with normal bloom (Eigenbrode and Shelton, 1990). These results suggested that glossy leaf surface waxes conditioned resistance to *P. xylostella* by eliciting reduced acceptance of the plants by neonates.

In this paper we report quantitative descriptions of the behaviors of *P. xylostella* neonates on glossy-leafed resistant NY 8329 and normal bloom susceptible Round-Up, in order to define better the host acceptance behaviors of the larvae. The role of leaf waxes in host acceptance was examined by quantifying behaviors on leaf surface wax extracts of these two cabbage genotypes. The composition of the leaf wax extracts used in these assays was determined by GC-MS analysis and chemical differences examined as the possible basis of behavioral discrimination by the larvae.

METHODS AND MATERIALS

Plants and Insects. Seeds of glossy cabbage NY 8329 were provided by M.H. Dickson (Department of Horticulture, New York State Agricultural Experiment Station) and those of a susceptible hybrid cabbage, Round-Up, were obtained from Seedway (Hall, New York). Seedlings were started in the greenhouse on April 15, 1989, and transplanted to field plots on June 16, 1989. Bioassays and extractions were performed using plants six weeks after transplant. Neonate *P. xylostella* were obtained from a laboratory colony, established in the autumn preceding the bioassay studies and maintained through the winter in the laboratory. These insects were reared on the same meridic diet used to rear larvae used in determining resistance levels of the plants.

Bioassay. Before being used in bioassays, neonate larvae were subjected to a two-stage screening procedure to reduce variability in their level of activity. Batches of approximately 1000 eggs freshly laid on cabbage extract-treated aluminum foil sheets were incubated at 28°C. Soon after the larvae hatched, they descended from the foil sheet on a silken thread. The first stage of the screening accepted only those larvae descending 3 cm to 6 cm from the sheet in a 5-min period. This group then was placed in the center of a 10-cm circle on glass, and those larvae reaching the perimeter of the circle in an additional 5 min were used in the bioassays. Thus the larvae used were neonates that had not yet fed, were < 1 hr old, and were similar in activity levels.

Larvae were placed on the test substrate (plant material or wax extract) and their movement and behaviors were quantified for 5 min. Larval movement rates, orientation, and degrees of turn per centimeter of track, as well as time and number of occurrences of specific behaviors were recorded using a computer-assisted monitoring device (Eigenbrode et al., 1989). This device permits simultaneous recording of larval location and specific behaviors observed at 30 $\times$ magnification with a dissecting microscope. Larvae were confined for observation in a 3 $\times$ 5-cm arena by a barrier of silicone grease (Dow-Corning, Corning, New York). If a larva left the arena or became entrapped in the silicone grease barrier, the observation was terminated.

Average speed was defined as the average distance traveled per minute during the entire 5-min observation. Average speed is therefore determined by the speed of walking and amount of time spent walking. This variable is identical with movement rate reported in previous studies of *P. xylostella* on resistant plants (Eigenbrode and Shelton, 1990; Eigenbrode et al., 1991). Walking speed was defined as the average distance traveled per minute, calculated only while the insect was walking. The direction of travel was calculated every 0.25 cm of path. The change in the direction of travel from the previous determination (degrees of turn) then was calculated. The accumulated degrees of turn during a 5-min observation were divided by the total path length of that observation to determine the average degrees of turn per centimeter of path (turning). The specific behaviors recorded were biting, palpating, searching, spinning silk, and walking. Biting was defined as contraction of the mandibular muscles, visible through the larva's cuticle, while the mouthparts were in contact with the substrate. Palpating was defined as touching the mouthparts repeatedly to the substrate but not biting. Searching was defined as raising the front half of the body from the substrate and moving it from side to side. Spinning silk was defined as the deliberate side to side movement of the head while spinning a silken strand and anchoring the strand at the extremes of this movement. Walking was defined as forward movement by the larva.

Test Substrates. Tests were performed on fresh leaves or on leaf surface wax extracts deposited on glass. Leaves were cut from plants in the field and

the petioles were immediately placed in water-filled tubes. The leaves were brought to the lab within 15 min, and observations were made on the underside of the leaves [the leaf surface preferred by larvae in the field (personal observations; Salinas, 1984)]. Leaves selected for study were the sixth to eighth fully expanded leaves on the plant. Each leaf was used for recording for no more than 1 hr (approximately five larvae could be recorded during this time period).

Waxes were removed from the leaf surfaces by washing the leaves in either hexane or dichloromethane. Approximately 30 leaves (sixth to eighth fully expanded) of each of the two genotypes were washed for 10 sec in three consecutive baths of the respective solvent. The three washings were combined, dried over sodium sulfite, and filtered. The amount of wax in each pooled extract was determined by evaporating the samples and weighing the residues. The amount of wax/cm^2 of leaf surface of each cabbage genotype was calculated, using the combined area of the leaves used to prepare the extract. Volumes of extract were applied to clean glass slides and evaporated so that a film of waxes was deposited on the slide. The amount of extract applied was adjusted to produce a wax deposit of approximately 60 μg/cm^2, which is the wax load on the susceptible cultivar, Round-Up. Preliminary studies with different wax loads indicated that this was the best procedure to detect larval discrimination. Leaf waxes deposited on slides did not differ substantially between the two genotypes in morphology, as determined by SEM, unlike the waxes on intact leaves, which have extremely different crystal structures (Eigenbrode et al., 1991). The assays thus detect differences in larval response to wax qualities, independent of amount or morphology of wax on the leaves of the two cabbage genotypes. The wax coated slides were held at -20°C until they were used in the bioassays. Barriers were constructed and larval behaviors were recorded as on intact leaves.

Design of Tests and Analysis. For the fresh leaf tests, 10 leaves of NY 8329 and Round-Up were used and a total of 45 and 52 larvae were recorded on each of the two lines. The recordings took place over a four-day period using larvae from two consecutive cohorts of larvae. The cabbage genotype was alternated hourly during the recording.

The test design was similar on deposited wax extracts. For each solvent $\times$ genotype combination, 30 larvae were observed, five on each of six replicate slides. The treatments were alternated and the replicate slides were used in rotation during recording. The tests for each solvent were conducted and analyzed as separate experiments comparing cabbage varieties, and each experiment used larvae from a single cohort and was conducted over a three-day period.

The behavioral data described above were determined for each larva in the tests. Differences in the mean values of each behavior or category on the leaves or waxes of the two cabbage genotypes were evaluated with Student's t test.

GC-MS Analysis. Extracts were treated with *N,O*-bis(trimethylsilyl)-

acetamide at 110°C for 10 min. Samples were dried under a stream of N_2 and resuspended in hexane. Aliquots were analyzed by combined gas chromatography–mass spectrometry (Hewlett Packard 5890A/5970). The capillary column (25-m cross-linked methyl silicone) was held at 55°C for 3 min after injection (splitless), and the oven temperature was then raised to 305°C at a rate of 15°C/min and held at this temperature for 20 min. Individual peaks were identified by their mass spectra (Holloway et al., 1976; Heupel, 1985; Espelie and Bernays, 1989), which were recorded at 70 eV at intervals of 0.8 sec. Quantitation was based upon the integration of total ion chromatograms.

The trimethylsilyl ether derivatives of standard samples of α-amyrin (K&K Chemicals) and β-amyrin (Pfaltz & Bauer) were found to have retention times and mass spectra identical to those of the components recovered from NY 8329 cabbage leaves, and the mass spectra were matched by computer search with the National Bureau of Standards Mass Spectral Library.

RESULTS

Behaviors of neonate *P. xylostella* differed on fresh leaves of the two cabbage genotypes (Table 1). The larvae spent significantly more time walking, walked more frequently, and walked significantly faster on leaves of NY 8329 than on leaves of susceptible Round-Up. As a result, average speed and walking speed were both significantly greater on the glossy genotype. Neonates also accumulated more degrees of turn per centimeter of path length on Round-Up than on NY 8329, and this difference was marginally significant. The larvae searched significantly more often on resistant leaves and spent more time searching (although the latter difference is not significant at $\alpha = 0.05$). The larvae also spent less time palpating resistant leaves and palpated them less often than susceptible leaves (although the latter difference is not significant at $\alpha = 0.05$). Larvae spent less time biting and spinning and engaged in these two behaviors less often on the resistant leaves than on susceptible leaves (although these differences are also not significant at $\alpha = 0.05$).

Larval behaviors also differed on the extracted leaf surface waxes of the two cabbage genotypes (Table 1). Relative responses of the larvae to waxes of the two genotypes were very similar to the responses to intact leaves from these genotypes. The cabbage genotype eliciting the greater response (occurrences or time) in the intact leaf bioassay almost always also elicited a greater response in the bioassay on the wax extracts. Exceptions were palpations (on both wax extracts) and searching (on hexane extracts), in which there were no differences between the two genotypes. However, the size of the behavioral differences and statistical separations were not as great on leaf wax extracts as on fresh leaves. Statistical separation was greatest on leaves, less on hexane-extracted waxes,

TABLE 1. NEONATE *P. xylostella* BEHAVIORS ON INTACT LEAVES AND LEAF SURFACE WAX EXTRACTS OF NY 8329 AND ROUND-UP CABBAGE GENOTYPES.

Behavior	On Intact Leaf Surface			On Leaf Waves Extracted with Hexane			On Leaf Waxes Extracted with Dichloromethane		
	Round-up	NY 8329	P^a	Round-up	NY 8329	P	Round-up	NY 8329	P
Average time in seconds spent in each behavior (±SEM)									
Searching	45.55 ± 4.00	50.23 ± 2.74	0.3359	20.78 ± 2.92	20.28 ± 2.20	0.9146	14.42 ± 2.24	20.47 ± 2.62	0.1975
Walking	178.4 ± 10.32	214.9 ± 5.69	0.0032	154.43 ± 13.23	196.11 ± 9.68	0.0200	180.56 ± 12.08	200.13 ± 8.92	0.2899
Biting	10.57 ± 4.09	3.73 ± 1.42	0.1205	48.01 ± 8.85	18.66 ± 3.44	0.0001	35.18 ± 6.83	28.03 ± 5.18	0.3104
Palpating	44.69 ± 6.08	21.95 ± 3.02	0.0011	37.15 ± 3.66	28.92 ± 3.04	0.2853	37.25 ± 3.43	29.10 ± 2.76	0.0725
Spinning	9.61 ± 2.63	5.83 ± 1.87	0.2442	29.56 ± 6.70	15.79 ± 4.29	0.0658	27.19 ± 6.50	15.45 ± 3.89	0.1163
Average number of occurrences of each behavior (±SEM)									
Searches	8.73 ± 0.69	15.42 ± 0.66	0.0001	11.53 ± 1.51	13.06 ± 1.21	0.4939	8.80 ± 1.17	13.97 ± 1.50	0.0217
Walks	12.54 ± 0.63	17.62 ± 0.62	0.0001	23.53 ± 2.04	27.93 ± 1.56	0.2352	21.50 ± 1.72	27.37 ± 1.82	0.0315
Bites	0.82 ± 0.26	0.40 ± 0.13	0.1592	7.60 ± 1.00	5.66 ± 0.92	0.0630	6.07 ± 0.93	4.43 ± 0.58	0.1160
Palpations	5.38 ± 0.57	4.03 ± 0.42	0.0602	16.93 ± 1.21	17.33 ± 1.45	0.8250	17.23 ± 1.11	17.67 ± 1.30	0.8107
Spins	1.30 ± 0.28	0.89 ± 0.27	0.3005	6.33 ± 1.04	3.80 ± 0.91	0.0234	4.07 ± 0.68	3.40 ± 0.68	0.5493
Average speed and walking speed (cm/min) and turning (degrees/cm of path ±SEM)									
Average speed	0.54 ± 0.04	1.09 ± 0.06	0.0001	2.83 ± 0.38	3.98 ± 0.38	0.0323	2.92 ± 0.32	4.15 ± 0.45	0.0224
Walking speed	0.88 ± 0.05	1.49 ± 0.06	0.0001	5.37 ± 0.56	5.67 ± 0.51	0.6789	4.98 ± 0.46	5.93 ± 0.54	0.1860
Turning	384.6 ± 33.3	311.5 ± 16.3	0.0521	532.39 ± 95.33	440.52 ± 47.04	0.0523	483.04 ± 54.23	445.54 ± 68.57	0.1885
N	52	45		30	30		30	30	

[a] P = probability of a greater value of Student's t.

and least on dichloromethane-extracted waxes, both in the number of differences significant at $\alpha = 0.05$ (6 vs. 4 vs. 3) and the size of the P values in general. One exception to this trend was biting; on hexane extracts, biting was much greater on Round-Up relative to NY 8329 than on whole plants.

Average speed, but not walking speed, was significantly greater on waxes from resistant NY 8329 than on waxes from susceptible Round-Up. Average speed and walking speed are both more than four times greater on waxes than on leaves of both genotypes (average speed = 3.47 cm/min on waxes vs. 0.82 on leaves; walking speed = 5.46 cm/min on waxes vs. 1.19 on leaves). It is possible that this difference is due to the differences in the larval cohorts used in these experiments. This is unlikely, however, since variation between cohorts in other behaviors is minimal. The mean values for all other behavioral statistics are comparable on fresh leaves and wax extracts.

The wax extracts from the two genotypes were shown by GC-MS to be very different chemically (Table 2). The epicuticular lipids recovered from leaves of Round-Up were dominated by a few components: *n*-nonacosane, 14- and 15-nonacosanol, 15-nonacosanone, and *n*-hentriacontane. These compounds comprised 91% of the surface lipids in the dichloromethane extract of Round-Up leaves, but only 13% of the surface lipids in the dichloromethane extract of NY 8329 leaves (Figure 1). Primary fatty alcohols were the major components (37% in the dichloromethane extract and 45% in the hexane extract) of the NY 8329 leaf wax, with hexacosanol the dominant homolog (Table 3). The NY 8329 leaves had a much lower proportion of *n*-alkanes than did the Round-Up leaves, and within this class of compounds the NY 8329 surface lipids had a shorter average chain length (more C_{25} and C_{27}; no detectable C_{31}). The secondary alcohols, 13- and 14-heptacosanol, were found only in the extracts of NY 8329 leaves, while the secondary diol, 14,15-nonacosandiol, was more prominent in the Round-Up leaf wax extracts (Table 2). The triterpenols, α- and β-amyrin, were detected only in the surface lipids of the NY 8329 leaves.

The hexane and dichloromethane extracts of Round-Up leaves were similar in chemical composition. There were greater differences between the two extracts of NY 8329 leaves. The dichloromethane extracts had a larger proportion of fatty acids (10%) than did the hexane extracts (2%) and hexacosanoic acid was found only in the dichloromethane extract (Table 2). There was also a greater amount of triterpenols in the dichloromethane extract (9%) of NY 8329 leaves than in the hexane extract (3%).

DISCUSSION

The differences in neonate *P. xylostella* behavior on intact leaves of resistant glossy NY 8329 and susceptible normal bloom Round-Up cabbage support the hypothesis that larvae reject the resistant glossy plants. Increased larval

TABLE 2. COMPOSITION (%) OF CUTICULAR LIPIDS RECOVERED BY HEXANE OR DICHLOROMETHANE EXTRACTON OF ROUNDUP AND NY 8329 CABBAGE LEAVES

Peak	Component	Hexane		Dichloromethane	
		Round-up	NY 8329	Round-up	NY 8329
1	Tetradecanoic acid		0.5	0.1	1.1
2	Pentadecanoic acid				0.5
3	Hexadecanoic acid	0.3	0.9	0.2	3.4
4	Octadecenoic acid		0.2		0.8
5	Octadecanoic acid		0.3		2.1
6	*n*-Pentacosane		1.1		2.6
7	Docosanol		0.8		1.9
8	*n*-Heptacosane	3.0	5.9	0.9	4.7
9	Tetracosanol		10.7	0.2	4.3
10	Pentacosanol		5.9		3.8
11	13- and 14-Heptacosanol		3.5		5.9
12	*n*-Nonacosane	27.4	3.7	38.3	4.7
13	Hexacosanol	1.6	21.4	1.5	19.2
14	Heptacosanol	1.2	5.3	1.2	6.7
15	Hexacosanoic acid				1.7
16	14- and 15-Nonacosanol	14.7	2.4	19.4	3.7
17	15-Nonacosanone[a]	15.9	3.0	21.1	4.8
18	*n*-Hentriacontane	7.7		12.1	
19	Octacosanol		0.8		0.8
20	14, 15-Nonacosandiol	4.1		2.8	0.3
21	Triacontanol	1.5		0.8	0.4
22	β-Amyrin		0.8		2.5
23	α-Amyrin		2.0		6.2

[a] Elutes with 14- and 15-nonacosanol; estimated by integration of selected ion chromatograms.

movement rates, which had been interpreted as indicators of larval nonacceptance of glossy resistant *Brassica oleracea* (Eigenbrode and Shelton, 1990; Eigenbrode et al., 1991), are shown here to be associated with increased searching and reduced biting and silk spinning on these plants, as compared with the susceptible normal bloom type.

The epicuticular lipid composition found for the leaves of Round-Up was similar to that previously reported for *Brassica oleracea* (Netting et al., 1972; Baker, 1974). In each class of components, C_{29} was consistently the dominant homolog. NY 8329 lipids were not dominated by the C_{29} homologs and had a more diverse composition. The higher percentage of free fatty acids and primary alcohols and lower percentage in alkanes, secondary alcohols, and ketones in

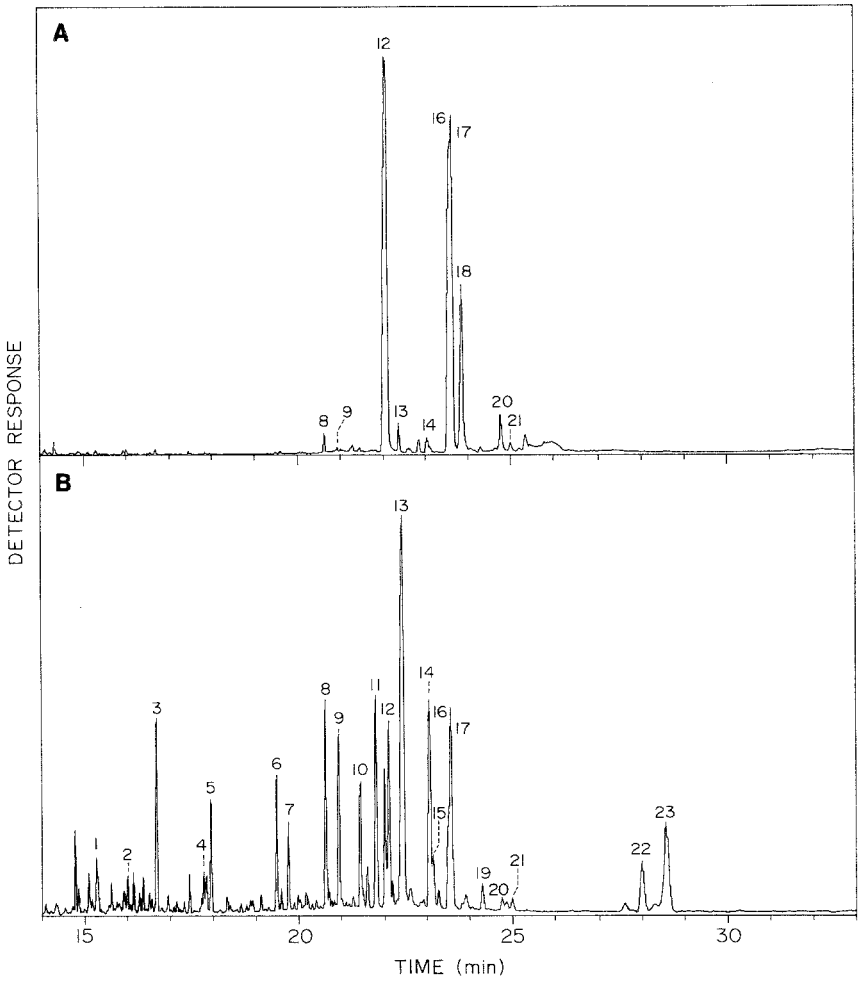


FIG. 1. Total ion chromatograms of the derivitized lipids recovered from the leaves of Round-Up (A) and NY 8329 (B) cabbage by dichloromethane extraction. Numbered peaks are identified in Table 2.

NY 8329 epicuticular lipids, as compared with the lipids of normal genotypes, resembles previously studied glossy mutants of *Brassica oleracea* (Baker, 1974).

The similarity of neonate *P. xylostella* behaviors on leaf wax extracts and intact leaves of NY 8329 and Round-Up implicate the leaf surface waxes in eliciting reduced acceptance of intact glossy plants. The substantial chemical differences between the wax extracts evidently produced the observed behavioral differences. It appears likely that discrimination by the larvae is a response

TABLE 3. CUTICULAR LIPID COMPOSITION (%) OF ROUNDUP AND NY 8329 CABBAGE LEAVES

Class of component	Hexane		Dichlormethane	
	Round-up	NY 8329	Round-up	NY 8329
n-Alkanes	38.1	10.7	51.3	12.0
Primary alcohols	4.3	44.9	3.7	37.1
Secondary alcohols	18.8	5.9	22.2	9.9
Ketones	15.9	3.0	21.1	4.8
Free fatty acids	0.3	1.9	0.3	9.6
Triterpenols	0.0	2.8	0.0	8.7

to a combination of stimulants and deterrents. Waxes from resistant and susceptible plants stimulate more biting and spinning than untreated glass or paraffin-treated glass substrates (unpublished data). In the experiments we report here, all larvae did some biting or spinning on resistant waxes and some searching on susceptible waxes. Those compounds comprising a lower percentage of the epicuticular waxes of NY 8329 than of Round-Up (Table 2, peaks 12, 16, 17, 20, and 21) are candidates as feeding stimulants or arrestants. This group contains the C_{29} *n*-alkane, alcohols, ketone, and a diol, as well as the C_{30} alcohol. Those components that comprise a higher percentage of (or are only present in) NY 8329 waxes (Table 2, peaks 1–11, 13–15, 22, and 23) may be deterrents. This group contains the free fatty acids, all the *n*-alkanes and fatty alcohols with C number <29, and α- and β-amyrin. Of particular interest are α- and β-amyrin, which together comprise 9% of the cuticular lipids of the NY 8329 leaves (Table 2) and had been found previously in the leaf wax of *Brassica napus* (Holloway et al., 1977). The triterpenols comprised only 0.4% of the total cuticular lipids in *B. napus*, but the α- and β-amyrin were found in the same ratio (2.5 : 1) as they were in the leaves of NY 8329. The palmitate ester of α-amyrin has been shown to inhibit growth of several lepidopteran species (Shankaranarayana et al., 1980). The structural similarity of α- and β-amyrin (which have the ursane and oleanane ring skeletons, respectively) to sterols may cause them to be toxic to some phytogphagous insects, and the presence of these compounds in the cuticle of NY 8329 leaves may play a role in the observed resistance to diamondback moth.

Hexane extracts of the cabbage leaves elicited greater behavioral differences with better statistical separation than did the dichloromethane extracts. Discrimination on the basis of biting was more clear between hexane extracts than intact plants. This suggests that the nonpolar constituents of the leaf waxes,

which are more dominant in the hexane extract, may be important in eliciting the behavioral differences. Those possible deterrents in higher relative concentrations in hexane extracts than in dichloromethane extracts (Table 2, peaks 1, 9, 10, and 13; tetradecanoic acid, tetracosanol, pentacosanol, and hexacosanol) and those possible stimulants in lower relative concentrations in hexane extracts (Table 2, peaks 16, 17, 20, 21; 14- and 15-nonacosanol, 15-nonacosanone, 14,15-nonacosandiol, and triacontanol) could be important allelochemics in the leaf waxes.

Although the physiological mechanisms by which insects detect nonpolar compounds with low volatility remain obscure, there is sufficient evidence that leaf surface wax components act as allelochemics. Plant surface wax extracts (Blaney and Chapman, 1970; Bernays et al., 1976, 1985; Albert and Parisella, 1983; Woodhead, 1983, 1987; Maloney et al., 1988; Woodhead and Padgham, 1988; Varela and Bernays, 1988; Chapman and Bernays, 1989), and specific compounds typically found in leaf waxes, including long chain alkanes (Klingauf et al., 1971, 1978), alcohols (Nayar and Fraenkel, 1962; Mori, 1982) esters (Woodhead, 1983; McKibben et al., 1985), and carboxylic acids (Greenway et al., 1978), can act as allelochemics affecting insect herbivore behavior. Thus, it is not unreasonable to conclude that specific compounds in *Brassica* waxes influence *P. xylostella* larval behaviors. Additional bioassays with chromatographic fractions of the crude wax extracts and with pure samples of the specific compounds listed above should determine the most active components.

The ability of *P. xylostella* larvae to discriminate between host-plant genotypes and leaf waxes is innate; the animals in these tests were neonates with no experience with potential food sources. In only a few other cases has innate discrimination on the basis of leaf surface lipids been demonstrated (Mori, 1982; Bernays et al., 1985; Varela and Bernays, 1988). In certain ecological settings, innate neonate discrimination at the leaf surface may permit larvae to locate the most suitable host plants, or host-plant tissues, before beginning to feed. Discrimination by neonates therefore can be adaptive and possibly occurs in many herbivores. However, increased searching and walking and reduced feeding behaviors on glossy cabbages in an agricultural monoculture apparently prevent timely establishment by *P. xylostella* larvae and result in the observed higher mortality on these plants.

The differences between neonate behaviors on the surface wax extracts of NY 8329 and Round-Up in this study were less distinct statistically than those on intact leaves of the resistant and susceptible cabbages. On intact leaves, the larvae apparently respond to other factors in addition to leaf wax composition. Our previous work has indicated that physical characteristics of the leaf surface waxes affect larval behavior and survival. Intact leaves of glossy resistant plants have reduced densities of wax crystallites and reduced amounts of wax/cm^2 (wax load) on their leaf surfaces, as compared with susceptible *Brassica* geno-

types (Eigenbrode et al., 1991). Crystallite density and wax load explained 69% of the variation in larval survival on a collection of 18 *Brassica* genotypes.

This statistical relationship between *P. xylostella* survival and movement and leaf wax physical characteristics may, however, only indicate a behavioral response to wax chemistry. This is possible because the morphology of leaf surface waxes on *Brassica* is largely determined by leaf wax chemistry (Jeffree et al., 1975; Holloway et al., 1977; Jeffree, 1986). Our data suggest, however, that chemical and physical attributes of leaf waxes act together, possibly with other leaf characteristics, to influence neonate *P. xylostella* behaviors. Differences in larval behavior occurred on pure wax extracts with identical morphology but were more pronounced on leaves with morphologically intact waxes. The much higher movement rates of larvae on wax extracts than on intact leaves, and the smaller differences in walking speed on extracts as compared with intact leaves (Table 1), suggest that wax morphology has particularly strong effects on larval locomotion.

This system represents one of the best examples of surface wax-mediated host-plant suitability, since survival of *P. xylostella* on glossy cabbages descended from PI 234599 is so much less than survival on normal bloom genotypes ($< 1\%$). It is therefore important to elucidate the mechanisms of this interaction. Better understanding of reduced acceptance and survival of *P. xylostella* on glossy resistant *Brassica* also will improve screening procedures for the development of cultivars resistant to this important pest.

Acknowledgments—We thank Wendy C. Kain for assistance with behavioral monitoring, and Glen W. Chapman from the Richard B. Russell Research Center for generous assistance in this project. We also thank John T. Trumble and Gregory P. Walker for critical reviews of drafts of the manuscript. This research was partly supported by the New York State Cabbage Growers Association, the Cornell Biotechnology Program, and by HATCH project No. 610 allocated to the Georgia Agricultural Experiment Station.

REFERENCES

ALBERT, P.J., and PARISELLA, S. 1983. Chemical bases of host-plant selection by eastern spruce budworm (*Choristoneura fumiferana* Clem.) (Lepidoptera: Tortricidae). Proceedings, Forest Defoliator-Host Interactions: A Comparison Between Gypsy Moth and Spruce Budworm, USDA Forest Service, General Technical Report NE-85 9-14.

BAKER, E.A. 1974. The influence of environment on leaf wax development in *Brassica oleracea* var. *gemmifera*. *New Phytol.* 73:955–966.

BERNAYS, E.A., BLANEY, W.M., CHAPMAN, R.F., and COOK, A.G. 1976. The ability of *Locusta migratoria* L. to perceive plant surface waxes, pp. 35–40, *in* T. Jermy and A. Szentesi (eds.). The Host-Plant in Relation to Insect Behavior and Reproduction. Symposia Biologica Hungarica, Budapest.

BERNAYS, E.A., WOODHEAD, S., and HAINES, L. 1985. Climbing by newly hatched larvae of the

spotted stalk borer *Chilo partellus* to the top of sorghum plants. *Entomol. Exp. Appl.* 39:73–79.

BLANEY, W.M., and CHAPMAN, R.F. 1970. The function of the maxillary palps of Acrididae. *Entomol. Exp. Appl.* 13:363–376.

CHAPMAN, R.F., and BERNAYS, E.A. 1989. Insect behavior at the leaf surface and learning as aspects of host plant selection. *Experientia* 45:215–222.

DICKSON, M.H., and ECKENRODE, C.J. 1980. Breeding for resistance in cabbage and cauliflower to cabbage looper, imported cabbageworm and diamondback moth. *J. Am. Soc. Hortic. Sci.* 105:782–785.

DICKSON, M.H., ECKENRODE, C.J., and BLAMBLE, A.E. 1984. NYIR 9602, NYIR 9605, and NYIR 8329, lepidopterous pest resistant cabbages. *HortSci.* 19:311–312.

DICKSON, M.H., SHELTON, A.M., EIGENBRODE, S.D., VAMOSY, M.L., and MORA, M. 1990. Selection for resistance to diamondback moth (*Plutella xylostella*) in cabbage. *HortSci.* 25:1643–1646.

EIGENBRODE, S.D., and SHELTON, A.M. 1990. Behavior of neonate diamondback moth larvae (Lepidoptera: Plutellidae) on glossy-leaved resistant genotypes of *Brassica oleracea*. *Environ. Entomol.* 19:566–1571.

EIGENBRODE, S.D., BARNARD, J., and SHELTON, A.M. 1989. A system for quantifying behavior of neonate caterpillars and other small, slow-moving animals. *Can. Entomol.* 121:1125–1126.

EIGENBRODE, S.D., SHELTON, A.M., and DICKSON, M.H. 1990. Two types of resistance to the diamondback moth (Lepidoptera: Plutellidae) in cabbage. *Environ. Entomol.* 19:1086–1090.

EIGENBRODE, S.D., STONER, K.A., SHELTON, A.M., and KAIN, W.C. 1991. Role of leaf waxes in resistance to diamondback moth larvae in glossy-leaved *Brassica oleracea*. *J. Econ. Entomol.* In press.

ESPELIE, K.E., and BERNAYS, E.A. 1989. Diet-related differences in the cuticular lipids of *Manduca sexta* larvae. *J. Chem. Ecol.* 15:2003–2017.

GREENWAY, A.C., GRIFFITHS, D.C., and LLOYD, S.L. 1978. Response of *Myzus persicae* to components of aphid extracts and to carboxylic acids. *Entomol. Exp. Appl.* 24:369–374.

HEUPEL, R.C. 1985. Varietal similarities and differences in the polycyclic isopentenoid composition of sorghum. *Phytochemistry* 24:2929–2937.

HOLLOWAY, P.J., JEFFREE, C.E., and BAKER, E.A. 1976. Structural determination of secondary alcohols from plant epicuticular waxes. *Phytochemistry* 15:1768–1770.

HOLLOWAY, P.J., BROWN, G.A., BAKER, E.A., and MACEY, M.J.K. 1977. Chemical composition and ultrastructure of the epicuticular wax in three lines of *Brassica napus* (L.). *Chem. Phys. Lipids* 19:114–127.

JEFFREE, C.E. 1986. The cuticle, epicuticular waxes and trichomes of plants, with reference to their structure, functions and evolution, pp. 23–64, *in* B.E. Juniper and T.R.E. Southwood (eds.). Insects and the Plant Surface. Edward Arnold, London.

JEFFREE, C.E., BAKER, E.A., and HOLLOWAY, P.J. 1975. Ultrastructure and recrystallization of plant epicuticular waxes. *New Phytol.* 75:539–549.

KLINGAUF, F., NÖCKER-WENZEL, K., and KLEIN, W. 1971. Einfluss einiger Wachskomponenten von *Vicia faba* L. auf das Wirtswahlverhalten von *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae). *Z. Pflanzenkrank. Pflanzensch.* 78:641–648.

KLINGAUF, F., NÖCKER -WENZEL, K., and RÖTTGER, U. 1978. Die Rolle peripherer Phlanzenwachse fr den Befall durch phytophage Insekten. *Z. Pflanzenkrank. Pflanzensch.* 85:228–237.

LIN, J., ECKENRODE, C.J., and DICKSON, M.H. 1983. Variation in *Brassica oleracea* resistance to diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* 76: 1423–1427.

LIN, J., DICKSON, M.H., and ECKENRODE, C.J. 1984. Resistance of *Brassica* lines to the diamondback moth (Lepidoptera: Yponomeutidae) in the field, and inheritance of resistance. *J. Econ. Entomol.* 77:1293–1296.

MALONEY, P.J., ALBERT, P.J., and TULLOCH, A.P. 1988. Influence of epicuticular waxes from white spruce and balsam fir on feeding behavior of the eastern spruce budworm. *J. Insect Behav.* 1:197–208.

MCKIBBEN, G.H., THOMPSON, M.J., PARROTT, W.L., THOMPSON, A.C., and LUSBY, W.R. 1985. Identification of feeding stimulants for boll weevils *Anthonomus grandis* from cotton buds and anthers. *J. Chem. Ecol.* 11:1229–1238.

MORI, M. 1982. *n*-Hexacosanol and *n*-octacosonol; feeding stimulants for larvae of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 28:969–973.

NAYAR, J.K., and FRAENKEL, G. 1962. The chemical basis of hostplant selection in the silkworm, *Bombyx mori* (L.). *J. Insect Physiol.* 8:505–525.

NETTING, A.G., MACEY, M.J.K., and BARBER, H.N. 1972. Chemical genetics of a subglaucous mutant of *Brassica oleracea*. *Phytochemistry* 11:579–585.

SALINAS, P.J. 1984. Studies on the behavior of the larvae of *Plutella xylostella* (Linnaeus)(Lepidoptera: Plutellidae), a world pest of cruciferous crops: Normal and spacing behavior. *Turrialba* 34:77–84.

SHANKARANARAYANA, K.H., AYYAR, K.S., and KRISHNA RAO, G.S. 1980. Insect growth inhibitor from the bark of *Santalum album*. *Phytochemistry* 19:1239–1240.

SHELTON, A.M., WYMAN, J.A., CUSHING, N.L., APFELBECK, K., DENNEHY, T.J., MAHR, S.E.R., and EIGENBRODE, S.D. 1991. Resistance of diamondback moth to insecticides in North America. *J. Econ. Entomol.* In press.

TALEKAR, N.S., and GRIGGS, T.D. (eds.). 1986. Diamondback moth management. Proceedings of the First International Workshop, Tainan, Taiwan. AVRDC, Shanhua.

VARELA, L., and BERNAYS, E.A. 1988. Behavior of newly hatched potato tuber moth larvae, *Phthorimaea operuclella* Zell. (Lepidoptera: Gelechiidae), in relation to their host plants. *J. Insect Behav.* 1:261–275.

WOODHEAD, S. 1983. Surface chemistry of *Sorghum bicolor* and its importance in feeding by *Locusta migratoria*. *Physiol. Entomol.* 8:345–352.

WOODHEAD, S. 1987. The influence of surface chemicals of sorghum on the behavior of the stemborer *Chilo partellus* (Swinhoe), p. 425, *in* V. Labeyrie, G. Fabres, and D. Lachaise (eds.). Insects–Plants: Proceedings, 6th International Symposium on Insect–Plant Relationships. Junk, Dordrecht.

WOODHEAD, S., and PADGHAM, D.E. 1988. The effect of plant surface characteristics on resistance of rice to the brown planthopper, *Nilaparvata lugens*. *Entomol. Exp. Appl.* 47:15–22.

Journal of Chemical Ecology, Vol. 17, No. 8, 1991

VARIATION IN SEMIOCHEMICAL-MEDIATED PREY–PREDATOR INTERACTION: *Ips pini* (Scolytidae) AND *Thanasimus dubius* (Cleridae)[4]

DANIEL A. HERMS,[1,*] ROBERT A. HAACK,[2] and BRUCE D. AYRES[3]

[1]*The Dow Gardens*
1018 W. Main St.,
Midland, Michigan 48640

[2]*USDA Forest Service, North Central Forest Experiment Station*
1407 S. Harrison Rd., East Lansing, Michigan 48823

[3]*Woods Run Forest Products*
Box 520, Colfax, Wisconsin 54730

(Received August 17, 1990, accepted October 29, 1990)

Abstract—The bark beetle *Ips pini* (Say) displays variation in its response to and production of enantiomeric blends of its pheromone ipsdienol. One of the principal predators of *Ips pini* is *Thanasimus dubius* (F.), which uses ipsdienol as a kairomone for prey location. During 1988 and 1989, in Wisconsin and Michigan, the response of both species to a range of enantiomeric blends of ipsdienol was investigated. Blends tested had the following ratios of the (*S*)-(+) to (*R*)-(−) enantiomers: 3%:97%, 25%:75%, 50%:50%, 75%:25%, and 97%:3%. Either 75% (+):25% (−) or 50% (+):50% (−) ipsdienol captured the most *Ips pini* in both years at both sites. The 25% (+):75% (−) blend also caught more *Ips pini* than the control during both years at both sites. All blends tested were attractive to *Thanasimus dubius* in both years at both locations. Blend preferences of both species were variable and labile at both sites. Response patterns of both species in Wisconsin were different from those in Michigan each year. Furthermore, response patterns of both species to the ipsdienol blends changed from 1988 to 1989 at both locations. A genetic component to this variation would permit predator-prey coevolution, as well as the development of resistance by *Ips pini* to management strategies based on mass-trapping with single blends.

*To whom correspondence should be addressed.

[4]Editor's Note: This paper originally appeared in *Journal of Chemical Ecology*, Volume 17, Number 3 (March 1991), pp. 515–524. Due to several significant printer's errors, the paper is reprinted here in entirety in its corrected form.

0098-0331/91/0800-1705$06.50/0

Key Words—*Ips pini*, *Thanasimus dubius*, Coleoptera, Scolytidae, Cleridae, bark beetle, ipsdienol, aggregation pheromone, kairomone, coevolution, mass-trapping, intraspecific variation, prey-predator interaction.

INTRODUCTION

Bark beetles (Coleoptera: Scolytidae) are the most destructive insect pests of North American forests. The pine engraver, *Ips pini* (Say), colonizes pine and spruce throughout much of North America (Lanier et al., 1972; Birch et al., 1980; Wood, 1982) and is the most common bark beetle in the Great Lakes region (Schenk and Benjamin, 1969). Adults and larvae feed on the phloem of weakened and recently killed trees. Upon colonization of a suitable host, adult males emit a pheromone containing primarily ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol), which attracts other males and females to the tree (Vité et al., 1972; Birch, 1978; Birch et al., 1980; Borden, 1982; Miller et al., 1989).

Ipsdienol occurs as (*R*)-(−) and (*S*)-(+) enantiomers, the relative proportion of which determines response specificity in *Ips pini*. There is geographic variation among North American populations of *Ips pini* in their response to and production of pheromones (Lanier et al., 1972; Birch, 1978; Miller et al., 1989). Western North American populations respond to 97% (−)-and are inhibited by (+)-ipsdienol (Birch et al., 1980). New York populations respond preferentially to a racemic mixture, i.e., 50% (+):50% (−) (Lanier et al., 1980). In Wisconsin, traps baited with 75% (+):25% (−) or 50% (+):50% (−)-ipsdienol caught significantly more beetles than 100% (−), 25% (+), or 100% (+) (Raffa and Klepzig, 1989). The 75% (+)-ipsdienol caught more than three times the number of beetles than did the racemic mixture during a two-week test, but differences were not significant.

Thanasimus dubius (F.) (Coleoptera: Cleridae) is a principal predator of *Ips pini* in the Great Lakes region (Schenk and Benjamin, 1969). *Thanasimus dubius* and other *Thanasimus* species use bark beetle pheromones as kairomones for prey location (Vité and Williamson, 1970; Bakke and Kvamme, 1980; Mizell et al., 1982; Hansen, 1983; Payne et al., 1984; Raffa and Klepzig, 1989). Enantiomeric specificity in their responses (Hansen, 1983; Payne et al., 1984; Raffa and Klepzig, 1989) is considered evidence that clerid predators have coevolved with their scolytid prey (Payne et al., 1984; Raffa and Klepzig, 1989). For example, predation may select for changes in the pheromone system of *Ips pini*, allowing temporary escape from its natural enemy. *Thanasimus dubius* then could be under strong selection to track these changes, permitting location of its primary prey (e.g., Price, 1981). Coevolution, however, requires heritable variation in the selected traits within populations of both species (Janzen, 1980). Miller et al. (1989) concluded that intrapopulation variation in the pro-

duction of ipsdienol enantiomeric blends by *Ips pini* most likely had a genetic basis; however, this variation has not been shown to be heritable. Kairomonal response preferences by *Thanasimus dubius* to *Ips* and *Dendroctonus* pheromones have been observed to vary from year to year, depending on the relative abundances of the different species of prey (Billings and Cameron, 1984).

Bark beetle suppression through mass-trapping, using aggregation pheromones as bait, is receiving much attention, but requires detailed knowledge of the pheromone preferences of target populations (Bakke, 1982, 1989). A high level of genetic variation within populations of *Ips pini* would create the potential for rapid development of "resistance" to pheromone-based programs that rely on only one pheromone blend (Lanier et al., 1972; Slessor et al., 1985; Borden et al., 1986; Miller et al., 1989).

The objectives of this study were: (1) to further characterize the preferences of *Ips pini* and *Thanasimus dubius* to enantiomeric blends of ipsdienol in the Great Lakes region, (2) to determine the degree of intrapopulational variation in the response of both species to the ipsdienol mixtures, and (3) to compare preferences between populations of the two species within the Great Lakes Region.

METHODS AND MATERIALS

Blend Preferences. The response of *Ips pini* and *Thanasimus dubius* populations to enantiomeric mixtures of ipsdienol was monitored in the field during 1988 and 1989, near Colfax, Wisconsin, and in Midland, Michigan. The Wisconsin study was conducted in an even-aged, 18-ha, pure stand of red pine (*Pinus resinosa* Ait.) planted in 1958. The Michigan study was conducted in a mature (ca. 75-year-old), naturally regenerated, 8-ha, mixed stand of red pine and eastern white pine (*Pinus strobus* L.), with a deciduous understory.

Populations were sampled using eight-unit Lindgren funnel traps (Lindgren, 1983) baited with 8-mg, C-flex, ipsdienol dispensers provided by Phero Tech, Inc., Delta, British Columbia. The lures had a pheromone release rate of 0.2 mg/day, and were replaced every four weeks. The following enantiomeric blends of ipsdienol were tested during 1989 in Wisconsin and both years in Michigan: 3% (+):97% (−), 25% (+):75% (−), 50% (+):50% (−), 75% (+):25% (−), 97% (+):3% (−), and a blank control. Only the 25% (+):75% (−), 50% (+):50% (−), and 75% (+):25% (−) mixtures were tested in Wisconsin in 1988.

In Wisconsin during 1988, and in Michigan during both years, each treatment was randomly assigned to one trap. Traps were separated by 15–25 m. Captured insects were removed and counted weekly. Traps remained in place throughout the season, but the locations of the pheromone baits were rerandom-

ized weekly to guard against spurious effects due to position. Thus, the experimental design was a randomized complete block, with blocks replicated in time. The 1989 Wisconsin experiment was designed as the previously described experiments but with traps deployed in three spatially separated blocks. Within each block, traps were separated by 15–25 m, and each treatment was represented once. The three blocks were equally spaced throughout the stand.

In Wisconsin, sampling was conducted from June 5 until October 16, during 1988, and April 9 until October 22, during 1989. In Michigan, sampling was conducted from April 13 until October 20, in 1988, and March 13 until November 20, in 1989.

Statistical Analysis. Data were analyzed separately by year and site using analysis of variance (PROC GLM; SAS Institute, 1988). Because variances were proportional to means, data were square-root transformed before analysis. Means were separated using the protected LSD test. Weeks with less than two insect captures were excluded from analyses.

Chi-square analyses were used to test for changes in pheromone preference between years. (PROC CATMOD, log-linear model; SAS Institute, 1988). Only the 25% (+), 50% (+), and 75% (+) blends were used in the chi-square analysis of the Wisconsin and Michigan *Ips* data, and the Wisconsin *Thanasimus* data. The other blends were not tested in Wisconsin in 1988. In Michigan, they did not capture enough *Ips* to affect the analysis. All blends were used in the chi-square analysis of Michigan *Thanasimus* data.

RESULTS

Ips pini *in Wisconsin.* There were differences in the 1988 blend preferences of *Ips pini* in Wisconsin ($F = 4.2$; $df = 2,36$; $P = 0.022$). The racemic blend tended to capture more insects than the 75% (+):25% (−) blend, but differences were not significant (Table 1). Both mixtures captured significantly more insects than did 25% (+):75% (−) ipsdienol (Table 1).

There were also differences in the number of *Ips* beetles captured by the ipsdienol blends in 1989. The analysis of variance indicated that a much greater proportion of the total variance was due to the pheromone blends than effects due to site, date, or any interactions ($F = 321.1$ for blend compared to $F = 19.7$ for date and $F \leq 4.4$ for all other effects) (Table 2). The significant blend × date interaction was due to a reduction in the absolute difference between blends in weeks when few beetles were captured. The relative proportion of beetles captured by the different blends tended to remain constant across the season. The 75% (+):25% (−)-ipsdienol captured significantly more *Ips* beetles than all other blends, followed by 50% (+):50% (−) and 25% (+):75% (−)-ipsdienol, respectively (Table 1). The 97% (+):3% (−) and 3% (+):97%

TABLE 1. COMPARISON OF *Ips pini* AND *Thanasimus dubius* RESPONSE TO ENANTIOMERIC MIXTURES OF IPSDIENOL IN MICHIGAN AND WISCONSIN DURING 1988 AND 1989

Ipsdienol enantiomeric blend	Insects/trap/week ($\bar{X} \pm$ SEM)[a]			
	Ips pini			
	Wisconsin		Michigan	
	1988 (N = 19)	1989 (N = 75)	1988 (N = 22)	1989 (N = 22)
3%(+):97%(−)	not tested	0.9 ± 0.3d	0.1 ± 0.1c	0.0 ± 0.0c
25%(+):75%(−)	12.8 ± 4.5b	5.0 ± 0.5c	11.5 ± 7.8b	1.8 ± 0.5b
50%(+):50%(−)	56.6 ± 22.2a	17.3 ± 1.7b	46.5 ± 30.3a	6.5 ± 1.9a
75%(+):25%(−)	40.3 ± 20.6a	30.6 ± 3.0a	89.2 ± 60.4a	8.7 ± 3.8a
97%(+): 3%(−)	not tested	1.0 ± 0.2d	1.1 ± 0.8c	0.3 ± 0.1c
Control	not tested	0.1 ± 0.0e	0.2 ± 0.2c	0.1 ± 0.1c
	Thanasimus dubius			
	1988 (N = 12)	1989 (N = 75)	1988 (N = 14)	1989 (N = 15)
3%(+):97%(−)	not tested	4.1 ± 0.6d	5.9 ± 2.7b	0.5 ± 0.4bc
25%(+):75%(−)	2.1 ± 0.7a	8.6 ± 1.0c	7.5 ± 2.1ab	1.1 ± 0.5b
50%(+):50%(−)	5.2 ± 1.9a	12.9 ± 1.5b	11.8 ± 4.9a	1.3 ± 0.5b
75%(+):25%(−)	2.3 ± 1.1a	18.7 ± 2.7a	9.9 ± 5.3ab	1.6 ± 1.0b
97%(+): 3%(−)	not tested	16.2 ± 1.8a	9.2 ± 2.7ab	4.8 ± 2.3a
Control	not tested	0.1 ± 0.1e	0.3 ± 0.3c	0.0 ± 0.0c

[a] Means within a column followed by the same letter are not significantly different ($P > 0.05$; protected LSD test applied to square-root transformed data).

(−) blends caught significantly more *Ips* than the control traps, but far fewer than the preferred blends.

Patterns of response by *Ips pini* to ipsdienol enantiomers changed from 1988 to 1989. The proportion of total beetles captured by 50% (+)-ipsdienol dropped from 52% in 1988 to 32% in 1989, while the proportion captured by the 75% (+) blend increased from 36% in 1988 to 56% in 1989 (Table 3). There was little change in the proportion of insects captured by 25% (+)-ipsdienol.

Ips pini *in Michigan.* Pheromone blends differed in *Ips* capture rates in 1988 ($F = 15.4$, $df = 5,105$; $P < 0.001$) and 1989 ($F = 10.3$; $df = 5,105$; $P < 0.001$). During both years, 75% (+)-ipsdienol captured more *Ips* than the

TABLE 2. ANOVA OF *Ips pini* AND *Thanasimus dubius* TRAP CAPTURE DATA NEAR COLFAX, WISCONSIN, 1989

Source	*df*	*Ips pini*			*Thanasimus dubius*		
		MS	*F*	*P*	MS	*F*	*P*
Blend (B)	5	214.3	321.1	<0.001	97.1	105.4	<0.001
Site (S)	2	1.5	2.2	0.114	13.4	14.7	<0.001
Date (D)	25	13.1	19.7	<0.001	21.4	23.2	<0.001
B × S	10	1.2	1.7	0.074	1.2	1.3	0.207
B × D	125	2.9	4.4	<0.001	2.3	2.5	<0.001
S × D	50	1.1	1.6	0.013	1.7	1.8	0.002

[a]MS = Mean Square.

TABLE 3. PERCENT OF TOTAL NUMBER OF *Ips pini* AND *Thanasimus dubius* CAPTURED BY DIFFERENT IPSDIENOL ENANTIOMERS NEAR COLFAX, WISCONSIN, AND MIDLAND, MICHIGAN, IN 1988 AND 1989

Location	Year	Percent (*S*) − (+)-Ipsdienol						Total insects	Trap weeks[a]
		3%(+)	25%(+)	50%(+)	75%(+)	97%(+)	Control		
Ips pini									
Wisconsin	1988		11.6	51.6	35.8			2081	19
	1989	1.6	9.1	31.5	55.7	1.8	0.2	4118	75
Michigan	1988	0.1	7.7	31.3	60.0	0.7	0.1	3272	22
	1989	0.0	10.3	37.0	50.3	1.7	0.5	385	22
Thanasimus dubius									
Wisconsin	1988		22.0	54.0	24.0			115	12
	1989	6.7	14.3	21.4	30.8	26.6	0.2	4560	75
Michigan	1988	13.2	16.7	26.6	22.1	20.8	0.7	627	14
	1989	5.4	11.8	14.0	17.2	51.6	0.0	139	15

[a]Trap weeks = the number of weeks in which insects were captured × the number of traps baited with each pheromone blend.

racemic mixture, although not significantly more; the 25% (+) mixture captured significantly fewer insects than the two preferred blends, but significantly more than the controls (Table 1). This response pattern differed from that observed for *Ips pini* in Wisconsin during the same two years.

In Michigan, the response pattern of *Ips pini* to ipsdienol enantiomers also changed significantly from 1988 to 1989 (Table 4). However, the magnitude of change was less than that observed in Wisconsin. The proportion of the total number of beetles captured by 75% (+)-ipsdienol dropped from 60% in 1988 to 50% in 1989, while the proportion captured by 50% (+)-ipsdienol increased from 31% in 1988 to 37% in 1989. There was little change in the proportion of *Ips* captured by the other pheromone blends (Table 3).

Thanasimus dubius *in Wisconsin.* In 1988, there also were apparent differences in the numbers of *Thanasimus* captured by different ipsdienol blends ($F = 3.0$; $df = 2,16$; $P = 0.079$). The racemic mixture captured more insects than the other two blends tested (Table 1).

There were highly significant differences among ipsdienol mixtures in numbers of *Thanasimus* beetles captured in Wisconsin in 1989 (Table 1). As with *Ips pini*, the variation accounted for by date, site, and all interactions was small compared with the variation due to pheromone blend (Table 2). The 75% (+)- and 97% (+)-ipsdienol blends were the most preferred; the racemic mixture captured significantly more *Thanasimus* than did 25% (+)-ipsdienol, which in turn was preferred over the 3% (+) blend (Table 1).

In Wisconsin, the pattern of response by *Thanasimus dubius* to ipsdienol enantiomers also changed significantly from 1988 to 1989 (Table 4), with the pattern of change similar to that of *Ips pini* at the same location. During 1988, the racemic mixture captured 54% of the total number of *Thanasimus*, compared to 24% and 22% for 75% (+)- and 25% (+)-ipsdienol, respectively. In

TABLE 4. CONTINGENCY ANALYSES COMPARING RESPONSE PATTERNS OF *Ips pini* AND *Thanasimus dubius* TO IPSDIENOL ENANTIOMERIC BLENDS FOR 1988 AND 1989 NEAR COLFAX, WISCONSIN, AND MIDLAND, MICHIGAN[a]

Species	Location	*df*	chi-square	*P*
Ips pini[b]	Wisconsin	2	453.1	<0.001
Ips pini[b]	Michigan	2	11.9	0.003
Thanasimus dubius[b]	Wisconsin	2	20.3	<0.001
Thanasimus dubius[c]	Michigan	4	49.1	<0.001

[a] The null hypothesis tested was that relative attractiveness of ipsdienol blends did not differ between years.
[b] Compares 25%, 50%, and 75% (+)-ipsdienol.
[c] Compares 3%, 25%, 50%, 75%, and 97% (+)-ipsdienol.

1989, however, the racemic mixture captured only 21% of the total *Thanasimus*, while the proportion captured by 75% (+)-ipsdienol increased to 31%. The 97% (+) blend, which was not tested in 1988, captured 27% of the total number of insects in 1989.

Thanasimus dubius *in Michigan.* In Michigan, all ipsdienol blends captured significantly more *Thanasimus* than the control during 1988 ($F = 8.2$; $df = 5,70$; $P < 0.001$) and 1989 ($F = 6.5$; $df = 5,60$; $P < 0.001$). The racemic blend captured significantly more *Thanasimus* than 3% (+)-ipsdienol, but there were no significant differences among the other blends (Table 1). In 1989, however, 97% (+)-ipsdienol was favored over all other blends with no differences observed among the remaining blends (Table 1).

As with the other three populations studied, the response pattern of *Thanasimus dubius* in Michigan changed significantly from 1988 to 1989 (Table 4). The proportion of the total number of *Thanasimus dubius* captured by 97% (+)-ipsdienol increased from 21% in 1988 to 52% in 1989; all other blends captured a greater proportion of the total number of *Thanasimus* beetles in 1988 than in 1989 (Table 3).

DISCUSSION

Ips pini population in Wisconsin and Michigan responded most strongly to 75% (+)- or 50% (+)-ipsdienol during both years, which agrees with an earlier report from Wisconsin (Raffa and Klepzig, 1989). This preference is more similar to New York populations, which respond preferentially to 50% (+)-ipsdienol (Lanier et al., 1980), than to western North American populations, which respond preferentially to the 97% (−) enantiomer (Birch et al., 1980). However, our experiment found substantial variation in response to ipsdienol within *Ips pini* populations from both Michigan and Wisconsin. Miller et al. (1989) demonstrated variation in the production of ipsdienol enantiomers among individual *Ips pini* within New York, California, and British Columbia populations. This indicates that variation exists among individuals in both the response and production components of the pheromone system of *Ips pini*. We also found substantial intrapopulation variation in the response of the clerid predator *Thanasimus dubius* to ipsdienol in both Michigan and Wisconsin.

The pheromone preferences within *Ips pini* and *Thanasimus dubius* populations in Wisconsin and Michigan were labile, changing from 1988 to 1989. Furthermore, these species exhibited geographic variation in their response to ipsdienol on a relatively local scale. The pattern of response by the Michigan *Ips pini* population to ipsdienol was different than that of the Wisconsin population both years. The same was true of the *Thanasimus dubius* populations. In fact, our 1988 data for *Thanasimus dubius* in Wisconsin differed from that collected by Raffa and Klepzig (1989) during the same year only 150 km away.

It is unknown to what degree, if any, intrapopulation variation in the response of *Thanasimus dubius* or *Ips pini* to ipsdienol is heritable. However, hybridization studies with *Ips pini* indicate a genetic basis to the interpopulation variation in response to ipsdienol (Piston and Lanier, 1974; Lanier et al., 1980). The spatial and temporal variation that we observed suggests a possible genetic component to the intrapopulation variation as well. High genetic variation within both predator and prey species would provide the potential for coevolutionary change. *Thanasimus dubius* may select for changes in the pheromone system of *Ips pini*, resulting in reduced predation (Raffa and Klepzig, 1989). The high variation observed in the response of *Thanasimus dubius* to ipsdienol may facilitate rapid counter adaptation to changes in *Ips pini* populations.

The potential capacity of *Ips pini* for evolutionary change in its pheromone system has important implications for pest management strategies. In particular, *Ips pini* may develop resistance to mass-trapping programs, adapting to ipsdienol blends other than those used as bait (Lanier et al., 1972; Slessor et al., 1985; Borden et al., 1986; Miller et al., 1989). Variation within populations of *Ips pini* may require that blend preferences of local populations be well characterized before and monitored during the course of mass-trapping programs. Several blends may have to be used simultaneously and/or rotated as part of a resistance management program.

Acknowledgments—We thank Herbert H. Dow for allowing us to conduct the Michigan experiments on his property. We are grateful to Matt Ayres for his substantial contributions to the analysis and interpretation of the data. Dave Petty and Tom Young assisted with field collections in Michigan. Cathy Infante and Dave Petty counted the insects. Earlier versions of the manuscript were criticized and improved by Matt Ayres, Ronald P. Billings, Kelly S. Johnson, Daniel R. Miller, James R. Miller, David G. Nielsen, James K. Nitao, Kenneth F. Raffa, and Stephen Teale. This study was conducted in cooperation with the Department of Entomology and Pesticide Research Center, Michigan State University.

REFERENCES

BAKKE, A. 1982. Mass trapping of the spruce bark beetle *Ips typographus* in Norway as part of an integrated control program, Vol. 2, pp. 17–25, *in* A.F. Kydonieus, M. Beroza, and G. Zweig (eds.). Insect Suppression with Controlled Release Pheromone Systems. CRC Press, Boca Raton, Florida.

BAKKE, A. 1989. The recent *Ips typographus* outbreak in Norway—experiences from a control program. *Holarc. Ecol.* 12:515–519.

BAKKE, A., and KVAMME, T. 1981. Kairomone response in *Thanasimus* predators to pheromone components of *Ips typographus*. *J. Chem. Ecol.* 7:305–312.

BILLINGS, R.F., and CAMERON, R.S. 1984. Kairomonal responses of Coleoptera, *Monochamus titillator* (Cerambycidae), *Thanasimus dubius* (Cleridae), and *Temnochila virescens* (Trogositidae), to behavioral chemicals of southern pine bark beetles (Coleoptera: Scolytidae). *Environ. Entomol.* 13:1542–1548.

BIRCH, M.C. 1978. Chemical communication in pine bark beetles. *Am. Sci.* 66:409–419.

BIRCH, M.C., LIGHT, D.M., WOOD, D.L., BROWNE, L.E., SILVERSTEIN, R.M., BERGOT, B.J., OHLOFF, G., WEST, J.R., and YOUNG, J.C. 1980. Pheromonal attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. *J. Chem. Ecol.* 6:703–717.

BORDEN, J.H. 1982. Aggregation pheromones, pp. 74–139, *In* J.B. Mitton and K.B. Sturgeon (eds.). Bark Beetles in North American Conifers: A System for the Study of Evolutionary Biology. University of Texas Press, Austin.

BORDEN, J.H., HUNT, D.W.A., MILLER, D.R., and SLESSOR, K.N. 1986. Orientation in forest Coleoptera: An uncertain outcome of responses by individual beetles to variable stimuli, pp. 97–109, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

HANSEN, K. 1983. Reception of bark beetle pheromone in the predaceous clerid beetle, *Thanasimus dubius* (Coleoptera: Cleridae). *J. Comp. Physiol.* 150:371–378.

JANZEN, D.H. 1980. When is it coevolution? *Evolution* 34:611–612.

LINDGREN, B.S. 1983. A multiple funnel trap for scolytid beetles (Coleoptera: Scolytidae). *Can. Entomol.* 115:299–302.

LANIER, G.N., BIRCH, M.C., SCHMITZ, R.F., and FURNISS, M.M. 1972. Pheromones of *Ips pini* (Coleoptera: Scolytidae): Variation in response among three populations. *Can. Entomol.* 104:1917–1923.

LANIER, G.N., CLASSON, A., STEWART, J.J., PISTON, J.J., and SILVERSTEIN, R.M. 1980. *Ips pini:* The basis for interpopulational differences in pheromone biology. *J. Chem. Ecol.* 6:677–687.

MILLER, D.R., BORDEN, J.H., and SLESSOR, K.N. 1989. Inter- and intrapopulation variation of the pheromone, ipsdienol produced by male pine engravers, *Ips pini* (Say) (Coleoptera: Scolytidae). *J. Chem. Ecol.* 15:233–247.

MIZELL, R.F., III, FRAZIER, J.L., and NEBEKER, T.E. 1982. Response of the clerid predator *Thanasimus dubius* (F.) to bark beetle pheromones and tree volatiles in a wind tunnel. *J. Chem. Ecol.* 10:177–187.

PAYNE, T.L., DICKENS, J.C., and RICHERSON, J.V. 1984. Insect predator-prey coevolution via enantiomeric specificity in a kairomone-pheromone system. *J. Chem. Ecol.* 10:487–492.

PISTON, J.J., and LANIER, G.N. 1974. Pheromones of *Ips pini* (Coleoptera: Scolytidae), response to interpopulational hybrids and relative attractiveness of males boring in two host species. *Can. Entomol.* 106:247–251.

PRICE, P.W. 1981. Semiochemicals in evolutionary time, pp. 251–279, *in* D.A. Nordlund, R.L. Jones, and W.J. Lewis (eds.). *Semiochemicals: Their Role in Pest Control.* Wiley, New York.

RAFFA, K.F., and KLEPZIG, K.D. 1989. Chiral escape of bark beetles from predators responding to a bark beetle pheromone. *Oecologia* 80:566–569.

SAS Institute. 1988. SAS/STAT User's Guide. Release 6.03. SAS Institute, Cary, North Carolina.

SCHENK, J.A., and BENJAMIN, D.M. 1969. Notes on the biology of *Ips pini* in central Wisconsin jack pine forests. *Ann. Entomol. Soc. Am.* 62:480–485.

SLESSOR, K.N., KING, G.G.S., MILLER, D.R., WINSTON, M.L., and CUTFORTH, T.L. 1985. Determination of chirality of alcohol or latent alcohol semiochemicals in individual insects. *J. Chem. Ecol.* 11:1659–1666.

VITÉ, J.P., and WILLIAMSON, D.L. 1970. *Thanasimus dubius:* Prey perception. *J. Insect. Physiol.* 16:233–239.

VITÉ, J.P., BAKKE, A. and J.A.A. RENWICK, 1972. Pheromones in *Ips* (Coleoptera: Scolytidae): Occurrence and production. *Can. Entomol.* 104:1967–1975.

WOOD, D.L. 1982. The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles. *Annu. Rev. Entomol.* 27:411–446.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

PROTECTIVE ACTION OF MIDGUT CATALASE IN LEPIDOPTERAN LARVAE AGAINST OXIDATIVE PLANT DEFENSES

GARY W. FELTON[1,*] and SEAN S. DUFFEY

Department of Entomology
University of California
Davis, California 95616

(Received March 12, 1991; accepted April 25, 1991)

Abstract—Catalase activity was detected in the midgut tissues and regurgitate of several lepidopteran pests of the tomato plant. Greatest activity in the midgut was detected in larval *Helicoverpa zea*, followed by *Spodoptera exigua, Manduca sexta*, and *Heliothis virescens*. We present evidence that catalase, in addition to removing toxic hydrogen peroxide, may inhibit the oxidation of plant phenolics mediated by plant peroxidases. Small amounts of larval regurgitate significantly inhibited foliar peroxidase activity via removal of hydrogen peroxide. Treatment of foliage with purified catalase nearly eliminated peroxidase activity and was superior as a larval food source compared to untreated foliage. Tomato foliar peroxidases oxidize an array of endogenous compounds including caffeic acid, chlorogenic acid, rutin, coumaric acid, cinnamic acid, and guaiacol. The oxidized forms of these compounds are potent alkylators of dietary and/or cellular nucleophiles (e.g., thiol and amino functions of proteins, peptides, and amines). When tomato foliar protein was pretreated with peroxidase and chlorogenic acid and incorporated in artificial diet, larval growth was reduced compared to larvae fed untreated protein. Thus, the diminution of peroxidase activity and removal of hydrogen peroxide by catalase may represent an important adaptation to leaf-feeding. The secretion of catalase in salivary fluid during insect feeding is also suggested to be a potential mechanism for reducing hydrogen peroxide formation as an elicitor of inducible plant defenses.

Key Words—*Helicoverpa zea, Heliothis virescens, Spodoptera exigua, Manduca sexta, Lycopersicon esculentum*, Lepidoptera, Noctuidae, Sphin-

*To whom correspondence should be addressed.
[1] Present address: Department of Entomology, University of Arkansas, Fayetteville, Arkansas 72701.

0098-0331/91/0900-1715$06.50/0

gidae, catalase, peroxidase, hydrogen peroxide, chlorogenic acid, plant phenolics, antioxidant defenses, insect–plant interactions.

INTRODUCTION

The presence of hydrogen peroxide in plant tissues may pose a significant impediment to the growth and survival of herbivores. Hydrogen peroxide is a powerful oxidant (Cadenas, 1989; Fridovich, 1989). The utilization and/or function of proteins, enzymes, and glutathione may be impaired due to oxidation of thiols and decarboxylation of amino acids by hydrogen peroxide (H_2O_2) (Slump and Schreuder, 1973; Berlett et al., 1990). In the presence of catalytic amounts of ferrous or manganese ions, H_2O_2 can degrade to form the hydroxy radical ($HO\cdot$), the most powerful oxidant known (Fridovich, 1989; Cadenas, 1989; Yim et al., 1990). This radical can then react with DNA, membrane lipids, and other essential cellular components (Halliwell and Gutteridge, 1984; Fridovich, 1989).

In conjunction with plant peroxidases (POD), H_2O_2 catalyzes the oxidation of diphenols to *o*-quinones. The quinones are susceptible to attack by nucleophilic amino and thiol groups of proteins (Matheis and Whitaker, 1984a, 1987). The enzymatic oxidation of the diphenol chlorogenic acid to chlorogenoquinone by polyphenol oxidase has been shown to impair the growth of *Helicoverpa* (=*Heliothis*) *zea* and *Spodoptera exigua* (Felton et al., 1989).

Peroxidase with H_2O_2 can directly oxidize tyrosine residues of proteins, causing substantial cross-linking (Matheis and Whitaker, 1984b). Plant peroxidases have multiple oxidative activities with broad substrate specificity; an array of phytochemicals including simple monophenols (e.g., coumaric acid), alcohols (e.g., coniferyl alcohol), coumarins (e.g., esculetin), and dihydroxyphenols (e.g., chlorogenic acid) may be oxidized by peroxidase (Gaspar et al., 1982).

Furthermore, H_2O_2 and POD are known to catalyze the cross-linking of hydroxyproline-rich glycoproteins in cell walls that stabilize cell walls against pathogen invasion (van Huystee, 1987). Moreover, POD and H_2O_2 are involved in the formation of lignin (van Huystee, 1987). The role of these cell-wall strengthening reactions in impeding utilization of plant tissues by insect herbivores is largely unknown (Stamopoulos, 1988; Wainhouse et al., 1990). POD may also be involved in lipid peroxidation and the formation of reactive molecules such as linoleic hydroperoxide (Garner, 1984; Keppler and Novacky, 1987). Both the primary oxidative products, fatty acid hydroperoxides, and the secondary products, such as malondialdehyde, form addition products with nucleophilic groups of protein (Garner, 1984). These lipid oxidation products have been implicated as defense mechanisms against herbivores (Shukle and

Murdock, 1983; Hildebrand et al., 1986, 1989; Duffey and Felton, 1989; Mohri et al., 1990).

Plants produce H_2O_2 by a variety of processes. Hydrogen peroxide may be formed from the reduction of oxygen directly by two-electron transfer reactions catalyzed by flavoproteins or via an initial one-electron step to O_2^- followed by dismutation (Cadenas, 1989). Hydrogen peroxide is a by-product of photosynthesis and several oxidative enzymes including glycollate oxidase, diamine oxidases, uricase, glucose oxidase, etc. (Choudhuri, 1988).

A number of plant compounds, implicated in defense against herbivores, may provide a source of H_2O_2, including photodynamically activated furanocoumarins, thiophenes, polyacetylenes, and extended quinones (Joshi and Pathak, 1983; Downum and Rodriguez, 1986). Moreover, ubiquitous dihydroxyphenolic compounds such as caffeic acid and chlorogenic acid autoxidize under basic conditions to form H_2O_2 (Hanham et al., 1983; Cilliers and Singleton, 1989; Tulyathan et al., 1989; Cheynier et al., 1989). Because the midgut pH of many lepidopteran larvae is basic, H_2O_2 may be formed from the autoxidation of phenolics in the digestive system of these species.

The tomato plant *Lycopersicon esculentum* contains substantial levels of the diphenols, rutin, and chlorogenic acid, that are sources of H_2O_2 in basic environments (Felton et al., 1989). Tomato is attacked by several lepidopteran larvae including *H. zea, Heliothis virescens, S. exigua, Trichoplusia ni*, and *Manduca sexta*. The midgut pH of these species, in all instances, is alkaline and ranges from 8.0 to 11.3 (Dow, 1984; Martin et al., 1987; Felton et al., 1989). Thus, the formation of H_2O_2 in the midguts of these species is likely and may adversely affect larval growth and survival. Consequently, the avoidance or removal of exogenous H_2O_2 is desirable.

The first line of defense against H_2O_2 toxicity is via enzymatic removal by catalase or glutathione peroxidase. Catalase has been well characterized recently in a number of lepidopteran larvae (Ahmad et al., 1987, 1988a,b; Pritsos et al., 1988a,b). In this investigation, we report on the presence of high levels of catalase in the midgut tissues and digestive fluid of several common lepidopteran herbivores of the tomato plant. Evidence for the particular advantages of possessing catalase activity in the digestive systems of these herbivores is presented.

METHODS AND MATERIALS

Insects. Eggs of *Helicoverpa zea, Heliothis virescens*, and *Spodoptera exigua* were obtained from the USDA Laboratory, Stoneville, Mississippi. Eggs of *Manduca sexta* were obtained from Dr. Bruce Hammock, University of California, Davis. In order to exclude differences in larval enzyme activity asso-

ciated with feeding on varying artificial diets, larvae for all species were reared on the same artificial diet containing 5% casein (wet wt) following Chippendale (1970).

Enzyme Preparation. For detection of catalase activity in midgut tissue, groups of five midguts were dissected from actively feeding 2-day-old fifth-instar larvae. After removal of midgut contents, the midgut was washed in 1.15% KCl and homogenized in ice-cold sodium phosphate buffer, pH 7.0 for all assays. The homogenate was centrifuged at 15,000*g* for 15 min. The soluble fraction was used immediately as the enzyme source.

To assay catalase in digestive fluid, larvae were gently held behind the head with forceps, and regurgitate was collected in a tube on Dry Ice. Fluid from at least 50 larvae was collected for each species except *M. sexta*. Digestive fluid was mixed 1:1 with 0.9% NaCl and centrifuged at 15,000*g* for 15 min. Supernatant was used as enzyme source.

To assay tomato foliar catalase, chlorogenic acid oxidase, or peroxidase activity, ca. 2-g leaflets from field-grown *Lycopersicon esculentum* var. Ace were excised with a razor blade. Leaflets were homogenized in ice-cold sodium phosphate buffer, pH 7.0, containing 5% polyvinylpolypyrrolidone. Homogenate was centrifuged as above and used immediately for enzyme assay.

Assay and Characterization of Larval Catalase. Catalase activity was monitored by following the loss of hydrogen peroxide at 240 nm (Aebi, 1984). Enzyme activity was determined from the linear portion of the rate curve and one unit of catalase activity expressed as the decomposition of 1 μmol H_2O_2/min/mg protein. Protein concentration was determined following Bradford (1976) with bovine serum albumin as a standard.

To determine the effect of pH on midgut catalase activity from different insect species, assay was performed at a pH range of 4.7 to 10.0 for most species. Three replicates in duplicate were performed for each species at each pH level tested. The buffer used in all assays was 0.1 M sodium phosphate. The catalase activity of regurgitate was assayed at pH 5.5 and pH 6.5. The experiment was replicated three times.

To determine the Michaelis constant (K_m) and V_{max} of catalase and H_2O_2 for the different insect species, varying substrate concentrations were used. In all instances at least seven concentrations of H_2O_2 were used. The initial absorbance was kept constant at ca. 0.500 by adjusting the concentration of H_2O_2 in the reference and sample cuvettes. The K_m was determined by a regression curve of a Lineweaver-Burk plot, and in all instances the correlation coefficient exceeded 0.96. Three replicates were performed for each species, and 95% confidence limits were used to determine statistical significance.

Characterization of Foliar Catalase and Peroxidase. Catalase activity for foliage was assayed as described above for insect larvae. Tomato plants (var. Ace) were grown in the field, and foliage was excised at the flowering stage.

Enzyme was prepared as described above. Catalase activity of tomato foliage was assessed across the pH range of 5.5 to 10.0. Three replicates for each pH unit were performed. Protein concentration was determined following Jones et al. (1989).

Peroxidase activity was determined following Ryan et al. (1982) by recording the increase in absorbance at 470 nm. The following substrates were tested: chlorogenic acid, rutin, guaiacol, coumaric acid, ferulic acid, esculetin, caffeic acid, cinnamic acid, and 3,4-dimethoxybenzyl alcohol. Four replicates per test chemical were performed. Peroxidase activity was then calculated by subtracting the difference in mean activity between treatments with H_2O_2 and without H_2O_2.

The peroxidase activity of tomato foliage was measured using 3 mM guaiacol across the pH range of 5.5 to 10.0. Protein concentration was determined following Jones et al. (1989). Three replicates for each pH unit were performed.

Effect of Foliage-Feeding on Midgut Catalase Activity. To determine if the ingestion of tomato foliage affects larval midgut catalase activity in *H. zea*, newly molted fifth-instar larvae were either given freshly excised tomato leaflets or maintained on artificial diet. Leaflets were removed from field-grown *L. esculentum* var. Ace. After 48 hr of feeding on foliage or artificial diet, larvae were sacrificed for catalase assay. For each treatment, 12–80 μg of midgut protein was assayed for activity. Activity was assessed at the enzyme optima of pH 6.5 and at the midgut pH 8.5. Differences between treatments were compared by the regression plot of catalase activity against micrograms of midgut protein. Three replicates of five larvae per treatment were performed.

Effect of Larval Regurgitate on Foliar Peroxidase Activity. Leaflets were excised from greenhouse-grown tomato plants (var. Castlemart) at the six-leaf stage. One-gram aliquots of leaflets were homogenized in 20 ml ice-cold buffer. Homogenates were centrifuged for 15 min at 15,000*g*. Substrate for peroxidase activity was 2 mM guaiacol with 5 mM H_2O_2.

Regurgitate from fifth-instar *H. zea* was obtained as described above. Regurgitate was collected from individual larvae on preweighed parafilm, weighed to the nearest 0.1 mg, and immediately mixed with 1.5 ml enzyme substrate. A 20-μl aliquot of the foliar peroxidase was quickly added, and peroxidase activity was recorded as described. Twenty-one samples were tested with 0 to ca. 25 mg of regurgitate in each.

Effects of Hydrogen Peroxide, Peroxidase, and Chlorogenic Acid on Larval Growth. To determine if larval growth was affected by the presence of dietary peroxidase, H_2O_2, and/or chlorogenic acid, tomato foliar protein was treated with combinations of the above chemicals. Foliar protein was obtained from field-grown var. Castlemart plants at the mature fruit stage. Protein was extracted and semipurified following Broadway and Duffey (1988) and Felton et al. (1989), free from protease inhibitor contamination. A 35-ml aqueous solu-

tion of 500 mg tomato foliar protein was incubated with various combinations of 125 mg chlorogenic acid, 34 μl 30% H_2O_2, and/or 28 mg purified horseradish peroxidase (Sigma Chemical Co., St. Louis, Missouri). The treatments included H_2O_2; H_2O_2 and peroxidase; H_2O_2, peroxidase, and chlorogenic acid; chlorogenic acid alone; and a control with untreated protein. Solutions were adjusted to pH 8.0 with 2 N NaOH and mechanically stirred for 60 min at room temperature.

Following incubation, solutions were added to the remaining dry portion of an artificial diet (Broadway and Duffey, 1988). The diets were then prepared with hot aqueous agar solution to give final fresh weight concentrations of 0.5% foliar protein, 10 mM H_2O_2, 3.5 mM chlorogenic acid, and 180 units peroxidase activity per gram of diet. Peroxidase activity was determined using guaiacol and activity expressed as 1 unit = 0.001 change in absorbance/min. The levels of these chemicals are consistent with what occurs commonly in tomato foliage (Felton et al., 1989; unpublished data).

Bioassays with *H. zea* larvae were conducted following Broadway and Duffey (1986) with a 16:8 light–dark cycle at 27°C. Neonate larvae were placed on the diet treatments, and fresh diet was provided every 48 hr. Larvae were weighed to the nearest 0.1 mg after 16 days. A total of 50 larvae were tested for each treatment, and larval mortality or escape did not exceed 4% for any treatment.

Effects of Catalase Activity on Ability of Tomato Foliage To Support Larval Growth. To determine if catalase activity protects tomato foliage from oxidative damage, Ace variety tomato plants were grown in the field. At the immature fruit stage, foliage was excised with a razor blade. The foliage was equally divided into one of two treatments. In the first treatment, 100 g foliage was homogenized in 200 ml ice-cold 0.01 M sodium phosphate (adjusted to pH 8.0 with 2 N NaOH) with 40 mg heat-inactivated catalase (from horseradish, Sigma Chemical Co.) and then mechanically stirred for 1 hr at 25°. Homogenate was then frozen and lyophilized. An artificial diet was prepared with agar from the dried leaf powder (Felton et al., 1989).

In the second treatment, 100 g foliage was homogenized with 40 mg of purified, active catalase. Otherwise, foliage was treated identically and diet was prepared as described above. Twenty-five second-instar *H. zea* per treatment were placed on the respective diet following conditions described above. Larval weight was recorded daily for 12 days. The experiment was replicated three times.

Catalase activity of foliage for both treatments was determined from several aliquots of the homogenate immediately following homogenization. Oxidation of chlorogenic acid was also followed spectrophotometrically for each aliquot (Felton et al., 1989). The formation of the lipid oxidation product, malondialdehyde, was determined with thiobarbituric acid from aliquots of

extracts of dried leaf powder following the method of Stewart and Bewley (1980).

RESULTS

Characterization of Larval Midgut Catalase. pH 6.5 was optimum for larval midgut catalase from *M. sexta, S. exigua*, and *H. zea* (Figure 1). *H. virescens* had much lower activity than the other species with a peak at pH 6.5–8.0 (Figure 1). Activity for *H. virescens* was not significantly different between 6.5 and 8.0.

The highest V_{max} and K_m were found in *H. zea* larvae followed by *S. exigua* (Table 1). *H. virescens* had the lowest V_{max}, whereas *M. sexta* had the lowest K_m (Table 1).

Catalase Activity in Larval Regurgitate. The presence of catalase activity in larval regurgitate was detected in the three species examined: *S. exigua, H. zea*, and *H. virescens* (Table 2). The highest activity was detected in *S. exigua* followed by *H. virescens* and *H. zea*. The activity of catalase was determined at pH 6.5, the pH optimum for catalase from midgut tissue (Table 1), and at pH 5.5, the pH of crushed foliage. In all three species examined, regurgitated catalase was less active at pH 5.5 than at pH 6.5 (Table 2).

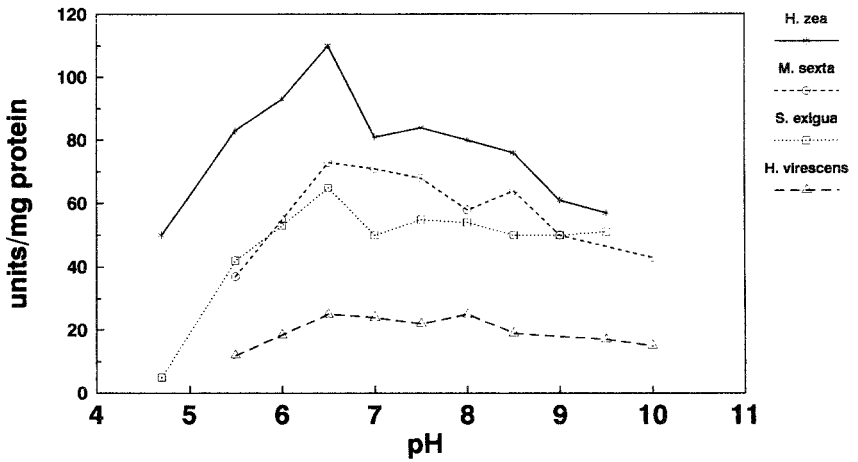


FIG. 1. Effect of pH on lepidopteran midgut catalase activity. Each point represents the mean of three replicates performed in duplicate. Activity for *H. virescens* was significantly lower ($P < 0.05$) than all other larvae at all pH levels tested. The activity of *H. zea* was significantly higher than other species for pH 4.7–8.0. Activities for *M. sexta* and *S. exigua* were not significantly different from each other except at pH 7.0. Significance is based on nonoverlap of 95% confidence limits.

TABLE 1. CHARACTERIZATION OF CATALASE ACTIVITY OF MIDGUT TISSUE OF SEVERAL LARVAL LEPIDOPTERAN HERBIVORES OF TOMATO PLANT[a]

Species	K_m constant (mM)	V_{max} (μmols/min/mg protein)
Helicoverpa zea	136.8a	291.7a
Heliothis virescens	37.7c	35.9c
Spodoptera exigua	71.9b	80.9b
Manduca sexta	22.3d	38.3c

[a] Means in columns not followed by the same letter are significantly different at $P < 0.05$ following ANOVA and 95% confidence limits.

TABLE 2. CATALASE ACTIVITY IN DIGESTIVE REGURGITATE OF SEVERAL LEPIDOPTERA LARVAE

Species	Activity (μmols/min/mg protein)[a]	
	pH 5.5	pH 6.5
Heliothis virescens	26.9b	15.8b
Spodoptera exigua	140.8a	97.5a
Helicoverpa zea	3.5c	3.1c

[a] Means in columns not followed by the same letter are significantly different at $p < 0.05$ following ANOVA and 95% confidence limits.

Characterization of Foliar Catalase and Peroxidase. Tomato foliar catalase was active across the range of pH 5.5–10.0 with a sharp peak of activity at pH 7.0 (Figure 2). The level of foliar catalase activity was substantially lower than that of any insect species with the exception of *H. virescens*.

Tomato foliar POD was also active from pH 5.5 to 10.0 but did not exhibit as sharp a peak as catalase (Figure 3). The optimum was at pH 5.5 with a secondary peak at pH 7.0.

Tomato POD oxidized a wide variety of phytochemicals known to occur in tomato foliage (Table 3). Greatest activity was towards caffeic acid followed by guaiacol, chlorogenic acid, esculetin, ferulic acid, coumaric acid, rutin, cinnamic acid, and 3,4-dimethoxybenzyl alcohol (Table 3). Relative activities are only approximations because the estimation of activities was based on the assumption that extinction coefficients of oxidative products were similar. Chlorogenic acid and caffeic acid were the only substrates oxidized by polyphenol

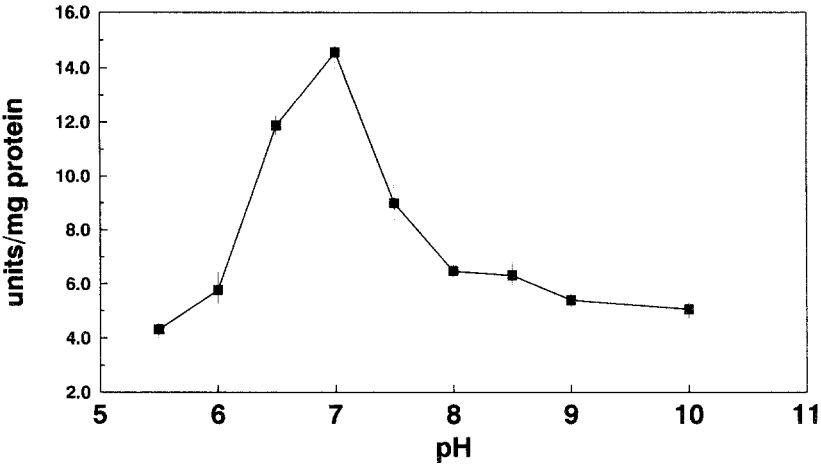


FIG. 2. Effect of pH on foliar catalase activity. Each point represents the mean of three replicates performed in duplicate. Bars represent standard error of the mean.

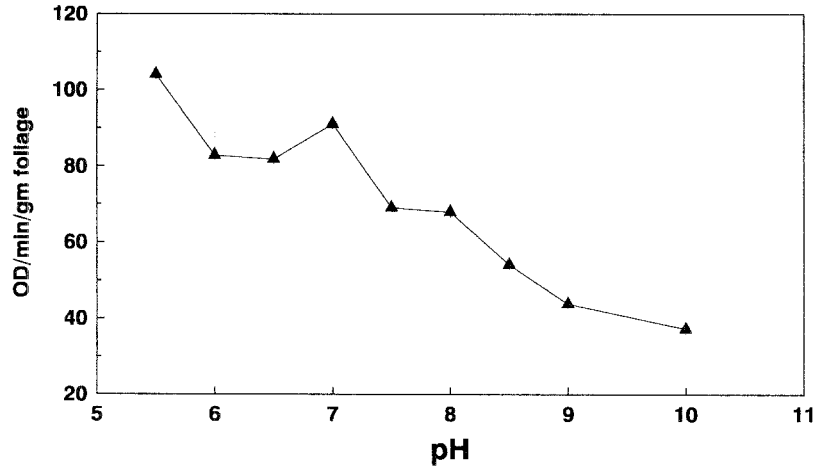


FIG. 3. Effect of pH on foliar peroxidase activity. Each point represents the mean of three replicates performed in duplicate. Bars represent standard error of the mean.

oxidase; peroxidase activity towards these substrates was five- to sixfold higher than polyphenol oxidase activity (data not shown).

Effect of Foliage-Feeding on Midgut Catalase Activity. The ingestion of tomato foliage had no significant effect on midgut catalase activity (Figures 4 and 5). Regression lines for data from larvae feeding on artificial diet or tomato foliage were parallel for both pH 6.5 (Figure 4) and pH 8.5 (Figure 5). The

TABLE 3. RELATIVE FOLIAR PEROXIDASE ACTIVITY TOWARDS VARIOUS SUBSTRATES

Substrate	Relative activity (%)[a]
Caffeic acid	100.0
Guaiacol	89.5
Chlorogenic acid	77.6
Esculetin	47.5
Ferulic acid	30.1
Coumaric acid	5.9
Rutin	5.1
Cinnamic acid	3.3
3,4-Dimethoxybenzyl alcohol	2.9

[a] 100% activity = change in OD_{470} = 70.0/min/g foliage wet weight.

TABLE 4. EFFECT OF EXOGENOUS CATALASE ON ACTIVITIES OF TOMATO FOLIAR ENZYMES AND LARVAL GROWTH RATE OF *Helicoverpa zea*[a]

Treatment	CAT[b]	POD[c]	PPO[d]	MAL[e]	RGR[f]
Without catalase	11.29a	65.19a	27.15a	4.44a	0.254a
With catalase	3570.60b	1.84b	22.00b	3.88b	0.294b

[a] Means in columns not followed by the same letter are significantly different at $P < 0.01$ following ANOVA and 99% confidence limits.
[b] CAT = catalase activity expressed as units/min/g foliage.
[c] POD = peroxidase activity expressed as change in OD/min/g foliage.
[d] PPO = rate of total oxidation of chlorogenic acid catalyzed by peroxidase and polyphenol oxidase expressed as change in OD/min/g foliage.
[e] MAL = malondialdehyde equivalents expressed as μmol/g foliage wet weight.
[f] RGR = relative growth rate (mg/day/mg larva) based on weight gain for 12 days.

slopes between treatments were not significantly different ($P > 0.05$) for either pH level. Catalase activity was greater at pH 6.5 than at pH 8.5 for both treatments.

Effect of Larval Regurgitate on Foliar Peroxidase Activity. Larval regurgitate significantly reduced foliar peroxidase activity (Figure 6). The slope of the regression curve of POD activity vs. regurgitate was significantly less than zero ($P < 0.01$). The addition of relatively small amounts of regurgitate (e.g., 5–10 μl) to the enzyme preparation caused greater than 50% reduction in peroxidase activity.

Reduction in peroxidase activity is apparently due to the removal of H_2O_2 by catalase activity in the regurgitate. Addition of 75 μl 3% H_2O_2 restored most peroxidase activity in samples containing regurgitate. Typically at least 20 μl

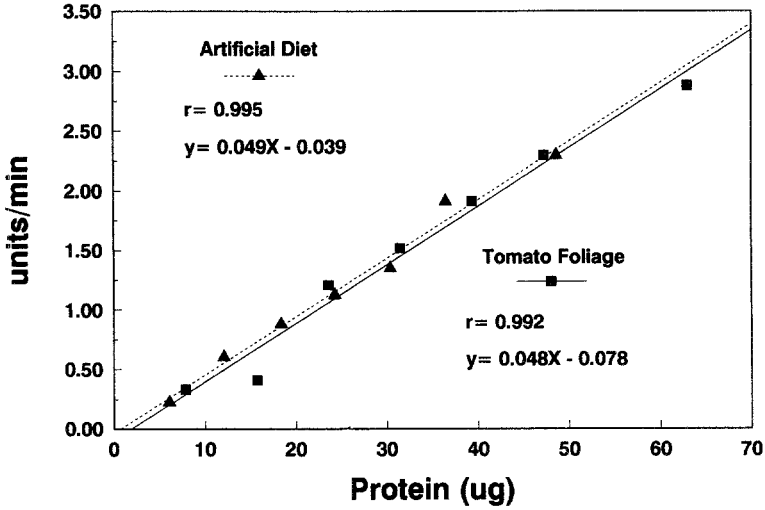


FIG. 4. Effect of foliar feeding on midgut catalase in *Helicoverpa zea* at pH 6.5. Each point represents the mean of three replicates performed in duplicate.

of regurgitate could be collected per larva with an average catalase activity of 3.50 μmol/min/mg protein. Regurgitate boiled in an equal volume of H_2O_2 and added to foliar enzyme preparation had no effect on peroxidase activity.

Effects of Hydrogen Peroxide, Peroxidase, and Chlorogenic Acid on Larval Growth. The growth of larvae was significantly reduced in all treatments relative to the control (Figure 7). The greatest growth reduction occurred with the peroxidase + chlorogenic acid + H_2O_2 treatment, with larvae weighing less than 28% of the control (Figure 7). In the control treatment, 60% of the larvae were in the fourth instar and 40% in the fifth instar. In the treatment with peroxidase + chlorogenic acid + H_2O_2, 26% were second instars, 65% third instars, and the remainder fourth instars. The presence of H_2O_2, chlorogenic acid, or peroxidase + H_2O_2 reduced larval growth by approximately 30–50% for each treatment. Preliminary experiments with heat-inactivated peroxidase added to diet showed that larval growth was not affected by the addition of the small amount of extra protein.

Effects of Catalase Activity on Ability of Tomato Foliage to Support Larval Growth. Supplementing the leaf homogenate with catalase effectively removed most of the foliar peroxidase activity (Table 4). Addition of purified catalase increased total catalase activity by over 300-fold (Table 4). Furthermore, the total rate of oxidation of chlorogenic acid was reduced when catalase was added to the homogenate (Table 4).

When first instar larvae were placed on the respective diets, greater than

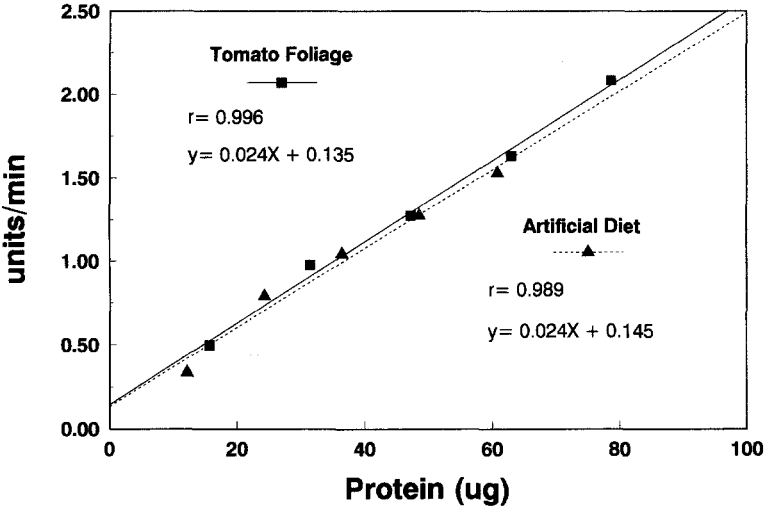


FIG. 5. Effect of foliar feeding on midgut catalase in *Helicoverpa zea* at pH 8.5. Each point represents the mean of three replicates performed in duplicate.

50% mortality occurred in both treatments. Subsequent bioassays used newly molted second-instar larvae that had fed on artificial diet for the first instar. Larvae that were fed on diet prepared from catalase-treated foliage grew significantly faster than those on diet prepared without catalase (Table 4). In addition to the alterations in foliar enzymes shown in Table 4, the presence of malonidialdehyde was reduced by the catalase treatment. These data suggest that foliar peroxidases contribute to lipid peroxidation.

DISCUSSION

Feeding on plant tissues, and tomato foliage in particular, may pose a significant barrier to herbivores not adapted to dietary H_2O_2. The potential adverse effects of H_2O_2 are many: (1) the utilization of amino acids may be directly impaired due to oxidative decarboxylation and oxidation of thiol groups by H_2O_2 (Slump and Schreuder, 1973; Berlett et al., 1990; Yim et al., 1990); (2) protein and enzyme function may be disrupted by H_2O_2 (Cadenas, 1989); (3) lipid peroxidation may be initiated (Garner, 1984); and (4) H_2O_2 is genotoxic and may induce cross-linking of DNA, base destruction, and single- and double-strand breaks in DNA (Hanham et al., 1983).

The combined action of H_2O_2 and peroxidases may: (1) cross-link proteins by formation of dityrosine bridges (Matheis and Whitaker, 1984b); (2) oxidize lignin precursors (e.g., coniferyl alcohol) that may bind to dietary protein

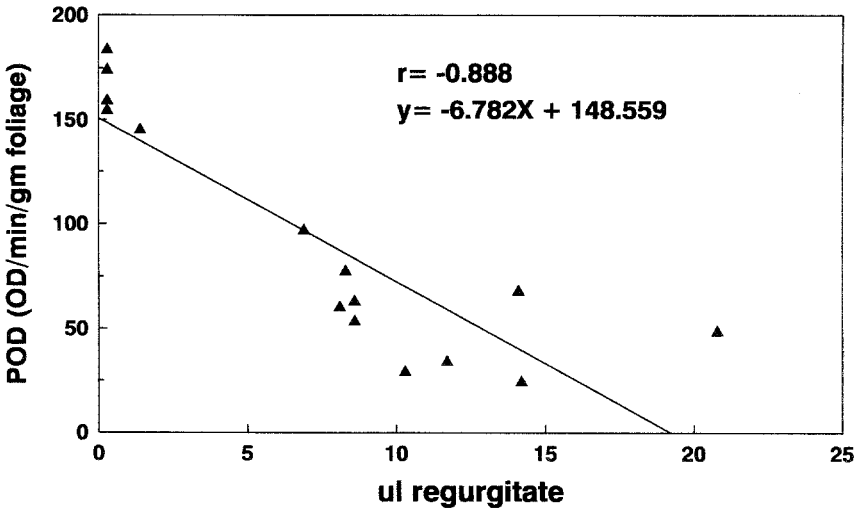


FIG. 6. Effect of *Helicoverpa zea* regurgitate on foliar peroxidase (POD) activity. Each point represents regurgitate collected from one larva feeding on artificial diet.

(Whitmore, 1982) or strengthen cell wall structures, impeding further attack by pathogens and herbivores; (3) oxidize diphenols to produce reactive *o*-quinones that covalently bind to dietary amino acids, peptides, and protein (Kalyanaraman et al., 1987; Duffey and Felton, 1989; Felton et al., 1989); (4) oxidize carotenoids and free fatty acids to form reactive lipid oxidation products (Garner, 1984); (5) oxidatively deaminate protein-bound lysine to form intermolecular cross-links between lysine residues (Stahmann et al., 1977); (6) oxidize ascorbic acid to dehydroascorbic acid (Gaspar et al., 1982); and (7) catalyze production of hydroxyproline-rich proteins that stabilize cell walls (van Huystee, 1987). Thus, the H_2O_2-dependent peroxidases have broad substrate specificity and, depending upon the substrate and cooxidant, may have monophenolase, diphenolase, tyrosinase, lipoxygenase, alcohol dehyrogenase, ascorbic acid oxidase, or lysyl oxidase activities. Considering the broad toxicological implications cited above, it is surprising that the defensive roles of plant peroxidases against insect herbivores have not been more widely studied.

The activity of peroxidases in foliage and in the insect digestive system will depend upon the balance between H_2O_2-generating and competing H_2O_2-degrading systems (i.e., catalase). The generation of H_2O_2 in foliage may result from: (1) photosynthetic reactions (Mehler reaction); (2) autooxidation of diphenols in alkaline fluids; and (3) a number of plant oxidative enzymes (e.g., uric acid oxidase, glycollate oxidase, glucose oxidase, diamine oxidase). Constitutive levels of H_2O_2 in plant tissues may be fairly high (e.g., ca. 15 mM;

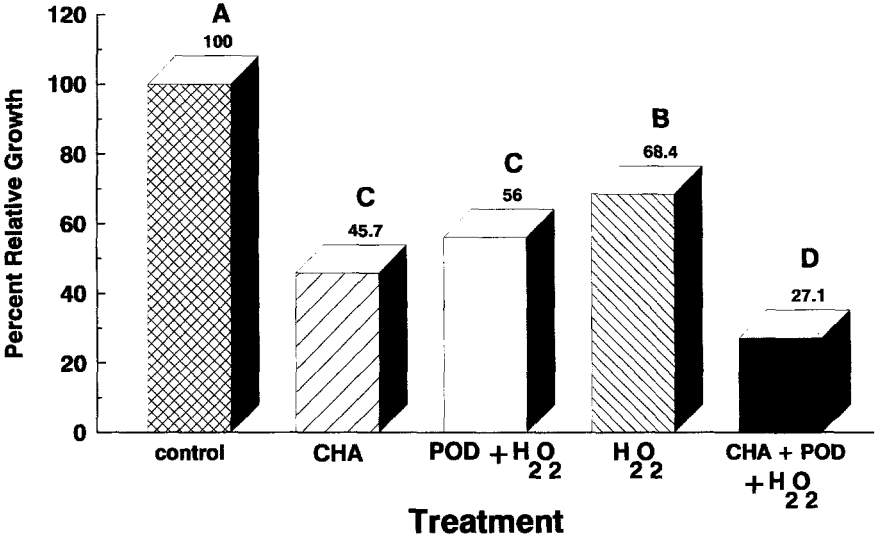


FIG. 7. Effect of peroxidase, hydrogen peroxide, and chlorogenic acid on growth of *Helicoverpa zea*. Means followed by the same letter are not significantly different at $P < 0.05$. CHA = chlorogenic acid; POD = peroxidase.

Choudhuri, 1988), and there is evidence that a rapid burst of H_2O_2 production occurs immediately upon wounding of plant tissues (Apostol et al., 1989). Our data with tomato foliage suggest that endogenous levels of H_2O_2 are sufficient for POD activity to contribute to reduced larval growth and development (Table 4).

Eukaryotic cells possess a variety of mechanisms to avoid H_2O_2 toxicity including: (1) catalase activity; (2) ascorbic acid at alkaline pH to reduce H_2O_2 to water; (3) ascorbate peroxidase; (4) glutathione peroxidase; and (5) peroxidases in the presence of hydrogen donors (Elstner, 1987). Catalase may be the most efficient mechanism because there is no requirement for glutathione or ascorbate; furthermore, there is no danger resulting from the formation of oxidized substrates by peroxidase (Figure 4).

Despite substantial catalase activity in the *H. zea* midgut, sufficient H_2O_2 for plant POD activity remains as indicated by the finding that approximately 25% of ingested rutin is oxidized and bound to excreted protein (J. Workman, G. Felton, and S. Duffey, unpublished data). Rutin is not a substrate for tomato foliar polyphenol oxidase. The presence of midgut POD activity may be a consequence of the great difference in apparent K_m values for H_2O_2 with catalase (i.e., millimolar range) vs POD (micromolar range, unpublished data), which would allow limited POD activity at low concentrations of H_2O_2. The potential

toxicity resulting from high levels of POD is apparently greater than that for polyphenol oxidase because of the wider array of POD substrates in tomato foliage including monophenols, diphenols, coumarins, ascorbates, and fatty acids (Table 3) (Garner, 1984). Whereas rutin is a substrate only for POD, chlorogenic acid is oxidized by both POD and polyphenol oxidase, and hence, a much greater percentage (ca. 45%) of ingested chlorogenic acid is bound to excreted protein (Felton et al., 1989).

Under certain conditions (e.g., low plant POD activity and/or low H_2O_2 concentration), the enzymatic oxidation of chlorogenic acid or rutin has minimal impact upon the nutritional quality of dietary protein (G. Felton, J. Workman, D. Sternberg, and S. Duffey, unpublished data). Hence, the presence of high catalase activity with resulting attenuated POD activity in the digestive system may be a desirable adaptation for overcoming dietary oxidative stresses. However, because of the large number of variables (e.g., substrates, reactants, competing enzymes), it is extremely difficult to meaningfully simulate conditions occurring at the plant–herbivore interface by artificial diet experiments. At best our bioassays represent only first order approximations of antibiosis.

It is worth noting that in certain herbivores, high levels of POD may be desirable because POD may function to simultaneously eliminate H_2O_2 and detoxify plant phenolics (Miles and Peng, 1989). We suggest that this may be limited to insects with an acidic pH in their digestive system (e.g., acridids) or those that form a salivary sheath while feeding on vascular tissues (e.g., aphidids). Under acidic conditions, oxidized plant phenolics do not bind covalently to amino acids but instead polymerize, forming nontoxic polymers (Felton et al., 1989; Duffey and Felton, 1989). Hence, damage to the nutritional quality of protein is avoided.

In addition to the diminution of H_2O_2 toxicity by catalase, this detoxicative enzyme may have other important functions. Recently, H_2O_2 has been implicated in transduction of defense signals in plants due to its exceedingly rapid production in cells following elicitor application and its ability to stimulate phytoalexin production (Apostol et al., 1989). Application of catalase to *Glycine max* plant cells significantly inhibited the formation of the phytoalexin glyceollin (Apostol et al., 1989). If these recent results prove to represent a generalized mechanism of defense signaling in plants, then the implications of our findings are more significant. If catalase is released in herbivore saliva during feeding, the transduction of certain defense signals in plant cells could be disrupted. Such disruption may offset, in part, the decline in leaf quality often observed following wounding by herbivores (e.g., Schultz and Baldwin, 1982).

In summary, the presence of catalase in the digestive system of herbivores may offer an extremely important protective mechanism against: (1) direct H_2O_2 toxicity; (2) the toxicity resulting from the formation of free radicals, quinones, and lipid oxidation products by peroxidases; (3) loss of nutrients such as amino

acids, linoleic acid, thiamine, and ascorbic acid; and (4) the induction of active plant defenses by interference with transduction of defense signals.

Acknowledgments—We thank Dr. J. Ruberson and Mr. J. Workman for careful review of the manuscript. This project was supported by USDA competitive grants awarded to SSD (87-CRC-1-2371) and SSD and GWF (89-37250-4639). The project was approved by the Director of the Arkansas Agricultural Experiment Station.

REFERENCES

AEBI, H. 1984. Catalase in vitro. *Methods Enzymol.* 105:121–126.

AHMAD, S., PRITSOS, C.A., BOWEN, S.M., KIRKLAND, K.E., BLOMQUIST, G.J., and PARDINI, R.S. 1987. Activities of enzymes that detoxify superoxide anion and related toxic oxyradicals in *Trichoplusia ni*. *Arch. Insect Biochem. Physiol.* 6:85–96.

AHMAD, S., PRITSOS, C.A., BOWEN, S.M., HEISLER, C.R., BLOMQUIST, G.J., and PARDINI, R.S. 1988a. Antioxidant enzymes of larvae of the cabbage moth, *Trichoplusia ni*: Subcellular distribution and activities of superoxide dismutase, catalase and glutathione reductase. *Free Rad. Res. Commun.* 4:403–408.

AHMAD, S., PRITSOS, C.A., BOWEN, S.M., HEISLER, C.R., BLOMQUIST, G.J., and PARDINI, R.S. 1988b. Subcellular distribution and activities of superoxide dismutase, catalase, glutathione reductase in the southern armyworm, *Spodoptera exigua*. *Arch. Insect Biochem. Physiol.* 7:173–186.

APOSTOL, I., HEINSTEIN, P.F., and LOW, P.S. 1989. Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. *Plant Physiol.* 90:109–116.

BERLETT, B.S., CHOCK, P.B., YIM, M.B., and STADTMAN, E.R. 1990. Manganese (II) catalyzes the bicarbonate-dependent oxidation of amino acids by hydrogen peroxide and the amino acid-dismutation of hydrogen peroxide. *Proc. Natl. Acad. Sci. U.S.A.* 87:389–393.

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

BROADWAY, R.M., and DUFFEY, S.S. 1986. The effect of dietary protein on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. *J. Insect Physiol.* 32:673–680.

BROADWAY, R.M., and DUFFEY, S.S. 1988. The effect of plant protein quality on insect digestive physiology and the toxicity of plant proteinase inhibitors. *J. Insect Physiol.* 34:1111–1117.

CADENAS, E. 1989. Biochemistry of oxygen toxicity. *Annu. Rev. Biochem.* 58:79–110.

CHEYNIER, V., BASIRE, N., and RIGAUD, J. 1989. Mechanism of *trans*-caffeoyltartaric acid and catechin oxidation in model solutions containing grape polyphenoloxidase. *J. Agric. Food Chem.* 37:1069–1071.

CHIPPENDALE, G.M. 1970. Metamorphic changes in fat body proteins of the southwestern corn borer *Diatraea grandiosella*. *J. Insect Physiol.* 16:1057–1068.

CHOUDHURI, M.A. 1988. Free radicals and leaf senescence—a review. *Plant Physiol. Biochem.* 15:18–29.

CILLIERS, J.J.L., and SINGLETON, V.L. 1989. Nonenzymic autoxidative phenolic browning reactions in a caffeic acid model system. *J. Agric. Food Chem.* 37:890–896.

DOW, J.A.T. 1984. Extremely high pH in biological systems: a model for carbonate transport. *Am. J. Physiol.* 246R:633–635.

DOWNUM, K.R., and RODRIGUEZ, E. 1986. Toxicological action and ecological importance of plant photosensitizers. *J. Chem. Ecol.* 12:823–834.

DUFFEY, S.S., and FELTON, G.W. 1989. Role of plant enzymes in resistance to insects, pp. 289–313, *in* J. Whitaker and P. Sonnet (eds.). Biocatalysis in Agricultural Biotechnology. American Chemical Society, Washington, D.C.

ELSTNER, E.F. 1987. Metabolism of activated oxygen species, pp. 253–315, *in* D.D. Davies (ed.). The Biochemistry of Plants, Vol. 11. Biochemistry of Metabolism. Academic Press, New York.

FELTON, G.W., DONATO, K., DEL VECCHIO, R.J., and DUFFEY, S.S. 1989. Activation of plant foliar oxidases by insect feeding reduces the nutritive quality of foliage for herbivores. *J. Chem. Ecol.* 15:2667–2694.

FRIDOVICH, I. 1989. Superoxide dismutases—an adaptation to a paramagnetic gas. *J. Biol. Chem.* 264:7761–7764.

GARNER, C.W. 1984. Peroxidation of free and esterified fatty acids by horseradish peroxidase. *Lipids* 19:863–868.

GASPAR, T., PENEL, C., THORPE, T., and GREPPIN, H. 1982. Peroxidases 1970–1980. Universite de Geneve-Centre de Botanique. 324 pp.

HALLIWELL, B., and GUTTERIDGE, J.M.C. 1984. The role of iron in oxygen radical reactions. *Methods Enzymol.* 105:47–56.

HANHAM, A.F., DUNN, B.P., and STICH, H.F. 1983. Clastogenic activity of caffeic acid and its relationship to hydrogen peroxide generated during autooxidation. *Mutat. Res.* 116:333–339.

HILDEBRAND, D.F., RODRIGUEZ, J.G., BROWN, G.C., LUU, K.T., and VOLDEN, C.S. 1986. Peroxidative response of leaves in two soybean genotypes injured by twospotted spider mites (Acari: Tetranychidae). *J. Econ. Entomol.* 79:1459–1465.

HILDEBRAND, D.F., RODRIGUEZ, J.G., LEGG, C.S., and BROWN, G.C. 1989. The effects of wounding and mite infestation on soybean leaf lipoxygenase levels. *Z. Naturforch.* 44:655–659.

JONES, C.G., HARE, J.D., and COMPTON, S.J. 1989. Measuring plant protein with the Bradford assay. 1. Evaluation and standard method. *J. Chem. Ecol.* 15:979–992.

JOSHI, P.C., and PATHAK, M.A. 1983. Production of singlet oxygen and superoxide radicals by psoralens and their biological significance. *Biochem. Biophys. Res. Commun.* 112:638–646.

KALYANARAMAN, B., PREMOVICH, P.I., and SEALY, R.C. 1987. Semiquinone anion radicals from addition of amino acids, peptides, and proteins to quinones derived from oxidation of catechols and catecholamines. *J. Biol. Chem.* 262:11080–11087.

KEPPLER, L.D. and NOVACKY, A. 1987. The initiation of membrane lipid peroxidation during bacteria-induced hypersensitive reaction. *Physiol. Mol. Plant Pathol.* 30:233–246.

MARTIN, J.S., MARTIN, M.M., and BERNAYS, E.A. 1987. Failure of tannic acid to inhibit digestion or reduce digestibility of plant protein in gut fluids of insect herbivores: Implications for theories of plant defense. *J. Chem. Ecol.* 13:605–621.

MATHEIS, G., and WHITAKER, J.R. 1984a. Modification of proteins by polyphenol oxidase and peroxidase and their products. *J. Food Biochem.* 8:137–162.

MATHEIS, G., and WHITAKER, J.R. 1984b. Peroxidase-catalyzed cross linking of proteins. *J. Protein Chem.* 3:35–48.

MATHEIS, G., and WHITAKER, J.R. 1987. A review: Enzymatic cross-linking of proteins applicable to foods. *J. Food Biochem.* 11:309–327.

MILES, P.W., and PENG, Z. 1989. Studies on the salivary physiology of plant bugs: Detoxication of phytochemicals by the salivary peroxidase of aphids. *J. Insect Physiol.* 35:865–872.

MOHRI, S., ENDO, Y., MATSUDA, K., KITAMURA, K., and FUJIMOTO, K. 1990. Physiological effects of soybean seed lipoxygenase in insects. *Agric. Biol. Chem.* 54:2265–2270.

PRITSOS, C.A., AHMAD, S., BOWEN, S.M., ELLIOT, A.J., BLOMQUIST, G.J., and PARDINI, R.S. 1988a. Antioxidant enzymes of the black swallowtail butterfly, *Papilio polyxenes*, and their response to the prooxidant allelochemical, quercetin. *Arch. Insect Biochem. Physiol.* 8:101–112.

PRITSOS, C.A., AHMAD, S., BOWEN, S.M., BLOMQUIST, G.J., and PARDINI, R.S. 1988b. Antioxidant enzyme activities in the southern armyworm, *Spodoptera exigua*. *Comp. Biochem. Physiol.* 90C:423–427.

RYAN, J.D., GREGORY, P., and TINGEY, W.M. 1982. Phenolic oxidase activities in glandular trichomes of *Solanum berthaultii*. *Phytochemistry* 8:1885–1887.

SCHULTZ, J.C., and BALDWIN, I.T. 1982. Oak leaf quality declines in response to defoliation by gypsy moth larvae. *Science* 217:149–151.

SHUKLE, R.H., and MURDOCK, L.L. 1983. Lipoxygenase, trypsin inhibitor, and lectin from soybeans: effects on larval growth of *Manduca sexta* (Lepidoptera: Sphingidae). *Environ. Entomol.* 12:787–791.

SLUMP, P., and SCHREUDER, H.A.W. 1973. Oxidation of methionine and cysteine in foods treated with hydrogen peroxide. *J. Sci. Food Agric.* 24:657–661.

STAHMANN, M.A., SPENCER, A.K., and HONOLD, G.R. 1977. Cross linking of proteins in vitro by peroxidase. *Biopolymers* 16:1307–1318.

STAMOPOULOS, D.C. 1988. Toxic effect of lignin extracted from the tegument of *Phaseolus vulgaris* seeds on the larvae of *Acanthoscelides obtectus* (Say) (Col., Bruchidae). *J. Appl. Entomol.* 105:317–320.

STEWART, R.R.C., and BEWLEY, J.D. 1980. Lipid peroxidation associated with accelerated aging of soybean axes. *Plant Physiol.* 65:245–248.

TULYATHAN, V., BOULTON, R.B., and SINGLETON, V.L. 1989. Oxygen uptake by gallic acid as a model for similar reactions in wines. *J. Agric. Food Chem.* 37:844–849.

VAN HUYSTEE, R.B. 1987. Some molecular aspects of plant peroxidase biosynthetic studies. *Annu. Rev. Plant Physiol.* 38:205–219.

WAINHOUSE, D., CROSS, D.J., and HOWELL, R.S. 1990. The role of lignin against the spruce bark beetle *Dendroctonus micans*: Effect on larvae and adults. *Oecologia* 85:257–265.

WHITMORE, F.W. 1982. Lignin-protein complex in cell walls of *Pinus elliotii*: Amino acid constituents. *Phytochemistry* 21:315–318.

YIM, M.B., BERLETT, B.S., CHOCK, P.B., and STADTMAN, E.R. 1990. Manganese (II)-bicarbonate-mediated catalytic activity for hydrogen peroxide dismutation and amino acid oxidation: Detection of free radical intermediates. *Proc. Natl. Acad. Sci. U.S.A.* 87:394–398.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

EFFECTS OF BIRCH PHENOLICS ON A GRAZING AND A BROWSING MAMMAL: A COMPARISON OF HARES

G.R. IASON[1,3,*] and R.T. PALO[1,2,4]

[1]*Grimsö Wildlife Research Station*
S-770 31 Riddarhyttan, Sweden

[2]*Department of Animal Physiology*
Swedish University of Agricultural Sciences
S-750 07 Uppsala, Sweden

(Received December 18, 1990; accepted April 26, 1991)

Abstract—Mountain hares (*Lepus timidus* L.) commonly have high proportions of birch (*Betula* spp.) in their winter diets, whereas European hares (*Lepus europaeus*) do not. The effects of a birch extract added to laboratory diets offered to mountain hares and European hares on the digestibility and sodium balance were measured. The extract added contained total phenolics equivalent in amount to that occurring in diets containing 0, 40, 60 and 80% dry matter of birch twigs. Increasing dietary phenolic concentration led to reduced voluntary food intake and apparent protein digestibility in both hare species. No effects on apparent dry matter digestibility were observed. The highest concentration of birch extract caused severe sodium losses via the urine by European hares but not by mountain hares. The effects of the birch extract within the digestive system appear to be similar in the two hare species, whereas the mountain hare appears to be better adapted to the toxic effects that disrupted sodium balance in the European hare. These preliminary results suggest that detoxification rather than digestive ability *per se* may contribute to different mammalian herbivores' feeding strategies.

Key Words—*Lepus timidus, Lepus europaeus, Betula pendula*, hares, birch, phenolic, digestion, chemical defense, sodium balance.

*To whom correspondence should be addressed.
[3]Present address: Macaulay Land Use Research Institute, Pentlandfield, Roslin, Midlothian EH25 9RF, U.K.
[4]Present address: Department of Wildlife Ecology, Swedish University of Agricultural Sciences, S-90183 Umeå, Sweden.

0098-0331/91/0900-1733$06.50/0

INTRODUCTION

Secondary plant compounds were originally named because they had no known function within the plant. In recent years, many studies have shown that certain secondary compounds or groups of them can influence herbivores' food selection or intake (Bryant, 1981; Tahvanainen et al., 1985; Iason and Waterman, 1988; Sinclair et al., 1988a). However, the theory of antiherbivore plant chemistry assumes that a chemical's action as an antifeedant is associated with a negative effect on the herbivore, either toxicity or a reduction in the herbivore's digestive ability (Rhoades, 1979). Members of a common and diverse group of secondary compounds, the phenolics, which includes different types of tannins and glycosides, show a range of antiherbivore effects. Phenolic plant constituents not only reduce *in vivo* digestibility (Lindroth et al., 1986; Sinclair et al., 1988b; Robbins et al., 1987a) but can also reduce the efficiency of nutrient utilization (Mole et al., in preparation). Both low- and high-molecular-weight phenolics can reduce ruminant in vitro digestibility (Risenhoover et al., 1985; Palo, 1985; Sunnerheim et al., 1988) and have been implicated as causing sodium loss in mountain hares, rabbits, and mice (Freeland et al., 1985; Palo and Knutsson, 1987; Palo et al., 1983, 1989). It is paradoxical that despite this array of possible negative effects, many herbivores continue to ingest large amounts of forages which contain phenolics.

Mammalian herbivores may be classified along a continuum from concentrate selectors, many of which utilize woody plant tissue, the browsers, to a less selective grazing or bulk-feeding strategy (Van Soest, 1982; Hofmann, 1989). Browsers feeding on leaves, twigs, and buds of woody shrubs or trees ingest more phenolics than the grazers, which take more graminoids, few of which contain phenolics in their vegetative parts (Jung et al., 1979; Rhoades and Cates, 1976; Hagerman and Butler, 1989). The browsers are hypothesized to be able to counter any detrimental effects of phenolics, either by bearing the costs of the toxic effect, the detoxification process, or the reduction in digestibility. They may also possess counteradaptations to nullify such effects, e.g., by secretion of tannin-binding salivary proteins (Robbins et al., 1987b). The present study aims to compare the effects of phenolic extracts from one plant species, the silver birch (*Betula pendula*) on two mammalian herbivores with contrasting feeding strategies.

An appropriate pair of mammalian herbivores for the comparison are the mountain hare (*Lepus timidus*) and the European hare (*Lepus europaeus*). These species are congeneric and have a cecal fermentation digestive system. In the northern temperate latitudes where their distribution overlaps, they are of similar body size and life-history strategy (Flux, 1981; Swihart, 1984). However, their feeding strategies are quite different; the mountain hare is a summer grazer reverting to woody browse in the winter (Lindlöf et al., 1974; Iason and Water-

man, 1988). The European hare also eats graminoids and herbs in summer (Seiskari, 1963; Helminen et al., 1966) but continues to do so in winter (Hyytinen, 1974). In boreal and arctic ecosystems, including at the northern limit of the distribution of European hares in Fennoscandia (see Lindström et al., 1989), the birch is an important food source to mountain hares but not European hares (Hyytinen, 1974; Barikmo, 1976). The constituents of extracts of *Betula pendula* are well characterized both chemically (Palo, 1984; Sunnerheim et al., 1988) and in terms of their effects on lagomorphs in which they cause sodium imbalance (Palo et al., 1983; Palo and Knutsson, 1987). Birch extracts have also been shown to reduce the *in vivo* digestibility of dry matter and protein in snowshoe hares (Sinclair et al., 1988b). The specific aims of this study are to compare the effect of a phenolic birch extract on the digestion and sodium retention of mountain hares and European hares. This approach should permit the elucidation of differences in digestion and sodium metabolism between them and identification of possible adaptations of browsing hind-gut fermenters to phenolic rich diets.

METHODS AND MATERIALS

Twigs were collected between April 7 and April 28, 1987, from previously unbrowsed shoots of *Betula pendula* around the Grimsö research station in southern-central Sweden. This was milled to approximately 5-mm particles and 1 kg aliquots were soaked for 1–2 hr in 6 liters 96% ethanol, after which time it was homogenized for 2 × 10 min. The ethanolic extract was decanted and filtered through a No. 3 filter (Munktells Swedish). A volume of 96% ethanol equivalent to that originally decanted was added to the residue. This procedure was repeated and the extracts pooled. The solvent was evaporated in a rotary evaporator and the residue concentrated. The residue was then redissolved in ethanol and applied as a fine spray in an enclosed space to commercially available pelleted animal food (Gödför Nötför, Lantmännen, Örebro; Energy 13.2 MJ/kg dry matter; crude protein 15.8% dry matter; neutral detergent fiber 19.6% dry matter).

The concentration of the ethanol-diluted extract was quantitatively adjusted such that the amount of total phenolics added was equivalent to that predicted to be found in a diet consisting of 40, 60, or 80% dry matter of fine (1-mm-diameter) birch twigs. Total phenolics were defined as those measured by the Folin-Ciocalteau assay and the predicted concentration in 1-mm-diameter birch twigs was according to Palo (1985). Control pellets were prepared with an equivalent volume of 96% ethanol. All pellets were dried to constant weight in an air draft at 30°C.

Three adult mountain hares, one nonreproducing female and two males,

and two adult male European hares were individually housed in large cages equipped with a device for the immediate separation and collection of urine and feces. The experiment was conducted in July–August 1987. After two weeks of feeding on only untreated pellets, they were offered the pelleted diets treated with ethanol only or 40, 60, or 80% phenolics. Each diet was offered to each hare for three days. This period was considered long enough to observe measurable effects on sodium balance (Palo and Knutsson, 1987) and digestibility. Reichardt et al. (1984) began fecal collections on the third day of treatment. The intention of this study was to compare only the short-term effects of phenolics without confounding experimental results with long-term adaptation that may occur at different rates in the two species. Between each of these treatments, at least three days of only untreated pellets were given; hence the basal diet was consistent throughout the whole experiment and long-term adaptation to phenolics was prevented.

Water was freely available throughout the experiments. Total collections of urine and feces were made, and hares were weighed daily. The hares were not trained to use laboratory drinking bottles and two of the individuals persistently tipped over their water supply; hence urine volume produced could not be determined precisely for these hares. Feces and food samples were air dried at 40°C and milled to pass a 1-mm mesh. Nitrogen in food and feces was determined colorimetrically with a Technicon autoanalyzer and converted to protein concentration ($\times 6.25$). Sodium was determined in food, feces, and urine samples by flame photometry. Apparent digestibility of protein or dry matter was calculated on a daily basis as the difference between the amounts consumed and excreted in the feces, expressed as a percentage of the amount consumed.

For food intakes, digestibility, and sodium balance, each variable was analyzed using a three-way factorial analysis of variance (Winer, 1971) with species, phenolic treatment, and day of measurement as fixed factors; individuals were treated as replicates. Only measurements on the second and third days of each treatment were included, although the intake on all three days of the treatments was also analyzed and presented separately to investigate possible aversion to the new food types.

RESULTS

Food Intake. When phenolic extracts were added to the pellets at 40, 60, and 80% levels, there was a tendency, although not significant, for a lower intake on day 1 than days 2 and 3 of the treatments (day 1: 30.07 g/kg/day; day 2: 34.11 g/kg/day; day 3: 33.09 g/kg/day, $F = 2.92$, $df = 2,27$, $P < 0.1$). The aversion to the phenolic treated pellets was particularly marked for the 80% phenolic treated pellets (day 1: 22.91 g/kg/day; day 2: 31.29 g/kg/

day; day 3: 33.42 g/kg/day; $F = 4.76$, $df = 2,9$, $P < 0.05$); there was no significant variation in food intake between days of the control treatment ($F = 0.26$, $df = 2,9$, NS).

Food intake varied significantly between treatments ($F = 4.481$, $df = 3,24$, $P < 0.05$), decreasing progressively with increasing dietary phenolic concentration (Figure 1). Food intakes on 60% and 80% phenol treatments were significantly lower than the control diet ($P < 0.05$, Fisher's protected LSD). Intake did not differ between species (mountain hares: 36.29 g/kg/day; European hares: 37.18 g/kg/day; $F = 0.155$, $df = 1,24$,NS) or between days 2 and 3 ($F = 0.008$, $df = 1,24$, NS).

Apparent Digestibility. Although the second and third successive days of treatment were included separately in all statistical analyses of digestibility, there were no differences between days for any of the digestibility measurements; hence data for different days are pooled.

Mean digestibility of dry matter was reduced when the birch phenolic extract was added to the pellets as compared to the control pellets, with the exception of the 80% treatment for European hares (Figure 2A). However, the errors associated with these measurements were large and dry matter digesti-

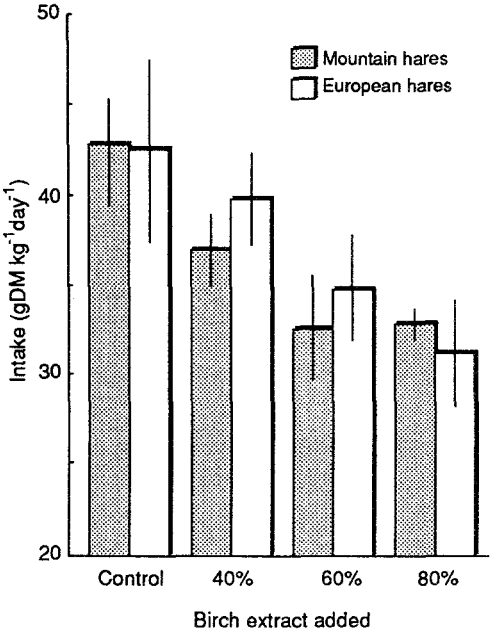


FIG. 1. The voluntary food intake by mountain hares and European hares offered control (solvent treated) commercial animal pelleted diet or the pellets treated with the phenolic birch extract equivalent to 40, 60, or 80% dry matter of birch (means ± SE).

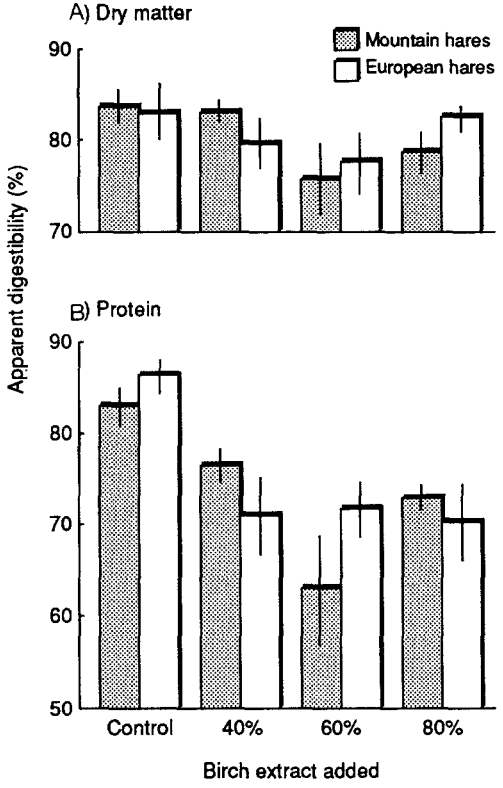


FIG. 2. The apparent digestibility of (A) dry matter and (B) protein of the artificial diet treated with increasing amounts of birch extract (mean ± SE).

bility did not vary significantly with phenolics added to the diet ($F = 1.79$, $df = 3,24$, NS); nor did it differ between species ($F = 0.03$, $df = 1,24$, NS).

Protein digestibility did not vary between species (mountain hares: 74.3%, European hares 75.2%, $F = 0.118$, $df = 1,24$, NS) but was significantly different between phenol treatments ($F = 6.266$, $df = 3,24$, $P < 0.01$); all levels of phenol treatment had lower protein digestibility than the control diet ($P < 0.05$, Fisher's protected LSD, Figure 2B).

Sodium Balance. Previous studies on domestic rabbits showed that phenolic-induced sodium imbalance occurred rapidly on the first day of treatment (Palo and Knutsson, 1987). A preliminary analysis of the data showed that this was also the case for European hares; on each of the three phenolic-treated diets, sodium balance on day 1 was negative. On days two and three this trend was reversed on the 40 and 60% phenol-treated diets, but it was reinforced, becoming even more strongly negative on days 2 and 3, when the 80% phenol-

treated diet was offered to European hares. The analyses reported here include only sodium balances on days 2 and 3 of treatments, which did not differ from each other ($F = 2.675$, $df = 1,24$, NS). The sodium balance of mountain hares was consistently positive regardless of treatment, whereas the European hares' sodium balance was around zero or was positive in all treatments except the 80% phenolic treatment when it was strongly negative (Figure 3). Although the species × treatment interaction was not significant ($F = 2.641$, $df = 3,24$, $P < 0.1$), the sodium balance was significantly different between treatments ($F = 3.726$, $df = 3,24$, $P < 0.05$) and overall was negative for European hares (−0.013 mmol/kg/day) but not mountain hares (1.233 mmol/kg/day) ($F = 9.828$, $df = 1,24$, $P < 0.01$). On average, only 1.7% of total sodium excreted was via the feces, 98.3% was excreted in the urine.

The mean weight changes of the hares were slight and not different between species (mountain hares: +0.3%/day; European hares: +0.5%/day, $F = 0.058$, $df = 1,24$, NS) or between treatments (control: −1.5%/day; 40% pheonlics: + 1.8%/day; 60% phenolics: −0.4%/day; 80% phenolics: +1.1%/day, $F = 2.726$, $df = 3,23$, NS). There was no correlation between weight change and sodium balance (mountain hares: $r = 0.16$, $df = 22$, NS; European hares: $r = 0.19$, $df = 14$, NS).

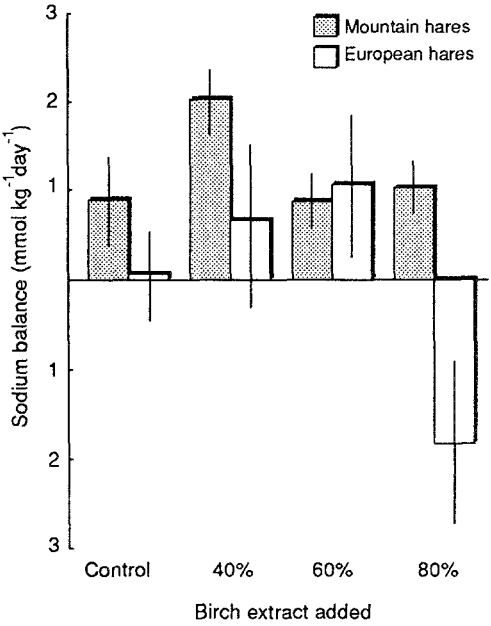


FIG. 3. The sodium balance of mountain and European hares fed with artificial diet treated with increasing amounts of birch extract (mean ± SE).

DISCUSSION

The theory of antiherbivore plant chemistry assumes the avoidance or reduced intake of a plant by a herbivore is due to the plant secondary compounds exerting some harmful effect on the animal (Rhoades, 1979). This study confirms others, which have shown that extracts of birch trees reduce the digestibility of food by hares (Sinclair et al., 1988b). Although phenolics have the property of complexation with both proteins and polysaccharides (Swain, 1979), the extract of birch used in this experiment inhibited only protein digestibility; total dry matter digestibility was unaffected. Some fractions of birch extracts have been shown to reduce organic matter digestibility in *in vitro* rumen systems (Palo et al., 1985; Sunnerheim et al., 1988). Sunnerheim et al. (1988) showed that polyphenolics had only a slight depressive effect on *in vitro* organic matter digestibility and that a low-molecular-weight fraction actually increased digestibility. Another *in vitro* study suggested that tannins may increase the digestibility of proteins (Mole and Waterman, 1985). However, the results here show that even at relatively low concentrations the *in vivo* effects of birch phenolics were to reduce the digestibility of proteins. The strength of this effect, combined with the absence of a reduction in *in vivo* dry matter digestibility suggests that in cecal fermenters the most significant antidigestive effect of birch phenolics is via their association with proteins. It suggests that interactions with plant cell wall constituents or any general antibiotic effect against the digestive system microorganisms are less important. Palo (1987) suggested that the type of digestive system of a herbivore, e.g., foregut or hindgut fermentation, will govern the pathway and action of certain plant secondary metabolites. In hindgut fermenters such as hares, it is more likely that low-molecular-weight compounds are rapidly absorbed prior to the site of microbial fermentation, whereas in foregut fermenters they might be transformed to a greater extent. Their action in the digestive tract would thus be relatively less.

The effect of high-molecular-weight plant chemical substances, such as tannins, on digestion might be expected to be similar in the two hare species, as a result of their similar digestive systems. However, differences in specific digestive physiological functions have been shown to exist between species with different feeding strategies but that have the same digestive system, e.g., qualitative differences of saliva produced by browsing and grazing ruminants that permit superior digestion of tannin-rich diets by the browsers (Robbins et al., 1987b). No differences were detected between the two hare species in the apparent digestibility of any of the dietary constituents measured. However, this does not preclude the possibility that differences in ability to digest different food components may develop given longer periods of exposure to the birch extract than permitted by this experiment. In contrast, there was a marked difference between the mountain and European hares in the effect of birch phenolics on

sodium balance. The sodium balance was strongly negative in European hares on the 80% phenol treatment, primarily due to a greatly increased urinary excretion, while no effect was observed in mountain hares. Sodium balance was not merely a correlate of weight loss, since there was no association between them in either species of hare. Previous studies of mountain hares and snowshoe hares (*Lepus americanus*) have found that a diet of pure narrow-clipped birch twigs, fed in winter, induces net sodium loss and loss of weight (Pehrson, 1983; Reichardt et al., 1984). This was not observed in mountain hares in this study, probably because the highest concentration of phenolics used was lower than that in a diet of pure birch twigs and, secondly, the basal diet was considerably more energy-rich than a twig diet. Furthermore, this experiment was conducted in summer with nonreproductive adults, circumstances that again may have led to a favorable energy balance that permitted any costs of detoxification to be met. Nevertheless, a significantly higher sodium excretion that increased with phenolic concentration of the diet was observed in European hares. This indicates a relatively lower tolerance of birch phenolics by this species as compared to mountain hares.

We conclude that although birch phenolics depressed apparent protein digestibility, they did so equally in both mountain and European hares. Apparent digestibility of dry matter was not significantly affected in either species. This experiment provides no support for the hypothesis that the reported differences in feeding strategy between the two species are associated with differences in the effects of phenolic secondary compounds on their digestive ability. We cannot, however, dismiss the possibility that longer-term adaptation may be different in the two species. In contrast, with only relatively short-term exposure to concentrations of birch phenolics equivalent to that in a diet of 80% birch, the sodium balance of European hares was severely impaired whereas that of mountain hares was unaffected. It is hypothesized that some component of the birch extract interfered with a systemic metabolic process or possibly kidney regulation of sodium balance, since most sodium is excreted via the urine. A parallel study showed that the extent and nature of biotransformation and urine excretion of catabolites of platyphylloside, a major birch phenolic constituent (Sunnherheim et al., 1988), also varied between the two hare species (Palo et al., 1989). The preliminary results reported here, lead to the hypothesis that metabolic differences and toxic effects, rather than differences solely in digestive function, may underlie the categorization of mammalian herbivores into those that commonly eat phenolic-rich foods (browsers) and those that do not (grazers).

Acknowledgments—This work was supported by a Royal Society European Exchange Fellowship to G.R.I. and a grant from the Swedish Council for Forestry and Agricultural Research to R.T.P. We are grateful to Åke Pehrson for advice and discussion, Emilia Fuchs for laboratory help, and Iain Gordon and two anonymous referees for comments.

REFERENCES

BARIKMO, J. 1976. Harens utnyttjelse av bjork som vinterfode. Hovedoppgave ved Institut for naturfovaltning. NHL PhD dissertation. As, Norway.

BRYANT, J.P. 1981. The regulation of snowshoe hare feeding behaviour during winter by plant anti-herbivore chemistry, pp. 720–731, *in* K. Myers and C.D. McInnes (eds.). Proceedings of the World Lagomorph Conference, Guelph, IUCN, Gland, Switzerland.

FLUX, J.E.C. 1981. Reproductive strategies in the genus *Lepus*, pp. 155–173, *in* K. Myers and D.C. McInnes (eds.). Proceedings of the World Lagomorph Conference, Guelph, Gland, Switzerland.

FREELAND, W.J., CALCOTT, P.H., and GEISS, D.P. 1985. Allelochemicals, minerals and herbivore population size. *Biochem. Syst. Ecol.* 13:195–206.

HAGERMAN, A.E., and BUTLER, L.G. 1989. Choosing appropriate methods and standards for assaying tannin. *J. Chem. Ecol.* 15:1795–1810.

HELMINEN, M., VALANNE, K., PIRKOLA, A., and STEN, I. 1966. Summer feeding habits of the mountain hare and of the brown hare (Finnish with English summary). *Suomen Riista* 18:133–144.

HOFMANN, R. 1989. Evolutionary steps of ecophysiological adaptation and diversification of ruminants: A comparative view of their digestive system. *Oecologia* 78:443–457.

HYYTINEN, T. 1974. Winter nutrition of the brown hare (*Lepus europaeus* Pallas) in western central Finland (Finnish with English summary). *Suomen Riista* 25:42–49.

IASON, G.R., and WATERMAN, P.G. 1988. Avoidance of plant phenolics by juvenile and reproducing female mountain hares in summer. *Funct. Ecol.* 2:433–440.

JUNG, H.-J.G., BATZLI, G.O., and SIEGLER, D.S. 1979. Patterns in the phytochemistry of arctic plants. *Biochem. Syst. Ecol.* 7:203–209.

LINDLÖF, B., LINDSTRÖM, E., and PEHRSON, Å. 1974. On the activity, habitat selection and diet of the mountain hare (*Lepus timidus*) in winter. *Viltrevy* 9:27–42.

LINDROTH, R.L., BATZLI, G.O., and AVILDSEN, S.I. 1986. *Lespedeza* phenolics and *Penstemon* alkaloids: Effects on digestion efficiencies and growth of voles. *J. Chem. Ecol.* 12:713–728.

LINDSTRÖM, E., ANGELSTAM, P., and WIDÉN, P. 1989. Niche separation in hares—habitat, climate and predation. XVI International Congress on Game Biology, 1983. Strblske Pleso.

MOLE, S., and WATERMAN, P.G. 1985. Stimulatory effects of tannins and cholic acid on tryptic hydrolysis of proteins: Ecological implications. *J. Chem. Ecol.* 11:1323–1331.

MOLE, S., ROGLER, J.C., MORRELL, C.J., and BUTLER, L.G. 1990. Herbivore growth reduction by tannins: Use of Waldbauer ratio techniques and manipulation of salivary protein production to elucidate mechanisms of action. *Biochem. Syst. Ecol.* 18:183–198.

PALO, R.T. 1984. Distribution of birch (*Betula* spp.), willow (*Salix* spp.), and poplar (*Populus* spp.) secondary metabolites and their potential role as chemical defense against herbivores. *J. Chem. Ecol.* 10:499–520.

PALO, R.T. 1985. Chemical defense in birch: Inhibition of digestibility in ruminants by phenolic extracts. *Oecologia* 68:10–14.

PALO, R.T. 1987. Chemical defense in a woody plant and the role of digestive systems of herbivores, pp. 103–107, *in* F.D. Provenza, J.T. Flinders, and E.D. McArthur (eds.). Inter-mountain Research Station Gen Tech Rep Int-222.

PALO, R.T., and KNUTSSON, P.-G. 1987. Possible effects of birch phenols on sodium retention in the rabbit, pp. 47–50, *in* M. Rose (ed.). Herbivore Nutrition Research. Australian Society of Animal Production, Brisbane.

PALO, R.T., PEHRSON, Å., and KNUTSSON, P.-G. 1983. Can birch phenolics be of importance in the defense against browsing vertebrates? *Finn. Game Res.* 41:77–80.

PALO, R.T., SUNNERHEIM, K., and THEANDER, O. 1985. Seasonal variation of phenols, crude protein and cell wall content of birch (*Betula pendula*) in relation to ruminant in vitro digestibility. *Oecologia* 65:314–318.

PALO, R.T., IASON, G.R., and KNUTSSON, P.-G. 1989. Phenols as defensive compounds in birch (*Betula* spp.): Metabolism of platyphylloside in goats, rabbits and hares, pp. 939–940, *in* Proceedings, XVI International Grasslands Congress. Nice.

PEHRSON, Å. 1983. Digestibility and retension of food components in caged mountain hares (*Lepus timidus*) during the winter. *Holarct. Ecol.* 6:395–403.

REICHARDT, P.B., BRYANT, J.P., CLAUSEN, T.P., and WIELAND, G.D. 1984. Defense of winter-dormant Alaska paper birch against snowshoe hares. *Oecologia* 65:58–69.

RISENHOOVER, K.L., RENECKER, L.A., and MORGANTINI, L.E. 1985. Effects of secondary metabolites from balsam poplar and paper birch on cellulose digestion. *J. Range Manage.* 38:370–372.

RHOADES, D.F. 1979. Evolution of plant chemical defense against herbivores, pp. 3–54, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

RHOADES, D.F., and CATES, R.G. 1976. A general theory of plant antiherbivore chemistry, pp. 168–213, *in* J.W. Wallace and R.L. Mansell (eds.). Biochemical interaction between plants and insects, Recent Advances in Phytochemistry, Vol. 10. Plenum, New York.

ROBBINS, C.T., HANLEY, T.A., HAGERMAN, A.E., HJELJORD, O., BAKER, D.L., SCHWARTZ, C.C. and MAUTZ, W.W. 1987a. Role of tannins in defending plants against ruminants: Reduction in protein availability. *Ecology* 68:98–107.

ROBBINS, C.T., MOLE, S., HAGERMAN, A.E., and HANLEY, T.A. 1987b. Role of tannins in defending plants against ruminants: Reduction in dry matter digestion. *Ecology* 68:1606–1615.

SEISKARI, P. 1963. Summer food of the snow hare (Finnish with English summary). *Suomen Riista* 16:46–55.

SINCLAIR, A.R.E., JOGIA, M.K., and ANDERSON, R.J. 1988a. Camphor from juvenile white spruce as an antifeedant for snowshoe hares. *J. Chem. Ecol.* 14:1505–1514.

SINCLAIR, A.R.E., KREBS, C.J., SMITH, J.N.M., and BOUTIN, S. 1988b. Population biology of snowshoe hares. III Nutrition, plant secondary compounds and food limitation. *J. Anim. Ecol.* 57:787–806.

SUNNERHEIM, K., PALO, R.T., THEANDER, O., and KNUTSSON, P.-G. 1988. Chemical defense in birch. II Platyphylloside, a phenol from *Betula pendula* inhibiting digestibility. *J. Chem. Ecol.* 14:549–561.

SWAIN, T. 1979. Tannins and lignins, pp. 657–683, *in* G.A. Rosenthal and E.D. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

SWIHART, R.K. 1984. Body size, breeding season length and life history tactics of lagomorphs. *Oikos* 43:282–290.

TAHVANAINEN, J., HELLE, E., JULKUNEN-TIITTO, R., and LAVOLA, A. 1985. Phenolic compounds of willow bark as deterrents against feeding by mountain hares. *Oecologia* 65:319–323.

VAN SOEST, P.J. 1982. Nutritional Ecology of the Ruminant. O and B Books Inc., Corvallis, Oregon.

WINER, B.J. 1971. Statistical Principles in Experimental Design. McGraw-Hill, New York, 907 pp.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

METABOLICALLY BLOCKED ANALOGS OF HOUSEFLY SEX PHEROMONE: I. SYNTHESIS OF ALTERNATIVE SUBSTRATES FOR THE CUTICULAR MONOOXYGENASES

BACHIR LATLI and GLENN D. PRESTWICH*

Department of Chemistry
State University of New York
Stony Brook, New York 11794-3400

(Received January 15, 1991; accepted April 26, 1991)

Abstract—Cuticular monooxygenases in the cuticle of the female housefly *Musca domestica* oxidize (*Z*)-9-tricosene to *cis*-9,10-epoxytricosane and to (*Z*)-9-tricosen-14-one. One possible explanation for these two products is that a single monooxygenase accepts the alkene substrate in two different orientations. Eleven analogs of (*Z*)-9-tricosene bearing methyl substituents, cyclopropyl groups, fluorine substituents, deuterium substituents, and additional double bonds were synthesized to probe the substrate requirements of this monooxygenase system. In addition, the 11 corresponding epoxides were prepared to aid in identification of the metabolites of each modified alkene.

Key Words—*Musca domestica*, housefly, Diptera, Muscidae, monooxygenase, (*Z*)-9-tricosene analog, *cis*-9,10-epoxytricosane, (*Z*)-9-tricosen-14-one, synthesis, cyclopropyl, inhibition, isotope effect, fluorination, epoxidation.

INTRODUCTION

The sex pheromone of the housefly *Musca domestica* was first demonstrated by Rogoff et al. (1964) and was identified as (*Z*)-9-tricosene and called muscalure (Carlson et al., 1971). It was shown that this compound attracts males in an olfactometer at doses as low as 0.07 μg (Adams et al., 1984). In a study to determine the effects of structural variation of (*Z*)-9-tricosene on activity (Carlson et al., 1974), a variety of compounds with different double bond positions

*To whom correspondence should be addressed.

0098-0331/91/0900-1745$06.50/0

and geometry or even with a triple bond and with methyl branching at different points in the molecule or with different carbon chain and length of carbon chain linked to the unsaturated bond were synthesized and tested in an olfactometer and a pseudofly test. The structural requirements for activity were found to be not very stringent. *cis*-9,10-Epoxytricosane and (*Z*)-14-tricosen-10-one, along with a series of C_{28}–C_{30} methyl alkanes, are also components of the pheromone. Each of these components has a precise role; (*Z*)-9-tricosene increases male mating strike activity toward females and other males. The epoxide and ketone decrease the number of homosexual mating strikes, thus acting as a sex recognition factor. The methyl alkanes act as arrestants and increase the length of time that a male spends with a treated model (Adams and Holt, 1987).

Newly emerged males and females have similar cuticular lipid profiles; the major hydrocarbon components are (*Z*)-9-alkenes of 27 carbons and longer. From stages 4 through 8 (vitellogenic stages of the female), both the percentage of alkenes and the percentage of (*Z*)-9-heptacosene in the alkene fraction decrease while the percentage of (*Z*)-9-tricosene increases. Since (*Z*)-9-tricosene (I) is readily converted to both the epoxide (II) the ketone (III) (Figure 1) (Blomquist et al., 1984), the increase of (*Z*)-9-tricosene is mirrored by a concomitant increase in the C_{23} epoxide and ketone.

The cytochrome P-450 monooxygenases present in housefly cuticle have been found to be responsible for the conversion of (*Z*)-9-tricosene into 9,10-epoxide and to 14-keto metabolites (Ahmad et al., 1987). These reactions correspond to oxygen insertion into an alkene pi electron system and to oxygen insertion into an alkane C—H bond, respectively. Formation of the ketone requires a second oxidation of a carbinol C—H bond, presumably giving the hydrate of the ketone. An alternative is the existence of a specific alcohol dehydrogenase that rapidly converts all alcohol formed to the ketone (cf. Prestwich,

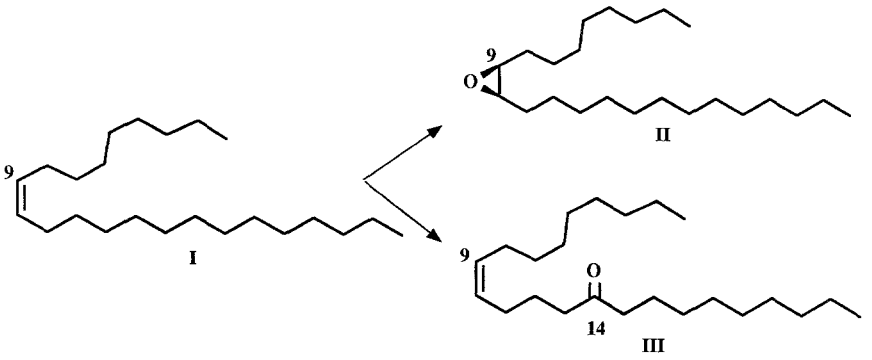


FIG. 1. Oxidation of (*Z*)-9-tricosene by cuticular polysubstrate monooxygenase of the housefly.

1987). It seems likely that both epoxide and ketone metabolite can arise by the action of a single monooxygenase; the different products would result from different orientations of the substrate in the active site (Figure 2).

To explore these possibilities, we synthesized a selection of compounds (and their expected metabolites) possessing modifications in the 14 position and in the alkyl chains of (*Z*)-9-tricosene (Figures 3 and 4). First, an additional alkene was introduced in the chain or as an *exo* methylene at C-14, providing an alternative site of epoxidation (compounds **1a, 2a**). Second, the C-14 position was blocked with only methyl to allow observation of the expected alcohol intermediate in ketone production (compounds **3a, 4a**). Third, the C-14 position was blocked with two methyl groups or with a spiro-fused cyclopropane ring, producing compounds resistant to oxidation at this site, yet still capable of 9,10-epoxidation (compounds **5a, 6a**). Fourth, the C-14 position was blocked with either one or two fluorine atoms, thereby altering the C—H oxidation (compounds **7a, 8a**). Fifth, one or two deuterium atoms were introduced at C-14 with the goal of retarding the second C—H oxidation and to permit measurement of kinetic isotope effect (compounds **9a, 10a**). Sixth, a perfluorooctyl chain was used to replace the octyl chain to probe the importance of hydrophobicity of the alkyl chain and of the nucleophilicity of the olefin on the epoxidation reaction (compound **11a**). Finally, the metabolism of these analogs in vivo was investigated and will be described in the following paper in this issue (Guo et al., 1991).

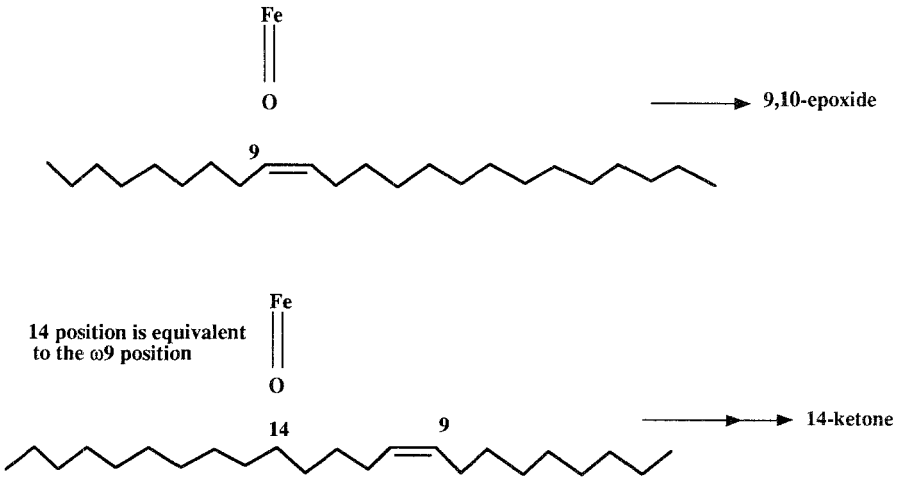


FIG. 2. Proposed model for monooxygenase action, showing the putative equivalency of the oxidations at C-14 and C-9.

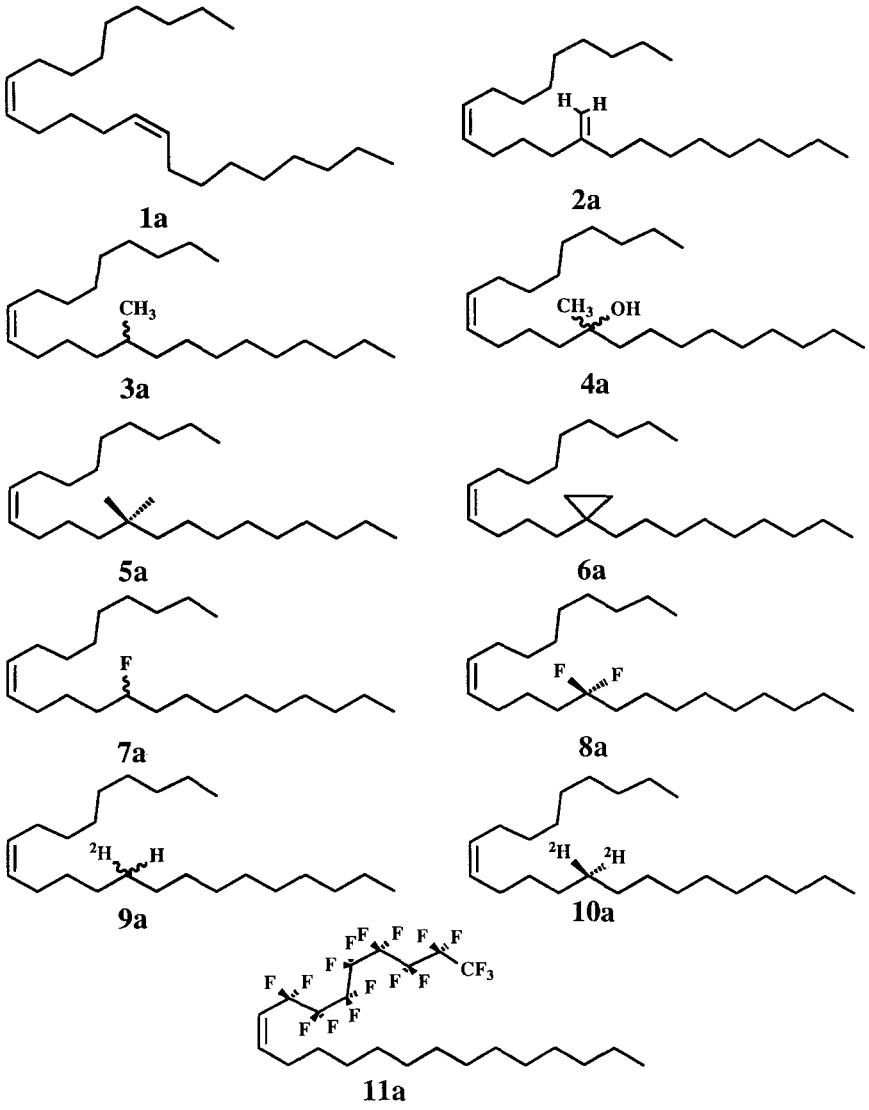


FIG. 3. Structures of the pheromone analogs prepared for biochemical experiments.

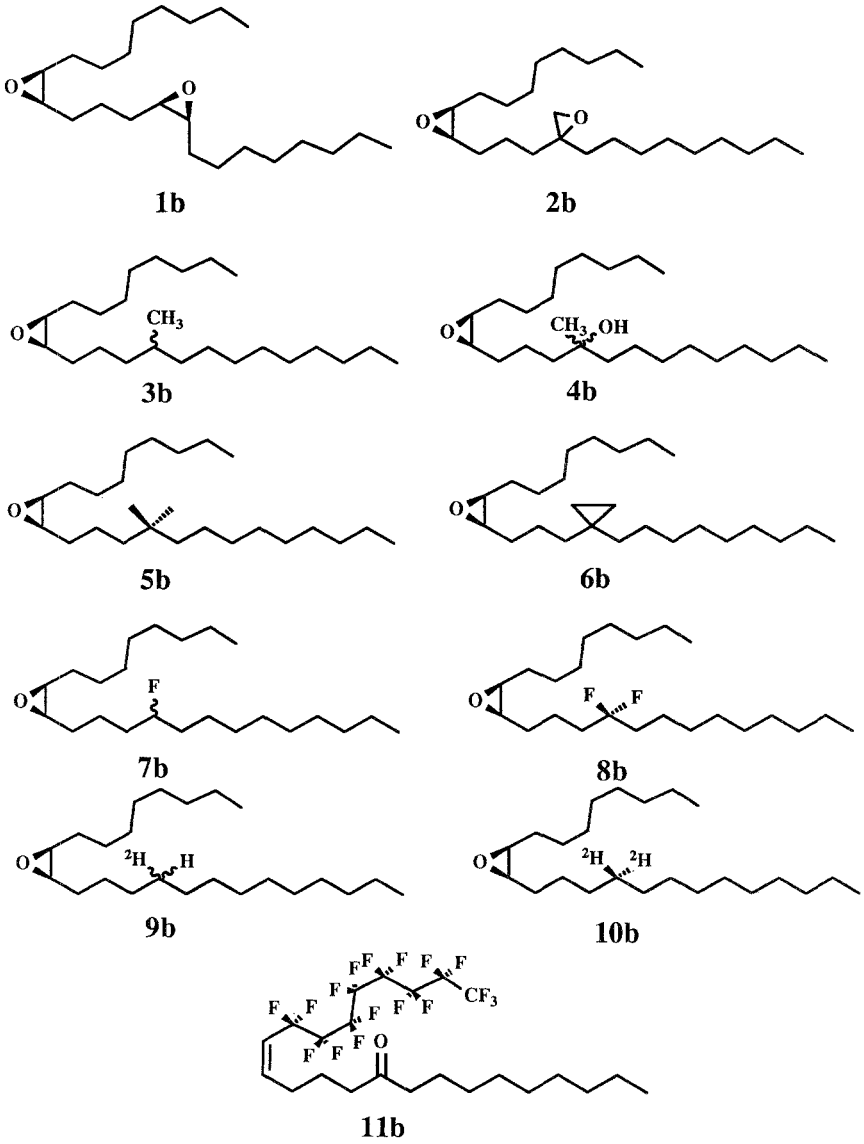


FIG. 4. Structures of the epoxides of the pheromone analogs prepared to verify metabolic processing.

METHODS AND MATERIALS

General

Air-sensitive materials and reactions were employed using standard techniques. All glassware was dried in the oven or with a flame. The glassware and syringes were assembled hot and allowed to cool under nitrogen atmosphere. Reactions were carried out under nitrogen atmosphere unless otherwise indicated. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were distilled from sodium benzophenone under nitrogen in a recirculating still, with a deep purple or blue color maintained in the distillation pot. Methylene chloride, HMPA, and triethylamine were distilled from calcium hydride (CaH_2). Benzene and toluene were dried by azeotropic distillation and stored over activated 3 Å molecular sieves. Ethyl acetate (EA) and hexane (H) were purchased from Fisher and distilled before use. Starting materials were obtained from commercial suppliers and were used without further purification, unless otherwise noted. All solvent transfers were accomplished using syringe or double-ended needle technique. "Isolated as usual" indicates that the organic extractions were combined, washed with aq. $NaHCO_3$, satd. NaCl, dried over $MgSO_4$, filtered, and concentrated in vacuo at room temperature (rt). Silica gel (SiO_2) chromatography was performed using 30 to 63-μm "flash" grade SiO_2 under nitrogen pressure.

Apparatus

Infrared (IR) spectra were obtained with a Perkin Elmer 1600 series FT-IR as neat liquid films or as solutions in carbon tetrachloride (CCl_4). UV spectra were recorded on a Perkin Elmer Lambda 5 UV/VIS spectrophotometer. Analytical gas chromatography (GC) was carried out on a Varian model 3700 chromatograph equipped with FID and using He as the carrier gas. The following columns were used: DB-5 (30 m $\times$ 0.25 mm) or DB-5 Megabore (15 m $\times$ 0.5 mm) (both J & W Scientific). The retention times were reported using the following program T_i = 50°C, T_f = 250°C, T_p = 10°C/min, with a delay at T_i for 2 min, unless otherwise indicated. Thin-layer chromatography (TLC) was performed with MN Polygram Sil G/UV 254 (4 $\times$ 8 cm). Visualization was accomplished by UV light, iodine, an ethanol–vanillin–H_2SO_4 solution, or an ethanol–phosphomolybdic acid solution, R_f = retention factor. Flash chromatographic purifications were carried out on Woelm Silica 32–63 μm or on alumina Woelm N-Super 1, using an ethyl acetate–hexane (EA/H) solution unless otherwise indicated. Fraction distillation or bulb to bulb Kugelrohr distillation at reduced pressure was also used for the purification of the crude products. Mass spectra (MS) were obtained at 70-eV ionization potential [^{1}H]NMR spectra were recorded at 300 MHz on a QE 300: s, singlet; d, doublet; dd,

doublet of doublets; t, triplet; q, quartet; m, multiplet; br, broadened. [^{13}C]NMR spectra were obtained at 75 MHz with off-resonance decoupling [^{19}F]NMR and [^{2}H]NMR spectra were recorded on an NT-300 using trichlorofluoromethane ($CFCl_3$) as an internal standard in C_6D_6 in case of [^{19}F]NMR and CCl_4 plus one drop of $CDCl_3$ for [^{2}H]NMR. Chemical shifts (ϕ) were expressed as parts per million (ppm) upfield from $CFCl_3$ (ϕ = 0 ppm) for fluorinated compounds. All other NMR samples were prepared in $CDCl_3$ or in $CDCl_3$ with 0.03% TMS, and shifts reported in ppm relative to δ ($CDCl_3$) = 7.259 ppm (^{1}H) or 77.00 ppm (^{13}C). Only diagnostic resonances are reported.

Synthesis of Alkenyl Ketone

*3-*n*-Nonyl-2-cyclohexenone (12).* A solution of 12.76 g (0.09 mol) of 3-ethoxy-2-cyclohexenone (Mori et al., 1977) and 23.3 g (0.11 mol) of freshly distilled *n*-nonylbromide was added dropwise to a stirred suspension of 6.26 g of lithium (25% wt dispersion in oil) in 40 ml of dry THF at 0°C under N_2 atmosphere (Figure 5). The mixture was stirred at rt for 2 hr, excess lithium was removed, and the solution was filtered by suction through a short pad of Celite. The filtrate was poured into 5% ice-diluted H_2SO_4, extracted with ether, and the product was isolated as usual. The pure product was isolated by fractional distillation at 0.6–0.8 mm Hg and 145–150°C as a colorless oil in 90% yield (17 g), which solidified upon refrigeration; R_f = 0.2 in 10% EA/H. FT-IR (neat): 2926, 2854, 1672, 1625, 1456, 1428, 1416 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.70 (s, 1H), 2.34 (t, J = 6.7 Hz, 2H), 2.27 (t, J = 5.8 Hz, 2H), 2.19 (t, J = 7.6 Hz, 2H), 1.97 (quintet, J = 6.3 Hz, 2H), 1.47 (quintet, J = 7.1 Hz, 2H); [^{13}C]NMR ($CDCl_3$); δ 199.74, 166.59, 125.49.

*3-*n*-Nonyl-2,3-Epoxycyclohexanone (13).* To a solution of 12 g of 3-*n*-nonyl-2-cyclohexenone (54 mmol) in 55 ml of MeOH, 26 ml of 30% H_2O_2 was added dropwise followed by 4.7 ml of 6 N NaOH during a 10-min period. After stirring at rt for 18 hr, the mixture was poured in 150 ml of water, extracted with ether, and purified (SiO_2) to give 12 g of a colorless oil in 93% yield, R_f = 0.40 in 10% EA/H. FT-IR (neat): 2929, 2854, 1715, 1458, 1401 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 2.90 (s, 1H), 1.50–2.40 (m, 6H); [^{13}C]NMR ($CDCl_3$): δ 206.74, 65.29, 61.08.

1-Pentadecyn-6-one (14). *p*-Tosylhydrazide (8.4 g, 43.7 mmol) was added in one portion to a stirred, ice-cold solution of 10.4 g (43.7 mmol) of the epoxide (13) in 50 ml of dry CH_2Cl_2 and 25 ml of acetic acid. The mixture was stirred for 3 hr at 0°C, 14 hr at rt, poured into water and extracted with hexane. The crude product was isolated as usual with hexane and purified (SiO_2) to give a colorless oil in 67% yield (6.6 g), R_f = 0.52 in 10% EA/H. FT-IR (neat): 3313, 2925, 2854, 1718, 1459, 1411 cm^{-1}; [^{1}H]NMR ($CDCl_3$); δ 2.55 (t, J = 7.2 Hz, 2H), 2.40 (t, J = 7.4 Hz, 2H), 2.22 (br t, 2H), 1.50–2.00 (m, 3H); [^{13}C]NMR ($CDCl_3$): δ 210.48, 83.56, 68.94.

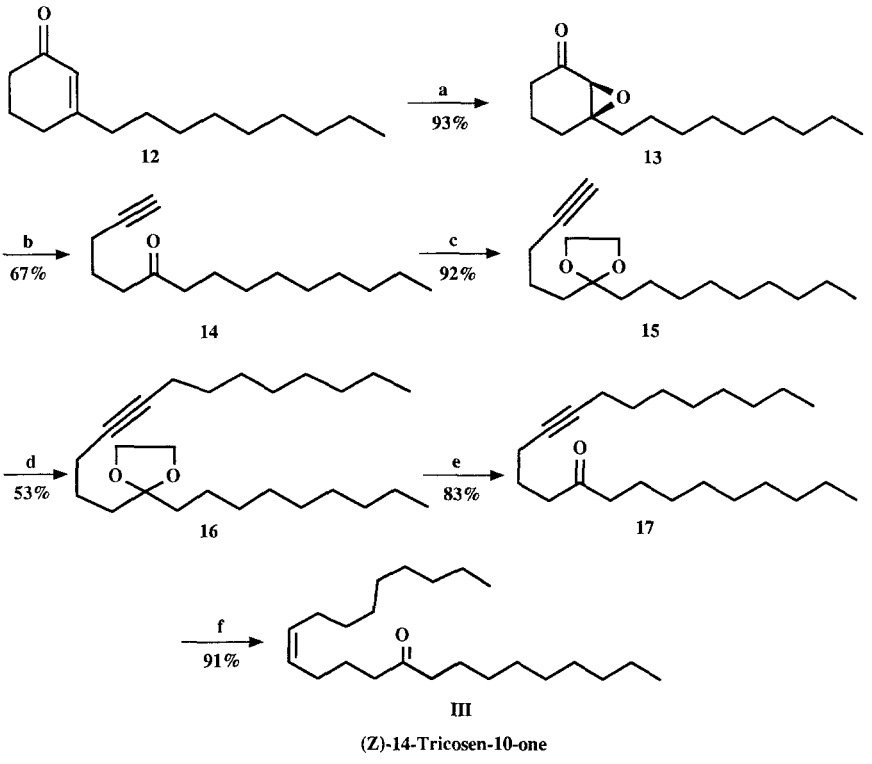


FIG. 5. Synthesis of (Z)-14-tricosen-10-one, a key precursor to several analogs: (a) H_2O_2, 6N NaOH, 25°C; (b) $TsNHNH_2$, CH_2Cl_2, AcOH, 0 to 25°C; (c) $HOCH_2CH_2OH$, $TsOH-H_2O$, C_6H_6, reflux; (d) *n*-BuLi, HMPA, *n*-$C_8H_{17}Br$, THF, −78°C; (e) 3N $HClO_4$, THF, 25°C; (f) H_2, 5% $Pd/BaSO_4$, EtOH, quinoline.

6-Ethylenedioxy-1-pentadecyne (15). A mixture of 1-pentadecyn-6-one (3 g, 13.5 mmol), an excess of ethylene glycol (20-fold molar excess), and 160 mg of *p*-toluenesulfonic acid monohydrate in 100 ml of benzene was heated under reflux with continuous removal of water for 3 hr. After cooling to rt, the mixture was washed with aq. $NaHCO_3$, the aqueous layer was extracted with hexane, and the product was isolated as usual. The crude product was chromatographed (SiO_2) to give 3.3 g of a colorless oil in 92% yield, R_f = 0.45 in 10% EA/H. FT-IR (neat): 3312, 2924, 2854, 1728, 1459 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 3.93 (s, 4H), 2.20 (t, 2H), 1.94 (s, 1H), 1.52–1.84 (m, 6H); [^{13}C]NMR ($CDCl_3$): δ 111.52, 77.43, 67.14, 64.90.

14-Ethylenedioxy-9-tricosyne (16). A solution of *n*-BuLi in hexane (5.6 ml, 2.5 M solution) was added dropwise to a solution of 2.66 g (10 mmol) of 6-ethylenedioxy-1-pentadecyne in 20 ml of dry THF stirred at −78°C under

N_2 atmosphere. The resulting yellow solution was stirred for 1 hr, 5 ml of dry HMPA was added, the red solution was stirred for an additional 30 min at −78°C, and then 1-bromooctane (2.2 g, 11 mmol) was added dropwise. The reaction solution was warmed to 0°C using an ice bath and stirred for 2 hr as the ice melted. The resulting clear solution was poured into water, extracted with hexane, and the product isolated as usual to give, after SiO_2 chromatography, 2 g of a colorless oil (53%), R_f = 0.57 in 10% EA/H. FT-IR (neat): 2925, 2854, 1466 cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.88 (s, 4H), 2.00–2.20 (m, 4H), 1.40–1.98 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 111.53, 81.12, 79.05, 64.90.

14-Tricosyn-10-one (17). A mixture of 1.5 g (4 mmol) of 14-ethylenedioxy-9-tricosyne in 15 ml of THF and excess of 3 N $HClO_4$ solution was stirred at rt for 24 hr, diluted with water, and the product isolated as usual. Purification of the product (SiO_2) gave 1.10 g in 83% yield, R_f = 0.51 in 10% EA/H. FT-IR (neat): 2955, 2927, 2855, 1716, 1466, 1411 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.51 (t, J = 7.3 Hz, 2H), 2.38 (t, J = 7.4 Hz, 2H), 2.16 (m, 4H), 1.72 (m, 2H); [^{13}C]NMR ($CDCl_3$): δ 210.91, 81.13, 79.04.

(Z)-14-Tricosen-10-one (III). A mixture of the above acetylenic ketone (0.80 g, 2.4 mmol), quinoline (0.80 ml of a solution of 100 mg : 1.0 ml, quinoline–hexane) and palladium on barium sulfate (5%, 100 mg) in absolute ethanol (15 mL) was stirred under hydrogen atmosphere. The progress of the reaction was monitored by GC. When no alkyne remained, the mixture was filtered through a short pad of Celite, washed with ether, concentrated in vacuo, and purified on SiO_2 by elution with 1–15% EA/H to give 732 mg of a colorless oil (91% yield), R_f = 0.65 in 10% EA/H. R_t = 14.74 (capillary GC, T_i = 140°C, 2 min; 7°C/min; T_f = 215°C). FT-IR (neat): 3004, 2924, 2853, 1716, 1465, 1409 cm^{-1}; ^1H NMR ($CDCl_3$): δ 5.34 (m, 2H), 2.37 (m, 4H), 2.01 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 211.01, 130.82, 128.56; LR-EI-MS, m/z (rel. abund.): 337 (M^+-1, 0.9).

Synthesis of Diene Analogs

5-Tetradecyn-1-ol THP ether (18). To a solution of 5-hexyn-1-yl-tetrahydropyranyl ether (1.0 g, 5.5 mmol) in dry THF (12 ml), stirred at −78°C, was added dropwise 4 ml of *n*-BuLi (1.6 M solution in hexanes) (Figure 7). The resulting yellow solution was stirred at −78°C for 1 hr, 3 ml of dry HMPA was added, and the stirring was continued for another 30 min. A solution of *n*-bromooctane (1.0 ml, 5.5 mmol) in THF (5 ml) was added dropwise, the mixture was warmed gradually to rt, and the product isolated as usual. The crude product was purified (SiO_2) to give 1.45 g as a colorless oil (90%), R_f = 0.51 in 10% EA/H. FT-IR (neat): 2927, 2856, 1454, 1440 cm^{-1}; [^1H]NMR ($CDCl_3$): δ 4.57 (m, 1H), 3.85 (m, 1H), 3.72 (m, 1H), 3.50 (m, 1H), 3.40 (m, 1H), 2.12 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 98.67, 80.40, 79.70, 66.99, 62.14.

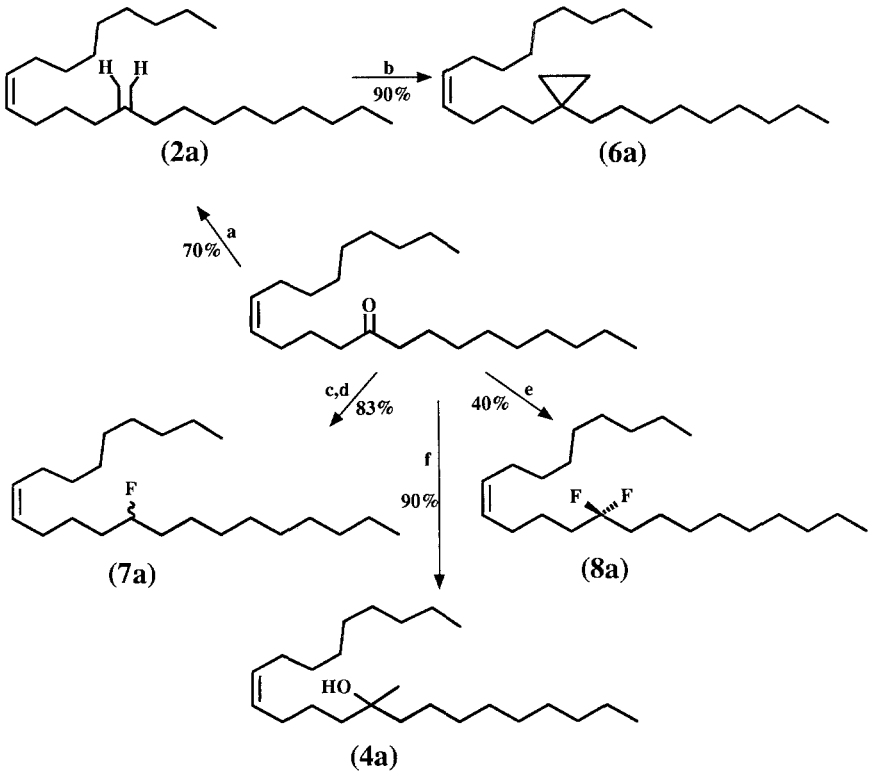


FIG. 6. Transformation of the enone **(III)** to the diene, cyclopropyl, tertiary alcohol, and mono and difluoro analogs: (a) $CH_3(C_6H_5)_3PBr$, *n*-BuLi, THF −78°C; (b) CH_2N_2, $Pd(OAc)_2$, Et_2O, 0°C; (c) $NaBH_4$, MeOH, 0°C; (d) DAST, CH_2Cl_2, −78°C; (e) DAST, CH_2Cl_2, 35°C; (f) CH_3MgBr, THF, 0°C.

(Z)-*5-Tetradecen-1-ol (19).* A solution of the alkynyl ether, **18** (0.70 g, 2.40 mmol), and PPTS (75 mg, 0.30 mmol) in ethanol (25 ml) was stirred at 55°C overnight, concentrated in vacuo, and purified (SiO_2) to give 0.64 g (92%) of a colorless oil, $R_f = 0.14$ in 10% EA/H. Next, a mixture of the alkynol (315 mg, 1.5 mmol), one drop of quinoline, and 20 mg of $Pd/BaSO_4$ (5%) in ethanol (10 ml) was stirred under a slight positive pressure of hydrogen. The mixture was filtered through Celite, concentrated *in vacuo*, and purified (SiO_2) to give 300 mg (94%) of a colorless oil, $R_f = 0.17$ in 10% EA/H. FT-IR (neat): 3329, 3004, 2925, 2854, 1459, 1404 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.36 (m, 2H), 3.64 (t, $J = 6.4$ Hz, 2H); [^{13}C]NMR ($CDCl_3$): δ (130.76, 129.70 8% *trans* impurity), 130.26, 129.23, 62.58.

(Z)-*5-Tetradecenal (20).* To a suspension of pyridinium chlorochromate

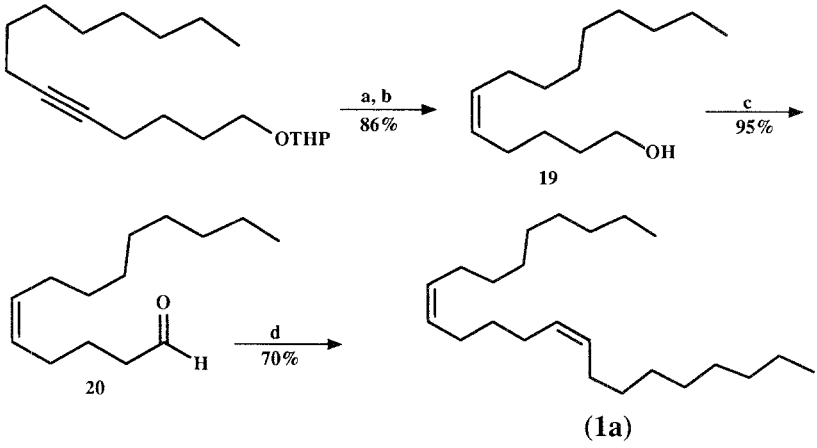


FIG. 7. Synthesis of the (Z,Z)-9,14-diene analog: (a) PPTS, EtOH, 60°C; (b) H_2, 5% $Pd/BaSO_4$, EtOH; (c) PDC, CH_2Cl_2; (d) n-$C_9H_{19}P(C_6H_5)_3Br$, n-BuLi, THF, −78°C.

(330 mg, 1.5 mmol) and sodium acetate (10 mg) in dry CH_2Cl_2 (10 ml) was added alcohol **19** (212 mg, 1 mmol) in dry CH_2Cl_2 (5 ml). After stirring the mixture for 2 hr at rt, it was diluted with ether, filtered through Florisil, concentrated, and purified (SiO_2) to give 200 mg of pure product as a colorless oil (95%), R_f = 0.5 in 10% EA/H. FT-IR (neat): 3004, 2925, 2854, 1728 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 9.77 (t, J = 1.7 Hz, 1H), 5.34 (m, 2H), 2.43 (dt, J = 1.6, 7.4 Hz, 2H); [^{13}C]NMR ($CDCl_3$): δ 202.50, 131.35, 128.11.

(Z,Z)-9,14-Tricosadiene (1a). To a solution of triphenylphosphine n-nonyl bromide, prepared from n-nonyl bromide and triphenylphosphine (470 mg, 1 mmol) in dry THF (5 ml) stirred at −78°C under nitrogen atmosphere, a solution of n-BuLi (0.5 ml, 1.6 M solution in hexanes) was added dropwise. The orange solution was stirred at −78°C for 1 hr, then (Z)-5-tetradecenal (105 mg, 0.5 mmol) in dry THF (2 ml) was added dropwise. After stirring 14 hr at rt, the product was isolated as usual with hexane and purified (SiO_2) to give 112 mg of a colorless oil (70%), R_f = 0.88 in 10% EA/H; R_t = 12.20 (T_i = 140°C, 2 min; 7°C/min; T_f = 220°C). FT-IR (neat): 3004, 2956, 2923, 2854, 1466 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.36 (m, 4H), 2.02 (m, 8H); [^{13}C]NMR ($CDCl_3$): δ 130.23, 129.51; EI-MS m/z 320 (M^+, 2.9).

cis,cis-9,10,14,15-Bisepoxy Tricosane (1b). To a solution of the above diene (5 mg, 15.6 mmol) in CH_2Cl_2 (1 ml) was added m-chloroperbenzoic acid (1 mg), stirred at 0°C for 1 hr, and the product isolated as usual from a hexane–water partition. Purification on SiO_2 afforded 5 mg (90%), R_f = 0.43 in 10% EA/H; R_t = 17.51 (T_i = 140°C, 2 min; 7°C/min; 250°C). [^{1}H]NMR ($CDCl_3$): δ 2.92 (m, 4H).

(Z)-2-(n-Nonyl)-1,6-pentadecadiene (2a). To a solution of methyltriphenyl-phosphonium bromide (107 mg, 0.30 mmol) in THF (5 ml), stirred at −78°C, was added *n*-BuLi (0.14 ml, 1.6 M solution in hexanes) dropwise. The red solution was stirred at −78°C for 1 hr, (Z)-14-tricosen-10-one (50 mg, 0.15 mmol) in THF (1 ml) was added, and the solution was stirred 1 hr as it warmed to rt. The mixture was poured into water, the product was isolated with hexane, and purified on SiO_2 to give 35 mg (70%) of a colorless oil, $R_f = 0.85$ in 10% EA/H; $R_t = 15.37$ ($T_i = 140$°C, 2 min; 7°C/min; $T_f = 250$°C). FT-IR (neat): 3071, 2957, 2925, 2855, 1644, 1466 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.36 (m, 2H), 4.69 (s, 2H); [^{13}C]NMR ($CDCl_3$): δ 150.10, 130.25, 129.48, 108.51.

2-n-Nonyl-1,2,9,10-bisepoxypentadecane (2b). The epoxide was prepared from 5 mg of diene, **2a**, in 91% yield as described above, $R_f = 0.43$ in 10% EA/H; $R_t = 19.78$ ($T_i = 140$°C, 2 min; 7°C/min; 250°C). [^{1}H]NMR ($CDCl_3$): δ 2.91 (m, 2H), 2.58 (s, 2H).

Synthesis of 14-methyl-(Z)-9-tricosene

5-Bromo-2-pentanone (21). A mixture of CuCl (1.0 g, 10 mmol), $PdCl_2$ (176 mg, 1 mmol), water (0.5 ml), dimethyl formamide (3.0 ml), and 5-bromo-1-pentene (1.5 g, 10 mmol) were stirred under an atmosphere of oxygen overnight at rt (Tsuji et al., 1976) (Figure 8). The dark mixture was poured into 3 N HCl and the product was isolated by extraction with CH_2Cl_2 followed by SiO_2 purification to give 1.0 g (60% yield) of pure product as a colorless oil that decomposes if left at rt, $R_f = 0.12$ in 10% EA/H. FT-IR (neat): 1715 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 3.35 (t, $J = 6.4$ Hz, 2H), 2.59 (t, $J = 7.0$ Hz, 2H),

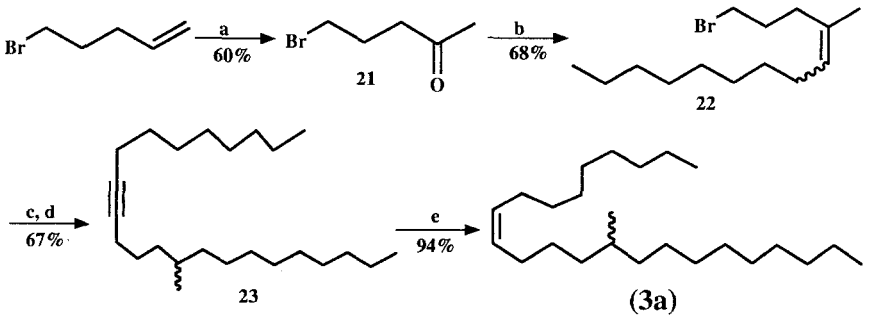


FIG. 8. Synthesis of 14-methyl-(Z)-9-tricosene: (a) $PdCl_2$, CuCl, DMF, H_2O, O_2; (b) n-$C_9H_{19}P(C_6H_5)_3Br$, n-BuLi, THF, −78°C; (c) H_2, 10% Pd/C, EtOH; (d) n-C_8H_{17}-C≡CH, n-BuLi, HMPA, THF, −78°C; (e) H_2, 5% $Pd/BaSO_4$, EtOH.

2.07 (s, 3H), 2.01 (dt; J = 6.7, 13.3 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 207.24, 41.31, 33.22, 31.77, 30.00, 26.25.

1-Bromo-4-methyl-4-tridecene (22). To a solution of *n*-nonyltriphenylphosphonium bromide (1.7 g, 3.6 mmol) in THF (10 ml), stirred at −78°C, was added dropwise *n*-BuLi (1.7 ml, 1.6 M in hexanes). The orange solution was stirred for 1 hr at −78°C, and then a solution of the above bromo ketone (300 mg, 1.8 mmol) in THF (5 ml) was added to give a colorless solution that was stirred at −78°C for 2 hr. The mixture was poured into water and the product isolated with hexane and purified (SiO_2) to give 340 mg (68% yield) of a 1:1 mixture (E:Z), R_f = 0.72 in 10% EA/H. FT-IR (neat): 2956, 2924, 2853, 1666, 1457 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.21 (br t, 1H); 3.39 (br t, 2H); 2.36 (m, 4H); 1.68 and 1.59 (s, s, 1:1 ratio, 3H); ^{13}C NMR ($CDCl_3$): δ 128.14 (127.08), 126.27 (125.24).

14-Methyl-9-tricosyne (23). To a solution of 1-decyne (50 mg, 0.30 mmol) in dry THF (4 ml) stirred at −78°C under nitrogen atmosphere was added *n*-BuLi (0.23 ml, 1.6 M solution in hexanes). After stirring for 1 hr, HMPA (1 ml) was added, the yellow solution was stirred for 30 min, a solution of 4-methyl-1-bromotridecane (70 mg, 0.25 mmol) (obtained by reducing the double bond in compound **22**) in THF (1 ml) was added, and the solution was stirred for 2 hr at −78°C. The reaction was poured into water and the product isolated with hexane and purified (SiO_2) to give 59 mg of a colorless oil (70%), R_f = 0.68 in 10% EA/H. FT-IR (neat): 3315, 2924, 2854, 1465 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 2.14 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 84.78, 80.25.

14-Methyl-(Z)-9-tricosene (3a). A mixture of 14-methyl-9-tricosyne (25 mg, 0.074 mmol), 5% Pd/$BaSO_4$ (5 mg), and one drop of quinoline in absolute ethanol was stirred under a slight positive pressure of hydrogen until no alkyne remained (GC). The mixture was filtered through Celite, concentrated, and purified on 20% $AgNO_3$–SiO_2 to give 25 mg (94%) of >98% *cis* alkene as a colorless oil, R_f = 0.80 in 10% EA/H; R_t = 14.48 (T_i = 140°C, 2 min; 7°C/min; T_f = 250°C). FT-IR (neat): 3004, 2955, 2923, 2853, 1652, 1465, 1403 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 5.35 (m, 2H), 2.02 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 129.89.

14-Methyl-9,10-epoxy Tricosane (3b). Epoxidation as above of 5 mg of 14-methyl-(Z)-9-tricosene afforded 5 mg of the corresponding epoxide after purification, R_f = 0.71 in 10% EA/H; R_t = 17.24 (T_i = 140°C, 2 min; 7°C/min; 250°C); [^{1}H]NMR ($CDCl_3$): δ 2.98 (m, 2H).

Synthesis of (Z)-*10-Methyl-(Z)-14-tricos-14-en-10-ol (4a).* To a solution of (*Z*)-14-tricosen-10-one (126 mg, 0.375 mmol) in dry THF (5 ml) stirred at 0°C under nitrogen atmosphere was added dropwise methylmagnesium bromide (0.36 ml, 3.0 M solution in ether). The resulting mixture was stirred at 0°C for 1 hr, poured into 0°C satd. aq. NH_4Cl, and the product was isolated with 5%

0.41 in 10% EA/H; R_t = 16.23 (T_i = 140°C, 2 min; 7°C/min; T_f = 250°C). FT-IR (neat): 3374, 3004, 2923, 2852, 145 cm^{-1}; ^1H NMR ($CDCl_3$): δ 5.36 (m, 4H), 2.02 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 130.26, 129.40, 72.66.

cis-*10-Methyl-14,15-epoxytricosan-10-ol (4b).* To a stirred solution of *m*-CPBA (15 mg, 0.07 mmol) in methylene chloride (2 ml) was added the above compound (20 mg, 0.058 mmol) in CH_2Cl_2 (1 ml) at 0°C. The solution was stirred at 0°C for 2 hr, quenched with 0.8 ml of 5% solution of Na_2SO_3, washed with 1 ml 5% solution of NaOH followed by 3 ml of water, filtered through Florisil, concentrated, and purified (SiO_2) to give 20 mg of the colorless oil (95% yield), R_f = 0.11 in 10% EA/H. This product rearranges at room temperature to a product without the double bond and seems to decompose during gas chromatography. ^1H NMR ($CDCl_3$): δ 2.91 (m, 2H), 1.51 (m, 8H), 1.26 (br s, 28H), 1.16 (s, 3H), 0.87 (t, J = 6.7 Hz, 6H); ^{13}C NMR ($CDCl_3$): δ 72.63, 57.18, 57.09.

Synthesis of Dimethyl Analog

1-Cyano-3,3-dimethyl-4-dodecene (24). To a solution of *n*-octyltriphenylphosphonium bromide (1.45 g, 3.2 mmol), prepared from *n*-octyl bromide and triphenylphosphine, in dry THF (10 ml) was added *n*-BuLi (1.5 ml, 1.6 M solution in hexanes) dropwise at −78°C. The orange solution was stirred for 1 hr, then 4-cyano-2,2-dimethylbutanal (0.20 g, 1.6 mmol) (prepared by the procedure described by Poidevin et al., 1979) was added in dry THF (2.0 ml) (Figure 9). The reaction was warmed to rt, poured into water, and the product was isolated with hexane and purified (SiO_2) to give 0.318 g (90% yield) of a colorless oil. FT-IR (neat): 2957, 2925, 2855, 2246, 1466 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.30 (dt, J = 12.1, 7.4 Hz, 1H), 5.09 (d, J = 12.0 Hz, 1H), 2.29 (t, J = 8.2 Hz, 2H), 2.09 (m, 2H), 1.76 (t, J = 8.2 Hz, 2H); [^{13}C]NMR ($CDCl_3$): δ 135.28, 131.87, 120.36.

4,4-Dimethyl-5-tridecenal (25). A solution of the above nitrile (0.30 g, 1.35 mmol) in dry ether (10 ml) was stirred at 0°C under nitrogen atmosphere, then DiBAL-H (2 ml, 1.0 M solution in hexanes) was added dropwise (Zakharkin and Khorlina, 1964). The reaction was stirred for 4 hr at 35°C and then poured into ice cold water (2 ml) and 10% H_2SO_4 solution (1 ml) and stirred for 30 min. The product was isolated with hexane and purified (SiO_2) to give 195 mg (64% yield) of a colorless oil. R_f = 0.53 in 10% EA/H. FT-IR (neat): 2957, 2924, 2855, 1727, 1647, 1466 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 9.76 (t, J = 1.8 Hz, 1H), 5.30 (dt, J = 7.3, 12.0 Hz, 1H), 5.08 (d, J = 12.0 Hz, 1H), 2.42 (t, J = 7.9 Hz, 2H), 2.07 (m, 2H), 1.78 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 202.92, 135.37, 132.00.

4,4-Dimethyl-1-bromotridecane (26). A solution of 4,4-dimethyl-1-tri-

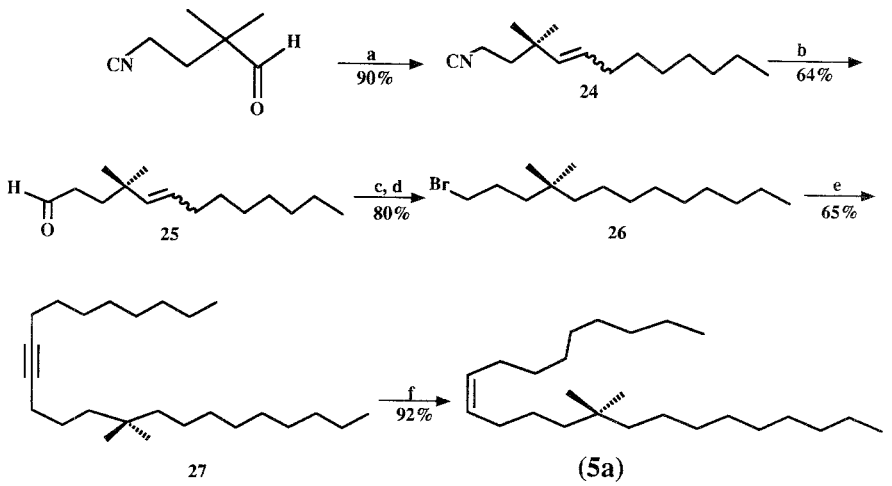


FIG. 9. Synthesis of 14,14-dimethyl (Z)-9-tricosene: (a) n-$C_8H_{17}P(C_6H_5)_3Br$, n-BuLi, THF, −78°C; (b) DIBAL-H, Et_2O, 35°C; (c) $LiAlH_4$, Et_2O, 0°C; (d) H_2, 10% Pd/C, MeOH; (e) Ph_3P, CBr_4, CH_2Cl_2, 0°C; (f) n-$C_8H_{17}CCH$, n-BuLi, HMPA, THF, −78°C (g) H_2, 5% $Pd/BaSO_4$, EtOH.

decanol (obtained into steps from the aldehyde, **14,** by reducing the double bond then the aldehyde to an alcohol) (40 mg, 0.17 mmol) and carbon tetrabromide (64 mg) in dry CH_2Cl_2 (5 ml) was added dropwise to a stirred, 0°C solution of triphenylphosphine in CH_2Cl_2 (60 mg, 1 ml) (Kociénski et al., 1977). The solution was stirred for 2 hr at 0°C and 1 hr at rt. The solvent was removed in vacuo; the white residue was diluted in hexane and filtered, and the product was purified (SiO_2) to give 49 mg of a colorless oil (95%), R_f = 0.8 in 10% EA/H. FT-IR (neat): 2956, 2925, 2853, 1467 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 3.37 (t, J = 7.0 Hz, 2H), 1.80 (m, 2H); [^{13}C]NMR ($CDCl_3$): δ 41.88, 40.42.

14,14-Dimethyl-9-tricosyne (27). A solution of 1-decyne (50 mg, 0.34 mmol) in dry THF (4 ml) was stirred at −78°C under nitrogen atmosphere, and n-Buli (0.3 ml, 1.0 M solution in hexanes) was added dropwise. The solution was stirred for 1 hr, HMPA (1 ml) was added, and the yellowish solution was stirred for 0.5 hr. 14,14-Dimethyl-1-bromotridecane (45 mg, 0.15 mmol) in THF (1 ml) was added, the solution was warmed to rt, poured into water, and the product was isolated with hexane and purified (SiO_2) to give 35 mg of a colorless oil (65%), R_f = 0.85 in 10% EA/H. FT-IR (neat): 3315, 2924, 2854, 1466 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 2.15 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 80.35, 80.24.

14,14-Dimethyl-(Z)-9-tricosene (5a). A mixture of the above tricosyne (30 mg, 0.086 mmol), 5 mg of 5% $Pd/BaSO_4$ and one drop of quinoline in ethanol

(5 ml) was stirred under a slight pressure of hydrogen until no alkyne remained (GC). The mixture was filtered, concentrated, and purified (20% $AgNO_3$–SiO_2) to give 27 mg of >95% *Z* isomer (92% yield), R_f = 0.85 in 10% EA/H; R_t = 13.60 (T_i = 140°C, 2 min; 7°C/min; T_f = 220°C). FT-IR (neat): 3004, 2956, 2923, 2853, 1465, 1403 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.32 (m, 2H), 2.02 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 129.89.

cis-*14,14-Dimethyl-9,10-epoxytricosane (5b).* The above alkene, **5a** (5 mg, 0.014 mmol), was stirred with *m*-CPBA (1 mg) in dry methylene chloride. After SiO_2 purification, 5 mg of the pure epoxide was obtained (95%); R_f = 0.70 in 10% EA/H; R_t = 16.44 (T_i = 140°C, 2 min; 7°C/min; T_f = 220°C). [^{1}H]NMR ($CDCl_3$): δ 2.87 (m, 2H).

Synthesis of Cyclopropyl Analog

Synthesis of 1-nonyl-1-[(Z)*-4-tetradecen-1-yl*]*-cyclopropane (6a).* A solution of 14-methylene-(Z)-9-tricosene **(2a),** (20 mg, 0.06 mmol) and an ethereal solution of diazomethane (1.5 ml, more than 10 equiv.) in dry ether (5 ml) was cooled in an ice-water bath, and palladium(II) acetate (10 mg) was added (Suda, 1981) (Figure 6). Vigorous gas production was observed. After 15 min of stirring, the ether was removed in vacuo, and the residue was purified on SiO_2 using hexane as eluent to give 19 mg (90%) of a colorless oil, R_f = 0.88 in 10% EA/H; R_t = 16.33 (T_i = 140°C, 2 min; 7°C/min; T_f = 250°C). FT-IR (neat): 3067, 2956, 2923, 2854, 1644, 1464, cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.34 (m, 2H), 2.01 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 129.98, 129.85, 104.94, 11.96.

1-Nonyl-1-[cis*-4,5-epoxy tetradec-1-yl*]*-cyclopropane (6b).* The above cyclopropane (5 mg, 0.014 mmol) and *m*-CPBA (1 mg) in dry methylene chloride (1 ml) were stirred at 20°C for 1 hr. After the usual work-up and SiO_2 purification, 5 mg (95%) of the epoxide as a colorless oil was obtained, R_f = 0.65 in 10% EA/H; R_t = 19.02 (T_i = 140°C, 2 min; 7°C/min; T_f = 250°C). [^{1}H]NMR ($CDCl_3$): δ 2.91 (m, 2H).

Synthesis of Fluorinated Analogs

(Z)*-10-Fluorotricos-14-ene (7a).* A solution of (Z)-14-tricosen-10-ol (30 mg, 0.088 mmol) in dry CH_2Cl_2 (2.0 ml) was stirred at −78°C under nitrogen atmosphere; to this solution diethylaminosulfur trifluoride (20 μl) was added (Figure 6). After stirring at −78°C for 1 hr, water was added (0.1 ml), the mixture was warmed to rt, the product was isolated with hexane, and purified (SiO_2) to give 27 mg of a colorless oil (88% yield), R_f = 0.77 in 10% EA/H; R_t = 15.84 (T_i = 140°C, 2 min; 7°C/min; T_f = 250°C). FT-IR (neat): 3004, 2925, 2854, 1655, 1466, cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.32 (m, 2H), 4.54, 4.37 (m, $J_{H\text{-}F}$ = 45.9 Hz, 1H), 2.04 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 130.51, 129.14, 95.52, 93.31; [^{19}F]NMR (C_6D_6): (φ) −184.76 (m, 1F).

cis-*10-Fluoro-14,15-epoxytricosane (7b).* Using *m*-CPBA oxidation, the epoxide was obtained in 95% yield as a colorless oil, R_f = 0.53 in 10% EA/H. GC analysis showed that we obtained two stereoisomers in about 1 : 1 mixture: R_t = 18.05, 18.13 (T_i = 140°C, 2 min; 7°C/min; T_f = 150°C). [^{1}H]NMR ($CDCl_3$): δ 4.56, 4.39 (m, $J_{H\text{-}F}$ = 50.7 Hz, 1H), 2.91 (m, 2H).

(Z)-10,10-Difluoro-14-tricos-14-ene (8a). A solution of (Z)-14-tricosen-10-one (100 mg, 0.30 mmol) in dry CH_2Cl_2 (3 ml) was stirred with DAST (80 μl) in a sealed vial at 35°C for four days (Middleton, 1975). After cooling to −78°C, methanol was added, the mixture was warmed to rt, concentrated, and the product was purified on a short pad of neutral alumina to give 42 mg (40%) of a colorless oil, R_f = 0.80 in 10% EA/H; R_t = 14.89, and about 50 mg of the starting material. FT-IR (neat): 2926, 2855, 1708, 1655, 1463 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.34 (m, 2H), 2.04 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 130.73, 128.46, 125.35 (t, J = 239.9 Hz), 36.20 (t, J = 25.5 Hz); [^{19}F]NMR (C_6D_6) ϕ (ppm): − 102.43 (quintet, J = 16.4 Hz, 2F).

cis-*10,10-Difluoro-14,15-epoxytricosane (8b).* This compound was obtained in more than 90% yield from the above alkene by epoxidation as described above, R_f = 0.57 in 10% EA/H; R_t = 17.69. [^{1}H]NMR ($CDCl_3$): δ 2.87 (m, 2H).

Synthesis of [14,14-^{2}H]- and [14-^{2}H]-(Z)-9-tricosene

(Z)-14-Tricosen-10-ol (28). To a solution of (Z)-14-tricosen-10-one (100 mg, 0.3 mmol) in methanol (5 ml) was added sodium borohydride (10 mg) at 0°C (Figure 10). The resulting solution was stirred for 2 hr, quenched with acetic acid, concentrated, and the product was purified (SiO_2) to give 95 mg

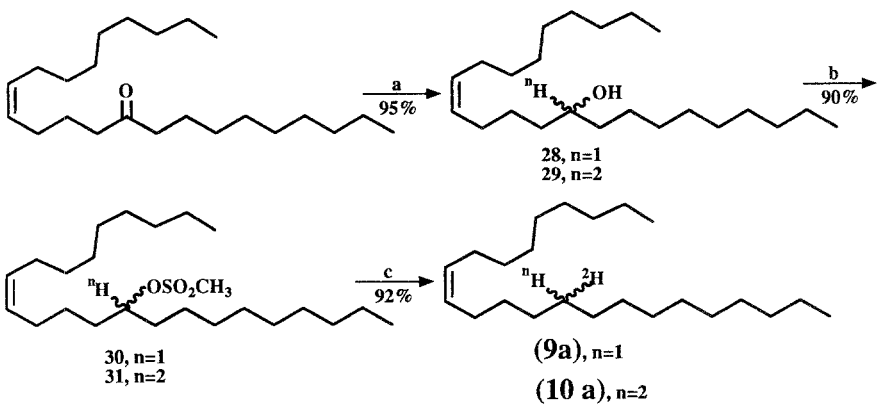


FIG. 10. Synthesis of [14-^{2}H]- and [14,14-^{2}H]-(Z)-9-tricosenes: (a) NaB^{n}H$_4$, CH_3O^nH (n = 1 or 2); (b) CH_3SO_2Cl, Et_3N, Et_2O, 0°C; (c) LiAl^{2}H$_4$, Et_2O, 0°C.

(95%) of a colorless oil, R_f = 0.31 in 10% EA/H; R_t = 15.26. FT-IR (neat): 3375, 3004, 2922, 2854, 1466 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.36 (m, 2H), 3.58 (m, 1H), 2.03 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 130.31, 129.36, 71.85.

[10-^{2}H]-(Z)-14-Tricosen-10-ol (29). To 97 mg (0.29 mmol) of (Z)-14-tricosen-10-one in 2 ml of MeO^2H was added sodium borodeuteride (10 mg, 98 atom % ^{2}H) at 0°C. After purification by flash chromatography, 92 mg (95%) of a colorless oil was obtained. [^{1}H]NMR ($CDCl_3$): δ 5.30 (m, 2H), 2.02 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 130.31, 129.36, 71.39 (t, $J_{C\text{-}H}$ = 20.9 Hz); [^{2}H]NMR (CCl_4 + one drop of $CDCl_3$): δ 3.51 ppm.

10-Mesyloxy-(Z)-14-tricosene (30). To an ice-cold and stirred solution of (Z)-14-tricosen-10-ol (12 mg, 35 μmol) and triethylamine (15 μl, 105 μmol) in CH_2Cl_2 (2 ml) was added methanesulfonyl chloride (3.4 μl, 42 μmol) (Kelkar et al., 1989). The mixture was stirred for 30 min at 0°C and for 4 hr at rt, concentrated, and the product was isolated with ether and purified (SiO_2) to give 13 mg of a colorless oil (90%), R_f = 0.31 in 10% EA/H. FT-IR (neat): 2926, 2855, 1466 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 5.36 (m, 2H), 4.70 (m, 1H), 2.99 (s, 3H), 2.04 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 130.90, 128.67, 84.20.

[10-^{2}H]-10-Mesyloxy-(Z)-14-tricosene (31). To an ice-cold and stirred solution of [10-^{2}H]-(Z)-14-tricosen-10-ol (50 mg, 0.146 mmol) and triethylamine (61 μl, 0.438 mmol) in CH_2Cl_2 (2 ml) was added methanesulfonyl chloride (14 ml, 0.175 mmol). Deuterium oxide was used (1 ml) for the work-up. Purification (SiO_2) gave 55 mg of a colorless oil (90%). [^{1}H]NMR ($CDCl_3$): δ 5.34 (m, 4H), 2.98 (s, 3H), 2.04 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 130.86, 128.64, 82.97; [^{2}H]NMR (CCl_4 + one drop of $CDCl_3$): 4.64 ppm.

[14-^{2}H]-(Z)-9-Tricosene (9a). To an ice-cold and stirred suspension of lithium aluminum deuteride (2 mg, 98 atom % ^{2}H) in dry ether (1 ml) was added a solution of the mesylate **(30)** (14 mg, 33.36 mmol) in ether (1 ml). The reaction mixture was stirred for 30 min at 0°C then for 1 hr at room temperature. Excess of $LiAl^2H_4$ was destroyed by adding one drop of ethyl acetate, and the mixture was filtered and concentrated in vacuo. The residue was chromatographed (20% $AgNO_3$–SiO_2) to give 10 mg (92%) of a colorless oil (>98% *cis*, detected by capillary GC), R_f = 0.83 in 10% EA/H; R_t = 13.27. [^{1}H]NMR ($CDCl_3$): δ 5.34 (m, 2H), 2.04 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 129.89; [^{2}H]NMR (CCl_4 + one drop of $CDCl_3$): 1.30 ppm.

[14-^{2}H]-(Z)-9,10-Epoxy Tricosane (9b). This compound was prepared from the corresponding alkene **(9a)**, R_f = 0.68 in 10% EA/H; R_t = 15.19. [^{1}H]NMR ($CDCl_3$): δ 2.90 (m, 2H).

[14,14-$^2H_2$]-(Z)-9-Tricosene (10a). To an ice-cold and stirred suspension of lithium aluminum deuteride (8 mg, 98 atom % ^{2}H) in dry ether (1 ml) was added a solution of the mesylate **(31)** (48 mg, 0.115 mmol) in dry ether (1 ml). The crude product was purified by chromatography (20% $AgNO_3$–SiO_2) to give 30 mg of >98% *cis* product as a colorless oil, R_t = 12.59. [^{1}H]NMR ($CDCl_3$):

δ 5.35 (m, 2H), 2.02 (m, 4H); [^{13}C]NMR ($CDCl_3$): δ 129.90; [^{2}H]NMR (CCl_4 + one drop of $CDCl_3$): 1.26 ppm.

cis-[*14,14-$^2H_2$*]-*9,10-Epoxytricosane (10b).* Epoxidation of alkene **10a** with *m*-CPBA gave epoxide **10b**. R_f = 0.71 in 10% EA/H; R_t = 14.86. [^{1}H]NMR ($CDCl_3$): δ 2.91 (m, 2H).

Synthesis of Perfluoro Analogs

1,1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8-Heptadecafluoro-10-iodo-9-tricosene (32). 1-Pentadecyne (418 mg, 2.0 mmol), *n*-perfluorooctyl iodide (1.0 g, 2.0 mmol), and azobis(isobutyronitrile) (AIBN, 100 mg) were placed in a 5-ml round-bottom flask equipped with a short condenser and a magnetic stirrer. The mixture was frozen using a bath of liquid nitrogen, degassed, and thawed under nitrogen atmosphere to eliminate oxygen (Sun and Prestwich, 1990); this process was repeated twice (Figure 11). The mixture was slowly warmed to rt,

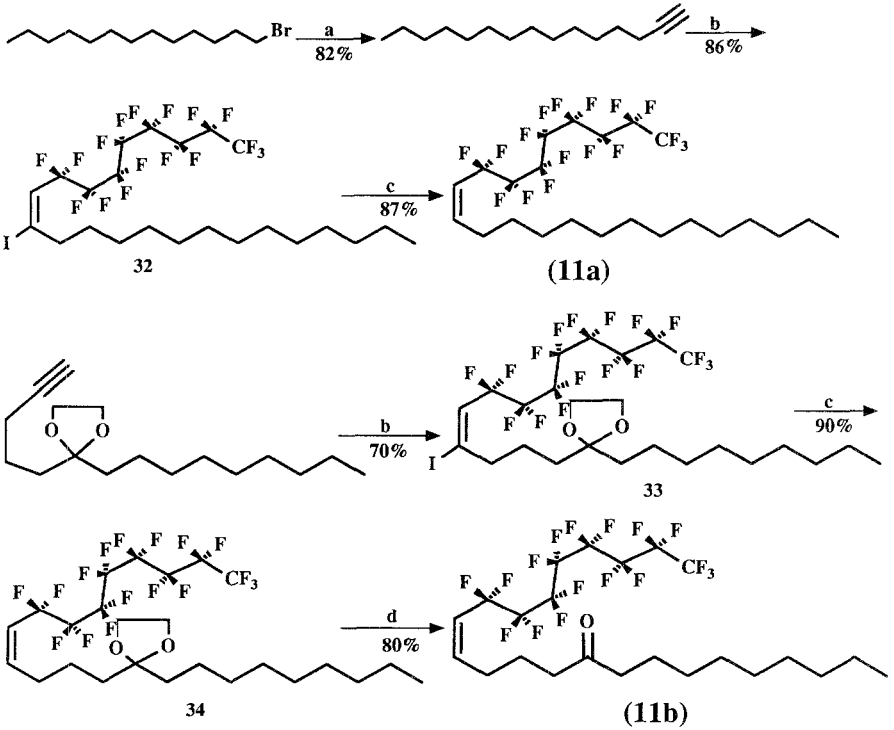


FIG. 11. Synthesis of perfluoroalkyl analog and its expected ketone metabolite: (a) HC≡CLi-EDA, DMSO; (b) *n*-$C_8F_{17}I$, AIBN; (c) *n*-BuLi, THF, MeOH; (d) 3N $HClO_4$, THF, rt.

then heated at 80°C for 20 hr. After cooling to rt the crude brown solution was purified (SiO_2) to give 1.4 g of a colorless oil (93%), R_f = 0.84 in 10% EA/H. GC analysis showed an *E/Z* ratio of 92:8. FT-IR (neat): 2926, 2855, 1634, 1467 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 6.31 (t, J= 14.4 Hz, 1H), 2.62 (dist t, 2H), 1.55 (m, 2H), 1.26 (br s, 20H), 0.88 (t, J = 6.5 Hz, 3H); [^{13}C]NMR ($CDCl_3$): δ 126.56 (t, J = 47.3 Hz), 123.06 t, J = 5.8 Hz); [^{19}F]NMR (C_6D_6): ϕ −85.80, −111.00, −115.60, −126.07, −126.62, −127.50, −128.03, −128.25, −130.96.

(Z)-*1,1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8-Heptadecafluoro-9-tricosene (11a).* The above iodoperfluorooctyl alkene (180 mg, 0.24 mmol) was stirred in dry ether (5.0 ml) at −78°C, while *n*-BuLi (1.0 ml of 1.6 M solution in hexanes) was added dropwise; the resulting solution was stirred at −78°C for 1 hr. Methanol (1 ml) was precooled at −78°C and added slowly; the mixture was allowed to warm to room temperature. The reaction was poured into saturated ammonium chloride (4 ml) and the product was isolated with ether and purified (SiO_2) to give 130 mg (87%) of a colorless oil, R_f = 0.80 in 10% EA/H; R_t = 5.86. FT-IR (neat): 2926, 2856, 1664, 1467, 1411 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 6.10 (m, 1H), 5.49 (m, 1H), 2.40 (m, 2H); [^{13}C]NMR ($CDCl_3$): δ 145.63 (t, J = 4.7 Hz), 115.86 (d, J = 25.2 Hz); [^{19}F]NMR (C_6D_6): ϕ −85.77, −109.82, −112.74, −126.15, −126.62, −127.50, −127.88, −130.96.

(Z)-*1,1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8-Heptadecafluoro-10-iodo-14-ethylenedioxy-1-tricosyne (33).* A mixture of 6-ethylenedioxy-1-pentadecyne **(15)** (0.25 g, 0.93 mmol), *n*-perfluorooctyl iodide (0.77 g, 1.4 mmol), and AIBN (30 mg) was heated at 120°C for 24 hr. Purification (SiO_2) gave 534 mg (70% yield) of a colorless oil, which solidified on refrigeration, R_f = 0.50 in 10% EA/H. (*E/Z* 90:10). FT-IR (neat): 2926, 2856, 1718, 1635, 1460, 1413 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 6.34 (t, J = 14.4 Hz, 1H), 3.90 (s, 4H), 2.64 (br t, 2H); [^{13}C]NMR ($CDCl_3$): δ 126.87 (t, J = 2.3 Hz), 122.65, 111.36, 64.94.

(Z)-*1,1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8-Heptadecafluoro-14-ethylenedioxy-9-pentadecene (34).* The protected iodo ketone **(33)** (120 mg, 0.147 mmol) was stirred at −78°C in dry ether (5 ml), and *n*-BuLi (0.4 ml of 1.6 M solution in hexanes) was added. After quenching with precooled methanol and the usual work-up, the crude product was purified (SiO_2) to give 90 mg (90% yield) of a colorless oil, R_f = 0.48 in 10% EA/H, which solidified upon refrigeration. [^{1}H]NMR ($CDCl_3$): δ 6.32 (m, 1H), 5.46 (m, 1H), 3.92 (s, 4H), 2.2 (m, 2H); [^{13}C]NMR ($CDCl_3$): δ 145.62 (t, J = 4.7 Hz), 115.85, 111.38, 64.95; [^{19}F]NMR (C_6D_6): ϕ: −85.73, −109.90, −126.10, −126.65, −127.50, −127.84, −130.90.

(Z)-*1,1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8-Heptadecafluoro-9-pentadecen-14-one (11b).* A mixture of the perfluoro protected ketone (40 mg, 0.058 mmol) and $HClO_4$ (0.5 ml of 3 N solution) in THF (3.0 ml) was stirred at room tem-

perature for 48 hr, and the product was isolated with ether, concentrated, and purified (SiO_2) to give 37 mg (80% yield) of a white solid, mp = 26–27°C, R_f = 0.54 in 10% EA/H; R_t = 10.80. FT-IR (neat): 2958, 2928, 2857, 1717, 1664, 1461, 1412 cm^{-1}; [^{1}H]NMR ($CDCl_3$): δ 6.20 (dtt, J = 14.0, 7.8, 2.3 Hz, 1H), 5.50 (m, 1H), 2.42 (t, J = 6.5 Hz, 2H), 2.37 (t, J = 6.5 Hz, 2H), 1.71 (m, 2H), 1.54 (m, 2H); [^{13}C]NMR ($CDCl_3$): δ 211.01, 146.35 (t, J = 4.8 Hz), 116.04 (d, J = 25.3 Hz); [^{19}F]NMR (C_6D_6): ϕ −85.73 (t, J = 9.9 Hz), −109.90 (q, J = 13.3 Hz), (−126.10, −126.65), (−127.50, −127.84), −130.90.

RESULTS AND DISCUSSION

(*Z*)-14-Tricosen-10-one was a suitable starting material in the synthesis of most of the compounds. As shown in Figure 5, the enone **(III)** was most conveniently synthesized in 13.5% overall yield from 1,3-cyclohexanedione by an Eschenmoser fragmentation reaction (Mori et al., 1977; Latli and Prestwich, 1988). Except for compound **1a**, which was synthesized from 5-hexyn-1-ol, the rest of the analogs were obtained or attempted from the enone **(III)**. Transformations of this enone are summarized in Figure 6. First, the diene **(2a)** was obtained from enone **(III)** by Wittig methylenation. Treatment of **2a** with diazomethane using palladium diacetate in catalytic amount gave the cyclopropyl analog **(6a)**. Next, the tertiary alcohol **(4a)** was obtained using the Grignard reagent methylmagnesium bromide. The fluorinated analogs were obtained from the ketone or the alcohol as follows. Reaction of the enone **(III)** with diethylamino sulfur trifluoride to produce the difluoro analog **(8a)** proceeded slowly at 35°C to give the desired difluoro compound. Attempts to obtain this analog from the corresponding 1,3-dithiolane, 1,3-dibromo-5,5-dimethylhydantoin, and HF-pyridine had failed (Sondej and Katzenellenbogen, 1986). In contrast, the reaction of the alcohol was complete in less than an hour at −78°C, to give the monofluorinated analog **(7a)** in quantitative yield.

The 9,14-diene analog (**1a,** Figure 7) was obtained in 51% overall yield, by first protecting 5-hexyn-1-ol followed by alkylation of the lithium acetylide. After reducing the triple bond to a *cis* double bond, deprotecting the alcohol, and then oxidizing it to the aldehyde, the diene was obtained by Wittig reaction.

The 14-methyl compound (**3a,** Figure 8) was prepared in 26% overall yield in five steps from 5-bromo-1-pentene. First, the terminal double bond was oxidized to a methyl ketone using cuprous chloride and palladium dichloride in DMF under an oxygen atmosphere. The resulting 5-bromo-2-pentanone was unstable and decomposed when left at rt. Next, Wittig reaction of this com-

pound with nonyltriphenylphosphine bromide followed by reduction of the double bond gave the methyl-branched alkyl halide, which was coupled with lithium-1-decyne and reduced to the *cis* alkene. Attempts to synthesize 14-methyl-(Z)-9-tricosene from 14-bromo-(Z)-9-tricosene by coupling with dimethylcopper lithium (Corey and Posner, 1967) or by refluxing (Z)-14-tricosen-10-ol with trimethylaluminum in toluene (Harney et al., 1974) had failed. Also tried without success was the reaction of 14-methylene-9-tricosyne with catecholborane (Brown and Gupta, 1975).

The 14,14-dimethyl analog (**5a,** Figure 9) was prepared in 17% overall yield by reacting isobutyraldehyde and acrylonitrile using a solution of 5% NaOH and dioxane as a solvent. The resulting 4-cyano-2,2-dimethyl butanal was used to prepare the dimethyl alkyl halide, which was coupled with acetylenic lithium as described before. An attempted reaction of dimethyl zinc and titanium tetrachloride with the enone **(III)** (Reetz et al., 1985) in order to produce the *gem*-dimethyl analog gave a mixture of nonpolar, unidentified products.

The deuterated analogs (**9a** and **10a,** Figure 10) were synthesized by reduction of the enone **(III)** to the alcohols followed by deuteride displacement on the corresponding mesylates to the desired mono- and dideuterated analogs. The reduction of (Z)-14-tricosen-10-ethylene dithioketal (Fujita et al., 1978) using deuterated Raney nickel (Djerassi and Williams, 1963) gave a mixture of inseparable products.

The perfluoro analog (**11a,** Figure 11) was prepared in 61% in three steps. Bromotridecane was transformed to 1-pentadecyne using lithium acetylide-ethylenediamine complex in DMSO (Novis Smith and Beumel, 1974). Reacting this alkyne with *n*-perfluorooctyliodide and AIBN as a catalyst gave the alkenyl iodides as a 92 : 8 *E*/*Z* mixture. Lithium iodide exchange using butyllithium and quenching at −78°C with methanol (Sun et al., 1990) gave the perfluoro analog, which was very volatile compared to the (Z)-9-tricosene. We were unable to obtain the epoxide of this analog even when using ten equivalents of *m*-CPBA at rt. The other expected metabolite of the perfluoro analog was synthesized from 6-ethylenedioxy-1-pentadecyne **(15)** in 50% yield in three steps. Attempts to prepare the analog **11a** from **11b** using Wolff-Kishner type reduction failed (Kabalka et al., 1979).

The expected epoxide metabolites (Figure 4) were obtained by treatment of each analog with *m*-CPBA acid in methylene chloride. Epoxides were characterized spectroscopically and used to identify metabolic products for in vivo experiments (Guo et al., 1991).

Acknowledgments—We thank the Herman Frasch Foundation for a grant to G.D.P. The intellectual exchange with the Blomquist group during this project was also greatly appreciated.

REFERENCES

ADAMS, T.S., and HOLT, G.G. 1987. Effect of pheromone components when applied to different models on male sexual behavior in the housefly, *Musca domestica*. *J. Insect. Physiol.* 33:9–18.

ADAMS, T.S., DILLWITH, J.W., and BLOMQUIST, G.J. 1984. The role of 20-hydroxyecdysone in housefly sex pheromone biosynthesis. *J. Insect Physiol.* 30:287–294.

AHMAD, S., KIRKLAND, K.E., and BLOMQUIST, G.J. 1987. Evidence for a sex pheromone metabolizing cytochrome P-450 mono-oxygenase in the housefly. *Arch. Insect Biochem. Physiol.* 6:121–140.

BLOMQUIST, G.J., DILLWITH, J.W., and POMONIS, J.G. 1984. Sex pheromone of the housefly. Metabolism of (*Z*)-9-tricosene to (*Z*)-9,10-epoxytricosene and (*Z*)-14-tricosen-10-one. *Insect Biochem.* 14:279–284.

BROWN, H.C., and GUPTA, S.K. 1975. Hydroboration XXXIX. 1,3,2-Benzodioxaborale (catecholborane) as a new hydroboration reagent for alkenes and alkynes. A general synthesis of alkane- and alkeneboronic acids and esters via hydroboration of alkenes and alkynes with catecholborane, *J. Am. Chem. Soc.* 97:5249–5255.

CARLSON, D.A., MAYER, M.S., SILHACEK, D.L., BEROZA, M., and BIERL, B.A. 1971. Sex attractant pheromone of the housefly: Isolation, identification and synthesis. *Science* 174:76–78.

CARLSON, D.A., DOOLITTLE, R.E., BEROZA, M., ROGOFF, W.M., and GRETZ, G.H. 1974. Muscalure and related compounds. I. Response of houseflies in olfactometer and pseudofly tests. *J. Agric. Food Chem.* 22:194–197.

COREY, E.J., and POSNER, G.H. 1967. Selective formation of carbon–carbon bonds between unlike groups using organocopper reagents. *J. Am. Chem. Soc.* 89:3911–3912.

DJERASSI, C., and WILLIAMS, D.H. 1963. Studies in organic sulphur compounds. Part XIV. Formation of olefins on desulphurisation of ethylene thioketals by Raney nickel. *J. Chem. Soc.* 1963:4046–4051.

FUJITA, E., NAGAO, Y., and KANEKO, K. 1978. Useful dethioacetalization with soft acid metal salts: Thallium trinitrate and mercuric perchlorate. *Chem. Pharm. Bull.* 26(12):3743–3751.

GUO, L., G., LATLI, B., PRESTWICH, G.D., and BLOMQUIST, G.J. 1991. Metabolically blocked analogs of housefly sex pheromone II. Biochemical metabolism studies. *J. Chem. Ecol.* 17:1769–1782.

HARNEY, D.W., MEISTERS, A., and MOLE, T. 1974. C-Methylation of alcohols by trimethylaluminum. *Aust. J. Chem.* 27:1639–1653.

KABALKA, G.W., HUTCHINS, R., NATALE, N.R., YANG, D.-T.C., and BROACH, V. 1979. Conjugate reduction of α,β-unsaturated *p*-toluenesulfonylhydrazones to alkenes with catecholborane: 5 β-Cholest-3-ene. *Org. Synth.* 59:42–48.

KELKAR, S.V., JOSHI, G.S., REDDY, B., and KULKARNI, G.H. 1989. A novel approach to the synthesis of 7(*Z*),11(*Z*)-nonacosadiene, pheromone of *Drosophila melanogaster* and 9(*Z*)-tricosene, pheromone of *Musca domestica*. *Syn. Commun.* 19(7 and 8):1369–1379.

KOCIÉNSKI, P.J., CERNIGLIARO, G., and FELDSTEIN, G. 1977. A synthesis of (+,−)-methyl *n*-tetradeca-*trans*-2,4,5-trienoate, an allenic ester produced by the male dried bean beetle *Acanthoscelides obtectus* (Say). *J. Org. Chem.* 42:353–355.

LATLI, B., and PRESTWICH, G.D. 1988. Synthesis of a tritium-labeled photoaffinity analog of the tussock moth pheromone: Tritium NMR of vinyl tritons of (*E*)- and (*Z*)-alkene isomers. *J. Org. Chem.* 53:4603–4605.

MIDDLETON, W.J. 1975. New fluorinating reagents. Dialkylaminosulfur fluorides. *J. Org. Chem.* 40:574–578.

MORI, K., UCHIDA, M., and MATSUI, M. 1977. Synthesis of aliphatic insect pheromones from alicyclic starting materials. *Tetrahedron* 33:385–387.

NOVIS SMITH, W., and BEUMEL, D.F., JR. 1974. Preparation of alkynes and dialkynes by reaction of monohalo- and dihaloalkanes with lithium acetylenide–ethylenediamine complex. *Synthesis* 1974:441–442.

POIDEVIN, G., FOY, P., and RULL, T. 1979. Une nouvelle synthèse de la diméthyl-3,3-hexaméthylène diamine-1,6 à partir de l'acrylonitrile, du butyraldéhyde et du nitrométhane. *Bull. Soc. Chim. Fr.* II:196–198.

PRESTWICH, G.D. 1987. Chemical studies of pheromone reception and catabolism. pp. 473–527. *In* G.D. Prestwich and G.J. Blomquist, (eds.). Pheromone Biochemistry. Academic Press, New York.

REETZ, M.T., WESTERMAN, J., and KYUNG, S.-H. 1985. Direct geminal dimethylation of ketones and exhaustive methylation of carboxylic acid chlorides using dichlorodimethyltitanium. *Chem. Ber.* 118:1050–1057.

ROGOFF, W.M., BELTZ, A.D., JOHNSEN, J.O., and PLAPP, F.W. 1964. A sex pheromone in the housefly, *Musca domestica* L. *J. Insect Physiol.* 10:239–246.

SONDEJ, S.C., and KATZENELLENBOGEN, J.A. 1986. *gem*-Difluoro compounds: A convenient preparation from ketones and aldehydes by halogen fluoride treatment of 1,3-dithiolanes. *J. Org. Chem.* 51:3508–3513.

SUDA, M. 1981. Cyclopropanation of terminal olefins using diazomethane/palladium (II) acetate, *Synthesis* 1981:714.

SUN, W.-C., and PRESTWICH, G.D. 1990. Partially fluorinated analogs of (*Z*)-9-dodecenyl acetate: Probes for pheromone hydrophobicity requirements. *Tetrahedron Lett.* 31:801–804.

TSUJI, J., SHIMIZU, I., and YAMAMOTO, K. 1976. Convenient general synthetic method for 1,4- and 1,5-diketones by palladium catalyzed oxidation of α-allyl and α-3-butenyl ketones. *Tetrahedron Lett.* 34:2975–2976.

ZAKHARKIN, L.I., and KHORLINA, I.M. 1964. Preparation of aldehydes by the reduction of nitriles with diisobutylaluminum hydride. *Izv. Akad. Nauk SSSR. Ser. Khim.* 1964:465–466.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

METABOLICALLY BLOCKED ANALOGS OF HOUSEFLY SEX PHEROMONE: II. METABOLISM STUDIES

LIN GUO,[1,*] BACHIR LATLI,[2] GLENN D. PRESTWICH,[2] and GARY J. BLOMQUIST[1]

[1]*Department of Biochemistry*
University of Nevada
Reno, Nevada 89557-0014

[2]*Department of Chemistry*
State University of New York at Stony Brook
Stony Brook, New York 11794-3400

(Received January 15, 1991; accepted May 9, 1991)

Abstract—Analogs of (*Z*)-9-tricosene (*Z*9–23 : Hy) bearing methyl substituents, cyclopropyl groups, fluorine substituents, and additional double bonds were used to probe the substrate requirements for the monooxygenase system that converts *Z*9–23 : Hy to the corresponding epoxide and ketone. Three of the seven analogs tested, 10-fluoro-(*Z*)-14-tricosene, 10,10-difluoro-(*Z*)-14-tricosene, and 14-methyl-(*Z*)-9-tricosene, were metabolized to the corresponding epoxide. Compounds with two methyl groups, a cyclopropane group, a hydroxy group, or an additional double bond at the 14 position were not epoxidized at the 9,10 position. This suggests that only minimal structural change at the 14-position of *Z*9–23 : Hy is allowed with retention of metabolic activity. None of the analogs tested were hydroxylated at the position equivalent to the 14 position of *Z*9–23 : Hy. Of the 13 analogs tested as inhibitors of *Z*9–23 : Hy metabolism, the two compounds that were the most effective inhibitors in both male and female houseflies were (*Z*)-14-tricosen-10-one and 1-nonyl-1-[(*Z*)-4-tetradecen-1-yl]-cyclopropane. These data show that the polysubstrate monooxygenase that metabolizes *Z*9–23 : Hy in the housefly has very strict structural requirements for the substrate.

Key Words—Sex pheromone, sex pheromone analogs, (*Z*)-9-tricosene, housefly, *Musca domestica*, Diptera, Muscidae, polysubstrate monooxygenase.

*To whom correspondence should be addressed.

0098-0331/91/0900-1769$06.50/0

INTRODUCTION

The sex pheromone components of the housefly, *Musca domestica*, consist of (*Z*)-9-tricosene (Z9–23 : Hy) (Carlson et al., 1971), *cis*-9,10-epoxytricosane (C_{23} epoxide), (*Z*)-14-tricosen-10-one (C_{23} ketone) (Uebel et al., 1978) and C_{28}–C_{30} methylalkanes (Uebel et al., 1976). Z9–23 : Hy is a short-range attractant and elicits the mating strike, the C_{23} epoxide and ketone act as sex recognition factors, and the methylalkanes promote and extend sexual contact as arrestant factors (Adams and Holt, 1987). The effects of structural variations of Z9–23 : Hy (originally named muscalure) on biological activity were examined in olfactometer and pseudofly tests (Carlson et al., 1974). The structural variations included double-bond position, *cis–trans* isomerism, substitution of the double bond with a triple bond, methyl branching at different points in the molecule, carbon chain length, and length of carbon chain linked to the unsaturated bond. The results of those experiments indicated that the structure requirements for activity were less stringent than those for most other sex pheromones.

The C_{23} sex pheromone components on the female housefly first appear on day 2 postemergence during the early vitellogenic stages and accumulate as a function of age and ovarian development (Dillwith et al., 1983; Ahmad et al., 1989). All female body parts contain the sex pheromone components, with the legs and abdomen containing the largest amount (Blomquist et al., 1984b). Male houseflies, which normally do not produce any of the C_{23} sex pheromone components (Adams et al., 1984), can be induced to produce female-specific pheromones after ovary implants or injections of 20-hydroxyecdysone (Blomquist et al., 1984a).

The biosynthesis of the C_{23} sex pheromone components have been studied (Blomquist et al., 1987). Z9–23 : Hy formation in female houseflies occurs by the microsomal elongation of oleoyl-CoA to a 24-carbon fatty acyl moiety that is then converted to an alkene one carbon shorter (Blomquist et al., 1987). The C_{23} epoxide and ketone arise from metabolism of Z9–23 : Hy (Blomquist et al., 1984b). Direct evidence was provided for the role of a cytochrome P-450 polysubstrate monooxygenase (PSMO) in the conversion of the alkene to the epoxide at the 9,10 positions and ketone (Ahmad et al., 1987). The latter is apparently formed by hydroxylation at the methylene carbon 10 carbons from the other end of the molecule with subsequent oxidation to the ketone by another enzyme, which has not been characterized. Males also readily convert exogenous Z9–23 : Hy to the epoxide and ketone and show a relatively higher rate of metabolism than females.

All major body parts of both male and female houseflies metabolized Z9–23 : Hy when it was applied to the surface of the insect (Blomquist et al., 1984b), which suggested that this PSMO is generally distributed. To study the molecular basis of pheromone metabolism requires detailed knowledge of the inter-

action between the pheromone and enzyme, in which the substrate specificity of the enzyme is one of the key points. Radiolabeled pheromones and pheromone analogs have been used to isolate and characterize both the macromolecules and pheromone metabolites in a variety of insect species (Prestwich, 1987; Prestwich et al., 1989), providing new insights into pheromone reception and catabolism.

In this paper, we present data on the metabolism of a variety of Z9-23 : Hy analogs in the male housefly and the effect of the analogs on the metabolism of [^{3}H]Z9-23 : Hy in both male and female houseflies. The chemical synthesis of the Z9-23 : Hy analogs and the justification for choosing the structures synthesized were described in the prior paper in this issue (Latli and Prestwich, 1991).

METHODS AND MATERIALS

Insects. Pupae of the Fales T-II strain houseflies, *Musca domestica*, were provided by the Biology Section, S.C. Johnson & Sons, Racine, Wisconsin. Within 12 hr after emergence, male and female insects were separated, and adult insects were maintained separately according to sex and fed ad libitum on sucrose–low-fat powdered milk (1 : 1, w/w) and water at 25°C.

Chemicals. Analogs of Z9-23 : Hy (Figure 1) were prepared as described in Latli and Prestwich (1991). Z9-23 : Hy was synthesized as described (Carlson et al., 1974).

[9,10-$^3H_2$]-(Z)-9-tricosene. Tritium-labeled pheromone was obtained by hydrogenation of 9-tricosyne using tritium gas and the conditions described above for the unlabeled reaction. The tritium gas reaction was performed by E. Do at Dupont-NEN, and provided a mixture of 40% (Z)-alkene, 37% (E)-alkene, and 23% saturated alkane, as determined by autoradiography of an argentated TLC plate using hexane as eluent. The desired [9,10-$^3H_2$]-Z9-23 : Hy was isolated by chromatography on 20% $AgNO_3$–SiO_2 by gradient elution with ethyl acetate in hexane. [^{3}H]NMR of the purified product was performed and showed that about three fifths of the ^{3}H was present at the 9,10 positions, one fifth at the allylic positions, and one fifth on other methylene carbons. [^{3}H]NMR ($CDCl_3$): δ 5.42 (57.9%), 1.95 (20.9%), 1.25 (21.2%).

(Z)-9,10-Epoxytricosane. To a stirred solution of *m*-chloroperoxybenzoic acid (*m*-CPBA, 13 mg, Aldrich, 80–85% technical grade) in CH_2Cl_2 (1.0 ml) was added (Z)-9-tricosene (20 mg, 0.06 mmol) in CH_2Cl_2 (1.0 ml). After stirring for 2 hr, the solution was stirred with 0.2 ml of 10% Na_2SO_3 for 15 min and then washed with 0.2 ml of 5% NaOH and 0.5 ml of water. The organic solution was concentrated in vacuo and purified by flash chromatography to give 20 mg (95% yield) as a colorless oil of 90 : 10 *cis-trans* by GC. R_f = 0.71 in 10% EtOAc–Hex. R_t = 15.30 (T_i = 140°C, 2 min; 7°C/min; T_f = 250°C).

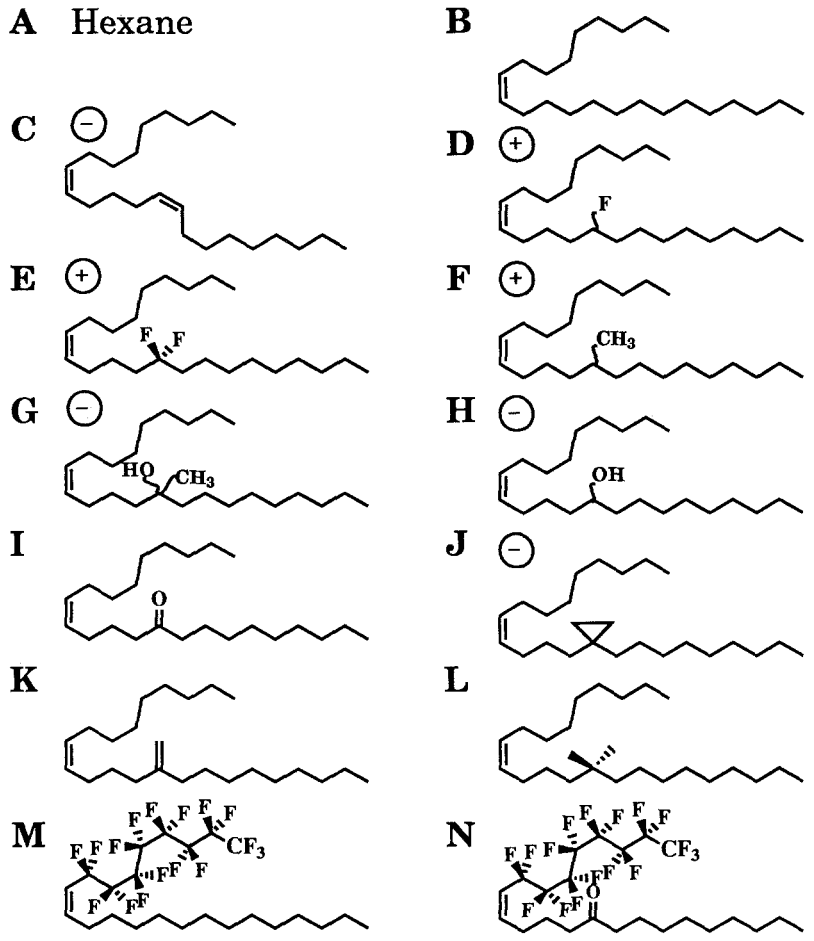


FIG. 1. Z9-23:Hy analogs used in these studies. +, epoxidized by male housefly; −, not epoxidized. Four-day-old unmated male houseflies were treated with (Z)-9-tricosene analogs on the ventral side of the abdomens as described in Methods and Materials. After a 6-hr incubation at 25°C, three groups of 10 flies each were killed, lipids were extracted, and the nonhydrocarbon fraction was analyzed by GC and GC-MS as described in Methods and Materials. + and − are based on GC and GC-MS analysis.

^{1}H NMR ($CDCl_3$): δ 2.86 (m, 2H), 1.44 (m, 4H), 1.26 (br s, 26H), 0.88 (t, J = 6.5 Hz, 6H); ^{13}C NMR ($CDCl_3$): δ 57.28, 31.93, 31.87, 31.60, 29.66, 29.57, 29.37, 29.23, 27.82, 26.62, 22.66, 14.12.

In Vivo Studies. For the studies with Z9–23 : Hy analogs, 4-day-old unmated males were treated on the ventral side of the abdomens with 1 μl of an analog in hexane (5 μg/μl) per insect. The treated flies were held at 25°C in a 200-ml urine bottle covered with a piece of organdy cloth and a rubber band. After 3 and 6 hr, flies were killed by freezing at −20°C and stored at −20°C. Three groups of 10 flies each were used for each data point.

For the studies on the effect of Z9–23 : Hy analogs on [^{3}H]Z9–23 : Hy metabolism, both 4-day-old males and females were used. Insects were treated with 0.5 μl of each analog in hexane (2 μg/μl) on the ventral side of the abdomen. After 10 min, [^{3}H]Z9–23 : Hy (60 Ci/mmol) in 0.5 μl hexane (0.18 μCi) was applied on each housefly on the same location. The amount of the analogs applied (1 μg) was comparable to the amount of Z9–23 : Hy on the cuticular of the female housefly (Ahmad et al., 1989). The amount (mass) of [^{3}H]Z9–23 : Hy applied was small enough that it would have minimal affect on the amount of Z9–23 : Hy on the female housefly (Ahmad et al., 1989). After 2 hr at 25°C, flies were killed by freezing at −20°C and stored at −20°C. Three groups of five flies each were used for each data point for both male and female houseflies.

Extraction and Separation. Houseflies were extracted by rinsing in 5 ml redistilled hexane for 10 min, removing the solvent, and then adding 2 ml of hexane for an additional 1 min. The extracts were combined and reduced in volume under a stream of N_2.

The extracts were separated into hydrocarbon and nonhydrocarbon fractions by chromatography on a minicolumn (6 × 0.5 cm) of Biosil-A (Bio-Rad Labs, Richmond, California). The hydrocarbons were eluted with 7 ml of hexane and the nonhydrocarbons were eluted with 7 ml hexane–diethyl ether 1 : 1.

GC and GC-MS Analysis. The nonhydrocarbon fractions from the Z9–23 : Hy analog metabolism studies were analyzed by gas chromatography (GC) and GC-mass spectrometry (GC-MS) to study the formation of products. Chemically synthesized epoxides (Latli and Prestwich, 1991) were used as standards for GC and GC-MS analyses. All GC analyses were performed on a Hewlett-Packard 5890 controlled by a Hewlett-Packard 3393A integrator fitted with a 30 m × 0.32 mm ID, 0.5 μm stationary phase DB-5 column. The oven was temperature programmed from 200 to 310°C at 5°C/min, then held at 310°C for 15 min. The carrier gas was He, at a flow rate of 2.5 ml/min, and the carrier gas plus makeup gas was at a total flow rate of 30 ml/min. The amount of epoxide formed was determined by comparing the epoxide peak areas with external standards (a known concentration of Z9–23 : Hy solution was used as the external standard).

GC-MS analyses were performed on a Finnigan 4023 mass spectrometer

interfaced with an INCOS data system at 70 eV. A 30-m DB-5 column as above was used. The GC oven was temperature programmed from 30 to 200°C at 20°C/min, 200–280°C at 5°C/min, then held at 280°C for 15 min. The carrier gas was He at 8 psi head pressure, linear flow velocity about 30 cm/sec.

Determination of Radioactivity. Radioactivity was assayed on a Beckman LS 1701 scintillation counter in a 10-ml fluor solution containing 0.4% 2,5-diphenyloxazole (PPO) in toluene at an efficiency of ca. 53% for ^{3}H.

RESULTS AND DISCUSSION

Metabolism of Z9–23 : Hy Analogs. Both female and male houseflies readily metabolize *Z*9–23 : Hy to the corresponding epoxide, with males showing an apparent higher rate of conversion of exogenous substrate than females (Blomquist et al., 1984b; Ahmad et al., 1987). After day 2 postemergence, the female produces large amounts of *Z*9–23 : Hy, whereas the male does not, thus suggesting that the apparent difference in the rates of metabolism of exogenous *Z*9–23 : Hy in part could be due to dilution and thus lowering of the specific activity of the substrate applied to the female. Because of the apparent higher rates of epoxide and ketone formation, male insects were used to examine the metabolism of metabolically blocked analogs of *Z*9–23 : Hy. In addition, the use of males for these studies simplified product analysis as limited endogenous epoxides were present.

Of the seven *Z*9–23 : Hy analogs studied, three were converted to the corresponding epoxides by male houseflies (Figure 1). These include 10-fluoro-(*Z*)-14-tricosene, 10,10-difluoro-(*Z*)-14-tricosene, and 14-methyl-(*Z*)-9-tricosene. Products were characterized by comparison with synthetic epoxide standards using both GC and GC-MS. The mass spectra of the resulting epoxides are shown in Figure 2, and are essentially identical to those of the synthetic standards. All three spectra show a strong ion at *m/z* 155, which arises from cleavage between carbons 10 and 11, with the charge retained on the fragment containing the oxygen, and is diagnostic for the 9,10-epoxide.

Analysis of the metabolic products of 10-fluoro-(*Z*)-14-tricosene showed two GC peaks that were not completely baseline separated (Figure 3). Both peaks gave identical mass spectra as represented in Figure 2A. The most likely explanation for this phenomena is that two racemic pairs of diastereomers are formed, i.e., (9*S**,10*R**,14*R**)-9,10-epoxy-14-fluorotricosane and the 9*S**,10*R**,14*S**) isomer.

Modifying the C-14 position with either one fluorine or two fluorine atoms did not prevent the epoxidation at the C-9, C-10 position. Although introduction of the C–F bond significantly increases the electronegativity of the bond at the C-14 position, only limited steric properties change with the addition of fluorine

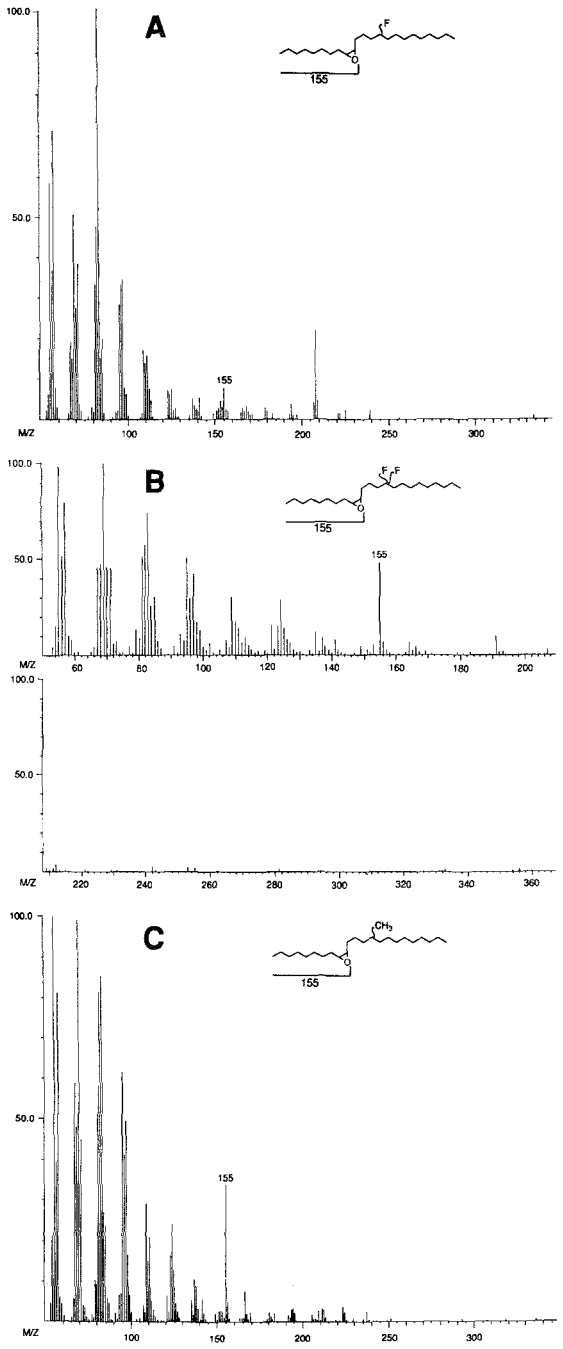


FIG. 2. Mass spectra of the epoxides formed by male houseflies treated with 10-fluoro-(Z)-14-tricosene (A), 10,10-difluoro-(Z)-14-tricosene (B), and 14-methyl-(Z)-9-tricosene (C).

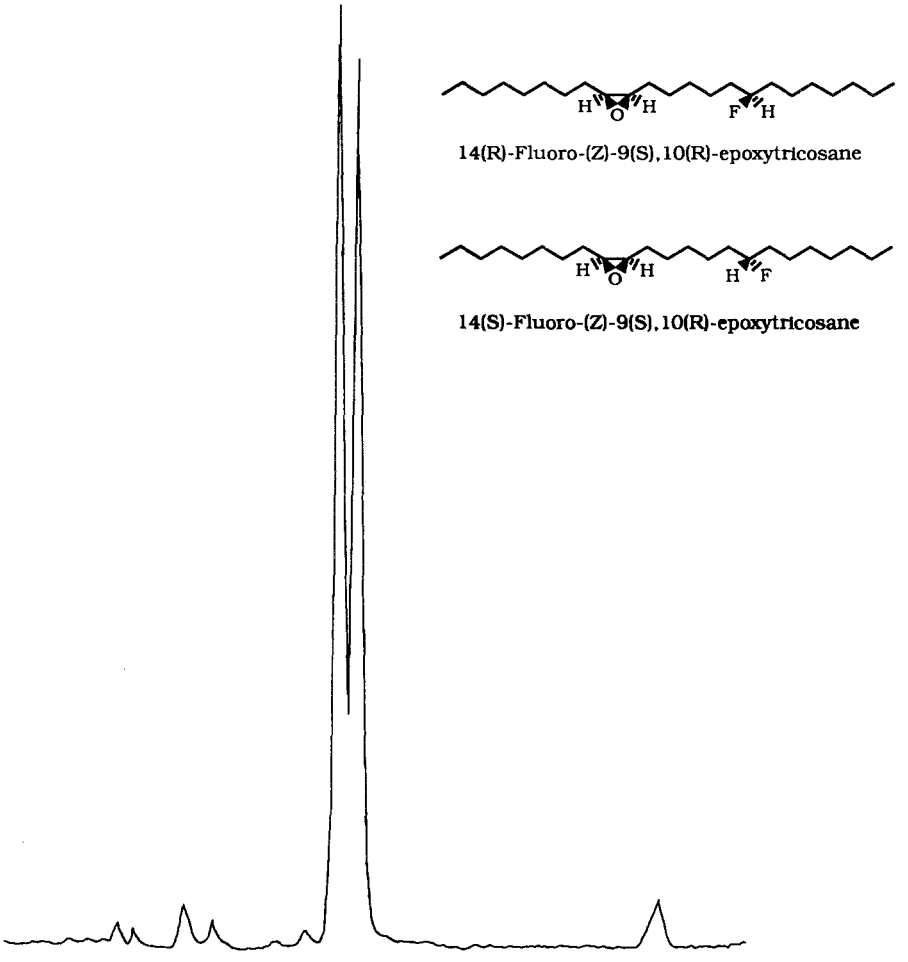


FIG. 3. Portion of the total ion GC-MS trace showing the two GC peaks that yielded identical mass spectra as shown in Figure 1A. The data are interpreted that two epoxide diastereomers were formed.

(Patrick, 1979; Welch, 1987; Sun and Prestwich, 1990). When the C-14 position was modified with the addition of one or two methyl groups, only the monomethyl derivative was converted to the epoxide. This suggests that steric hindrance at the C-14 position markedly effects the PSMO epoxidation at carbons 9 and 10 and that the addition of the second methyl group prevents a productive binding of the analog to the enzyme. Our observation that 1-nonyl-1-[(Z)-9-tetradecenyl]-cyclopropane, an analog with 14,14-dimethyl groups fused into a cyclopropyl ring, is not epoxidized further supports this hypothesis.

No epoxidation was observed for (Z,Z)-9,14-tricosadiene (Figure 1), suggesting that introduction of a double bond at the C-14 position changes the three dimensional structure of the molecule such that it no longer fits the steric requirements for this PSMO. Likewise, introducing a hydroxyl group at the 14 position of Z9–23 : Hy prevents epoxidation, probably by increasing the polarity such that binding in a hydrophobic site is prevented. This is consistent with the absence of a 14-keto 9,10 epoxide on the female housefly. Apparently once the Z9–23 : Hy is modified at either the 9, 10, or the 14 position, it will not serve as a substrate for further oxygenation.

Different rates of epoxidation were observed among the three Z9–23 : Hy analogs that were converted to the corresponding epoxides by male houseflies (Figure 4). Relatively high amounts of each analog were applied (2 μg/housefly); thus, the epoxidation reactions were performed under saturating conditions. The rate of 10-fluoro-*cis*-14,15-epoxytricosane formation was about two times higher than the rate of 10,10-difluoro-*cis*-14,15-epoxytricosane formation, and the rate of 14-methyl-(Z)-9,10-epoxytricosane formation was the lowest (Figure 4). This suggests that the rate of epoxidation is very closely related to the steric barrier size at the C-14 position. Thus, increased steric hindrance or electronic perturbation at C-14 results in a lower rate of epoxidation at the C-9,C-10 position.

None of the metabolically blocked Z9–23 : Hy analogs underwent hydroxylation at the 14-position. Adding any substituent at carbon 14, even a single methyl group, is apparently sufficient to prevent this hydroxylation.

Direct evidence was obtained for the conversion of (Z)-14-tricosen-10-ol to the C_{23} ketone in male houseflies by analysis of the C_{23} ketone produced

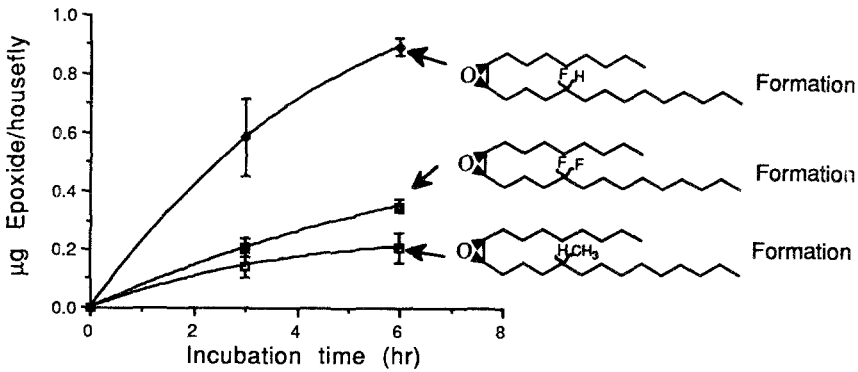


FIG. 4. The formation of epoxides as a function of incubation time. Z9–23 : Hy analogs were applied to the male houseflies (5 μg/housefly) on the ventral side of the abdomens. After the time indicated, houseflies were killed, and the cuticular lipids were extracted and analyzed as described in Methods and Materials.

from the C_{23} secondary alcohol. GC-MS was used and the spectrum compared to the spectrum of the corresponding ketone standard. Previous work (Ahmad et al., 1987) using only TLC R_f values had suggested that the secondary alcohol was an intermediate in the metabolism of the alkene to ketone.

Selective ion monitoring of the GC-MS trace at *m/z* 155 showed that trace amounts of *cis*-9,10-epoxyheptacosane were present in all samples. The mass spectrum of this component showed ions at *m/z* 155 and 267, consistent with cleavage of C–C bonds adjacent to the epoxide with retention of the charge on the oxygen-containing fragment. This is the first identification of *cis*-9,10-epoxyheptacosane in the housefly and suggests that the chain length specificity of the epoxidation enzyme is not absolute for the 23-carbon alkene. It will apparently accept as substrate, although at a much lower rate, the 27-carbon alkene. This is the major alkene in control males and previtellogenic females. Neither males nor females produce appreciable amounts of the 25-carbon alkene (Nelson et al., 1981), and it is not known if shorter chain Z9-alkenes or alkenes with double bonds in different positions are epoxidized.

Effect of Z9–23 : Hy Analogs on [^{3}H]Z9–23 : Hy Metabolism. Twelve synthetic Z9–23 : Hy analogs were applied to both male and female houseflies to study their effect on the metabolism of [^{3}H]Z9–23 : Hy (Table 1). [^{3}H]Z9–23 : Hy (>98% pure as determined by radio-GLC) was used to monitor the effect of the analogs on Z9–23 : Hy metabolism. The percentage of radioactivity recovered from the nonhydrocarbon fraction was used to estimate [^{3}H]Z9–23 : Hy metabolism (Figure 5). Previous work showed that most of the oxygenated material in the nonhydrocarbon fraction was the C_{23} epoxide and C_{23} ketone (Blomquist et al., 1984b). Hexane and nonradioactive Z9–23 : Hy in separate experiments were used as control groups. To study the significance of the inhibition effect *t* tests at the 0.005 level were used (Table 1).

The results show that the analogs studied can be separated into three groups. Group I compounds had no significant inhibition when compared to control groups, suggesting that group I compounds do not interact with the PSMO. Group II compounds were significantly different from the hexane-treated group, but showed no significant inhibition when compared to the nonradioactive Z9–23 : Hy-treated group. Because the percentage of Z9–23 : Hy metabolized is based on the percentage of radioactivity recovered in the nonhydrocarbon fraction, the nonradioactive Z9–23 : Hy-treated group had a lower percentage of substrate metabolized than did the hexane-treated group. The nonradioactive Z9–23 : Hy simply dilutes the specific activity of the [^{3}H]Z9–23 : Hy as substrate and therefore appears as an inhibitor. Therefore, for group II compounds, the results suggest that they do interact in some way with PSMO, but do not significantly inhibit the enzyme activity. Group III compounds significantly inhibit Z9–23 : Hy metabolism when compared with the nonradioactive Z9–23 : Hy-treated group.

Male and female houseflies respond to the analogs somewhat differently.

TABLE 1. STATISTICAL INTERPRETATION OF EFFECTS OF Z9-23 : Hy ANALOGS ON [^{3}H]Z9-23 : Hy METABOLISM IN MALE[a] AND FEMALE HOUSEFLIES

		Significance of the inhibition effect on [^{3}H]Z9-23 : Hy metabolism (α = 0.005)					
		Male			Female		
	Compound applied[b]	Hexane as control	nonradioactive Z9-23 : Hy as control	Group[c]	Hexane as control	nonradioactive Z9-23 : Hy as control	Group[c]
A	Hexane (control)	−	−		−	−	
B	(Z)-9-Tricosene (control)	+	−		−	−	
C	(Z,Z)-9,14-Tricosadiene	+	−	II	−	−	I
D	10-Fluoro-(Z)-14-tricosene	+	−	II	−	−	I
E	10,10-Difluoro-(Z)-14-tricosene	+	+	III	−	−	I
F	14-Methyl-(Z)-9-tricosene	−	−	I	−	−	I
G	10-Methyl-(Z)-14-tricosen-10-ol	+	−	II	+	−	II
H	(Z)-14-Tricosene-10-ol	+	+	III	−	−	I
I	(Z)-14-Tricosen-10-one	+	+	III	+	+	III
J	1-Nonyl-1-[(Z)-4-tetradecen-1-yl]-cyclopropane	+	+	III	+	+	III
K	14-Methylene-(Z)-9-Tricosene	+	−	II	−	−	I
L	14,14-Dimethyl-(Z)-9-tricosene	+	−	II	−	−	I
M	1-Perfluorooctyl-1-pentadecene	+	−	II	−	−	I
N	1-Perfluorooctyl-1-pentadecen-6-one	−	−	I	−	−	I

[a] Statistical calculation was based on data presented on Figure 5.
[b] Compound symbols same as Figure 1 and Figure 5.
[c] The classification of the group is described in the text.

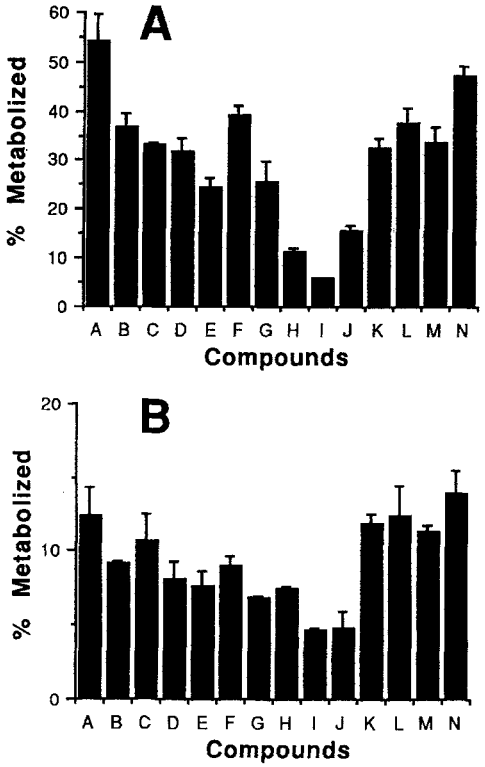


FIG. 5. Effect of Z9-23:Hy analogs on [^{3}H]Z9-23:Hy metabolism. Analogs and [^{3}H]Z9-23:Hy were applied to both male and female houseflies on the abdomens as described in Methods and Materials. After a 2-hr incubation, cuticular lipids were extracted. The percentage of radioactivity recovered from the nonhydrocarbon fraction is presented as the percent [^{3}H]Z9-23:Hy metabolized. Table 1 lists the names of the applied compounds used. See Figure 1 for the structures of these compounds.

Females normally produce C_{23} sex pheromone, and it is therefore not surprising that Z9-23:Hy metabolism in females is less affected by addition of unlabeled Z9-23:Hy than males (Table 1). Nine compounds applied to females are group I compounds (Table 1). For the males on the other hand, only two compounds belong to group I. Some of the group III compounds that inhibit Z9-23:Hy metabolism in females also act as inhibitors in males. These include (Z)-14-tricosene-10-one (I) and 1-nonyl-1-[(Z)-9-tetradecen-1-yl]-cyclopropane (J) (Table 1). Some group III compounds for males, such as 10,10-difluoro-(Z)-14-tricosene (E) and 10-methyl-(Z)-14-tricosene-10-ol (F), do not act as inhibitors in female (Table 1).

The compound that inhibits PSMO activity in both males and females most

significantly is (*Z*)-14-tricosene-10-one (Figure 5, Table 1). This is particularly interesting since it is one of the metabolites of Z9–23 : Hy, suggesting that it may have a role in end-product inhibition.

Acknowledgments—Supported in part by National Science Foundation grant DCB-8900088 to G.J.B. and by a Herman Frasch Foundation award to G.D.P. We thank Robin Eppard for technical assistance and Murray Hackett for GC-MS analysis. A contribution of the Nevada Agricultural Experiment Station.

REFERENCES

ADAMS, T.S., and HOLT, G.G. 1987. Effect of pheromone components when applied to different models on male sexual behavior in the housefly, *Musca domestica*. *J. Insect Physiol.* 33:9–18.

ADAMS, T.S., HOLT, G.G., and BLOMQUIST, G.J. 1984. Endocrine control of pheromone biosynthesis and mating behavior in the housefly, *Musca domestica*, pp. 441–457, *in* Advances in Invertebrate Reproduction. W. Engels, W.H. Clark, A. Fisher, P.G. Olive, and D.F. Went (eds.). Elsevier, Amsterdam.

AHMAD, S., KIRKLAND, K.E., and BLOMQUIST, G.J. 1987. Evidence for a sex pheromone metabolizing cytochrome P-450 mono-oxygenase in the housefly. *Arch. Insect Biochem. Physiol.* 6:121–140.

AHMAD, S., MACKAY, M.E., and BLOMQUIST, G.J. 1989. Accumulation of the female sex pheromone and its transfer to, and metabolism in, the male housefly, *Musca domestica* L., during courtship and mating. *J. Insect Physiol.* 3:775–780.

BLOMQUIST, G.J., ADAMS, T.S., and DILLWITH, J.W. 1984a. Induction of female sex pheromone production in male houseflies by ovarian implants or 20-hydroxyecdysone. *J. Insect Physiol.* 30:295–302.

BLOMQUIST, G.J., DILLWITH, J.W., and POMONIS, J.G. 1984b. Sex pheromone of the housefly: Metabolism of (*Z*)-9-tricosene to (*Z*)-9-epoxytricosane and (*Z*)-14-tricosene-10-one. *Insect Biochem.* 14:279–284.

BLOMQUIST, G.J., DILLWITH, J.W., and ADAMS, T.S. 1987. Biosynthesis and endocrine regulation of sex pheromone production in Diptera, pp. 217–250, *in* G.D. Prestwich and G.J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, New York.

CARLSON, D.A., MAYER, M.S., SILHACEK, D.L., JAMES, J.H., BEROZA, M., and BIERL, B. 1971. Sex attractant pheromone of the housefly: Isolation, identification and synthesis. *Science* 174:76–77.

CARLSON, D.A., DOOLITTLE, R.E., BEROZA, M., ROGOFF, W.M., and CRETZ, G.H. 1974. Muscalure and related compounds. I. Response of houseflies in olfactometer and pseudofly tests. *J. Agric. Food Chem.* 22:193–197.

DILLWITH, J.W., ADAMS, T.S., and BLOMQUIST, G.J. 1983. Correlation of housefly sex pheromone production with ovarian development. *J. Insect Physiol.* 29:377–386.

LATLI, B., and PRESTWICH, G.D. 1991. Metabolically blocked analogs of housefly sex pheromone. I. Synthesis of alternative substrates for cuticular monooxygenases. *J. Chem. Ecol.* 17:1745–1768.

NELSON, D.R., DILLWITH, J.W., and BLOMQUIST, G.J. 1981. Cuticular hydrocarbons of the housefly, *Musca domestica*. *Insect Biochem.* 11:186–197.

PATRICK, T.B. 1979. Fluoro-organic biochemistry. *J. Chem. Educ.* 56:228–230.

PRESTWICH, G.D. 1987. Chemical studies of pheromone reception and catabolism. pp. 473–527,

in G.D. Prestwich and G.J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, New York.

PRESTWICH, G.D., GRAHAM, S.G., HANDLEY, M., LATLI, B., STREINZ, L., and TASAYCO, J.M.L. 1989. Enzymatic processing of pheromones and pheromone analogs. *Experientia* 45:263–270.

SUN, W.-C., and PRESTWICH, G.D. 1990. Partially fluorinated analogs of (*Z*)-9-dodecenyl acetate: Probes for pheromone hydrophobicity requirements. *Tetrahedron Lett.* 31:801–804.

UEBEL, E.C., SONEET, P.E., and MILLER, R.W. 1976. Housefly sex pheromone: Enhancement of mating strike activity by combination of (*Z*)-9-tricosene with branched saturated hydrocarbons. *J. Econ. Entomol.* 5:905–908.

UEBEL, E.C., SCHWARZ, M., LUSBY, W.R., MILLER, R.W., and SONNET, P.E. 1978. Cuticular non-hydrocarbons of the female housefly and their evaluation as mating stimulants. *Lloydia* 41:63–67.

WELCH, J.T. 1987. Advances in the preparation of biologically active organofluorine compounds. *Tetrahedron* 43:3123–3197.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

WHITE ALDER AND DOUGLAS-FIR FOLIAGE QUALITY AND INTEREGG-MASS INFLUENCES ON LARVAL DEVELOPMENT OF GYPSY MOTH, *Lymantria dispar*

G. JOSEPH,[1,*] J.C. MILLER, R.E. BERRY, J. WERNZ, A.F. MOLDENKE, and R.G. KELSEY[2]

Department of Entomology
Oregon State University
Corvallis, Oregon 97331-2907

(Received February 11, 1991; accepted April 29, 1991)

Abstract—Individual families of gypsy moth collected from a single population exhibited different degrees of fitness when fed diets of white alder, a suitable broadleaf host, and Douglas-fir, an unsuitable conifer host. Members of families on diets of Douglas-fir had significantly lower survival, longer larval periods, lower pupal weights, and shorter pupal periods than members of the same families fed alder. Foliar nutritional quality, including nitrogen level and allelochemical composition (terpenes and phenols), was considered the key factor responsible for these differences. Growth parameters differed significantly for families within diet treatments, indicating that the genetic resources of a family did affect performance somewhat. The influence of a family's genetic resources on larval survival was most notable when larvae were under the greatest nutritional stress.

Key Words—Douglas-fir, *Pseudotsuga menziesii*, gypsy moth, *Lymantria dispar*, Lepidoptera, Lymantriidae, insect-host plant relations, phenolics, terpenes, white alder, *Alnus rhombifolia*.

INTRODUCTION

Developmental rate and survival of the gypsy moth are influenced by many factors, including host species (Hough and Pimentel, 1978; Barbosa et al.,

*To whom correspondence should be addressed.
[1]Present address: Department of Forest Science, School of Forestry, Oregon State University, Corvallis, Oregon 97331-2907.
[2]Present address: USDA, Forest Service, Pacific Northwest Research Station, Forestry Sciences Laboratory, Corvallis, Oregon 97331-2907.

0098-0331/91/0900-1783$06.50/0

1986); constitutive and induced chemical variation within the host species (Rhoades, 1983; Rossiter et al., 1988); genetic variation among and within insect populations (Leonard, 1966); and environmental variables, such as temperature and humidity (Elkinton and Liebhold, 1990). Previous studies relating the suitability of hosts to gypsy moth fitness have suggested that certain allelochemicals, such as alkaloids, influence fitness parameters, such as larval development (Doskotch et al., 1981; Barbosa et al., 1983, 1990a,b; Barbosa and Krischik, 1987; Miller and Hanson, 1989a). Plants containing iridoids and alkaloids appear to be poor hosts (Barbosa and Krischik, 1987; Miller and Hanson, 1989a, Barbosa et al., 1990a), but many other plants containing a wide array of allelochemicals permit successful development (Miller and Hanson, 1989a). Lechowicz (1983) suggested that suitable plants are characterized by precipitable (hydrolyzable) tannins and sclerophylly (a combined measure of leaf toughness and water content).

Goldschmidt (1934) and Leonard (1966, 1969) noted significant intra- and interpopulation variance in larval development that was related to the parental source (egg mass) of the larvae. Variation in growth among individuals in a population may be influenced by genetic differences between families that also may influence suitability of hosts, particularly those newly encountered.

The gypsy moth is not an established pest in the forests of the Pacific Northwest of North America. Isolated infestations were detected in Pacific Coast states in the early 1980s, when egg masses were accidentally transported from sites with major infestations in the northeastern United States. Oregon had a serious problem in 1984 (Daterman et al., 1986). Spraying with *Bacillus thuringiensis* in 1985 and 1986 successfully controlled the outbreak. As long as large infestations exist in the United States, new introductions of gypsy moth into western forests remains a possibility.

The objective of this research was to determine the range of certain developmental parameters relative to the parental source of larvae and the allelochemical content of the diet. Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco], a conifer, was selected for study because of its prominent status in forests of the Pacific Northwest, and its limited suitability as a host (Miller and Hanson, 1989b; Miller et al., 1991). White alder (*Alnus rhombifolia* Nutt.) was chosen to represent the highly suitable woody angiosperm hosts that commonly grow intermixed or adjacent to Douglas-fir forests. The differences in host suitability of these two species was believed to be due to significant differences in the allelochemical contents of their foliage.

METHODS AND MATERIALS

Insects. Egg masses were obtained at the end of January from an oak woodland in Seneca Creek State Park, Montgomery County, Maryland, and stored at 4°C until May. Eight large egg masses (each containing ca. 700–1000

eggs) were selected for evaluation of larval performance on Douglas-fir; eggs from four of these masses also were evaluated on alder. Large egg masses were chosen to ensure against selecting two egg masses from the same parents (Doane and McManus, 1981), thus assuring that each egg mass was of a separate family line.

Foliage Collection. Foliage was collected from three alder trees initially selected for their differences in nitrogen content after a preliminary analysis; two trees had higher nitrogen levels (tree-type A) than the third (tree-type B). These differences in nitrogen did not persist, however, resulting in nearly identical nitrogen contents for the two alder types when foliage collection began. Douglas-fir foliage was collected from five trees. Two were 10 years old and had high levels of foliar nitrogen resulting from fertilization (tree-type A); the other three, ranging in age from 10 to 15 years old, were unfertilized and had low foliar nitrogen (tree-type B).

Collections for laboratory feeding trials began in May and were completed by the end of June, coincident with the natural life cycle of gypsy moth larvae in Oregon. Only one tree of each type was harvested on collection days, with the same tree harvested on subsequent days until the foliage became limited; then a new tree of the same type was selected. The freshly gathered foliage was immediately transported to the laboratory, where it was subsampled for chemical analysis and then prepared for feeding by surface sterilizing in 0.25% sodium hypochlorite, rinsing in distilled water, and briefly air-drying.

Larval Feeding Experiment. Upon eclosion, 80 larvae from each of eight families were randomly selected for rearing on Douglas-fir foliage; an additional 42 larvae were selected from each of four of these families and reared on alder foliage. Larvae on Douglas-fir diets were reared in 145-ml cups, 10 per cup until the third instar and three per cup for the remaining instars. Larvae on alder diets were reared in groups of three per cup throughout.

Larvae were reared at 24°C, 45–50% relative humidity, and 16:8 (light–dark) hr photoperiod. Foliage was replaced every two days for early instars and daily for later instars. Larvae were observed every three days to determine percent survival. Pupae were weighed 48 hr after pupation. Days to pupation and pupal period also were determined by daily observations.

Nitrogen Analysis. Subsamples of foliage for nitrogen analysis were washed in dilute soap solution, rinsed three times with distilled H_2O, oven-dried at 60°C for 48 hr, and ground in a Wiley mill to pass a 20-mesh screen. Nitrogen content was determined by a micro-Kjeldahl technique with an automated Technicon Autoanalyzer II (Anonymous, 1975).

Terpene Analysis. Five to twelve branch tips of Douglas-fir, similar to those used to feed larvae, were selected. One-year-old needles were detached, combined into a composite sample, sealed in double air-tight plastic bags, and stored frozen until processed for analysis.

Before analysis, samples were warmed to room temperature inside the bags.

A subsample was withdrawn, frozen with liquid N_2, and ground with a mortar and pestle. Ground tissue was transferred to a capped scintillation vial. Water content was determined on triplicate samples dried at 105°C overnight.

Terpenes were extracted from 0.5 g of the freshly ground subsamples in 1 ml MeOH–H_2O (2 : 1) and 2 ml of pentane containing fenchone (0.1 mg/ml) as an internal standard (Brooks et al., 1987). Samples were shaken mechanically for 60 min and then centrifuged at room temperature in a IEC HN-SII centrifuge at approximately 1000 rpm for 3–4 min. The pentane, containing the terpenes, was removed and stored at −16°C.

Terpenes were analyzed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector and a Supelcowax 10 fused silica capillary column (30 m × 0.32 mm ID, 0.25-μm film thickness, 1 : 50 split). Injector and detector were at 250°C. Oven temperature was programmed from 60 to 220°C at 5°/min with a 15-min pause at 220°C. Peak areas were obtained with a Hewlett-Packard 3390A integrator. Compounds were identified by a combination of gas chromatography–mass spectrometry and peak enrichment with standards. Separate response factors for hydrocarbons and monooxygenated and dioxygenated compounds were determined with standards relative to fenchone.

Total Phenol Analysis. A subsample of alder leaves was air-dried after collection and stored in plastic bags at room temperature. A portion of the fresh-frozen Douglas-fir needles analyzed for terpenes was subsampled for phenolic analysis. Before analysis, alder and Douglas-fir foliage were oven-dried for 24 hr at 60°C, ground to pass a 40-mesh screen, and redried overnight. Phenols were extracted from a 100-mg subsample in 8 ml (Douglas-fir) or 10 ml (alder) MeOH–H_2O (7 : 3) on a shaker for 1 hr. The mixture was centrifuged at 1000 rpm in an IEC HN-SII centrifuge at room temperature for 5 min and analyzed with Folin-Ciocalteu phenol reagent as described by Julkunen-Tiitto (1985). Percent transmittance was measured at 700 nm with a Bausch and Lomb Spectronic 21 set to zero with distilled H_2O. A standard curve was prepared with catechin containing a MeOH concentration equivalent to the samples.

Water Content. Water content of foliage samples was measured gravimetrically from freshly cut alder leaves and from frozen Douglas-fir needles stored for terpene analysis.

Statistical Analyses. One-way analysis of variance (ANOVA) was used to determine if the foliage diets contained different concentrations of nitrogen during the feeding experiment. Fisher's least significant difference (LSD) was used to compare the means. Concentrations of total phenols and terpenes were compared only for diets within species by the Student's t test. In order to test the effect of diet on the performance of families, each growth parameter was analyzed separately for males and females with a nested ANOVA; family within treatment was the error term for treatment, and individual larvae within families

was the error term for families. Both the family and larvae were considered random effects. Treatment means were compared by Fisher's LSD. The effect of diet on percent family survival had to be analyzed with a one-way ANOVA because there was a single value for each family; data transformation was not necessary. Significantly different means were identified by Fisher's LSD.

RESULTS

Foliar Quality. The chemical composition of alder foliage differed substantially from that of Douglas-fir throughout the feeding experiment, as illustrated by the seasonal trends (Figure 1) and averages (Table 1). The alder foliage contained higher quantities of both nitrogen and total phenols, but contained no volatile terpenes. The nitrogen contents were within expected ranges for angiosperms and conifers (Mattson, 1980); the two alder types each averaged more than 2% through the sampling period and the two Douglas-fir types averaged 1–2% (Figure 1).

Steam distillation confirmed the absence of terpenes in alder. A bulk fresh leaf sample failed to produce an oil layer above a column water trap; the water was not cloudy, and no characteristic terpene odor was detectable (Farnsworth, 1966). A fresh leaf sample extracted and analyzed like the Douglas-fir needles also exhibited no appreciable quantities of any volatile compounds. Total terpenes in Douglas-fir foliage made up about 1.75% of the dry weight (Table 1), with monoterpenes representing more than 90% of this quantity. α-Pinene, β-pinene, and sabinene were the most abundant monoterpenes.

In addition to the differences in foliar quality between species, foliar quality differed between tree types within a species. When the alder trees were selected for study, they differed substantially in their nitrogen content (3.7% tree-type A, and 2.8% tree-type B), but, as the growing season progressed, their nitrogen contents rapidly converged. By the time the feeding experiment began, their nitrogen contents were the same (Figure 1), and they did not differ when averaged over the season (Table 1). Total phenol concentrations were consistently lower in alder tree-type A (LP), than in tree-type B (HP) throughout the collection period (Figure 1), resulting in a significant difference when averaged for the season (Table 1).

In Douglas-fir, the nitrogen level was consistently higher for tree-type A (Figure 1), and the average content over the collection period differed significantly from that of tree-type B. Total phenols were most similar between the two fir types early in the collection period, May 17–25, (Figure 1); during the rest of May and nearly all of June, they generally were much higher for tree-type B, which had a significantly higher seasonal average (Table 1). Concentrations of total terpenes in the fir varied considerably (Figure 1), and there

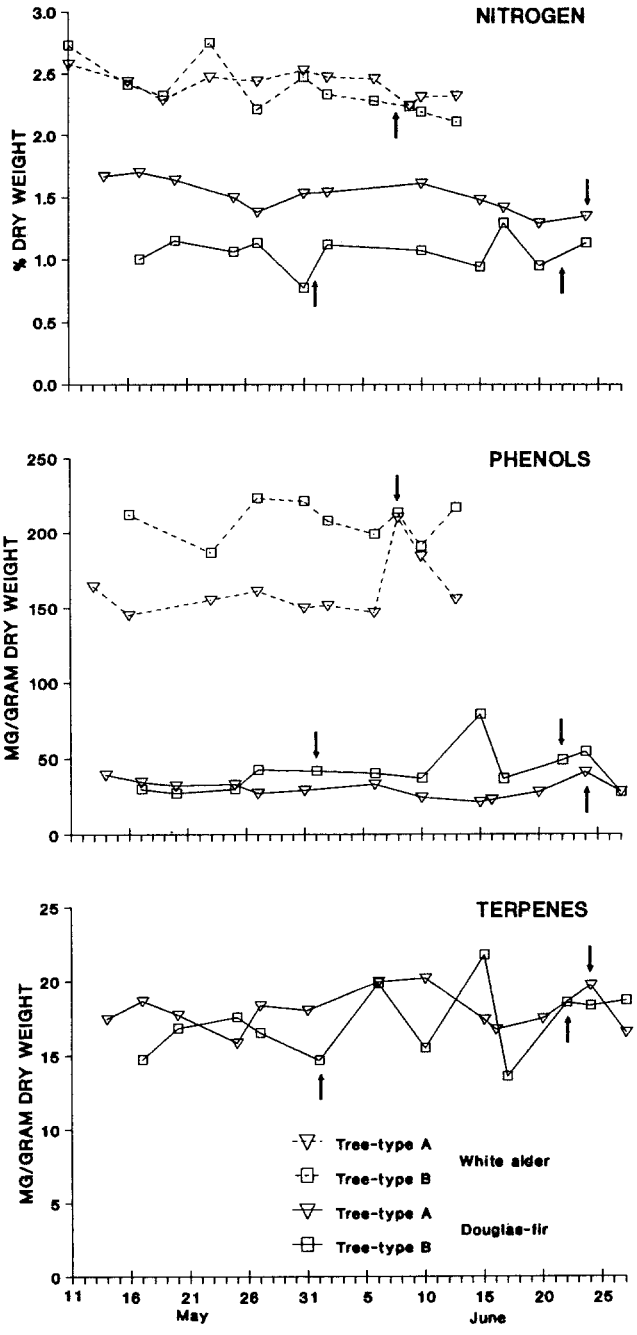


FIG. 1. Seasonal levels (1988) of foliar nitrogen and allelochemicals in white alder and Douglas-fir diets fed to gypsy moth larvae. Vertical arrows indicate when a new tree was selected for harvest within a type.

TABLE 1. SEASONAL MEAN CONCENTRATIONS (±SE) OF NITROGEN AND ALLELOCHEMICALS IN WHITE ALDER AND DOUGLAS-FIR FOLIAGE AND DEVELOPMENTAL PARAMETERS OF GYPSY MOTH FAMILIES FED THESE TISSUES[a]

		White alder[b]		Douglas-fir[c]	
	Sex	Type A LP	Type B HP	Type A HNLP	Type B LNHP
Foliar quality					
Nitrogen (%)		2.41a (±0.03)	2.36a (±0.06)	1.51b (±0.03)	1.05c (±0.04)
Total phenols (mg catechin equiv/g)[d]		162.38 (±6.37)	207.78 (±4.35)	30.40 (±1.63)	36.94 (±2.45)
Terpenes (mg/g)[e]		0.0	0.0	17.21 (±0.69)	18.01 (±0.37)
Developmental parameters					
Survival (%)		88.3a (±6.0)	84.5a (±3.9)	16.8b (±2.7)	7.5c (±2.3)
Larval period (days)	F	38.5a (±0.7)	36.9a (±0.7)	49.7b (±1.6)	51.1b (±1.3)
	M	33.5a (±0.6)	32.3a (±0.6)	46.4b (±1.0)	47.8b (±0.2)
Pupal weight (mg)	F	1295a (±73)	1447b (±27)	846c (±27)	708c (±87)
	M	459a (±7)	510b (±16)	344c (±7)	317c (±1)
Pupal period (days)	F	11.0a (±0.1)	11.1a (±0.1)	10.4b (±0.1)	10.3b (±0.0)
	M	13.5a (±0.2)	13.7a (±0.3)	12.1b (±0.1)	12.2b (±0.1)

[a] Nitrogen and survival analyzed with a one-way ANOVA; all other larval parameters were analyzed with a nested ANOVA. Multiple comparisons between means were computed with a standard LSD. Means followed by the same letters are not significantly different at $P \leq 0.05$

[b] LP = low phenols; HP = high phenols.

[c] HNLP = high nitrogen, low phenols; LNHP = low nitrogen, high phenols.

[d] Only intraspecific concentrations were compared statistically by the Student t test; alder significantly different at $P \leq 0.001$, Douglas-fir significantly different at $P \leq 0.05$.

[e] Douglas-fir was compared by the Student t test, not significant at $P \leq 0.05$.

were no significant differences between the two tree types over the course of the experiment (Table 1). Chemically, Douglas-fir tree-type A contained higher nitrogen and lower phenolic concentrations (HNLP) than tree-type B (LNHP).

Water content of the LNHP fir diet was lower than that of the HNLP diet most of the season, averaging 55.3% and 57.1%, respectively (Joseph, 1989). There were no differences in the water content of the alder diets. In May, Doug-

las-fir diets had a lower average water content (56.2%) than alder (63.6%), whereas in June, Douglas-fir contained more water (56.2%) than alder (41.3%).

The morphology and texture of alder leaves differed substantially from that of Douglas-fir needles. Simple puncture tests in our laboratory indicated that 1-year-old needles of Douglas-fir were tougher than white alder leaves.

Larval Survival and Development. Growth and development of families fed alder foliage was much better than that of families fed Douglas-fir foliage (Table 1). Survival was significantly lower on Douglas-fir foliage than on alder; mortality in the first instar accounted for over 90% of the losses. Those families with larvae surviving through pupation on Douglas-fir had significantly longer larval periods, significantly lower pupal weights, and significantly shorter pupal periods than did families reared on alder (Table 1). Longer larval periods and lower pupal weights decrease the fitness of insect herbivores (Rhoades, 1983).

Intraspecific differences in tree nutritional quality affected family performance to some extent. The only difference between families reared on HP and LP alder foliage was the lower pupal weights of families fed the latter (Table 1). Survival, larval periods, and pupal periods did not differ. Survival of families reared on HNLP Douglas-fir was more than twice as high as survival for families reared on LNHP fir. Pupal weights and larval and pupal periods did not differ for larvae in families fed the two types of fir foliage. *F* ratios and *P* values from the nested ANOVA (Table 2) indicated significant differences between families for all parameters, except for female pupal period and male pupal weight. Nevertheless, there were even greater differences caused by the treatment, demonstrating that variation in all growth parameters was most

TABLE 2. *F* RATIOS AND *P* VALUES FOR NESTED ANOVA FOR GYPSY MOTH DEVELOPMENTAL PARAMETERS

Sex	Source of variation	Developmental parameters					
		Larval period		Pupal weight		Pupal period	
		F[a]	*P*	*F*	*P*	*F*	*P*
Female	Host species and quality	31.8	0.0001	29.2	0.0001	10.9	0.0010
	Family (within treatment)	9.2	0.0001	2.1	0.0208	1.4	0.1730
Male	Host species and quality	75.5	0.0001	163.3	0.0001	11.5	0.0008
	Family (within treatment)	4.2	0.0001	0.8	0.6317	3.4	0.0002

[a] *F* = *F* ratio, *P* = level of significance.

strongly influenced by the species of host tree fed to the larvae. Survival, with an F ratio of 151.4, also was greatly influenced by the host species and quality (Table 1).

Although host species strongly influenced development, differences in larval performance were associated with interegg-mass differences (Table 2), especially on diets of Douglas-fir foliage (Table 3). For the four families (I, V, VI, VII) subjected to all diets, survival and growth of each was superior on alder foliage. Only two families (I, V) had sufficient genetic resources to survive and complete development (Tables 3) on the foliage of HNLP Douglas-fir, nutritionally the more suitable of the two Douglas-fir types tested. None of the four families completed their life cycles on the most unsuitable diet, LNHP fir. Six of eight families reared on Douglas-fir survived and completed development on the HNLP foliage, but only two of the eight families were successful on the LNHP foliage, the least nutritious of the two types. Coefficients of variation for family survival on each of the four diets confirmed that survival varied least for families fed the two alder diets (CV 13.6% and 9.4% for LP and HP alder, respectively), and increased inversely as the nutritional quality of the diet declined (CV 54.0% and 168.3% for HNLP and LNHP fir, respectively). Therefore, genetic differences among families are probably most critical to survival when the nutritional quality of the diet is least satisfactory.

The performance of some families relative to the other families varied substantially with the diet (Table 3). For example, survival in family I was the lowest of all four families on both diets of alder; its survival on LNHP fir was low. On fir HNLP diets, however, family I survival was intermediate to high (Table 3) compared to the three other families in this group. Female pupal weights in family I showed similar responses. On LP alder diets, family I females had significantly greater pupal weights than females from the other three families, and family I had one of the two highest pupal weights on HP alder diets (Table 3). Only two of the four families had sufficient larval survival when fed HNLP Douglas-fir to permit measurement of pupal weights, but there were pupae from four additional families reared only on Douglas-fir. Females of family I had the lowest pupal weights of all six families measured. Not enough females survived on LNHP Douglas-fir diet for measurements. Performance of a family on a suitable host diet probably has limited utility for predicting its success and potential adaptability to a new, less suitable host.

DISCUSSION

Both genetic background and host species clearly were critical in determining survival, development, and overall fitness of gypsy moth larvae in this study. Chemical analysis confirmed the nutritional and allelochemical differ-

TABLE 3. DEVELOPMENTAL PARAMETERS (MEAN ± SE)[a] OF GYPSY MOTH LARVAE FROM EGG MASSES REARED IN LABORATORY ON FIELD-COLLECTED FOLIAGE OF WHITE ALDER AND DOUGLAS-FIR

Egg mass	Larval survival (%)	Larval period (days)		Pupal weight (mg)[b]		Pupal period (days)	
		Female	Male	Female	Male	Female	Male
Alder type A (LP)							
I	71	39.1 (±0.6)	34.3 (±0.8)	1493 (±64)	480 (±14)	11.2 (±0.2)	14.1 (±0.2)
V	99	38.5 (±0.5)	33.5 (±0.6)	1299 (±57)	454 (±11)	10.8 (±0.1)	13.3 (±0.2)
VI	91	38.8 (±0.6)	34.3 (±0.6)	1245 (±62)	456 (±11)	11.3 (±0.2)	13.7 (±0.2)
VII	92	35.8 (±0.7)	31.9 (±0.6)	1142 (±66)	446 (±10)	10.8 (±0.2)	13.0 (±0.2)
Alder type B (HP)							
I	76	36.0 (±0.7)	31.6 (±0.7)	1485 (±64)	512 (±12)	11.1 (±0.2)	13.8 (±0.2)
V	90	38.7 (±0.6)	32.5 (±0.6)	1497 (±62)	513 (±11)	11.3 (±0.2)	13.8 (±0.2)
VI	80	37.3 (±0.7)	33.8 (±0.7)	1432 (±71)	515 (±13)	11.3 (±0.2)	14.2 (±0.2)
VII	92	35.5 (±0.5)	31.3 (±0.7)	1375 (±51)	500 (±19)	10.8 (±0.1)	12.8 (±0.2)
Douglas-fir type A (HNLP)							
I	14	43.0 (±1.0)	41.0 (±1.2)	750 (±104)	313 (±21)	10.5 (±0.3)	12.0 (±0.4)
II	26	50.1 (±0.8)	47.5 (±0.8)	757 (±77)	350 (±13)	10.4 (±0.2)	12.3 (±0.2)
III	21	50.3 (±1.0)	45.6 (±0.8)	828 (±104)	343 (±15)	10.5 (±0.3)	12.2 (±0.3)
IV	15	53.3 (±1.3)	47.7 (±1.1)	940 (±127)	370 (±19)	10.3 (±0.3)	12.0 (±0.3)
V	19	48.4 (±0.9)	49.2 (±1.1)	835 (±85)	346 (±19)	10.3 (±0.2)	12.0 (±0.3)
VI	4						
VII	10						
VIII	25	53.2 (±0.7)	47.5 (±1.1)	954 (±68)	347 (±19)	10.3 (±0.2)	12.0 (±0.3)
Douglas-fir type B (LNHP)							
I	1						
II	11						
III	15	49.8 (±1.3)	48.0 (±1.5)	631 (±127)	323 (±23)	10.3 (±0.3)	12.3 (±0.5)
IV	16	52.3 (±1.3)	47.6 (±1.0)	784 (±127)	311 (±19)	10.3 (±0.3)	12.1 (±0.3)
V	0						
VI	0						
VII	7						
VIII	10						

[a]Because family was used as a random effect in the nested ANOVA, no mean separation was performed.
[b]Fresh weight two days after pupal formation.

ences between alder and Douglas-fir and the poor nutritional quality and limited suitability of Douglas-fir for gypsy moth development (Miller and Hanson, 1989b; Miller et al., 1991).

To what extent did each of the chemical components in the diets contribute to the differences in family performance? Comparing foliar chemistry of alder and Douglas-fir is difficult, because three principal groups of compounds that differ both quantitatively and qualitatively are involved. Relatively modest differences in nitrogen and phenols in the two Douglas-fir diets had significant effects on survival. The alder diets contained much more nitrogen and total phenols than the Douglas-fir diets. The greater nitrogen content of alder undoubtedly made it a better diet nutritionally (Mattson, 1980; Scriber and Slansky, 1981; Mattson and Scriber, 1987) and contributed to the higher survival and better larval growth.

The differences in total phenol concentrations between Douglas-fir and alder must be interpreted with caution, because the types of tannins and phenols may be quite different in the two species. Conifers, including Douglas-fir, produce condensed tannins (Swain, 1979; Stafford and Lester, 1981). Angiosperms can synthesize hydrolyzable as well as condensed tannins (Swain, 1979), and the proportion of each can differ substantially between species (Bate-Smith, 1977). Tannin and phenol composition of white alder apparently has not been reported.

In the Folin-Denis assay for total phenols, a procedure similar to that used in our study, the absorbance of tannic acid (a standard for hydrolyzable tannins) was 2.2 times greater than that of catechin (the same standard for condensed tannins that we used) when normalized (Mole and Waterman, 1987). Consequently, if two plant tissues contained equal quantities of tannins and phenols, one having predominantly hydrolyzable tannins and the other predominantly condensed tannins, the one with hydrolyzable tannins would appear to have twice the total phenol concentration, as calculated from a catechin standard curve. If alder tissues contain a significant proportion of hydrolyzable tannins, a 2.2-fold difference in concentration relative to Douglas-fir could represent no difference at all in total phenols. Total phenol concentrations in alder, however, were 4.4–6.8 times greater than in Douglas-fir. Thus, concentrations of total phenols in the alder diets probably were greater than in Douglas-fir diets, even though the precise structure and composition of the alder tannins are unknown.

The species effect of the greater phenolic concentration in alder and the interactions of phenolics with nitrogen are difficult to evaluate, because the actual tannin structures and toxicities and how toxicities may interact with nitrogen level are unknown. The two alder diets we tested differed only in their concentration of total phenols; this comparison is not complicated by interspecific differences in tannin structures or nitrogen concentrations. The greater pupal weights produced by feeding on HP alder may have resulted from increases in

the relative consumption rate, the efficiencies of conversion of ingested or digested food, or some combination of these (Waldbauer, 1968), since the larval period was unchanged. Plants containing tannins are more acceptable hosts for gypsy moth than are plants containing other allelochemicals—iridoids, sesquiterpenes, and particularly alkaloids (Barbosa and Krischik, 1987; Miller and Hanson 1989a; Barbosa et al., 1990a), and host acceptance in the field is more closely correlated with tannin content than with total phenol content of the leaves (Lechowicz, 1983). Hydrolyzable tannins may act as phagostimulants for adapted species, such as gypsy moth (Bernays, 1981; Lechowicz, 1983; Kleiner et al., 1989). The alder phenolics appear to have acted as phagostimulants in this study.

The combined effect of low nitrogen and high phenol concentrations in Douglas-fir had a greater adverse impact on survival than did either high quantities of phenols alone in alder or the HNLP diet in Douglas-fir. Part of the difference between Douglas-fir and alder, or between the two Douglas-fir diets, may be attributable to the relative amounts of condensed tannins, which are detrimental to several species of herbivorous insects (Bernays, 1981; Klocke and Chan, 1982; Reese et al., 1982; Berenbaum, 1983; Manuwoto et al., 1985; Manuwoto and Scriber, 1986). This detrimental effect has often been ascribed to formation of insoluble complexes between tannins and proteins that diminish metabolizable nitrogen (Feeny, 1976; Rhoades and Cates, 1976). This mechanism, however, does not appear to function in insect herbivores (Bernays, 1981; Manuwoto et al., 1985; J.S. Martin et al., 1985; Manuwoto and Scriber, 1986; M.M. Martin et al. 1987), including gypsy moth (Schultz and Lechowicz, 1986). Survival of gypsy moth larvae was 77% of controls, and larval weights were 40% of controls after 20 days of exposure to tannin- and phenol-rich extract of Douglas-fir (with the terpenes removed) incorporated into artificial diet (Joseph, 1989). When the nitrogen contents of the treatment and control diets were lowered, survival did not change, but weights of larvae fed low-nitrogen, phenol-rich diets were approximately 20% of the controls.

The presence of terpenes in Douglas-fir and their absence in alder is probably the greatest difference in the allelochemistry of the two host species. Two of the three major terpenes in Douglas-fir foliage, α-pinene and β-pinene, were strong phagodeterrents when applied in pure form to food of the gypsy moth (Meisner and Skatulla, 1975). Other monoterpenes, sesquiterpenes, sesquiterpene lactones, and diterpenes also have feeding deterrent activity (Doskotch et al., 1980a,b; El-Naggar et al., 1980).

The impact of terpenes alone on gypsy moth growth and development cannot be evaluated from our data, because their effects are inseparable from the influences of nitrogen and phenols. However, induction of detoxification enzymes in insects ingesting monoterpenes in their diet indicates that these compounds are nutritionally undesirable (Brattsten, 1986; Yu, 1986, 1987; Har-

wood et al., 1990). Douglas-fir terpenes isolated by steam distillation and incorporated into artificial diet at natural concentrations did not affect survival or weights of gypsy moth larvae reared on these diets for 20 days (Joseph, 1989). When the terpenes were combined in artificial diet with an extract containing Douglas-fir phenols, survival and pupal weights were greatly reduced below those on diet containing only the phenol extract, suggesting an interaction or possible synergism between these two groups of compounds.

Most larval mortality occurred during the first instar, when the small larvae were more likely to be repelled by physical barriers such as leaf toughness (Hough and Pimentel, 1978; Ohmart et al., 1985). Barbosa et al. (1986) reported mechanical barriers to biting, and consequent starvation, as a probable cause of mortality for first instars fed conifer foliage. In our experiments leaf toughness probably did affect mortality of first-instar larvae on fir, but not alder. However, the magnitude of the effect relative to foliar chemistry is not determinable from these data. We can conclude that toughness did not contribute to the differences in family performance observed between tree types within a species. Furthermore, incorporation of Douglas-fir foliage extracts into artificial diets, where needle toughness was not a factor, resulted in low survival and decreased larval weight (Joseph, 1989), as observed with our fresh foliage.

Water content is closely associated with leaf toughness; together they provide a measure of sclerophylly (Lechowicz, 1983). Foliar water content in Douglas-fir differed little from that of alder and probably did not contribute much to the differences in family performances between the two host species.

Host adaptability has important implications for potential establishment of gypsy moth in the forests of the Pacific Northwest, where preferred hosts such as Oregon white oak and alder species often are situated in or adjacent to Douglas-fir stands (Daterman et al., 1986). The genetic constitution of some families of gypsy moth clearly permits survival and successful development on an unsuitable host, such as Douglas-fir, even when this host is of low nutritional value. Furthermore, the likelihood of survival increases as the nutritional value of the Douglas-fir increases, as would be the case in fertilized plantations.

In the northeastern United States, gypsy moths effectively utilize conifers such as white pine (*Pinus strobus* L.), pitch pine (*P. rigida* Mill.), and Norway spruce [*Picea abies* (L.) Karst] as secondary hosts during low-density population phases (Rossiter, 1987), and performance and fitness improve on a two-species diet of black oak (*Quercus velutina* Lam.) and either Virginia pine (*Pinus virginiana* Mill.) or loblolly pine (*P. taeda* L.), relative to black oak alone (Barbosa et al., 1986). Utilization of secondary hosts may help to release populations from a low-density stable phase into a higher-density building phase (Campbell, 1981). Therefore, establishment of gypsy moth on Douglas-fir even at low levels might pose a serious threat, especially from larvae in later instars, which survive switching from alder to Douglas-fir (Joseph, 1989). In addition,

establishment on Douglas-fir might give rise to populations with enhanced behavioral or metabolic resistance to pathogens (Rossiter, 1987) or pesticides (Moldenke et al., unpublished data).

In summary, although nutritional quality is a primary determinant of host suitability, the genetic makeup of each family influences the degree of suitability to family members. Genetic differences between families are particularly important when the nutritional quality of the host is limited. If gypsy moth becomes established at low population densities in mixed conifer–broadleaf stands in the Pacific Northwest, some utilization of Douglas-fir can be anticipated.

Acknowledgments—We thank P. Barbosa and P. Gross for supplying gypsy moth egg masses, G.E. Daterman and T.D. Schowalter for reading an earlier draft, T.E. Sabin for help with the statistics, and L. Gerson and A. Berry for laboratory and field assistance. Work leading to this publication was funded by USDA, CRGO, Pest science grant 87-CRCR-1-2478 and by cooperative agreement 87-409 with the USDA, Forest Service, Pacific Northwest Research Station.

REFERENCES

ANONYMOUS. 1975. Technicon Autoanalyzer II Methodology. Individual Simultaneous Determination of Nitrogen & Phosphorus in BD Acid Digests. Industrial Method No. 334-74A. Technicon Corporation, New York.

BARBOSA, P., and KRISCHIK, V.A. 1987. Influence of alkaloids on feeding preference of eastern deciduous forest trees by the gypsy moth *Lymantria dispar*. *Am. Nat.* 130:53–69.

BARBOSA, P., WALDVOGEL, M., MARTINAT, P., and DOUGLASS, L.W. 1983. Developmental and reproductive performance of the gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) on selected hosts common to mid-Atlantic and southern forests. *Environ. Entomol.* 12:1858–1862.

BARBOSA, P., MARTINAT, P., and WALDVOGEL, M. 1986. Development, fecundity and survival of the herbivore *Lymantria dispar* and the number of plant species in its diet. *Ecol. Entomol.* 11:1–6.

BARBOSA, P., GROSS, P., PROVAN, G.J., PACHECO, D.Y., and STERMITZ, F.R. 1990a. Allelochemicals in foliage of unfavored tree hosts of the gypsy moth, *Lymantria dispar* L. 1. Alkaloids and other components of *Liriodendron tulipifera* L. (Magnoliaceae), *Acer rubrum* L. (Aceraceae), and *Cornus florida* L. (Cornaceae). *J. Chem. Ecol.* 16:1719–1730.

BARBOSA, P., GROSS, P., PROVAN, G.J., and STERMITZ, F.R. 1990b. Allelochemicals in foliage of unfavored tree hosts of the gypsy moth, *Lymantria dispar* L. 2. Seasonal variation of saponins in *Ilex opaca* and identification of saponin aglycones. *J. Chem. Ecol.* 16:1731–1738.

BATE-SMITH, E.C. 1977. Astringent tannins of *Acer* species. *Phytochemistry* 16:1421–1426.

BERENBAUM, M.R. 1983. Effects of tannins on growth and digestion in two species of papilionids. *Entomol. Exp. Appl.* 34:245–250.

BERNAYS, E.A. 1981. Plant tannins and insect herbivores: An appraisal. *Ecol. Entomol.* 6:353–360.

BRATTSTEN, L.B. 1986. Fate of ingested plant allelochemicals in herbivorous insects, pp. 211–255, *in* L.B. Brattsten and S. Ahmad (eds.). Molecular Aspects of Insect-Plant Associations. Plenum Press, New York.

BROOKS, J.E., BORDEN, J.H., PIERCE, H.D., JR., and LISTER, G.R. 1987. Seasonal variation in foliar and bud monoterpenes in sitka spruce. *Can. J. Bot.* 65:1249–1252.

CAMPBELL, R.W. 1981. Population dynamics, pp. 65–86, *in* C.C. Doane and M.L. McManus (eds.). The Gypsy Moth: Research Toward Integrated Pest Management. USDA, Forest Service, Science Education Agency Technical Bulletin 1584. Washington, D.C.

DATERMAN, G.E., MILLER, J.C., and HANSON, P.E. 1986. Potential for gypsy moth problems in southwest Oregon, pp. 37–40, *in* O.T. Helgerson (ed.). Forest Pest Management in Southwest Oregon. Forest Research Laboratory, Oregon State University, Corvallis.

DOANE, C.C., and MCMANUS, M.L. 1981. The Gypsy Moth: Research Toward Integrated Pest Management. USDA, Forest Service, Science Education Agency Technical Bulletin 1584. Washington D.C. 757 pp.

DOSKOTCH, R.W., CHENG, H.Y., O'DELL, T.M., and GIRARD, L. 1980a. Nerolidol: An antifeeding sesquiterpene alcohol for gypsy moth larvae from *Melaleuca leucadendron*. *J. Chem. Ecol.* 6:845–851.

DOSKOTCH, R.W., FAIRCHILD, E.H., HUANG, C.T., WILTON, J.H., BENO, M.A., and CHRISTOPH, G.G. 1980b. Tulirinol, an antifeedant sesquiterpene lactone for the gypsy moth larvae from *Liriodendron tulipifera*. *J. Org. Chem.* 45:1441–1446.

DOSKOTCH, R.W., O'DELL, T.M., and GIRARD, L. 1981. Phytochemicals and feeding behavior of gypsy moth larvae, pp. 657–666, *in* C.C. Doane and M.L. McManus (eds.). The Gypsy Moth: Research Toward Integrated Pest Management. USDA, Forest Service, Science Education Agency Technical Bulletin 1584. Washington, D.C.

ELKINTON, J.S., and LIEBHOLD, A.M. 1990. Population dynamics of gypsy moth in North America. *Annu. Rev. Entomol.* 35:571–596.

EL-NAGGAR, S.F., DOSKOTCH, R.W., O'DELL, T.M., and GIRARD, L. 1980. Antifeedant diterpenes for the gypsy moth larvae from *Kalmia latifolia*: Isolation and characterization of ten grayanoids. *J. Nat. Prod.* 43:617–631.

FARNSWORTH, N.R. 1966. Biological and phytochemical screening of plants. *J. Pharm. Sci.* 55:225–276.

FEENY, P. 1976. Plant apparency and chemical defense. *Recent Adv. Phytochem.* 10:1–40.

GOLDSCHMIDT, R. 1934. *Lymantria. Biblio. Genet.* 11:1–186.

HARWOOD, S.H., MOLDENKE, A.F., and BERRY, R.E. 1990. Toxicity of peppermint monoterpenes to the variegated cutworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 83:1761–1767.

HOUGH, J.A., and PIMENTEL, D. 1978. Influence of host foliage on development survival and fecundity of the gypsy moth. *Environ. Entomol.* 7:97–102.

JOSEPH, G. 1989. Host suitability studies of Douglas-fir and white alder to the gypsy moth. Masters thesis. Oregon State University, Corvallis. 103 pp.

JULKUNEN-TIITTO, R. 1985. Phenolic constituents in the leaves of northern willows: Methods for the analysis of certain phenolics. *J. Agric. Food Chem.* 33:213–217.

KLEINER, K.W., MONTGOMERY, M.E., and SCHULTZ, J.C. 1989. Variation in leaf quality of two oak species: Implications for stand susceptibility to gypsy moth defoliation. *Can. J. For. Sci.* 19:1445–1450.

KLOCKE, J.A., and CHAN, B.G. 1982. Effects of cotton condensed tannin on feeding and digestion in the cotton pest, *Heliothis zea*. *J. Insect Physiol.* 28:911–915.

LECHOWICZ, M.J. 1983. Leaf quality and the host preferences of gypsy moth in the northern deciduous forest, pp. 67–82, *in* Forest Defoliater–Host Interactions: A Comparison Between Gypsy Moth and Spruce Budworms. USDA, Forest Service, Northeastern Station General Technical Report NE-85.

LEONARD, D.E. 1966. Differences in development of strains of the gypsy moth *Porthetria dispar* (L.). Connecticut Agriculture Experiment Station Bulletin 680. 31 pp.

LEONARD, D.E. 1969. Intrinsic factors causing qualitative changes in populations of gypsy moth. *Proc. Entomol. Soc. Ont.* 100:195–199.

MANUWOTO, S., and SCRIBER, J.M. 1986. Effects of hydrolyzable and condensed tannin on growth and development of two species of polyphagous lepidoptera: *Spodoptera eridania* and *Callosamia promethea*. *Oecologia* 69:225–230.

MANUWOTO, S., SCRIBER, J.M., HSIA, M.T., and SUNARJO, P. 1985. Antibiosis/antixenosis in tulip tree and quaking aspen leaves against the polyphagous southern armyworm, *Spodoptera eridania*. *Oecologia* 67:1–7.

MARTIN, J.S., MARTIN, M.M., and BERNAYS, E.A. 1987. Failure of tannic acid to inhibit digestion or reduce digestibility of plant protein in gut fluids of insect herbivores: Implications for theories of plant defense. *J. Chem. Ecol.* 13:605–621.

MARTIN, M.M., ROCKHOLM, D.C., and MARTIN, J.S. 1985. Effects of surfactants, pH and certain cations on precipitation of proteins by tannins. *J. Chem. Ecol.* 11:485–494.

MATTSON, W.J., JR. 1980. Herbivory in relation to plant nitrogen content. *Annu. Rev. Ecol. Syst.* 11:119–161.

MATTSON, W.J., and SCRIBER, J.M. 1987. Nutritional ecology of insect folivores of woody plants: Nitrogen, water, fiber, and mineral considerations, pp. 105–146, *in* F. Slansky, Jr. and J.G. Rodriguez (eds.). Nutritional Ecology of Insects, Mites, Spiders, and Related Invertebrates. Wiley-Interscience, New York.

MEISNER, J., and SKATULLA, U. 1975. Phagostimulation and phagodeterrency in the larva of the gypsy moth, *Porthetria dispar* L. *Phytoparasitica* 3:19–26.

MILLER, J.C., and HANSON, P.E. 1989a. Laboratory feeding tests on the development of gypsy moth larvae with reference to plant taxa and allelochemicals. Oregon State University Agriculture Experiment Station Bulletin 674. Corvallis, Oregon. 63 pp.

MILLER, J.C., and HANSON, P.E. 1989b. Laboratory studies on development of gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), larvae on foliage of gymnosperms. *Can. Entomol.* 121:425–429.

MILLER, J.C., HANSON, P.E., and KIMBERLING, D.N. 1991. Development of the gypsy moth on Douglas-fir foliage. *J. Econ. Entomol.* 84:461–465.

MOLE, S., and WATERMAN, P.G. 1987. A critical analysis of techniques for measuring tannins in ecological studies. I. Techniques for chemically defining tannins. *Oecologia* 72:137–147.

OHMART, C.P., STEWART, L.G., and THOMAS, J.R. 1985. Effects of food quality, particularly nitrogen concentrations, of *Eucalyptus blakelyi* foliage on the growth of *Paropsis atomaria* larvae (Coleoptera: Chrysomelidae). *Oecologia* 65:543–549.

REESE, J.C., CHAN, B.G., and WAISS, A.C., JR., 1982. Effects of cotton condensed tannin, maysin (corn) and pinitol (soybeans) on *Heliothis zea* growth and development. *J. Chem. Ecol.* 8:1429–1436.

RHOADES, D.F. 1983. Herbivore population dynamics and plant chemistry, pp. 155–220, *in* R.F. Denno and M.S. McClure (eds.). Variable Plants and Herbivores in Natural and Managed Systems. Academic Press, New York.

RHOADES, D.F., and CATES, R.G. 1976. Toward a general theory of plant antiherbivore chemistry. *Recent Adv. Phytochem.* 10:168–213.

ROSSITER, M.C. 1987. Use of a secondary host by non-outbreak populations of the gypsy moth. *Ecology* 68:857–868.

ROSSITER, M.C., SCHULTZ, J.C., and BALDWIN, I.T. 1988. Relationships among defoliation, red oak phenolics, and gypsy moth growth and reproduction. *Ecology* 69:267–277.

SCHULTZ, J.C., and LECHOWICZ, M.J. 1986. Host plant, larval age, and feeding behavior influence midgut pH in the gypsy moth (*Lymantria dispar*). *Oecologia* 71:133–137.

SCRIBER, J.M., and SLANSKY, F., JR. 1981. The nutritional ecology of immature insects. *Annu. Rev. Entomol.* 26:183–211.

STAFFORD, H.A., and LESTER, H.H. 1981. Proanthocyanidins and potential precursors in needles of Douglas fir and in cell suspension cultures derived from seedling shoot tissues. *Plant Physiol.* 68:1035–1040.

SWAIN, T. 1979. Tannins and lignins, pp. 657–682, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

WALDBAUER, G.P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol.* 5:229–288.

YU, S.J. 1986. Consequences of induction of foreign compound-metabolizing enzymes in insects, pp. 153–174, *in* L.B. Brattsten and S. Ahmad (eds.). Molecular Aspects of Insect–Plant Associations. Plenum Press, New York.

YU, S.J. 1987. Microsomal oxidation of allelochemicals in generalist (*Spodoptera frugiperda*) and semispecialist (*Anticarsia gemmatalis*) insect. *J. Chem. Ecol.* 13:423–436.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

PSORALENS IN SENESCING LEAVES OF *Ruta graveolens*

ALICJA M. ZOBEL* and STEWART A. BROWN

Department of Chemistry
Trent University
Peterborough, Ontario, Canada K9J 7B8

(Received March 6, 1991; accepted April 30, 1991)

Abstract—Concentrations of three furanocoumarins, psoralen, xanthotoxin, and bergapten, were measured on the surface and within mature whole leaves of two groups of *Ruta graveolens* L. late autumn plants, 2 and 6 years old, which contained green, yellow, and dry yellow leaves. Upper green leaves contained higher concentrations of these coumarins than lower green leaves, green leaves contained several times as much as yellow leaves, and dry leaves contained even smaller amounts than yellow ones. The dry yellow leaves contained only a very small percentage of furanocoumarins on the surface, suggesting that extrusion to the surface of yellow leaves was slower or had stopped, while loss from the surface continued. The loss of psoralen was the most dramatic in and on the dry leaves. Bergapten's ratio to the other coumarins increased during senescence. Xanthotoxin was always the predominant furanocoumarin in this species.

Key Words—*Ruta graveolens*, Rutaceae, rue, senescence, furanocoumarins, leaf surface, plant defense.

INTRODUCTION

Senescence, a normal process in a plant (Simon, 1967; Mothes, 1980; Janzen and Waterman, 1986) occurs during tissue ontogenesis (Wiermann, 1980), and is characterized by very complex processes differing from those in juvenile tissues (Woolhouse, 1967). Changes occur both in primary metabolism and structure (Malik, 1987; Fobel et al., 1987; Braber, 1980; Patra and Mishra, 1979) and in secondary metabolism (Mann, 1980; Haslam, 1985). Senescence coin-

*To whom correspondence should be addressed.

0098-0331/91/0900-1801$06.50/0

cides with a change in the action of hormones (Osborne, 1968, Audus 1972), which can be involved in regulation of secondary product metabolism (Ebel, 1979).

At the end of the normal senescence process, leaves dry out and fall to the ground, where leakage both from the leaf surface and its interior occurs, followed by decay. Because some secondary products do not undergo ready turnover (Margna and Vainjaero, 1981) and others do (Barz and Hoesel, 1979; Barz and Koester, 1981), it is important to know what compounds are retained in senescent and dry leaves, because such compounds can potentially enter the environment. Although some secondary metabolites appear to accumulate in plant tissues simply as waste products, others are metabolized and therefore do not accumulate. Some that do accumulate can be allelochemicals (Barz et al., 1985; Jacques et al., 1985) influencing hormone action (Hale and Orcutt, 1987) or serving as natural pest and disease control agents. Certain secondary products that have survived the senescing process may be released by the dead plants into the soil (Barz and Weltring, 1985).

Among products of secondary metabolism, furanocoumarins have long been known to act as growth inhibitors or growth retardants (Rodighiero, 1954; Shina-Roy and Chakraborty, 1976; Friedman et al., 1982). We have found psoralens (linear furanocoumarins) on the surface of rutaceous and umbelliferous plants (Zobel and Brown, 1990a), sometimes in amounts of micrograms per gram fresh weight within the green leaves of *Ruta graveolens* and on the leaf surface (Zobel and Brown, 1989). As with other compounds, these coumarins may pass into the soil both from living leaves on the plant (through the action of rain and other physical weathering processes) and dead ones on the ground. Because furanocoumarins have a broad spectrum of biological activity (Murray et al., 1982; Ivie, 1987a,b), the possibility of effects on the growth of neighboring plants cannot be excluded.

The behavior of coumarins during senescence and their possible role in that process have not yet been explored. In this study we have investigated the extent to which psoralens remain on and within the leaves during the process of senescence.

METHODS AND MATERIALS

Plant Material. R. graveolens L. plants, growing outdoors in the soil during the summer growing season of 1988, were used. Leaf samples of 2- and 6-year-old specimens, each containing about 20 shoots, each with about 30 leaves, were collected for analysis November 1, just before the autumn frosts. We wished to obtain all the necessary stages of leaf development on each plant and to avoid the influence of variations in the vegetative period that we had

observed in *Heracleum lanatum* (Zobel and Brown, 1990b), as well as changing environmental conditions, knowing that frost can cause a 100-fold increase in furanocoumarins. On the plant there were four distinct populations of leaves, all mature: group I, upper green, which were smaller in size; II, lower green, which were larger and older; III, lower yellow, which were still turgid, showing a decline in the amount of chlorophyll; and IV, the lowest, yellow, which had dried on the plant.

Two green leaves from each shoot were removed: the third from the shoot apex (called here "upper green") and the third green leaf from the bottom ("lower green"). The upper leaves were not new growth, but small-sized (2–4 cm) mature, "hard" leaves. Lower green leaves (7–12 cm) showed no signs of damage or yellowing. Below the green leaves on the shoot were yellowish leaves, and the lowest location had dry yellow leaves, collected as "lower yellow" and "lower dry" samples, respectively. Separate samples were collected from the 2- and 6-year-old plants, and each of the four different leaf samples, containing 12–14 leaves, was duplicated.

Analyses. The leaves of each sample were weighed as a group and surface coumarins removed from the surface by a brief dipping in almost-boiling water (Zobel and Brown, 1988). After extraction, the dry weights of these leaves were determined for comparison with those of the senescent leaves. Because there were two groups of leaves, dry and turgid, there were alternative ways of expressing concentrations, on the basis of either dry or fresh weight. For comparison with our previous papers, we chose to calculate on the basis of fresh weight, as the values for only one group of leaves (yellow, dry) then had to be adjusted. Groups III and IV were the most similar, because they developed at the beginning of the vegetative period when all the leaves were growing very actively and were of similar size. Thus calculation of fresh weight of group IV was done according to the equation:

$$\frac{\text{Fresh weight of group III}}{\text{Dry weight of group III}} = \frac{\text{Fresh weight of group IV}}{\text{Dry weight of group IV}}$$

The furanocoumarins in the extracts were purified and determined by previously described procedures (Thompson and Brown, 1984; Zobel and Brown, 1988). The error of the method was $\leq 10\%$.

RESULTS

Table 1 compares the sum of the concentrations (Σ values) of psoralen, xanthotoxin, and bergapten (P + X + B).

Whole Leaves. The whole leaves of the younger group of plants contained higher concentrations than the older: 40% (4200 vs. 2500) in the upper leaves

TABLE 1. CONCENTRATIONS[a] OF TOTAL FURANOCOUMARINS IN AND ON THE SURFACE OF *Ruta graveolens* PLANTS

	Younger plants (2-year-old)			Older plants (6-year-old)		
	$\Sigma P + X + B$[b]			$\Sigma P + X + B$		
Leaves	Whole leaf	On surface	On surface (%)	Whole leaf	On surface	On surface (%)
Upper, green[c]	4200 ± 100	830 ± 30	20	2500 ± 100	760 ± 40	30
Lower, green[c]	1400 ± 100	520 ± 50	37	980 ± 60	190 ± 10	19
Lower, yellow[c]	580 ± 10	130 ± 10	23	1100 ± 100	140 ± 10	13
Yellow, dry[c]	580 ± 40	25 ± 2	4.3	680 ± 40	20 ± 2	2.9
Yellow, dry[d]	70 ± 5	3 ± 0.5	4.3	76 ± 5	2.2 ± 0.2	2.9

[a] Mean of two samples.
[b] P: psoralen, X: xanthotoxin, B: bergapten.
[c] μg/g fresh weight.
[d] μg/g dry weight.

and 30% (1400 vs. 980) in the lower green leaves. In both age groups the upper leaves had over twice as much as did the lower leaves, in spite of the fact that all these leaves were mature, differing only in size and location on the shoot.

In the yellowish senescent leaves of the younger plants (becoming senescent but still not dry), the Σ value was about 40% of that of the lower green ones (580 vs. 1400). In the fully dry leaves, this value was still of similar magnitude, indicating that in the course of the drying stage of senescence there was little if any diminution of furanocoumarins. In the yellowish leaves of the older plants, the Σ value was much higher than in the young plants, but in the dry leaves the two values were comparable. In both groups of plants the old, dry leaves, the lowest on the plant, contained a much lower concentration than the younger, upper leaves and the lower, green leaves, although the difference was less pronounced in the older plants. In younger plants the upper green leaves contained over seven times, (and in older plants over three times) the concentration of furanocoumarins as dry ones.

Leaf Surface. In both younger and older plants the total surface concentrations showed similar tendencies, decreasing down the shoot in absolute amounts. Differences between upper green and dry leaves were even more pronounced than in the whole leaf: on the upper green leaves of both age groups, concentrations were >33 times (830/25 and 760/20) as high as on dry ones. In younger plants the percentage of furanocoumarins on the surface decreased toward the base of the shoot from 20% to 4.3%, and on the older plants from 30% to 2.9%. The lower percentage in the case of younger plants reflects the very high

concentration in the whole leaf (4200 μg/g). Except for the upper green leaves, the younger plants had a higher percentage on the surface than the older.

Changes in the individual furanocoumarins during the experiment are shown in Table 2.

Xanthotoxin. Xanthotoxin was the predominant furanocoumarin in each age group, both in the whole leaf and on the surface. Of the three coumarins, xanthotoxin had the highest percentage on the surface only in the case of the lower, yellow leaves of both age groups. In terms of absolute concentrations, the amounts on the surface followed similar trends to those described above for the sum of the three coumarins. In both groups only a small fraction remained on the surface of the lower dry leaves compared to the upper green leaves.

Psoralen. Psoralen concentrations, both in the whole green leaf and on the surface, were always lower than those of xanthotoxin, especially on the surface of the yellow leaves, and throughout the dry leaves. In the whole lower green leaves there were three (200/75) to 10 times (370/35) as much psoralen as in

TABLE 2. CONCENTRATION[a] OF PSORALENS ON SURFACE AND IN WHOLE LEAF OF *Ruta graveolens*

	On surface			In leaf			Whole leaf			On surface (%)		
Leaves	P[b]	X[b]	B[b]	P	X	B	P	X	B	P	X	B
Younger plants												
Upper	280	460	120	1100	1900	600	1300	2300	710			
green	240	440	120	900	1700	520	1200	2200	650	21	20	18
Lower	210	230	54	160	460	220	400	810	280			
green	170	300	70	200	540	180	340	730	240	51	35	23
Lower	3.4	110	30	35	190	240	40	300	270			
yellow	3.0	90	28	29	170	210	30	260	250	9.1	36	11
Lower dry	1.2	21	4.6	20	440	120	23	460	125			
	1.2	19	4.2	20	420	120	19	430	125	5.7	4.5	3.7
Older plants												
Upper	220	450	150	490	990	310	700	1500	470			
green	200	370	130	450	890	290	660	1300	410	31	30	32
Lower	60	85	55	150	500	160	210	610	230			
green	60	75	45	130	480	160	190	530	190	30	14	24
Lower	9.4	100	40	70	700	300	79	700	360			
yellow	9.0	88	34	58	600	300	69	580	330	12	15	11
Lower dry	1.5	14	5.9	30	460	180	30	480	180			
	1.5	12	5.0	26	460	160	27	460	170	5.1	2.3	3.1

[a] μg/g fresh weight; duplicate values shown.
[b] P: psoralen; X: xanthotoxin; B: bergapten.

the yellow, and seven (200/29) to 17 times (370/21) as much as in the dry; on the leaf surface of the younger plants this factor reached 160 (190/1.2).

Bergapten. Bergapten was always found in smaller concentrations than xanthotoxin. Compared to psoralen, it was lower in the upper green leaves, at a similar level in the lower green leaves, but much higher in both yellow and dry leaves. Smaller surface concentrations of bergapten were noted on both upper and lower green leaves, but on yellow and dry leaves the bergapten concentration was several times as high as that of psoralen. Despite lower absolute amounts, the percentage of bergapten on the surface was high—in most cases at least comparable to those of the other two.

The ratios of surface and whole-leaf concentrations of the three coumarins of the upper green leaves and the dry leaves of the same age group are compared in Table 3. This table shows the factors by which concentrations of furanocoumarins are lower in the yellow, dry leaves compared to the upper green, emphasizing the decline in furanocoumarin concentrations from the uppermost leaves to the dry ones at the base. The two groups showed similar tendencies: surface concentrations decreased more than those of the whole leaf, e.g., for younger plants, psoralens decreased on the surface 217 times, but only 60 times in the whole leaf; xanthotoxin 23 times vs 5; bergapten 27 vs 5. Younger plants showed higher ratios of decrease than older ones; the sharpest decrease was observed for psoralen, and the other two were similar. Psoralen appears more susceptible to changes in senescing leaves, showing ratios up to 12 times (60/5) those of xanthotoxin and bergapten.

Ratio of Psoralen to Xanthotoxin and Bergapten. Table 4 shows the proportions of the three furanocoumarins in the leaf and on the leaf surface, with the value for psoralen taken as unity. In both the younger and older plants the concentrations of xanthotoxin were higher than those of psoralen (i.e., >1), most notably in the yellow and dry leaves. Again in both groups, in almost all

TABLE 3. DIFFERENCES[a] IN CONCENTRATIONS OF FURANOCOUMARINS IN GREEN UPPER LEAVES AND DRY LEAVES OF *Ruta graveolens*

	Surface			Whole leaf		
Collection date	P[b]	X[b]	B[b]	P	X	B
November 1						
Younger plants	217	23	27	60	5	5
Older plants	140	32	26	23	3	3

[a] Concentrations of psoralens in and on upper green leaves/concentrations of psoralens in and on yellow dry leaves.
[b] P: psoralen, X: xanthotoxin, B: bergapten.

TABLE 4. RATIOS[a] OF CONCENTRATIONS OF FURANOCOUMARINS IN WHOLE LEAF AND ON SURFACE OF *Ruta graveolens* PLANTS

	Younger plants				Older Plants			
	Whole leaf		Surface		Whole leaf		Surface	
Leaves	X[b]	B[b]	X	B	X	B	X	B
Upper, green	1.8	0.54	1.7	0.46	2.0	0.65	2.0	0.67
Lower, green	2.1	0.71	1.4	0.33	5.9	1.0	1.3	0.83
Lower, yellow	8.0	7.4	31	9.0	8.6	4.6	10	4.0
Lower, dry	21	6.0	17	3.7	16	6.2	8.7	3.6

[a] Based on psoralen = 1.
[b] X: xanthotoxin, B: bergapten.

cases, the variations on the surface were greater than in the whole leaf. In the green leaves of the younger plants, bergapten was lower than psoralen, but in the yellow and dry leaves it was several times as high. In older plants, although bergapten was less in the upper leaves, there was at least as much in the lower, green leaves, and it was several times as high as psoralen in yellow or dry ones. On the surface, bergapten showed the same tendencies in the green leaves of both groups of plants, being lower than psoralen, but again it was much higher than psoralen in both yellow and dry leaves.

In yellow and dry leaves, both xanthotoxin and bergapten were higher than psoralen, but xanthotoxin was more so, by factors of ca. 8–30, both in the whole leaf and on the surface, in contrast to ca. 4–9 times for bergapten. Bergapten on green leaves, however, was in smaller absolute amounts than psoralen, as shown in Table 2. These findings point to very marked changes in concentrations and proportions of particular furanocoumarins during leaf aging.

DISCUSSION

The process of leaf aging is associated with changes in the physiology of the cells and, therefore, in the concentrations of the products stored in them. Senescence can be either a complex natural process, occurring over the course of the vegetative period, or may be caused by environmental changes. In view of the changes in furanocoumarin concentrations known to occur during the vegetative period in *Heracleum lanatum* (Zobel and Brown, 1990b), autumn leaves, all on the same plant, were chosen so that all the leaves would be mature without new growth, and the naturally senescing leaves would exhibit a range of stages simultaneously. In each of the two groups of plants examined, younger

and older, we distinguished four kinds of leaves, using as a marker their color and location on the shoot.

Younger and older plants tended to exhibit the same changes during senescence. Marked diminutions in furanocoumarin concentrations observed both in and on senescing leaves indicated lower production and extrusion rates during senescence. Changes of surface concentrations in each of these four groups of leaves showed the same tendencies: decreasing concentrations of both total furanocoumarins and each individual furanocoumarin in the whole leaf and, even more strikingly, on the surface. This suggests decreased extrusion as one possible explanation for the decline. Whether it is connected with the presumed need for less protection by leaves located lower on the plant must remain only speculation at this point.

The drop in the total concentration of the three furanocoumarins of aging leaves, in the whole leaf, and on the surface, was followed by a decrease in each individual concentration, but not in the same proportions. That of bergapten on the dry leaves was not so extreme as that of psoralen, whose concentrations decreased drastically by factors of >100. Psoralen was the most susceptible compound to changes due to senescence, both in the whole leaf and on the surface. Xanthotoxin always predominated in absolute amounts, but in the range of changes between the upper green leaves and dry ones it was comparable to that of bergapten. Bergapten might be termed a "senescing compound" because it was found in higher concentrations on and within aging callus cells (Zobel and Brown, 1991). We wish to emphasize the importance of visualizing changes in concentrations of particular coumarins as well as their ratios, in the knowledge that they can react synergistically (Berenbaum and Neal, 1985). Further investigations on the coexistence of other coumarins in the plant and on its surface, both qualitative and quantitative, are indicated.

In yellow, dry leaves there remained only a small fraction of furanocoumarins, which could eventually enter the soil, but the absolute amounts were quite substantial (580–680 μg/g fresh weight, or >70 μg/g dry weight). From the surface of the leaves, during the process of senescence, the loss was from 800 to 25 μg/g. More investigation is needed to measure the extent to which these compounds were washed by rain into the soil, vaporized into the air, or degraded chemically or biologically. Also needed is more study to evaluate the influence of changing environmental conditions on surface furanocoumarin concentrations and to distinguish these from genetically dependent changes such as natural physiological aging.

Acknowledgments—We are grateful to the Natural Sciences and Engineering Research Council of Canada for financial support through operating grant A2487 to S.A.B.

REFERENCES

AUDUS, L.J. 1972. Plant Growth Substances, Vol. 1: Chemistry and Physiology. Leonard Hill, London.

BARZ, W., and HOESEL, W. 1979. Metabolism and degradation of phenolic compounds in plants. *Recent Adv. Phytochem.* 12:339–369.

BARZ, W., and KOESTER, J. 1981. Turnover and degradation of secondary (natural) products, pp. 35–84, *in* P.K. Stumpf and E.E. Conn (eds.). The Biochemistry of Plants, Vol. 7. Academic Press, New York.

BARZ, W., and WELTRING, K.-M. 1985. Biodegradation of aromatic extractives of wood, pp. 607–666, *in* T. Higuchi (ed.). Biosynthesis and Biodegradation of Wood Compounds. Academic Press, New York.

BARZ, W., KOESTER, J., WELTRING, K.-M., and STRACK, D. 1985. Recent advances in the metabolism and degradation of phenolic compounds in plants and animals, *Ann. Proc. Phytochem. Soc. Eur.* 25:307–347.

BERENBAUM, M., and NEAL, J.J. 1985. Synergism between myristicin and xanthotoxin, a naturally coocurring plant toxicant. *J. Chem. Ecol.* 11:1349–1358.

BRABER, J.M. 1980. Catalase and peroxidase in primary bean leaves during development and senescence. *Z. Pflanzenphysiol.* 97:135–144.

EBEL, J. 1979. Elicitor-induced phytoalexin synthesis in soybean (*Glycine max*), pp. 155–162, *in* M. Luckner and K. Schreiber (eds.). Regulation of Secondary Product and Plant Hormone Metabolism. Pergamon, Oxford.

FOBEL, M., LYNCH, D.V., and THOMPSON, J.E. 1987. Membrane deterioration in senescing carnation flowers. *Plant Physiol.* 85:204–221.

FRIEDMAN, J., RUSHKIN, E., and WALLER, G.R. 1982. Highly potent germination inhibitors in aqueous eluate of fruits of bishop's weed (*Ammi majus* L.) and avoidance of autoinhibition. *J. Chem. Ecol.* 8:55–65.

HALE, M.G., and ORCUTT, J.M. 1987. The Physiology of Plants under Stress. Wiley, New York.

HASLAM, E. 1985. Metabolites and Metabolism. Clarendon, Oxford. Chap. 6.

IVIE, G.W. 1987a. The chemistry of plant furanocoumarins and their medical, toxicological, environmental, and coevolutionary significance. *Rev. Latinoam. Quim.* 18(1):1–6.

IVIE, G.W. 1987b. Biological actions and metabolic transformations of furanocoumarins, pp. 217–230, *in* J.R. Heitz and K.R. Downum (eds.). Light-activated pesticides. American Chemical Society Symposium Series No. 339. American Chemical Society, Washington, D.C.

JANZEN, D.H., and WATERMAN, P.G. 1986. A seasonal census of phenolics, fiber and alkaloids in foliage of forest trees in Costa Rica. *Biol. J. Linn. Soc.* 21:439–454.

JACQUES, V., KOESTER, J., and BARZ, W. 1985. Differential turnover of isoflavone-7-*O*-glucoside-6″-malonates in *Cicer arientinum* L. roots. *Phytochemistry* 24:949–951.

MALIK, N.S.A. 1987. Senescence in oat leaves: Changes in translatable mRNA. *Physiol. Plant.* 70:438–446.

MANN, J. 1980. Secondary Metabolism. Clarendon, Oxford.

MARGNA, V., and VAINJAERO, T. 1981. Buckwheat seedling flavonoids do not undergo rapid turnover. *Biochem. Physiol. Pflanzen* 176:44–53.

MOTHES, K. 1980. Secondary plant products: A historical introduction, pp. 1–11, *in* E.A. Bell and B.V. Charlwood (eds.). Encyclopedia of Plant Physiology (New Series). Springer-Verlag, Berlin.

MURRAY, R.D.H., MÉNDEZ, J., and BROWN, S.A., 1982. The Natural Coumarins: Occurrence, Chemistry and Biochemistry, Wiley, Chichester, U.K.

OSBORNE, D.J., 1968. Hormonal mechanisms regulating senescence and abscission, pp. 815–840,

in F. Wightman and G. Setterfield (eds.). The Biochemistry and Physiology of Plant Growth Substances. Runge Press, Ottawa.

PATRA, H.K., and MISHRA, D. 1979. Pyrophosphatase, peroxidase and polyphenoloxidase activities during leaf development and senescence. *Plant Physiol.* 63:318-323.

RODIGHIERO, G. 1954. Influence of natural furanocoumarins on the germination of seeds and on the growth of lettuce sprouts and roots. *Giorn. Biochem.* 3:138-146.

SHINA-ROY, S.P., and CHAKRABORTY, D.P. 1976. Psoralen, a powerful germination inhibitor. *Phytochemistry* 15:2005-2007.

SIMON, E.W. 1967. Types of leaf senescence. *Symp. Soc. Exp. Biol.* 21:215-230.

THOMPSON, H.J., and BROWN, S.A. 1984. Separations of some coumarins of higher plants by liquid chromatography. *J. Chromatogr.* 314:323-336.

WIERMANN, R., 1980. Secondary plant products and cell and tissue differentiation, pp. 86-116, *in* P.K. Stumpf and E.E. Conn (eds.). The Biochemistry of Plants, Vol. 7. Academic Press, New York.

WOOLHOUSE, H.W., 1967. The nature of senescence in plants. *Symp. Soc. Exp. Biol.* 21:869-932.

ZOBEL, A.M., and BROWN, S.A. 1988. Determination of furanocoumarins on the leaf surface of *Ruta graveolens* with an improved extraction technique. *J. Nat. Prod.* 51:941-946.

ZOBEL, A.M., and BROWN, S.A. 1989. Histological localization of furanocoumarins in *Ruta graveolens*. *Can. J. Bot.* 67:915-921.

ZOBEL, A.M., and BROWN, S.A. 1990a. Dermatitis-inducing furanocoumarins on the leaf surfaces of eight species of rutaceous and umbelliferous plants. *J. Chem. Ecol.* 16:693-700.

ZOBEL, A.M., and BROWN, S.A. 1990b. Seasonal changes of furanocoumarin concentrations in leaves of *Heracleum lanatum*. *J. Chem. Ecol.* 16:1623-1634.

ZOBEL, A.M., and BROWN, S.A. 1991. Furanocoumarins on the surface of callus cultures from species of the Rutaceae and Umbelliferae. *Can. J. Bot.* Submitted.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

DIFFERENTIAL EFFECTS OF CONDENSED AND HYDROLYZABLE TANNIN ON POLYPHENOL OXIDASE ACTIVITY OF ATTINE SYMBIOTIC FUNGUS

COLIN NICHOLS-ORIANS[1]

Department of Entomology
Pennsylvania State University
University Park, Pennsylvania 16802

(Received February 19, 1991; accepted May 6, 1991)

Abstract—The leaf-cutting ant *Atta cephalotes* is a generalist herbivore of the neotropics and collects leaf material to cultivate a fungus. It appears that this fungus, a Basidiomycete, is responsible for the ability of the ants to utilize most of the available woody plant species. Tannins and other phenolics are ubiquitous secondary chemicals in woody plants, and Basidiomycete fungi produce enzymes, such as polyphenol oxidase, that are capable of polymerizing and inactivating the phenolics. This study evaluates the effects of a condensed and a hydrolyzable tannin on the activity of polyphenol oxidase and the growth of the fungus. I hypothesized that low concentrations of tannin would not inhibit polyphenol oxidase activity but high concentrations would inhibit the enzyme. Consequently, I predicted that only high concentrations of tannin would inhibit fungal growth. Laboratory assays with the fungus indicated that hydrolyzable tannin (tannic acid) and condensed tannin (quebracho tannin) differ in the mechanism of inhibition. Tannic acid does not inhibit polyphenol oxidase activity but does inhibit fungal growth. Quebracho tannin, however, inhibits both polyphenol oxidase activity and fungal growth. As predicted, both tannic acid and quebracho tannin primarily inhibit the fungus at high concentrations.

Key Words—*Atta cephalotes*, Hymenoptera, Formicidae, attine fungus, fungal performance, condensed tannin, hydrolyzable tannin, polyphenol oxidase.

INTRODUCTION

The leaf-cutting ant, *Atta cephalotes* (L.) (Hymenoptera: Formicidae: Attini), is a dominant herbivore in Central America (Fennah, 1950; Cherrett, 1968;

[1] *Present Address:* Department of Biology, Vassar College, Poughkeepsie, New York 12601.

0098-0331/91/0900-1811$06.50/0

Rockwood, 1976, 1977) and may harvest up to 77% of the plant species in their habitat (Rockwood, 1976). No other insect in the neotropics appears to be so polyphagous (Cherrett et al., 1989). Rather than consuming the leaf material directly, the ants use the harvested leaf material to support the growth of a symbiotic fungus. This fungus is the sole food source of the developing larvae (Quinlan and Cherrett, 1979). The fungus, a white-rotting Basidiomycete, may be responsible for the polyphagous nature of leaf-cutting ants because the fungus produces an array of enzymes that are capable of detoxifying secondary chemicals in leaves (Cherrett et al., 1989). For example, the fungus produces polyphenol oxidase, an enzyme capable of polymerizing and inactivating tannins and other phenolics (Powell, 1984). Yet tannins can inhibit the ants (Kawanashi and Raffauf, 1986; Howard, 1990; but see Littledyke and Cherrett, 1976). Field studies indicate that only high concentrations of tannin inhibit the ants (Nichols-Orians and Schultz, 1990; Nichols-Orians, 1991a–c). Perhaps this is because only high concentrations of tannin inhibit the fungus and its enzymes.

Tannins do inhibit fungal growth and fungal enzyme activity (Seaman, 1984; Cherrett et al., 1989; Nichols-Orians and Schultz, 1990; Nichols-Orians, 1991a). Hydrolyzable tannins have been shown to inhibit the growth of an attine fungus (Seaman, 1984), and condensed tannins appear to be even more inhibitory (Cherrett et al., 1989). This result is consistent with the observation that condensed tannins are very effective inhibitors of fungi and their enzymes (Zucker, 1983). Only high concentrations of tannin appears to inhibit fungal growth (Seaman, 1984). I hypothesized that only high concentrations of tannin inhibit fungal growth because, at high concentrations, the tannin would inactivate the polyphenol oxidases, thereby limiting the degradation of the tannin.

Thus this research investigated how two types of tannins at different concentrations affect the activity of a polyphenol oxidase and the performance of the fungus. Evidence is presented indicating that tannic acid and quebracho tannin differ in their inhibition of polyphenol oxidase activity, yet both inhibit fungal growth.

METHODS AND MATERIALS

Ants–Fungus. Laboratory assays with the fungus were conducted at Penn State in 1989–1990 with fungus obtained from an *Atta cephalotes* colony collected from La Selva, Costa Rica, in January 1987. The colony was maintained at Penn State on a diet of frozen *Forsythia* and fresh cabbage prior to removal of the fungus.

The attine fungus was isolated, maintained on potato dextrose agar plates, transferred to Sabaroud's dextrose broth (SDB), and later grown in a liquid medium designed to test for the effects of tannins on fungal performance (Sea-

man, 1984). This medium (modified FPT) contained the following components per liter of distilled water: (A) major components (g)—KH_2PO_4 (1); $MgSO_4$–$7H_2O$ (0.5); NaCl (0.1); $CaCl_2$–$2H_2O$ (0.1); NH_4 tartrate (5.0); and dextrose (20); (B) minor components (mg)—$ZnSO_4$–$4H_2O$ (4); $MnSO_4$–$4H_2O$ (4); and ferric tartrate (4); (C) organic supplement (mg)—4-Methyl-5-thiazoleethanol (0.180); and 4-amino-5-aminomethyl-2-methylpyrimidine dihydrochloride (0.250). In these experiments, 500 ml of water was replaced with 500 ml of 0.05 M dimethylglutaric acid buffer (pH 5.5) (Powell and Stradling, 1986).

Three basic experiments were conducted. First, I quantified the relationship between the concentration of condensed or hydrolyzable tannin and the polyphenol oxidase (PPO) activity at different points in time (10, 30, 60, or 120 min and 2, 7, or 21 days). There was one replication per concentration at each point in time, except at day 21 where there were three replicates per tannin concentration. Second, I determined whether tannic acid and quebracho tannin inhibit the enzymes directly or whether inhibition is merely a function of reduced fungal growth. Finally, I assessed how the two types of tannin affect fungal growth.

Fungal Preparation. Fungus obtained from the agar plates was placed in 65 ml of SDB and blended for 1 min in a Vertis blender at low speed. Five milliliters of the fungal suspension was pipetted into Erlenmeyer flasks containing 45 ml of SDB. The cultures were then grown for 10 weeks in the dark. After 10 weeks, the fungus was filtered, resuspended in modified FPT (50 ml FPT/flask of fungus), and blended for 1 min at low speed. The fungus was kept evenly suspended using a stir bar and magnetic plate.

One milliliter of fungal suspension was added to 8 ml of modified FPT in sterile test tubes, and placed for four days in the dark at room temperature. After four days, 1 ml of one of four tannin solutions, tannic acid, or quebracho tannin was added to the suspension to give a final concentration of 0, 0.0025, 0.025, and 0.25% tannin. At various time intervals from 10 min to 21 days the activity of polyphenol oxidase (PPO) was determined (using the protocol described below). At day 21, the biomass of fungus was measured.

PPO Activity. Catechol is commonly used as a substrate to measure PPO activity (Flurkey and Jen, 1978). Liquid cultures of fungus were centrifuged at 1000 rpm for 10 min and the supernatants placed on ice. In test tubes, 0.75 ml of the supernatant from the fungal cultures were combined with 2 ml 0.1 M citric acid–sodium citrate buffer (pH 5.5). Following the addition of 0.25 ml of catechol (or distilled water for controls), test tubes were vortexed and incubated at 30°C for 30 min, and then the final absorbance was determined (420 nm) with a spectrophotometer. After subtracting the absorbance of the control, a relative PPO activity was obtained for each treatment. PPO activity is expressed as relative optical density (o.d.).

Statistical Analyses. All statistical analyses were done using SAS (SAS

Institute, 1985). The effect of increasing the concentration of tannic acid or quebracho tannin on the activity of PPO or the biomass of fungus was analyzed with an two-way ANOVA model with tannin type and tannin concentration as the main effects. Bonferroni's test was used to determine whether differences in PPO activity were significant at $P < 0.05$.

RESULTS

Tannic acid and quebracho tannin have different effects on PPO activity. Initially, PPO activity was identical across the different concentrations of tannic acid (Figure 1), but an increase in quebracho tannin concentration resulted in a decrease in PPO activity after only 10 min (Figure 2). These results suggest that tannic acid does not inhibit the enzymes directly but quebracho tannin does.

Because I obtained these results by incubating the tannin with the fungus, the differences in PPO activity could have been due to direct effects on the fungus not on the enzymes directly. Consequently, I used supernatants from

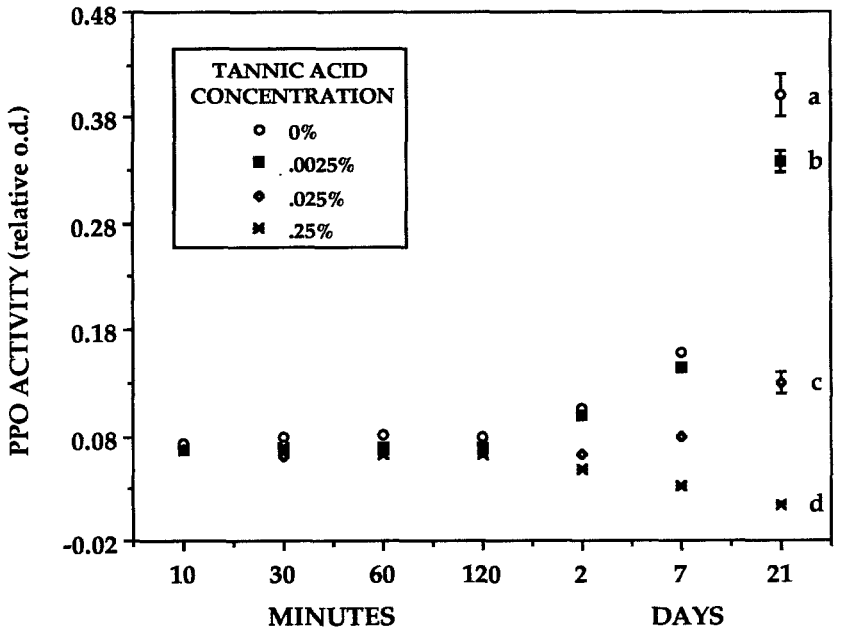


FIG. 1. The effects of tannic acid on the polyphenol oxidase (PPO) activity in liquid cultures at different points in time. $N = 1$ for each concentration of tannin at each point in time except at day 21 where $N = 3$. At day 21, values with different letters are different at $P < 0.05$.

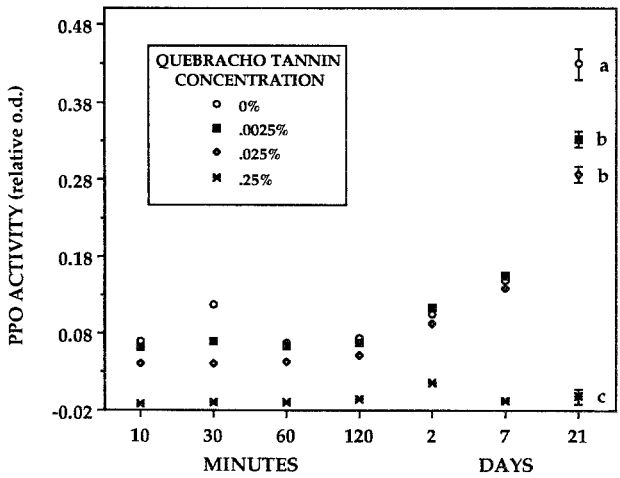


FIG. 2. The effects of quebracho tannin on the polyphenol oxidase (PPO) activity in liquid cultures at different points in time. $N = 1$ for each concentration of tannin at each point in time except at day 21 where $N = 3$. At day 21, values with different letters are different at $P < 0.05$. (Reprinted from Nichols-Orians, 1991a.)

tannin-free cultures to determine whether the addition of either type of tannin inhibited PPO activity directly. As indicated before, tannic acid did not inhibit PPO activity while quebracho tannin did inhibit PPO activity directly (Figure 3).

However, by day 2 it was evident that PPO activity in cultures with tannin was lower for tannic acid as well as quebracho tannin (Figures 1 and 2). By day 21 it was clear that an increase in tannic acid resulted in a decrease in PPO activity ($P < 0.05$) (Figure 1). For quebracho tannin, the highest concentration caused a severe reduction in PPO activity while the lower concentrations caused only a minimal reduction ($P < 0.05$) (Figure 2). Previously, I found that as fungal biomass increases so does PPO activity (Nichols-Orians, 1991a). Therefore, I suggest that the reason tannic acid affected PPO activity beginning around day 2 was due to the direct inhibition of fungal growth, not of enzyme activity.

Indeed, both types of tannin can inhibit fungal growth. At day 21, an increase in tannin concentration appeared to result in a decrease in fungal biomass ($P < 0.001$) (Figure 4). These differences in biomass are conservative estimates because it was not possible to accurately measure fungal biomass in cultures with tannin due to binding between the tannin and the fungus. In fact, at 0.25% tannic acid, I was unable to even approximate fungal biomass because much of the tannic acid had bound to the fungus and precipitated out of solution.

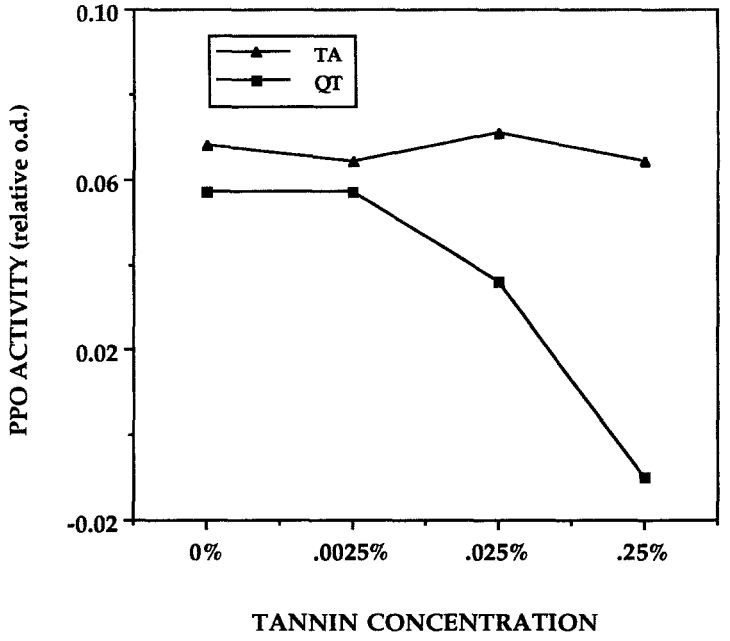


FIG. 3. Direct inhibition of polyphenol oxidase (PPO) activity by tannic acid and quebracho tannin. $N = 1$ at each concentration of tannin.

DISCUSSION

Tannins are ubiquitous secondary chemicals in plants and have a putative effect against many herbivores (see Schultz, 1989, for review). Tannins are most reactive as quinones. The fungus cultivated by the ants produces an enzyme, polyphenol oxidase, that appears capable of oxidizing quinones to a less reactive state (Cherrett et al., 1989). The effectiveness of the PPO may depend on the type and concentration of tannin (Zucker, 1983; Clausen et al., 1990), since tannins can inhibit polyphenol oxidases (Lyr, 1962).

Tannin type is important. According to Cherrett et al. (1989), Powell (1984) found that condensed tannins are more inhibitory of the fungus than hydrolyzable tannins. Here, I found that quebracho tannin (a condensed tannin) inhibits PPO activity directly while tannic acid (a hydrolyzable tannin) does not (Figure 1 and 2). This result is consistent with Zucker's (1983) claim that condensed tannins are more effective inhibitors of fungal enzymes than hydrolyzable tannins. However, these results do not indicate that quebracho tannin is more inhibitory of fungal growth than tannic acid (Figure 4).

Tannin concentration is important as well. Seaman (1984) found that concentrations of tannic acid below 0.03% had little effect on the performance of

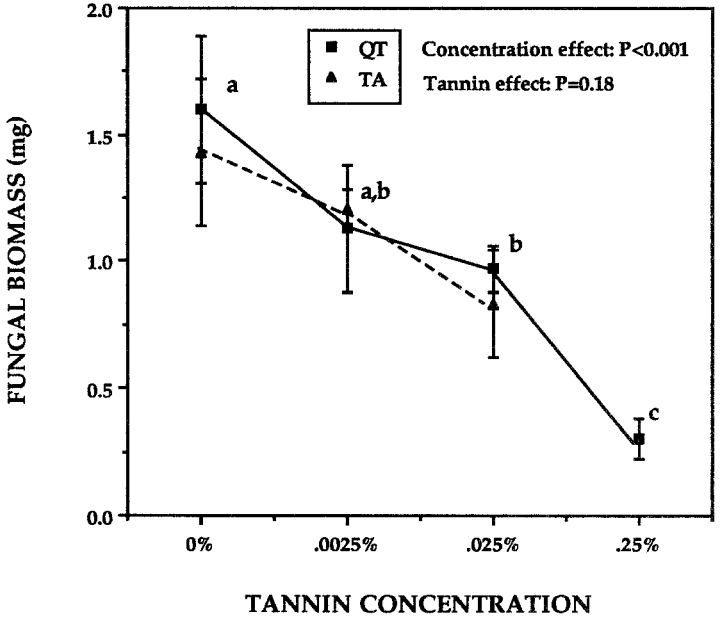


FIG. 4. The relationship between tannin concentration and the biomass of fungus at day 21. $N = 3$ at each concentration of tannin. Values with different letters are different at $P < 0.05$.

a related attine fungus. My results lend support to those of Seaman (1984). I found no difference in fungal biomass for 0% and 0.0025% tannic acid but did measure a reduction in biomass at 0.025% tannic acid (Figure 4). At 0.25% tannic acid the fungus appeared dead. However, a change in the concentration of tannic acid did not affect PPO activity; there was no inhibition at all. Quebracho tannin, on the other hand, inhibits both PPO activity and fungal growth but mostly at the highest concentration (Figures 2 and 4).

In a previous study on the effects of quebracho tannin on the ants and the fungus (Nichols-Orians, 1991a), I had hypothesized that the PPO would be induced by the presence of quebracho tannin (sensu Mayer, 1987), so that in the absence of quebracho tannin there would be no PPO activity. It appeared from that study that PPO was produced constitutively and was not induced by the presence of quebracho tannin, yet the results were not definitive because quebracho tannin inhibited the enzymes directly. This could have made it impossible to detect elevated levels. In this study, it is possible to test for induction because tannic acid does not inhibit PPO (Figure 3). PPO is not induced by tannic acid; PPO activity in control (no tannic acid) cultures is as high or higher than in cultures with tannic acid at all sampling times (Figure 1).

Growing attine fungus in liquid culture is ideal for testing how different types of tannin affect PPO activity. It is not ideal, however, for testing how tannins would affect fungal performance under natural conditions. Fungal hyphae secrete PPOs externally, and presumably the enzymes begin degrading any tannin present in the leaves before the tannin contacts the fungus. Consequently, a particular type of tannin would only come into contact with the fungus if it (1) inactivates the PPO, (2) is unaffected by the PPO, or (3) is present at extremely high concentrations and diffuses rapidly toward the fungal hyphae. Clearly, it is necessary to determine which wins, tannins or PPOs, under natural conditions.

Nevertheless, there are a couple of conclusions that can be drawn from this study. First, different types of tannin will differ in their effects on the ant–fungus system. If quebracho tannin and tannic acid are at all indicative of other condensed and hydrolyzable tannins, respectively, then it appears that only condensed tannins would limit the ability of the fungus to inactivate tannins. Hydrolyzable tannins would only be important if unaffected by the PPOs or if at such high concentration that they satiate the enzymes and subsequently contact the fungus.

Second, tannins are not usually the only phenolics present in woody plants, and nontannin phenolics can have antimicrobial effects also (Harborne, 1988). Thus, the presence of PPO-inhibiting tannins may limit the ability of the fungus to inactivate other phenolics. Many terpenoids are toxic to the fungus (Hubbell et al., 1983; Howard et al., 1988), and perhaps the presence of certain tannins could inhibit enzymes responsible for detoxifying a toxic terpenoid as well. This could enhance the toxicity of that terpenoid. Clearly, comparative studies on the effects of different types of tannin, with and without other secondary chemicals, on the activity of detoxification enzymes and the growth of the fungus are warranted. Future experiments should try to mimic natural conditions as much as possible.

Acknowledgments—H. Appel, J. Cazin, W. McCarthy, and C. Mullin provided suggestions for the experimental protocol. Early versions of this paper benefited from the comments of H. Appel, S. Keating, and two anonymous reviewers. This research was supported by a Jesse Smith Noyes Fellowship Grant awarded by the Organization for Tropical Studies, and National Science Foundation grants BSR-8605106 (to J. Schultz), BSR-8815648 (Dissertation Improvement Grant to C. Nichols-Orians), and BSR-8917752 (to R. Fritz).

REFERENCES

CHERRETT, J.M. 1968. The foraging behavior of *Atta cephalotes* L. (Hymenoptera, Formicidae). 1. Foraging pattern and plant species attacked in tropical rain forest. *J. Anim. Ecol.* 37:387–403.

CHERRETT, J.M., POWELL, R.J., and STRADLING, D.J. 1989. The mutualism between leaf-cutting ants and their fungus, pp. 93–120, *in* N. Wilding, N.M. Collins, P.M. Hammond and J.F. Webber (eds.). Insect-Fungus Interactions. Academic Press, London.

CLAUSEN, T.P., PROVENZA, F.D., BURRITT, E.A., REICHARDT, P.B., and BRYANT, J.P. 1990. Ecological implications of condensed tannin structure: A case study. *J. Chem. Ecol.* 16:2381-2392.

FENNAH, R.G. 1950. Parasol ants, their life history and methods for their control. *Proc. Agric. Soc. Trinidad* 50:312-326.

FLURKEY, W.H., and JEN, J.J. 1978. Peroxidase and polyphenoloxidase activities in developing peaches. *J. Food Sci.* 43:1828-1831.

HARBORNE, J.B. 1988. Introduction to Ecological Biochemistry. Academic Press, San Diego.

HOWARD, J.J. 1990. Infidelity of leafcutting ants to host plants: Resource heterogeneity or defense induction? *Oecologia* 82:394-401.

HOWARD, J.J., CAZIN, J. JR., and WIEMER, D.F. 1988. Toxicity of terpenoid deterrents to the leafcutting ant *Atta cephalotes* and its mutualistic fungus. *J. Chem. Ecol.* 14:59-69.

HUBBELL, S.P., WIEMER, D.F., and ADEJARE, A. 1983. An antifungal terpenoid defends a neotropical tree (*Hymenaea*) against attack by fungus growing ants (*Atta*). *Oecologia (Berlin)* 60:321-327.

KAWANASHI, K., and RAFFAUF, R.F. 1986. *Caryocar microcarpum*: An ant repellent and fish poison of the northwest Amazon. *J. Nat. Prod.* 49:1167-1169.

LITTLEDYKE, M., and CHERRETT, J.M. 1976. Direct ingestion of plant sap from cut leaves by the leaf-cutting ants *Atta cephalotes* (L.) and *Acromyrmex octospinosus* (Reich) (Formicidae, Attini). *Bull. Entomol. Res.* 66:205-217.

LYR, H. 1962. Detoxification of heartwood toxins and chlorophenols by higher fungi. *Nature* 195:289-230.

MAYER, A.M. 1987. Polyphenol oxidases in plants—recent progress. *Phytochemistry* 26:11-20.

NICHOLS-ORIANS, C. 1991a. Condensed tannins, attine ants, and the performance of a symbiotic fungus. *J. Chem. Ecol.* 17:1177 -1195.

NICHOLS-ORIANS, C.M. 1991b. The effects of light on foliar chemistry, growth and susceptibility of seedlings of a canopy tree to an attine ant. *Oecologia* 86:552-560.

NICHOLS-ORIANS, C.M. 1991c. Environmentally induced differences in plant traits: Consequences for susceptibility to a leaf-cutter ant. *Ecology* 72(5):In press.

NICHOLS-ORIANS, C.M., and SCHULTZ, J.C. 1990. Interactions among leaf toughness, chemistry and harvesting by attine ants. *Ecol. Entomol.* 15:311-320.

POWELL, R.J. 1984. The influence of substrate quality on fungus cultivation by some attine ants. Ph.D. thesis. University of Exeter, U.K. (unpublished).

POWELL, R.J., and STRADLING, D.J. 1986. Factors influencing the growth of *Attamyces bromatificus*, a symbiont of attine ants. *Trans. Br. Mycol. Soc.* 87:205-213.

QUINLAN, R.J., and CHERRETT, J.M. 1979. The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L.). *Ecol. Entomol.* 4:151-160.

ROCKWOOD, L.L. 1976. Plant selection and foraging patterns in two species of leaf-cutting ants (*Atta*). *Ecology* 57:48-61.

ROCKWOOD, L.L. 1977. Foraging patterns and plant selection in Costa Rican leaf-cutting ants. *J. N.Y. Entomol. Soc.* 85:222-233.

SAS Institute. 1985. SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina.

SCHULTZ, J.C. 1989. Tannin-Insect Interactions, pp. 417-433, *in* R.W. Hemingway and J.J. Karchesy (eds.). Chemistry and Significance of Condensed Tannins. Plenum Press, New York.

SEAMAN, F.C. 1984. The effects of tannic acid and other phenolics on the growth of the fungus cultivated by the leaf-cutting ant, *Myrmicocrypta buenzlii*. *Biochem. System. Ecol.* 12:155-158.

ZUCKER, W.V. 1983. Tannins: does structure determine function? An ecological perspective. *Am. Nat.* 121:335-365.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

REASSESSMENT OF THE ROLE OF GUT ALKALINITY AND DETERGENCY IN INSECT HERBIVORY[1]

GARY W. FELTON[2,*] and SEAN S. DUFFEY

Department of Entomology
University of California
Davis, California 95616

(Received February 28, 1991; accepted May 8, 1991)

Abstract—Previously it was reported that significant amounts of the tomato phenolic, chlorogenic acid, were oxidized in the digestive system of generalist feeders *Spodoptera exigua* and *Helicoverpa zea*. The covalent binding of the oxidized phenolic (i.e., quinone) to dietary protein exerts a strong antinutritive effect against larvae. In this study, we examined the fate of ingested chlorogenic acid in larval *Manduca sexta*, a leaf-feeding specialist of solanaceous plants. Significant amounts of chlorogenic acid were bound to excreted protein by *M. sexta* when larvae fed on tomato foliage. However, in the case of *M. sexta* we suggest that the strong alkalinity and detergency of the midgut may minimize the antinutritive effects of oxidized phenolics. The solubility of tomato leaf protein is significantly greater at pH 9.7, representative of the midgut of *M. sexta*, than at pH 8.0, representative of the midguts of *H. zea* and *S. exigua*. We suggest that this increase in solubility would compensate for any loss in bioavailability of essential amino acids caused by the covalent binding of chlorogenic acid to amino acids. Furthermore, lysolecithin, a surfactant likely to contribute to the detergent properties of the midgut fluid, was shown to enhance protein solubility as well as inhibit polyphenol oxidase activity. The adaptive significance of gut alkalinity and detergency is discussed.

Key Words—*Manduca sexta, Lycopersicon*, phenolics, chlorogenic acid, polyphenol oxidase, midgut pH, surfactants, detergency, alkalinity, herbivore adaptations, plant defense, lysolecithin, insect nutrition.

*To whom correspondence should be addressed.
[1] Approved by the Director of the Arkansas Agricultural Experiment Station.
[2] Current address: Department of Entomology, University of Arkansas, Fayetteville, Arkansas 72701.

0098-0331/91/0900-1821$06.50/0

INTRODUCTION

Investigations continue to be conducted on the impact of plant phenolics and polyphenol oxidases on insect growth using the tomato plant, *Lycopersicon esculentum*, and the generalist noctuids *Helicoverpa* (=*Heliothis*) *zea* and *Spodoptera exigua* as a model system (Felton et al., 1989a,b; Felton and Duffey, 1990; Duffey and Felton, 1989). Upon damage to leaf tissue, liberated polyphenol oxidase oxidizes orthodihydroxyphenolics such as chlorogenic acid to the corresponding reactive, electrophilic quinone (Mayer, 1987). The quinones undergo rapid addition reactions with nucleophiles such as the thiol and amino groups of proteins (Hurrell et al., 1982; Pierpoint, 1983; Kalyanaraman et al., 1987). These reactions occur in the digestive systems of both *H. zea* and *S. exigua*, and, as a consequence, the utilization of dietary protein is impaired (Felton et al., 1989a,b; Duffey and Felton, 1989).

In addition to *H. zea* and *S. exigua*, which consume a variety of plant tissues, the tomato herbivore complex includes other lepidopteran larvae such as the oligophagous *Manduca sexta* and the polyphagous *Trichoplusia ni* (Lange and Bronson, 1981). Of these species, only *M. sexta* and *T. ni* normally restrict their feeding to leaves (Lange and Bronson, 1981). The leaf-specialist *M. sexta* grows relatively more rapidly on tomato foliage than *H. zea* (Hare, 1983; Felton et al., 1989a). Larval *H. zea* is better adapted to feeding on fruit where polyphenol oxidase activity is several orders of magnitude less than foliage (Felton et al., 1989a).

One of the most striking differences in the digestive systems between the leaf specialists and the fruit-feeding lepidopteran species is the alkalinity of the midgut. The leaf-feeders, *M. sexta* and *T. ni*, have a gut pH (ca. 10.0 to 11.0) much greater than the generalist feeders *Spodoptera* and *Helicoverpa* (ca. 8.0) (Dow, 1984; Martin et al., 1987; Felton et al., 1989a). Our contention is that the highly alkaline gut conditions of the leaf-feeding herbivores represent one physiological strategy to enhance the acquisition of dietary protein, thus minimizing the impact of oxidative enzymes and their substrates on dietary protein.

In this paper, we examine the effects of alkalinity and detergency upon the solubility of leaf protein, the activity of foliar oxidative enzymes, and the binding of phenolics to protein. The adaptive significance of gut alkalinity and detergency to leaf-feeding herbivores is discussed.

METHODS AND MATERIALS

Insects. Eggs of *M. sexta* were obtained from Dr. Bruce Hammock, University of California, Davis. Neonate larvae were placed on artificial diet containing 4% fresh weight (fwt) casein (Chippendale, 1970).

Isotope. Tritiated chlorogenic acid prepared by catalytic exchange was

obtained from Amersham Corp. and purified by thin-layer chromatography to remove labile tritium as described (Isman and Duffey, 1983) with a specific activity of 135 mCi/mmol.

Extraction of Leaf Protein. To determine the effect of pH and oxidation on amino acid composition of extracted protein, four leaves were excised at the petiole with a razor blade from each flowering-stage, greenhouse-grown, tomato plant (var. Ace). From each leaf, four leaflets were excised, weighed, and randomly placed in one of four extraction treatments. Each of the four treatments contained eight leaflets weighing approximately 3.0 g (fwt). Leaflets were frozen at −20°C for 1 hr prior to homogenization and separately homogenized for 2 min in a Waring blender containing 100 ml ice-cold buffer from one of four treatments: 0.1 M sodium phosphate buffer at pH 8.0 and at pH 9.7 without diethyldithiocarbamate and at pH 8.0 and pH 9.7 with 2 mg/ml diethyldithiocarbamate. Diethyldithiocarbamate is an effective inhibitor of polyphenol oxidases (Mayer, 1987).

Protein was extracted in the following manner. Following homogenization, the treatments were allowed to sit at 30°C for 2 hr to enhance solubilization of protein and to allow oxidation of phenolics. Each treatment was filtered through a single layer of cheesecloth and centrifuged at 10,000*g* for 30 min at 0–4°C. The supernatant was used for all further enzyme and protein assays. A 50-ml aliquot of the supernatant was removed from each treatment and placed on ice. Ten milliliters of 30% trichloroacetic acid was added to each aliquot followed by centrifugation for 15 min at 10,000*g*. The pellets were washed with 90% methanol and recentrifuged, a process that was repeated several times. The methanol supernatant was discarded in each case. Finally, the pellets were frozen, lyophilized, and weighed to the nearest 0.1 mg.

The extraction and quantification of protein was replicated three times after which the protein was pooled for each of the four treatments. Samples (ca. 50 mg) of the leaf protein were hydrolyzed in 6 N HCl (1 mg protein/ml) for 24 hr at 100°C in vacuo and analyzed for amino acid content by automated amino acid analysis at the Protein Structure Laboratory, University of California, Davis. The total amount of amino acid per gram of foliage was calculated by multiplying the amino acid content of the protein by the average weight of precipitated protein per gram of foliage.

Polyphenol oxidase and peroxidase activities from aliquots of the supernatant from the four treatment regimes were determined with chlorogenic acid and guaiacol–H_2O_2 as substrates, respectively (Ryan et al., 1982). One unit of polyphenol oxidase or peroxidase activity was defined as a change in OD_{470}/min equal to 0.001/min.

To examine the effect of pH on the solubility of leaf protein, leaves were excised from field-grown tomato plants (var. Castlemart) and immediately frozen at −20°C for 1 hr. Leaves were lyophilized and ground to a fine powder.

Forty-milligram aliquots of leaf powder were added to centrifuge tubes containing 1 ml of 0.2 M sodium phosphate buffer at pH 7.0, 8.0, 9.0, or 10.0. The samples were placed on a shaking water bath for 60 min at 30°C and centrifuged for 15 min at 16,000*g*. Ten-microliter aliquots from each sample were assayed for protein with 1.5 ml Coomassie blue protein reagent (BioRad Laboratories, Richmond, California) following the procedure of Jones et al. (1989). Ribulose-1,5-bisphosphate carboxylase/oxygenase (Sigma Chemical Co.) was used as a protein standard. Five replicates per pH concentration were tested.

Effect of Alkalinity and Tomato Polyphenol Oxidase Activity on Binding of Chlorogenic Acid to Leaf Protein. To determine the effect of pH and polyphenol oxidase activity on binding of chlorogenic acid to leaf protein, partially purified tomato polyphenol oxidase was isolated from tomato foliage by precipitation with 35% ammonium sulfate following the method of Gentile et al. (1988). The enzyme preparation contained 9600 units/mg protein of chlorogenic acid oxidase activity. Less than 1% of the total peroxidase activity remained in the polyphenol oxidase enzyme preparation.

Four treatments were performed: pH 8.0 with active polyphenol oxidase, pH 8.0 with heat-denatured polyphenol oxidase, pH 9.7 with polyphenol oxidase, and pH 9.7 with heat-denatured polyphenol oxidase. Each treatment contained 4 ml 0.1 M sodium phosphate buffer containing 14 μmol unlabeled chlorogenic acid, 100,000 dpm [^{3}H]chlorogenic acid, and 5 mg purified ribulose-1,5-bisphosphate carboxylase/oxygenase. At zero time, 0.3 mg of active or inactive polyphenol oxidase was added to the respective treatments. Treatments were placed on a water bath at 30°C and gently shaken for 2 hr. Reactions were stopped with the addition of ammonium sulfate to 95% saturation. After vortexing, samples were centrifuged at 10,000*g* for 30 min. Pelleted protein was washed repeatedly with 80% methanol and centrifuged until radioactivity remaining in the supernatant was equal to background levels. Finally, the protein pellet was solubilized in 0.1% SDS and added to ACS liquid scintillant for direct counting to calculate nanomoles of chlorogenic acid bound per milligram of protein.

Determination of Binding of Chlorogenic Acid to Protein in Insect Gut. To determine if chlorogenic acid binds to protein in the digestive system when *M. sexta* larvae feed on tomato foliage, a 1.0 μCi aliquot of [^{3}H]chlorogenic acid in 50% methanol was applied to each excised tomato leaflet weighing 75–100 mg and the leaflet was allowed to air dry. A leaflet contained on average 2.2 μmol chlorogenic acid/g fwt as determined spectrophotometrically (Broadway et al., 1986). Individual leaflets treated with the isotope were fed to individual day-old fifth instar *M. sexta* larvae that had been starved for 12 hr.

After 3 hr of feeding each larva had ingested an entire leaflet. Feces were

collected as produced, pooled on an individual larval basis, and placed immediately in 5 ml ice-cold double distilled H_2O containing 15 mg diethyldithiocarbamic acid to inhibit residual polyphenol oxidase activity. All larvae ceased defecating within 10 hr.

After larvae ($N = 10$) stopped defecating, the pooled feces of each larva were homogenized for 1 min, centrifuged for 10 min at 20,000*g*, and the supernatant was removed. Each pellet was reextracted in double distilled H_2O containing diethyldithiocarbamic acid and 1% Triton X-100. The supernatants of a given pellet were pooled and placed on ice, and ammonium sulfate to 90% saturation was added to precipitate protein. After 30 min, samples were centrifuged for 30 min at 20,000*g*, and the supernatant was removed for direct counting of [^{3}H]chlorogenic acid by liquid scintillation. Each pellet was washed with 80% methanol and centrifuged to remove residual noncovalently bound chlorogenic acid. After several successive washings, the supernatants were pooled for each sample, and radioactivity was counted by liquid scintillation. Finally, the pelleted protein was solubilized in 0.1% sodium dodecylsulfate, and covalently bound [^{3}H]chlorogenic acid was counted. The unbound chlorogenic acid represented combined radioactivity of the supernatants from the aqueous and methanolic extracts. A small aliquot also was applied to cellulose thin-layer plates and chromatographed as described (Felton et al., 1989a) to verify the formation of a chlorogenic acid–amino acid conjugate.

A 50-μl aliquot of hemolymph was removed from each larva 24 hr after it had ingested the leaflet. Radioactivity of the hemolymph was determined directly by liquid scintillation.

Effect of Surfactant on Foliar Oxidases and Protein Solubility. To determine if lysolecithin, a surfactant that may contribute to midgut detergency, affects foliar polyphenol oxidase activity, tomato leaflets (16 g) were divided into four equal parts and homogenized separately in ice-cold 0.1 M potassium phosphate buffer, pH 6.8, containing one of the following treatments: 0.02%, 0.04%, 0.08% lysolecithin, and a control lacking added surfactant. Following homogenization, the treatments were centrifuged for 20 min at 10,000*g* and assayed in triplicate for polyphenol oxidase activity with chlorogenic acid by measuring increase in absorbance at 470 nm (Ryan et al., 1982). The experiment was replicated five times.

To determine if lysolecithin affects protein solubility, 40 mg lyophilized leaf powder was added to 1 ml 0.1M sodium phosphate buffer, pH 7.0, with and without 0.04% lysolecithin. The treatments were treated similarly to above experiments on effects of pH on protein extractability. The concentration of lysolecithin was selected because it stimulates the surfactant properties of *M. sexta* midgut fluid (Martin and Martin, 1984). Protein was quantified following Jones et al. (1989).

RESULTS

Effect of pH and Oxidase Activity on Amount and Composition of Amino Acids in Extracted Protein. Both pH and polyphenol oxidase activity significantly affected the amount of extractable protein indexed as TCA-precipitable protein (Figure 1). Total amino acids are defined as the total molar content of amino acids in hydrolyzed protein. Essential amino acids are defined as essential amino acids required for larval growth of *Helicoverpa zea* (Rock and Hodgson, 1971). Significantly more total amino acids were extracted at pH 9.7 in both treatments (with or without polyphenol oxidase activity) than at the lower pH of 8.0 (Figure 1). Likewise, the inhibition of polyphenol oxidase resulted in greater amounts of extracted protein amino acids at both pH values. In treatments with polyphenol oxidase activity, nearly 42% more total amino acids and 60% more essential amino acids were obtained at pH 9.7 than at 8.0 (Figure 1). Similar effects were observed in treatments lacking polyphenol oxidase activity, in which approximately 43% more total and essential amino acids were extracted at the higher pH. The method of amino acid analysis does not take into account the essential amino acid, tryptophan, because it is destroyed during hydrolysis.

The effects of pH and polyphenol oxidase treatments on individual amino acids are shown in Table 1. Without exception, greater absolute amounts of all amino acids were obtained at pH 9.7 than at 8.0. In the case of the two most

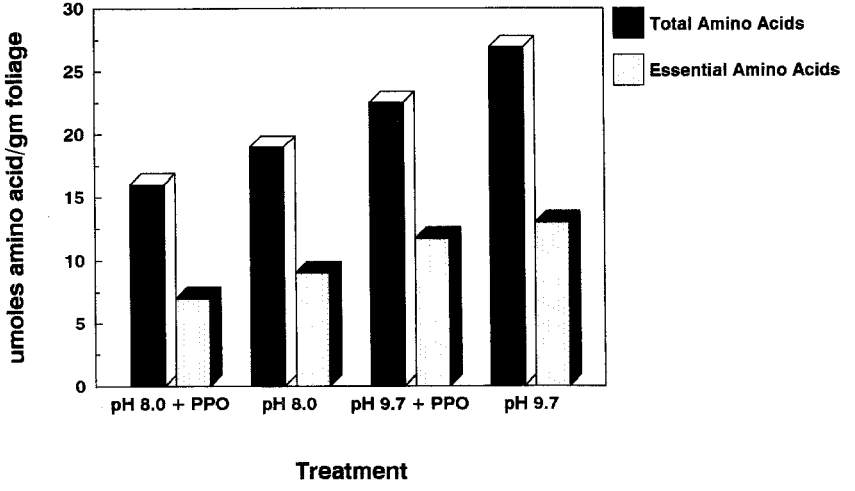


FIG. 1. The effect of pH and polyphenol oxidase activity on the extraction of essential and total amino acids from tomato foliage. PPO = polyphenol oxidase activity. Values are based upon single analyses of pooled replicates.

TABLE 1. EFFECT OF pH AND POLYPHENOL OXIDASE ACTIVITY ON EXTRACTABILITY OF TRICHLOROACETIC ACID-INSOLUBLE AMINO ACIDS[a]

Amino acid	pH 8.0 + PPO	pH 8.0	pH 9.7 + PPO	pH 9.7
ASP	1615	1837	2205	2595
THR	896	1034	1245	1479
SER	772	882	1095	1250
GLU	1757	2156	2435	3109
PRO	694	885	1133	1296
GLY	1457	1431	2486	2061
ALA	1394	1544	1911	2155
VAL	1210	1329	1684	1925
MET	281	307	327	513
ILE	766	788	1078	1170
LEU	1474	1712	2013	2510
TYR	600	687	853	1005
PHE	684	803	978	1179
LYS	1068	1390	1385	1909
ARG	848	1007	1174	1332
HIS	490	617	672	860

[a] Values for amino acids are expressed as nanomoles/per gram of foliage (fresh weight).

limiting amino acids (i.e., essential amino acids at the lowest concentrations), 16% more methionine and 37% more histidine were obtained at the more alkaline pH. The presence of polyphenol oxidase activity reduced the total quantity of amino acids at both pH levels. Apparently, the primary cause of this reduction is decreased solubility of phenolic-bound protein (Betschart and Kinsella, 1973).

Oxidized chlorogenic acid is known to bind covalently to amino acids such as lysine, histidine, and methionine (Pierpoint, 1983; Barbeau and Kinsella, 1983). The presence of polyphenol oxidase activity reduced the relative amounts of these amines. When the relative mole percent of amino acids for each protein treatment was taken into account, only methionine, lysine, and histidine were negatively affected by polyphenol oxidase activity (Figure 2). Methionine was reduced by 26%, lysine by nearly 16%, and histidine by 8% at pH 9.7 in the presence of polyphenol oxidase activity. At pH 8.0, lysine was reduced by 11.7%, histidine by 8%, and methionine apparently was unaffected by oxidative activity. However, despite the greater relative loss in methionine, histidine, and lysine at pH 9.7, a greater absolute amount of these amino acids was soluble at the higher pH. Our amino acid analyses do not take into account potential loss of methionine to methionine sulfoxide formation via chlorogenoquinone-mediated oxidation (Igarashi and Yasui, 1985).

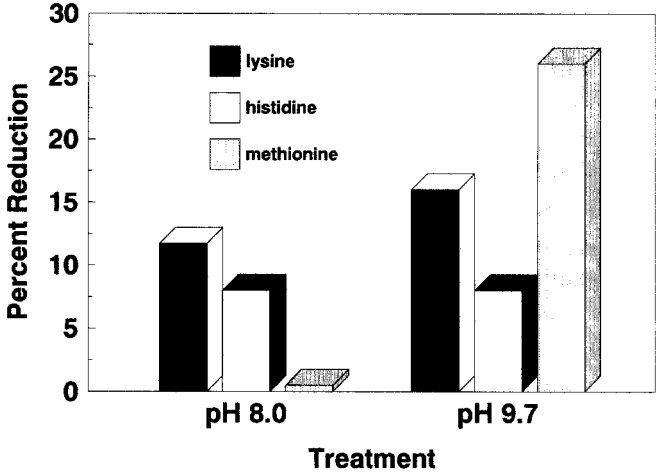


FIG. 2. The effect of polyphenol oxidase activity on the loss of lysine, histidine, and methionine in tomato foliage. Reductions are based upon comparison of molar ratios of amino acids between treatments with and without polyphenol oxidase activity. Values are based upon single analyses of pooled replicates.

The addition of the inhibitor, diethyldithiocarbamate, abolished polyphenol oxidase activity and reduced peroxidase activity by greater than 85% at both pH levels (Table 2). The activity of both enzymes was markedly reduced by the higher pH, with nearly 35% and 65% reductions in polyphenol oxidase and peroxidase, respectively.

When leaf powder was extracted at neutral and at alkaline pH levels, significantly more protein was soluble at alkaline pH (Figure 3; $r = 0.880$, $P < 0.01$). The relationship between pH of extraction buffer and percent protein was significantly correlated, and the slope of the regression line was significantly greater than zero ($P < 0.01$). Nearly 25% more soluble protein on a wet weight basis was observed at pH 10.0 compared to pH 7.0. Furthermore, in the bell pepper plant, *Capsicum annuum*, another solanaceous plant host of *M. sexta*, 45% more soluble foliar protein was obtained at the higher pH (unpublished data).

Effect of Alkalinity and Tomato Polyphenol Oxidase Activity on Binding of Chlorogenic Acid to Leaf Protein. Significantly more chlorogenic acid was bound to protein in the presence of polyphenol oxidase (Figure 4). In treatments containing polyphenol oxidase activity, pH had little effect on the amount of chlorogenic acid bound to protein (58.9 vs. 64.3 nmol/mg protein for pH 8.0 vs. 9.7, respectively). In the absence of polyphenol oxidase activity, pH significantly affected binding with nearly 60% more chlorogenic acid bound per milligram of protein at the higher pH of 9.7.

TABLE 2. EFFECTS OF pH AND DIETHYLDITHIOCARBAMIC ACID ON ACTIVITY OF FOLIAR OXIDASES

Treatment	PPO activity[a]	POD activity[b]
8.0	100	100
8.0 + inhibitor[c]	0	15.0
9.7	65.5	33.8
9.7 + inhibitor	0	4.1

[a] PPO activity = relative polyphenol oxidase activity measured with chlorogenic acid as substrate.
[b] POD activity = relative peroxidase activity measured with guaiacol and hydrogen peroxide.
[c] Inhibitor = diethyldithiocarbamic acid at 2 mg/ml buffer.

Determination of Binding of Chlorogenic Acid to Protein in Insect Gut. Nearly 39% of the excreted [^{3}H]chlorogenic acid was bound to protein when larvae were fed [^{3}H]chlorogenic acid-treated leaflets (Figure 5). Thin-layer chromatographic analyses of aqueous aliquots of the protein precipitate also indicated that chlorogenic acid was bound to protein because greater than 90% of the radioactivity was associated with a single band (Rf = 0.85–0.95) that was both phenolic and amine positive (Felton et al., 1989a).

A relatively large amount (ca. 14%) of the ingested radioactivity was retained in the hemolymph 24 hr following ingestion of the treated leaflet (Figure 5). Two to three times as much radioactivity was found in the hemolymph of *M. sexta* compared to *S. exigua* and *H. zea* larvae (Felton et al., 1989a). Radioactivity recovered in feces and hemolymph accounted for ca. 64% of the ingested radioactivity.

Effect of Surfactant on Foliar Oxidases and Protein Solubility. The addition of the gut surfactant lysolecithin significantly decreased polyphenol oxidase activity (Figure 6).At a concentration of 0.04% lysolecithin, the activity of polyphenol oxidase was reduced by 24%. This concentration of lysolecithin corresponds to a critical micelle concentration equal to that reported in the midgut fluids of *M. sexta* larvae (Martin and Martin, 1984). Peroxidase activity was not affected at the surfactant concentrations tested (data not shown).

The addition of 0.04% lysolecithin to the extraction buffer enhanced the solubility and/or extractability of leaf protein (Table 3). Approximately 9% more soluble protein occurred in the treatment with 0.04% lysolecithin.

DISCUSSION

Alkalinity and detergency have been proposed as adaptations to avoid the antidigestive effects of tannins (Feeny, 1970; Berenbaum, 1980; Martin et al., 1987). Alkalinity reduces hydrogen bonding of tannins with proteins, thus pre-

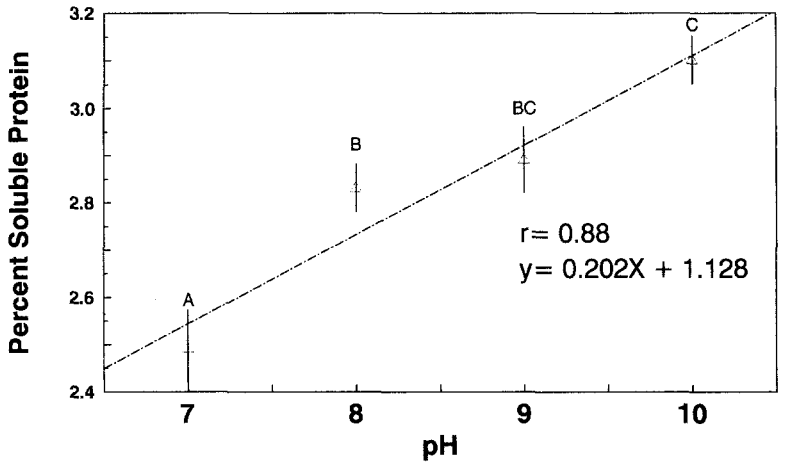


FIG. 3. Effect of pH on the extraction and solubility of tomato leaf protein. Each point represents the mean of three determinations. Error bars represent 95% confidence limits. Points not followed by the same letter are significantly different at $P < 0.05$.

venting protein precipitation (Martin and Martin, 1983; Pierpoint, 1983; Zucker, 1983; Haslam, 1988). Surfactants such as lysolecithin are highly effective at inhibiting the formation of insoluble tannin–protein complexes and appear to be widely present in insect midguts (Martin and Martin, 1984; Martin et al., 1987; Blytt et al., 1988). However, many lepidopteran larvae possess strongly alkaline, detergent gut fluids despite the apparent absence of tannins from their host plants (e.g., *M. sexta*; Martin et al., 1987). In these instances, it cannot be argued that alkalinity or detergency are adaptations for circumventing the antidigestive effects of tannins. We do not question the importance of alkalinity and detergency as adaptive factors mitigating tannin toxicity. These properties may play a significant role in enhancing the solubilization of dietary protein in the digestive tract. This is especially important considering that leaves are considered a protein-limited food source for most insect herbivores (White, 1978; Mattson, 1980; Brodbeck and Strong, 1987).

The plant biochemistry literature is replete with examples that support our findings that alkalinity enhances solubility of leaf nitrogen (e.g., Singer et al., 1952; Betschart and Kinsella, 1973). Leaf protein is generally more soluble and chloroplasts are more effectively disrupted at higher pH than at acidic or neutral pH (Betschart and Kinsella, 1973; Jones et al., 1989). Moreover, alkali extraction is essential for solubilizing cytoplasmic proteins in tobacco leaves, resulting in 25–50% more protein than at neutral or acidic pH (Singer et al., 1952). We have observed that increased levels of dietary protein in *H. zea* or *S. exigua* may substantially or completely alleviate the growth inhibition caused by the

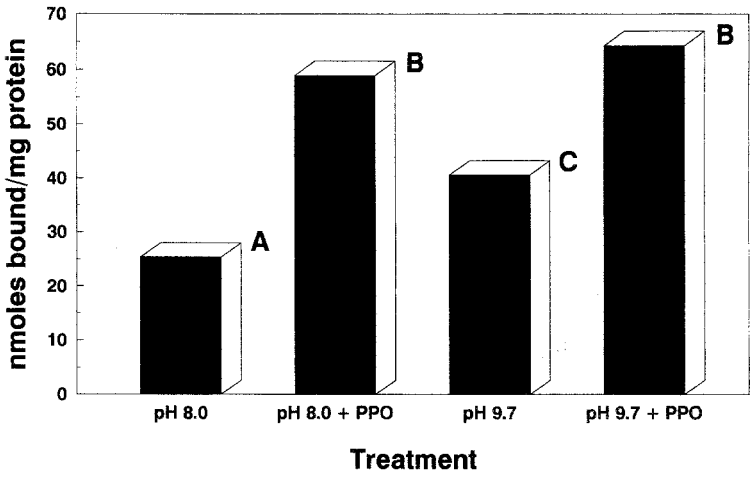


FIG. 4. Effect of pH and polyphenol oxidase activity on the binding of chlorogenic acid to ribulose-1,5-bisphosphate carboxylase/oxygenase. Each bar represents the mean of three determinations. Bars not followed by the same letter are significantly different at $P < 0.05$. PPO = polyphenol oxidase activity.

binding of chlorogenoquinone to protein (Duffey and Felton, 1989; Felton et al., 1989b, unpublished data). Moreover, the nutritional quality of protein extracted at alkaline pH (8.0–12.0) is often superior to protein extracted at neutral pH (Woodham, 1983).

Polyphenol oxidase activity is markedly reduced at alkaline pH from many solanaceous host plants of *M. sexta*, e.g., tomato, bell pepper, eggplant, and potato (see Table 1) (Felton et al., 1989a; Fujita and Tono, 1988; Sharma and Ali, 1980; Kang et al., 1983). Although the enzyme is active across a broad pH spectrum in many plant species, the pH optimum for most species is in the acidic to neutral range (i.e., 5.0–7.0; Mayer and Harel, 1979). It is noteworthy that none of the major lepidopteran herbivores of the tomato plant have a gut pH near the optimum of ca. 7.0 for enzyme activity (Felton et al., 1989a). Thus, alkalinity may improve herbivore performance on marginal food sources by reducing the activities of polyphenol oxidase and enhancing the acquisition of greater amounts of dietary protein.

Although alkaline pH reduces polyphenol oxidase activity, the formation of chlorogenoquinone can occur nonenzymatically in basic conditions (Hurrell et al., 1982; Barbeau and Kinsella, 1983, 1985; Cilliers and Singleton, 1989). However, Hurrell et al. (1982) found that significant nutritional damage to protein by nonenzymatically produced chlorogenoquinone was only observable under conditions of continuous oxygenation and stirring. Recently, Appel and Martin (1990) reported that the midgut of *M. sexta* larvae possessed strong

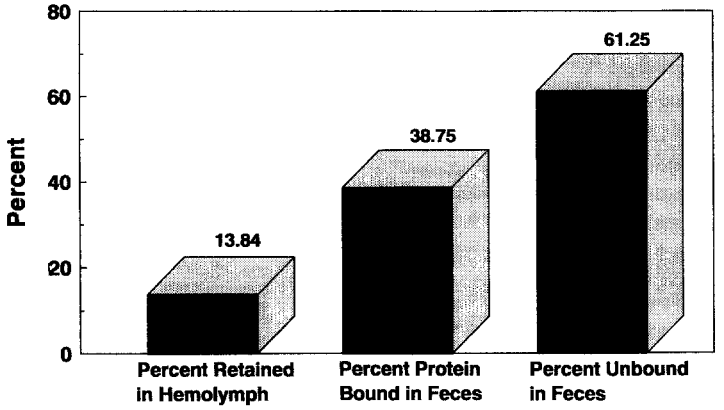


FIG. 5. The uptake and excretion of [^{3}H]chlorogenic acid by fifth-instar *Manduca sexta* larvae.

reducing properties (redox potential = −136 mV) and a slightly anaerobic environment, which may further minimize the antinutritive effects induced by the oxidation of phenolics such as chlorogenic acid. However, whether a specific quinone species will be reduced depends upon the difference in redox potentials of the midgut and the quinone. Also, it must be reiterated that once the quinones are formed and bound to nucleophilic portions of amino acids, changes in redox potentials will not disrupt the irreversible covalent bonds that form. The fore- and hindguts of *M. sexta* were reported to be oxidizing, and it is possible that much of the binding that we observed took place in these regions of the gut. Binding occurring in the foregut will still impair the utilization of amino acids. Furthermore, the activity of tomato polyphenol oxidase is so great that it is likely much of the phenolic binding to protein occurs in the initial stages of ingestion. The midgut reducing conditions may still serve to minimize nonenzymatic oxidations of other phenols.

The amount of chlorogenic acid bound to excreted protein by *M. sexta* (i.e., 38.8%; Figure 4) was similar to that observed in *S. exigua* and *H. zea* (38% and 49%, respectively, Felton et al., 1989a). In the case of *M. sexta*, the antinutritive effect of quinone binding may be mitigated by the increased levels of soluble amino acids occurring at the higher midgut pH (Figure 1, Table 1). Our estimates on protein solubility in the digestive system of *M. sexta* may be quite conservative because the midgut pH has been reported as high as 11.3 (Dow, 1984).

The upper limit in gut pH is restricted by the energetic costs required for maintaining a high pH and the negative consequences of high pH on protein quality and enzyme function. At pH levels of 12 or more, protein nutritional

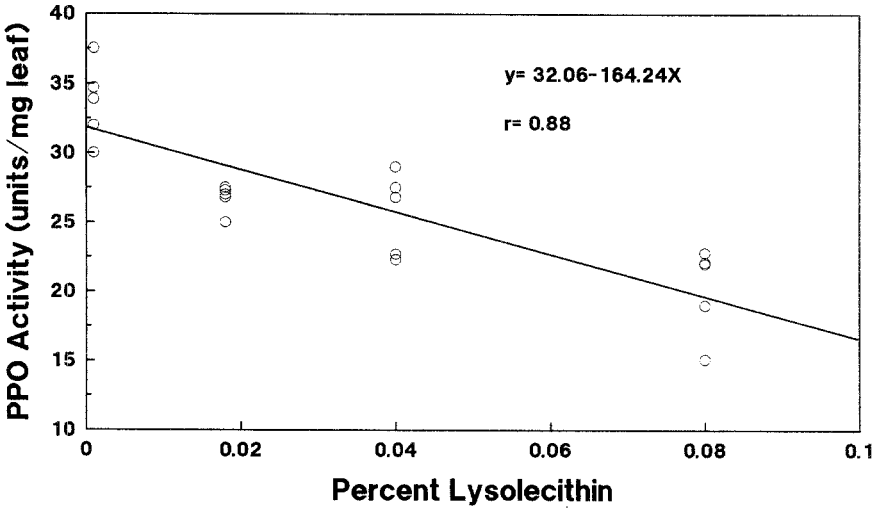


FIG. 6. Effect of the surfactant, lysolecithin, on tomato foliar polyphenol oxidase activity. Each point represents the mean of one replicate performed in triplicate. PPO = polyphenol oxidase activity.

quality is degraded as a consequence of amino acid racemization, thiol oxidation, and formation of toxic cross-linked amines such as lysinoalanine (Friedman, 1982; Pierpoint, 1983; Whitaker and Feeney, 1983; Finot, 1983; Hurrell and Finot, 1985). At such a high pH, digestive enzymes would likely denature and be unable to function. These restrictions, therefore, would partially determine the upper limit for the evolution of gut pH.

The surfactant lysolecithin also has the ability to enhance protein solubility (Table 3) and diminish polyphenol oxidase activity (Figure 6). The reduction in polyphenol oxidase activity may be due to protein denaturation or conformational changes. Certain detergents are known to inactivate polyphenol oxidase activity (Meyer and Biehl, 1981). The ability of surfactants to enhance extraction of leaf protein is due to the disruption of chloroplasts and other protein bodies (Betschart and Kinsella, 1973). Lysolecithin also has been shown to solubilize membrane-bound protein and phytosterols in plant cells (Sandstrom and Cleland, 1989). In addition, surfactants may play an important role in protein digestion by denaturing substrate protein and exposing more binding sites for proteolytic enzymes (Mole and Waterman, 1985).

In conclusion, we concur with the theory that the alkalinity and detergency of midgut fluids of lepidopteran larvae represent adaptations to leaf-feeding. Whereas previous investigations have advocated these properties as adaptations to avoid toxicity from tannin–protein complexes (Feeny, 1970; Martin and Mar-

TABLE 3. EFFECT OF LYSOLECITHIN ON PROTEIN EXTRACTION

Treatment	Protein (%)[a]	95% confidence limit
Control	2.35	2.29-2.42
0.04% lysolecithin	2.59	2.51-2.66

[a] Percent protein expressed as dry weight percent.

tin, 1984; Martin et al., 1987), we contend that they may have other significant functions for folivores. Both detergency and alkalinity were shown to reduce foliar polyphenol oxidase activity and enhance the extraction and/or solubility of leaf proteins. Moreover, surfactants may aid in the digestion of protein and in the acquisition of other key nutrients such as sterols (Sandstrom and Cleland, 1989). Gut alkalinity also may be important for extracting hemicelluloses of cell walls (Terra, 1988). Thus, gut alkalinity and detergency may represent important nutritional adaptations for facilitating the uptake of essential or limiting nutrients from host plants.

Acknowledgments—Research was supported by USDA competitive grants awarded to S.S.D. (87-CRCR-1-2371) and S.S.D. and G.W.F. (89-37250-4639). The authors wish to thank Dr. D.T. Johnson and Mr. J. Workman for critical review of the manuscript. We also thank Dr. M.M. Martin for kindly providing an unpublished manuscript by Appel and Martin. This manuscript was published with the approval of the Director of the Arkansas Experiment Station.

REFERENCES

APPEL, H.M. and MARTIN, M.M. 1990. Gut redox conditions in herbivorous lepidopteran larvae. *J. Chem. Ecol.* 16:3277-3290.

BARBEAU, W.E., and KINSELLA, J.E. 1983. Factors affecting the binding of chlorogenic acid to fraction 1 leaf protein. *J. Agric. Food Chem.* 31:993-998.

BARBEAU, W.E., and KINSELLA, J.E. 1985. Effects of free and bound chlorogenic acid on the in vitro digestibility of ribulose bisphosphate carboxylase from spinach. *J. Food Sci.* 50:1083-1100.

BERENBAUM, M. 1980. Adaptive significance of midgut pH in larval lepidoptera. *Am. Nat.* 115:138-146.

BETSCHART, A., and KINSELLA, J.E. 1973. Extractability and solubility of leaf protein. *J. Agric. Food Chem.* 21:60-64.

BLYTT, H.J., GUSCAR, T.K., and BUTLER, L.G. 1988. Antinutritional effects and ecological significance of dietary condensed tannins may not be due to binding and inhibiting digestive enzymes. *J. Chem. Ecol.* 14:1455-1465.

BROADWAY, R.M., DUFFEY, S.S., PEARCE, G., and RYAN, C.A. 1986. Plant proteinase inhibitors: A defence against herbivorous insects? *Entomol. Exp. Appl.* 24:94-100.

BRODBECK, B.V., and STRONG, D.R. 1987. Amino acid nutrition of herbivorous insects and stress to host plants, pp. 347-364, *in* P. Barbosa and J.C. Schultz (eds.). Insect Outbreaks. Academic Press, New York.

CHIPPENDALE, G.M. 1970. Metamorphic changes in fat body proteins of the southwestern corn borer *Diatraea grandiosella*. *J. Insect Physiol.* 16:1057–1068.

CILLIERS, J.J.L., and SINGLETON, V.L. 1989. Nonenzymic autoxidative phenolic browning reactions in a caffeic acid model system. *J. Agric. Food Chem.* 37:890–896.

DOW, J.A.T. 1984. Extremely high pH in biological systems: a model for carbonate transport. *Am. J. Physiol.* 246R:633–635.

DUFFEY, S.S., and FELTON, G.W. 1989. Role of plant enzymes in resistance to insects, pp. 289–313, *in* J. Whitaker and P. Sonnet (eds.). Biocatalysis in Agricultural Biotechnology. American Chemical Society, Washington, D.C.

FEENY, P.P. 1970. Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* 51:561–581.

FELTON, G.W., and DUFFEY, S.S. 1990. Inactivation of a baculovirus by quinones formed in insect-damaged plant tissues. *J. Chem. Ecol.* 16:1221–1236.

FELTON, G.W., DONATO, K., DEL VECCHIO, R.J., and DUFFEY, S.S. 1989a. Activation of plant foliar oxidases by insect feeding reduces the nutritive quality of foliage for noctuid herbivores. *J. Chem. Ecol.* 15:2667–2694.

FELTON, G.W., BROADWAY, R.M., and DUFFEY, S.S. 1989b. Inactivation of protease inhibitors by plant-derived quinones: Complications for host-plant resistance against noctuid herbivores. *J. Insect Physiol.* 35:981–990.

FINOT, P.A. 1983. Influence of processing on the nutritional value of proteins. *Qual. Plant Foods Hum. Nutr.* 32:439–453.

FRIEDMAN, M. 1982. Lysinoalanine formation in soybean proteins: Kinetics and mechanisms, pp. 231–273, *in* Food Protein Deterioration. Mechanisms and Functionality. J.P. Cherry (ed.). American Chemical Society, Washington, D.C.

FUJITA, S., and TONO, T. 1988. Purification and some properties of polyphenol oxidase in eggplant (*Solanum melongela*). *J. Sci. Food Agric.* 46:115–123.

GENTILE, I.A., FERRARIS, L., and MATTA, A. 1988. Variations of polyphenoloxidase activities as a consequence of stresses that induce resistance to *Fusarium* wilt of tomato. *J. Phytopathol.* 122:45–53.

HARE, J.D. 1983. Manipulation of host suitability for herbivore pest management, pp. 655–680, *in* R.F. Denno and M.S. McClure (eds.). Variable Plants and Herbivores in Natural and Managed Systems. Academic Press, New York.

HASLAM, E. 1988. Plant polyphenols (syn. vegetable tannins) and chemical defense—a reappraisal. *J. Chem. Ecol.* 14:1789–1805.

HURRELL, R.F., and FINOT, P.A. 1985. Effects of food processing on protein digestibility and amino acid availability, pp. 233–246, *in* J.W. Finley and D.T. Hopkins (eds.). Digestibility and Amino Acid Availability in Cereals and Oilseeds. American Association of Cereal Chemists, St. Paul, Minnesota.

HURRELL, R.F., FINOT, P.A., and CUQ, J.L. 1982. Protein-polyphenol reactions. 1. Nutritional and metabolic consequences of the reaction between oxidized caffeic acid and the lysine residues of casein. *J. Nutr.* 47:191–211.

IGARASHI, K., and YASUI, T. 1985. Oxidation of free methionine and methionine residues in protein involved in the browning reaction of phenolic compounds. *Agric. Biol. Chem.* 49:2309–2315.

ISMAN, M.B., and DUFFEY, S.S. 1983. Pharmacokinetics of chlorogenic acid and rutin in larvae of *Heliothis zea*. *J. Insect Physiol.* 29:295–300.

JONES, C.G., HARE, J.D., and COMPTON, S.J. 1989. Measuring plant protein with the Bradford assay. 1. Evaluation and standard method. *J. Chem. Ecol.* 15:979–992.

KALYANARAMAN, B., PREMOVIC, P.I., and SEALY, R.C. 1987. Semiquinone anion radicals from the addition of amino acids, peptides, and proteins to quinones derived from the oxidation of catechols and catecholamines. *J. Biol. Chem.* 262:11080–11087.

KANG, K., CHOO, Y., and KIM, K. 1983. Enzymatic discoloration of raw potato tubers: With special reference to the formation and inhibition of dopachrome. *Am. Potato J.* 60:451–460.

LANGE, W.H., and BRONSON, L. 1981. Insect pests of tomato. *Annu. Rev. Entomol.* 26:345–371.

MARTIN, J.S., and MARTIN, M.M. 1983. Tannin assays in ecological studies: Precipitation of ribulose-1,5-bisphosphate carboxylase/oxygenase by tannic acid, quebracho, and oak foliage extracts. *J. Chem. Ecol.* 9:285–294.

MARTIN, J.S., MARTIN, M.M., and BERNAYS, E.A. 1987. Failure of tannic acid to inhibit digestion or reduce digestibility of plant protein in gut fluids of insect herbivores. *J. Chem. Ecol.* 13:605–621.

MARTIN, M.M., and MARTIN, J.S. 1984. Surfactants: Their role in preventing the precipitation of proteins by tannins in insect guts. *Oecologia* 61:342–345.

MATTSON, W.J. 1980. Herbivory in relation to nitrogen content. *Annu. Rev. Ecol. Syst.* 11:119–161.

MAYER, A.M. 1987. Polyphenol oxidases in plants-recent progress. *Phytochemistry* 26:11–20.

MAYER, A.M., and HAREL, E. 1979. Polyphenol oxidases in plants. *Phytochemistry* 18:193–215.

MEYER, H.-U., and BIEHL, B. 1981. Activation of latent phenolase during spinach leaf senescence. *Phytochemistry* 20:955–959.

MOLE, S., and WATERMAN, P.G. 1985. Stimulatory effects of tannins and cholic acid on tryptic hydrolysis of proteins: Ecological implications. *J. Chem. Ecol.* 11:1323–1332.

PIERPOINT, W.S. 1983. Reactions of phenolic compounds with proteins, and their relevance to the production of leaf protein, pp. 235–267, *in* L. Telek and H.D. Graham, (eds.). Leaf Protein Concentrates. Avi Publ. Westport, Connecticut.

ROCK, G.C., and HODGSON, E. 1971. Dietary amino acid requirements for *Heliothis zea* determined by dietary deletion and radiometric techniques. *J. Insect Physiol.* 17:1087–1097.

RYAN, J.D., GREGORY, P., and TINGEY, W.M. 1982. Phenolic oxidase activities in glandular trichomes of *Solanum berthaultii*. *Phytochemistry* 21:1885–1887.

SANDSTROM, R.P., and CLELAND, R.E. 1989. Selective delipidation of plasma membrane by surfactants. *Plant Physiol.* 90:1524–1531.

SHARMA, R.C., and ALI, R. 1980. Isolation and characterization of catechol oxidase from *Solanum melongena*. *Phytochemistry* 19:1597–1600.

SINGER, S.J., EGGMAN, L., CAMPBELL, J.M., and WILDMAN, S.G. 1952. The proteins of green leaves. IV. A high molecular weight protein comprising a large part of the cytoplasmic proteins. *J. Biol. Chem.* 197:233–239.

TERRA, W.R. 1988. Physiology and biochemistry of insect digestion: An evolutionary perspective. *Braz. J. Med. Biol. Res.* 21:675–734.

WHITAKER, J.R., and FEENEY, R.E. 1983. Chemical and physical modification of proteins by the hydroxide ion. *CRC Crit. Rev. Food Sci. Nutr.* 19:173–212.

WHITE, T.C.R. 1978. The importance of a relative shortage of food in animal ecology. *Oecologia* 33:71–86.

WOODHAM, A.A. 1983. The nutritional evaluation of leaf protein concentrates, pp. 415–433, *in* L. Telek and H.D. Graham (eds.). Leaf Protein Concentrates. Avi Publ., Westport, Connecticut.

ZUCKER, W.V. 1983. Tannins: does structure determine function? An ecological perspective. *Am. Nat.* 121:335–365.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

EFFECT OF SOME LEAF ESSENTIAL OIL PHENOTYPES FROM COASTAL REDWOOD ON GROWTH OF ITS PREDOMINANT ENDOPHYTIC FUNGUS, *Pleuroplaconema* sp.

FRANCISCO J. ESPINOSA-GARCÍA[1] and JEAN H. LANGENHEIM*

Department of Biology
University of California
Santa Cruz, California 95064

(Received February 8, 1991; accepted May 15, 1991)

Abstract—Experiments were performed to assess the effect of four foliar essential oil phenotypes from a coastal redwood (*Sequoia sempervirens*) population on isolates of *Pleuroplaconema* sp., its ubiquitous endophytic fungus. Isolates were exposed to essential oils extracted from their trees of origin and from other trees. The hypotheses tested were: (1) redwood leaf essential oils extracted from distinct trees would have a differential effect on *Pleuroplaconema* sp. growth, and (2) growth of isolates from a particular tree would be differentially affected when exposed to essential oil phenotypes different from that of their tree of origin. The essential oil phenotypes were differentially inhibitory, but the pattern of inhibition did not support the second hypothesis. *Pleuroplaconema* sp. showed low average tolerance to all of the essential oils; two phenotypes reduced growth 70–80% and the other two 50–60% at the dose tested. The overall growth response of individual isolates to all treatments suggests that more than one fungus genotype per tree was represented in the experiment. The variability in tolerance of individual isolates to the essential oils was low for three phenotypes. The low tolerance of *Pleuroplaconema* sp. to redwood essential oils, in spite of its predominance and specialization in this conifer, is discussed considering: (1) the possible pathogenic ancestry of this fungus, and (2) that essential oil phenotypes may be important in controlling the activity of *Pleuroplaconema* sp. after it colonizes the leaf.

Key Words—*Sequoia sempervirens, Pleuroplaconema* sp., essential oil

*To whom correspondence should be addressed.
[1] Present address: Centro de Ecología, Universidad Nacional Autónoma de México. Apartado Postal 70-275, 04510, México, D.F.

0098-0331/91/0900-1837$06.50/0

phenotypes, leaf fungus endophytic control, tolerance variability, terpenoids, latent infections, symbiosis.

INTRODUCTION

Volatile terpenoids occurring in complex mixtures, also known as essential oils, are ubiquitous in conifers. Studies on the effect of essential oils, or their components, on food-spoiling fungi or human pathogenic fungi frequently show the antifungus nature of these chemicals (e.g., Benjalili et al., 1982; Pauli and Knobloch, 1987). These compounds have been associated with differential susceptibility of some conifers to fungus pathogens (Rockwood, 1973, 1974; Thibault-Balesdent and Delatour, 1985) or to protective reactions after pathogen attack (Gibbs, 1968; Raffa and Berryman, 1982; Gambliel et al., 1985; Miller et al., 1986). Furthermore, many pathogenic or wood-inhabiting fungi may be inhibited by saturated atmospheres of conifer monoterpenes of their hosts (Cobb et al., 1968; Hintikka, 1970; De Groot, 1972; Väisälä, 1974; Flodin and Fries, 1978; Bridges, 1987; Ennos and Swales, 1988). On the other hand, some of these volatile terpenoids alone or in combination have neutral or even stimulatory effects on certain pathogenic fungi (Fries, 1973; Flodin and Fries, 1978; Franich et al., 1982; Bridges, 1987).

Terpenoid variability within and among populations of some plants tends to be multimodal, i.e., few or several terpenoid mixture phenotypes are distributed among many individuals (Langenheim and Stubblebine, 1983; Squillance et al., 1985; Leather et al., 1987). However, few studies have considered terpenoid phenotypes and fungi. Furthermore, most of these have: (1) included only one phenotype or a pooled sample of phenotypes and apparently one isolate per fungus species, or (2) various terpenoid phenotypes and only one fungus isolate (Cobb et al., 1968; Shrimpton and Whitney, 1968; Flodin and Fries, 1978; Franich et al., 1982; Arrhenius and Langenheim, 1983; Tripathi et al., 1985).

Fungi with endophytic stages colonizing aerial tissues are ubiquitous in conifers (Carroll, 1986; Petrini, 1986). They even have been proposed to be "as common among plants as are mycorrhizae" (Carroll, 1988). However, little is known about their relationships with terpenoid mixtures. Although some of these fungus symbionts can be latent pathogens, many fungi do not cause apparent harm to the plant in their endophytic stages and can be isolated from symptomless leaves. In this paper, the term "endophyte" is used to refer to these kinds of fungi.

Some endophytes antagonize herbivores and possibly pathogens of the plant that they inhabit (Clay et al., 1985; Carroll, 1986, 1988). For this reason mutualism has been proposed in some endophyte–plant systems (Carroll, 1988; Clay, 1988). These mutualisms have been postulated to have evolved from

plant–pathogen relationships, since most proven endophytic mutualists are close relatives of virulent pathogens and some otherwise harmless endophytes can cause disease when the plant is under stress (Carroll, 1988; Clay, 1988). Most leaf endophytes in conifers apparently remain inactive after colonizing the leaf, and active fungus growth and reproduction occur when the leaf senesces or is damaged (Carroll, 1986). The mechanisms by which this apparent inactivity is maintained and by which growth is resumed are not known, although the mechanisms discussed by Verhoeff (1974) for latent fungus pathogenic infections (i.e., control by secondary chemicals of the host, changes in the nutritional quality of the tissues or a combination of both factors) may be similar for all endophytes.

The purpose of this research was to explore the relationships between foliar essential oil phenotypes in a coastal redwood [*Sequoia sempervirens* (D. Don. ex Lamb.) Endl.] population and *Pleuroplaconema* sp., the most frequent and ubiquitous endophyte in redwood leaves (Rollinger and Langenheim, 1989; Espinosa-García and Langenheim, 1990). We tested specifically the hypotheses that (1) some of the frequent essential oil phenotypes in a redwood population would differentially affect *Pleuroplaconema* sp. growth; and (2) growth of isolates would be differentially affected when exposed to essential oil phenotypes different from that of their tree of origin.

Pleuroplaconema sp. [possible asexual state of *Chloroscypha chloromela* (Phil. and Hk.) Seaver, that is known only from coastal redwood (Petrini, 1982)], has never been associated with disease in this host. According to Carroll's criteria (1986), this is a fungus anticipated to form a mutualistic association with redwood. *Pleuroplaconema* sp. spores are apparently dispersed by water-splashing, which results in colonization of a great proportion of the redwood foliage (Rollinger and Langenheim, 1989; Espinosa-García and Langenheim, 1990). The fungus then appears to cease growth until the leaf senesces or dies.

Coastal redwood leaves have a relatively high amount of essential oils. Monoterpenes constitute 60–88% of the mixture, accounting for 0.1–6.6% of the leaf dry weight (Hall, 1985). The other components are sesquiterpenes, oxygenated terpenoids and, in trace amounts, allyl phenylethers (Gregonis et al., 1968; Hilal et al., 1977; Okamoto et al., 1981). Redwood leaf essential oil is variable within the population and developmentally variable within the individual, both in yield (i.e., total amount) as well as in compositional pattern (i.e., percentage of individual compounds relative to the total amount). The essential oil compositional pattern has been characterized by the predominant monoterpene fraction throughout its geographic distribution (Hall and Langenheim, 1987). Both this compositional pattern and yield change during the first six months of leaf development but then stabilize until the beginning of senescence (Hall and Langenheim, 1986b); The compositional pattern is constant

throughout the mature leaves from a tree, but the yield of the leaves on the uppermost part of the tree is higher than that of the other leaves (Hall and Langenheim, 1986a).

Five essential oil compositional patterns were recognized from a sample of 36 adult trees from a Santa Cruz population (Espinosa-García, 1991). Four trees, each representing a compositional pattern, which in this paper are called "essential oil phenotypes," were used in the experiment (Figure 1). Fourteen monoterpenes and one sesquiterpene are represented, although each of the four phenotypes was characterized by its own spectrum of five predominant monoterpenes (sabinene, limonene, β-phellandrene, γ-terpinene, and α-pinene) and the sesquiterpene caryophyllene. In three of the phenotypes (I, II, and IV), sabinene is the most abundant compound, followed by relatively similar but characteristically different amounts of limonene, γ-terpinene, β-phellandrene, and α-pinene. In phenotype III, however, β-phellandrene occurs in the highest amount with α-pinene, sabinene, and limonene being relatively similar in con-

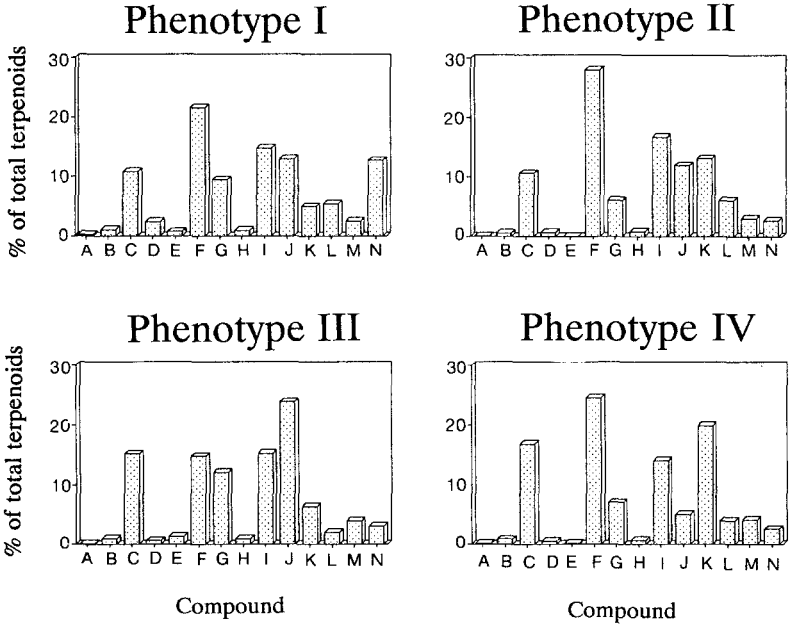


FIG. 1. Essential oil phenotypes used in the experiment. Bars represent percent of individual compounds relative to the total amount of monoterpenes plus caryophyllene. Each terpenoid profile was obtained from an individual representing an essential oil compositional pattern group. A, tricyclene; B, thujene; C, α-pinene; D, camphene; E, β-pinene; F, sabinene; G, myrcene; H, α-phellandrene; I, limonene; J, β-phellandrene; K, γ-terpinene; L, p-cymene; M, terpinolene; N, caryophyllene.

centration. The relatively high amount of caryophyllene also distinguishes phenotype I.

METHODS AND MATERIALS

Four trees with distinctive essential oil phenotypes were the source of both foliage for essential oil extraction and *Pleuroplaconema* sp. isolates. An additional tree from a compositional pattern not represented in the experiment was used as a source of isolates to have a better representation of the fungus population. The trees were part of a ca. 85-year-old second-growth forest dominated by redwood. The stand was apparently even-aged with no old-growth forest in the vicinity.

Essential Oil Extraction. Foliage from each tree was collected the second week of November 1989. Leaves 1–3 years old were detached from twigs and extracted by two methods. The first was a small-scale extraction where 2 g of leaves per tree were ground in pentane with tetradecane added as internal standard. This pentane extract was dried with sodium sulfate, concentrated to 0.1 ml under a mild nitrogen stream, and analyzed by gas chromatography (GC). The terpenoid profiles were compared with those obtained in a larger-scale extraction procedure. In this second extraction, 300–400 g of leaves per tree were soaked in pentane for five days at room temperature in the dark. Each extract from soaked leaves was fractionate-distilled, the residue saved, and the distillate returned to the jars where the soaked leaves remained. This procedure was repeated three times. Each time the residue was stored in amber bottles in the freezer. After the fourth distillation, distillate and liquid from cold traps were combined (ca. 1 liter), dried and concentrated to 50 ml under nitrogen. The concentrate was added to the previously collected residues, and this was dried and distilled to remove the solvent. Pentane at concentrations higher than 0.17 mg pentane/ml air are known to be inhibitory to the growth of some *Pleuroplaconema* sp. isolates (Espinosa-García, unpublished results). Complete removal of pentane was not possible without loss of some monoterpenes; therefore, lost monoterpenes were replaced with commercially prepared compounds (Aldrich, Co. and ICN Chemicals). The more than 95% purity of these chemicals was confirmed by GC. Because β-phellandrene was not available commercially, the remaining amount of this compound in the extracts was used as a base to adjust the relative proportions of the other monoterpenes determined from the small scale extraction. These reconstituted concentrates were analyzed with GC and their patterns were compared with those obtained with the small-scale method. No significant differences existed in the monoterpene fraction, and the pentane concentrations were below levels known to inhibit *Pleuroplaconema* sp. growth. Pentane and volatile terpenoids in the extracts were quan-

tified by adding 5 μl of 99% tetradecane to 60 μl of extract in a tightly closed minivial. After mixing thoroughly, an aliquot was injected and analyzed by GC.

Gas Chromatographic Analysis. A Perkin-Elmer Sigma 300 gas chromatograph with glass capillary column 0.9 mm ID and 86 m long coated with 12% Carbowax 20 on Chromosorb and flame ionization detector was used. Injections were split to a 40:1 ratio with He as carrier gas at a 2 ml/sec flux, with the following temperature program: 65°C held 5 min, 6°C/min increase up to 180°C and held for 10 min. Injector temperature was 210°C, and the detector temperature was 250°C. Monoterpenes and caryophyllene were identified by retention time and the peak enrichment method.

Fungus Isolates. Leaves 1–3 years old were collected from a lower canopy branch from each tree and placed separately on water–agar tubes following surface sterilization with serial immersion in 95% EtOH for 1 min, 65% household bleach for 10 min, and 35% EtOH for 30 sec twice. One *Pleuroplaconema* sp. isolate was taken from each leaf age class per tree to increase the probability of isolating different fungus genotypes. Because the isolates were not of single spore origin, they may have been multiclonal. A total of 15 isolates, three per tree, was used. The isolates were transferred and incubated at room temperature (ca. 20–25°C) in redwood broth agar [macerated redwood foliage extract (equivalent to 50 g foliage/liter), filtered through Whatman No. 1 paper, and autoclaved; filter-sterilized glucose (5 g/liter), ampicillin (100 mg/liter), and streptomycin (25 mg/liter), and autoclaved water agar (15 g/liter) were added]. These cultures were used as inoculum source for colonies that provided plugs from colony edges for tests.

Experimental Design and Statistical Analysis. The experiment had a nested factorial design of extract type by isolates (5 × 15) nested by isolation origin. All *Pleuroplaconema* sp. isolates were exposed to the essential oil from all extract types. In the control treatment isolates grew with no volatiles. Isolate exposure to the treatments was replicated four times. Results were analyzed using analysis of variance (ANOVA) and means were compared with Duncan's multiple-range test. ANOVA was performed for the entire group of isolates as well as for isolates grouped by tree. Pearson correlation coefficients were calculated among isolate tolerance values to phenotypes:

$$\text{Tolerance} = \frac{\text{average growth exposed to volatiles}}{\text{average growth with no volatiles}}$$

Stepwise multiple regression was performed with: (1) growth in absence of essential oil and tolerance to all phenotypes, and (2) tolerance and concentration of terpenoids in the phenotypes (Table 1). Tolerance and the sum of the concentrations of sabinene and γ-terpinene were regressed because these monoterpenes inhibit the growth of *Pleuroplaconema* sp. in an additive fashion (Espinosa-García, 1991).

TABLE 1. CONCENTRATION (mg/vial) OF MOST MONOTERPENES, CARYOPHYLLENE, AND OTHER VOLATILE COMPOUNDS DELIVERED IN EXTRACTS FROM REDWOOD FOLIAGE USED IN EXPERIMENT

	Tree 1	Tree 2	Tree 3	Tree 4
Tricyclene	0.004	0.000	0.003	0.002
Thujene	0.029	0.014	0.026	0.025
α-Pinene	0.383	0.313	0.439	0.521
Camphene	0.079	0.019	0.016	0.017
β-Pinene	0.021	0.000	0.038	0.003
Sabinene	0.758	0.827	0.426	0.756
Myrcene	0.350	0.177	0.350	0.217
α-Phellandrene	0.033	0.021	0.025	0.020
Limonene	0.529	0.492	0.442	0.437
β-Phellandrene	0.479	0.351	0.686	0.154
γ-Terpinene	0.192	0.386	0.181	0.615
p-Cymene	0.183	0.177	0.056	0.121
Terpinolene	0.096	0.088	0.114	0.125
Caryophyllene	0.462	0.076	0.086	0.079
Monoterpene total	3.137	2.865	2.801	3.013
Other compounds[a]	1.937	2.138	2.193	1.984

[a] Total of sesquiterpenes, oxygenated terpenoids and other minor components.

Statistical analyses were carried out using the SAS statistical package, version 5 (SAS Institute Inc., Cary, North Carolina).

Procedure. Plugs (4 mm diameter) of actively growing colonies were inoculated into 7-ml glass vials with ca. 1 ml of medium that solidified when the vial was horizontal. A filter paper disk (7 mm diameter) then was put on the inside of the vial's screw cap, 10–20 μl of extract were dispensed on the paper, and the vial was immediately closed very tightly. When applicable, the vials received ca. 5.0 mg of volatile terpenoids (Table 1), enough to saturate the vial's atmosphere. Once the vials were closed, they were grouped according to essential oil phenotype and put in tightly closed 1-liter jars sealed with parafilm. Cultures were incubated at 25°C in the dark for 15 days. At the end of the incubation period, linear growth approximated to the nearest 0.5 mm was measured under a dissecting microscope.

RESULTS

All the essential oil phenotypes showed statistically significant inhibitory effects on growth (Table 2). Phenotype IV was the most inhibitory, followed closely by phenotypes II and III. The least inhibitory was phenotype I (Table 2).

TABLE 2. RESULTS FOR NESTED ANOVA WITH TREATMENTS (ESSENTIAL OIL PHENOTYPES AND CONTROL), ISOLATE GROUPS, AND ISOLATES NESTED BY TREE OF ORIGIN[a]

Variation source	*DF*	MS	*F*	*P*
Treatments (T)	4	0.2676	35.4	≤0.005
Isolate groups (G)	4	0.0075	0.6	NS
T × G	16	0.0031	1.3	NS

	Overall growth average (mm) for the treatments (standard error in parentheses)
Control	9.55a (±0.50)
Phenotype I	4.70b (±0.36)
Phenotype II	2.74c,d (±0.22)
Phenotype III	3.86b,c (±0.33)
Phenotype IV	2.02d (±0.18)

[a] ANOVA was performed with arcsin of the square root of percent growth of the control divided by 1000. Means followed by the same letter are not significantly different according to Duncan's multiple-range test with $\alpha = 0.05$.

Stepwise multiple regression between isolate tolerance and the concentration of compounds in the essential oils showed that γ-terpinene was the only compound sufficiently significant to be included in the regression model. This monoterpene had a negative correlation with tolerance ($R^2 = 0.33$ $F = 24.19$, $P < 0.0001$). According to the model, γ-terpinene alone explains one third of the variance in tolerance. However, the sum of the concentrations of sabinene and γ-terpinene was correlated with 56% of the growth reduction of *Pleuroplaconema* sp. (Figure 2). On the other hand, phenotype III produced a growth reduction higher than expected according to the sum of these monoterpenes; phenotype I, with more sabinene and γ-terpinene than phenotype III, was less inhibitory (Figure 2).

Isolates from different trees did not grow uniformly as a group when exposed to volatiles of the phenotypes (Table 3). Isolate groups 2 and 4 reacted similarly to phenotypes I and III. Isolates from trees 1, 3, and 5 were similarly inhibited by phenotypes II, III, and IV. Groups from trees 2 and 4 were most inhibited by phenotypes IV and II. The standardized average response (as per-

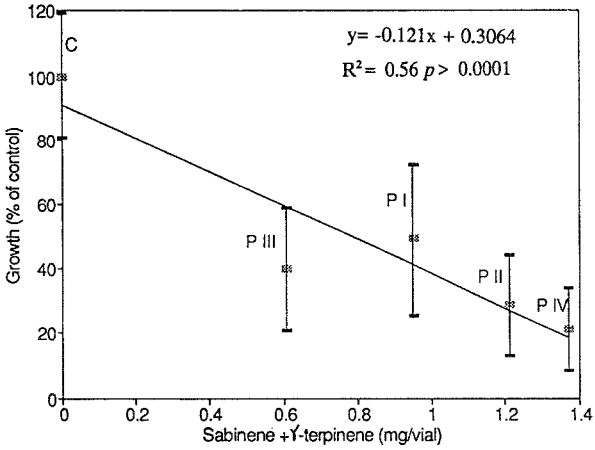


FIG. 2. Growth of *Pleuroplaconema* sp. isolates according to the sum of the concentrations of sabinene and γ-terpinene per vial. Bars represent standard deviations. C = control, P = phenotype. The line represents expected values obtained with the regression equation. The regression analysis was performed with arcsin of the square root of the percent growth of the control divided by 1000. $N = 276$.

cent of growth of control) shows that *Pleuroplaconema* sp. isolates grouped by origin vary relatively little in their strong inhibition response (Figure 3). Performance of the isolate groups exposed to volatiles of their original tree was not uniformly higher or lower compared to their performance with other phenotypes (Figure 3, Table 2). Contrary to our second hypothesis, all isolate groups were strongly inhibited when exposed to their original tree volatiles.

To determine the possibility that the isolates from each tree differed in growth rate both with and without essential oils, two-way ANOVAs were performed with the isolates grouped by origin in relation to their response to the essential oil phenotypes (Table 4). In all cases the phenotypes had a highly significant effect on the growth of *Pleuroplaconema* sp. Within each tree, all isolates (except the ones from tree 3) had a statistically significant different growth rate in the control and essential oil phenotype treatments combined (Table 5). Furthermore, the variance explained by isolates and the interaction term isolate by treatment in the ANOVAs were significant (Table 4). The interaction term indicates that at least one isolate within each tree was reacting differently to the essential oils. Although the amount of variance of these interaction terms is not large, the possibility that various genotypes of *Pleuroplaconema* sp. are producing this effect is high.

The relative tolerance of each *Pleuroplaconema* sp. isolate to the volatiles varied according to phenotype (Figure 4). This parameter was calculated by

TABLE 3. AVERAGE LINEAR GROWTH (mm) OF *Pleuroplaconema* sp. EXPOSED TO VOLATILES FROM FOUR REDWOOD PHENOTYPES[a]

Treatment	Isolate group origin				
	Tree 1	Tree 2	Tree 3[b]	Tree 4	Tree 5
Control	9.36a[c] (±0.93)	9.18a[c] (±1.45)	8.00a (±0.63)	11.75a (±1.07)	8.87a (±0.91)
Phenotype I	3.92b (±0.50)	6.08b[c] (±1.17)	3.25b (±1.65)	5.08b (±0.57)	4.67b (±0.76)
Phenotype II	1.83c (±0.22)	3.45c[c] (±0.61)	2.37b,c (±0.68)	3.21c (±0.34)	2.75c (±0.54)
Phenotype II	2.73c[c] (±0.52)	5.71b (±1.65)	2.87b,c (±0.31)	4.62b (±0.57)	2.96c (±0.48)
Phenotype IV	2.00c (±0.41)	1.50d (±0.28)	1.44c (±0.45)	2.92c (±0.84)	2.07c (±0.47)

[a] Means in each category come from 12 measurements belonging to three isolates. Means in columns with the same letter are not significantly different at $\alpha = 0.05$ according to Duncan's multiple-range test. Standard errors are in parentheses.
[b] Averages from eight measurements in each category belonging to two isolates.
[c] Averages of 11 measurements belonging to three isolates.

dividing growth in the presence of the volatiles from a phenotype by growth with no volatiles. The variabilities in response to volatiles from phenotypes IV and II were the most restricted, with tolerance values mostly below 0.5. Tolerances to volatiles from phenotypes III and I were more evenly spread, with some isolates scoring responses in the 0.71–0.8 category, i.e., a mild growth inhibition. Pearson correlation coefficients among isolate performances with each essential oil phenotype showed that tolerances to phenotypes I, II, and III are highly correlated (Table 6). However, tolerance to phenotype IV showed no statistically significant correlation with that of other phenotypes. Stepwise multiple regression between growth in absence of volatiles and tolerance to the essential oil phenotypes showed that tolerance to phenotypes alone or in combination was never significant in explaining growth without volatiles.

DISCUSSION

Results from this experiment agree with previous work, finding that volatile terpenoids, alone and in mixtures, generally inhibit fungus growth (Cobb et al., 1968; Shrimpton and Whitney, 1968; De Groot, 1972; Väisälä, 1974; Benjalili et al., 1984; Thibault-Balesdent and Delatour, 1985; Tripathi et al., 1985; Bridges, 1987; Ennos and Swales, 1988). The essential oil phenotypes

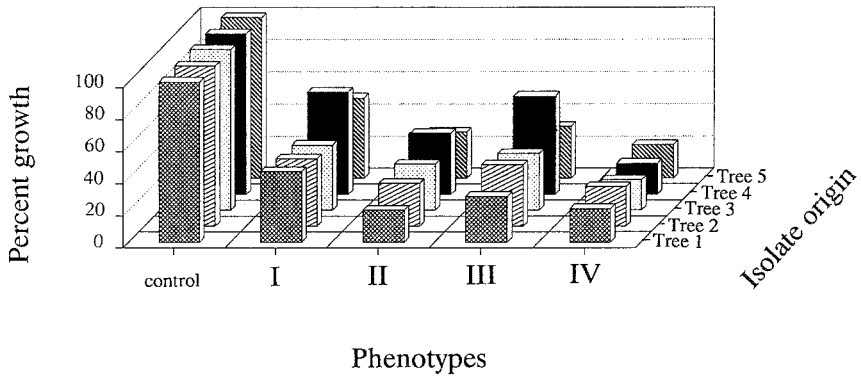


FIG. 3. Average growth response of groups of *Pleuroplaconema* sp. isolates to essential oil phenotypes from redwood. Isolates were grouped according to their tree of origin.

in this experiment have the same terpenoids, but some of them occurred in different concentrations. For example, the γ-terpinene concentration range in the phenotypes was sufficient to explain one third of the growth inhibition of *Pleuroplaconema* sp. in the multiple-regression analysis. Thus, this agrees with other results showing that growth inhibition intensity can be dose dependent (Väisälä, 1974; Flodin and Fries, 1978; Benjalili et al., 1984; Hall, 1985; Tripathi et al., 1985).

Two thirds of the variance in growth of *Pleuroplaconema* sp. were not explained by single components of the essential oils in the multiple regression analysis. This indicates that more than one terpenoid is important in affecting the endophyte or that other important components did not show sufficient variation to be detected by this analysis. In fact, the sum of the concentrations of sabinene and γ-terpinene correlated with the growth reduction of *Pleuroplaconema* sp.; the variance explained by the summed concentrations was almost twice as large as that explained by γ-terpinene alone. Fungi and other plant consumers generally encounter terpenoids in mixtures as in redwood. Single terpenoids assayed alone can be inhibitory, and some have been linked to resistance or susceptibility to fungus pathogens in conifers (Rockwood, 1974; Thibault-Balesdent and Delatour, 1985). In some cases, a single component of a terpenoid mixture may explain the growth inhibition of a fungus (Arrhenius and Langenheim, 1983). However, each of several terpenoids constituting such a mixture may frequently be able to inhibit the same fungus species (Cobb et al., 1968; De Groot, 1972; Flodin and Fries, 1978; Tripathi et al., 1985; Bridges, 1987). Thus, several compounds may be important when a fungus or other plant consumer encounters a terpenoid mixture, and synergistic, antagonistic, or additive effects of these compounds on consumers are possible (e.g., Chararas et al., 1982; Espinosa-García, 1991).

TABLE 4. RESULTS OF TWO-WAY ANOVA APPLIED TO *Pleuroplaconema* sp. GROWTH RESPONSE TO ESSENTIAL OILS FROM FOUR REDWOOD PHENOTYPES[a]

Source of variation	Isolates from tree 1			Isolates from tree 2			Isolates from tree 3			Isolates from tree 4			Isolates from tree 5		
	DF	Var %	*F*	*DF*	Var %	*F*	*DF*	Var %	*F*	*DF*	Var %	*F*	*DF*	Var %	*F*
Isolates (I)	2	12.4	30.9***[b]	2	36.8	77.2***	1	1.5	2.1 NS	2	9.8	23.0***	2	30.5	72.2***
EOP and C (E)	4	73.3	91.2***	4	37.6	37.3***	4	71.5	23.9***	4	77.1	90.5***	4	55.9	66.0***
I × E	8	9.3	5.8***	8	15.5	8.1***	4	4.5	1.5 NS	8	3.5	2.0[c]	8	3.9	2.3*
Residual	43	1.2		42	2.2		30	2.1		45	1.4		45	1.4	

[a] Analyses were performed grouping isolates by tree of origin. Var. % = percent of variance accounted by the source of variation. EOP and C = essential oil phenotypes and control.

[b] $*P < 0.05$; $***P < 0.0001$.

[c] $P < 0.06$; NS, nonsignificant.

TABLE 5. OVERALL LINEAR GROWTH RESPONSE (mm) TO ALL TREATMENTS OF *Pleuroplaconema* sp. ISOLATES GROUPED BY TREE OF ORIGIN[a]

Isolate	Tree of origin				
	1	2	3	4	5
A	5.39a[b] (±0.84)	8.40a (±1.15)	[c]	5.47b (±0.82)	2.39c (±0.58)
B	3.47b (±0.80)	4.37b[d] (±0.41)	3.25a (±0.54)	4.25c (±0.71)	3.67b (±0.45)
C	2.97c (±0.43)	2.25c[b] (±0.40)	3.95a (±0.66)	7.32a (±0.94)	6.72a (±0.76)

[a] Averages from 20 measurements per isolate. Means in a column followed by the same letter are not significantly different at $\alpha = 0.05$ according to Duncan's multiple-range test. Standard errors in parenthesis.
[b] Average from 18 measurements.
[c] Isolate lost to contamination.
[d] Average from 19 measurements.

The redwood essential oil phenotypes were characterized by combinations of four or five compounds with relatively similar concentrations. However, the greater inhibition by phenotypes IV and II may partially result from greater concentrations of sabinene and γ-terpinene here than in the other types. Both compounds were equally inhibitory to *Pleuroplaconema* sp. in single-compound trials, and mixtures of these compounds inhibited the fungus in an additive way (Espinosa-García, 1991). Although these two monoterpenes combined may explain 56% of the inhibition by the phenotypes, discordant results with phenotypes I and III and the 44% unexplained growth reduction indicate that more compounds are important in the *Pleuroplaconema* sp. inhibition. Phenotype III, with the lowest concentration of sabinene and γ-terpinene, produced stronger inhibition than did phenotype I, which had a higher concentration of these monoterpenes. Perhaps the high proportion of β-phellandrene or its combination with other monoterpenes in phenotype III is producing this effect. Thus, additive effects or synergism of the redwood essential oils components acting on the endophyte would seem to provide a reasonable explanation for our results.

Water dispersal makes host reinfection by the same *Pleuroplaconema* sp. strains highly probable, and its endophytic habit greatly increases the likelihood of contact with the host's leaf volatiles. For these reasons, we hypothesized that strains of the endophyte would be adapted to specific phenotypes. Adaptation to single hosts has been proposed for a pine canker fungus (Ennos and Swales, 1988) and pine leaf-scale insects (Edmunds and Alstad, 1978). Our results, however, do not support this hypothesis, because the isolates were strongly

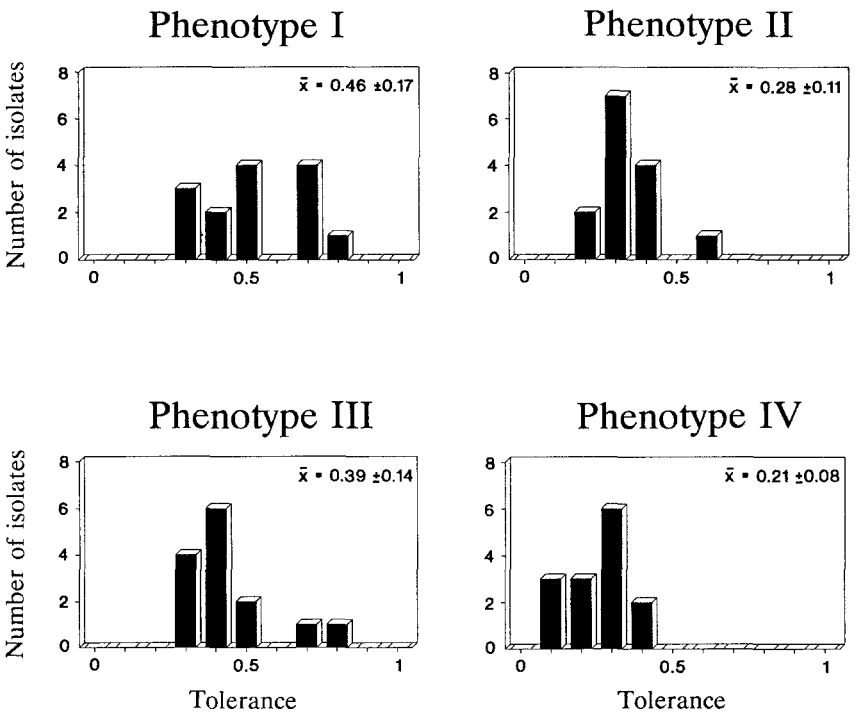


FIG. 4. Tolerance of individual isolates of *Pleuroplaconema* sp. to essential oil phenotypes from redwood foliage. Tolerance = growth with essential oil/growth without essential oil.

inhibited when exposed to the essential oil phenotypes from their original trees. In fact, the ubiquitous and abundant redwood symbiont showed a rather uniformly high susceptibility to its host's volatile terpenoids. This result is puzzling considering the possible pathogenic ancestry of *Pleuroplaconema* sp. and the fact that growth of some fungus pathogens is differentially affected (inhibited or stimulated) by the terpenoids of their hosts (Bridges, 1987). This variable response occurs in single strains of the pine canker pathogen *Crumenulopsis sororia*, which react differently to single monoterpenes of their host (Ennos and Swales, 1988). This further suggests that a host population that has individuals with different essential oil phenotypes will be "perceived" differently by fungi; some trees would be suitable for some fungus individuals whereas some others would be unsuitable. Moreover, variability in resistance traits (e.g., monoterpenes) and virulence traits (e.g., monoterpene tolerance) has been suggested to be common in plant–fungi pathosystems (Ennos and Swales, 1988). Therefore,

TABLE 6. PEARSON CORRELATION COEFFICIENTS (r) AMONG ISOLATE PERFORMANCES WITH EACH ESSENTIAL OIL PHENOTYPE (P)

	P I	P II	P III	P IV
P I		0.67[a]	0.57[b]	0.40 NS[d]
P II			0.74[c]	0.37 NS
P III				0.12 NS
P IV				

[a] $P < 0.01$.
[b] $P < 0.05$.
[c] $P < 0.005$.
[d] NS = nonsignificant.

Pleuroplaconema sp. may be "perceiving" redwood individuals as similar, thus reacting to essential oils as a nonpathogenic fungus.

The different overall reaction of the isolates from a tree to all treatments and the variance explained by the isolates alone and in combination with the treatments suggest that different genotypes of *Pleuroplaconema* were colonizing single branches of redwood. Carroll (1990) anticipated this kind of multiple simultaneous infection of the same host by different strains of the same endophyte that has been demonstrated for fungus pathogens (Day, 1980; Ennos and Swales, 1988).

Only a few examples have documented variability in susceptibility to terpenoids within populations of fungi. Ennos and Swales (1988) showed that *Crumenulopsis sororia* displays either a wide or narrow variability in tolerance to single monoterpenes of its host. This differential tolerance was heritable, and for some compounds heritability was very high. They thus suggested that these traits have adaptive values. Mean tolerance values to five monoterpenes in *C. sororia* were never below 0.55 and frequently above 0.65. The mean tolerance values of *Pleuroplaconema* sp. to the volatiles of the redwood phenotypes were always below 0.5 (Figure 4). The distribution of tolerance values to the phenotypes was skewed toward the high susceptibility side for three phenotypes. Tolerance to phenotype I was the only one with a wider range. If tolerance to terpenoids is heritable, with adaptive value in conifer pathogens, and *Pleuroplaconema* sp. has a pathogenic ancestry, we might speculate that tolerance to terpenoid mixtures would have been selected against in this redwood symbiont in its evolutionary history.

Pleuroplaconema sp. tolerance to three essential oil phenotypes was correlated, but growth performance with phenotype IV was not correlated with other phenotypes. Tolerance to monoterpenes in some pathogenic fungi has been suggested to be controlled by one or a few related mechanisms (Thibault-

Balesdent and Delatour, 1985; DraczynskaLusiak, 1987; Ennos and Swales, 1988). Analogously, *Pleuroplaconema* sp. might have a common tolerance mechanism in dealing with essential oils phenotypes, although some components or a particular combination in phenotype IV would require a different tolerance mechanism.

The absence of correlation between growth with no terpenoids and tolerance to the essential oil phenotypes probably indicates that this trait is not costly in terms of linear growth to *Pleuroplaconema* sp. This lack of association also has been shown for *Crumenulopsis sororia* and its host's monoterpenes (Ennos and Swales, 1988), but its significance is not clear to us.

Pleuroplaconema sp. shows a relatively high susceptibility to redwood volatile terpenoids when compared with other endophytic fungi. Fungus species that can have endophytic stages, but that are known to be pathogenic to redwood, are less inhibited by redwood essential oil phenotypes than *Pleuroplaconema* sp. (Espinosa-García, 1991). Low virulence in some pathogenic fungi has been associated with inability to break down host secondary compounds formed after fungus infection, i.e., phytoalexins (VanEtten et al., 1989). Although the phytoalexins in those pathosystems were phenolic compounds, a similar process might occur with either preformed terpenoids or those that are induced. The higher sensitivity of *Pleuroplaconema* sp. to redwood terpenoids may be associated with a process of increased susceptibility to the host secondary chemicals with concurrent pathogenicity attenuation. In this scenario, terpenoids must be defensive against pathogens, and selection against this tolerance in the fungus symbiont would be viable. Otherwise, the balance would be tilted toward the pathogenic side of the symbiosis. A possible relationship between increased specialization in endophytic lifestyle, with low or null pathogenic potential, and high susceptibility to the host secondary chemicals may be worth exploring in plant–endophyte systems where mutualism is known or suspected.

The sensitivity of *Pleuroplaconema* sp. to its host terpenoids also may be explained if these compounds are important in maintaining the apparent inactivity of the fungus after colonization. Krupa and Fries (1971) demonstrated that *Boletus variegatus*, an ectomycorrhizal fungus, induces high amounts of volatile terpenoids when it colonizes roots of *Pinus sylvestris*. This induction, as well as the antifungus properties of terpenoids, led these authors to propose a model in which host terpenoids, and other nonvolatile secondary compounds, would restrict the growth of the fungus symbiont and maintain the mutualistic state. The redwood–*Pleuroplaconema* sp. relationship might fit a similar model, but with preformed terpenoids as controlling agents.

To consider that *Pleuroplaconema* sp. activity is controlled by terpenoid volatiles would require that their concentrations in mature leaves would suppress fungus growth. This could be accomplished by the activity of the volatiles regardless of the leaf nutritional quality for the fungus or because a low nutri-

tional quality induces a higher susceptibility in the endophyte. Although the 5 mg of volatiles per vial used in the experiment were enough to initially saturate the vial's atmosphere (1.4–1.5 mg of a monoterpene are enough to saturate 6 ml of air), they did not completely halt the growth of any isolate. Experiments performed with the same incubation vials and a constantly maintained saturated atmosphere of sabinene or γ-terpinene completely stopped *Pleuroplaconema* sp. growth (Espinosa-García, 1991). Concentrations of these compounds, which were sufficient to saturate initially the vial atmosphere, did not halt the growth of this fungus. Hence, essential oil loss or inactivation probably prevented a stronger inhibition of *Pleuroplaconema* sp. in this experiment, and a saturated atmosphere that was maintained longer would completely halt the growth of this endophyte. The considerable emission of volatile terpenoids to the atmosphere by conifer foliage (e.g., Yokouchi and Ambe, 1984; Schindler and Kotzias, 1989) indicates that internal atmospheres of conifer leaves, such as redwood, are likely to be saturated with terpenoids. In spite of monoterpene loss from the redwood leaf, the total amount of monoterpenes and their profile apparently vary little after the leaf reaches maturity (Hall and Langenheim, 1986a). Thus, the internal atmospheres of redwood leaves are probably supplied constantly with volatile terpenoids escaping from the resin ducts, and these compounds are affecting *Pleuroplaconema* sp. The total amount of leaf redwood monoterpenes decreases by half in senescent leaves (Hall and Langenheim, 1986b). This drop could be attributed to the failure of the leaf to replace compounds lost to the atmosphere or possibly to some catabolism (Croteau, 1988; Njar et al., 1989). In this context, *Pleuroplaconema* sp. may be kept inactive by redwood's terpenoids at certain levels, but the fungus may be released when these decrease to lower amounts. This possibility would require a relatively uniform effectivity of the essential oil phenotypes against all genotypes of the fungus, which appears to be the case. This experiment simulated internal atmospheres of redwood leaves, but other conditions of living leaves are more difficult or impossible to manipulate. Although these factors within living leaves could influence the response of endophytes to essential oils, this experiment is the first approach that will lead to further investigations on the relationships between *Pleuroplaconema* sp. and redwood leaves.

The apparent inactivity of other endophytes within leaves may be maintained or influenced by secondary chemicals. *Colletotrichum gloesporioides*, the anthracnose fungus of citrus, infects a leaf and after a long period can colonize one cell or remain apparently inactive as a small intercellular hypha. The infections are asymptomatic unless leaves are detached or the plant is put under stress (Tokunaga and Ohira, 1973). *Pestalotia subcuticularis* also can form asymptomatic infections in attached leaves of *Hymenaea courbaril* seedlings and can be isolated from apparently healthy leaves. However, it readily produces necrotic spots in detached or senescent leaves and also can be found in a

pathogenic state attacking mature leaves (Nascimento, 1980; Fail and Langenheim, 1990). Furthermore, *P. subcuticularis* produced asymptomatic infections in greenhouse experiments with seedlings of *H. courbaril* and *Copaifera pubiflora*, but 12-year-old saplings developed visible symptoms (Arrhenius and Langenheim, 1986). Seedlings of these tropical tree genera typically show higher yields of sesquiterpenoid resin than their parent trees, and generally fungus attack is much heavier on leaves of parent trees than on the seedlings (Nascimento, 1980; Langenheim, unpublished results). These cases, as well as those documenting pathogenicity of endophytes when plants are under stress (Millar, 1980; Andrews et al., 1985) and where endophytes are activated after herbivore attack (Carroll, 1986) may indicate that plant endogenous factors, actively kept at certain levels by the plant, are controlling the activity of endophytic fungi. Although changes in nutritional quality of plant tissues to the fungi probably are important, secondary chemicals, such as essential oils tested in this experiment, are possibly one of the proximate and/or ultimate causes of the apparent inactivity in some endophytes.

Acknowledgments—We wish to thank Dr. G.C. Carroll, Dr. F. Cobb, Jr., Dr. G.R. Lazo, J. Rollinger, R. Goralka, Dr. S. Schwab, and two anonymous reviewers for their comments on the manuscript. We also appreciate E. García-Márquez's help during the setup of the experiment. We are also grateful to G. Hall and S. Wood for the help with statistical analysis and the extraction process of some essential oils, respectively. This work was partially funded by U.C.S.C. Faculty Research funds grant to J.H.L., and graduate student grants to F.J.E. from the U.C.S.C. Biology Department. F.J.E. wishes to thank the Universidad Nacional Autónoma de México and México's Consejo Nacional de Ciencia y Tecnología for supporting him with a fellowship during the period of this study.

REFERENCES

ANDREWS, J.H., HETCH, E.P., and BASHIRIAN, S. 1985. Association between the fungus *Acremonium curvulum* and Eurasian water milfoil, *Myriophyllum spicatum*. *Can. J. Bot.* 60:1216–1221.

ARRHENIUS, S.P., and LANGENHEIM, J.H. 1983. Inhibitory effects of *Hymenaea* and *Copaifera* leaf resins on the leaf fungus, *Pestalotia subcuticularis*. *Biochem. Syst. Ecol.* 11:361–366.

ARRHENIUS, S.P., and LANGENHEIM, J.H. 1986. The association of *Pestalotia* species with members of the leguminous tree genera *Hymenaea* and *Copaifera* in the neotropics. *Mycologia* 78:673–676.

BENJALILI, B., TANTAOUI-ELARAKI, A., AYADI, A., and IHLAL, M. 1984. Methods to study antimicrobial effects of essential oils: Application to the antifungal activity of six Moroccan essences. *J. Food Prot.* 47:748–742.

BRIDGES, J.R. 1987. Effects of terpenoids compounds on growth of symbiotic fungi associated with the southern pine beetle. *Phytopathology* 77:83–85.

CARROLL, G.C. 1986. The biology of endophytism in plants with particular reference to woody plants, pp. 205–222, *in* N. Fokkema and J. van den Heuvel (eds.). Microbiology of the Phyllosphere. Cambridge University Press, London.

CARROLL, G.C. 1988. Fungal endophytes in stems and leaves: From latent pathogen to mutualistic symbiont. *Ecology* 69:2–9.

CARROLL, G.C. 1990. Fungal associates of woody plants as insect-antagonists, *in* P. Barbosa, V.A. Krischik, and C.G. Jones (eds.). Mediation of Herbivore–Plant Interactions. John Wiley & Sons, New York. In press.

CHARARAS, C., REVOLON, C., FEINBERG, M., and DUCAUZE, C. 1982. Preference of certain Scolytidae for different conifers. A statistical approach. *J. Chem. Ecol.* 8:1093–1109.

CLAY, K. 1988. Clavicipitaceous fungal endophytes of grasses: Coevolution and the change from parasitism to mutualism, pp. 79–105, *in* K.A. Pirozinski and D.L. Hawksworth (eds.). Coevolution of Fungi with Plants and Animals. Academic Press, London.

CLAY, K., HARDY, T.N., and HAMMOND, A.M., JR. 1985. Fungal endophytes of grasses and their effects on an insect herbivore. *Oecologia* 66:1–5.

COBB, F.W., JR., KRSTIC, M., ZAVARIN, E., and BARBER, H.W. 1968. Inhibitory effects of volatile oleoresin components on *Fomes annosus* and four *Ceratocystis* species. *Phytopathology* 58:1327–1335.

CROTEAU, R. 1988. Catabolism of monoterpenes in essential oil plants, pp. 65–84, *in* B.M. Lawrence, B.D. Mookherjee, and B.J. Willis (eds.). Flavors and Fragrances: A World Perspective. Elsevier Science Publishers, Amsterdam.

DAY, A.W. 1980. Competition and distribution of genetically marked strains of *Ustilago violacea* in the same host plant. *Bot. Gaz.* 141:313–320.

DE GROOT, R.C. 1972. Growth of wood inhabiting fungi in saturated atmospheres of monoterpenoids. *Mycologia* 64:863–870.

DRACZYNSKALUSIAK, B. 1987. Oxidation of selected p-menthane derivatives by means of *Armillaria mellea* (honey fungus), a parasite of woodlands. *J. Basic Microbiol.* 27:191–196.

EDMUNDS, G.F., JR., and ALSTAD, D.N. 1978. Coevolution in insect herbivores and conifers. *Science* 199:941–945.

ENNOS, R.A., and SWALES, K.W. 1988. Genetic variation in tolerance to host monoterpenes in a population of the ascomycete canker pathogen *Crumenulopsis sororia*. *Plant Pathol.* 37:407–416.

ESPINOSA-GARCÍA, F.J. 1991. Studies of the leaf essential oils of coastal redwood (*Sequoia sempervirens*) in relation to its fungal endophytic community. PhD dissertation. University of California, Santa Cruz, California.

ESPINOSA-GARCÍA, F.J., and LANGENHEIM, J.H. 1990. The endophytic fungal community in leaves of a coastal redwood population—diversity and spatial patterns. *N. Phytol.* 116:89–97.

FAIL, G.L., and LANGENHEIM, J.H. 1990. Infection process of *Pestalotia subcuticularis* on leaves of *Hymenaea courbaril*. *Phytopathology* 80:1259–1265.

FLODIN, K., and FRIES, N. 1978. Studies on volatile compounds of *Pinus sylvestris* and their effect on wood decomposing fungi. II. Effects of some volatile compounds on fungal growth. *Eur. J. For. Pathol.* 8:300–310.

FRANICH, R.A., GASKIN, R.E., WELLS, L.G., and ZABKIEWICZ, J.A. 1982. Effect of *Pinus radiata* needle monoterpenes on spore germination and mycelial growth of *Dothiostroma pini* in vitro in relation to mature tree resistance. *Physiol. Plant Pathol.* 21:55–63.

FRIES, N. 1973. The growth promoting activity of terpenoids on wood decomposing fungi. *Eur. J. For. Pathol.* 3:169–180.

GAMBLIEL, H.A., CATES, R.G., CAFFEY-MOQUIN, M.K., and PAINE, T.D. 1985. Variation in the chemistry of loblolly pine in relation to infection by the blue-stain fungus, pp. 177–185, *in* S.J. Branham and R.C. Thatcher (eds.). Integrated Pest Management Research Symposium: The Proceedings. U.S. Department of Agriculture Forest Service Southern Forest Experiment Station General Technical Report SO-56.

GIBBS, J.N. 1968. Resin and resistance of conifers to *Fomes annosus*. *Ann. Bot.* 32:649–665.

GREGONIS, D.E., PORTWOOD, R.D., DAVIDSON, W.H., DURFEE, D.A., and LEVINSON, A.S. 1968. Volatile oils from foliage of coast redwood and big tree. *Phytochemistry* 7:975–981.

HALL, G.D. 1985. Leaf monoterpenes of coast redwood (*Sequoia sempervirens*). PhD dissertation. University of California, Santa Cruz, California.

HALL, G.D., and LANGENHEIM, J.H. 1986a. Within-tree spatial variation in the leaf monoterpenes of *Sequoia sempervirens*. *Biochem. Syst. Ecol.* 14:625–632.

HALL, G.D., and LANGENHEIM, J.H. 1986b. Temporal changes in the leaf monoterpenes of *Sequoia sempervirens*. *Biochem. Syst. Ecol.* 14:61–69.

HALL, G.D., and LANGENHEIM, J.H. 1987. Geographic variation in leaf monoterpenes of *Sequoia sempervirens*. *Biochem. Syst. Ecol.* 15:31–43.

HILAL, S.H., ZEDAN, H.H., HAGGUG, M.Y., and SOLIMAN, F.M. 1977. Volatile oil of *Sequoia sempervirens* Endl. *Egypt J. Pharm. Sci.* 18:85–95.

HINTIKKA, V. 1970. Selective effects of terpenes on wood decomposing Hymenomycetes. *Karstenia* 11:28–32.

KRUPA, S., and FRIES, N. 1971. Studies on ectomycorrhizae of pine. I. Production of volatile organic compounds. *Can. J. Bot.* 49:1425–1431.

LANGENHEIM, J.H., and STUBBLEBINE, W.H. 1983. Variation in leaf resin composition between parent tree and progeny in *Hymenaea*: Implications for herbivory in the humid tropics. *Biochem. Syst. Ecol.* 11:97–106.

LEATHER, S.R., WATT, A.D., and FORREST, G.I. 1987. Insect induced chemical changes in young lodgepole pine (*Pinus contorta*): the effect of previous defoliation on oviposition, growth and survival of the pine beauty moth, *Panolis flammea*. *Ecol. Entomol.* 12:275–281.

MILLAR, C.S. 1980. Infection processes on conifer needles, pp. 185–209, *in* J.P. Blakeman (ed.). Microbial Ecology of the Phylloplane. Academic Press, London.

MILLER, R.H., BERRYMAN, A.A., and RYAN, C.A. 1986. Biotic elicitors of defense reactions in lodgepole pine. *Phytochemistry* 25:611–612.

NASCIMENTO, J.C. 1980. Ecological studies of sesquiterpenes and phenolic compounds in leaves of *Copaifera multijuga* Hayne (Leguminosae) in a central Amazonian rainforest. PhD dissertation, University of California, Santa Cruz, California.

NJAR, V.C.O., ARNOLD, L., BANTHORPE, D.V., BRANCH, S.A., CHRISTIE, A.C., and MARSH, D.C. 1989. Metabolism of exogenous monoterpenes and their epoxides in seedlings of *Pinus pineaster* Ait. *J. Plant Physiol.* 135:628–630.

OKAMOTO, R.A., ELLISON, B.O., and KEPNER, R.E. 1981. Volatile terpenes in *Sequoia sempervirens* foliage. Changes in composition during maturation. *J. Agric. Food Chem.* 29:324–326.

PAULI, A., and KNOBLOCH, K. 1987. Inhibitory effects of essential oil components on growth of food contaminating fungi. *Z. Lebensm. Unters. Forsch.* 185:10–13.

PETRINI, O. 1982. Notes on some species of *Chloroscypha* endophytic in Cupressaceae of Europe and North America. *Sydowia* 35:206–222.

PETRINI, O. 1986. Taxonomy of endophytic fungi of aerial plant tissues, pp. 175–187, *in* N. Fokkema and J. van den Heuvel (eds.). Microbiology of the Phyllosphere. Cambridge University Press, London.

RAFFA, K.F., and BERRYMAN, A.A. 1982. Accumulation of monoterpenes and associated volatiles following inoculation of grand fir with a fungus transmitted by the fir engraver, *Scolytus ventralis* (Coleoptera: Scolytidae). *Can. Entomol.* 114:797–810.

ROCKWOOD, D.L. 1973. Monoterpene–fusiform rust relationships in loblolly pine. *Phytopathology* 63:551 –553.

ROCKWOOD, D.L. 1974. Cortical monoterpene and fusiform rust resistance relationships in slash pine. *Phytopathology* 64:976–979.

ROLLINGER, J., and LANGENHEIM, J.H. 1989. Geographic variation in fungal endophyte community composition in leaves of *Sequoia sempervirens* (D. Don ex Lamb) Endl. *Proc. Pacific Div. Am. Assoc. Adv. Sci.* 8 (Part 1):33 (abstract).

SCHINDLER, T., and KOTZIAS, D. 1989. Comparison monoterpene volatilization and leaf-oil composition of conifers. *Naturwissenschaften* 76:475–476.

SHRIMPTON, D.M., and WHITNEY, H.S. 1968. Inhibition of growth of blue stain fungi by wood extractives. *Can. J. Bot.* 46:757–761.

SQUILLANCE, A.H., POWERS, H.R., JR., and KOSSUTH, S.V. 1985. Monoterpene phenotypes in loblolly pine populations: natural selection trends and implications. *Proc. 18th South. For. Tree Improve. Conf.* 299–308.

THIBAULT-BALESDENT, M., and DELATOUR, C. 1985. Variabilité du comportament de *Heterobasidion annosum* (Fr.) Bref. a trois monoterpenes. *Eur. J. For. Pathol.* 15:301–307.

TOKUNAGA, Y., and OHIRA, I. 1973. Latent infection of anthracnose on *Citrus* in Japan. *Rep. Tottori Mycol. Inst. (Jpn.)* 10:693–702.

TRIPATHI, R.D., BANERJI, R., SHARMA, M.L., BALASUBRAHMANYAM, V.R., and NIGAM, S.K. 1985. Toxicity of essential oil from a new strain of *Ocimum gratissimum* (Clocimum) against betelvine pathogenic fungi. *Agric. Biol. Chem.* 49:2277–2282.

VÄISÄLA, L. 1974. Effects of terpene compounds on the growth of wood-decomposing fungi. *Ann. Bot. Fenn.* 11:275–278.

VANETTEN, D.E., MATTHEWS, D.E., and MATTHEWS, P.S. 1989. Phytoalexin detoxification: Importance for pathogenicity and practical implications. *Annu. Rev. Phytopathol.* 27:143–164.

VERHOEFF, K. 1974. Latent infections by fungi. *Annu. Rev. Phytopathol.* 12:99–110.

YOKOUCHI, Y., and AMBE, Y. 1984. Factors affecting the emission of monoterpenes from red pine (*Pinus densiflora*). *Plant Physiol.* 75:1009–1012.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

IDENTIFICATION OF EIGHT COUMARINS OCCURRING WITH PSORALEN, XANTHOTOXIN, AND BERGAPTEN ON LEAF SURFACES

ALICJA M. ZOBEL,* JIANYAO WANG, RAYMOND E. MARCH, and STEWART A. BROWN

Department of Chemistry
Trent University
Peterborough, Ontario, Canada K9J 7B8

(Received January 17, 1991; accepted May 16, 1991)

Abstract—Surface extracts of the leaves of five species in the Umbelliferae, *Citrus limon* (Rutaceae), and *Psoralea bituminosa* (Leguminosae) were examined for the presence of coumarins, after a previous study had shown the presence of three psoralens. In the current investigation eight more coumarins were identified by mass spectrometric techniques: the simple coumarins scopoletin, scoparone, and osthol, the linear furanocoumarins imperatorin and phellopterin, the angular furanocoumarins angelicin and pimpinellin, and the pyranocoumarin seselin. Five of these occur in *Apium graveolens*, and scopoletin, scoparone, and imperatorin were each found in three of the species examined. The co-occurrence of all these coumarins on the surface may be significant in communication between the plant and its environment.

Key Words—Furanocoumarins, coumarins, mass spectrometry, leaf surface.

INTRODUCTION

Psoralen, xanthotoxin, and bergapten are the most common linear furanocoumarins (psoralens) of plants in the families Umbelliferae, Rutaceae, and Leguminosae, and recent studies in our laboratory have demonstrated their presence, to some extent, on the surface of leaves (Zobel and Brown, 1988b, 1989, 1990a) and fruits and seeds (Zobel and Brown, 1991). Their existence within the seeds and fruits of these families has long been known (Murray et

*To whom correspondence should be addressed.

0098-0331/91/0900-1859$06.50/0

al., 1982), with new compounds continually being found (Ceska et al., 1987, 1988). The concentrations and proportions of the three main psoralens on plant surfaces vary during the vegetative periods (Zobel and Brown, 1990b) and are influenced by air pollution (Zobel et al., 1991), as is the concentration in the whole leaf (Dercks et al., 1990; Zobel et al., 1991). Concentrations of furanocoumarins are also increased by other environmental stress (Beier and Oertli, 1983; Zangerl and Berenbaum, 1987), as well as by fungal infections (Ashwood-Smith et al., 1985; Surico et al., 1987), or treatment with elicitors (Matern et al., 1988).

Many toxins formed within a plant can be extruded outside the cells where they are synthesized and are thus prevented from being toxic to these cells. Both quality and quantity of these extruded compounds must be important for a defensive role in the plant (Beier and Oertli, 1983; Nitao and Zangerl, 1987; Städler and Buser, 1984; Towers, 1987; Zobel and Brown, 1989).

An extraction method has been developed recently in this laboratory for removing compounds from the plant surface without damaging epidermal cells (Zobel and Brown, 1988a). Research utilizing this approach has laid new stress on the plant surface as a compartment for keeping phenolic compounds isolated from sites of potential damage and as the first barrier in the contact between the environment and the plant body (Zobel and Brown, 1989). During analysis of surface extracts of plants causing dermatitis (Zobel and Brown, 1990a), a number of unidentified compounds were found in addition to psoralen, xanthotoxin, and bergapten. The aim of the present work has been to identify those compounds for which quantities and chromatographic separation were adequate.

METHODS AND MATERIALS

Plant Material. Leaves of *Psoralea bituminosa, Ferula communis* var. Glauca, *Pastinaca sativa, Apium graveolens, Pimpinella anisum*, and *Citrus limon* were obtained from the Royal Botanical Gardens, Hamilton, Ontario; *Heracleum lanatum* was collected from the campus of Trent University, in July 1989.

Analytical Techniques. Relatively clean extracts of surface deposits were obtained by briefly dipping leaves in almost boiling water as previously described (Zobel and Brown, 1988a, 1989). HPLC analysis was performed as described by Thompson and Brown (1984), on a 7.5-cm Nova-Pak C_{18} column eluted with 15% aqueous acetonitrile. Up to 2 ml of eluate was concentrated to 30 μl by evaporation at 70°C, and the residue was transferred to a capillary tube and dried at room temperature. Each capillary tube was inserted directly into the solids probe of a ZAB-2FQ mass spectrometer. The probe temperature was 100°C and the source temperature was 250°C. Chemical ionization (CI)

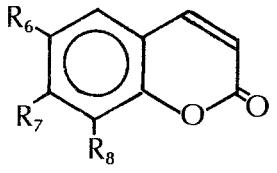
mass spectra were obtained with isobutane as the CI agent, at an indicated pressure in the source housing of 3×10^{-5} mbar (3×10^{-3} Pa), which corresponds to a source pressure of ca. 1 mbar (100 Pa). Helium was used as the collision gas for collision-induced dissociation (CID) with a beam attenuation of ca. 20%.

RESULTS AND DISCUSSION

Seven plant species previously investigated by us for localization of psoralen, xanthotoxin, and bergapten on the surface (Zobel and Brown, 1988b, 1990a) contained other coumarins, unidentified at the time, which also had been extruded to the surface of the cuticle covering their leaves (unpublished). Eight additional coumarins have now been identified by means of CI mass spectrometry (Munson and Field, 1966) with isobutane as the CI agent (Harrison, 1983).

Mass Spectrometric Analyses. Pure samples of eight coumarins from our collection were examined mass spectrometrically, and the electron impact (EI), CI, CID and mass-analyzed ion kinetic energy (MIKE) mass spectra (Cooks et al., 1973) of each were examined. The relative ion intensities of these mass spectra are shown in Tables 1–4.

The relative ion signal intensities for four types of mass spectra are given in Table 1 for each of scopoletin (Scheme 1, **1a**), and osthol **(1b).** For each species, the base peak in the EI mass spectrum is the molecular ion. The principal fragment ions observed for **1b** and the corresponding neutral losses were *m/z* 213 ($CH_3\cdot$), *m/z* 201 (CO + $CH_3\cdot$), *m/z* 189 (55 amu, possibly $C_4H_7\cdot$), and *m/z* 175 ($C_5H_9\cdot$). For **1a** the principal fragment ions were *m/z* 177 ($CH_3\cdot$), *m/z* 164 (CO), and *m/z* 149 ($CH_3\cdot$ + CO).



a R_6 = OMe, R_7 = OH, R_8 = H

b R_6 = H, R_7 = OMe, R_8 = CH_2-CH=CMe_2

c R_6 = R_7 = OMe, R_8 = H

SCHEME 1.

TABLE 1. RELATIVE INTENSITIES OF ELECTRON IMPACT, CHEMICAL IONIZATION, COLLISION-INDUCED DISSOCIATION, AND MASS-ANALYZED ION KINETIC ENERGY MASS SPECTRA OF SCOPOLETIN AND OSTHOL

	Scopoletin (**1a**)				Osthol (**1b**)			
m/z	EI[a]	CI[b]	CID[c]	M[d]	EI	CI	CID	M
245					10.8	100		
244					100	44.8		
229					87.0	22.4	6.6	5.6
227					1.0	1.0		3.2
215					8.7	1.0	4.2	3.2
214					7.8			4.2
213					34.8	10.4		
211					11.3			
201					60.9	20.2		
200					1.0	1.1	4.8	2.8
194	1.0	12.5						
193	11.1	100						
192	100	39.8	56.9					
191	2.0		33.0					
189					61.7	28.4	100	100
186	1.0				1.0	1.1		2.0
185	4.0							
178	5.5		100	100				
177	52.5	8.0						
176					12.1			
175					18.6			
171	3.7							
165	4.2		5.0[e]	35.5[e]				
164	21.6	5.0	9.1[e]	17.9[e]				
163			11.5[e]					
161			10.0	24.4				
152		2.8						
149	47.2	7.8	17.0					
146		6.1	5.5					
145			3.2					
130					34.8	1.0	3.6	

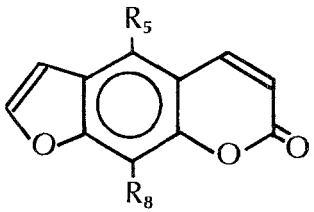
[a] 70-eV electron impact mass spectrum.
[b] Chemical ionization mass spectrum with isobutane as a CI agent.
[c] Collision-induced dissociation mass spectrum of $(M + 1)^+$ using He as collision gas.
[d] Mass-analyzed ion kinetic energy spectrum of $(M + 1)^+$.
[e] Overlapping.

Isobutane CI produced ion signals due to $(M + 1)^+$ for each of **1a** and **1b;** the signal intensities of $(M + 1)^+$ accounted for ca. 50% of the total ion intensity, with the remaining ion signals corresponding to the principal components of the EI mass spectrum. The CID mass spectrum of $(M + 1)^+$ for **1a** was dominated by losses of $H\cdot$, H_2, and $CH_3\cdot$, with the ion of *m/z* 178 forming the base peak both in the CID and MIKE mass spectra. In each mass spectrum, neutral losses of ca. 30 amu, that is, CO, HCO, and possibly H_2CO, were accompanied by relatively large releases of kinetic energy, which produced overlapping fragment ion peaks. The CID and MIKE mass spectra were dominated by the loss of a moiety of 56 amu leading to formation of *m/z* 189.

The mass spectra of imperatorin (Scheme 2, **2a**) and phellopterin **(2b)** as shown in Table 2 exhibit the effect of 5-methoxylation on the stability of the prenylated coumarins. The molecular ion is weak in the EI mass spectrum of each compound, and the base peak corresponds in each case to loss of the prenyl ($C_5H_9\cdot$) moiety. Further loss of CO to form *m/z* 173 is a dominant process for **2a,** whereas further loss of CH_3 to form *m/z* 216 is favored for **2b.** As the $(M + 1)^+$ is twice as intense relative to the base peak of the CI mass spectrum as is the $(M + 1)^+$ ion for **2a,** it appears that methoxylation may stabilize the protonated coumarin with respect to prenyl loss. The CID and MIKE mass spectra of the $(M + 1)^+$ ions of **2a** and **2b** are dominated by the loss of C_5H_8.

The mass spectra of seselin (Scheme 3, **3**), shown in Table 3, are dominated by the formation of *m/z* 213 due to $CH_3\cdot$ loss from the molecular ion and CH_4 loss from $(M + 1)^+$. Rearrangement of the dimethylpyran structure to lose methyl and formation of a seven-membered ring is known. Further loss of CO to form *m/z* 185 is prevalent.

The mass spectra of angelicin (Scheme 4, **4a**) and pimpinellin **(4b)** shown in Table 4 once more indicate enhanced stabilization due to methoxy substitution: the molecular ion of **4b,** dimethoxy substituted **4a,** is the base peak in the



a R_5 = H, R_8 = CH_2-CH=CMe_2

b R_5 = OMe, R_8 = CH_2-CH=CMe_2

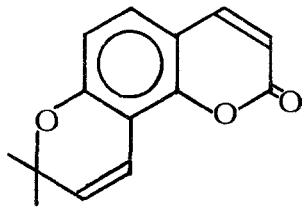
SCHEME 2

TABLE 2. RELATIVE INTENSITIES OF ELECTRON IMPACT, CHEMICAL IONIZATION, COLLISION-INDUCED DISSOCIATION, AND MASS-ANALYZED ION KINETIC ENERGY MASS SPECTRA OF IMPERATORIN AND PHELLOPTERIN

	Imperatorin (**2a**)				Phellopterin (**2b**)			
m/z	EI[a]	CI[b]	CID[c]	M[d]	EI	CI	CID	M
301						76.7		
300					1.0			
271	1.0	35.5						
270	5.4	9.6						
269	5.6							
260					1.0	1.0		
245					1.0	1.1	10.9	1.6
234					2.1	1.0	5.0	5.5
233						88.3	100	100
232						100		
231					100			
230					6.3			
229	1.2	1.5	1.6	1.4				
219						8.6		
217					4.0	15.7	30.9	
216					74.5	1.2		
215	1.2	1.0	2.4	2.8				
203		49.2	100	100		4.0		
202	16.9	100			4.2			
201	100							
189					12.5	3.8		
187	1.0	1.0	1.0					
174	2.8	18.6						
173	23.5							
172	4.9							
161					7.3	4.0		
147					1.0	1.0		
145	5.6							
144	6.1							

[a] 70-eV electron impact mass spectrum.
[b] Chemical ionization mass spectrum with isobutane as a CI agent.
[c] Collision-induced dissociation mass spectrum of $(M + 1)^+$ using He as collision gas.
[d] Mass-analyzed ion kinetic energy spectrum of $(M + 1)^+$.

EI mass spectrum, whereas the base peak for **4a** corresponds to loss of CO from the molecular ion. In the CI mass spectra the dominant species for both **4a** and **4b** is the pseudomolecular ion $(M + 1)^+$. The CID and MIKE mass spectra of $(M + 1)^+$ for **4a** both show loss of HCO, their only common spectral feature. CID for **4a** leads to the loss of HCO + CO (*m/z* 130). The MIKE spectrum



SCHEME 3

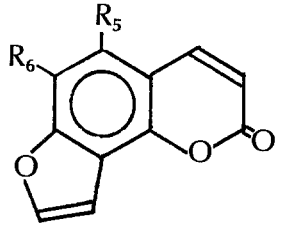
TABLE 3. RELATIVE INTENSITIES OF ELECTRON IMPACT, CHEMICAL IONIZATION, COLLISION-INDUCED DISSOCIATION, AND MASS-ANALYZED ION KINETIC ENERGY MASS SPECTRA OF SESELIN

	Seselin (3)			
m/z	EI[a]	CI[b]	CID[c]	M[d]
229	3.0	58.7		
228	38.0	27.5		
213	100	100	100	100
199	1.0	1.0	14.0	1.0
185	18.3	24.7	46.5	2.1
177	1.1	1.0	36.8	1.2
169	1.0	1.0	14.2	
158	1.0	1.0	13.5	
128	8.1	1.0	11.2	
127	3.7			
115	1.0	1.0	15.5	

[a] 70-eV electron impact mass spectrum.
[b] Chemical ionization mass spectrum with isobutane as a CI agent.
[c] Collision-induced dissociation mass spectrum of $(M + 1)^+$ using He as collision gas.
[d] Mass-analyzed ion kinetic energy spectrum of $(M + 1)^+$.

indicates the somewhat unusual unimolecular losses of 13, 44, and 56 amu to yield fragment ions of *m/z* 174, 143, and 131, respectively. Loss of $CH_3\cdot$ to form *m/z* 232 is the dominant feature of both the CID and MIKE mass spectra of **4b.** Additional decomposition channels involved losses of HCO to form *m/z* 218 and (HCO + CH_3) to yield *m/z* 203.

Distribution. The distribution of the eight coumarins is as follows: (1) *Apium graveolens:* scopoletin **(1a),** osthol **(1b),** scoparone **(1c),** imperatorin **(2a),** phellopterin **(2b);** (2) *Pimpinella anisum:* scopoletin, imperatorin, seselin **(3),** scoparone **(1c);** (3) *Psoralea bituminosa:* angelicin **(4a);** (4) *Citrus limon:* scopoletin, scoparone, seselin; (5) *Heracleum lanatum:* phellopterin, pimpi-



a $R_5 = R_6 = H$

b $R_5 = R_6 = OMe$

SCHEME 4

nellin **(4b)**; (6) *Ferula communis* var. Glauca: imperatorin; (7) *Pastinaca sativa:* imperatorin.

As shown, we identified only one additional coumarin in the last three species, and the highest number was five in *A. graveolens*. Imperatorin, scopoletin, and scoparone were the most often identified, although some additional peaks remain to be characterized. Scopoletin, an acidic coumarin, should have been removed completely by extraction with dilute alkali during the purification (Zobel and Brown, 1988a), and its presence in this fraction suggests carryover from a high concentration on the surface.

Tissues of some of these species are known to contain the above-mentioned coumarins (Murray et al., 1982): angelicin in *P. bituminosa* (Samylina, quoted by Murray et al., 1982), imperatorin in *Pastinaca sativa* (Soine et al., 1956; Beyrich, 1966), pimpinellin and phellopterin in *H. lanatum* (Steck, 1970), and scopoletin in *Pimpinella anisum* and *Citrus limon* (Kartnig et al., 1975; Feldman and Hanks, 1965). Some other compounds were found for the first time in the seven species examined here but had been known to occur in other species of these genera (Murray et al., 1982). Thus we foresee the identification of more coumarins on plant surfaces, not only on leaves and fruits, but perhaps even on root surfaces.

While there are still some unidentified peaks in the plant surface extracts, the complexity of the mixtures extruded to the surface is clearly revealed even by the number of coumarins already shown to be present. Quantitative and possibly qualitative changes, such as those already demonstrated on the surface of *H. lanatum* for psoralen, xanthotoxin, and bergapten, may also occur during the vegetative period. Some compounds may be in such small concentrations on the surface that only very sophisticated methods could show their presence, e.g., bioassay for the identification of psoralens on *Daucus carota* leaves, as performed by Ashwood-Smith's group (Ashwood-Smith et al., 1983; Ceska et

TABLE 4. RELATIVE INTENSITIES OF ELECTRON IMPACT, CHEMICAL IONIZATION, COLLISION-INDUCED DISSOCIATION, AND MASS-ANALYZED ION KINETIC ENERGY MASS SPECTRA OF ANGELICIN AND PIMPINELLIN

	Angelicin (**4a**)				Pimpinellin (**4b**)			
m/z	EI[a]	CI[b]	CID[c]	M[d]	EI	CI	CID	M
247					19.1	100		
246					100	57.0		
232					14.0		100	100
231					90.0	20.0		
223						26.6		
222					9.0			
221					34.0			
220						42.3		
219					1.0	1.0		4.5
218					1.2	1.0	35.8	8.5
215					1.1	1.0		2.2
208					14.0			
205					1.0	1.1		1.8
203					12.0	1.1	10.2	10.2
199						13.8		
198						13.9		
197						57.7		
188					13.2			
187		100			1.0	1.0	5.2	
186	85.0	41.8						
175					22.4			
174	1.0	1.0		14.5				
170	1.0	1.0	8.5					
160					10.1			
158	100	23.4	88.5	100				
149						31.5		
148					10.1	13.8		
147	41.5	7.3	17.5		62.3	57.1		
143	1.0	1.0		28.8				
131	1.0	1.0		13.9				
130	1.5	1.1	100					
129	19.0							
114	1.0	1.1	8.5					
101	46.6	1.0	14.1					
88	1.0	1.0	3.5					

[a] 70-eV electron impact mass spectrum.
[b] Chemical ionization mass spectrum with isobutane as a CI agent.
[c] Collision-induced dissociation mass spectrum of $(M + 1)^+$ using He as collision gas.
[d] Mass-analyzed ion kinetic energy spectrum of $(M + 1)^+$.

al., 1986), but they could still play an important role because of possible synergistic reactions, as has been shown for xanthotoxin and myristicin (Berenbaum and Neal, 1985).

Ecological Implications. Furanocoumarins develop in a plant qualitatively and quantitatively in coevolution with certain insects (Berenbaum, 1978; Berenbaum et al., 1986; Feeny, 1987) and can act not only as insect repellents, but also as attractants for some insects, stimulating oviposition, as by the carrot fly on *Daucus carota* (Ashwood-Smith et al., 1983; Städler and Buser, 1984; Ceska et al., 1986). Besides furanocoumarins, which were in very low concentrations, these authors found other compounds on carrot leaves in higher concentrations than xanthotoxin and psoralen.

The present study shows that this newly recognized compartment, the plant surface, is a repository for numerous compounds. Flavonoids (Wollenweber and Dietz, 1981; Wollenweber, 1986) are the only other large group of phenolic compounds proved to be in substantial concentrations on the surface of many plant species. The concentrations, proportions, and degree of volatility of substances on the plant surface may prove to be a key to explaining communication among plants, and between plants and insects, as well as other herbivores. They could well be concerned, too, in self-protection against changing environmental conditions, as their concentrations change with varying environmental conditions (Zobel, 1991). Our investigations to date represent only the first steps in probing the complexity of the protective epicuticular wax layer and the atmosphere surrounding the plant.

Acknowledgments—This study was financially supported by the Natural Sciences and Engineering Research Council of Canada operating grants A2502 to R.E.M. and A2487 to S.A.B. We are grateful to Dr. James Pringle of the Royal Botanical Gardens for his assistance in collecting plant material.

REFERENCES

ASHWOOD-SMITH, M.J., POULTON, G.A., CESKA, O., LIN, M., and FURNISS, E. 1983. An ultrasensitive bioassay for the detection of furocoumarins and other photosensitizing molecules. *Photochem. Photobiol.* 38:113–118.

ASHWOOD-SMITH, M.J., CESKA, O., and CHAUDHARY, S.K. 1985. Mechanism of photosensitivity reactions to diseased celery. *Br. Med. J.* 290:1249.

BEIER, R.C., and OERTLI, E.H. 1983. Psoralen and other linear furanocoumarins as phytoalexins in celery (*Apium graveolens*). *Phytochemistry* 22:2595–2597.

BERENBAUM, M. 1978. Toxicity of a furanocoumarin to armyworms: A case of biosynthetic escape from insect herbivores. *Science* 201:532–534.

BERENBAUM, M., and NEAL, J.J. 1985. Synergism between myristicin and xanthotoxin, a naturally occurring plant toxicant. *J. Chem. Ecol.* 11:1349–1358.

BERENBAUM, M.R., ZANGERL, A.R., and NITAO, J.K. 1986. Constraints on chemical coevolution: The parsnip webworm and wild parsnip. *Evolution* 40:1215–1228.

BEYRICH, T. 1966. Die Furanocumarine von *Pastinaca sativa* L. *Pharmazie* 21:365–373.

CESKA, O., CHAUDHARY, S.K., WARRINGTON, R.J., and ASHWOOD-SMITH, M.J. 1986. Furanocoumarins in the cultivated carrot, *Daucus carota*. *Phytochemistry* 25:81–83.

CESKA, O., CHAUDHARY, S.K., WARRINGTON, P., and ASHWOOD-SMITH, M.J. 1987. Photoactive furocoumarins in fruits of some umbellifers. *Phytochemistry* 26:165–169.

CESKA, O., CHAUDHARY, S.K., WARRINGTON, P., ASHWOOD-SMITH, M.J., BUSHNELL, G.W., and POULTON, G.A. 1988. Coriandrin, a novel highly photoactive compound isolated from *Coriandrum sativum*. *Phytochemistry* 27:2083–2087.

COOKS, R.G., BEYNON, J.H., CAPRIOLI, R.M., and LESTER, G.R. 1973. Metastable Ions. Elsevier, Amsterdam.

DERCKS, W., TRUMBLE, J., and WINTER, C. 1990. Impact of atmospheric pollution on linear furanocoumarin content of celery. *J. Chem. Ecol.* 16:443–454.

FEENY, P.P. 1987. The roles of plant chemistry in associations between swallowtail butterflies and their host plants, pp. 353–359, *in* V. Labeyrie, G. Fabes, and D. Lachaise (eds.). Insects–Plants. Junk, The Hague.

FELDMAN, A.W., and HANKS, R.W. 1965. Phenolic compounds in roots and leaves of 4 citrus cultivars. *Nature* 207:985–986.

HARRISON, A.G. 1983. Chemical Ionization Mass Spectrometry. CRC Press, Boca Raton, Florida.

KARTNIG, T., MAECKEL, H., and MAUNZ, B. 1975. Occurrence of coumarins and sterols in tissue culture of roots of *Anethum graveolens* and *Pimpinella anisum*. *Planta Med.* 27:1–4.

MATERN, U., STRASSER, H., WENDORFF, H., and HAMERSKI, D. 1988. Coumarins and furanocoumarins, pp. 3–21, *in* I. Vasil and P. Constabel (eds.). Cell Culture and Somatic Cell Genetics of Plants, Vol. 5. Academic Press, New York.

MUNSON, M.S.B., and FIELD, F.H. 1966. Chemical ionization mass spectrometry. I. General introduction. *J. Am. Chem. Soc.* 88:2621–2630.

MURRAY, R.D.H., MÉNDEZ, J., and BROWN, S.A. 1982. The Natural Coumarins: Occurrence, Chemistry and Biochemistry. Wiley, Chichester.

NITAO, J.K., and ZANGERL, A.R. 1987. Floral development and chemical defense allocation in wild parsnip (*Pastinaca sativa*). *Ecology* 68:521–529.

SOINE, T.O., ABU-SHADY, H., and DI GANGI, F.M. 1956. The isolation of bergapten and imperatorin from the fruits of *Pastinaca sativa*. *J. Am. Pharm. Assoc.* 45:426–427.

STÄDLER, E., and BUSER, H.-R. 1984. Defense chemicals in the leaf surface wax synergistically stimulate oviposition by a phytophagous insect. *Experientia* 40:1157–1159.

STECK, W. 1970. Leaf furanocoumarins of *Heracleum lanatum*. *Phytochemistry* 9:1145–1146.

SURICO, G., VARVANO, L., and SOLFRIZZO, M. 1987. Linear furanocoumarins accumulation in celery plants infected with *Erwinia carotovera* pathovar *carotovera*. *J. Agric. Food Chem.* 35:406–409.

THOMPSON, H.J., and BROWN, S.A. 1984. Separations of some coumarins of higher plants by liquid chromatography. *J. Chromatogr.* 314:323–336.

TOWERS, G.H.N. 1987. Fungicidal activity of naturally occurring photosensitizers. *Am. Chem. Soc. Symp. Ser.* 339:231–240.

WOLLENWEBER, E. 1986. Flavonoid aglycones in leaf exudate constituents in higher plants, pp. 155–169, *in* L. Farkas, M. Gábor, and F. Kállay (eds.) Studies in Organic Chemistry, Flavonoids, Bioflavonoids, 1985. Elsevier, Amsterdam.

WOLLENWEBER, E., and DIETZ, U.H. 1981. Occurrence and distribution of free flavonoid aglycones in plants. *Phytochemistry* 20:869–932.

ZANGERL, A.R., and BERENBAUM, M. 1987. Furanocoumarins in wild parsnip. Effects of photosynthetically active radiation, ultraviolet light and nutrients. *Ecology* 68:516–520.

ZOBEL, A.M. 1991. A comparison of furanocoumarin concentrations of greenhouse-grown *Ruta chalepensis* with outdoor plants later transferred to a greenhouse. *J. Chem. Ecol.* 17:21–27.

ZOBEL, A.M., and BROWN, S.A. 1988a. Determination of furanocoumarins on the leaf surface of *Ruta graveolens* with an improved extraction technique. *J. Nat. Prod.* 51:941–946.

ZOBEL, A.M., and BROWN, S.A. 1988b. Furanocoumarins on plant surfaces. Bulletin de Liaison No. 14 du Groupe Polyphenols. Compte-rendu des Journées Internationales d'Étude et de l'Assemblée Générale. pp. 65–68.

ZOBEL, A.M., and BROWN, S.A. 1989. Histological localization of furanocoumarins in *Ruta graveolens* shoots. *Can. J. Bot.* 67:915–921.

ZOBEL, A.M., and BROWN, S.A. 1990a. Dermatitis-inducing furanocoumarins on the leaf surfaces of rutaceous and umbelliferous plants. *J. Chem. Ecol.* 16:693–700.

ZOBEL, A.M., and BROWN, S.A. 1990b. Seasonal changes of furanocoumarin concentrations in leaves of *Heracleum lanatum*. *J. Chem. Ecol.* 16:1623–1634.

ZOBEL, A.M., and BROWN, S.A. 1991. Psoralens on the surface of seeds of Rutaceae and fruits of Umbelliferae and Leguminosae. *Can. J. Bot.* In press.

ZOBEL, A.M., BROWN, S.A., and NIGHSWANDER, J.E. 1991. Influence of acid and salt sprays on furanocoumarin concentrations on the *Ruta graveolens* leaf surface. *Ann. Bot.* 167:213–218.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

DISTRIBUTION OF AMATHAMIDE ALKALOIDS WITHIN SINGLE COLONIES OF THE BRYOZOAN *Amathia wilsoni*

JUSTIN T. WALLS,[1,*] ADRIAN J. BLACKMAN,[2] and DAVID A. RITZ[1]

[1]*Zoology Department*

[2]*Chemistry Department*
University of Tasmania
Box 252C, GPO
Hobart, Tasmania, Australia, 7001

(Received February 4, 1991; accepted May 16, 1991)

Abstract—The content and distribution of amathamide alkaloids within single colonies of the bryozoan *Amathia wilsoni* (Ctenostomata) varied depending on the location in the colony. Three colonies in all, collected from the same site at the same time, were analyzed and gave very similar results. The outermost, more exposed, tips of the colony had an alkaloid content of nearly 9% of dry weight, while basal parts were apparently devoid of alkaloids. Samples taken midway between tips and base yielded intermediate concentrations of about 1%. Very little variation in the proportions of individual amathamides A, B, C, E occurred between exposed tips of the colonies. However, some differences in ratios were found between tips from exposed and more protected regions.

Key Words—*Amathia wilsoni*, bryozoans, amathamides A, B, C, D, E, alkaloid distribution.

INTRODUCTION

Much of the interest in marine chemical ecology has developed from the study of marine natural products. These organic compounds, which are also known

*To whom correspondence should be addressed.

0098-0331/91/0900-1871$06.50/0

as secondary metabolites, are often postulated to be mediators in marine ecological interactions. The roles played by secondary metabolites in the terrestrial environment have been relatively well studied, particularly in the case of compounds from higher plants. This is not so for marine natural products. During the last three decades a considerable number of compounds, having a wide variety of novel functional groups and structures, have been discovered from marine organisms (Faulkner, 1990). Although some of these compounds have biological activity that is of potential pharmacological value, only a few of them have been investigated for their ecological functions (Bakus et al., 1986). Most studied have been the secondary metabolites from marine algae and their involvement in predator–prey relationships (Norris and Fenical, 1982; Van Alstyne, 1988). The ecological significance of compounds originating from marine invertebrates has been reviewed recently (Coll and Sammarco, 1988). Several functions have been ascribed to metabolites from soft corals, while antifouling agents from sponges (Sears et al., 1990), gorgonians (Gerhart, 1986; Rittschoff et al., 1985), and ascidians (Davis and Wright, 1990) have been reported also (Bakus et al., 1986).

The secondary metabolites of bryozoans have been relatively poorly investigated; one reason for this is collection constraints (Faulkner, 1990). Most bryozoan natural products are alkaloids. A wide range of structural types has been described, and some compounds have pronounced biological activity such as the antitumor action of the bryostatins (Suffness et al., 1989). In one of the very few investigations of the ecological significance of bryozoan metabolites, the roles played by tambjamine alkaloids have been described. These compounds are produced by the bryozoan *Sessibugula translucens* and have a range of activities including fish feeding inhibition and antimicrobial action. The tambjamines were also shown to be sequestered and utilized by two species of nudibranchs that grazed on a bryozoan and by a third nudibranch, which in turn preyed upon the two other bryozoan-eating nudibranchs (Carté and Faulkner, 1983).

Amathia wilsoni (Ctenostomata) is the largest and most common bryozoan occurring in Tasmanian coastal waters. Previously we have shown that this bryozoan gives rise to a series of amathamide alkaloids (A–F) and a possible biosynthetic precursor 2-(2,4-dibromo-5-methoxyphenyl)ethanamine (Blackman and Matthews, 1985; Blackman and Green, 1987; Blackman and Fu, 1991). The proportion of total alkaloid and relative amounts of alkaloids A–F from different colonies at the same locality was essentially identical and did not show any significant seasonal variation. Collections from different locations did, however, exhibit considerable variation in these characteristics. In this paper we report on the alkaloid distribution within single colonies collected at the same site and time.

METHODS AND MATERIALS

During September 1990, colonies of *Amathia wilsoni* were collected from waters 5–10 m deep, surrounding Arch Island, SE Tasmania (43°17′S, 147°10′E). After collection, colonies were cleaned of any visible fouling organisms, individually packed, and kept at 0°C until transported to the laboratory, where they were stored at −20°C.

Eight samples of colonial material (3–4 cm) were selected from varying parts of two colonies. Dry weight was recorded after freeze drying for 48 hr. Before extraction, an internal standard (eicosane) was added to each sample. Each sample was extracted with 1 ml of freshly distilled dichloromethane three times. The resultant combined crude extracts were stored at 0°C until analysis using GC-MS. Five amathamides (A–E) were identified from these samples, F was below the detection level.

A third colony was used to develop a more detailed map of the amathamide distribution. The colony was placed on graph paper, and the size and arrangement of the main and side branches traced. Sequential samples comprising approximately 2 cm of colony material then were removed from one third of the colony. Their orientation and position were recorded. Each sample then was freeze-dried before extraction using the method described above. The smaller portions for individual analyses were removed from this colony in order to obtain higher spatial resolution of amathamide distribution within the colony. As a consequence, only four amathamides were identified from these samples; amathamides D and F were below the detection level.

The various crude extracts were analyzed by GC-MS. The amathamides do not give strong molecular ions. Mass spectra of these compounds are therefore dominated by the *N*-methylpyrolidine fragment (*m/z* 84 base peak). The lack of fragmentation makes identification using mass spectra difficult, and so to identify the bases in the crude extract GC retention times of the amathamides were compared with those of isolated pure amathamides. For greater resolution and for targeting of specific ions, the selected ion monitoring facility of the Hewlett Packard 5970 mass selective detector (Sweeley et al., 1966) was used.

Gas chromatography was performed on a Hewlett Packard 5890A gas chromatograph using a 18-m methylsilicone column with a head pressure of 10 psi. An initial temperature of 50°C was used in the oven program. This was followed by a ramp of 30°C every minute up to 150°C with a subsequent reduction to 10°C/minute up to a final temperature of 300°C. The ions chosen for the selective monitoring were the characteristic amathamide base peak and internal standard fragment ions. The peak areas were calculated using the integration software on the HP5970.

We have assumed that the behavior of the various amathamides is con-

sistent during GC. A calibration curve of the internal standard peak area to the peak area of a chosen amathamide at varying concentrations was calculated to allow for the nonlinear relationship between internal standard peak area and amathamide peak area. In this way absolute concentrations of alkaloids were calculated on a percentage dry weight basis.

RESULTS

Amathamide concentrations of different sections of two colonies of *A. wilsoni* are shown in Table 1. From the table it can be seen that total concentrations and proportions of the five amathamides are very constant in samples from the tips of the two colonies. Samples from the bases of the colonies differed markedly from the tips in yielding no detectable amathamides. In general the middle portion of a colony yielded a concentration approximately 0.1 times that in the tip. Colony tips yielded an average of 0.087 g/g, middle portions 0.0087 g/g, and base regions 0 g/g dry weight total amathamide. Figure 1 traces 1–3 show typical GC-MS traces for samples from a colony tip, mid-portion, and base.

Figure 2 shows in greater detail the distribution of total alkaloid content and proportions of the four major amathamides in portions of two branches of a single colony. Again, the increase in alkaloid concentration towards the exposed tips is evident. Proportions of the major amathamides vary in ways that bear no clear relation to location in the colony. Amathamide C, although varying from 37.7% to 68% of the total, is always the major component. The greatest variability is in the proportion of B, which ranges from 3% to 26.4% of the total, with the lowest values occurring in exposed tip samples. Amathamide A

TABLE 1. AMATHAMIDE CONCENTRATIONS OF DIFFERENT SECTIONS OF TWO COLONIES OF *A. wilsoni*

	Amathamide concentration (% dry weight)								
	Colony one				Colony two				
Amathamide	Base	Tips			Base	Middle	Tips		
	1–3	1	2	3	1–3	1	1	2	3
B	"	0.86	0.18	1.15	"	0.07	0.55	0.32	0.41
D	"	0.60	0.24	0.38	"	"	0.29	0.24	0.34
A	"	0.29	0.33	0.36	"	"	0.47	0.36	0.31
C	"	5.30	5.08	3.98	"	0.50	5.48	4.24	4.61
E	"	3.00	0.62	3.02	"	0.30	3.49	2.32	3.19
Total	"	10.05	6.45	8.89	"	0.87	10.28	7.48	8.86

"Below detection level.

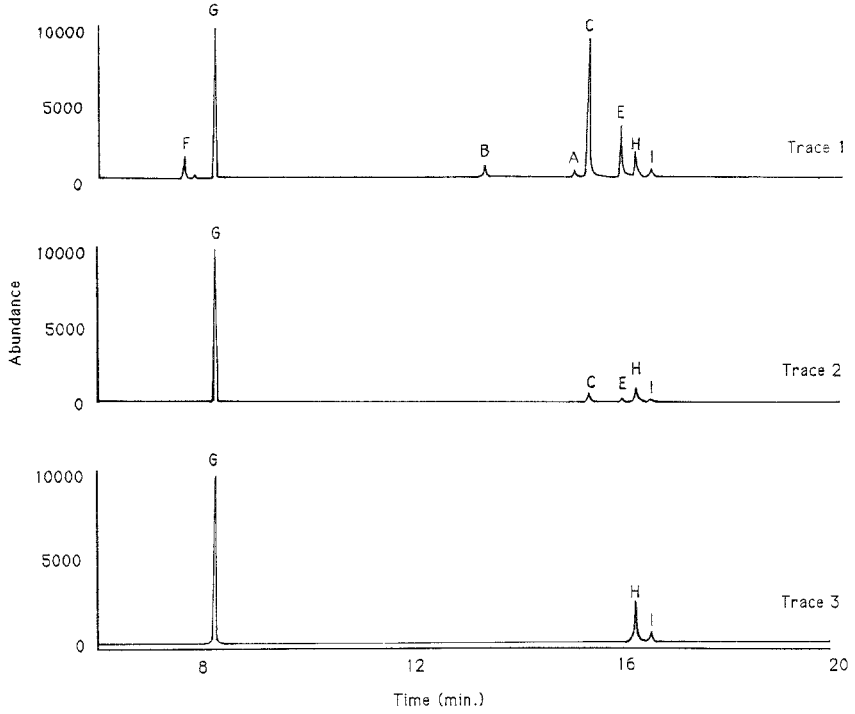


FIG. 1. Typical full-scan GC traces of sections of an *A. wilsoni* colony. Trace 1, tip sample; trace 2, intermediate, and trace 3, basal. A: amathamide A, B: amathamide B, C: amathamide C, E: amathamide E, F unidentified peak, G: internal standard, H: cholesterol, and I: unidentified sterol.

proportions in general vary in the same way as those of B, and concentrations of these two are inversely related to that of C. Proportions of amathamide E vary only from 17.1% to 27.7%. These data with additional measurements from other parts of the colony are given in Table 2 and their locations shown in Fig. 3.

Primary metabolites were found consistently in all GC traces from tip, intermediate, and base samples in similar amounts. This suggests that the proportion of living to inert material was comparable in each of the regions sampled with the exception of the basal attachment structure. These metabolites are detailed in Figure 1.

DISCUSSION

We have demonstrated a gradient in concentration of alkaloid secondary metabolites from high levels in exposed tips to undetectable at the base within three colonies of *A. wilsoni* collected at the same time and at the same location.

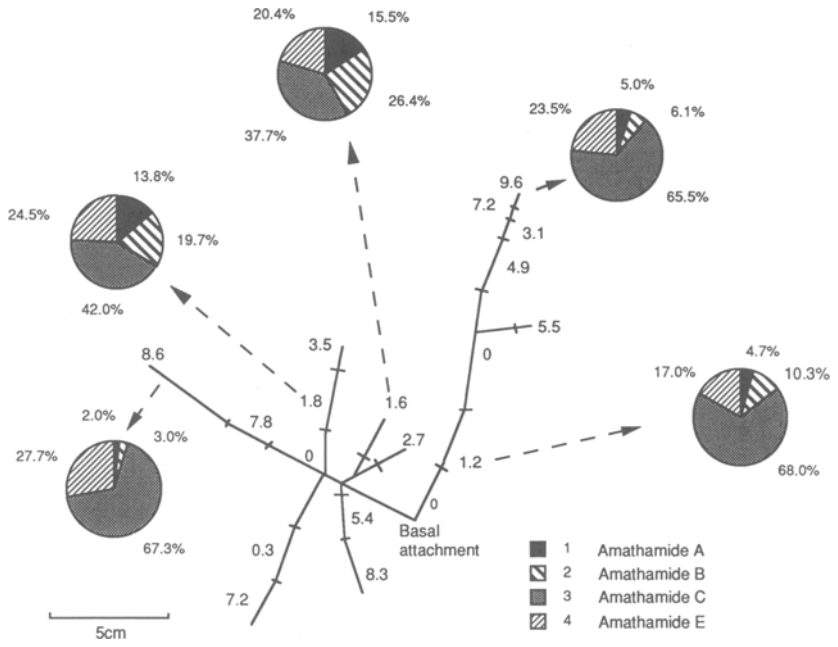


FIG. 2. Schematic of a section of a colony of *A. wilsoni* showing two major branches with associated subbranching. Numbers represent total amathamide as a percentage dry weight at each location. Pie charts represent the proportion of the major amathamides at the position indicated by the arrows. (Branch angles are schematic only.)

Samples taken midway between tips and base of a colony yielded concentrations approximately 0.1 times that of the tips. Exposed tips (those found at the periphery of the colony) exhibited a higher overall alkaloid content and different alkaloid proportions when compared to tips sampled from the interior of the colony. The concentrations reported here are the highest recorded for amathamides and the highest found for any alkaloid compounds in bryozoans. We calculate that total alkaloid concentration for a whole *A. wilsoni* colony would have been about 0.9% of dry weight. Macroalgae commonly yield secondary metabolites in comparable concentrations to those described in the present paper (e.g., phlorotannins in brown algae up to 12% dry weight; Steinberg, 1986).

Blackman and Green (1987) recovered 0.25% (of dry weight) crude alkaloid material from *A. wilsoni* and a range of 1×10^{-3} to 8×10^{-2}% for isolated individual amathamides. They used whole colonies in their procedure, and this may explain why their yields were substantially lower than in the present paper. Other secondary metabolites recovered from bryozoans have been generally in the range of 8×10^{-5} to 0.45% (flustramines); 0.45% (tambjamines); and 7.5

TABLE 2. DISTRIBUTION OF TOTAL ALKALOID AND PROPORTIONS OF MAJOR AMATHAMIDES IN PORTIONS OF TWO BRANCHES OF A SINGLE COLONY

	Amathamide concentration (% dry weight), position of sample within colony																		
Amathamide	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
B	0.17	0.18	[a]	0.03	0.37	0.25	0.36	0.24	0.28	0.24	0.17	[a]	0.06	[a]	0.31	0.49	0.61	0.63	0.48
D	[a]	[a]	[a]	[a]	0.08	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	0.87	[a]	[a]	[a]
A	0.26	0.36	[a]	0.06	0.69	0.35	0.70	[a]	0.43	0.41	[a]	[a]	0.13	[a]	0.55	1.55	[a]	1.00	0.58
C	5.75	5.68	[a]	0.12	4.08	0.76	1.63	3.96	5.53	0.59	2.00	[a]	0.85	[a]	3.51	1.42	1.03	4.30	6.28
E	2.37	1.55	[a]	0.06	2.02	0.44	0.77	1.18	2.08	0.32	0.55	[a]	0.21	[a]	1.17	0.54	1.45	1.31	2.25
Total	8.55	7.77	[a]	0.27	7.24	1.80	3.46	5.38	8.32	1.56	2.72	[a]	1.24	[a]	5.54	4.87	3.09	7.24	9.59

[a]Below detection level.

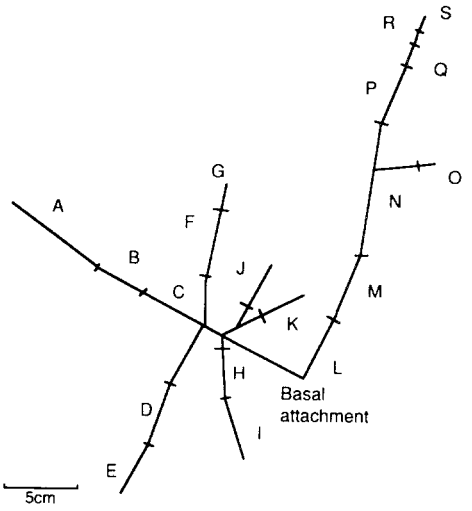


FIG. 3. Schematic of a section of a colony of *A. wilsoni* showing two major branches with associated subbranching. Numbers refer to location within the colony. Amathamide proportions at each location are detailed in Table 2. (Branch angles are schematic only.)

$\times 10^{-3}$ to 1.14×10^{-2}% (bromogramine-derived alkaloids) (Christophersen, 1985). The figures quoted are for isolated compounds, and considerable losses are experienced during the purification process. Thus many of these compounds may be similarly differentially distributed within colonies, and much higher yields from selected regions can be anticipated.

The ecological significance of this nonuniform distribution in alkaloid concentration is not yet clear. Several secondary metabolites of bryozoans have been implicated in protection from grazers, and in antifouling. For example, extracts of *Flustra foliacea* were shown to be effective antifoulants (Christophersen, 1985) and to be acutely toxic to larvae of another bryozoan (Dyrinda, 1983). *Sessibugula translucens* produces tambjamines that are believed to be defense secretions acting to deter grazers, although some nudibranch predators are able to exploit these metabolites for their own defense (Carté and Faulkner, 1983).

Spatial differences in distribution of secondary metabolites have been inferred in marine algae and in a bryozoan by reference to antibiotic properties (Al-Ogily and Knight-Jones, 1977). They showed that older parts of a colony of *Flustra foliacea* were more effective in producing inhibition zones in bacterial cultures than younger growing surfaces. Noting an inverse correlation between antibiotic activity and larval settlement, they suggested that settling

larvae of spirorbids and another bryozoan were avoiding the areas of antibacterial activity. By settling preferentially on the youngest sections, larvae would gain the advantage of less intense spatial competition, although other explanations of the observed distribution are possible (Davis et al., 1989). In contrast, other workers have shown that actively growing tips of some algal species display maximal antibacterial activity (Sieburth and Conover, 1965).

Concentration of secondary metabolites in one area of the colony does not necessarily imply that these chemicals are synthesized in this area. Best and Thorpe (1985) demonstrated transport of metabolites between polypides within a colony of *Membranipora membranacea*. Moreover, most of the tracer ^{14}C, taken in with the food, accumulated in the rapidly growing edges of the colony. On the other hand, specialization of function within colonies is common in the Bryozoa (Silen, 1977). We could detect no gross morphological differences between zooids situated in the exposed tips compared to those more remote from the periphery of colonies of *A. wilsoni* that might have signalled such specialization. However, basal regions clearly had considerably fewer feeding zooids than any other region sampled.

Hay et al. (1988) found that tropical seaweeds in the genus *Halimeda* could gain protection from diurnal fish grazers by producing more herbivore-susceptible new growth at night and also providing it with enhanced chemical defense. New growth contained high concentrations of predator-deterrent terpenoids (2–4.5% of ash-free dry weight) compared to plant portions older than about 24 hr. This form of rapidly mobilizable defense allied with other morphological defenses is very effective where cycles of grazing pressure are very predictable, e.g., with diurnal herbivorous fish on many coral reefs. We are not aware of any comparable data on growth of bryozoans but suggest that such a cycle would not provide protection from the range of grazers on colonial animals in temperate waters (both invertebrate and vertebrate). However, a comparison of the amathamide concentration in colony tips collected at night and during the day would be interesting if the amathamides proved to have predator deterrent properties.

Anthoni et al. (1990) hypothesized that some of the secondary metabolites isolated from bryozoans, perhaps including the amathamides, are manufactured by associated microorganisms. Failure to detect amathamides in two species of bryozoans growing in close proximity to amathamide-containing colonies of *Amathia wilsoni* was taken by Blackman and Green (1987) as evidence arguing against a dietary origin for these chemicals. They proposed that *A. wilsoni* synthesized the amathamides, although the possibility remains that they are synthesized by symbionts. We are planning further work to compare the surface microflora of these colonies and also the microflora associated with different regions of the same colony. We also plan to search for endosymbiotic zoox-

anthellae, although, to our knowledge, none has ever been described from bryozoans. It remains to be established whether the amathamides have antimicrobial properties or provide protection against grazers.

Acknowledgments—We thank Mr. N. Davies for assistance with the GC-MS analysis, Mr. P. Bock for positive identification of the bryozoans, and an anonymous referee for constructive comments. Financial support from ARC grant 889/010 is gratefully acknowledged.

REFERENCES

AL-OGILY, S.M., and KNIGHT-JONES, E.W. 1977. Antifouling role of antibiotics produced by marine algae and bryozoans. *Nature* 265:728–729.

ANTHONI, U., NIELSEN, P.H., PEREIRA, M., and CHRISTOPHERSEN, C. 1990. Bryozoan secondary metabolites: A chemotaxonomical challenge. *Comp. Biochem. Physiol.* 96B:431–437.

BAKUS, G.J., TARGETT, N.M., and SCHULTE, B. 1986. Chemical ecology of marine organisms: An overview. *J. Chem. Ecol.* 12:951–985.

BEST, M.A., and THORPE, J.P. 1985. Autoradiographic study of feeding and the colonial transport of metabolites in the bryozoan *Membranipora membranacea*. *Mar. Biol.* 84:295–300.

BLACKMAN, A.J., and FU, S.-L. 1991. A β-phenylethylamine derived possible biosynthetic precursor to the amathamides, alkaloids from the bryozoan *Amathia wilsoni*. *J. Nat. Prod.* 52:436.

BLACKMAN, A.J., and GREEN, R.D. 1987. Further amathamide alkaloids from the bryozoan *Amathia wilsoni*. *Aust. J. Chem.* 40:1655–1662.

BLACKMAN, A.J., and MATHEWS, D.J. 1985. Amathamide alkaloids from the marine bryozoan *Amathia wilsoni* Kirkpatrick. *Heterocycles* 23:2829.

CARTÉ, B., and FAULKNER, D.J. 1983. Defensive metabolites from three nembrothid nudibranchs. *J. Org. Chem.* 48:2314–2318.

CHRISTOPHERSEN, C. 1985. Secondary metabolites from marine bryozoans. A review. *Acta. Chem. Scand.* 39B:517–529.

COLL, J.C., and SAMMARCO, P.W. 1988. The role of secondary metabolites in the chemical ecology of marine invertebrates. A meeting ground for biologists and chemists. *Proc. 6th Int. Coral Reef Symp. Aust.* 1:167–174.

DAVIS, A.R., and WRIGHT, A.E. 1990. Inhibition of larval settlement by natural products from the ascidian, *Eudistoma olivaceum* (*Van Name*). *J. Chem. Ecol.* 16:1349–1357.

DAVIS, A.R., TARGETT, N.M., MCCONNEL, O.J., and YOUNG, C.M. 1989. Epibiosis of marine algae and benthic invertebrates: Natural products chemistry and other mechanisms inhibiting settlement and overgrowth, pp. 85–114, *in* P.J. Scheuer (ed.). *Bioorganic Marine Chemistry*, Vol. 3. Springer-Verlag, Berlin.

DYRINDA, P.E.J. 1983. Modular sessile invertebrates contain larvatoxic allelochemicals. *Dev. Comp. Immunol.* 7:621–624.

FAULKNER, D.J. 1990. Marine natural products. *Nat. Prod. Rep.* 7:269–309.

GERHART, D.J. 1986. Prostaglandin A_2 in the Caribbean gorgonian *Plexaura homomalla*. Evidence against allelopathic and antifouling roles. *Biochem. Syst. Ecol.* 14:417–422.

HAY, M.E., PAUL, V.J., LEWIS, S.M., GUSTAFSON, K., TUCKER, J., and TINDELL, R.N. 1988. Can tropical seaweeds reduce herbivory growing at night? Diel patterns of growth, nitrogen content and chemical versus morphological defenses. *Oecologia* (*Berlin*) 75:233–265.

NORRIS, J.N., and FENICAL, W. 1982. Chemical defense in tropical marine algae. *Smithson. Contrib. Mar. Sci.* 12:417–431.

RITTSCHOF, D., HOOPER, I.R., BRANSCOMB, E.S., and COSTLOW J.D., 1985. Inhibition of barnacle

settlement and behaviour by natural products from whip corals, *L. virgulata* (Lamarck, 1815). *J. Chem. Ecol.* 11:551–563.

SEARS, M.A., GERHART, D.J., and RITTSCHOF, D., 1990. Antifouling agents from the marine sponge *Lissodendoryx isodictyalis* Carter. *J. Chem. Ecol.* 16:131–168.

SIEBURTH, J., and CONOVER, J.T. 1965. *Sargassum* tannin, an antibiotic which retards fouling. *Nature* 208:52–53.

SILEN, L. 1977. Polymorphism in bryozoa, pp. 184–227, *in* R.M. Woollacott and R.L. Zimmer (eds.). *Biology of Bryozoans*. Academic Press, New York.

STEINBURG, P.D. 1986. Chemical defenses and the susceptibility of tropical marine brown algae to herbivores. *Oecologia (Berlin)* 69:628–630.

SUFFNESS, M., NEWMAN, D.J., and SNADER, K., 1989. Discovery and development of antineoplastic agents from natural sources, pp. 132–168, *in* P. Scheuer (ed.). *Bioorganic Marine Chemistry*, Vol. 3. Springer-Verlag, Berlin.

SWEELEY, C.C., ELLIOTT, W.H., FRIES, I., and RYHLAGE, R. 1966. Mass spectrometric determination of unresolved components in gas chromatographic effluents. *Anal. Chem.* 38:1549.

VAN ALSTYNE, K.L. 1988. Herbivore grazing increases polyphenolic defences in intertidal brown alga *Fucus distichus*. *Ecology* 69:655–663.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

SEX PHEROMONE OF LUCERNE LEAFROLLER, *Merophyas divulsana* (WALKER) (LEPIDOPTERA: TORTRICIDAE): EVIDENCE FOR TWO DISTINCT POPULATIONS

C.P. WHITTLE,[1*] T.E. BELLAS,[1] and A.L. BISHOP[2]

[1]*Division of Entomology, CSIRO,*
GPO Box 1700
Canberra, A.C.T. 2601, Australia

[2]*Horticultural Research and Advisory Station*
N.S.W. Agriculture and Fisheries
PO Box 581
Gosford, N.S.W. 2250, Australia

(Received February 19, 1991; accepted May 20, 1991)

Abstract—When specimens of the lucerne leafroller, *Merophyas divulsana*, were sampled from an area with a history of crop damage, they were found to have (*E*)-11-tetradecenyl acetate, hexadecyl acetate, and tetradecyl acetate as principal components of the pheromone gland. A synthetic mixture of these compounds proved to be a successful lure in delta traps. On the other hand, apparently identical moths collected in areas with no history of crop damage were found to have (*Z*)-11-tetradecenyl acetate as the major component of the sex pheromone gland. The distribution of the two moths provides the basis for a plausible explanation of the regional pest activity reported for *M. divulsana*.

Key Words—Sex pheromones, pheromone blends, moth populations, lucerne, pest, *Merophyas divulsana*, Lepidoptera, Tortricidae.

INTRODUCTION

The lucerne leafroller, *Merophyas divulsana* (Walker) is a tortricid moth, native to Australia, and widely distributed (Common, 1963). It is a significant pest of cultivated lucerne (*Medicago sativa* L.) in parts of Queensland and New South

*To whom correspondence should be addressed.

0098-0331/91/0900-1883$06.50/0

Wales, especially the Lockyer (Qld) and Hunter (NSW) Valleys (Allsopp et al., 1983; Bishop, 1984; Turner, 1969). In the Hunter Valley, field populations of the moth can reach high numbers in late summer and autumn when successive discrete generations occur approximately five weeks apart. In instances of high infestation (95% of stems infested), crop losses up to 40% can occur (Bishop and O'Connell, 1988). Control methods have included applications of insecticides but not without problems associated with damage to beneficial predators and parasites or high mammalian toxicity. In recent years integrated control methods have been investigated (Franzmann and Rossiter, 1981; Allsopp et al., 1983; Bishop and McKenzie, 1986) in efforts to minimize interference with ecologically based pest management systems developed for control of the introduced aphid pests, spotted alfalfa, and bluegreen aphids. One promising approach suggests that careful selection of harvesting cycles can control *Merophyas divulsana* without the need for application of insecticide. In field trials in the Hunter Valley, harvesting of lucerne three weeks before expected peaks in the late-instar larval population was successful in keeping damage levels below an economic threshold of 10% yield loss (Bishop and McKenzie, 1986).

The present investigation into the sex pheromone of *M. divulsana* was initiated in the belief that traps baited with synthetic pheromone would be useful in monitoring population cycles and thus permit harvesting to be timed for best effect as a control method. It was also hoped that it might be possible to explain the apparent regional pest activity of the species.

METHODS AND MATERIALS

Late-instar larvae of *M. divulsana*, collected in eastern Australia, were reared on fresh lucerne in the laboratory. Male and female pupae were separated, and the adult moths were allowed to emerge in a 14:10 hr light–dark regime.

Extracts of pheromone glands for gas–liquid chromatographic (GLC) analysis were prepared from ovipositors excised from female moths taken at the beginning of scotophase and not less than 24 hr after eclosion. Usually a single ovipositor was allowed to stand in 10 μl of hexane (Waters Associates, HPLC grade) for at least 15 min. GLC analyses of extracts from individual moths were carried out on a Varian 3700 gas chromatograph (splitless injection) fitted with one of four columns: BP5, 5% phenyl methyl silicone, bonded phase, 12 m $\times$ 0.32 mm, 170°C; BP10, cyanopropyl silicone, bonded phase, 12 m $\times$ 0.32 mm, 170°C; BP20, polyethylene glycol, bonded phase, 25 m $\times$ 0.32 mm, 170°C; SCOT, SP1000, 43 m $\times$ 0.5 mm, 165°C; all obtained from SGE Scientific Pty. Ltd. Injections were made at 40°C and isothermal conditions main-

tained for 4 min (inlet purged after 2 min). The column oven was then rapidly heated (50°C/min) to the operating temperature (165° or 175°C) and the remainder of the run completed isothermally at that temperature.

Extracts to be used for chemical derivatizations were purified by evaporation and recondensation of the volatiles. The hexane extract, together with washings (2 × 5 μl), was injected in 3-μl aliquots, into a small glass tube (54 mm × 3 mm ID) packed with glass beads (250 mg, 60–80 mesh) and the hexane evaporated under a flow of nitrogen (25 ml/min, 40 sec/aliquot). Volatiles were removed from the glass beads in a flow of helium (2.5 ml/min) at 135°C during 10 min and trapped in a glass capillary cooled with dry ice. $LiAlH_4$ reductions of purified extracts were carried out in small tubes with 50 μl of dried ether and excess $LiAlH_4$. Wet ether was added to destroy the complex and excess reagent. Hexane (20 μl) was added and the organic phase removed with a syringe for GLC analysis. Ozonolyses were carried out using an oxygen–ozone mixture generated with a microozonizer similar to that described by Beroza and Bierl (1976). The ozone mixture was passed (2 ml/min) for 6 sec through 20 μl of hexane cooled in Dry Ice; 1–2 μl of this solution added to a purified gland extract was sufficient for complete ozonolysis. Splitless injection of the reaction mixture into a GLC inlet at >200°C was sufficient to decompose the ozonide to the respective aldehyde ester.

GLC data were recorded with a Hewlett Packard HP3392A integrator. Kovats indices (KI) for all sample peaks were calculated using the retention times for a set of normal hydrocarbon standards (C_{18}, C_{19}, C_{20}, C_{22}, and sometimes C_{24}) coinjected with the sample. Peaks were identified by comparison of KI values with those from a library of known compounds. Identifications were confirmed by the GLC analysis of the products from nanoscale ozonolysis and reduction ($LiAlH_4$) reactions.

Field-trapping experiments were carried out near Singleton in the Hunter Valley, NSW. Comparisons were made between live virgin females and candidate synthetic pheromone mixtures formulated on the basis of the chromatographic analyses. Lures, prepared by impregnating of small sections of medical-grade rubber tubing with blends of synthetic pheromone components or by using live female moths in small cages, were placed in modified Biotrap (delta type) traps. The treatments (including controls) were arranged in a 5 × 5 Latin square (trial A) or in four 3 × 3 balanced lattice squares (controls separate, trial B) with the traps fixed to pegs 30 cm above the ground at 20-m intervals.

Counts of male moths were made after three or four days. Analysis of variance was carried out on $\log_e$ transformed counts. Means were separated using Fisher's (protected) least-significant difference procedure. Smaller trials were also carried out at Wilton, NSW.

Voucher specimens of insect material have been placed with the Australian National Insect Collection (ANIC).

RESULTS

Initially *M. divulsana* larvae were collected from a site of moderate infestation (>25% of stems infested) at Singleton, NSW, and also from a site of low infestation (<1 infested stem/m^2) at Wilton, NSW, in 1987 during the January to April season when the pest is most active.

For the Singleton material, GLC analysis of individual gland extracts showed peaks consistent with tetradecyl acetate (*n*14:OAc, 19%), (*E*)-11-tetradecenyl acetate (*E*11-14:OAc, 42%), and hexadecyl acetate (*n*16:OAc, 38%) with small to trace amounts of (*Z*)-11-tetradecenyl acetate (*Z*11-14:OAc, 1%), (*E*)-11-tetradecenyl alcohol (*E*11-14:OH), heneicosane (C_{21}), and tricosane (C_{23}). The GLC peaks were identified by coincidence of KI values with those from a library of known compounds measured on at least two different columns (Table 1). Confirmation was obtained from the GLC analyses of reduction and ozonolysis products. $LiAlH_4$ reduction of a purified gland extract resulted in the disappearance of peaks assigned to acetates and the increase in or appearance of corresponding alcohol peaks (*n*14:OH, *E*11-14:OH, *Z*11-14:OH, and *n* 16:OH with KI on SP1000 of 2137.2, 2179.3, 2191.1, and 2338.1, respectively). Ozonolysis removed peaks for *E*11-14:OAc and *Z*11-14:OAc, and a new peak coincident with the ozonolysis product from a known sample of *Z*11-14:OAc (the acetate of 11-hydroxy-undecanal) was formed (KI on SP1000, 2305.2). In these reactions the C_{21} and C_{23} hydrocarbon peaks were unaffected.

Similar analyses were carried out on extracts from the low-density population found at Wilton where there was no evidence of crop damage. A distinctly different result was obtained. The major component of the pheromone

TABLE 1. KOVATS INDICES OBTAINED FOR COMPOUNDS FOUND IN EXTRACTS OF OVIPOSITOR TIPS FROM *Merophyas divulsana*

Compound	Kovats indices (±0.3)[a]			
	BP5 column	BP10 column	BP20 column	SP1000 column
*n*14:OAc	1810.2a	1890.1b	2105.4	2069.7
C_{21}	2100.0	2100.0	2100.0	2100.0
*E*11-14:OAc	1806.2	1892.3b	2151.4	2110.4
*Z*11-14:OAc	1810.2a	1900.1	2165.3	2121.7
*E*11-14:OH			2230.4	2179.3
*Z*11-14:OH			2244.4	2191.0
*n*16:OAc	2011.3	2090.7	2311.2	2270.2
C_{23}	2300.0	2300.0	2300.0	2300.0

[a]Within columns, KI with the same letter were not resolved.

gland was found to be Z11–14:OAc (80%) followed by *E*11–14:OAc (16.5%), *n*14:OAc (2%) and *n*16:OAc (1.5%) with trace amounts of Z11–14:OH and *E*11–14:OH. For convenience, the insects having *E*11–14:OAc as the major component are henceforth referred to as E-type and those with Z11–14:OAc as Z-type. Figure 1 shows GLC traces for typical specimens of each type.

M. divulsana larvae were subsequently collected between December 1987 and April 1988 from various locations (Figure 2) having either a high level of infestation with obvious crop damage or a very low density with no apparent crop damage. The sex pheromone gland extracts obtained from moths reared from this material were analyzed as above. The results are shown in Table 2. Without exception all moths obtained from low-level infestations were found to be Z-type. Similarly the moths obtained from locations with significant pest activity proved to be E-type. Only at Windsor were the E- and Z-type moths found to be coincident, and at this location, where pest activity was apparent, one of the 13 specimens (collected at the same time) was found to be Z-type.

Analyses of specimens taken monthly from Singleton during the course of a year (1987) were entirely consistent with the E-type moth and showed no seasonal variation. Similarly specimens taken from Wilton over the same time were all of the Z-type.

A morphological comparison of specimens from populations of the two

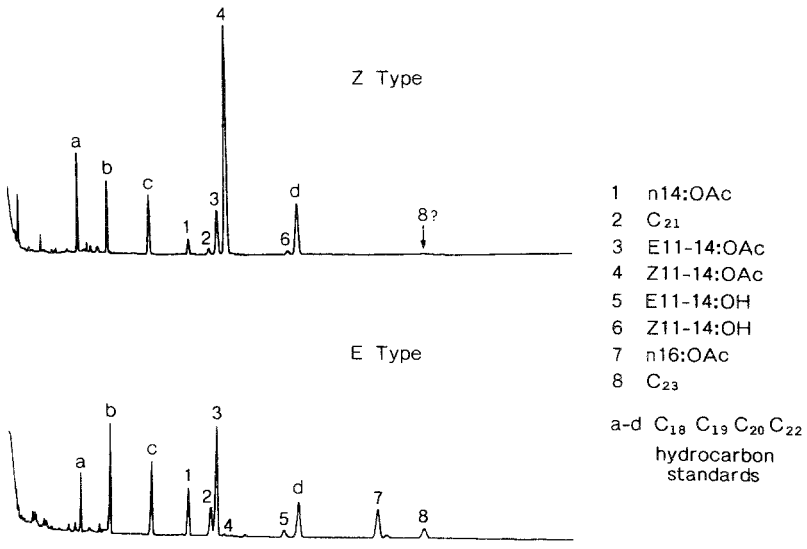


FIG. 1. Capillary gas–liquid chromatography traces from gland extracts of typical specimens of Z-type and E-type *Merophyas divulsana*, on a BP20 column.

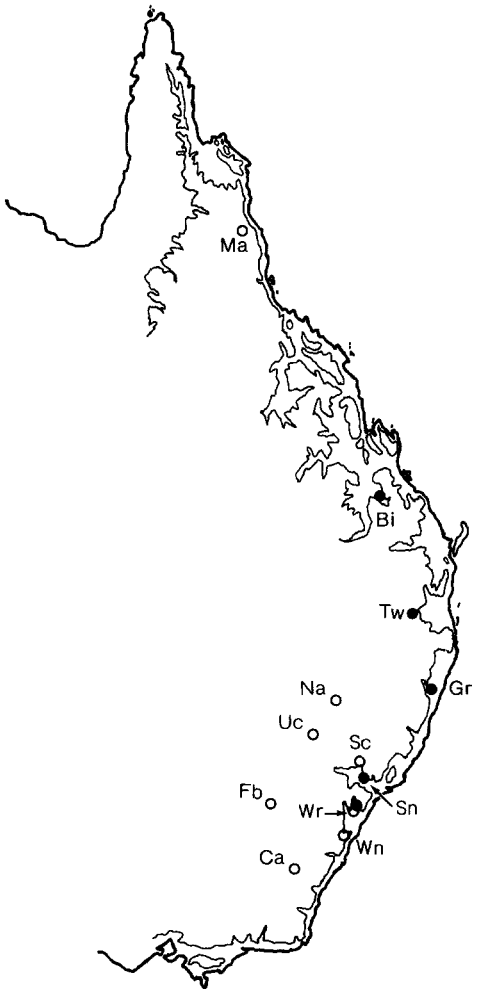


FIG. 2. Locations of sampling sites for the Z-type (○) and E-type (●) *Merophyas divulsana* showing the 200-m contour line. Place names corresponding to abbreviations on the map are given in Table 2.

pheromone types of *M. divulsana*, including a study of genitalia, failed to reveal any consistent differences (M. Horak, personal communication).

In field trials at Singleton, candidate synthetic pheromone blends were compared with live female E-type and Z-type moths as lures in delta traps (Table 3). The synthetic mixtures, labeled E1, E2, Z1, and Z2 for convenience, were based on the analytical results for the E- and Z-type moths from Singleton (E1)

TABLE 2. AVERAGE RELATIVE AMOUNTS OF PHEROMONE GLAND COMPONENTS FOR *Merophyas divulsana* FROM VARIOUS LOCALITIES (VALUES NORMALIZED TO 1000 FOR MAJOR UNSATURATED COMPONENT)

Location (abbrev.). (lat., long.)	*n*14:OAc (range)	*E*11–14:OAc (range)	Z11–14:OAc (range)	*n*16:OAc (range)	*N*[a]
Canberra (Ca) 36°16′S, 149°07′E	41.8[b] (18–58)	194.8[b] (185–224)	1000[b]	< 10[b]	11
Mareeba (Ma) 17°00′S, 145°26′E	44.8 (20–120)	214.7 (177–408)	1000	50 (< 10–292)	11
Narrabri (Na) 30°20′S, 149°47′E	50.4 (40–69)	257.8 (187–354)	1000	35.8 (11–84)	5
Windsor (Wr) 33°37′S, 150°47′E	67	311	1000	49	1
Wilton (Wn) 34°14′S, 150°42′E	25.8 (20–47)	208.0 (169–252)	1000	20 (< 10–77)	4
Uargon Creek (Uc) 31°28′S, 148°59′E	186	295	1000	< 10	1
Scone (Sc) 32°04′S, 150°52′E	34	184	1000		1
Forbes (Fb) 33°28′S, 148°13′E	26.5 (25, 28)	279.5 (259, 300)	1000	< 20	2
Toowoomba (Tw) 27°34′S, 151°57′E	132.6[b] (90–235)	1000[b]	3[b] (< 10–10)	684.5[b] (381–1058)	14
Singleton (Sn) 32°34′S, 151°10′E	448.1 (113–1928)	1000	22 (< 10–99)	913.4 (409–2427)	27
Windsor (Wr) 33°37′S, 150°47′E	370.5 (78–1082)	1000	35 (< 10–52)	487.7 (74–1389)	12

TABLE 2. Continued

Location (abbrev). (lat., long.)	*n*14:OAc (range)	*E*11–14:OAc (range)	Z11–14:OAc (range)	*n*16:OAc (range)	*N*[a]
Grafton (Gr) 29°38′S, 152°56′E	132.9 (37–419)	1000	3.5 (0–28)	561.8 (216–962)	8
Biloela (Bi) 24°24′S, 150°31′E	94.3 (60–141)	1000	4.21 (0–22)	241.6 (22–877)	11
$\bar{X}$ Z-type	47	236	1000	c. 29	35
$\bar{X}$ E-type	285	1000	c. 15	656	72

[a] Number of moths analyzed.
[b] These figures were obtained from glands taken 1–4 at a time.

and Wilton (Z1) and on overall averages for each type (E2 and Z2). The Z-type female moth attracted about one third as many males as the live E-type female. At a dose level of 1 mg/bait (trial A) the synthetic E1 mixture was also only one third as effective as the E-type female. When the dose of the synthetic baits was reduced (trial B), the baits based on GLC analysis of E-type moths (E1 and E2) proved to be equally effective as the live female moth. The synthetic Z-type baits (Z1 and Z2) were, by comparison, poor attractants.

A small trial was carried out at Wilton where Z-type moths had been found exclusively. The low incidence of moths at this location did not allow a comprehensive test; however, when E1 and Z1 lures (ten of each, 0.1 mg dose) were compared, after seven days 25 male *M. divulsana* were caught in the Z1 baited traps and no moths were found in the E1 baited traps.

DISCUSSION

Lucerne is an exotic species widely cultivated in Australia for animal fodder. The lucerne leafroller, *M. divulsana*, is a native tortricid largely known for its pest activity in lucerne crops, but it is a polyphagous species and has been found on a wide variety of exotic plants. Its native host range is unknown and Australian National Insect Collection host records list only four native plants, all herbaceous species, each from a different family. The present investigation was prompted by the significant pest activity of *M. divulsana* on lucerne in eastern Australia and the potential for the use of synthetic pheromones in its control. In this regard our initial results were promising.

TABLE 3. MEAN TRAP CATCHES OF MALE *Merophyas divulsana* IN THE FIELD AT SINGLETON, NSW

Lure	Mean No. male moths/trap[a] (Dose[b])		
	Trial A	Trial B	
E-type female	64.0a	175.6a	
E1	24.6a (1.0)	124.0a (0.1)	152.4a (0.01)
E2		176.2a (0.1)	155.5a (0.01)
Z-type female	26.2a		
Z1	3.0b (1.0)	26.4b (0.1)	34.7b (0.01)
Z2		25.8b (0.1)	11.6b (0.01)
Control	2.0b	6.3	

E-type female	caged live female moth, ex Singleton
Z-type female	caged live female moth, ex Canberra
E1	*E*11-14:OAc/*n*16:OAc/*n*14:OAc 10:20:7
E2	*E*11-14:OAc/*n*16:OAc/*n*14:OAc 10:6.6:2.8
Z1	*Z*11-14:OAc/*E*11-14:OAc/*n*14:OAc 10:2:0.8
Z2	*Z*11-14:OAc/*E*11-14:OAc/*n*14:OAc 10:2.4:0.5
Trial A	5 × 5 Latin square, 3 days
Trial B	4 3 × 3 balanced lattice squares, controls separate, 4 days

[a] Within trials, means identified by different letters are significantly different, $F_{4,19}$, $P < 0.001$ (trial A), $F_{8,7}$, $P < 0.001$ (trial B).
[b] Doses in milligrams of major unsaturated component per bait.

Female *M. divulsana*, reared from material collected in a lucerne crop at Singleton, NSW, during a period of high infestation, were found to have *E*11-14:OAc, *n*16:OAc, and *n*14:OAc as principal components in the sex pheromone gland. In a field trial carried out at Singleton, baits containing mixtures of these compounds were found to be as attractive to male moths as a single live virgin female moth taken from the same population.

However, the investigation of the sex pheromone of *M. divulsana* was complicated by the discovery of a second population at Wilton, NSW, also found on lucerne but at low density and having a distinctly different ratio of components in the sex pheromone gland. In this case *Z*11-14:OAc was found to be the major component with smaller amounts of *E*11-14:OAc and *n*14:OAc. The proportions of *E*11-14:OAc and *Z*11-14:OAc measured for individual moths were such that each moth could be unequivocally assigned to one or the other population. Individual GLC analyses showed ratios of *E*11-14:OAc to *Z*11-14:OAc clustered about values of 1000:16 (*Z*11-14:OAc range 0–99) for E-type moths and 236:1000 (*E*11-14:OAc range 169–408) for Z-type moths, for all of the moths examined.

Specimens taken from both locations at intervals during the course of a year were found to be consistently E-type at Singleton and Z-type at Wilton. When E-type and Z-type females and synthetic mimics were tested in comparative field trials at Singleton, and Z-type female and synthetic lure were both found to attract some male moths, although not as well as the corresponding E-type baits. At Wilton, in a test of the E-type and Z-type synthetic lures, the Z-type bait was successful whereas the E-type bait failed to attract moths.

When the study was extended to include the GLC analysis of specimens from a variety of locations, both within and outside the range of the observed pest activity of the moth, it became apparent that the E-type moth only occurs in locations where pest control measures for *M. divulsana* are always or sometimes necessary. In other locations, where the moth can be found in low numbers and crop damage does not occur, the Z-type moth is found. The locations of the sampling sites for the E-type and Z-type moths along the eastern seaboard of Australia are shown in Figure 2; the 200-m contour line is drawn to indicate the coastal topography. Significantly, the E-type moth appears to be restricted to the river valleys and coastal areas of the central eastern Australian coast, an area that includes the known range of pest activity. Thus the distribution data (Figure 2) provide the basis for an explanation of the apparent regional pest activity of the lucerne leafroller. The description of *M. divulsana* as a sporadic (Fitzgerald et al., 1980) or occasional (Holtkamp and Goodyer, 1982) pest of lucerne is probably inappropriate when considered in the context of the pest histories for specific localities. Although *M. divulsana* has a wide distribution in Australia (Figure 3), for most localities the insect occurs in low numbers on cultivated lucerne and control measures are seldom, if ever, necessary. This is not true for areas on the eastern coast of Australia, particularly southeast Queensland and the Hunter Valley, NSW, where *M. divulsana* is an annual problem and an important pest (Turner, 1969; Bishop, 1984). If the apparent correlation between the range of pest activity and the distribution of the E-type moth is sustained, the occasional pest behavior could be explained in terms of differing host preferences of the two types, and chemical analysis of the pheromone gland extracts will provide a means for assessing the likelihood of pest activity in any given area.

The two pheromone types of *M. divulsana* have been found to occur at the same time at one site (Windsor), and at another site, both moths have been detected but at different times during the January to April season. At Scone, NSW, a single specimen taken during a period of low population density and no crop damage proved to be Z-type. When this site was revisited at a later time (1989) when crop damage was occurring (and subsequent to the compilation of the data in Table 2), the E-type moth was found. Because of this and also the difficulty in detecting the presence of a low population of Z-type moths

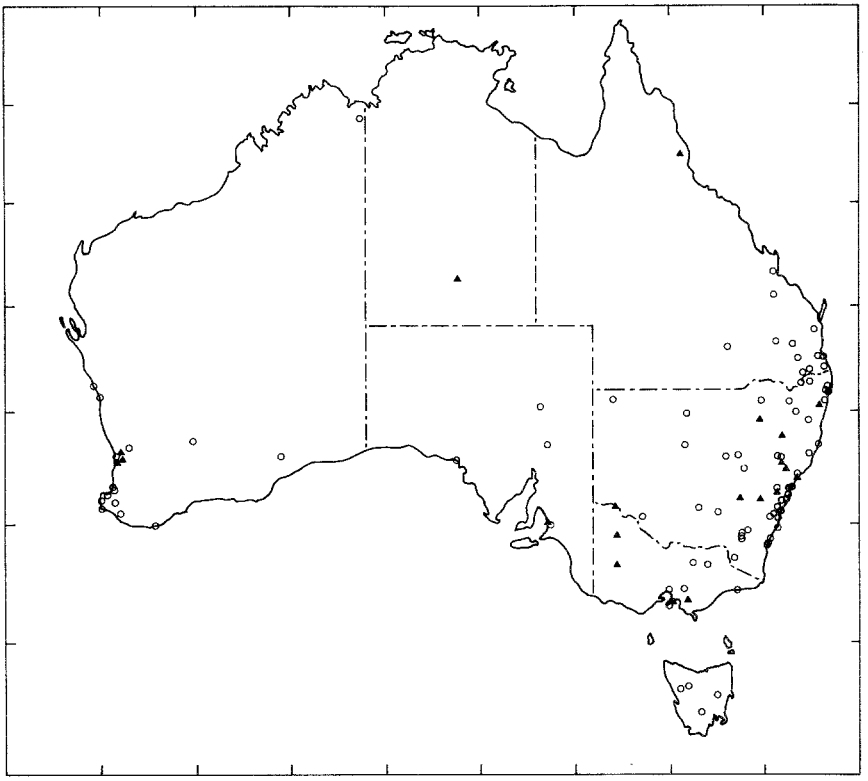


FIG. 3. Distribution of *Merophyas divulsana* in Australia taken from field notes (▲) and specimens (○) held by the Australian National Insect Collection.

in the presence of pest proportions of the E-type moth, it has not been possible to determine the extent of population overlap, if any.

Within species, considerable variation in the ratio of one or more of the saturated (Horak et al., 1988) or unsaturated (Guerin et al., 1986) components of the pheromone gland is a common occurrence. However, there have been relatively few reports of discretely different blends within populations of otherwise morphologically indistinguishable moths. Sex pheromone polymorphism has been described for the European corn borer in the United States and Europe (Roelofs et al., 1985; Klun and cooperators, 1975), the western avocado leafroller in California (Bailey et al., 1986), and the larch budworm in Europe (Guerin et al., 1984). In these species the different pheromone types are not reproductively isolated and have been described as races, types, or strains. On the other hand, sibling species have been proposed for some morphologically

indistinguishable New Zealand tortricids on the basis of discrete pheromone gland blends, the specificity of synthetic lures, and differences in host-plant preference (Foster et al., 1987; Foster and Roelofs, 1987). The differing pest status of the two *M. divulsana* types suggests that they might best be regarded as sibling species also; however, further studies are being carried out to determine other possible behavioral differences and the extent to which the two populations may be reproductively isolated.

Acknowledgments—The authors are grateful to Mr. P. Bowey, Mr. J. Feehan, and Ms. J. Fisk of the CSIRO Division of Entomology; Dr. B. Franzmann, Mr. B. Ingram, and Dr. I. Cunningham of the Queensland Department of Primary Industries; and Mr. J. Betts of NSW Agriculture and Fisheries for the collection of larval material.

REFERENCES

ALLSOPP, P.G., COWIE, B.A., and FRANZMANN, B.A. 1983. Development of immature stages of the lucerne leafroller *Merophyas divulsana* (Walker) (Lepidoptera: Tortricidae) under constant temperatures and on several larval diets. *J. Aust. Entomol. Soc.* 22:287–291.

BAILEY, J.B., MCDONOUGH, L.M., and HOFFMANN, M.P. 1986. Western avocado leafroller, *Amorbia cuneana* (Walsingham), (Lepidoptera: Tortricidae): Discovery of populations utilizing different ratios of sex pheromone components. *J. Chem. Ecol.* 12:1239–1245.

BISHOP, A.L. 1984. *Heliothis* spp. and *Merophyas divulsana* (Walker) in the seasonal damage of lucerne in the Hunter Valley, New South Wales. *Gen. Appl. Entomol.* 16:36–44.

BISHOP, A.L., and MCKENZIE, H.J. 1986. Development of *Merophyas divulsana* (Walker) (Lepidoptera: Tortricidae) in relation to lucerne harvesting as a control strategy. *J. Aust. Entomol. Soc.* 25:229–233.

BISHOP, A.L., and O'CONNELL, M.A. 1988. Relationships between the lucerne leafroller, *Merophyas divulsana* (Walker) (Lepidoptera: Tortricidae), and damage to lucerne. *Plant Protect. Q.* 3:97–99.

COMMON, I.F.B. 1963. A new genus for the Australian lucerne leaf roller (Lepidoptera: Tortricidae). *Proc. Linn. Soc. N.S.W.* 88:298–300.

FITZGERALD, R.D., SIMMONS, K.V., and SOUTHWOOD, O.R. 1980. Lucerne. Division of Plant Industry Bulletin P2.5.7. Department of Agriculture NSW, Sydney.

FOSTER, S.P., and ROELOFS, W.L. 1987. Sex pheromone differences in populations of the brown-headed leafroller, *Ctenopseustis obliquana*. *J. Chem. Ecol.* 13:623–629.

FOSTER, S.P., CLEARWATER, J.R., and ROELOFS, W.L. 1987. Sex pheromone of *Planotortrix species* found on mangrove. *J. Chem. Ecol.* 13:631–637.

FRANZMANN, B.A., and ROSSITER, P.D. 1981. Toxicity of insecticides to *Trioxys complanatus* Quilis (Hymenoptera: Braconidae) in lucerne. *J. Aust. Entomol. Soc.* 20:313–315.

GUERIN, P.M., BALTENSWEILER, W., ARN, H., and BUSER, H.-R. 1984. Host race pheromone polymorphism in the larch budmoth. *Experientia* 40:892–894.

GUERIN, P.M., ARN, H., BUSER, H.R., and CHARMILLOT, P.J. 1986. Sex pheromone of *Adoxophyes orana*: Additional components and variability in ratio of (Z)-9- and (Z)-11-tetradecenyl acetate. *J. Chem. Ecol.* 12:763–772.

HOLTKAMP, R.H., and GOODYER, G.J. 1982. Insect Pests of Lucerne. Agfact P2.AE.2, Department of Agriculture, New South Wales, Sydney.

HORAK, M., WHITTLE, C.P., BELLAS, T.E., and RUMBO, E.R. 1988. Pheromone gland components of some Australian tortricids in relation to their taxonomy. *J. Chem. Ecol.* 14:1163–1175.

KLUN, J.A., and cooperators 1975. Insect sex pheromones: intraspefic pheromonal variability of *Ostrinia nubilalis* in North America and Europe. *Environ. Entomol.* 4:891–894.

ROELOFS, W.L., DU, J.-W., TANG, X.-H., ROBBINS, P.S., and ECKENRODE, C.J. 1985. Three European corn borer populations in New York based on sex pheromones and voltinism. *J. Chem. Ecol.* 11:829–836.

TURNER, J.W. 1969. Control of lucerne leafroller. *Queensl. Agric. J.* 95:179–180.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

CHEMISTRY OF VENOM ALKALOIDS IN THE ANT GENUS *Megalomyrmex*

T.H. JONES,[1,*] M.S. BLUM,[2] H.M. FALES,[1] C.R.F. BRANDÃO,[3] and J. LATTKE[4]

[1]*Laboratory of Biophysical Chemistry*
National Heart, Lung, and Blood Institute
Bethesda, Maryland 20892

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

[3]*Museu de Zoologia da Universidade de São Paulo*
Caixa Postal 7172
01051 São Paulo, SP, Brazil

[4]*Fundacion Terramar S.C.*
Universidad Simon Bolivar
Pabellon 1, OFC 26, Apartado 80659
Caracas, Venezuela 1080

(Received March 13, 1991; accepted May 20, 1991)

Abstract—Chemical analyses of three species in the Neotropical ant genus *Megalomyrmex* have identified this taxon as the third myrmicine genus to produce alkaloids as major venom products. Workers of *M. leoninus* and workers and ergatoids of *M. goeldii* produce one or more of four *trans*-2,5-dialkylpyrrolidines previously identified in other myrmicine genera. *M. modestus*, on the other hand, is distinctive in producing the novel alkaloid (5*E*,8*E*)-3-butyl-5-hexylpyrrolizidine **(5d)**, whose structure was established using a micro-Hofmann degradation sequence. The relationship of *Megalomyrmex* to other alkaloid-producing ant genera is discussed along with the possible chemotaxonomic significance of the analyzed species when viewed in terms of the recognized species groups in this genus.

Key Words—*Megalomyrmex*, Hymenoptera, Formicidae, venom, alkaloids, ants, *trans*-2,5-dialkylpyrrolidine, 3,5-dialkylpyrrolizidine, Hofmann degradation.

*To whom correspondence should be addressed.

0098-0331/91/0900-1897$06.50/0

INTRODUCTION

The ant genera *Monomorium* and *Solenopsis* are distinctive in producing poison gland products dominated by alkaloids (Jones et al., 1982). Ants in no other taxa in the large subfamily Myrmicinae have been demonstrated to produce venoms in which nitrogen heterocycles are major constituents rather than proteins and peptides (Blum, 1985).

Monomorium and *Solenopsis* are far and away the largest of the 13 genera in the *Solenopsis* genus-group, an assemblage whose members are mostly found in the Old and New World tropics (Bolton, 1987). Ants in these genera are considered to form a taxonomic grouping that is isolated from all other ants in the Myrmicinae (Bolton, 1987). In view of the natural relationship of the alkaloid-producing genera *Monomorium* and *Solenopsis* with the other members of the *Solenopsis* genus-group, we consider all these genera as appropriate candidates for producers of alkaloidal venoms.

In the present report we describe the results of the analysis of three species in the genus *Megalomyrmex* and demonstrate that alkaloids are produced by each species. One of these, *M. modestus*, produces the novel (5*E*,8*E*)-3-butyl-5-hexylpyrrolizidine (**5d,** see Figure 2), whose structure and stereochemistry were assigned by microdegradative methods.

METHODS AND MATERIALS

Chemical Analyses

Gas chromatographic analyses were carried out using a Shimadzu GC-9A equipped with a 30-m × 0.5-mm-ID open DB-17 column (1-μm film thickness). The temperature for the analyses was programed from 60 to 215°C at 10°/min, and the carrier gas flow rate was 15 ml/min. On a given day, retention temperatures were reproducible to 1°C. Preparative gas chromatography was conducted with a Varian model 1400 gas chromatograph equipped with a 2-m × 5-mm-ID column packed with 10% OV-17 on 100–120 mesh Supelcoport. Gas-phase IR spectra were obtained using a Hewlett-Packard model 5965A FTIR. ^{1}H- and ^{13}C NMR spectra were obtained from $CDCl_3$ solutions using a Varian XL-200 spectrometer. Electron impact mass spectra were obtained using an LKB-9000 GC-MS equipped with a 30-m × 0.52-mm-ID open DB-17 column (1-μm film thickness), a LKB-2091 equipped with a 25-m × 0.31-mm-ID HP-5 column, or a Finnigan ion trap model 800 equipped with a 25-m × 0.31-mm-ID HP-5 column. Unless otherwise specified, all gas chromatographic retention times and elution patterns refer to use of the DB-17 column. High-resolution mass spectra were obtained using a VG 7070F instrument in the EI mode at an ionizing voltage of 70 eV. Melting points are uncorrected.

Ants

Megalomyrmex modestus workers were collected on the summit (1800 m) of Ayan Tepui, Estado Bolivar, Venezuela. *M. leoninus* workers were collected east of Caracas, Estado Miranda, Venezuela. Workers and ergatoids (reproductives) of *M. goeldii* were obtained in the south of Bahia, Brazil. Ants or venom (*M. leoninus*) were placed in methylene chloride, and these extracts were used directly for chemical analyses. Specimens of all ants were deposited in the collection of the Los Angeles County Museum of Natural History, Los Angeles, California, and have been accessed by Roy R. Snelling. The alkaloids found in the ants are listed in Table 1. The structures of **1–4** (Figure 1) were assigned by comparison of their mass spectra and gas chromatographic retention times with those of authentic samples. Analysis of the extracts of *M. modestus* revealed the presence of a single volatile component whose mass spectrum had important ions at *m/z* 251(M+), 194(80), and 166(100) and was unchanged after attempted hydrogenation (H_2, PtO_2 in ethanol). The gas chromatographic retention time, mass spectrum, and infrared spectrum of this compound were identical to those of **5c/5d** (Scheme 1) prepared below.

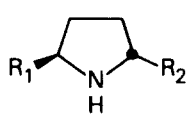
Hofmann Degradation of the M. modestus *Extract*

The solvent was removed from a small portion of the extract from *M. modestus*, and the residue was heated to 100°C in a sand bath in a closed vial with a few drops of methyl iodide for 1 hr. After removal of the methyl iodide in vacuo, the residue was treated with 0.2 ml of 1.6 M *n*-butyllithium in hexane and stirred for 24 hr. After the addition of three drops of saturated $NaHCO_3$, the mixture was stirred for 0.5 hr, and the hexane solution was added to 0.3 ml of hexane containing 25 mg of PtO_2, which had been freshly hydrogenated. Hydrogen was bubbled slowly through the mixture for 0.5 hr. GC-MS analysis of the resulting mixture using selected ion monitoring for **5**, **8**, and **10** revealed only an equivalent amount of **5** (*m/z* 194, 166) and the second eluting isomer

TABLE 1. ALKALOIDS FOUND IN *Megalomyrmex* SPECIES

Species	Alkaloids[a]				
	1	**2**	**3**	**4**	**5d**
M. leoninus	–	*	–	–	–
M. goeldii (workers)	0	*	+	*	–
M. goeldii (ergatoids)	*	+	*	+	–
M. modestus	–	–	–	–	*

[a] * = major component; + = minor component (< 15%); 0 = trace; – = not detected.



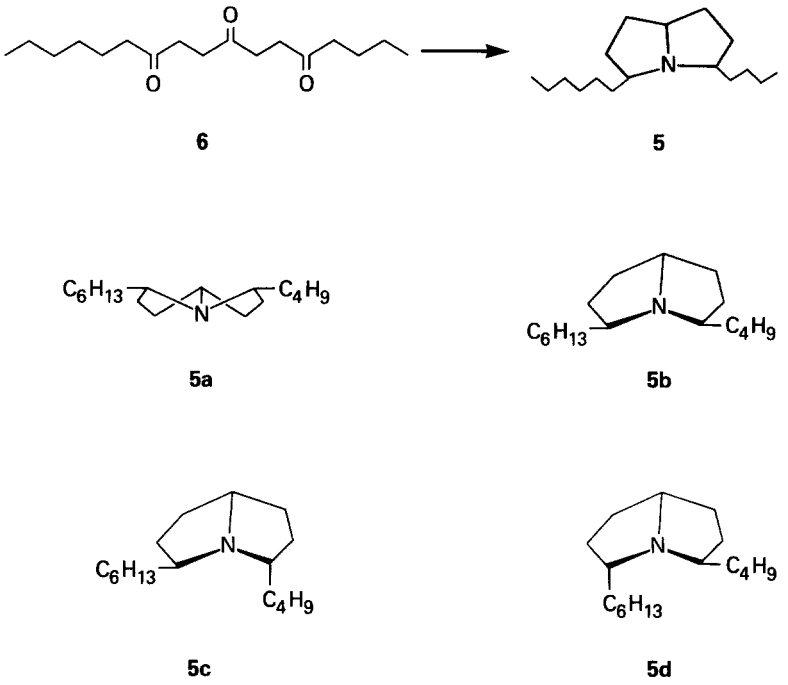
1 $R_1 = C_4H_9$, $R_2 = C_5H_{11}$

2 $R_1 = C_4H_9$, $R_2 = C_7H_{15}$

3 $R_1 = C_6H_{13}$, $R_2 = C_5H_{11}$

4 $R_1 = C_6H_{13}$, $R_2 = C_7H_{15}$

FIG. 1. *trans*-2,5-Dialkylpyrrolidines from *M. leoninus* and *M. goeldii*.



SCHEME 1.

of **8** (*m/z* 140, 210), no ions attributable to **10** were detected. The retention times and mass spectra of these peaks confirmed their identities.

Synthesis of 3-Butyl-5-hexylpyrrolizidine

5,8,11-Heptadecatrione (6). A solution containing 0.5 g of 4-oxooctanal (3.6 mmol), 0.5 g of 1-nonen-3-one (3.6 mmol), 0.1 g of 5-(2-hydroxymethyl)-4-methyl-3-benzylthiazolium chloride, and 2 ml of triethylamine was heated to reflux under a nitrogen atmosphere overnight. After cooling, the mixture was taken up in ether and filtered through a short Florisil column. The solvent was

removed, and Kugelrohr distillation at 250°C (0.05 mm Hg) gave 0.6 g (60% yield of **6**; [^{1}H]NMR δ = 2.7(8H, br s), 2.1(4H, t, J = 6 Hz), 1.55(4H, m), 1.25(8H, br m), 0.85(6H, m); [^{13}C]NMR δ = 209.73(2C), 208.16, 42.82, 42.51, 36.12(4C), 31.59, 28.89, 25.97, 23.83, 22.49, 22.33, 14.02, 13.83; MS *m/z* (relative intensity) 282(1, M+), 240(3), 225(3), 222(2), 212(6), 197(12), 194(5), 169(41), 141(42), 127(26), 113(30), 85(53), 57(64), 55(44), 43(100); HRMS *m/z* 282.2186 ($C_{17}H_{30}O_3$, M+, calcd. 282.2195).

3-Butyl-5-hexylpyrrolizidine ***(5).*** (A) A solution containing 0.35 g of triketone **6** (1.25 mmol) in 10 ml of methanol was subjected to the usual reductive amination conditions (0.1 g NH_4OAc, 0.1 g $NaCNBH_3$, 0.02 g KOH, 12 hr) and worked up to provide 0.2 g of a mixture of three components in a 1:6:1.5 ratio with identical mass spectra: MS *m/z* (relative intensity) 251(2, M+), 250(2), 222(2), 195(12), 194(80), 167(12), 166(100), 138(2), 126(2), 124(2), 122(2), 109(4), 108(4), 82(5), 81(6), 70(5), 69(5), 68(8), 67(8), 55(10), 43(5), and 41(8). A variety of gas chromatographic columns (DB-17, DB-1, DB-FFAP, and DB-wax) and conditions were employed in unsuccessful attempts to separate all four possible stereoisomers produced in this reaction. The FTIR spectrum of the first eluting components **5a** had distinctive bands at 2798 and 2707 cm^{-1}, and the following spectral data were also obtained: **(5a)** [^{1}H]NMR δ = 2.5(1H, m), 2.2(2H, m), 1.8–1.2(24H, complex m), 0.86(6H, br t); [^{13}C]NMR δ = 72.16, 60.02(2C), 35.78(4C), 31.89, 29.65, 29.00, 26.75, 25.59(2C), 23.02, 22.06, 14.05(2C); second eluting (major) component **(5b)**; [^{1}H]NMR δ = 3.55 (1H, m), 2.6(2H, m) 1.9(4H, m), 1.5–1.1(20H, m), 0.85(6H, br t); [^{13}C]NMR δ = 66.80(2C), 64.63, 37.06, 36.75, 31.95, 31.75(4C), 29.61, 29.37, 27.14, 22.99, 22.67, 14.13(2C), third eluting component **(5c** and **5d):** [^{1}H]NMR δ = 3.6(1H, m), 3.0(1H, m), 2.88(1H, m), 1.7–2.0(4H, m), 1.6–1.1(20H, m), 0.85(6H, br t); [^{13}C]NMR δ = 66.05(br), 64.01, 58.06(br), 38.2(br), 32–29(complex m), 28.23, 27.05, 22.96, 22.88, 22.61, and 14.06; HRMS *m/z* 251.2622 ($C_{17}H_{33}N$, M+, calcd. 251.2613). (B). A mixture containing 0.1 g of **6,** 0.5 g of ammonium acetate, 0.09 g of 5% rhodium on carbon catalyst, and 2 ml of conc. ammonium hydroxide in 50 ml of ethanol was hydrogenated at 3 atm of pressure for 12 hr. The mixture was filtered through celite, and the solvent removed in vacuo. The residue was taken up in ether and dried over anhydrous potassium carbonate. Analysis revealed the presence of ca. 60% of three isomeric components in a 14:5:1 ratio with gas chromatographic retention times, IR, and mass spectra identical to those prepared above.

Synthesis of Pyrrolidine Standards

*2-Butyl-*N*-methyl-5-nonylpyrrolidine* ***(8).*** A mixture containing 1.0 g of 16-heptadecen-5,8-dione (Jones et al., 1988) and 200 mg of 10% Pd/C in 50 ml of EtOH was hydrogenated at 3 atm of pressure for 24 hr. After filtration,

the solvent was removed to provide 0.74 g of 5,8-heptadecadione **(7)**, mp 53–55°C: [^{1}H]NMR δ = 2.68(4H, s), 2.44(4H, t, J = 7 Hz), 1.58(4H, m), 1.3 (14H, m), 0.84(6H, br t); [^{13}C]NMR δ = 209.76, 209.72, 42.91, 42.59, 36.02(2C), 31.86, 29.40(2C), 29.25(2C), 25.97, 23.88, 22.65, 22.33, 14.07, 13.81; MS *m/z* 268(1, M+), 239(2), 226(7), 211(8), 183(16), 169(9), 157(5), 156(40), 155(20), 141(59), 127(35), 114(51), 113(39), 95(27), 85(100), 71(77), 57(99), 55(6); HRMS *m/z* 268.2408 ($C_{17}H_{32}O_2$ M+, calcd. 268.2402). A solution containing 0.27 g (1 mmol) of **7**, 0.13 g of methylamine hydrochloride, and 0.15 g of $NaCNBH_3$ in 10 ml of MeOH was stirred for 48 hr. After the addition of 0.1 g of $NaBH_4$, the mixture was stirred for 0.5 hr and worked up in the usual manner. GC-MS analysis showed the presence of two components in a 5 : 1 ratio with identical mass spectra: MS *m/z* 267(1, M+), 266(2), 211(9), 210(54), 141(10), 140(100), 98(9), 83(8), 82(12), 69(3), 55(15); HRMS *m/z* 267.2932 ($C_{19}H_{37}N$ M+, calcd, 267.2926). Upon isolation by preparative gas chromatography, magnetic resonance spectra were obtained for each component. First eluting component: [^{1}H]NMR δ = 2.25(3H, s), 2.04(2H, m), 1.84(2H, m), 1.7(2H, m), 1.3(22H, m), 0.9 (6H, br t); [^{13}C]NMR δ = 67.80(2C), 39.02, 34.53, 34.20, 31.90, 30.04, 29.64, 29.59, 29.33, 29.04(2C), 28.94, 26.75, 23.08, 22.67, 14.08(2C); second eluting component: [^{1}H]NMR δ = 2.80(2H, m), 2.38(3H, s), 1.95(2H, m), 1.6–1.1(24H, m), 0.9(6H, br t); [^{13}C]NMR δ = 63.38(2C), 36.15, 31.90, 30.58, 30.27, 30.02, 29.68, 29.58, 29.33, 29.10, 28.73(2C), 26.89, 23.05, 22.67, 14.10(2C).

2-Heptyl-5-hexyl-N-*methylpyrrolidine* ***(10)***. A mixture containing 2.2 g (16 mmol) of 1-nonen-3-one (Jones et al., 1989), 2.04 g of octanal, 0.25 g of 5-(2-hydroxymethyl)-4-methyl-3-benzylthiazolium chloride and 3 ml of triethylamine was heated to reflux under a nitrogen atmosphere overnight. The cooled mixture was taken up in ether, filtered through a short Florisil column, and after removal of the solvent, the residue was recrystallized from cold MeOH to give 2.5 g (58% yield) of 7,10-heptadecadione **(9)**, mp 63°C: [^{1}H]NMR δ = 2.68(4H, s), 2.45(4H, t, J = 7.0 Hz), 1.6(4H, m), 1.3(14 H, m), 0.9(6H, br t); [^{13}C]NMR δ = 209.65(2C), 42.90(2C), 36.03(2C), 31.66, 31.58, 29.19, 29.03, 28.89, 23.90, 23.85, 22.58, 22.46, 14.0, 13.96; MS *m/z* 268(1, M+), 198(10), 184(15), 183(11), 155(17), 141(22), 137(50), 114(41), 113(38), 109(14), 85(25), 81(15), 71(40), 69(17), 57(100), 56(11), 55(43); HRMS *m/z* 268.2403 ($C_{17}H_{32}O_2$ M+, calcd. 268.2402). Reductive amination of 1 mmol of **9** with methylamine hydrochloride in the same manner as for **7** provided a similar mixture of two components with identical mass spectra; MS *m/z* 267(1,M+), 266(2), 183(13), 182(88), 169(13), 168(100), 96(13), 82(15), 70(9), 69(10), 55(15); HRMS *m/z* 267.2939 ($C_{18}H_{37}N$ M+, calcd. 267.2926). First eluting component: [^{1}H]NMR δ = 2.25(3H, s), 2.02(2H, m), 1.85(2H, m), 1.68(2H, m), 1.3(22H), 0.85(6H, br t); [^{13}C]NMR δ = 67.86(2C), 39.03, 34.51(2C), 31.90(2C), 30.03, 29.73, 29.33, 29.06(2C), 26.79, 26.75, 22.69,

22.65, 14.11(2C); second eluting component: [^{1}H]NMR δ = 2.8(2H, m), 2.40(3H, s), 1.95(2H, m), 1.6–1.2(24H, m), 0.9(6H, br t); [^{13}C]NMR δ = 63.43(2C), 35.12, 31.88, 31.84, 30.55(2C), 29.96, 29.65, 29.32, 28.71(2C), 26.88, 26.84, 22.62(2C), 14.06(2C).

RESULTS

Examination of methylene chloride extracts of *Megalomyrmex leoninus* and *M. goeldii* revealed the presence of known *trans*-2,5-dialkylpyrrolidines **1–4** (Table 1, Figure 1) (Jones et al., 1989), whose structures and stereochemistry were confirmed by comparison with available authentic samples. In the case of *M. goeldii*, collections of workers and ergatoids were available; the compositions of the mixtures of alkaloids from the castes were somewhat different. The workers of this species produce mostly *trans*-2-butyl-5-heptylpyrrolidine **(2)** and *trans*-2-heptyl-5-hexylpyrrolidine **(4),** with smaller amounts of **1** and **3,** while the ergatoids produce mostly *trans*-2-butyl-5-pentylpyrrolidine **(1)** and *trans*-2-hexyl-5-pentylpyrrolidine **(3)**, with smaller amounts of **2** and **4.**

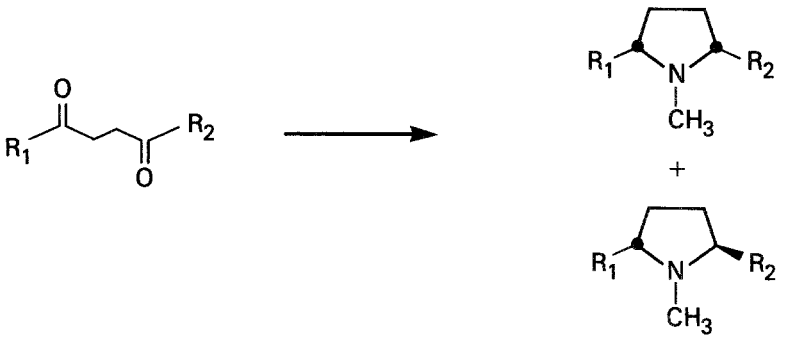
In contrast to the previously described alkaloids found in *M. leoninus* and *M. goeldii*, GC-MS analysis of the methylene chloride extract of *M. modestus* revealed the presence of one major volatile component whose mass spectrum had important ions at *m/z* 251 (M+), 194(80), and 166(100). These intense fragment ions show the loss of C_4H_9 and C_6H_{13} from a nucleus of $C_7H_{11}N$. This spectrum was unchanged after catalytic hydrogenation, indicating that the two units of unsaturation implicit in the nucleus are due to the presence of a bicyclic system. The 3,5-disubstituted pyrrolizidine ring system is typical of such compounds that occurs in other myrmicine species (Jones et al., 1980).

The overall carbon–nitrogen skeleton of the likely candidate, 3-butyl-5-hexylpyrrolizidine **(5)** was confirmed by synthesis (Scheme 1). The condensation of 4-oxooctanal (Kulinkovich et al., 1985) and 1-nonen-3-one (Jones et al., 1989) in the presence of a thiazolium salt catalyst provided 5,8,11-heptadecatrione **(6).** Reductive amination of **6** with sodium cyanoborohydride and ammonium acetate formed pyrrolizidine **5** in good yield. In the case of unsymmetrical triketones, this methodology produces all four possible pyrrolizidine stereoisomers (Jones et al., 1980, 1988) (Scheme 1); however, in the case of **5,** only three gas chromatographic peaks in a 1 : 6 : 1.5 ratio with identical mass spectra could be observed. The first and second eluting components of the synthetic mixture could be assigned as (5*Z*,8*Z*)-3-butyl-5-hexylpyrrolizidine **(5a)** and (5*Z*,8*E*)-3-butyl-5-hexylpyrrolizidine **(5b)** on the basis of their spectral data and relative retention times (Jones et al., 1980, 1988). The mass spectrum and gas chromatographic retention time of the alkaloid from *M. modestus* were identical to those of the third eluting component.

The [^{1}H]- and [^{13}C]NMR spectra of the third eluting component confirmed the presence of either or both of two diastereomers, (5*E*,8*Z*)-3-butyl-5-hexylpyrrolizidine **(5c)** and (5*E*,8*E*)-3-butyl-5-hexylpyrrolizidine **(5d).** Along with the usual signal at δ = 3.6 for the C-8 proton, the [^{1}H]NMR spectrum of this component had distinct signals at δ = 3.0 and 2.88 for the methine protons on C-3 and C-5 carbons attached, respectively, to alkyl groups *endo* and *exo* to the *cis*-fused pyrrolizidine ring system. In contrast with the two [^{13}C]NMR signals observed for **5b** at δ = 66.78 (C-3 and C-5) and 64.62 (C-8), in this component the three methine signals appeared at δ = 66.05, 64.01, and 58.06 ppm. While these data indicated that one of the alkyl groups was *endo* and the other *exo*, the lack of splitting of these signals due to the similarity of the butyl and hexyl groups at C-3 or C-5 made it impossible to distinguish **5c** and **5d** in this component.

In an unsuccessful attempt to prepare a higher proportion of **5c/5d,** the reductive amination of **6** was conducted under hydrogenation conditions using 5% Rh/C as a catalyst. It is noteworthy that this procedure formed the three pyrrolizidine components **5a, 5b,** and **5c/5d** in a 14:5:1 ratio and is the most efficient preparation of the *trans*-fused (5*Z*,8*Z*)-3,5-dialkylpyrrolizidines reported to date.

Since the gas chromatographic retention time of the alkaloid from *M. modestus* was identical to that of **5c** or **5d,** a selective Hofmann degradation was carried out on a small portion of the *M. modestus* extract in order to determine the stereochemistry of the natural pyrrolizidine. After quaternization with methyl iodide, cleavage of the one of either of two of the C—N bonds (Figure 2) would be expected to provide 2,5-dialkyl-*N*-methylpyrrolidines with side chain unsaturation, which could subsequently be removed by hydrogenation without altering their stereochemistry. With this in mind, the two possible final products, 2-butyl-*N*-methyl-5-nonylpyrrolidine **(8)** and 2-heptyl-5-hexyl-*N*-methylpyrrolidine **(10)** were prepared by reductive amination of the corresponding 1,4-diketones with methylamine in the presence of sodium cyanoborohydride (Borch, et al., 1971) (Scheme 2). The *cis* and *trans* isomers of **8** and **10** were isolated and their stereochemistry assigned from their magnetic resonance spectra. The ^{13}C spectra of the first eluting *cis* isomers of **8** and **10** show a resonance at δ = 67.8 for the methine carbons, while the later-eluting *trans* isomers show a resonance at δ = 63.4 for these carbons (Gessner et al., 1987). With the stereoisomers of **8** and **10** characterized, a small portion of the extract from *M. modestus* was quaternized and the residue was then stirred with an excess of *n*-butyllithium (Bach et al., 1974). After hydrogenation, GC-MS analysis using selected ion chromatography for the α-cleavage ions of **5, 8,** and **10,** revealed the presence of the original natural pyrrolizidine, possibly from the elimination of the quaternary methyl group, together with *trans*-**8.** Ions at *m/z* 168 and 182, indicating the presence of **10,** were not detected. These results



7: $R_1 = n-C_4H_9$, $R_2 = n-C_9H_{19}$ **8:** $R_1 = n-C_4H_9$, $R_2 = n-C_9H_{19}$

9: $R_1 = n-C_6H_{13}$, $R_2 = n-C_7H_{15}$ **10:** $R_1 = n-C_6H_{13}$, $R_2 = n-C_7H_{15}$

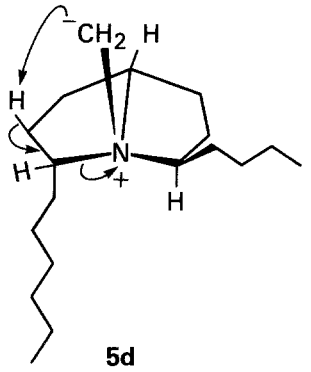
SCHEME 2.

show that the natural pyrrolizidine from *M. modestus* is (8*E*,5*E*)-3-butyl-5-hexylpyrrolizidine **(5d).**

On closer inspection, the regiospecificity as well as the stereoselectivity of the Hofmann degradation is evidence for the stereochemistry of **5d.** Thus, the stereoselectivity of the elimination reaction of quarternary ammonium salts with alkyllithium bases has been attributed to the formation of an ylid intermediate followed by intramolecular *syn* proton abstraction (Bach et al., 1974). In these elimination reactions, pyrrolidine ring protons would be expected to be more readily abstracted by the base than acyclic side chain protons (Jewers and McKenna, 1958), so that in 3,5-dialkylpyrrolizidines the base would attack the 2 or 6 positions. In **5d** the 2-*exo* proton is blocked by the *exo* 3-butyl group while the 6-*exo* proton is accessible to the ylid anion (Figure 2), resulting in exclusive formation of *trans*-**8** after hydrogenation.

DISCUSSION

Megalomyrmex joins the genera *Monomorium* and *Solenopsis* as the third myrmicine genus whose members are known to produce alkaloidal venoms. All three genera are members of the *Solenopsis* genus-group, and they constitute about 95% of the recognized species (Bolton, 1987). Significantly, alkaloids of the *Megalomyrmex* species are similar to that of species of both *Monomorium*



5d

FIG. 2. Regiospecific ring opening of the **5d** methiodide.

and *Solenopsis* (Jones et al., 1982). Some species in the *Solenopsis* subgenus *Diplorhoptrum* produce some of the 2,5-dialkylpyrrolidines identified from *Megalomyrmex goeldii* (Pedder et al., 1976), while other *Diplorhoptrum* species produce venoms containing single alkaloids **2** or **3** comparable to *Megalomyrmex leoninus* (Jones et al., 1979; Blum et al., 1980). On the other hand, the 2,5-dialkylpyrrolidines **1–4** identified in *M. goeldii* and *M. leoninus* recently have been identified in a *Monomorium* species (Jones et al., 1989). Indeed, *Monomorium* species characteristically produce 2,5-dialkylpyrrolidines in their venoms, and the occurrence of these compounds in *Megalomyrmex* species may be taxonomically significant.

Analyses of the alkaloids produced by workers and ergatoids of *M. goeldii* clearly demonstrate that each caste has a distinctive quantitative alkaloidal fingerprint (Table 1). The major compounds produced by workers of *M. goeldii*, **2** and **4**, are minor or trace constituents of the ergatoids. On the other hand, the venom of the ergatoid queens is dominated by **1** and **3** and contains **2** and **4** as minor components. Similar differences in the composition of worker and queen venoms have been observed previously in *Solenopsis* species.

Solenopsis (Solenopsis) species produce venoms containing *cis*- and *trans*-2,6-dialkylpiperidines in characteristic proportions (Brand et al., 1973a,b). In these ants, the venoms of queens typically contain the isomers of a single alkaloid in a relatively fixed ratio. On the other hand, the ratios of the isomers of this compound are very different in the venoms of the workers. Furthermore, in the case of some *Solenopsis* species, qualitative differences in alkaloids also distinguish the venoms of workers and queens from each other (Brand et al., 1973a,b).

In *Megalomyrmex* and *Solenopsis* species producing venoms that differ between workers and queens, the distinctive venoms produced by the two castes must reflect the emphasis that each places on selected alkaloids in the colonial milieu. For example, the alkaloids produced by the ergatoids of *M. goeldii* are

structurally similar to *trans*-2,5-dialkylpyrrolidines that have been demonstrated to have substantial insecticidal, antibiotic, and repellent activities (Escoubas and Blum, 1990; Cutler and Blum, unpublished data; Blum et al., 1991). The marked biological activities of these alkaloids could be highly adaptive in the nest environment and could provide ergatoid queens with a means of inoculating eggs with fungicides that offer protection against invasive spores for example. It will not prove surprising if the venom compositions of workers and queens are identified with the adaptiveness of these secretions vis-à-vis specific competitors.

The genus *Megalomyrmex* has been revised into four species groups (Brandão, 1990), and it seems worthwhile to compare the alkaloids produced by these species. For example, although *M. goeldii* and *M. modestus*, two species in the Modestus group, produce venoms that are qualitatively distinct, the pyrrolidine **4** may be regarded as a monocyclic analog of the pyrrolizidine **5d.** While **5d** occurs alone in *M. modestus*, its formation may be envisioned by the closure of its N—C-3 bond from a "*cis*-**4**"-like precursor; it may be significant that this species has been included in the same species group as *M. goeldii*, which produces *trans*-**4** (Brandão, 1990). In *Monomorium* species that produce 3,5-dialkylpyrrolizidines in their venom, concomitant *trans*-2,5-dialkylpyrrolidines, isomeric with the *cis*-2,5-dialkylpyrrolidines that would result from cleavage of one ring of the bicyclic system, have been detected (Jones et al., 1988). These observations suggest that the bicyclic alkaloids may be produced in ants when the initially formed ring is *cis* disubstituted (Jones et al., 1990). Significantly, the genera *Megalomyrmex* and *Monomorium* are considered to be closely related (Bolton, 1987), so that similarities in their venom biochemistry may not be unexpected. Obviously, in the absence of data on the alkaloids produced by other *Megalomyrmex* species, it is impossible to generalize about the chemistry of the species groups.

The production of alkaloids by *Megalomyrmex* species offers optimistic grounds for regarding other genera in the *Solenopsis* genus-group as sources of nitrogen heterocycles. Analyses of species in additional genera may provide valuable data on the biochemical evolution of alkaloidal venoms in the largest of the ant subfamilies, the Myrmicinae.

Acknowledgments—We thank Dr. R.J. Highet of the Laboratory of Biophysical Chemistry, NHLBI, for the NMR spectra, and Noel Whittaker of the Laboratory of Bioorganic Chemistry, NIDDK, for the high-resolution mass spectra.

REFERENCES

BACH, R.D., ANDRZEJEWSKI, D., and BAIR, K.W. 1974. Nitrogen ylid intermediates in elimination reactions of quaternary ammonium salts. *J. Chem. Soc. Chem. Commun.* 1974:820–822.

BLUM, M.S. 1985. Poisonous ants and their venoms, pp. 225–242, *in* A.T. Tu (ed.). Handbook

of Natural Toxins 2, Insect Poisons, Allergens, and Other Invertebrate Venoms. Marcel Dekker, New York.

BLUM, M.S., JONES, T.H., HOLLDOBLER, B., FALES, H.M., and JAOUNI, T. 1980. Alkaloidal venom mace: Offensive use by a thief ant. *Naturwissenschaften* 67:144–145.

BLUM, M.S., EVERETT, D.M., JONES, T.H., and FALES, H.M. 1991. Arthropod natural products as insect repellents, pp. 14–26, *in* P.A. Hedin (ed.). Naturally Occurring Pest Bioregulators. ACS Symposium Series 449. American Chemical Society, Washington, D.C.

BOLTON, B., 1987. A world review of the *Solenopsis* genus group and revision of afrotropical *Monomorium* Mayr. *Bull. Br. Mus. (Nat. Hist.) (Entomol.)* 54:263–452.

BORCH, R.F., BERNSTEIN, M.D., and DURST, H.D. 1971. The cyanohydrididoborate anion as a selective reducing agent. *J. Am. Chem. Soc.* 93:2897–2904.

BRAND, J.M., BLUM, M.S., and BARLIN, M.R. 1973a. Fire ant venoms: Intraspecific and interspecific variation among castes and individuals. *Toxicon* 11:325–331.

BRAND, J. M., BLUM, M.S., and ROSS, H.H. 1973b. Biochemical evolution in fire ant venoms. *Insect Biochem.* 3:45–51.

BRANDÃO, C.R.F. 1990. Systematic revision of the neotropical ant genus *Megalomyrmex* Forel (Hymenoptera: Formicidae: Myrmicinae). *Arq. Zool.* 31:411–481.

ESCOUBAS, P., and BLUM, M.S. 1990. The biological activities of ant-derived alkaloids, pp. 482–489, *in* R.K. Vander Meer, K. Jaffe, and A. Cedeno (eds.). Applied Myrmecology. Westview Press, Boulder, Colorado.

GESSNER, W., TAKAHASHI, K., BROSSI, A., KOWALSKI, M., and KALINER, M.A. 1987. Synthesis of (±)-*trans*-2,5-dialkylpyrrolidines from the Lukes-Sorm dilactam. *Acta Chim. Helv.* 70:2003–2010.

JEWERS, K., and MCKENNA, J. 1958. Stereochemical investigations of cyclic bases. *J. Chem. Soc.* 1958:2209–2217.

JONES, T.H., BLUM, M.S., and FALES, H.M. 1979. Synthesis of unsymmetrical 2,5-di-*n*-alkylpyrrolidines: 2-Hexyl-5-pentylpyrrolidine from the thief ants *Solenopsis molesta*, *S. texanus*, and its homologues. *Tetrahedron Lett.* 1979:1031–1034.

JONES, T.H., BLUM, M.S., FALES, H.M., and THOMPSON, C.R. 1980. (5*Z*,8*E*)-3-Heptyl-5-methylpyrrolizidine from a thief ant. *J. Org. Chem.* 45:4778–4780.

JONES, T.H., BLUM, M.S., and FALES, H.M. 1982. Ant venom alkaloids from *Solenopsis* and *Monomorium* species. *Tetrahedron* 38:1949–1958.

JONES, T.H., STAHLY, S.M., DON, A.W., and BLUM, M.S. 1988. Chemotaxonomic implications of the venom chemistry of some *Monomorium "antarcticum"* populations. *J. Chem. Ecol.* 14:2197–2212.

JONES, T.H., BLUM, M.S., ESCOUBAS, P., and MUSTHAK ALI, T.M. 1989. Novel pyrrolidines in the venom of the ant *Monomorium indicum*. *J. Nat. Prod.* 52:779–784.

JONES, T.H., LADDAGO, A., DON, A.W., and BLUM, M.S. 1990. A novel (5*E*,9*Z*)-dialkylindolizidine from the ant *Monomorium smithii*. *J. Nat. Prod.* 53:375–381.

KULINKOVICH, O.G., TISCHENKO, I.G., SOROKIN, V.L. 1985. A new and convenient method of synthesis of γ-ketoaldehydes. *Synthesis* 1985:1058–1059.

PEDDER, D.J., FALES, H.M., JAOUNI, T., and BLUM, M.S. 1976. Constituents of the venom of a South African fire ant (*Solenopsis punctaticeps*). Tetrahedron 32:2275–2279.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

CHEMICAL CONTENTS OF STINGING TRICHOMES OF *Cnidoscolus texanus*

S.E. LOOKADOO and A.J. POLLARD*

Department of Biology
Furman University
Greenville, South Carolina 29613

(Received January 14, 1991; accepted May 28, 1991)

Abstract—Plants known as nettles bear trichomes that deliver a painful sting when touched. Studies of members of the Urticaceae have suggested that their trichomes may contain histamine, acetylcholine, and serotonin. We report investigations using TLC, HPLC, and UV-VIS spectrophotometry to analyze the hair fluids of *Cnidoscolus texanus*, a member of the Euphorbiaceae, along with standards of known histamine, acetylcholine, and serotonin. Of these, only serotonin was detected in hair extracts. Up to four other compounds were detected and partially characterized, but they have not been identified.

Key Words—Serotonin, nettles, trichomes, stinging hairs, plant defense mechanisms, *Urtica dioica*, *Cnidoscolus texanus*.

INTRODUCTION

This study presents a partial analysis of the fluids contained within the stinging trichomes of *Cnidoscolus texanus* (Muell. Arg.) Small (Euphorbiaceae), a nettle native to the southwestern United States and adjacent Mexico. Nettles are plants that bear stinging trichomes (stinging hairs) and are found in four plant families: Urticaceae, Euphorbiaceae, Loasaceae, and Hydrophyllaceae. The basic structure of stinging trichomes consists of a multicellular pedestal upon which is a single elongate, tapering cell with a slightly swollen tip (Thurston and Lersten, 1969). These hairs are known to contain chemicals that serve as defenses against herbivores (Pollard and Briggs, 1984). When an animal brushes against the plant, the tip of the hair fractures, the broken end of the hair (often beveled like

*To whom correspondence should be addressed.

0098-0331/91/0900-1909$06.50/0

a hypodermic needle) penetrates the skin, and the contents are expelled. The results of the sting vary, depending on the species, from mild discomfort and swelling about the area of penetration, to much more serious effects (Lutz, 1914; Francis, quoted in MacFarlane, 1963) including reported human fatalities (Winkler, 1922).

Most studies of the chemical contents of nettle trichomes have focused on members of the Urticaceae. Early work using in vitro bioassay techniques (Emmelin and Feldberg, 1947, 1949; Collier and Chesher, 1956), concluded that hairs of *Urtica* and related genera contained histamine, acetylcholine, and serotonin (5-hydroxytryptamine). Although they are frequently cited, these conclusions have been questioned by studies using more modern instrumentation (Robertson and MacFarlane, 1957; MacFarlane, 1963; Willis, 1969; Oelrichs and Robertson, 1970; Thurston, 1974). Very little research has considered the stinging chemicals of plants outside the Urticaceae. A study of *Cnidoscolus oligandrus* in the Euphorbiaceae (Cordeiro et al., 1983) reports the presence of histamine. On the other hand, Willis (1969) concluded that neither *Cnidoscolus stimulosus* nor several species in the Urticaceae contained any of the above-mentioned compounds and suggested that all shared an unidentified high-molecular-weight compound that might be responsible for the sting.

This study considers another member of the Euphorbiaceae, *Cnidoscolus texanus*, commonly known as bull nettle or spurge nettle. The species was chosen for several reasons. Previous research has suggested that its stinging hairs represent a defensive adaptation against mammalian herbivores (Pollard, 1986). As stated above, the few existing studies of *Cnidoscolus* spp. have generated contradictory results. The plant has a high number of very large trichomes (3–8 mm in length) covering its entire aboveground surface area, making it possible to obtain extracts that consist only of chemicals from the trichomes, free of contamination from other plant structures. Characterization of extracts was attempted using thin-layer chromatography, high-performance liquid chromatrography, and UV–visible spectrophotometry, in comparison with known standards of histamine, acetylcholine, and serotonin.

METHODS AND MATERIALS

Chemicals. Standards of acetylcholine HCl, histamine, and 5-hydroxytryptamine HCl were all obtained from Sigma Chemical Company, as were TLC indicator solutions. All other chemicals and solvents were either HPLC- or reagent-grade materials. Stock solutions at 0.01 M were made using methanol for acetylcholine and serotonin, and 1 : 1 methanol–acetone for histamine. In UV-VIS spectrophotometry, 0.01 M aqueous solutions also were used.

Plant Extracts. *Cnidoscolus texanus* specimens originated in populations near the Cimarron and Red rivers in Oklahoma. Hairs were obtained from live specimens (three genotypes) maintained in the Furman University greenhouses or from dried field-collected specimens (massed collections from many individuals). Hairs were carefully scraped from the stems and petioles using a razor blade and sieved to remove any extraneous plant material. In order to provide sufficient material, hair samples from several genotypes were combined and ground using a mortar and pestle. Ground hairs were placed in 30 ml of extraction solvent, vortexed for 20 min, centrifuged for 5 min at 2400 rpm, and the supernatant pipetted off for concentration and analysis.

Three extraction protocols were used: (1) extraction in 1 : 1 methanol–acetone solvent and concentration by drying to 1.5 ml under nitrogen; (2) extraction in deionized water, lyophilization to dryness, and reconstitution in 1.5 ml of 1 : 1 methanol–acetone solvent; and (3) extraction in deionized water, lyophilization to dryness, and reconstitution in 1.5 ml of deionized water. Aqueous samples (protocol 3) were used in UV-VIS spectrophotometry. Organic samples (protocols 1 and 2) were used in chromatographic techniques, with the difference being that protocol 2 extracted only water-soluble compounds from the plants, while protocol 1 could extract organic-soluble materials not in solution in intact stinging trichomes (e.g., epicuticular waxes).

Thin-Layer Chromatography. TLC was performed using both silica gel and Avicel microcrystalline cellulose plates, both with a 250-μm coating and containing 254 nm fluorescent indicator, in a mobile phase of 12 : 3 : 5 *n*-butanol–acetic acid–water. Extracts were spotted in parallel with standards (diluted to 0.001 M) of histamine, acetylcholine, and serotonin. Specific indicator solutions used in development of plates were ninhydrin for histamine, Dragendorff's reagent for acetylcholine, and Ehrlich's reagent for serotonin. For more general visualization of spots, plates were examined under ultraviolet light and developed with iodine vapor. Tracings were made of all plates, and R_f values measured.

UV-VIS Spectrophotometry. Spectra were determined using a Beckman DU-50 spectrophotometer, for both organic and water extracts, along with standards also dissolved in organic solvents or water. Solvent background runs were also performed. All spectra were measured from 200 to 700 nm at a scan speed of 750 nm/min.

High-Performance Liquid Chromatography. HPLC was performed using a system of Beckman pumps and controller, with Hewlett-Packard UV diode array detector and data processing programs. In all cases the column used was a Beckman 5-μm C_{18} Ultrasphere all-purpose column. Two HPLC protocols were employed: (1) a 4.6-mm × 25-cm column with 1-μl injection loop, using a 60-min program, which gave a gradient of 100% water to 100% methanol, at a rate of 1 ml/min; and (2) a 10-mm × 25-cm column with 1-ml injection loop,

a rate of 1 ml/min; and (2) a 10-mm × 25-cm column with 1-ml injection loop, run for 60 min at 0.7 ml/min in a set eluent of 90% reagent alcohol (9:5:5 ethanol–methanol–2-propanol) plus 10% hexane.

RESULTS

Based on comparisons of fresh and dry weights, each *C. texanus* hair contained an average of 0.97 mg of fluid. The pH of a slurry of crushed hairs and deionized water was 5.0. In no case was any difference noted between the results for hairs from fresh specimens and those from dried specimens; removal of hairs from dried specimens was considerably easier.

Table 1 lists R_f values from TLC of standard solutions. Silica gel plates provided good identification of serotonin but poor separation of histamine and acetylcholine, while cellulose plates provided good identification of histamine but poor separation of acetylcholine and serotonin. Staining reactions using indicator solutions were as expected, with particularly clear visualization of acetylcholine. TLC of *Cnidoscolus* extracts gave three to four spots, with no consistent differences between aqueous and organic extraction protocols. Separations were generally clearer on silica gel than on cellulose. Table 2 shows R_f values and responses to indicator solutions.

UV-VIS spectrophotometry revealed, for both organically dissolved and aqueous histamine and acetylcholine, absorption maxima at approximately 220 nm. Organic solutions of serotonin revealed a more complex absorption pattern, with maxima at approximately 220 nm, 320 nm, and 360 nm. Aqueous serotonin, however, did not absorb above 320 nm. Spectra for *Cnidoscolus* extracts behaved much like those for serotonin, with similar peaks in organic solution and loss of the high-wavelength peaks in aqueous solution. Because UV-VIS spectra had no peaks in the visible range, and otherwise resembled very closely the spectra from the HPLC UV-diode array detector, only the latter have been reproduced here.

The two HPLC protocols produced similar results (elution times given here

TABLE 1. R_f VALUES[a] FROM TLC OF STANDARDS

Standard	Silica gel	Cellulose
Histamine	0.250 ± 0.004	0.645 ± 0.009
Acetylcholine	0.236 ± 0.022	0.799 ± 0.021
Serotonin	0.677 ± 0.011	0.755 ± 0.031

[a] Mean of three measurements, ± standard error.

TABLE 2. R_f VALUES AND STAINING RESPONSES FROM TLC OF *C. texanus* EXTRACTS

Adsorbent	R_f value[a] of observed spots	Standards with same R_f as spot[b]			Observed stain response of spots[c]		
		H	A	S	H	A	S
Silica Gel	0.226 ± 0.004	+	+	–	–	–	–
	0.650 ± 0.011	–	–	+	–	–	+
	1.000 ± 0.008	–	–	–	–	–	–
Cellulose	0.318 ± 0.046	–	–	–	–	–	–
	0.627 ± 0.045	+	+	+	–	–	–
	0.761 ± 0.019	–	+	+	–	–	+

[a] Mean of three measurements, ± standard error.
[b] A plus sign indicates correspondence (within 95% confidence intervals) with R_f of standard; H = histamine, A = acetylcholine, S = serotonin.
[c] A plus sign indicates positive staining response; H = histamine (ninhydrin), A = acetylcholine (Dragendorff's reagent), S = serotonin (Ehrlich's reagent).

are from protocol 1 and were longer under the slower flow rate of protocol 2). In all cases, chromatograms of standards displayed a single clear peak. Histamine and acetylcholine were poorly differentiated, with elution times of approximately 3 min and a spectrum showing a single absorption maximum at approximately 220 nm (Figure 1). Serotonin was retained much longer, with elution times of 25–30 min, and gave multiple absorptions at 230 nm, 280 nm, and 310 nm (Figure 1). Chromatograms of *Cnidoscolus* extracts showed several peaks early in the run; however, none of these had spectra similar to those of histamine and acetylcholine. At approximately 25–30 min, *Cnidoscolus* extracts gave a large, broad peak with a spectrum matching that of the serotonin standard (Figure 2).

DISCUSSION

Results indicate the presence of serotonin in the trichomes of *Cnidoscolus texanus*, a conclusion supported by all three techniques used in this study. No evidence was found to support the presence of histamine or acetylcholine in the stinging hairs of this species. Although TLC revealed some spots with R_f values similar to acetylcholine and histamine, none showed a corresponding reaction to indicator reagents. Histamine and acetylcholine were somewhat poorly resolved by the other techniques used in this study, with similar retention times in HPLC and UV absorption peaks that were suspiciously close to the instruments' limits of detection. However, to the extent that standards could be detected, no corresponding results were found for hair extracts.

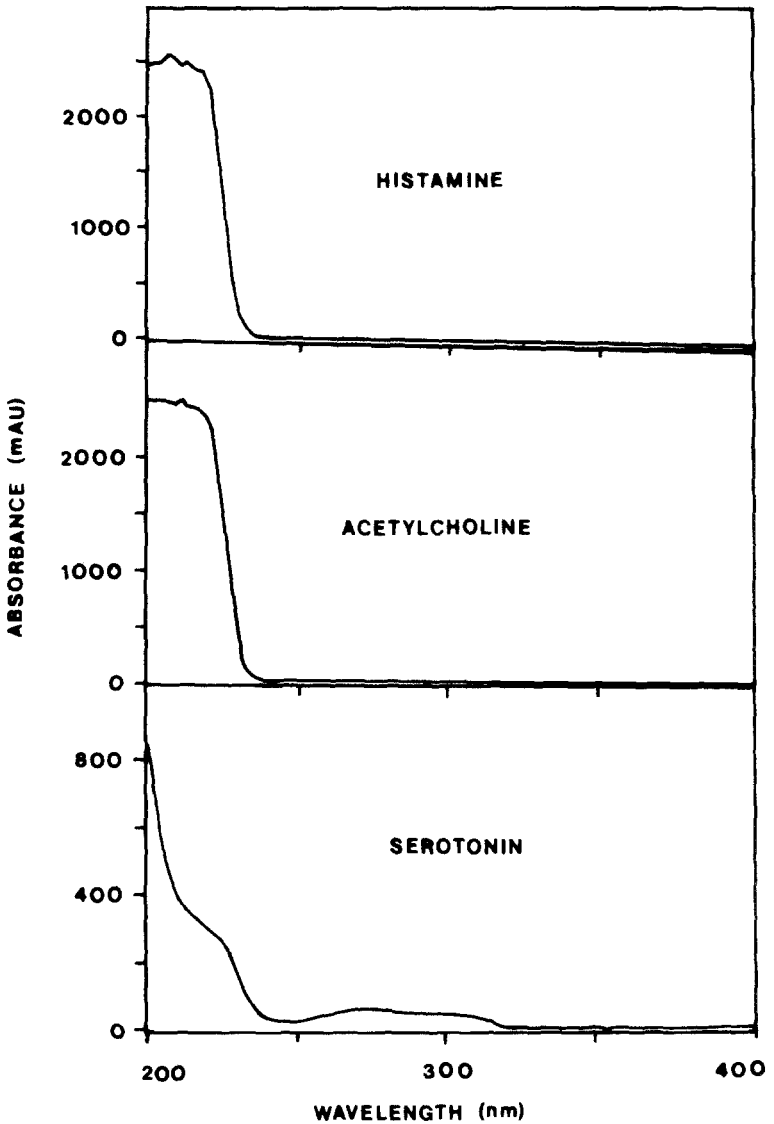


FIG. 1. Spectra of authentic standards (run separately) from HPLC UV diode array detector. Top: histamine (time = 2.95 min); middle: acetylcholine (time = 3.01 min); bottom: serotonin (time = 33.87 min).

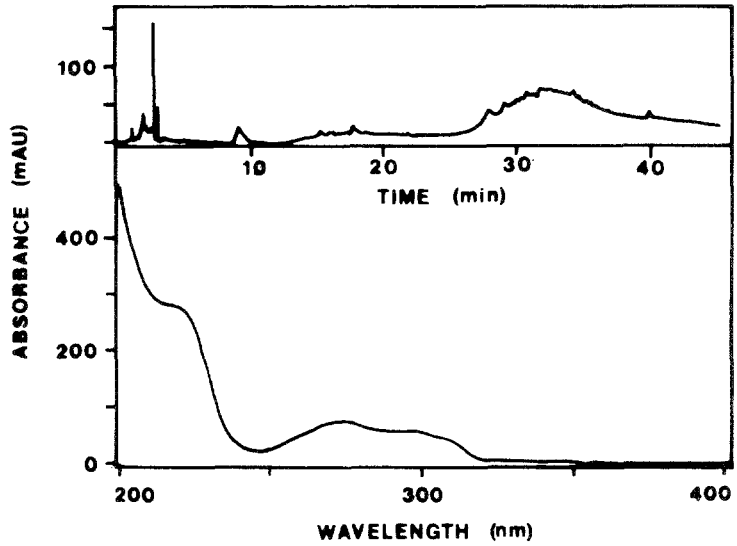


FIG. 2. Results of HPLC analysis of *Cnidoscolus texanus* hair extracts. Top: chromatogram scanned at 260 nm; bottom: Spectrum at 31.70 min.

There were several indications of unknown compounds in the trichomes, including a substance that repeatedly gave a strong spot at very high R_f values (0.99–1.00) in silica-gel TLC, and one or more substances rapidly eluted (2–5 min) in HPLC (Figure 2). It is quite possible that these may be the same substance detected with the two different chromatographic techniques. It is hoped that future studies may allow further characterization of the substance(s) and purification of sufficient quantities to examine biological activity. Earlier studies (e.g., Collier and Chesher, 1956; Robertson and MacFarlane, 1957; Willis, 1969; Thurston, 1974) have suggested that unknown substances, instead of or in addition to histamine, acetylcholine, and serotonin, may be responsible for the sting of various nettle species. Thus, identification of novel compounds in stinging hairs is an important subject for further research.

The results of this study on *C. texanus* are not in agreement with the findings of Cordeiro et al. (1983) on *C. oligandrus*, which was reported to contain histamine and not serotonin, nor with those of Willis (1969) on *C. stimulosus*, which was reported to contain neither. This sort of disagreement, which may represent real chemical differences between species or may be due to differences in methodology and difficult experimental material, is common in the literature of nettle stinging hair chemistry. Such questions will only be resolved by extensive further studies.

This is the first report of the presence of serotonin in nettles outside the

Urticaceae. Along with other literature mentioned above, this provides further support for the idea that nettles in two quite distantly related families show remarkable convergent evolution in both structural and chemical aspects of their defensive systems.

Acknowledgments—We thank R.J. Tyrl and M.J. Stone for supplying some of the plant material used in this study, and L.P. Stratton for comments on the manuscript. This material is based upon work supported by the National Science foundation under grant CSI-8650497. S.E.L. received support during the course of the study from the Furman Advantage Program.

REFERENCES

COLLIER, H.O.J., and CHESHER, G.B. 1956. Identification of 5-hydroxytryptamine in the sting of the nettle (*Urtica dioica*). *Br. J. Pharmacol.* 11:186–189.

CORDEIRO, R., ARAGAO, J., and MORHY, L. 1983. The presence of histamine in *Cnidosculus* [sic] *oligandrus* (Euphorbiaceae). *An. Acad. Brasil. Cienc.* 55:123–128.

EMMELIN, N., and FELDBERG, W. 1947. The mechanism of the sting of the common nettle (*Urtica dioica*). *J. Physiol.* 106:440–445.

EMMELIN, N., and FELDBERG, W. 1949. Distribution of acetylcholine and histamine in nettle plants. *New Phytol.* 48:143–148.

FLURY, F. 1927. Uber die chemische Natur der Nesselgifte. *Z. Gesamte Exp. Med.* 56:402–409.

LUTZ, D. 1914. The poisonous nature of the stinging hairs of *Jatropha* [= *Cnidoscolus*] *urens*. *Science* 40:609–610.

MACFARLANE, W.V. 1963. The stinging properties of Laportea. *Econ. Bot.* 17:303–311.

OELRICHS, P.B., and ROBERTSON, P.A. 1970. Purification of pain-producing substances from *Dendrocnide* (*Laportea*) *moroides*. *Toxicon* 8:89–90.

POLLARD, A.J. 1986. Variation in *Cnidoscolus texanus* in relation to herbivory. *Oecologia* 70:411–413.

POLLARD, A.J., and BRIGGS, D. 1984. Genecological studies of *Urtica dioica* L. III. Stinging hairs and plant-herbivore interactions. *New Phytol.* 97:507–522.

ROBERTSON, P.A., and MACFARLANE, W.V. 1957. Pain-producing substances from the stinging bush *Laportea moroides*. *Aust. J. Exp. Biol. Med. Sci.* 35:381–393.

THURSTON, E.L. 1974. Morphology, fine structure, and ontogeny of the stinging emergence of *Urtica dioica*. *Am. J. Bot.* 61:809–817.

THURSTON, E.L., and LERSTEN, N.R. 1969. The morphology and toxicology of plant stinging hairs. *Bot. Rev.* 35:393–412.

WILLIS, C.L. 1969. Toxic constituents of the stinging nettle. M.S. thesis. Iowa State University, Ames, Iowa.

WINKLER, H. 1922. Die Urticaceen Papuasiens. *Bot. Jahrb.* 57:501–608.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

INDIVIDUAL VARIATION IN SEX PHEROMONE OF SMALLER TEA TORTRIX MOTH, *Adoxophyes* sp. (LEPIDOPTERA: TORTRICIDAE)

R. KOU* and Y.S. CHOW

Institute of Zoology
Academia Sinica
Nankang 11529, Taipei, Taiwan

(Received January 28, 1991; accepted May 28, 1991)

Abstract—Female smaller tea tortrix moths *Adoxophyes* sp. (Lepidoptera: Tortricidae), which initiated calling at 1, 2, or 3 days old, respectively, were analyzed individually for (*Z*)-11-tetradecenyl acetate (Z11-14:OAc) and (*Z*)-9-tetradecenyl acetate (Z9-14:OAc) in the pheromone gland via GLC. Among different age groups, broad and similar distributions were found for pheromone quantity ($\bar{X}$ = 58.6 ± 52.9 ng/female; range 1.3–219.8 ng/female). The ratio of the two pheromone components averaged 65:35 but ranged from 84:16 to 40:60. The significance of the pheromone blend variation to the attraction of males was tested in a field experiment. The ratio of males trapped by the most attractive blend versus the least attractive one was 2.2.

Key Words—Sex pheromone, smaller tea tortrix moth, *Adoxophyes* sp., Lepidoptera, Tortricidae, (*Z*)-11-tetradecenyl acetate, (*Z*)-9-tetradecenyl acetate.

INTRODUCTION

In moths, intraspecific variation in relative quantities of the sex pheromone components have been reported in some species such as *Argyrotaenia velutinana* (Miller and Roelofs, 1980), *Heliothis virescens* (Pope et al., 1982), *Pectinophora gossypiella* (Haynes et al., 1984), *Agrotis segetum* (Löfstedt et al., 1985), and *Ephestia cautella* (Barrer et al., 1987). These variations are likely to be important at a number of evolutionary levels: sexual selection, allopatric speciation, and reproductive isolation between species (Cardé and Baker, 1984;

*To whom correspondence should be addressed.

0098-0331/91/0900-1917$06.50/0

Löfstedt, 1990). In our previous study of the *Adoxophyes* sp. (Kou et al., 1991), variation in the proportion of the sex pheromone components, (Z)-11-tetradecenyl acetate (Z11–14:OAc) and (Z)-9-tetradecenyl acetate (Z9–14:OAc), was found among different times of the photoperiod. In that study, pooled samples were used to quantify the sex pheromone blend variation, so the unusual proportion of sex pheromone components in the individual females might be overlooked. In the present study, individual variation of pheromone components in the gland was quantified by gas chromatography to determine the intraspecific variation in pheromone production in *Adoxophyes* sp. Field-trapping experiments also were conducted to obtain an estimate of the biological significance of the variation to mating success, as measured by trapping activity.

METHODS AND MATERIALS

Insects. Female pupae of the smaller tea tortrix moth used in this study were obtained from the Taiwan Tea Experiment Station (TTES), Hsinchu, Taiwan. The laboratory culture of *Adoxophyes* sp. in TTES had been reared for 10 generations, and each generation had been combined with the wild insects in a 1:1 ratio. Female pupae were maintained in environmental chambers at 23–26°C under 14:10 hr light–dark photoperiod. Emerged moths were fed with 10% aqueous sucrose solution.

Preparation of Pheromone Gland Extracts. Emerged females were put into 30 × 30 × 30-cm transparent plastic boxes and fed with 10% sucrose solution. Ovipositor extracts were prepared from individual females, which initiated calling at 1, 2, or 3 days old. The extraction was performed at the time of maximum calling activity (ca. 8 hr into the scotophase) (Kou et al., 1990, 1991; Tamaki et al., 1969) by soaking the tip in 5 μl hexane containing 50.0 ng (Z)-11-tetradecenyl acetate (Z11–16:OAc) as internal standard.

Quantification of Sex Pheromone Components. The ovipositor was soaked in the solvent for 10 min; then the extract was subsequently analyzed for Z11–14:OAc and Z9–14:OAc using the internal standard method of quantitative analysis. Two microliters of the extract was injected onto a 35-m × 0.25-mm-ID fused silica capillary column coated with a 0.25-μm film of Carbowax 20 M phase; the column was installed in a Shimadzu 14-A gas chromatograph (GC) equipped with a flame ionization detector. Chromatographic conditions: nitrogen carrier at a linear flow velocity of 20 cm/sec, column temperature was isothermal at 190°C. The proportion of the two pheromone components was calculated.

Field Trapping. The males' response specificity to alterations of the pheromone blend proportion observed in female gland extract was carried out in a tea plantation, during August 2 to November 2, 1990. Synthetic chemicals

(99.5% purity) of 12 different blend proportions of Z11–14:OAc and Z9–14:OAc (84:16, 80:20, 75:25, 72:28, 68:32, 66:34, 64:36, 62:38, 60:40, 56:44, 53:47, and 40:60) were prepared so as to cover most of the observed blend proportions from individual females. One milligram of synthetic chemicals was dissolved in hexane and loaded in plastic capsules (capsule for electron microscope, type C050, 8 mm ID × 17.8 mm, TAAB Ltd., Berkshire, England). The plastic capsule was hung on the inside top of a wing-shaped sticky trap. Traps were placed randomly, 10 m apart and 1.2 m above the ground. All field data were analyzed with one-way analysis of variance (ANOVA), then by Student-Newman-Keuls' test when a significant F value was found in the ANOVA (Steel and Torrie, 1980).

RESULTS

Relative Quantities of Sex Pheromone Components in Gland Extracts. A sample of 19, 21, and 9 females which initiated calling at 1, 2, and 3 days old, respectively, produced the distribution of pheromone proportions summarized in Figure 1. Within each age group, there was considerable variation between individual moths in the proportion of pheromone components (Figure 1A–C). Broad and similar distributions of relative quantities were found for each of these age groups. The ranges of the proportion of Z11–14:OAc for these three age group were 51–80%, 40–80%, and 54–84%, respectively. The mean proportions of Z11–14:OAc for the 1-, 2-, and 3-day-old groups were 65.3 ± 7.4%, 64.0 ± 8.3%, and 67.7 ± 8.6%, respectively. When the amount of variation in the relative proportion of Z11–14:OAc was measured with the coefficient of variation (CV), calculated as the standard deviation in percent of the mean [$CV = (SD/\overline{X}) \times 100$], a very constant coefficient of variation was found among the different age groups. The arcsin transformed CV was 8.1%, 9.3%, and 9.7% for the three age groups, respectively (Table 1).

The range for sex pheromone titer (Z11–14:OAc + Z9–14:OAc) also varied widely (4.0–219.8 ng/female, 1.3–200.8 ng/female, and 6.5–64.5 ng/female for the 1-day-old, 2-day-old, and 3-day-old age groups, respectively; with $\overline{X}$ = 58.6 ± 52.9 ng/female for all three age groups). The average titer of Z11–14:OAc was 42.0, 46.3, and 17.3 ng/female. Although the mean proportion of Z11–14:OAc increased a little bit with age (Table 1), both the mean value and total range of the proportion of Z11–14:OAc in the individual gland extracts were quite constant among the different age groups, so that results from all three age groups could be pooled (Figure 1D). The mean of the pooled age group was 65.2 ± 8.1% Z11–14:OAc and the range, 40–80% Z11–14:OAc.

Field Trapping. Male response to different pheromone blends of individual females in the field is shown in Table 2. No significant difference was found in

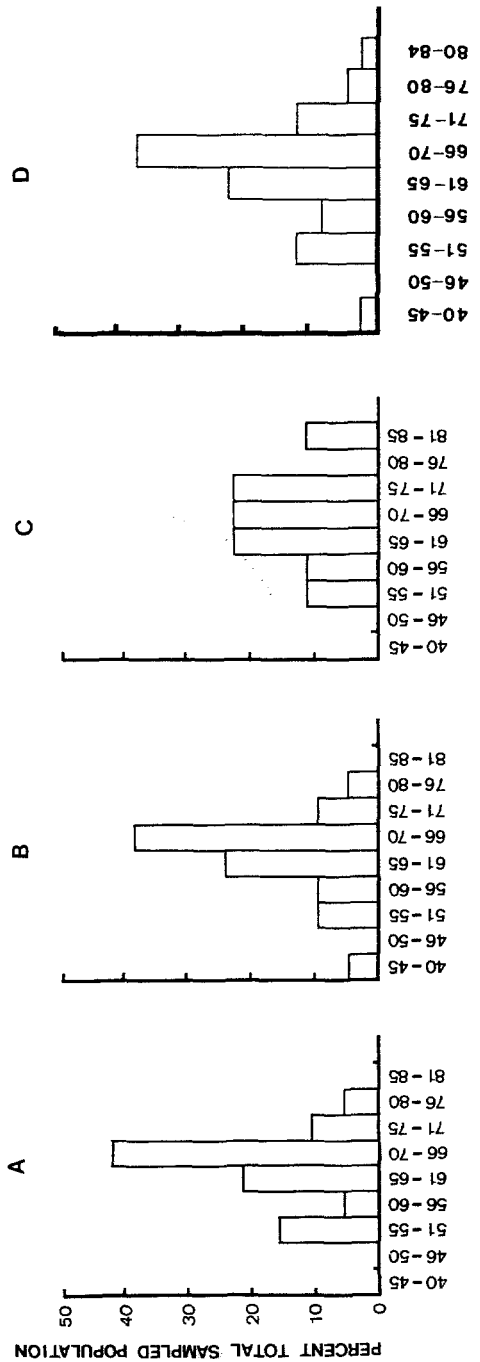


FIG. 1. Distribution of pheromone blend proportions in *Adoxophyes* sp. females that initiated calling (A) at 1 day old ($N = 19$), (B), 2 days old ($N = 21$), (C) 3 days old ($N = 9$), and (D) 1–3 days old ($N = 49$).

TABLE 1. AVERAGE AMOUNT OF Z11–14:OAc IN PHERMONE GLANDS OF FEMALE *Adoxophyes* sp. INITIATING CALLING AT (N = 19), 2 (N = 21), AND 3 Days Old (N = 9); AND COEFFICIENT OF VARIATION (CV)

Age group	Mean Percentage (%/female) $\bar{X} \pm$ SD	CV	CV (arcsin transformed)	Mean pheromone titer (ng/female), $\bar{X} \pm$ SD)
1 day old	65.3 ± 7.4	11	8.1	42.0 ± 43.8
2 day old	64.0 ± 8.3	13	9.3	46.3 ± 31.0
3 day old	67.7 ± 8.6	13	9.7	17.3 ± 13.8

the field attractivity among the 12 different blends (Table 2) (ANOVA, $P > 0.05$). Among the 12 different blends, the blends of 72:28, 56:44, and 53:47 (Z11–14:OAc/Z9–14:OAc) tended to be more attractive (although not statistically significantly so) than the other blends. The ratio of males trapped by the most attractive blend versus the least attractive one was 2.2.

DISCUSSION

The present result showed that there is a wide range of blend proportions within the population of *Adoxophyes* sp. The range of blend proportions of the main pheromone component (Figure 1, 40–84% Z11–14:OAc) in this species was three times broader than that reported for the *A. velutinana* (4–15%) (Miller and Roelofs, 1980), but was similar to that reported for *E. cautella* (60–99%) (Barrer et al., 1987). The coefficient of variation in this species (8.1%, 9.3%, and 9.7% for the 1-, 2-, and 3-day-old age groups, respectively) was close to that reported for *A. velutinana* (9.7%) (Miller and Roelofs, 1980). In *Adoxophyes* sp., in spite of the slight difference in the range of blend proportion among different age groups, the mean pheromone blend proportion tended to remain constant among different age groups (Figure 1, Table 1).

Attygalle et al. (1986) pointed out that the precise regulation of pheromone ratios might be a consequence of a great demand of selection pressure and isolation control for certain moths, brought about by the presence of closely related and sympatrically occurring species. In the tea plantation in Taiwan, no other sympatric species share the same two blend components with *Adoxophyes* sp. until now, so there would be little selective pressure for *Adoxophyes* sp. to maintain precise control over the relative quantities of Z11–14:OAc to Z9–14:OAc in each individual female. The mean and variation of blend proportion would be limited only by other, possibly intraspecific, factors as suggested by

TABLE 2. MEAN TRAP CATCHES (MALES/TRAP/WEEK) OF MALE *Adoxophyes*. SP. WITH 12 DIFFERENT BLENDS OF SEX PHEROMONE IN A TEA PLANTATION, AUGUST 2–NOVEMBER 2, 1990

Blend proportion of Z11-14:OAc/Z9-14:OAc	Mean catch[a]
84/16	2.5 ± 2.4a
80/20	3.3 ± 4.4a
75/25	3.0 ± 3.5a
72/28	5.9 ± 5.2a
68/32	3.9 ± 4.5a
66/34	3.2 ± 2.2a
64/36	3.1 ± 3.6a
62/38	3.4 ± 2.9a
60/40	3.9 ± 2.1a
56/44	5.0 ± 4.0a
53/47	5.2 ± 4.8a
40/60	3.0 ± 2.1a
Control	0.4 ± 0.6b

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

Barrer et al. (1987) in *E. cautella*. If *Adoxophyes* sp. was sometimes sympatric with other species that shared the same two blend components, then such populations could be expected to regulate blend proportions much more precisely and possibly centered on a different mean, as in *Archips argyrospilus* (Roelofs et al., 1974; Cardé et al., 1977).

In the field test, the ratio of males trapped by the most attractive blend versus the least attractive one was only 2.2; a similar ratio also had been obtained from *A. segetum* in a field test (Löfstedt et al., 1985). The result of males' field response to the broad range of pheromone blend proportions may suggest that either the majority of males were broadly tuned, responding almost equally to the different blend proportions (Barrer et al., 1987) or the broad response was due to the existence of different behavioral morphs in the population, responding to different isomer ratios (Hansson et al., 1990; Löfstedt, 1990). Actually, in *A. segetum*, the broad response profile of the male might be due to the sympatric occurrence of different male pheromone morphs (Hansson et al., 1990). Whether the *Adoxophyes* sp. was the same as *A. segetum* still need further study.

In this study, a wide range of pheromone quantities was obtained from strongly calling *Adoxophyes* sp. females; similar results also had been reported in other moths such as the false codling moth, *Cryptophlebia leucotreta* (Attygalle et. al., 1986); the turnip moth, *A. segetum* (Löfstedt et al., 1985); and *E. cautella* (Barrer et al., 1987). Whether this variation was a reflection of a dif-

ferent synthesizing rate in the gland or a different release rate still needs further study.

Acknowledgments—We thank the Taiwan Tea Experiment Station, Hsinchu, for supplying the smaller tea tortrix moth, and Miss S.L. Hsao and Miss R.S. Tsai for help during the field test.

REFERENCES

ATTYGALLE, A.B., SCHWARZ, J., VOSTROWSKY, O., and BESTMANN, H.J. 1986. Individual variation in the sex pheromone components of the false codling moth, *Cryptophlebia leucotreta* (Lepidoptera: Tortricidae). *J. Naturforsch.* 41c: 1077–1081.

BARRER , P.M., LACEY, M.J., and SHANI, A. 1987. Variation in relative quantities of airborne sex pheromone components from individual female *Ephestia cautella* (Lepidoptera: Pyralidae). *J. Chem. Ecol.* 13:639–653.

CARDÉ, R.T., and BAKER, T.C. 1984. Sexual communication with pheromones, pp. 355–383, *in* W.J. Bell and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

CARDÉ, R.T., CARDÉ, A.M., HILL, A.S., and ROELOFS, W. 1977. Sex pheromone specificity as a reproductive isolating mechanism among the sibling species *Archips argyrospilus* and *A. mortuanus* and other sympatric tortricine moths (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 3:71–84.

HANSSON, B.S., TOTH, M., LÖFSTEDT, C., SZOCS, G., SUBCHEV, M., and LOFQVIST, J. 1990. Pheromone variation among eastern european and a western asian population of the turnip moth *Agrotis segetum. J. Chem. Ecol.* 16(5):1611–1622.

HAYNES, K.F., GASTON, L.K., POPE, M.M., and BAKER, T.C. 1984. Potential for evolution of resistance to pheromones: Interindividual and interpopulational variation in chemical communication system of pink bollworm moth. *J. Chem. Ecol.* 10:1551–1565.

KOU, R., TANG, D.S., CHOW, Y.S., and TSENG, H.K. 1990. Sex pheromone components of female smaller tea tortrix moth, *Adoxophyes* sp. (Lepidoptera: Tortricidae) in Taiwan. *J. Chem. Ecol.* 16(4):1409–1415.

KOU, R., TANG, D.S., and CHOW, Y.S. 1991. Calling behavior and pheromone titre in the smaller tea tortrix moth, *Adoxophyes* sp. (Lepidoptera: Tortricidae). *Ann. Entomol. Soc. Am.* (Submitted).

LÖFSTEDT, C. 1990. Population variation and genetic control of pheromone communication systems in moths. *Entomol. Exp. Appl.* 54:199–218.

LÖFSTEDT, C., LANNE, B.S., LOFQVIST, J., APPELGREN, M., and BERGSTROM, G. 1985. Individual variation in pheromone of the turnip moth *Agrotis segetum. J. Chem. Ecol.* 11:1181–1196.

MILLER, J.R., and ROELOFS, W.L. 1980. Individual variation in sex pheromone component ratios in two populations of the redbanded leafroller moth *Argyrotaenia velutinana. Environ. Entomol.* 9:359–363.

POPE, M.M., GASTON, L.K., and BAKER, T.C. 1982. Composition, quantification, and periodicity of sex pheromone glad volatiles from *Heliothis virescens* females. *J. Chem. Ecol.* 8:1043–1055.

ROELOFS, W., HILL, A., CARDÉ, R., TETTE, J., MARSDEN, H., and VAKENTI, J. 1974. Sex pheromone of the fruit tree leafroller moth, *Archips argyrospilus. Environ. Entomol.* 3:747–751.

STEEL, R.G.D., and TORRIE, J.H. 1980. Principles and Procedures of Statistics, 2nd ed. McGraw-Hill, New York.

TAMAKI, Y., NOGUCHI, H., and YUSHIMA, T. 1969. Artificial control of mating activity of the smaller tea tortrix, *Adoxophyes orana* Fisher Von Roslerstamm, and quantitative bioassay for the sex pheromone. *Botyu-Kagaku* 34:107–110.

Journal of Chemical Ecology, Vol. 17, No. 9, 1991

ANALYSIS, SYNTHESIS, FORMULATION, AND FIELD TESTING OF THREE MAJOR COMPONENTS OF MALE MEDITERRANEAN FRUIT FLY PHEROMONE[1]

R.R. HEATH,* P.J. LANDOLT, J.H. TUMLINSON, D.L. CHAMBERS,[2] R.E. MURPHY, R.E. DOOLITTLE, B.D. DUEBEN, J. SIVINSKI, and C.O. CALKINS

Insect Attractants, Behavior, and Basic Biology Research Laboratory
Agricultural Research Service, U.S. Department of Agriculture
Gainesville, Florida 32604

(Received September 18, 1989; accepted May 30, 1991)

Abstract—Three major components, ethyl-(*E*)-3-octenoate, geranyl acetate, and (*E,E*)-α-farnesene, emitted as volatiles by laboratory-reared and wild male medflies were collected and analyzed qualitatively and quantitatively. Peak emission of these compounds occurred during the third to fifth hours of the photophase and differences were observed in the ratios of the three components emitted by male laboratory-reared and wild flies. These three compounds were synthesized, and a method was developed to formulate a synthetic blend that released the compounds in a ratio similar to that emitted by wild male medflies. Attractiveness of the blend to female medflies was demonstrated under field conditions by comparing trap catches. Black spherical traps, baited with the synthetic blend to release 1.6 male equivalents, caught significantly more females than blank traps and traps from which the blend released was 0.3, 3.2 or 6.4 male equivalents.

Key Words—Insecta, Diptera, Tephritidae, Mediterranean fruit fly, pheromone, attractant, *Ceratitis capitata*, ethyl-(*E*)-3-octenoate, geranyl acetate, (*E,E*)-α-farnesene.

*To whom correspondence should be addressed.

[1] This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or recommendation for its use by USDA.

[2] Guatemala Medfly Methods Station, APHIS, USDA, American Embassy, APO Miami, Florida 34024.

0098-0331/91/0900-1925$06.50/0

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wied.) (medfly), is a worldwide pest of many deciduous and tropical fruits. Although not established in the continental United States, small infestations in Florida and California have necessitated multimillion dollar eradication programs. It is estimated that in California an infestation of the medfly could result in a $206,000,000 loss in crops and would require a $156,000,000 control program, which would include the use of 1,818,000 kg of pesticide (Dowell and Wange, 1986). The most effective monitoring tool available for the medfly is trimedlure (Beroza et al., 1961), and it is routinely used in California, Texas, and Florida. Trimedlure predominantly attracts males, although a small percentage of females do respond to this chemical (Nakagawa et al., 1970). The lack of a useful attractant for female medflies prompted us to analyze the male-produced pheromone (Féron, 1962) and evaluate it as a lure for females.

Baker et al. (1985) reported the identification of nine compounds emitted by male medflies. However, they gave no behavioral data to substantiate pheromonal activity. Subsequently, Jang et al. (1989) determined electroantennogram responses of male and female medflies to those nine compounds and to an additional 56 compounds collected from air passed over male medflies. The latter were identified by gas chromatography–mass spectroscopy (GC-MS) by matching the spectra obtained with a mass spectral library. We report in this paper the qualitative and quantitative analyses of three principle components of the volatiles emitted by laboratory-reared and wild male medflies during the photophase. Additionally, we synthesized these compounds, developed a method to formulate each of the compounds individually so as to release these three compounds as a synthetic blend in a ratio similar to that emitted by wild male medflies, and investigated the attractiveness of the pheromone blend at four release rates by trapping wild female medflies in the field.

METHODS AND MATERIALS

Insects. Laboratory-reared *C. capitata* pupae were obtained from the USDA-APHIS mass-rearing facility located in Petapa, Guatemala. Sexually immature 2- to 3-day-old adults were sexed and maintained in 30 cm^3 screen cages containing a mixture of brown sugar and yeast hydrolysate. Flies were held indoors under natural light conditions and ambient temperature and humidity. Wild flies were obtained from infested coffee beans (*Coffee arabica*) collected near Antigua, Guatemala. Beans were held on a screen over soil until maggots left the fruit to pupate in the soil. Emerged flies were maintained in cardboard 1-liter cups with a cotton wick descending from the floor of the containers into a second, water-bearing, cup. When adults were 1–2 days old, they

were sexed and maintained as described above. The yeast hydrolysate food source was replaced with sugar water 24 hr prior to the collection of volatiles from the insects. Volatiles were collected on Porapak-Q (see later) from wild and laboratory-reared male medflies 5–10 days old in groups of 8–12 flies/chamber for 2-hr periods throughout the photophase. Similar collections of volatiles from female medflies were made.

Collection of Emitted Pheromone. The push–pull system used to collect volatiles emitted by the insects has been described previously (Landolt and Heath, 1987). Briefly, compressed air, purified by passing through two charcoal filters, entered two glass chambers 5 cm OD × 30 cm long, each containing 8–12 flies. A frit near the upwind end of the chamber produced a laminar airflow over the insects, and this flow was then pulled through a collector trap by vacuum. Each chamber contained three collector traps, any one of which could be selected for volatile collection without disturbing the flies. The airflow was 0.9 liters/min with a slight positive pressure (+0.2 cm H_2O) established in the insect holding chamber (incoming air to vacuum). Vent flow was monitored with a flowmeter from a vent in the chamber, and the vent flow was maintained at 2% of the airflow through the filter. Pressure drop in the insect chamber due to the collector trap was reduced to near zero by the use of vacuum downwind of the collector filter.

In preliminary experiments, volatiles emitted by male and female medflies were collected on traps using charcoal or Porapak-Q (Alltech Assoc., Deerfield, Illinois) as the adsorbent. Most collector traps initially were extracted in Guatemala as described below, and the extracts were sealed in glass ampoules and shipped to Gainesville, Florida. Some traps were sealed with Teflon tape and transported in glass vials to be extracted in Gainesville, Florida. Analyses of extracts obtained by both methods determined that the filters could be sealed with Teflon tape without loss of the collected volatiles. Based on results of this initial work, subsequent analyses were done on Porapak-Q collector traps that were sealed with Teflon tape and extracted after arrival in Gainesville, Florida.

Charcoal traps (2 mm × 4 mm ID) were prepared as described by Tumlinson et al. (1981). After collection of volatiles, each collector trap was extracted with three 20-μl portions of CH_2Cl_2 followed by three 20-μl extractions with hexane. A 200-ng internal standard of hexadecane was added, and each sample was concentrated to ca. 50 μl under argon for analysis as described below. Porapak-Q traps were prepared by packing ca. 60 mg of the adsorbent in 4-cm-long × 4.0-mm-ID glass tubes, resulting in a bed length of 15 mm. Silanized glass wool was used to contain the adsorbent. The Porapak-Q traps were cleaned by Soxhlet extraction with CH_2Cl_2 for 24 hr prior to use. Volatiles collected on the Porapak-Q traps were eluted with 500 μl of hexane–ether (90:10) and then 200 ng of hexadecane was added as internal standard for subsequent analyses. The average amount of each volatile component collected

per male medfly per hour, as determined by GC analyses (see later), was designated as one male-hour-equivalent (MHe).

Chemical Analyses. Gas chromatographic (GC) analyses were conducted using a Hewlett-Packard model 5890 gas chromatograph, equipped with splitless capillary and cool-on column injectors and flame ionization detectors. Helium was used as the carrier gas at a linear flow velocity of 18 cm/sec. Columns used in this investigation were: 50 m × 0.25 mm ID BP-1, 50 m × 0.25 mm ID Supelcowax-10, and 50 m × 0.25 mm ID CPS-1, a high-polarity cyano-silicone column. Each was operated at 60°C for 2 min, then temperature programmed at 30°C/min to 160°C. The chromatographic data were stored and analyzed in a Nelson 4000 data system.

The identities of the compounds were confirmed by GC-MS analyses using the BP-1 capillary column, operated as described above, coupled to a Nermag model R1010 mass spectrometer in the chemical ionization mode. The reagent gas in the mass spectrometer was either methane or isobutane. Spectra of the natural products were compared with those of candidate synthetic compounds. Additionally, 300-MHz proton magnetic resonance (PMR) spectra were obtained on both natural and synthetic compounds. Carbon-13 spectra (75.4 MHz) were obtained on all synthetic compounds.

Chemicals. Geranyl acetate was purchased from ICN Biomedicals Inc. (Plainview, New York). Prior to use, the material was purified by $AgNO_3$ high-performance liquid chromatography (HPLC) using previously described methods (Heath et al., 1977). Analysis by capillary GC on the columns described above indicated a purity of >99%.

The ester ethyl-(*E*)-3-octenoate was prepared from (*E*)-3-octenoic acid (ICN Biomedicals) by the following method. The acid (1.42 g, 10 mmol) was placed in a 50-ml flask together with 2.00 ml (30 mmol) of absolute ethanol, 0.120 g of *p*-toluenesulfonic acid, and 25 ml of benzene. This mixture was heated under reflux overnight, with the water produced collected in a Dean-Stark trap. When 10 mmol of water had been collected, the mixture was diluted with ether and washed (separating funnel) with water-saturated sodium bicarbonate and dried over anhydrous sodium sulfate. Removal of the drying agent and solvent left a yellow oil that was vacuum distilled, bp 80°C/8 mm Hg, to give a clear liquid (1.53 g, 90% yield). The ester was purified by $AgNO_3$ HPLC. Mass spectra and NMR spectra were consistent with the structure of ethyl-(*E*)-3-octenoate. Capillary GC analysis on columns described above indicated a purity of >98%.

(*E,E*)-α-Farnesene was prepared by the method described by Negishi and Matsushita (1984) with modification. The 1-buten-3-yne used in this procedure was either purchased from Wiley Organics (Columbus, Ohio) or prepared by the method of Brandsma (1971). The toluene solution of trimethylaluminum

used in this procedure was purchased from Aldrich Chemical Company (Milwaukee, Wisconsin). The geranyl chloride used in the preparation was prepared from geraniol (Aldrich Chemical Company) by the method of Calzada and Hooz (1974). Briefly, 1-buten-3-yne is carboaluminated with trimethylaluminum using a catalytic amount of dichlorobis-(N^5-cyclopentadienyl) zirconium. Without isolation, the resulting alkenylalane is coupled with geranyl chloride in the presence of a catalytic amount of tetrakis(triphenylphosphine) palladium. The ratio of 1-buten-3-yne to geranyl chloride should be at least 2 : 1. The use of a smaller ratio resulted in excessive amounts of geranyl chloride remaining after work-up. While it is possible to add the geranyl chloride as a solution in THF, it was more convenient to add the catalyst as a solid via a piece of Gooch tubing. It was necessary to chill the contents of the reaction flask in a dry ice–isopropanol bath during the trimethylaluminum addition as this step is exothermic. Because of difficulty in maintaining a temperature of 0°C with an ordinary ice bath during the hydrolysis with 3 N hydrochloric acid, the hydrolysis was begun with the flask contents at −25°C (CO_2/CCl_4). Due to the large amount of gas evolved during the hydrochloric acid addition, it was necessary to add the acid slowly while at the same time maintaining the low temperature.

The removal of unreacted geranyl chloride from the crude product was very important, since its presence during distillation causes extensive decomposition of the product. Geranyl chloride and catalyst residues were removed as follows: after concentration on a rotary evaporator at aspirator vacuum, the residue (about 125 ml for the scale described in the reference) was diluted 2 : 1 with hexane and allowed to stand in the refrigerator (6–7°C) overnight, which resulted in a yellow precipitate. This was removed by filtration and the filtrate passed first through a short (15–20 cm) silica-gel column (60–200 mesh, EM Science, New York, New York) to remove the catalyst residue, and then through a short (15–20 cm) basic alumina column (Woelm, 60–200 mesh, activity grade I) for removal of the unreacted geranyl chloride. The solvents then were removed and the residue vacuum distilled as a colorless or slightly yellow liquid (bp ≈60° at 0.05 mm). The yields were in the range of 60–70%, which is lower than that reported by Negishi and Matsushita (1984). It is suspected that the catalyst that we used may have deteriorated and thus resulted in reduced yields. The (*E,E*)-α-farnesene was found to be 96% geometrically pure with an overall purity of 86% based on analysis by capillary GC. This material was purified by $AgNO_3$ HPLC to provide a compound that was of >98% purity. Infrared and NMR spectra were in complete agreement with those reported by Negishi and Matsushita (1984). Infrared reported (Negishi and Matsushita, 1984) (neat) cm^{-1} 3080 (m), 2960(s), 2900(s), 1664(m), 1635(m), 1601(m), 981(m), 883(s); [^{1}H]NMR reported (Negishi and Matsushita, 1984) [$CDCl_3$, $(CH_3)_4Si$] δ = 1.59 (S, 3H), 1.63 (S, 3H), 1.66 (S, 3H), 1.74 (S, 3H), 2.03 (m, 4H), 2.82 (t, J =

6, 2H); [^{13}C]NMR [$CDCl_3$, $(CH_3)_4Si$] δ = 11.62, 16.07, 17.63, 25.69, 26.89, 27.35 , 39.88, 110.37, 122.36, 124.50, 131.10, 131.74, 133.79, 135.55, 141.69.

Formulations. In the first field test, all three synthetic components were formulated in glass capillaries of different inside diameters and with different lengths of the vapor–air column above the liquid in the capillary in a manner similar to that reported by Weatherston et al. (1984, 1985a,b). Capillaries (A.H. Thomas Company, Philadelphia, Pennsylvania #7707-B20 series) were sealed at the bottom and release rates were measured by the methods previously described using Porapak-Q as the adsorbent (Landolt and Heath, 1987). Regression analysis of the release rate versus the cross-sectional area of the capillary divided by vapor column height then was obtained. Using the resulting equation from this curve, we obtained the capillary inside diameter and vapor column height required for the desired release rate. Capillary formulations used in this study are reported in Table 1. Further validation of this method was obtained by the collection of volatiles emitted from capillaries formulated in the same manner as those loaded for field bioassays. Similarly, random samples of capillaries used in field bioassays were returned to the laboratory and release rates were measured and compared to the predicted release rate for the given exposure time. Release rates reported are based on measurement at an ambient room temperature of 22.5°C. No attempt was made to adjust for temperature effects on the release rate of the pheromone in the field.

(*E,E*)-α-Farnesene was formulated on rubber septa (Heath et al., 1986) for the second field test, while geranyl acetate and ethyl octenoate were formulated as described for the first field test. Septa were loaded with 10, 50, 100, 500, and 1000 μg of (*E,E*)-α-farnesene in 200 μl hexane and aired in the hood for two days. The load amount required for the desired release rate was determined from the linear equation obtained from release rate versus septum load.

Field Tests. Tests were conducted during March 1987 and March 1988. Traps used were 7.5-cm-diam. polypropylene hollow spheres painted black and coated with Stickum Special. Such a design was found to be very attractive visually to both sexes of medfly and was recommended by Nakagawa et al. (1978) for the testing of attractants. Pheromone dispensers were mounted on a 1-cm-long piece of 0.95-cm-ID Tygon tubing glued to the sphere ca. 2 cm down from the sphere top (trap hanger point). An "umbrella" also was mounted over pheromone dispensers on each trap to prevent rain from contaminating the capillary tubing. Each umbrella was a white plastic vial snap-cap (2 cm diam.) mounted above the lures with an insect pin stuck into the tubing. Traps were set up in north-to-south rows of coffee shrubs in a coffee farm near Antigua, Guatemala (Finca Portrero, at Ciudad Viejo). Dosages were initially randomized within each block and traps were rotated one position daily through the duration of the experiment. Each replicate block was placed in one row or hedge

TABLE 1. RELEASE RATE OF MEDFLY PHEROMONE FROM CAPILLARIES

	Capillary		Capillary formulations			
			Day 5 release rate		Day 30 release rate	
Release (MHe)	ID (mm)	Vapor height (mm)	Predicted[a] (ng/hr)	Measured laboratory[b] (ng/hr ± SD)	Predicted (ng/hr)	Measured field[c] (ng/hr ± SD)
	Ethyl octenoate					
0.3	0.59	20	198	167 ± 6	166	164 ± 8
1.6	0.84	9	881	862 ± 32	700	704 ± 12
3.2	1.19	9	1763	1627 ± 82	1290	1415 ± 120
6.4	1.68	9	3527	3360 ± 68	3280	
	Geranyl acetate					
0.3	1.19	20	68	76 ± 1	76	76 ± 1
1.6	1.68	8	339	351 ± 9	290	295 ± 16
3.2	1.68	4	677	700 ± 21	520	532 ± 23
6.4	2.49	4	1485	1500 ± 85	1380	
	(*E*,*E*)-α-Farnesene					
0.3	1.19	10	80	85 ± 13	78	95 ± 8
1.6	1.68	5	320	333 ± 34	310	410 ± 8
3.2[d]	1.68	5	640	642 ± 42	620	630 ± 68
6.4	3.49	6	1151	1130 ± 67	1090	

[a] Predicted based on regression analysis as follows: Ethyl octenoate release rate (ng/hr) = 14.3 × area of capillary ÷ length of vapor height (r^2 = 0.98, N = 10); geranyl acetate release rate (ng/hr) = 1.22 × area of capillary ÷ length of vapor height (r^2 = 0.97, N = 10); (*E*,*E*)-α-Farnesene release rate (ng/hr) = 0.72 × area of capillary ÷ length of vapor height (r^2 = 0.99, N = 10).

[b] Mean of three analyses.

[c] Mean of three analyses. Samples of 6.4 MHe used in field were lost.

[d] Release rate of 3.2 MHe was obtained by using two of the 1.6 MHe capillaries.

of coffee shrubs. Traps within blocks were 7.6 m apart. Blocks were roughly 8 m apart and traps were hung from coffee branches at heights of 1–1.5 m. Each day, flies were removed, sexed, and counted, and each days' catch was considered a replicate. In the first series of tests (1987), the attractiveness of the three major chemicals identified from wild medfly male volatiles was evaluated using a release rate equivalent to 0.3, 1.6, 3.2, and 6.4 MHe in the same ratio emitted by the wild male medflies. Spheres without chemicals were used as blanks. All chemicals were formulated in glass capillaries as described. Jackson traps (Harris et al., 1971) with a cotton wick containing 2 ml of standard trimedlure, used in USDA APHIS monitoring programs, were placed within test plots to provide an indication of fly populations. Traps and trimedlure were obtained from USDA, APHIS, PPQ, Guatemala City, Guatemala. Trimedlure baits are the

standard means of monitoring medfly populations. Three blocks of the six treatments were randomized daily for a six-day period. The second series of tests (1988) were set up in a similar manner with the following changes. The (*E,E*)-α-farnesene was dispensed from rubber septa, and no trimedlure-baited traps were placed in test plots. The duration of this test was eight days. Trap catch data were subjected to ANOVA and daily means separated using Duncan's (1955) multiple-range test.

RESULTS

Analysis of Volatile Constituents. Analysis of volatiles collected from 5- to 10-day-old virgin males and females indicated that three major compounds were present and were produced only by male medflies (Figure 1). These compounds were identified as geranyl acetate, ethyl-(*E*)-3-octenoate, and (*E,E*)-α-farnesene, based on retention times on the BP-1 and Supelcowax columns and the comparison of NMR and EI and CI mass spectra with synthetic compounds. These compounds accounted for 90% of total peak areas seen in the chromatograms and were previously reported by Baker et al. (1985) and Jang et al. (1989) to be present in the volatiles emitted by male medflies. Additionally, in

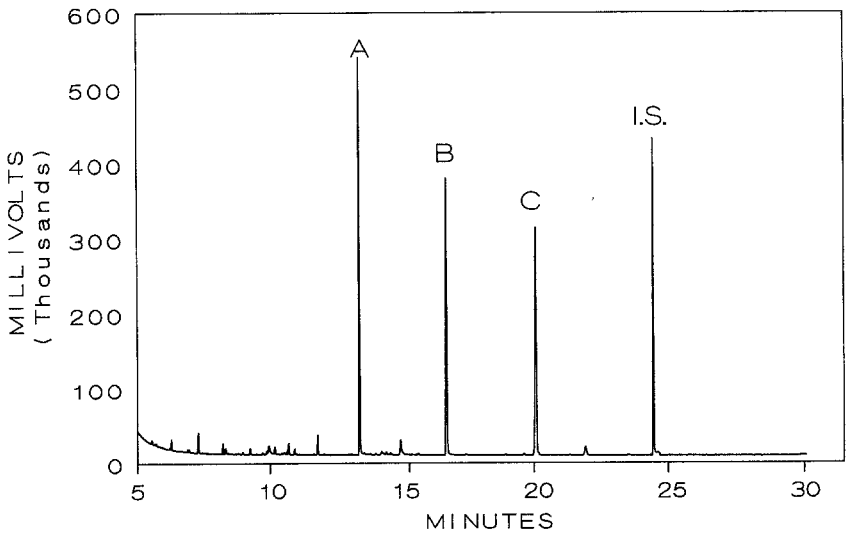


FIG. 1. Volatiles collected from eight wild male medflies during the third to fifth hour of the photophase, analyzed on a 50 m × 0.25 mm ID BP-1 (SGE) fused silica capillary GC column. Peak A = ethyl-(*E*)-3-octenoate, B = geranyl acetate, C = (*E,E*)-α-farnesene, and I.S. = internal standard, 8 μg of hexadecane.

the preliminary experiments, volatiles extracted from Porapak-Q traps immediately after collection from male medflies in Guatemala and sealed in ampoules and analyzed later in Gainesville were identical to those from collector traps sealed with Teflon tape, returned to Gainesville, and then extracted and analyzed. We confirmed that these compounds do not degrade when adsorbed on Porapak-Q for 20 days by analysis of synthetic material that was collected and extracted, and analyzed immediately compared with material that was collected, stored under ambient conditions in the laboratory for 20 days, and then extracted. It was noted during the preliminary experiment ($N = 10$) that less (E,E)-α-farnesene was recovered from volatiles collected on charcoal traps than from volatiles collected on Porapak-Q collector traps. This also was confirmed by the collection of volatilized synthetic compound, which indicated that a 40 $\pm$ 5.2% ($\bar{X} \pm$ SD) loss of (E,E)-α-farnesene occurred when charcoal was used in the collector trap compared with Porapak-Q ($N = 6$).

Periodicity of Pheromone Production. The results of the analyses of volatiles collected on Porapak-Q for 2-hr periods throughout the photophase from wild male medflies 5–10 days old is shown in Figure 2. Peak collection of volatiles occurred 3–5 hr into the photophase, with less material collected in the earlier and later 2-hr periods. No differences were observed in the patterns

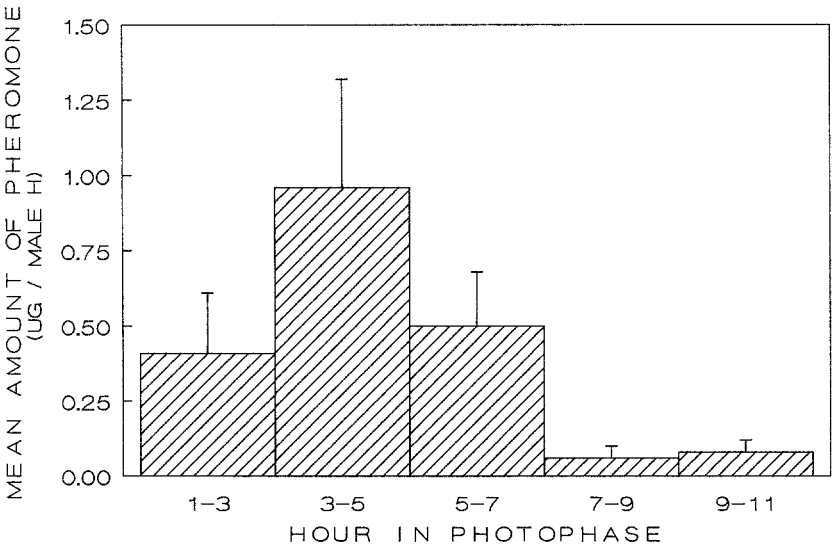


FIG. 2. Mean amount of 18 pheromone collections using Porapak-Q from wild male medflies 5–10 days old in groups of 8–12 flies/chamber for 2-hr periods throughout the photophase. Small bars indicate standard deviation.

of pheromone released by wild (N = 16) versus laboratory-reared (N = 11) flies.

Comparison of Volatiles. Significant differences in the ratios of ethyl-(E)-3-octenoate, geranyl acetate, and (E,E)-α-farnesene were found when the volatiles collected on Porapak-Q traps from wild medflies were compared with volatiles obtained from laboratory-reared flies (Figure 3) during the third to fifth hour of the photophase. Average percentages of ethyl-(E)-3-octenoate, geranyl acetate, and (E,E)-α-farnesene from wild males were 50.3 $\pm$ 5.6 SD, 24.7 $\pm$ 2.7 SD, and 25.0 $\pm$ 4.0 SD, respectively (N = 18), while average percentage releases from the laboratory-reared males were 29.6 $\pm$ 5.9 SD, 38.3 $\pm$ 2.7 SD, and 32.1 $\pm$ 3.8 SD, respectively (N = 10). The mean percent of ethyl-(E)-3-octenoate in wild fly volatiles was significantly greater than that in lab fly volatiles (t = 9.04, P = <0.00001), while the mean percent of geranyl acetate was significantly less in wild fly volatiles (t = 26.0, P < 0.001). The total amount of the three components released by the 5- to 10-day-old laboratory-reared flies during the 2-hr period of peak production was 360 $\pm$ 180 SD ng/male/hr (N = 6) while the 5- to 10-day-old wild flies released 960 $\pm$ 240 SD

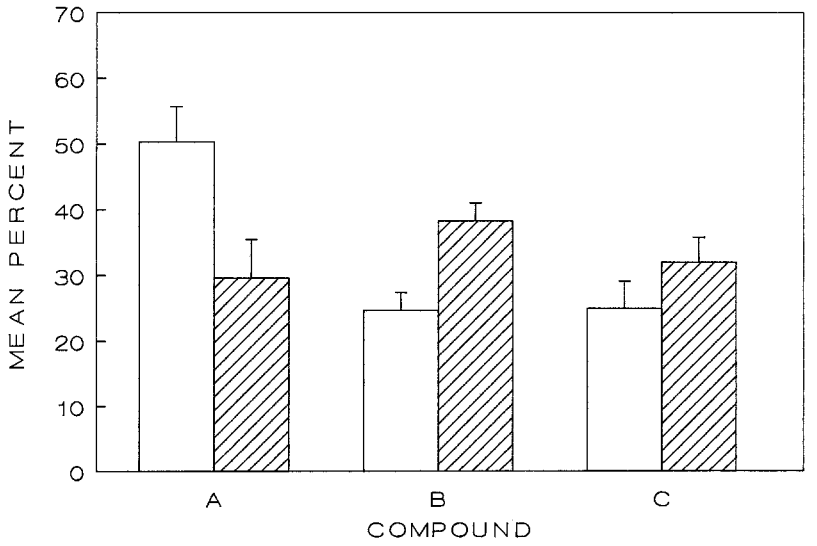


FIG. 3. Comparison of volatiles collected on Porapak-Q traps from wild medflies (N = 18) with volatiles obtained from laboratory reared flies (N = 10) during the 3- to 5-hr period of the photophase. Open bars indicate the percent of compound emitted by wild flies and slash bars the percent from laboratory reared flies. Percents are normalized to 100. Small bars indicate standard deviation. Letter designation as follows: A = ethyl-(E)-3-octenoate, B = geranyl acetate, and C = (E,E)-α-farnesene.

ng/male/hr ($N = 6$). The release rate used for subsequent formulation of synthetic material was based on the amount released by the wild flies (960 ng/hr).

Formulations. Regression analyses of release rate versus the area of the capillary–vapor column height was obtained for each of the three components used in the field bioassay. Results from the linear regression analyses are shown in Table 1. Capillaries then were formulated from these data to provide the desired release rate of each compound at approximate released percentages of 55.2 ± 0.6 SD, 23.9 ± 0.8 SD, and 21.0 ± 1.3 SD for ethyl-(*E*)-3-octenoate, geranyl acetate, and (*E,E*)-α-farnesene, respectively. Prior to use in the field, release rates and ratios of the formulations were determined in the laboratory by GC analysis of collected volatiles and compared to the desired predicted values (Table 1). Release rate and ratio data obtained from laboratory studies indicated that the longevity of the formulation was in excess of two weeks with less than a 5% decrease in emission rate of any of the three compounds investigated. After field use, capillaries were returned to the laboratory, and the change in vapor column height was measured. Additionally random samples of the capillaries used in the field were taken, and the release rates from these capillaries were measured and were found to be in close agreement with the predicted rate for the time they were used (Table 1). These measurements were in agreement with measurements obtained under laboratory conditions. It was noted that use of large (3.49 mm ID) capillaries for the formulation of (*E,E*)-α-farnesene resulted in what appeared to be movement of the compound towards the top of the capillary. Although the release rates from the capillaries were consistent with the predicted release rates, this observation prompted us to formulate (*E,E*)-α-farnesene in rubber septa for the second series of field tests. Based on regression analysis of release rate of (*E,E*)-α-farnesene versus increased load on rubber septa, a release rate approximately the same as that from the capillaries used the previous year was obtained. Release rates were measured in the laboratory from septa after two to nine days of exposure in the fume hood. The results of these analyses are shown in Table 2. Delays in shipment of the septa used in the field precluded the analysis of release rates from these septa.

Field Tests of Three Component Pheromone Blends. In the 1987 field tests, we compared a control unbaited sphere to spheres baited with the three component blend formulated to release at 0.3, 1.6, 3.2, and 6.4 MHe (Figure 4). A total of 259 female medflies were caught in this test. Females were trapped with all treatments and the control trap; however, the sphere baited with 1.6 MHe of pheromone was significantly more attractive. It should be noted in this test that traps with pheromone released at 0.3 and 3.2 MHe caught significantly fewer females than the spheres baited with pheromone that released 1.6 and 6.4 MHe. In field tests conducted during 1988, the same general trend in female trap captures on spheres loaded with different amounts of the three-component

TABLE 2. RELEASE RATE OF (*E*,*E*)-α-FARNESENE FROM RUBBER SEPTA

	Rubber septa formulations (*E*,*E*)-α-Farnesene			
			Measured[b] release rate (ng/hr ± SD)	
MHe	Desired release rate (ng/hr)	Septa[a] load (μg)	Day 2	Day 9
0.3	80	20	70 ± 7	65 ± 8
1.6	320	100	345 ± 40	326 ± 43
3.2	640	200	690 ± 50	652 ± 58
6.4	1151	400	1208 ± 72	1142 ± 67

[a]Septa load based on release rate (ng/hr) = 34.54 × load amount (μg), $r^2 = 0.991$ (septa were loaded with four increasing amounts of compound and release rates from each septum were determined three times).
[b]Mean of three analyses.

male pheromone was observed (Figure 4); traps baited with pheromone released at 1.6 and 6.4 MHe caught more females than the blank and the 0.3 and 3.2 MHe pheromone traps. In this test, a total of 368 females were trapped. Statistically, only the spheres baited with pheromone released at 1.6 MHe were significantly better than the other treatments. This may be due in part to the use of rubber septa formulation for (*E*,*E*)-α-farnesene in this test. Release rates from rubber septa are wind dependent, and this may account for the high variance in capture of flies in traps baited with pheromone as was found in the statistical analysis of data.

DISCUSSION

The first report of a putative medfly pheromone was published by Jacobson et al. (1973). Recent findings do not support their findings, and it is suggested that due to limited analytical methodology available at the time the report is incorrect. Baker et al. (1985) identified nine volatile compounds emitted and/or extracted from sexually mature male Mediterranean fruit flies. The identified components in addition to those reported here included 3,4-dihydro-2H-pyrrole, (*E*)-2-hexenoic acid, dihydro-3-methylfuran-2(3,H)-one, 2-ethyl-3,5-dimethylpyrazine, linalool, and ethyl acetate. It was claimed that the 3,4-dihydro-2H-pyrrole is active, but no data were reported to support pheromonal activity of this or other identified compounds. The amounts and ratios of the nine identified components released by male flies were not reported. Jang et al. (1989) detected 69 compounds using GC analysis of collections of headspace from sexually mature male laboratory reared medflies and identified 56 of these compounds.

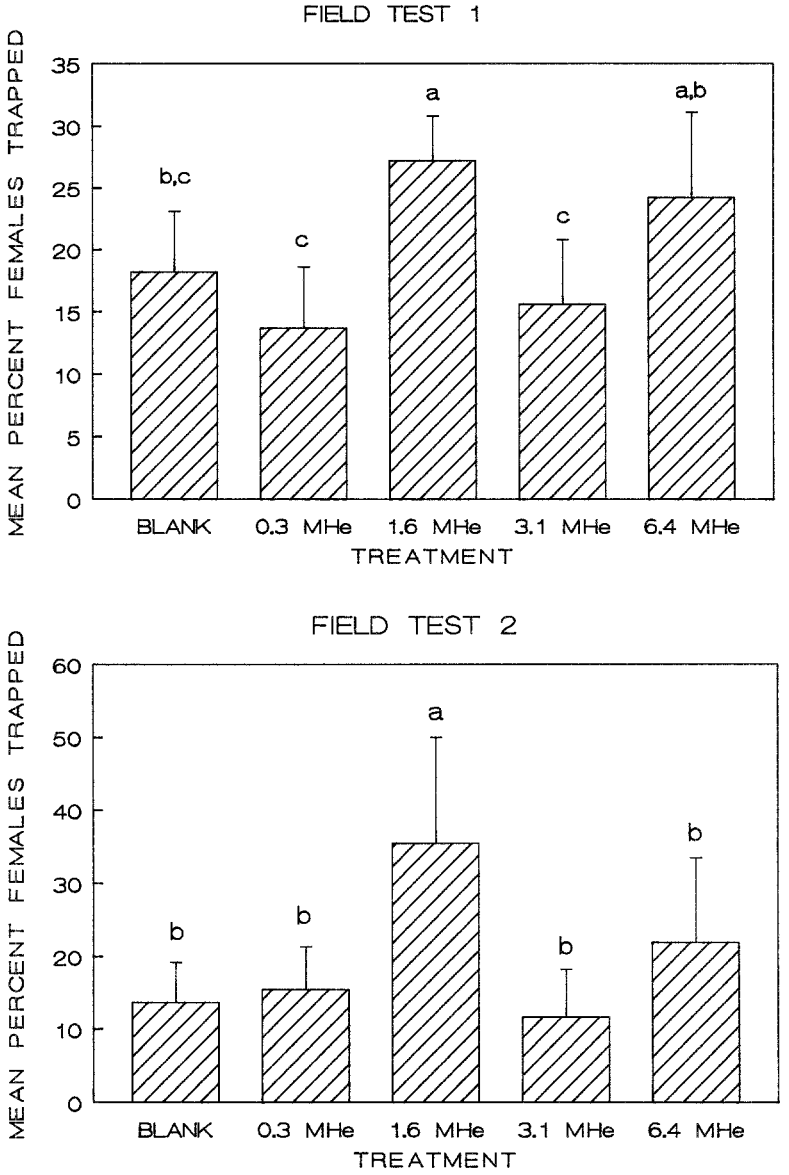


FIG. 4. Results of the field test 1 conducted in 1987 and field test 2 in 1988. Comparison of a blank sphere with spheres that had the three-component blend formulated to release pheromone in increased amounts. Release rate is in male hour equivalents (MHe) based on amount produced by wild male medflies. Bars with the same letter are not significantly different, Duncan's (1955) multiple range test ($P < 0.05$, $N = 6$ for test 1 and $N = 8$ for test 2).

Evaluation of six compounds [ethyl-(*E*)-3-octenoate, geranyl acetate, (*E,E*)-α-farnesene, 3,4-dihydro-2H-pyrrole, linalool, and ethyl acetate individually and as a blend] showed significant activity for all compounds and the blend compared to blanks. Baker et al. (1990), using sterile medflies, tested racemic linalool, 2,3-dimethylpyrazine, 2,5-dimethyl pyrazine, and geranyl acetate individually and in combination. Based on the results of female capture data using baited yellow delta traps and yellow squares, it was determined that the traps containing large amounts of the compound(s) singularly and in combination were more attractive to released female medflies than the control trap.

It is apparent from the studies reviewed that there are a considerable number of putative pheromonal compounds produced by male Mediterranean fruit flies. Our research effort was to provide some focus regarding the release, synthesis, formulation, and testing of three major compounds released by male medflies. A principal problem in interpreting the results of previous published studies on medfly pheromone is the lack of quantitative data and control over chemical release ratios and rates, both in analysis of male volatiles and in testing of compounds for pheromonal activity.

In this paper we demonstrated that ethyl-(*E*)-3-octenoate, geranyl acetate, and (*E,E*)-α-farnesene are three major compounds emitted by virgin male medflies and are released in a precise ratio within a population or group from one source, although this ratio differs significantly between laboratory-reared and wild flies. We have developed a formulation method that releases the three synthetic pheromone compounds in the same ratio released by wild males. Field tests demonstrated the attractiveness of the formulated blend to medfly females at 1.6 MHe but indicate a need for precise control of release rate. Thus we demonstrated that female medflies can be captured in the field with a three-component synthetic pheromone blend. In the first field evaluation of the pheromone, it was found that the black sphere baited with pheromone at a release equivalent to 1.6 MHe captured 25 times more female medflies than the trimedlure in the Jackson trap (11 females/pheromone trap/day versus 0.44 females/trimedlure trap/day). Conversely, the trimedlure-baited Jackson trap caught 14 times more male medflies than the pheromone-baited trap that released at 1.6 MHe (29 males/trimedlure trap/day versus 2.1 males/pheromone trap/day). Differences in lure type and trap design, however, preclude statistical comparison of this data.

The apparent bimodal response distribution observed on traps baited with pheromone at different release ratios is similar to that observed for the papaya fruit fly attraction to 2-methyl-6-vinylpyrazine (Landolt et al., 1988; Landolt and Heath, 1990). The apparent bimodal nature of the numbers of papaya fruit fly females captured at different pheromone release rates was likely due to different responses by mated versus virgin females to the pheromone (Landolt and Heath, 1990). Female medflies captured in these tests were not preserved and

dissected to determine their mating status. More field tests and behavioral studies are planned to investigate the nature of the dose–response relationship, the efficacy of the three-component pheromone system, and whether other compounds reported and yet to be identified as released by males will enhance attraction and capture of female medflies.

REFERENCES

BAKER, P.S., HOWSE, P.E., ONDARZA, R.N., and REYES, J. 1990. Field trials of synthetic sex pheromone components of the male Mediterranean fruit fly (Diptera: Trypetidae) in Mexico. *J. Econ. Entomol.* 6:2236–2245.

BAKER, R., HERBERT, R.H., and GRANT, G.G. 1985. Isolation and identification of the sex pheromone of the Mediterranean fruit fly, *Ceratitis capitata* (Wied.). *J. Chem. Soc. Chem. Commun.* 1985:824–825.

BEROZA, M., GREEN, N., GERTLER, S.I., STEINER, L.F., and MIYASHITA, D.H. 1961. Insect attractants. New attractants for the Mediterranean fruit fly. *J. Agric. Food Chem.* 9:361–365.

BRANDSMA, L. 1971. Page 120, *in* L. Brandsma (ed.). Preparative Acetylenic Chemistry. Elsevier, New York.

CALZADA, I.G., and HOOZ, I. 1974. Geranyl chloride [(*E*)-1-chloro-3,7-dimethyl-2,6-octadiene]. *J. Org. Synth.* 54:63–67.

DOWELL, R.V., and WANGE, L.K., 1986. Process analysis and failure avoidance in fruit fly programs, pp. 43–65, *in* M. Mangel, J.R. Carey, and R.E. Plant (eds.). Pest Control. NATO ASI Series, Springer-Verlag, New York.

DUNCAN, D.B., 1955. Multiple range and multiple *F* tests. *Biometrics* 11:1–41.

FÉRON, M. 1962. L'instinct de reproduction chez la mouche Mediterranéene des fruits *Ceratitis capitata* Wied. (Diptera: Trypetidae). Comportement sexuel-comportement de ponte. *Rev. Pathol. Veg. Entomol. Agric. Fr.* 41:1–129.

HARRIS, E.J., NAKAGAWA, S., and URAGO, T. 1971. Sticky traps for detection and survey of three tephritids. *J. Econ. Entomol.* 64:62–65.

HEATH, R.R., TUMLINSON, J.H., DOOLITTLE, R.E., and DUNCAN, J.H. 1977. Analytical and preparative separation of geometrical isomers by high efficiency silver nitrate liquid chromatography. *J. Chromatogr. Sci.* 15:10–13.

HEATH, R.R., TEAL, P.E.A., TUMLINSON, J.H., and MENGELKOCH, L.J. 1986. Prediction of release ratios of multicomponent pheromones from rubber septa. *J. Chem. Ecol.* 12(12):2133–2143.

JACOBSON, J., OHINATA, K., CHAMBERS, D.L., JONES, W.A., and FUJIMOTO, M.S. 1973. Insect sex attractants. 13. Isolation, identification and synthesis of sex pheromones of the male Mediterranean fruit fly. *J. Med. Chem.* 16:248–251.

JANG, E.G., LIGHT, D.M., FLATH, R.A., NAGATA, J.T., and MON, T.R. 1989. Electroantennogram responses of the Mediterranean fruit fly, *Ceratitis capitata* to identified volatile constituents from calling males. *Entomol. Exp. Appl.* 50(1):7–19.

LANDOLT, P.J., and HEATH, R.R. 1987. Role of female-produced sex pheromone in behavioral reproductive isolation between *Trichoplusia ni* (Hübner) and *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae, Plusiinae). *J. Chem. Ecol.* 13:1005–1018.

LANDOLT, P.J., and HEATH, R.R. 1990. Effects of pheromone release rate and time of day on catches of male and female papaya fruit flies (Diptera: Tephritidae) on fruit-model traps baited with pheromone. *J. Econ. Entomol.* 83:2040–2043.

LANDOLT, P.J., HEATH, R.R., AGEE, H.R., TUMLINSON, J.H., and CALKINS, C.O. 1988. A sex

pheromone-based trapping system for the papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker (Diptera: Tephritidae). *J. Econ. Entomol.* 81(4):1163–1169.

NAKAGAWA, S., FARIAS, G.J., and STEINER, L.F. 1970. Response of female Mediterranean fruit flies to male lures in the relative absence of males. *J. Econ. Entomol.* 63:227–229.

NAKAGAWA, S., PROKOPY, R.J., WONG, T.T., ZIEGLER, J.R., MITCHELL, S.M., URAGO, T., and HARRIS, E.J. 1978. Visual orientation of *Ceratitis capitata* flies to fruit models. *Entomol. Exp. Appl.* 24:193–198.

NEGISHI, E., and MATSUSHITA, H. 1984. Palladium-catalyzed synthesis of 1,4-dienes by allylation of alkenylalanes: α-Farnesene (1,2,6,10-dodecatetraene, 3,7,11-trimethyl). *Org. Synth.* 62:31–38.

TUMLINSON, J.H., TEAL, P.E.A., and HEATH, R.R. 1981. Chemical ethology: A holistic approach to the study of insect pheromones, pp. 19–31, *in* Proceedings 1st Japan/USA Symposium on IPM. Tsukuba, Japan, September 29–30.

WEATHERSTON, I., MILLER, D., and DOHSE, L. 1984. Release of volatile materials from glass capillaries—a reinvestigation, pp. 114–115, *in* Proceedings 11th International Symposium Controlled Release Bioactive Materials. Fort Lauderdale, Florida.

WEATHERSTON, I., MILLER, D., and DOHSE, L. 1985a. Capillaries as controlled release devices for insect pheromones and other volatile substances—a reevaluation: Part I. Kinetics and development of predictive model for glass capillaries. *J. Chem. Ecol.* 11:953–965.

WEATHERSTON, I., MILLER, D., and LAVOI-DORNIK, J. 1985b. Capillaries as controlled release devices for insect pheromones and other volatile substances—a reevaluation: Part II. Predicting release rates from Celcon and Teflon capillaries. *J. Chem. Ecol.* 11:967–968.

Journal of Chemical Ecology, Vol. 17, No. 10, 1991

α-TOCOPHEROL ALTERATION OF SOYBEAN ANTIHERBIVORY TO *Trichoplusia ni* LARVAE

FANINDRA P. NEUPANE[1] and DALE M. NORRIS[2,*]

[1]*Tribhuvan University*
Institute of Agriculture and Animal Science
Rampur, Chitwan, G.P.O. Box 984
Kathmandu, Nepal

[2]*Department of Entomology*
University of Wisconsin
Madison, Wisconsin 53706

(Received April 22, 1991; accepted June 3, 1991)

Abstract—The antioxidant vitamin E, α-tocopherol, was tested as a candidate elicitor of alterable antiherbivory in soybean plants against cabbage looper larvae. Although a nonspecific antioxidant, vitamin E proved elicitory to the involved sulfhydryl-dependent receptor-energy transducer protein in soybean plasma membrane. Effects of α-tocopherol were dependent on dosage, time, and space in the plant. The observed elicited effects were all decreases in herbivory. The best negative phytochemical correlate of looper feeding was the percentage of increased total HPLC peak area of extractables from elicited as compared to nonelicited leaves. Some specific compounds, e.g., glyceollins, were quantitatively major components of the total profile of secondary metabolites.

Key Words—α-Tocopherol, elicitor, alterable resistance, soybean, *Trichoplusia ni*, Lepidoptera, Noctuidae, antiherbivory.

INTRODUCTION

Alterable (inducible) resistance in plants against herbivores and other environmental stresses is a major investigate topic in chemical ecology. Such alterable resistance apparently involves a plant receptor system that receives and processes, from the extracellular environment of the plant, a signal indicating the presence of a stressful entity (e.g., insect defoliator or plant pathogen) (Sequeira,

*To whom correspondence should be addressed.

0098-0331/91/1000-1941$06.50/0

1983; Low and Heinstein, 1986; Norris, 1992). Such receptors may be plasma membrane-bound, may possess a transmembrane signal-transduction capability, and may interact with one or more intracellular effector molecules (e.g., second messengers) (Yoshikawa et al., 1983; Stössel, 1984; Schmidt and Ebel, 1987; Low and Heinstein, 1986; Norris, 1988). Various heavy metals, including mercuric compounds, and several sulfhydryl reagents are known to interact as messenger ligands with the plant receptor to elicit altered plant resistance similar to that induced by various stressful biotic factors (Stössel, 1984; Gustine, 1987; Norris, 1992). The classical sulfhydryl reagents, iodoacetic acid (IAA) and *N*-ethylmaleimide (NEM), were recently shown to elicit such changed resistance in soybean plants against cabbage looper defoliation (Neupane and Norris, 1990, 1991). Such results further support an interpretation that sulfhydryls are involved in signal transduction for alterable plant resistance to insect herbivory. Sulfhydryl involvement was shown previously in quite analogous signal transduction in insect chemoreception (Norris et al., 1970; Rozental and Norris, 1973, 1975; Norris, 1979, 1981, 1986, 1988). Thus, common sulfhydryl-dependent parameters of signal transduction apparently exist between environmental factors and both plant and animal cells. Such sulfhydryls in peptides and proteins may be susceptible to environmental oxidation to disulfides, and such disulfides may be reduced by other environmental conditions to sulfhydryls. Based on these facts, we hypothesized that application of an antioxidant to the soybean plant could alter its resistance to cabbage looper defoliation. The results from experimental testing of this hypothesis are presented in this paper.

METHODS AND MATERIALS

Insects. The cabbage looper (CL), *Trichoplusia ni* (Hübner), was reared in the laboratory on a pinto bean-based artificial diet (Shorey and Hale, 1965) at 27 ± 2°C and 50 ± 10% relative humidity with a 14-hr photophase (14 : 10 light–dark).

Plants. Seeds of an unnumbered soybean plant introduction obtained from Jacob Hartz Seed Co., Stuttgart, Arkansas, were germinated in moisture-sterilized vermiculite in plastic flats in a controlled climate in the University of Wisconsin Biotron. Young seedlings were transplanted at the first-leaf stage, one per pot, into 10.5-cm-diam. plastic pots containing a support medium (Griffy-mix). The plants then were provided with 30–40 ml of half-strength Hoagland's solution (Hammer et al., 1978) four times a day (i.e., every 6 hr). A 14-hr photophase involving 12 hr of full light intensity, i.e., 300–500 μE/m^2/sec; a day temperature of 27 ± 1°C; a night temperature of 20 ± 1°C; and a relative humidity of 65 ± 5% were maintained.

Soybean plants in the V3 stage of development (Fehr and Caviness, 1977) were used for experiments.

Chemical (α-Tocopherol) Elicitor Treatments. Soybean plants having a fully expanded second-trifoliolate (T2) leaf were treated with α-tocopherol (3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-timethyltridecyl)-2H-1-benzopyran-6-ol, vitamin E) [Sigma Chemical Co., St. Louis, Missouri; 670 mg (about 1000 IU) *d*-α-tocopherol per gram] as follows. Sealed plastic ampules containing 200 IU of active ingredient, α-tocopherol, were ruptured with a sterile razor blade, and the contents were squeezed into a measured volume of white oil (American White Oil-Heavy, No. 30 U.S.P., American Oil Co., Chicago, Illinois, to yield a final α-tocopherol concentration of 12.5 or 25 IU/ml of white oil. One-half milliliter of one of the two α-tocopherol formulations or the white oil (solvent) control was applied over the upper surface of the three leaflets of the first-trifoliolate (T1) leaf of each soybean plant with a clean camel's hair brush.

Harvest of Soybean Leaves. Leaves were removed from the plants at 48, 72, and 96 hr after treatment for immediate use in CL feeding bioassays or for immediate storage in 100% methanol at −20°C until further experimental use. The following specific procedures were used: (1) Of the two unifoliate leaves per plant, one was used for CL bioassay and the other for chemical analyses. (2) With the T1 leaf, the central leaflet was used for chemical analyses, and the two outside leaflets were used for CL bioassays. (3) With the T2 leaf, the use was the same as with the T1 leaf.

Bioassay of Leaves. The appropriate leaves, or leaflets, were placed between moistened paper towels in a plastic crisper immediately after removal from a plant and then brought within 15 min to the laboratory for CL bioassay. Leaf disks (12 cm diam.) were cut from each leaf using a No. 6 cork borer. Two leaf disks, one from a given leaf on a treated plant and the other from the same leaf on an untreated plant, were secured, with their abaxial surface up, in an opposed position, 2 mm apart in the center of a petri dish, using cut-off insect pins inserted through a filter paper covering an underlying layer of paraffin. Another piece of filter paper was attached to the inner surface of the Petri-dish lid and was moistened with water to maintain the turgidity of leaf disks. One third-instar CL larva, which has been previously starved for 2 hr, was placed in each prepared Petri-dish arena and allowed to make a choice in feeding between the two presented leaf disks at 27 ± 2°C and 65 ± 5% relative humidity. Each assay was run until at least 50% of one leaf disk was eaten or until 8 hr had passed. The number of replicate plants per treatment or control ranged from 15–25. The area eaten per leaf disk by the CL larva was measured in square centimeters, using an automated area meter (model LI-3100, LI-COR, Lincoln, Nebraska). Ten leaf disks were held in petri-dish arenas as if in assays,

but without the CL larva, and after 8 hr their mean square centimeter area was determined as the standard area per CL-unfed (control) leaf disk. The uneaten area of each assayed leaf disk was substracted from the determined standard control leaf-disk area to yield the experimental area eaten.

The statistical significance of the resultant difference in the two leaf areas (cm^2) consumed by CL in each choice bioassay was evaluated by the Student's *t* test.

Extraction and HPLC Separation of Leaf Chemicals. Individual soybean leaves that had been stored in 100% methanol at −20°C in darkness were removed from that solvent and homogenized in a new 50 ml of 100% methanol for 3 min using an Omni-mixer (Ivan Sorvall, Inc., Norwalk, Connecticut). The resultant homogenate and the storage methanol per leaf were filtered through Whatman No. 1 filter paper. The total filtrate per leaf was rotoevaporated to dryness using a 50°C water bath. Each dried sample of extractables was prepared for high performance liquid chromatography (HPLC) as follows: (1) Extractables per leaf were redissolved in 50 ml of a mixture of double-distilled (dd) water, ethyl acetate, and ethanol (6:5:1), and then partitioned four times in a separatory funnel between ethyl acetate and ethanol (25:5). (2) The combined ethyl acetate fractions per sample were rotoevaporated to dryness and redissolved in 10 ml ethyl acetate. (3) Each sample was then stored in a dried, preweighted, screw-cap septum vial and dried under a nitrogen stream. (4) The dried weight per sample was determined. (5) The dried sample in the closed vial was stored at −20°C in darkness inside a desiccator.

Before HPLC, each dried sample of leaf extractables was first dissolved in the containing vial in HPLC-grade ethyl alcohol at 1 ml/10 mg extractables. The resultant contents of each vial were vortexed on a Vortex-Genie for 5 min and then were filtered through a 5-μm Metrical membrane. In HPLC 20 μl of a filtrate were injected per run into a gradient system consisting of mixtures of hexane–2-propanol through a 5-μm, 4.6-μm × 25-cm Ultrasphere SI column (Beckman No. 235341) controlled by an Altex model 422 programming microprocessor and two Altex model 100A pumps. Data were recorded, reduced, and illustrated through a Spectra Physics 4100 computing integrator. Three replicate injections and analyses were conducted per sample of leaf extractables.

RESULTS

Insect Bioassays. α-Tocopherol at 12.5 IU caused CL larvae to feed significantly ($P < 0.05$) less on disks from all leaves of soybean plants, as compared to control soybeans, at 48 hr after treatment (Figure 1). The only other significant effect of the 12.5 IU α-tocopherol treatment on CL feeding was an induced preference for disks from control third-trifoliolate (T3) leaves at 96 hr after treatment.

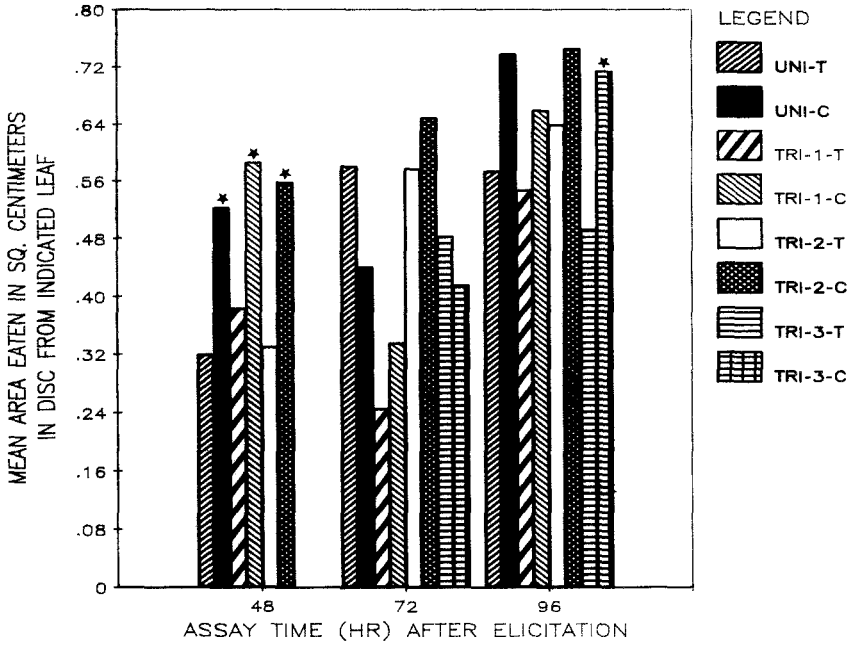


FIG. 1. α-Tocopherol (12.5 IU) elicited differences in cabbage looper feeding (cm^2) on disks cut from the indicated leaf of elicited versus nonelicited (control) soybean plants and presented to the insect in a two-choice assay (*significantly different, $p < 0.05$). UNI-T, unifoliate leaf on an elicited plant; UNI-C, such a leaf on a control plant; TRI-1-T, the first trifoliolate leaf on a treated (elicited) plant; TRI-1-C, the first trifoliolate leaf on a control plant; TRI-2 and TRI-3, ditto above.

α-Tocopherol at 25 IU did not significantly ($P < 0.05$ or greater) alter CL larval feeding preference for soybean leaves until 72 hr after treatment in first- (T1) and second- (T2) trifoliolate leaves (Fig. 2). There subsequently was also a highly significant ($P < 0.01$) induced preference for disks from control T2 and T3 leaves at 96 hr after treatment.

HPLC Resolution of Leaf Extractables. The HPLC-resolved profiles of compounds in the extractables from leaves of elicited (treated) and nonelicited (control) soybean plants were qualitatively similar (Figure 3A and 3B). Major chemicals in the mixture include glyceollins (peak 5, Figure 3A, 3B), coumestrol (peak 9), and daidzein (peak 12) (Neupane and Norris, 1991; Sharma and Norris, 1991). The percentage of total HPLC peak area attributable to individual resolved peaks did not differ ($P < 0.05$) between comparable samples from elicited versus nonelicited (control) soybeans either when highly significant

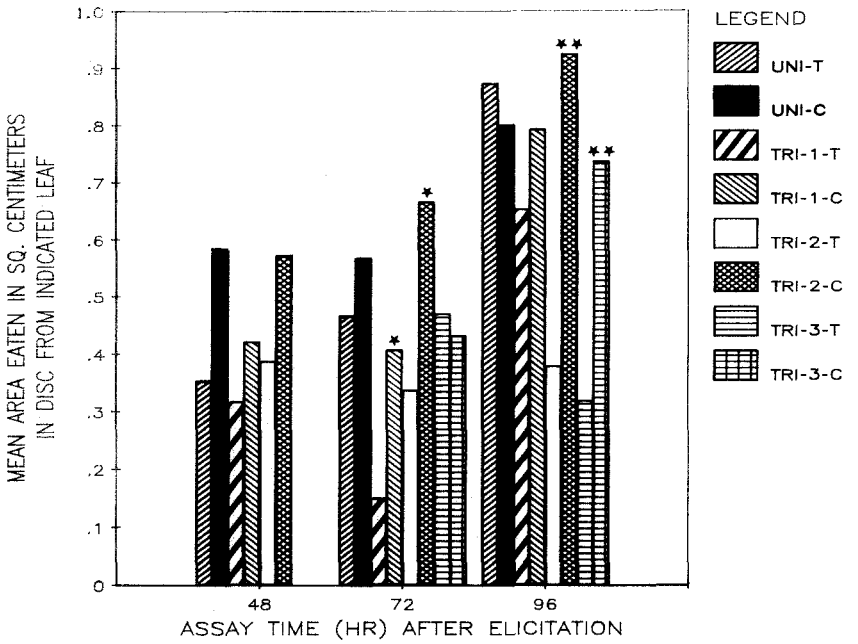


FIG. 2. α-Tocopherol (25 IU) elicited differences in cabbage looper feeding (cm^2) on disks cut from the indicated leaf of elicited versus nonelicited (control) soybean plants, and presented to the insect in a two-choice assay (*significantly different, $P < 0.05$; **$P < 0.01$).

antiherbivory was induced (Figure 4) or no significant ($P < 0.05$) change was elicited (Figure 5).

The mean difference in the area eaten (cm^2) per disk from the same leaf of paired elicited versus nonelicited (control) plants showed a strong positive correlation with the percentage of increased total area under the HPLC peaks of the involved samples from elicited as compared to control soybeans (Figure 6). A linear regression, $Y = -0.13 + 0.042X$, of the mean difference in leaf disk area eaten upon the percentage of increased total area under the HPLC peaks for elicited plants had $r = 0.937$.

DISCUSSION

An Antioxidant as Elicitor. The antioxidant vitamin E (α-tocopherol) proved to be an effective elicitor of alterable soybean resistance to CL herbivory. These results thus support our hypothesis that the application of an antiox-

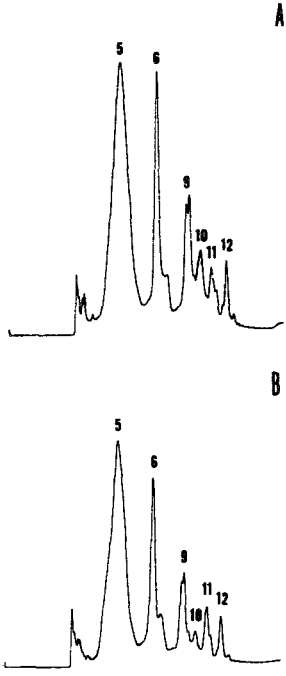


FIG. 3. The similar HPLC-resolved profiles of compounds in the described extractables from leaves of (A) vitamin E-elicited versus (B) nonelicited (control) soybean plants (peak 5, glyceollins; peak 9, coumestrol; and peak 12, daidzein).

idant to a soybean plant can alter its resistance to CL defoliation. We may therefore conclude that even though energy transduction between environmental stress factors and plant cells involves receptor sulfhydryl chemistry, nonspecific antioxidants such as α-tocopherol may alter the redox state of such receptor sulfhydryl chemistry sufficiently to act as elicitory entities regarding alterable soybean resistance to CL herbivory. The sulfhydryl-dependent receptor involved is located in the soybean plasma membrane (Liu and Norris, 1992). This is significant because such a cell membrane appears to be the major site of plant plastic strain, injury, and damage caused by environmental stresses (Levitt, 1980). The plant plasma membrane thus may be the common site where the elicitory effects of various environmental factors (e.g., insect herbivory and certain sulfhydryl reagents) may be mechanically interactive. If this proves to be true, it would support the earlier interpretation that successful (i.e., survivor) plants have evolved to cope with a variety of environmental stresses but rarely, if ever, to deal with a specific stress (e.g., insect herbivory) (Norris, 1988).

Some Parameters of α-Tocopherol Elicitation. The effectiveness of

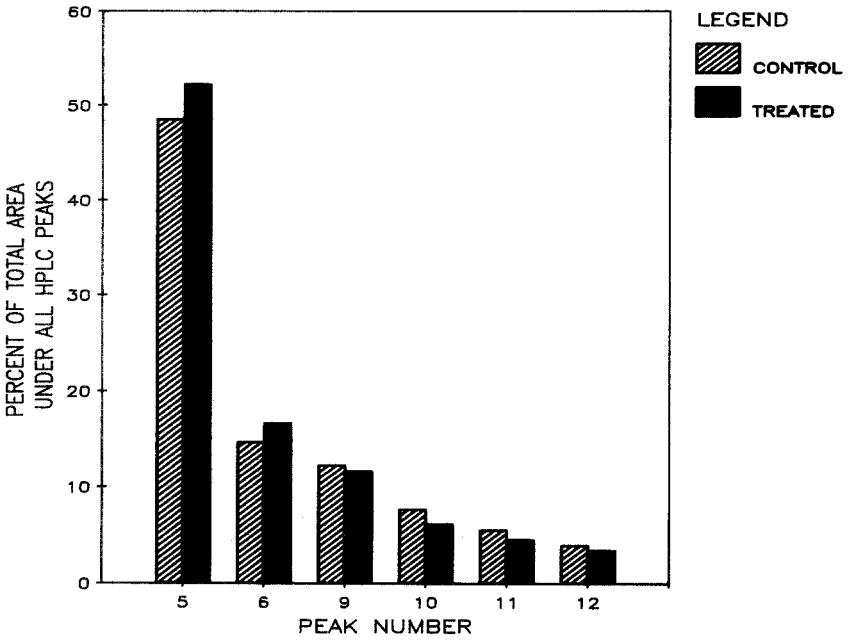


FIG. 4. The percentage of the total area (cm^2) under all HPLC peaks for a given extractable sample (i.e., T3 leaf, 96 hr) attributable to the indicated (numbered, Figure 3) peak did not differ ($P < 0.05$) between elicited (treated) versus control (nonelicited) soybean plants. CL showed a highly significant ($P < 0.01$) feeding preference for the leaf from the control plant in the choice bioassay.

α-tocopherol was clearly dependent on dosage, time, and space parameters. The observed treatment alterations of soybean leaves were all decreases regarding CL preference for herbivory. Significant effects were observed at 48, 72, and 96 hr after treatment. Such effects at 96 hr were limited to the younger T2 and T3 leaves. These leaves might be expected to be the more metabolically active ones on the plant. It is now clear from several previous studies (Chiang et al., 1987; Khan et al., 1987; Norris, 1988) and the present findings that alterable (inducible) soybean resistance to insect herbivory is very dynamic in response to elicitor dose, time since treatment, and space within a plant. At a given elicitor dosage and time after treatment, opposite induced effects on herbivory may even be expected in spatially separated leaves on a plant (Neupane and Norris, 1990; Haanstad and Norris, 1991).

Antifeedant Effects Correlate with Total Extractables. The best negative phytochemical correlate of the bioassayed CL feeding on treated foliage was the percentage of increased total HPLC peak area of the extractables from such

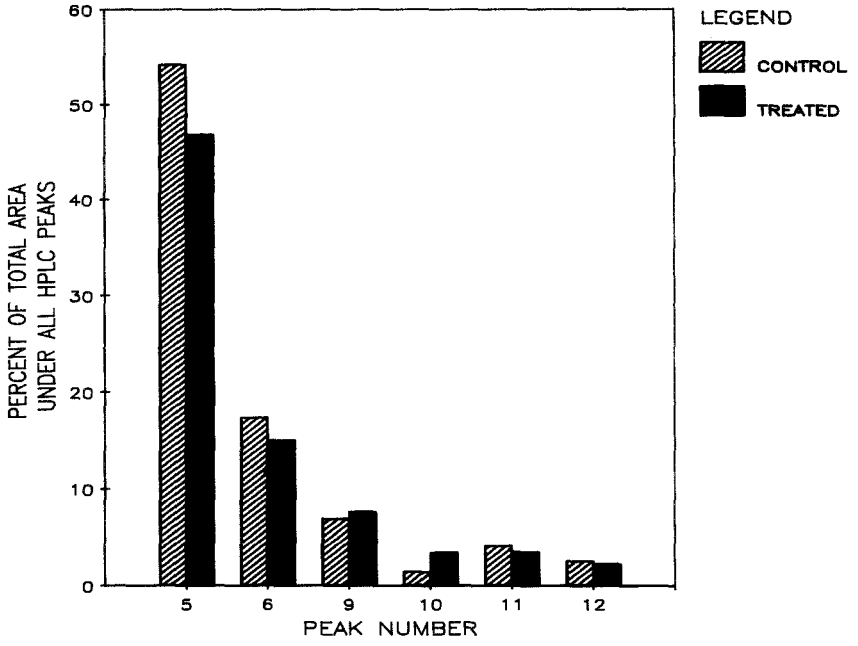


FIG. 5. The percentage of the total area (cm^2) under all HPLC peaks for a given extractable sample (i.e., T3 leaf, 72 h) attributable to the indicated (numbered, Figure 3) peak did not differ ($P < 0.05$) between elicited (treated) versus control (nonelicited) soybean plants. CL showed no significant ($P < 0.05$) feeding preference for the leaf from the control or elicited plant in the choice bioassay.

leaves as compared to that for the nonelicited control plants. Thus, in spite of the proven antifeedant and/or antibiotic effects (Sharma and Norris, 1991) of many of the specific chemicals resolved in the extractables, the findings in the current study support the quantity of the overall mix (i.e., profile) of compounds in the pertinent leaf extractables as especially responsible for the observed effects on CL feeding behavior. Concentrations of specific compounds (e.g., glyceollins) (Neupane and Norris, 1991) also have been highly positively correlated with induced antiherbivory in soybean leaves. Such specific chemicals are major contributors to the overall composition of the pertinent leaf extractables.

Other Effects of Phytoalexins. The results of this study and several others (Kogan and Paxton, 1983; Khan et al., 1987; Norris et al., 1988; Sharma and Norris, 1991) clearly show that phytoalexins, known primarily as antibiotics against microbial plant pathogens, actually are also major antifeedants and antibiotics to insect herbivores. Such findings support the interpretation that inducible defensive phytochemistry is not really specific for pathogens or pests.

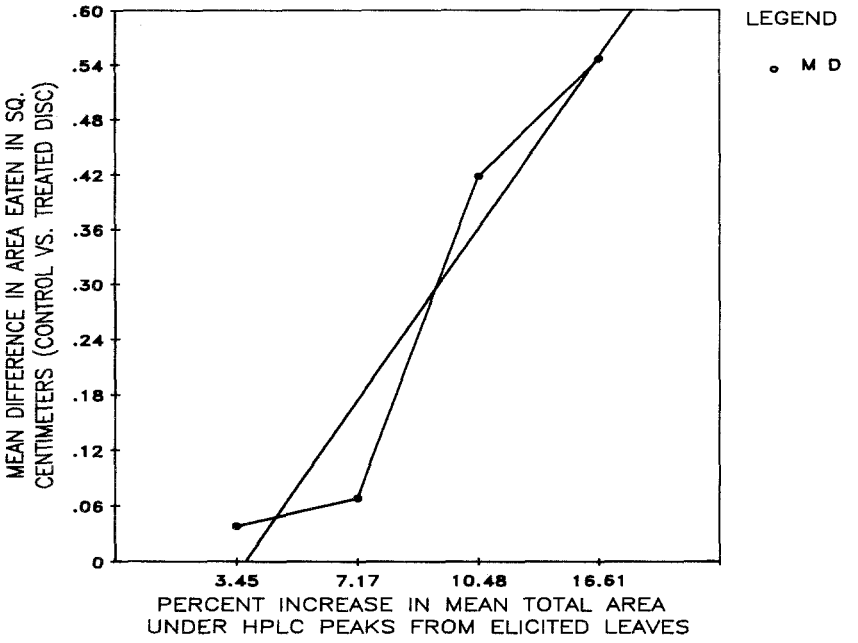


FIG. 6. Linear regression, $Y = -0.13 + 0.042X$ ($r = 0.937$), of the mean difference in the area eaten (cm^2) per disk from the same leaf on paired elicited versus nonelicited (control) soybean plants upon the percentage of the increased total area under the HPLC peaks of the extractables from the elicited as compared to the control leaf.

Acknowledgments—This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and in part by funds from U.S. Hatch Project No. 3040, CRGO/USDA Research Grants 84-CRCR-1-1501 and 88-37153-4043 and USAID-Utah State University-Institute of Agriculture and Animal Science (Tribhuvan University, Nepal)-II Project.

REFERENCES

CHIANG, H.S., NORRIS, D.M., CIEPIELA, A., SHAPIRO, P., and OOSTERWYK, A. 1987. Inducible versus constitutive PI 227687 soybean resistance to Mexican bean beetle, *Epilachna varivestis*. *J. Chem. Ecol.* 13:741–749.

FEHR, W.R., and CAVINESS, C.E. 1977. Stages of soybean development. Special Report 80. Cooperative Extension Service Agriculture and Home Economics, Home Economics Experiment Station. Iowa State University, Ames, Iowa.

GUSTINE, D.L. 1987. Induction of medicarpin biosynthesis in ladino clover calus by *p*-chloromercuribenzoic acid is reversed by dithiothreitol. *Plant Physiol.* 84:3–6.

HAANSTAD, J.O., and NORRIS, D.M. 1991. Altered elm resistance to smaller European elm bark beetle (Coleoptera: Scolytidae) and forest tent caterpillar (Lepidoptera: Lasiocampidae). *J. Econ. Entomol.* In press.

HAMMER, P.A., TIBBITTS, T.W., LAUGHANS, R.W., and MCFARLANE, J.C. 1978. Baseline growth studies of Grand Rapid lettuce in controlled environments. *J. Am. Soc. Hortic. Sci.* 103:649–655.

KHAN, Z.R., CIEPIELA, A., and NORRIS, D.M. 1987. Behavioral and physiological responses of cabbage looper, *Trichoplusia ni* (Hübner), to steam distillates from resistant versus susceptible soybean plants. *J. Chem. Ecol.* 13:1903–1915.

KOGAN, M., and PAXTON, J. 1983. Natural inducers of plant resistance to insects, pp. 153–171, *in* P. A. Hedin (ed.). Plant Resistance to Insects. American Chemical Society, Washington, D.C.

LEVITT, J. 1980. Responses of Plants to Environmental Stresses. Water, Radiation, Salt, and Other Stress, Vol. 2, Academic Press, New York.

LIU, S.H., and NORRIS, D.M. 1992. Sulfhydryl-protein receptor in soybean plasma membrane for a chemical elicitor of insect antifeedants and antibiotics. *Plant Physiol.* In press.

LOW, P. S., and HEINSTEIN, P.F. 1986. Elicitor stimulation of the defense response in cultured plant cells monitored by fluorescent dyes. *Arch. Biochem. Biophys.* 249:472–479.

NEUPANE, F.P., and NORRIS, D.M. 1990. Iodoacetic acid alteration of soybean resistance to the cabbage looper (Lepidoptera: Noctuidae) *Environ. Entomol.* 19:215–221.

NEUPANE, F. and NORRIS, D.M. 1991. Sulfhydryl-reagent alteration of soybean resistance to the cabbage looper, *Trichoplusia ni. Entomol. Exp. Appl.* In press.

NORRIS, D.M. 1979. Chemoreception proteins, pp. 59–77, *in* T. Narahashi (ed.). Neurotoxicology of Insecticides and Pheromones. Plenum Publishing, New York.

NORRIS, D.M. 1981. Possible unifying principles in energy transduction in the chemical senses, pp. 289–306, *in* D.M. Norris (ed.). Perception of Behavioral Chemicals. Elsevier/North-Holland Biomedical Press, Amsterdam.

NORRIS, D.M. 1986. Anti-feeding compounds, pp. 97–146, *in* G. Haug, and G. Hoffmann (eds.). Chemistry of Plant Protection, Vol. 1. Springer-Verlag, Berlin.

NORRIS, D.M. 1988. Sensitivity of insect-damaged plants to environmental stresses, pp. 341–361, *in* E.A. Heinrichs (ed.). Plant Stress-Insect Interactions. John Wiley & Sons, New York.

NORRIS, D.M. 1992. Information exchange among animals, plants, and their environments. Washington State and University Centennial Anniversary Celebration Distinguished Lecture Series 1988–90. Washington State University, Pullman. In press.

NORRIS, D.M., FERKOVICH, S.M., BAKER, J.E., ROZENTAL, J.M., and BORG, T.K. 1970. Energy transduction: Inhibition of cockroach feeding by naphthoquinones. *Science* 170:754–755.

ROZENTAL, J.M., and NORRIS, D.M. 1973. Chemosensory mechanism in American cockroach olfaction and gustation. *Nature* 244:370–371.

ROZENTAL, J.M., and NORRIS, D.M. 1975. Genetically variable olfactory receptor sensitivity in *Periplaneta americana. Life Sci.* 17:105–110.

SCHMIDT, W.E., and EBEL, J. 1987. Specific binding of a fungal glucan phytoalexin elicitor to membrane fractions from soybean *Glycine max. Proc. Natl. Acad. Sci. U.S.A.* 84:4117–4121.

SEQUEIRA, L. 1983. Mechanisms of induced resistance in plants. *Annu. Rev. Microbiol.* 37:51–79.

SHARMA, H.C., and NORRIS, D.M. 1991. Chemical basis of resistance in soy bean to cabbage looper, *Trichoplusia ni. J. Sci. Food Agric.* In press.

SHOREY, M.M., and HALE, R.L. 1965. Mass-rearing of the larvae of nine noctuid species on a simple artificial medium. *J. Econ. Entomol.* 58:522–524.

STÖSSEL, P. 1984. Regulation by sulfhydryl groups of glyceollin accumulation in soybean hypocotyls. *Planta* 160:314–319.

YOSHIKAWA, M., KEEN, N.T., and WANG, M.-C. 1983. A receptor on soybean membranes for a fungal elicitor of phytoalexin accumulation. *Plant Physiol.* 73:497–506.

Journal of Chemical Ecology, Vol. 17, No. 10, 1991

IDENTIFICATION AND DIRECTED BIOSYNTHESIS OF EFRAPEPTINS IN THE FUNGUS *Tolypocladium geodes* GAMS (DEUTEROMYCOTINA: HYPHOMYCETES)[1]

STUART B. KRASNOFF* and SANDEEP GUPTA[2]

USDA-ARS, U.S. Plant, Soil, and Nutrition Laboratory
Tower Road, Ithaca, New York 14853

(Received April 15, 1991; accepted June 11, 1991)

Abstract—HPLC analysis of crude dichloromethane extracts of shaken liquid cultures of the hyphomycetous fungus *Tolypocladium geodes* Gams revealed the presence of efrapeptins. These peptides, which have mitochondrial ATPase inhibitory activity as well as antifungal and insecticidal properties, are previously known only from the congeneric species, *T. niveum* Rostrup. The identity of efrapeptins was confirmed by fast atom bombardment mass spectrometry and by amino acid analysis. HPLC analyses of efrapeptins extracted from single isolates of both *T. geodes* and *T. niveum* indicated that both species produced the same efrapeptins but the profile of relative abundance of the compounds produced was diagnostic for the isolates examined. Efrapeptin F was the major component of the mixture from *T. geodes* with the order of abundance of the six efrapeptins detected being F > G > D ~ E > H > C. Efrapeptin D was the major component from *T. niveum* with the order of abundance of the six efrapeptins detected being D > E > F > C ~ G > H. Efrapeptin F differs from efrapeptin D by a single amino acid residue, efrapeptin F having an alanine where efrapeptin D has a glycine. Addition of alanine to the culture medium increased the relative abundance of efrapeptin F in the profile of both species. Conversely, addition of glycine increased the relative abundance of efrapeptin D in the profile of both species.

Key Words—*Tolypocladium geodes*, *T. niveum*, Deuteromycotina, Hyphomycetes, fungal toxins, efrapeptins, mitochondrial ATPase inhibition, antibiotic, directed biosynthesis.

*To whom correspondence should be addressed.
[1] Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.
[2] Present address: Boyce Thompson Institute, Tower Rd. Ithaca, New York 14853.

INTRODUCTION

The hyphomycetous fungal genus *Tolypocladium* contains a number of soil-inhabiting species, some of which are pathogenic to insects (Bisset, 1983). One species in the genus, *T. niveum* (Rostrup) is especially notable for its secondary metabolites. It produces the cyclosporins, a group of cyclic peptides with well-known immunosuppressive and antifungal activity (Dreyfuss et al., 1976, and references cited), and the efrapeptins (Figure 1), a comparatively lesser known group of antibiotics that have been shown to be potent inhibitors of ATPases from mitochondria, chloroplasts, and bacteria (Gupta et al., 1991a,b, and references cited).

Insecticidal activity has been reported for crude extracts of several species of *Tolypocladium*, including *T. geodes*, and has been attributed to an unidentified material referred to as "tolypin" (Matha et al., 1988). However, at present, the only chemically characterized compounds from *Tolypocladium* species with documented insecticidal activity are the cyclosporins (Weiser and Matha, 1988) and the efrapeptins (Krasnoff et al., 1991), and these two classes of compounds are known so far only from *T. niveum*.

As part of an ongoing investigation of the role that efrapeptins play in the biology of *Tolypocladium* species, we have been screening extracts of isolates of various species for insecticidal activity as a probe for the presence of efrapeptins. We recently detected insecticidal and feeding deterrent activity against adult tephritid fruit flies of several species (*Anastrepha suspensa*, *Ceratitis capitata*, and *Rhagoletis pomonella*) with crude dichloromethane extracts of culture broth from an isolate of *T. geodes* (Krasnoff and Gupta, unpublished data). In

C	Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-AIB-X	(FW=1606)
D	Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-IVA-X	(FW=1620)
E	Ac-PIP-AIB-PIP-IVA-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-IVA-X	(FW=1634)
F	Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-ALA-LEU-IVA-X	(FW=1634)
G	Ac-PIP-AIB-PIP-IVA-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-ALA-LEU-IVA-X	(FW=1648)

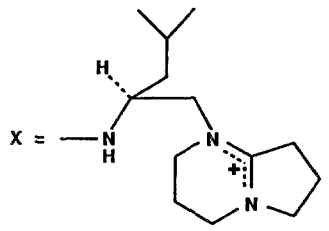


FIG. 1. Structure of efrapeptins C, D, E, F, and G (compounds C–G, respectively). Amino acid residues are abbreviated as follows: alanine (ALA), glycine (GLY), leucine (LEU), α-amino-isobutyric acid (AIB), β-alanine (β-ALA), isovaline (IVA), and pipecolic acid (PIP). Ac = acetyl, FW = formula weight.

this paper we present evidence that *T. geodes* produces efrapeptins and that the relative abundance of the efrapeptins produced by the strain studied is distinct from that produced by an isolate of *T. niveum*. We also present evidence that the amino-acid composition of the culture medium affects the profile of efrapeptins produced by both species.

METHODS AND MATERIALS

Growth of Fungi. Fungal inoculum was obtained from cultures maintained under liquid nitrogen in the USDA-ARS entomopathogenic fungi collection (ARSEF). To compare production of efrapeptins by *T. geodes* (ARSEF #2684) and *T. niveum* (ARSEF #616) under identical conditions, conidia were harvested in sterile 0.1% Tween-80 from 7- to 9-day-old cultures grown on slants of Sabouraud dextrose agar + 1% yeast extract (SDAY) at 22 ± 1°C. Concentrations of initial conidial suspensions were determined using an improved Neubauer hemacytometer. Suspensions of ca. 1×10^6 conidia/ml were made by dilution of initial suspensions in sterile 0.1% Tween-80. These were used to inoculate 100-ml batches of Czapek-Dox (Difco) liquid medium + 0.5% bactopeptone (Difco) in 250 Erlenmeyer flasks with 1×10^6 conidia. Liquid cultures were grown at 22 ± 1°C on a rotary shaker (160 rpm) for 11 days. Six replicates were produced for each species.

To investigate the effect of glycine and alanine on efrapeptin production, the fungi were grown in the above medium as well as media supplemented with either 1.0% L-alanine or 1.0% glycine (Sigma). Agar plugs from 13-to 14-day-old cultures grown on SDAY at 22 ± 1°C were used as inoculum for this experiment. Liquid cultures were grown at 22 ± 1°C on a rotary shaker (160 rpm) for 11 days. Each of the three treatments (control medium, alanine-supplemented medium, and glycine-supplemented medium) was duplicated.

Production of Extracts. Fungal mycelium, harvested after 11 days of growth, was separated from the broth by filtration through eight layers of cheese cloth. The broth was then centrifuged at 860 *g* for 10 mins. The pellet, containing conidia and mycelial fragments, was discarded and the supernatant was extracted three times with 100-ml portions of dichloromethane. The organic layer was concentrated *in vacuo*, then taken up in 60 ml of dichloromethane, washed twice with 10-ml portions of deionized water, dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo* once again. The extract was then transferred through a cotton filter to a vial in 3 × 2-ml portions of dichloromethane and concentrated to dryness with gentle heating under a stream of dry nitrogen. Extracts were stored at −20°C. Prior to HPLC analysis, samples were dissolved in methanol and filtered through a 0.45-μm membrane filter cartridge.

Isolation and Characterization of Efrapeptins. A sample of the crude dichloromethane extract of culture broth of the *T. geodes* isolate was analyzed on a reverse-phase C8 analytical column (0.4 × 15 cm, particle size 5 μm) eluted with MeCN–12.5 mM $(NH_4)_2SO_4$ (63:37) at a flow rate of 1.2 ml/min. Detection was by monitoring UV absorption at 225 nm (0.2 aufs). Material for fast atom bombardment mass spectrometry (FABMS) and amino acid analysis was obtained from repetitive semipreparative HPLC on a reverse-phase C8 column (0.9 × 50 cm) eluted with MeCN–12.5 mM $(NH_4)_2SO_4$ (75:25) at a flow rate of 5.5 ml/min. Detection was by monitoring UV absorption at 225 nm (0.5 aufs). Amino acid analysis was performed on an analyzer in which amino acid composition of the hydrolyzate was determined as the phenylthiohydantoin derivatives by comparing HPLC retention times with those of standards. Efrapeptin profiles were evaluated by HPLC analyses using an integrating chart recorder (Hewlett-Packard 3390 A) for quantitative comparison of peak areas. Semiquantitative comparisons among samples of relative amounts of efrapeptins produced per unit of crude extract were made by comparing total efrapeptin peak area and correcting for amount of crude extract injected (product of injection volume and concentration of extract solution). A standard curve was generated to verify that the response of the UV detector to efrapeptins was linear with respect to the amount of extract injected onto the HPLC.

RESULTS

Isolation and Characterization of Efrapeptins. HPLC analysis of crude dichloromethane extracts of *T. geodes* indicated the presence of a mixture of efrapeptins (Figure 2). Peaks from the *T. geodes* extracts cochromatographed with efrapeptins C, D, E, F, and G from a partially purified mixture from *T. niveum* (ARSEF #616) (Gupta et al., 1991b). A peak eluting after efrapeptin G possibly corresponds to efrapeptin H as shown by Jackson et al. (1979). Peak H made up ca. 6% of the total area of the efrapeptin fraction from *T. geodes*. The amino acid sequence of this peak remains to be determined.

Identification of efrapeptins from *T. geodes* extracts was confirmed by data from FABMS analysis and amino acid analysis of an HPLC-purified mixture of efrapeptins C, D, E, F, G, and H. FAB mass spectra showed molecular ions (M^+) at *m/z* 1606, 1620, 1634, 1648, and 1662. The quaternary nitrogen in the C-terminal blocking group of the efrapeptins (Figure 1) is responsible for producing unusually intense molecular ion peaks (M^+) instead of the usual $M+H^+$ under FABMS conditions (Gupta et al., 1991b). This spectrum is consistent with the presence of efrapeptins C (1606), D (1620), E (1634), F (1634), G (1648), and a sixth compound of molecular weight 1662, probably efrapeptin H.

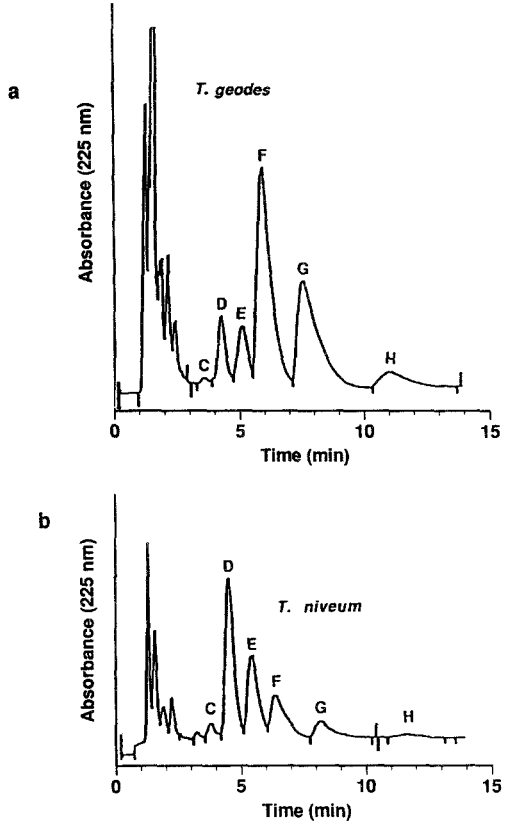


FIG. 2. HPLC chromatograms of crude extracts from *Tolypocladium geodes* (5 μl of 5 μg/μl solution in methanol) and *T. niveum*. (4 μl of 5 μg/μl solution in methanol). Samples were injected on a reverse-phase C8 analytical column (0.4 × 15 cm, particle size, 5 μm; solvent MeCN–12.5 mM $(NH_4)_2SO_4$ (63:37); flow 1.2 ml/min; detection UV 225 nm, 0.2 aufs. Fungi were grown on Czapek-Dox liquid medium + 0.5% bactopeptone.

Amino acid analysis of the HPLC-purified mixture confirmed the presence of α-aminoisobutyric acid (aib), leucine (leu), isovaline (iva), glycine (gly), β-alanine (β-ala), and alanine (ala) in a molar ratio of 672.2 (aib):257.3 (leu):203.2 (iva):185.1 (gly):148.8 (β-ala):123.8 (ala). This corresponds well with the molar ratio calculated for a mixture of efrapeptins C, D, E, F, and G, by taking into account their amino acid content (Figure 1), their relative abundance in *T. geodes* crude extracts as determined by HPLC analyses (see below), and standardizing to the molar estimate for leucine from the analysis: 717.0 (aib):257.3 (leu):183.6 (iva):153.2 (gly):128.7 (β-ala):104.1 (ala).

Comparison of Efrapeptin Profiles. Efrapeptin F was the major component of the efrapeptin profile of *T. geodes* with the order of abundance of the six detectable efrapeptins being F > G > D ~ E > H > C (Table 1, Figure 2a). Efrapeptins F and G accounted for >75% of the efrapeptin fraction of *T. geodes*. The major component in the efrapeptin profile of *T. niveum* was efrapeptin D with the order of abundance of the six efrapeptins being D > E > F > C ~ G > H (Table 1, Figure 2b). Efrapeptins D, E, and F accounted for >85% of the efrapeptin fraction of *T. niveum*. Efrapeptin G, a major component in the *T. geodes* profile, was a minor component in the *T. niveum* profile. Conversely, efrapeptin E, a major component in the *T. niveum* profile, was a minor component in the *T. geodes* profile.

Although *T. geodes* produced almost twice as much extract per unit of broth, 18.7 ± 1.2 mg/100 ml (±standard error for $N = 6$) compared with *T. niveum*, 8.9 ± 0.2 mg/100 ml (±standard error, $N = 6$), it produced only 0.63 times as much of the efrapeptins per unit mass of extract as *T. niveum*. Thus *T. geodes* produced 1.3 times more efrapeptins per unit volume of broth than *T. niveum*.

Effect of Supplemental Alanine and Glycine on Efrapeptin Profiles. Efrapeptins F and D differ only by an amino acid substitution at position 13 (Figure 1), D having a glycine and F having an alanine. With the standard medium (Czapek-Dox + 0.5% bactopeptone), *T. geodes* produced a ratio of efrapeptin F to D of 5.3:1 (ratios presented are based on the average of two replicates; Figure 3a). Cultured with 1% alanine this ratio increased to 11.3:1 (Figure

TABLE 1. RELATIVE ABUNDANCE OF EFRAPEPTINS C, D, E, F, G, H IN DICHLOROMETHANE EXTRACTS OF CULTURE BROTH FROM *T. geodes* AND *T. niveum* EXPRESSED AS AVERAGE PERCENTAGE OF TOTAL EFRAPEPTIN FRACTION BASED ON HPLC PEAK AREA $n = 6$ SAMPLES; (RANGE OF PERCENTAGES SHOWN IN PARENTHESES)[a]

Efrapeptin	Species	
	T. geodes	*T. niveum*
C	0.6(0.4–0.7)	6.0(4.8–7.7)
D	8.3(6.6–9.5)	39.9(37.1–41.8)
E	9.1(8.4–10.0)	24.5(22.3–26.1)
F	44.5(42.9–46.1)	21.9(20.3–23.1)
G	31.7(30.3–33.6)	6.8(4.4–9.4)
H	6.0(5.1–7.4)	1.0(0.0–0.4)

[a] Means for all six compounds were significantly different between species at $\alpha = .01$ (T-tests with α-levels adjusted by Bonferoni's inequality).

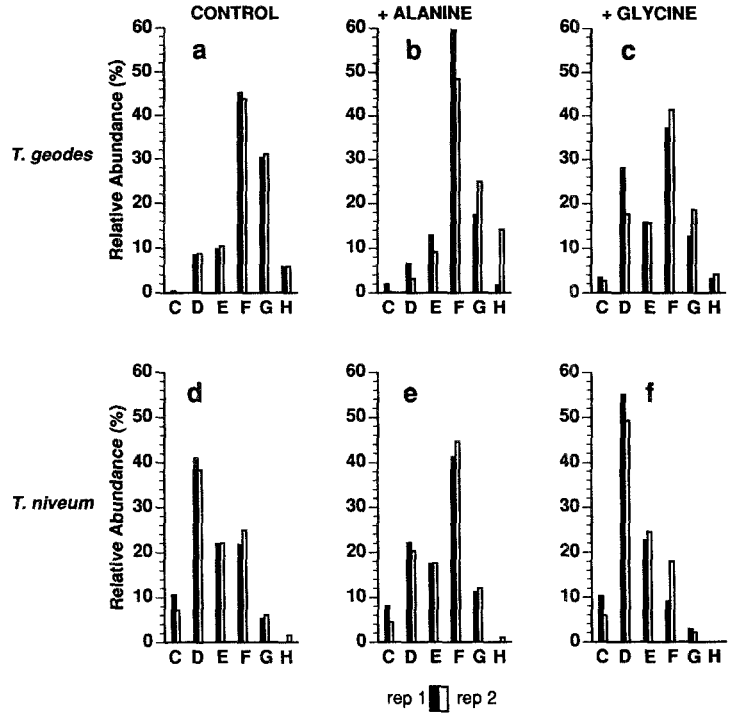


FIG. 3. Effect of supplementing standard growth medium (Czapek-Dox + 0.5% bactopeptone) with 1% alanine or 1% glycine on the efrapeptin profiles of *Tolypocladium geodes* and *T. niveum*. Relative abundance of efrapeptins C, D, E, F, G, and H (labeled on the *x* axes) in dichloromethane extracts of culture broth expressed as percentage of efrapeptin fraction based on HPLC peak area. Shown are results from two replicates.

3b). Cultured with 1% glycine, this ratio decreased to 1.7:1 (Figure 3c). With the standard medium, *T. niveum* produced a D to F ratio of 1.7:1 (Figure 3d). Grown with 1% alanine, this ratio decreased to 0.5:1 (Figure 3e). Grown with 1% glycine this ratio increased to 3.9:1 (Figure 3f). Thus in both species supplemental glycine had the effect of increasing the production of D relative to F while supplemental alanine had the effect of increasing the production of F relative to D.

Some analogous effects, although not as dramatic and consistent, were noted with the secondary components of the two species, efrapeptins E and G, which also differ from each other only by the replacement of glycine with alanine at position 13 (Figure 1). With additional alanine, the ratio of G to E in *T. geodes* extracts actually decreased from 3.1:1 to 1.9:1 counter to the expected pattern (Figure 3a,b). With additional glycine, however, the ratio decreased

predictably from 3.1:1 to 1:1 (Figure 3c). In *T. niveum* extracts the E to G ratio, which was 3.9:1 in the controls (Figure 3d), decreased predictably to 1.5:1 with additional alanine (Figure 3e) and increased predictably to 9.2:1 with additional glycine (Figure 3f).

DISCUSSION

We have documented the production of efrapeptins by *T. geodes* and have described a distinct difference between the efrapeptin profile of the isolate studied and that of an isolate of *T. niveum*. Two independent HPLC analyses of an efrapeptin fraction from another *T. niveum* isolate (Jackson et al., 1979; Krishna et al., 1990) show that efrapeptin D is the primary component with the order of abundance of efrapeptins essentially the same as reported here for *T. niveum*. This points to the possibility that the strain-specific differences we have documented here are representative of species-level characteristics. However, we recognize the need to evaluate a broader range of isolates of each species to support such a claim and are currently expanding our survey of isolates to provide a better overall picture of the intra- and interspecific patterns of variation in efrapeptin production in *T. geodes* and *T. niveum*.

The literature on antibiotics and mycotoxins is replete with reports of species-specific or strain-specific profiles based on the presence or absence of various compounds (e.g., Frisvad, 1986; Katz and Demain, 1977; Moss, 1982). However, documentation of strain- or species-specific differences of the type we have described here, i.e., where there is a diagnostic pattern of relative abundance in a microheterogeneous group of secondary metabolites, is relatively rare. Examples are the actinomycin profiles of *Streptomyces chrysomallus* and *S. antibioticus* (Katz, 1974) and the xanthomegnin/viomellein profiles of *Aspergillus ochraceus*, *Penicillium cyclopium*, and *P. viridicatum* (Stack and Mislivec, 1978).

There are precedents in the antibiotic literature for what has been called "directed" or "controlled" biosynthesis, i.e., the favoring of particular components of a microheterogeneous mixture by providing exogenous precursors (Katz, 1974). It is intriguing that this phenomenon has been previously reported to occur with cyclosporins, the other class of antibiotics known to be produced by *T. niveum* (Kobel and Traber, 1982).

As mentioned above, efrapeptin D, the primary product of *T. niveum*, differs from efrapeptin F, the primary product of *T. geodes*, by one amino acid substitution, efrapeptin D having a glycyl residue in position 13 where F possesses an alanyl residue. In analogous fashion, the second most abundant efrapeptins for each species, E in *T. niveum* and G in *T. geodes*, differ in precisely the same way, E having glycine in position 13 where G has an alanine. Although

nothing has been published regarding the biosynthesis of the efrapeptins, the difference observed here between the two species points to the existence of a mechanism whereby *T. niveum* favors the inclusion of glycine vs. alanine at position 13 and vice versa for *T. geodes*. This could involve differences in the levels of biosynthesis of the constituent amino acids, differences in the preference for one amino acid over another at a particular step in biosynthesis, or a combination of both scenarios. The only firm conclusion that can be made based on our findings regarding the biosynthesis of efrapeptins is that both fungi studied can incorporate exogenous amino acids into the peptide chains. This suggests the possibility that similar effects might be observed with α-aminoisobutyric acid and isovaline supplements in that several differences among the known efrapeptins are due to substitutions of these two amino acids at positions 4 and 15 (Figure 1). The effects of glycine and alanine supplements on the production of the major components in the efrapeptin profiles of the two species also have a practical implication; they point to the feasibility of directing biosynthesis toward either efrapeptin D or F, thus making purification more efficient in any large-scale effort to produce efrapeptins.

T. geodes is the second species in the genus *Tolypocladium* for which efrapeptin production has been documented. The presence of efrapeptins in *T. geodes* may account for the antifungal activity observed by Lundgren et al. (1978) in isolates of *T. geodes* and the insecticidal activity observed in extracts of *T. geodes* reported by Matha et al. (1988). Our results suggest the possibility that efrapeptins may occur elsewhere in the genus and their presence (and quantitative profiles) may be of taxonomic relevance. A more complete view of the pattern of efrapeptin distribution among species of *Tolypocladium* should offer insights into the evolution and function of these secondary metabolites.

Acknowledgments—We thank V. Macko, T.J. Poprawski, R.J. St. Leger, and an anonymous reviewer for critical comments on the manuscript, and M. Chapple for technical assistance.

REFERENCES

BISSET, J. 1983. Notes on *Tolypocladium* and related genera. *Can. J. Bot.* 61:1311–1329.

DREYFUSS, M., HARRI, E., HOFMANN, H., KOBEL, J., PACHE, W., and TSCHERTER, H. 1976. Cyclosporin A and C. *Eur. J. Appl. Microbiol.* 3:125–133.

FRISVAD, J.C. 1986. Taxonomic approaches to mycotoxin identification (taxonomic indication of mycotoxin content in foods), pp. 415–457, *in* R.J. Cole (ed.). Modern Methods in the Analysis and Structural Elucidation of Mycotoxins. Academic Press, Orlando.

GUPTA, S., KRASNOFF, S.B., ROBERTS, D.W., RENWICK, J.A.A., BRINEN, L., and CLARDY, J. 1991a. Structures of the efrapeptins—potent inhibitors of mitochondrial ATPase from the fungus *Tolypocladium niveum*. *J. Am. Chem. Soc.* 113:707–709.

GUPTA, S., KRASNOFF, S.B., ROBERTS, D.W., RENWICK, J.A.A., BRINEN, L., and CLARDY, J. 1991b. Structure of efrapeptins from the fungus *Tolypocladium niveum:* Peptide inhibitors of mitochondrial ATPase *J. Org. Chem.* Submitted.

JACKSON, C.G., LINNET, P.E., BEECHEY, R.B., and HENDERSON, P.J.F. 1979. Purification and preliminary structural analysis of the efrapeptins, a group of antibiotics that inhibit the mitochondrial adenosine triphosphatase. *Biochem. Soc. Trans.* 7:224–226.

KATZ, E. 1974. Controlled biosynthesis of actinomycins. *Cancer Chemother. Rep.* 58:83–91.

KATZ, E., and DEMAIN, A.L. 1977. The peptide antibiotics of *Bacillus:* Chemistry, biogenesis, and possible functions. *Bacteriol. Rev.* 41:449–474.

KOBEL, H., and TRABER, R. 1982. Directed biosynthesis of cyclosporins. *Eur. J. Appl. Microbiol Biotechnol.* 14:237–240.

KRASNOFF, S.B., GUPTA, S., ST. LEGER, R.J., RENWICK, J.A.A., and ROBERTS, D.W. 1991. Antifungal and insecticidal properties of the efrapeptins: Metabolites of the fungus *Tolypocladium niveum. J. Invert. Pathol.* 58:180–188.

KRISHNA, K., SUKUMAR, M., and BALARAM, P. 1990. Structural chemistry and membrane modifying activity of the fungal polypeptides zervamicins, antiamoebins and efrapeptins. *Pure Appl. Chem.* 62:1417–1420.

LUNDGREN, B., BAATH, E., and SODERSTROM, B.E. 1978. Antagonistic effects of *Tolypocladium* species. *Trans. Br. Mycol. Soc.* 70:305–307.

MATHA, V., WEISER, J., and REPOVA, A. 1988. Entomocidal activity of metabolites from strains of fungi of the genus *Tolypocladium* (Deuteromycetes). *Microbios Lett.* 38:71–73.

MOSS, M.O. 1982. The biosynthesis of *Fusarium* mycotoxins, pp. 195–213, *in* M.O. Moss and J.E. Smith (eds.). The Applied Mycology of *Fusarium*. Cambridge University Press, Cambridge.

STACK, M.E., and MISLIVEC, P.B. 1978. Production of xanthomegnin and viomellein by isolates of *Aspergillus ochraceus*, *Penicillium cyclopium*, and *Penicillium viridicatum. Appl. Environ. Microbiol.* 36:552–554.

WEISER, J., and MATHA, V. 1988. The insecticidal activity of cyclosporines on mosquito larvae. *J. Invert. Pathol.* 51:92–93.

Journal of Chemical Ecology, Vol. 17, No. 10, 1991

CHEMOORIENTATION OF EASTERN TENT CATERPILLARS TO TRAIL PHEROMONE, 5β-CHOLESTANE-3,24-DIONE

STEVEN C. PETERSON[1] and T.D. FITZGERALD[2,*]

[1]*Department of Biological Sciences*
University of Maryland, Baltimore County
Catonsville, Maryland 21228

[2]*Department of Biological Sciences*
State University of New York, College at Cortland
Cortland, New York 13045

(Received April 22, 1991; accepted June 18, 1991)

Abstract—Chemoorientation behavior of the larval eastern tent caterpillar, *Malacosoma americanum*, was studied using the synthetic trail pheromone 5β-cholestane-3,24-dione. Divergent arms of Y mazes were treated with various concentration ratios of the pheromone. At application rates of 10^{-10}–10^{-9} g/mm of trail, larvae showed a significant preference for stronger trails when concentration ratios differed by as little as 4 : 1. At application rates of 10^{-8} and greater there was no significant difference in trail choice even when trails differed in strength by a full order of magnitude. Other studies showed that the caterpillars abandon the pattern of choosing stronger over weaker trails when they repeatedly fail to find food at the end of a stronger trail. Experiments in which larvae were required to choose trails separated by a gap demonstrated orientation by chemoklinotaxis. Caterpillars that had one of the maxillary palps ablated looped in the direction of their intact chemoreceptor when placed on filter paper treated uniformly with pheromone, indicating that they may also orient by tropotaxis. The relevance of these findings to the tent caterpillar communication system is discussed.

Key Words—Chemoorientation, pheromone, eastern tent caterpillar, *Malacosoma americanum*, trail following, klinotaxis, tropotaxis, 5β-cholestane-3,24-dione, Lepidoptera, Lasiocamsidae.

*To whom correspondence should be addressed.

0098-0331/91/1000-1963$06.50/0

INTRODUCTION

Previous behavioral experiments with eastern tent caterpillars, *Malacosoma americanum* (Fitzgerald, 1976), and caterpillars of the lackey moth, *Malacosoma neustria* (Peterson, 1988), demonstrated that larvae deposit chemical markers to facilitate orientation to and from feeding sites. A major component of the pheromone system of the eastern tent caterpillar, 5β-cholestane-3,24-dione, was identified recently and shown to elicit trail following at threshold levels of 10^{-11} g/mm of trail (Crump et al., 1987). Caterpillars mark exploratory trails as they move to food by dragging a ventral secretory site against the substrate (Fitzgerald and Edgerly, 1982). Successful foragers overmark these trails as they return to the tent (Fitzgerald and Peterson, 1983). Overmarked trails are analogous to the recruitment trails used by ants and serve to lead siblings to food finds.

Observations made in earlier studies indicated that the trail system of the eastern tent caterpillar consists of chemical trails of variable age and strength. For example, both the availability and the quality of foliage affect the marking intensity of caterpillars (Fitzgerald and Peterson, 1983). Moreover, although the chemical marker is long-lived and may persist for weeks under field conditions, it declines in effectiveness as it ages (Fitzgerald and Gallagher, 1976; Peterson, 1988). As caterpillars move over this trail system, they must choose between the divergent trails that occur at forks in the branches. Previous studies showed that caterpillars choose newer over older trails at these choice points (Fitzgerald and Gallagher, 1976), but the effect of pheromone concentration on trail selection behavior has not been assessed previously. We therefore undertook studies to determine how caterpillars respond to trails containing different quantities of the synthetic marker.

A second study was undertaken to investigate the orientation mechanisms of trail-following tent caterpillars. 5β-Cholestane-3,24-dione is perceived by contact chemoreception mediated by the maxillary palps (Roessingh et al., 1988), which are bilateral mouthpart chemoreceptors (Schoonhoven, 1987). Generally, organisms that perceive stimuli via bilateral receptors may use tropotactic or klinotactic orientation mechanisms (Bell, 1984; Bell and Tobin, 1982; Kennedy, 1986). In tropotaxis, animals orient to spatial gradients of stimuli by sampling simultaneously left and right from the paired sensory receptors (instantaneous comparisons) (Bell and Tobin, 1982). In klinotaxis, sampling is done successively (temporal comparisons) (Bell and Tobin, 1982), either along a path (longitudinal klinotaxis) or from side to side (transverse klinotaxis). In both klinotaxis and tropotaxis, locomotory output may take the form of turning to the more stimulated side (Kennedy, 1986). Thus, manipulations of the sensory system, the stimulus field, or both, are necessary to determine if tropotactic or klinotactic mechanisms are being used.

METHODS AND MATERIALS

Malacosoma americanum larvae were collected as early instars from field colonies or reared from field-collected egg masses hatched in the laboratory. Laboratory colonies were kept as described previously (Fitzgerald and Peterson, 1983).

Tent caterpillar larvae readily orient to artificial trails made of synthetic 5β-cholestane-3,24-dione (Crump et al., 1987; Peterson, 1988). To determine if larvae distinguish a higher dosage pheromone trail from a lower dosage one (i.e., a stronger trail from a weaker one), we applied the synthetic pheromone to filter-paper Y mazes (Figure 1). The design of the maze enabled larvae to encounter discretely weaker and stronger pheromone stimuli at the fork. 5β-Cholestane-3,24-dione dissolved in hexane was applied to the paper strips using 5-μl capillary pipets. The runway of the maze was treated with the higher dosage of pheromone. Concentrations ranged from 2.5×10^{-10} g/mm to 2.5×10^{-6} g/mm. Twenty second- or third-instar caterpillars were used in each test. Each caterpillar was tested on a separate Y maze.

Large numbers of caterpillars frequently collect at newly discovered feeding sites and may exhaust them before all of the caterpillars in the colony have

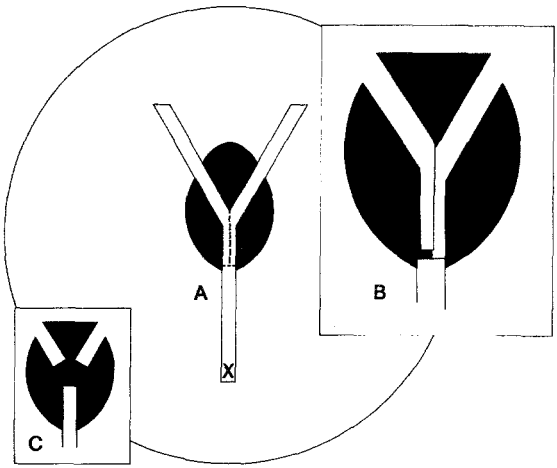


FIG. 1. (A) The Y mazes used in this study were drawn in pencil on filter paper. Black areas were cut out and removed and the stem of each maze was cut along the dotted line. As shown in (B), this allowed each arm to be bent slightly upward when applying the pheromone to prevent bleed-over. As the caterpillar moved down the stem of the maze from point (X), it contacted both treated arms before they diverged. (C) In some trials, sections were removed from the juncture of the stem and arms to prevent instantaneous comparison of trail strength as described in the text.

fed. Hungry caterpillars are likely to choose the heavily marked trails that lead to such sites initially, but they must be prepared to abandon them for weaker trails or unmarked branches when they discover that they no longer lead to food (Fitzgerald and Peterson, 1988). Studies were conducted to determine how long an individual persists in choosing the more heavily marked of two trails when the trail does not lead to food. The response of 20 third-instar larvae to the alternate arms of paper Y mazes was observed. Trails of the synthetic pheromone were established on the arms of the maze using a concentration of 2.5×10^{-9} g/mm for the stronger trail and a 1/10 dilution for the weaker trail. Each animal was allowed to move ad libitum back and forth over the maze for 5 min, and its choice was recorded each time it encountered the juncture of the strong and weak trail. The maze was oriented vertically and suspended from cardboard with insect pins to discourage the caterpillars from leaving it. A new maze was constructed for each animal.

To determine if larvae can orient chemoklinotactically, the Y maze was modified to force larvae to make temporal comparisons of two trails. A small gap was created in the maze by cutting away the junction of the runway and arms (Figure 1). The gap did not impede the forward progress of the caterpillars but was sufficiently large to ensure that the second-instar caterpillars were unable to sense both trails simultaneously, requiring them to swing their heads from trail to trail. The arms of the maze were treated with synthetic pheromone using a concentration of 2.5×10^{-9} g/mm for the stronger trail and a 1/10 dilution for the weaker. The responses of 20 second-instar caterpillars were observed.

To determine if tent caterpillars make instantaneous comparisons with their palps, we unilaterally ablated the palps by pinching with a fine forceps while gently holding the animal under a dissecting microscope. Insects were allowed to recover overnight and were examined prior to testing to ensure that the palp was completely removed. Control animals were handled in the same way but were not operated on.

Both surgically altered and control animals were tested on a uniform field of pheromone, which was prepared by placing a sheet of 11-cm Whatman No. 2 filter paper into a Petri dish and adding 2 ml of 10^{-9} g/ml of the synthetic pheromone dissolved in hexane. This technique produced a uniform wetting of the paper with pheromone solution. The filter paper was air-dried for 30 min. A caterpillar was placed in the center of the field and its activity was recorded on video tape. The test was terminated when the larva encountered the edge of the field. The caterpillar pathways were traced onto transparencies directly from the video. The pathways were digitized using SigmaScan (Jandel Scientific, Corte Madera, California), and the path length was measured. From these data we calculated an index of "search intensiveness" (Schal et al., 1983), by counting the number of times the animal crossed its own path (loops) clockwi. and counterclockwise, then dividing the number of loops by the distance traveled.

Thus, the index increases in magnitude with the number and compactness of the loops.

RESULTS

Tent caterpillars exhibited rhythmic side-to-side head movements (wig-wagging) (Bell and Tobin, 1982) as they walked on artificial trails. Encounters with discontinuities or Y-maze branches were often accompanied by a locomotory pause and head waving over a larger radius. Caterpillars typically checked both arms of the Y maze before proceeding.

In dosage-discrimination tests, tent caterpillar larvae preferred stronger to weaker trails (Figure 2). When the trail pheromone was applied at a dosage of 2.5×10^{-10} g/mm, all larvae tested at the 10:1 concentration ratio chose the stronger trail. The preference was less pronounced at a 4:1 concentration ratio, and there was no significant preference for the stronger trail at a 2:1 concentration ratio. At higher pheromone concentrations, preference for the stronger trail was diminished in tests of 10:1 concentration ratios. For example, there was no significant preference for stronger trails when the weak trail dosage was 10^{-8} or 10^{-7} g/mm (Figure 2). The results demonstrate that tent caterpillars

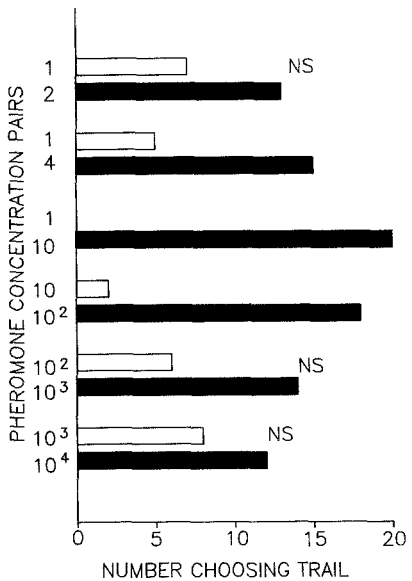


FIG. 2. Dosage dependency of eastern tent caterpillar trail following. The y axis values are multiples of the base concentration of 2.5×10^{-10} g of pheromone per millimeter of trail. NS = not significant, all others significant at $P < 0.05$ (G test).

can distinguish differences in trails of less than an order of magnitude when the marker is applied at near threshold strength, but orientation to stronger trails is less pronounced at higher absolute pheromone dosages. Pheromone concentrations in these tests were several orders of magnitude above threshold, levels unlikely to occur in nature. Such concentrations may lead to saturation of the maxillary chemoreceptors and prevent discrimination.

Observations of caterpillars allowed to make repeated choices between the weak and strong arms of a Y maze show that caterpillars initially prefer the stronger of the two but eventually move onto the weaker one (Figure 3). By the fourth encounter, greater than 50% of the larvae had chosen the weaker trail. The maximum number of encounters required for any individual caterpillar to choose the weaker trail was 16 ($N = 1$), and the minimum number was two ($N = 3$).

The ability of caterpillars to make temporal comparisons of stimulus intensity was demonstrated in tests involving Y mazes altered by removing a section of the trail at the choice point (Figure 1). Nineteen of the 20 larvae tested chose the stronger trail after crossing the gap ($P < 0.005$, G test).

The results of the ablation experiments are summarized in Figure 4. Caterpillars with the left palp removed looped clockwise, in the direction of their intact chemoreceptor. Caterpillars with the right palp removed looped counterclockwise, also in the direction of their intact chemoreceptor. Unoperated caterpillars traveled in loops, but there was no consistent directionality to their movements. Examples of looping behavior are given in Figure 5.

DISCUSSION

Our study shows that tent caterpillars can discriminate trails on the basis of relative pheromone concentration. Under field conditions, trails branch frequently and at each juncture caterpillars must chose between alternate pathways. At normal hunger levels and when trail recency is not a factor, caterpillars initially choose more heavily marked trails over weaker ones (Figure 2). Since the trail system of the caterpillar becomes weaker with distance from the tent due to progressive branching of its structure, caterpillars can return directly to the tent after feeding by selecting stronger over weaker trails at each juncture point. By applying the same rule, outbound foragers can move directly to the feeding sites marked by the largest numbers of successful foragers, even though the overall trail gradient declines with distance from the tent.

Discrimination of trails by strength may be only one form of discrimination used by the caterpillars. Previous studies showed that caterpillars can also discriminate trails by age (Fitzgerald and Gallagher, 1976). Although the ultimate basis for the discrimination of aged trails might also be relative trail strength,

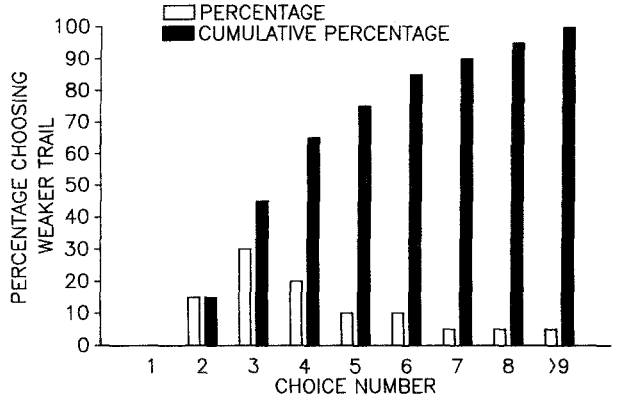


FIG. 3. Percentage of caterpillars choosing weaker trail (2.5×10^{-10} g/mm) over stronger trail (2.5×10^{-9} g/mm) during first through ninth (or greater) encounter with choice point of maze. See text for details.

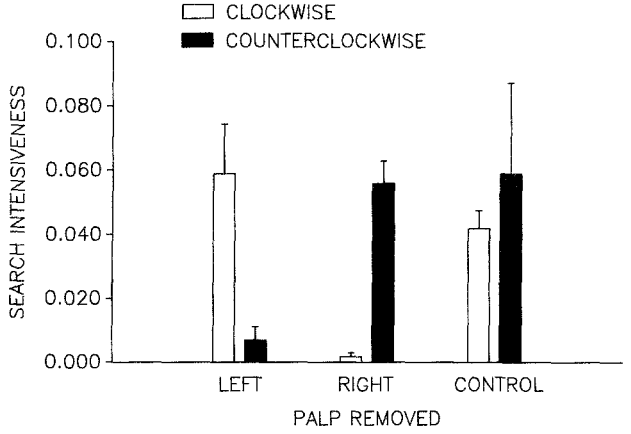


FIG. 4. Search intensiveness as a function of palp removal. Bar is the standard error.

the accumulation of a degradation product could be involved. In addition, the ability of caterpillars to discriminate between trails marked by hungry and sated caterpillars may be due to qualitative differences between the trail types (Crump et al., 1987).

The tendency of tent caterpillars to aggregate at feeding sites commonly leads to the local exhaustion of foliage and to the cessation of trail marking. In a similar circumstance, the volatile recruitment trails of many ants disappear quickly, facilitating the rapid abandonment of the site. In contrast, the nonvolatile trail marker of the tent caterpillar persists long after a feeding site has been

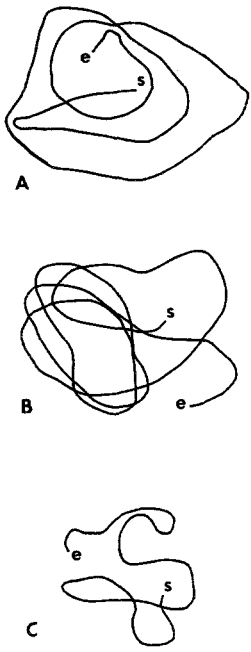


FIG. 5. Examples of looping behavior of caterpillars on a uniform pheromone field. s = start, e = end of run. (A) Right palp removed, (B) left palp removed, (C) both palps intact.

exhausted. We show here that tent caterpillars deal with this problem by abandoning the pattern of following the most heavily marked trails when such behavior fails to reward the forager. Indeed hungry caterpillars eventually break into an exploratory mode in which they leave established trails altogether, moving over unmarked branches in search of food (Fitzgerald and Peterson, 1988).

Our results show that tent caterpillars may orient by using either temporal or instantaneous comparisons of stimulus intensity. The ability of caterpillars to make temporal comparisons, clearly shown in the gap test, suggests that chemoklinotaxis is responsible for discrimination of trail differences at branch points. Moving caterpillars also wave their heads from side to side (wig-wagging) (Bell and Tobin, 1982; Kennedy, 1986), suggesting they employ klinotaxis (transverse klinotaxis) (Kennedy, 1986) as they move along unbranched trails. The pronounced circling response of caterpillars with unilaterally ablated palps shows that input biases also have the potential to influence orientation behavior as larvae move along trails. Studies of the ant *Lasius* (Hangartner, 1967), a species that exhibits marked zigzagging behavior as it moves along odor trails, have been similarly interpreted to demonstrate that the insect makes

simultaneous, bilateral comparisons of trail strength with its antennal chemoreceptors (Schöne, 1984).

Some species of ants regulate the number of individuals on trails by using a system of mass communication in which group transfer of information plays a prominent role (Wilson, 1971). Such systems are based on: (1) modulation of trail laying in response to variation in the quantity or quality of resources, and (2) the ability of trail followers to orient on the basis of the amount of trail pheromone produced by recruiters. Our present study and a previous study showing that tent caterpillars modulate their recruitment trail-laying in response to factors such as leaf quality or crowding at feeding sites (Fitzgerald and Peterson, 1983) indicate that eastern tent caterpillars employ a similar process.

Acknowledgments—P. Roessingh participated in preliminary experiments and discussions of tent caterpillar orientation mechanisms at Wageningen University, The Netherlands. Trail chemicals were provided by R. Silverstein and D. Crump. Supported by NSF grant BNS-8908610.

REFERENCES

BELL, W.J. 1984. Chemo-orientation in walking insects, pp. 93–109, *in* W.J. Bell and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, New York.

BELL, W.J., and TOBIN, T.R. 1982. Chemo-orientation. *Biol. Rev.* 57:219–260.

CRUMP, D., SILVERSTEIN, R.M., WILLIAMS, H.J., and FITZGERALD, T.D. 1987. Identification of trail pheromone of larva of eastern tent caterpillar *Malacosoma americanum* (Lepidoptera: Lasiocampidae). *J. Chem. Ecol.* 13:397–402.

FITZGERALD, T.D. 1976. Trail marking by larvae of the eastern tent caterpillar. *Science* 194:961–963.

FITZGERALD, T.D., and EDGERLY, J. 1982. Site of secretion of the trail marker of the eastern tent caterpillar. *J. Chem. Ecol.* 8:31–39.

FITZGERALD, T.D., and GALLAGHER, E. 1976. A Chemical trail factor from the silk of the eastern tent caterpillar *Malacosoma americanum* (Lepidoptera: Lasiocampidae). *J. Chem. Ecol.* 2:187–193.

FITZGERALD, T.D., and PETERSON, S.C. 1983. Elective recruitment by the eastern tent caterpillar (*Malacosoma americanum*). *Anim. Behav.* 31:417–423.

FITZGERALD, T.D., and PETERSON, S.C. 1988. Cooperative foraging and communication in caterpillars. *BioScience* 38:20–25.

HANGARTNER, W. 1967. Spezifität und Inaktivierung des Spurpheromons von *Lasius fuliginosus* Latr. und Orientierung der Arbeiterinnen im Duftfeld. *Z. Vergl. Physiol.* 57:103–136.

KENNEDY, J.S. 1986. Some current issues in orientation to odour sources, pp. 11–25, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction. Oxford University Press, New York.

PETERSON, S.C. 1988. Chemical trail marking and following by caterpillars of *Malacosoma neustria*. *J. Chem. Ecol.* 14:815–824.

ROESSINGH, P., PETERSON, S.C., and FITZGERALD, T.D. 1988. The sensory basis of trail following in some lepidopterous larvae: Contact chemoreception. *Physiol. Entomol.* 13:219–224.

SCHAL, C., TOBIN, T.R., SURBER, J.L., VOGEL, G., TOURTELLOT, M.K., LEBAN, R.A., SIZEMORE, R., and BELL, W.J. 1983. Search strategy of sex pheromone-stimulated male German cockroaches. *J. Insect Physiol.* 29:575–579.

SCHÖNE, H. 1984. Spatial Orientation: The Spatial Control of Behavior in Animals and Man. Princeton University Press, Princeton, New Jersey.

SCHOONHOVEN, L.M. 1987. What makes a caterpillar eat? The sensory code underlying feeding behavior, pp. 69–97, *in* R.F. Chapman, E.A. Bernays, and J.G. Stoffolano (eds.). Perspectives in Chemoreception and Behavior. Springer-Verlag, New York.

WILSON, E.O. 1971. The Insect Societies. Harvard University Press, Cambridge, Massachusetts.

Journal of Chemical Ecology, Vol. 17, No. 10, 1991

IDENTIFICATION AND BIOASSAY OF SEX PHEROMONE COMPONENTS OF CAROB MOTH, *Ectomyelois ceratoniae* (ZELLER)

T.C. BAKER,[1,*] W. FRANCKE,[2] J.G. MILLAR,[1] C. LÖFSTEDT,[3] B. HANSSON,[3] J.-W. DU,[1,5] P.L. PHELAN,[4] R.S. VETTER,[1] R. YOUNGMAN,[1,6] and J.L. TODD[1]

[1]*Department of Entomology*
University of California
Riverside, California 92521

[2]*Institut für Organische Chemie*
Universität Hamburg
Hamburg, Germany

[3]*Department of Animal Ecology*
University of Lund
S 223 62 Lund, Sweden

[4]*Department of Entomology*
OARDC/OSU
Wooster, Ohio 44691

(Received April 15, 1991; accepted June 17, 1991)

Abstract—Three sex pheromone components of the carob moth were isolated and identified from the extract of female pheromone glands, using a variety of techniques including coupled gas chromatographic–electroantennographic recordings, coupled gas chromatographic–mass spectrometric analysis, microozonolysis, electroantennographic assays of monounsaturated standards, wind-tunnel bioassays, and field trials. The major component was identified as (*Z,E*)-9,11,13-tetradecatrienal, a novel lepidopterous pheromone component structure. Two minor components, either one of which improves the upwind flight response of males when blended with the major component, were identified as (*Z,E*)-9,11-tetradecadienal, and (*Z*)-9-tetradecenal.

Key Words—Flight tunnel, gas chromatography–electrophysiology, mass

*To whom correspondence should be addressed.
[5]Current address: Shanghai Institute of Entomology, Academia Sinica, Shanghai, China.
[6]Current address: Department of Entomology, Virginia Polytechnic Institute, Blacksburg, Virginia 24061.

0098-0331/91/1000-1973$06.50/0

spectrometry, aldehydes, (*Z,E*)-9,11,13-tetradecatrienal, (*Z,E*)-9,11-tetradecadienal, (*Z*)-9-tetradecenal, pheromone, Lepidoptera, Pyralidae.

INTRODUCTION

The carob moth, *Ectomyelois ceratoniae*, is a worldwide pest of nuts and fruits, including carobs, almonds, and dates (Gothilf, 1984). It has been expanding its range into new parts of the world. For instance, in the United States this species is a primary pest of dates in the desert valleys of southern California, and it now threatens to move north into the vast almond and pistachio groves of the San Joaquin Valley (Warner, 1988). There is thus an urgent need for a sensitive means of monitoring endemic populations and for detecting new ones, such as is offered by traps with synthetic sex pheromone lures. Opportunities exist also for direct control using mating disruption, because the labor-intensive nature of the crop would allow for application of disruptants at minimal additional labor cost. To provide this needed tool, we undertook to identify the female sex pheromone of this species, and although we provided a brief report on the identification (Baker et al., 1989), the present paper presents more extensive data on the identification and new information about the laboratory and field activity of these compounds.

METHODS AND MATERIALS

Chemical Analysis of Female Pheromone Gland Extract. Moths were obtained from infested dates near Indio in the Coachella Valley of Riverside County, California, in 1985. Larvae were reared on a honey and wheat-bran diet in one-gallon jars in the laboratory (Warner, 1988), the pupae were segregated by sex, and the moths allowed to emerge on a 14:10 light–dark photoperiod regime. The sex pheromone glands of 2- to -5-day-old females were excised into ca. 50 μl of CS_2 in a micro-test-tube during the normal period of female pheromone emission in the fourth through sixth hours of scotophase. The glands from up to 100 females were extracted for 15–45 min, the solvent was recovered with a syringe, and the extract was pooled with other similarly collected samples and stored at −20°C.

Aliquots of the extract, usually 10–20 female equivalents (FE) were injected onto 30-m × 0.25-mm-ID capillary gas chromatographic (GC) columns, coated either with nonpolar DB-1 or polar DB-WAX or DB-225 (J & W Scientific, Folsom, California) for analyses by GC–mass spectrometry (GC-MS) and GC–electroantennography (GC-EAG). Column conditions for the DB-

1 and DB-WAX columns were as follows: H_2 carrier gas flow of 1.5 ml/min, injector temperature 250°C, oven temperature program, 2 min at 80°C, then 10°/min to 230°C, and flame ionization detector at 250°C. Conditions for the DB-225 column was: H_2 carrier gas flow of 1.5 ml/min, injector temperature 250°C, oven temperature program 2 min at 80°C, then 15°/min to 180°C, and flame ionization detector at 250°C. GC-MS analyses were performed using a Hewlett-Packard 5890 gas chromatograph with a direct interface to a Hewlett-Packard 5970 mass selective detector (electron impact, 70 eV). All NMR spectra of synthetic samples were obtained with a Bruker WM 400 spectrometer at 400 MHz.

Microozonolysis was performed using an adaptation of the previously published technique (Beroza, 1975). The unknown compound was first collected from the DB-1 column by turning off the flame ionization detector and placing a melting-point tube over the end of the detector jet. A small piece of Dry Ice was touched to the side of the tube so that the effluent would more readily condense onto the glass walls. Ozone was then generated in a 500-ml Ehrlenmeyer flask by introducing oxygen into the flask at ca. 50 ml/min and creating a continuous spark inside the flask with a Tesla coil (high-voltage source) applied to an electrode. The melting point tube then was placed at the outlet of the ozone-flask for no more than 2 sec to expose the adsorbed compound to the ozone, and within ca. 10 sec the tube was rinsed with 5 μl of CS_2 solution. The CS_2 was then removed and injected onto the GC to examine the sample for ozonolysis reaction products, which were identified by comparisons with ozonolysis products from synthetic standards.

Electroantennogram Analysis. To examine antennal sensitivity to geometric and positional isomers, as well as to functional groups, electroantennograms (EAGs) were recorded (Roelofs, 1984) using a series of synthetic standards of acetates and alcohols purchased from Dr. S. Voerman at the Institute for Pesticide Research, Wageningen, The Netherlands. Several 14- and 16-carbon aldehydes were available (Albany International) including tetradecanal, (*Z*)-9-tetradecenal, (*Z*)-9-hexadecenal, (*E*)-9-hexadecenal, (*Z*)-7-hexadecenal, and (*Z*)-11-hexadecenal. Serial dilutions of each compound were made in hexane such that 10 μg were applied to filter paper in 10 μl of solvent, and then the filter paper was placed inside a Pasteur pipet. A male carob moth antenna was placed between two saline-filled glass electrodes, and the signal amplified with a Hansson model 103 amplifier and displayed on a storage oscilloscope. Each sample pipet was inserted into the airstream in a glass tube upwind of the antenna, and 1 ml of air was puffed through the tube from a glass syringe whose plunger was depressed by hand in less than 30 msec; the resulting antennal depolarization was measured on the oscilloscope screen. After every three to four puffs of test compounds, a standard control compound, (*E*)-6-tetradecenyl acetate, was puffed in order to monitor possible deterioration of the preparation.

EAG amplitudes were standardized according to the responses to this internal standard by dividing the amplitude of the EAG generated from the test compound by that from the standard. Within a particular series of isomers having the same chain length and functional group, presentation of the test compounds was randomized.

Simultaneous GC-EAG analysis (Arn et al., 1975) was performed on the DB-1, DB-WAX, and DB-225 30-m × 0.25-mm-ID GC columns (J & W Scientific). Briefly, the effluent was split 1 : 1 so that it entered two 1-m-long deactivated fused silica columns just before they left the GC oven, and make-up gas was added just before the split point to improve splitting and to drive the effluent rapidly through the deactivated columns and to the flame and EAG detectors, respectively. Simultaneous GC and single-cell (GC-SC) analysis (Wadhams, 1984; Van der Pers and Löfstedt, 1983) was performed using the same splitting technique, except that instead of a whole antenna, an electrode was placed over a single sensillum trichodeum (see next section) to record the single neurons' responses to the GC effluent simultaneously with the FID.

Single Cell Antennal Recordings. Recordings from single antennal neurons were made using the cut-sensillum technique of Kaissling (1974; Van der Pers and Den Otter, 1978). An antenna was removed from a male carob moth and its base placed in a saline electrode serving as the ground. A single sensillum trichodeum was then positioned over the edge of a glass knife, and the sensillar tip excised with a second glass knife edge brought down over the first knife edge by means of a micromanipulator. A saline recording electrode was then maneuvered to contact the sensillum and to record from the neurons within after amplifying the signal using a Hansson model 103 amplifier. Recordings of both the AC and DC components of the signal were stored on VCR tape using a Vetter model 420 F four-channel FM recorder and displayed and printed on a Gould model 1604 (DSO) digital oscilloscope/printer.

Wind-Tunnel and Field-Trapping Tests. Behavioral assays were conducted in a 3.5 × 1.0 × 1.0-m wind tunnel previously described in detail by Kuenen and Baker (1982). Two- to five-day-old males were placed in the wind tunnel at least 2 hr before testing in order to acclimate them to the conditions (24–26°C, 0.3 lux, 30–70% relative humidity, 0.5 m/sec wind velocity). Bioassays were conducted 4–6 hr into the scotophase, the optimal sexual activity period for this species. Treatments were loaded onto filter-paper disks (Whatman No. 1) affixed to metal clips 6 cm high complete with metal base. The cork–wire holder for each filter paper was placed on a 15 × 15-cm sheet metal platform 15 cm above the floor of the tunnel, 30 cm from the tunnel's upwind end. Males were released one at a time from screen cages, 4 cm long × 3 cm diameter, 1 m from the odor source and 15 cm above the tunnel floor. The cages were placed on the metal platform in the plume until the male took flight or until 30

sec elapsed. Males were scored for taking flight, locking-on to the plume and progressing upwind, flying to within 50 cm or 10 cm of the source, and contacting the source. Tests were conducted using a randomized, complete-block design so that all treatments were presented on a given day.

In order to see whether or not the reduced attraction with synthetics was due to antagonistic contaminants, we tested the attraction of males to calling females in the wind tunnel compared to calling females plus the 8:1:1 (333 μg) synthetic blend in polyethylene Beem embedding capsules, size 3 (Ted Pella Inc., Redding, California) (see next section). Six females were placed in a cage in a Pherocon 1C trap just as they were in the field except the trap was placed in the wind tunnel. A second trap was also used, but was loaded with a cage containing six females, to which was also affixed the Beem capsule containing the synthetic blend. Under the same wind-tunnel conditions used in the other tests, 10 males were released downwind and allowed 2 min to approach and enter each trap, presented singly to the males. Males were scored for close approach to the trap (<10 cm), the duration of hovering in front of the trap, and whether or not they were captured on the sticky surface.

Field-trapping tests were conducted in date gardens in the Coachella Valley near Indio, California, beginning in 1989. Polyethylene Beem capsules, which were left unsealed, were loaded with 333 μg of the 8:1:1 mixture of synthetic (*Z,E*)-9,11,13-tetradecatrienal, (*Z,E*)-9,11-tetradecadienal, and (*Z*)-9-tetradecenal, and individual capsules were affixed to the bottoms of Pherocon 1C traps. Five virgin female carob moths were placed in screen cages in Pherocon 1C traps as a positive control. They were given continuous access to distilled water on a dental wick. Traps were counted and rebaited twice per week over several weeks. Males were removed and the traps rerandomized whenever counts were taken. Trap bottoms were replaced each week when the Stickem surfaces became coated with dust.

Synthesis. (*Z*)-9-Tetradecenal and (*Z,E*)-9,11-tetradecadienal were prepared from the corresponding alcohols by oxidation with pyridinium dichromate–molecular sieve (Herscovici et al., 1982) and by Swern oxidation (Mancuso et al., 1978). The crude products were purified by HPLC (RP-18; low-pressure gradient; methanol–water) or by flash chromatography on silica gel (5% ether in hexane) followed by Kugelrohr distillation (0.1 mm Hg, oven temperature 100°C). Syntheses of (*Z,E*)-9,11,13-tetradecatrienal have been published elsewhere (Baker et al., 1989; Millar, 1989, 1990).

The trienal was very unstable and usually trimerized or polymerized in the space of a few weeks even at -20°C under Ar, either neat or in dilute solution (hexane, benzene, ether). When freshly prepared, the trienal was >98% stereoisomerically pure by capillary GC (DB-5). However, the trienal was contaminated with 2–5% of a rearrangement product that coeluted with the trienal on

silica gel flash chromatography or HPLC. This rearrangement product has not yet been isolated or fully characterized. The trienal was also thermally unstable and decomposed when subjected to GC injector temperatures above 250°C.

RESULTS

EAGs of Monounsaturated Standards. Standardized EAG responses revealed that male antennae were far more responsive to 14-carbon aldehydes than to other molecules to which they were exposed. Although we had a limited array of aldehydes to test, by far the most active compound was (*Z*)-9-tetradecenal, generating a response 3.8 times (±0.91 SD; $N = 8$) as great as that to the standard compound, (*E*)-6-tetradecenyl acetate. In comparison, the next most active compound, either monounsaturated or saturated, was tetradecanal with a response of 2.3 (±0.45 SD; $N = 8$), followed by (*Z*)-9-tetradecenyl acetate with a response of 2.1 (±0.19 SD; $N = 5$) (Figure 1). Responses to an array of 16-carbon aldehydes, including (*Z*)-11-hexadecenal, hexadecanal, (*E*)-9-hexadecenal, (*Z*)-9-hexadecenal, and (*Z*)-7-hexadecenal all were below a standardized response of 1.3. Thus, the high responses to 14-carbon aldehydes suggested that at least one of the sex pheromone components contained an alde-

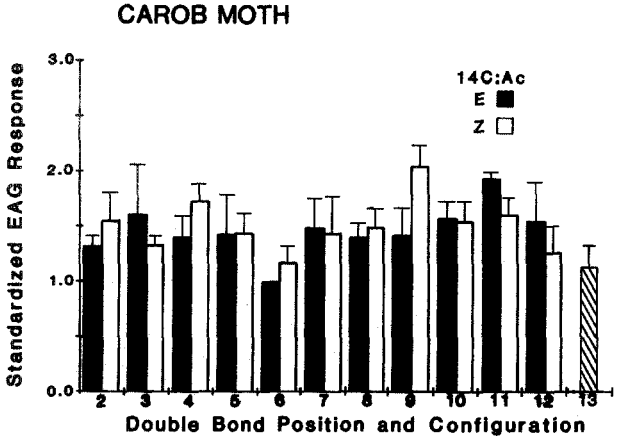


FIG. 1. Standardized EAG amplitudes in response to a series of tetradecenyl acetates ($N = 5$ antennae). Compounds were dispensed at a loading of 10 μg on a filter paper strip and placed in Pasteur pipets. One milliliter of air was puffed through each pipet into an airstream and over the male antenna, the series of compounds being tested in a randomized, complete-block design. The standard response was the millivolt response to a test compound divided by the millivolt response to the most recent puff of (*E*)-6-tetradecenyl acetate, such puffs being interspersed with the test puffs every three to four compounds.

hyde moiety and was 14 carbons in length. Although lower than those of the 14-carbon aldehydes, the EAG responses to the series of 14-carbon acetates (Figure 1) and alcohols, for which complete sets of standards from Δ4 to Δ12 were available, were illuminating, because they highlighted potential involvement of molecules having a (*Z*)-9 configuration and also an (*E*)-11 configuration.

Analysis of Pheromone-Gland Extract. Combined GC-EAG analysis of the extract from ca. 10 FE of pheromone-gland extract consistently revealed three peaks from the FID corresponding to EAG activity on either the DB-1 or DB-WAX columns in GC-EAG trials (Figures 2 and 3, respectively). These three peaks were labeled compounds I, II, and III; the order of elution, based on the amplitude of the EAG's, was the same on the two columns, with a compound corresponding to a small EAG peak eluting first, a compound evoking a larger EAG response eluting second, and the compound with the largest EAG response eluting last (Figures 2 and 3). The compounds were present in a ratio of 8 : 1 : 1

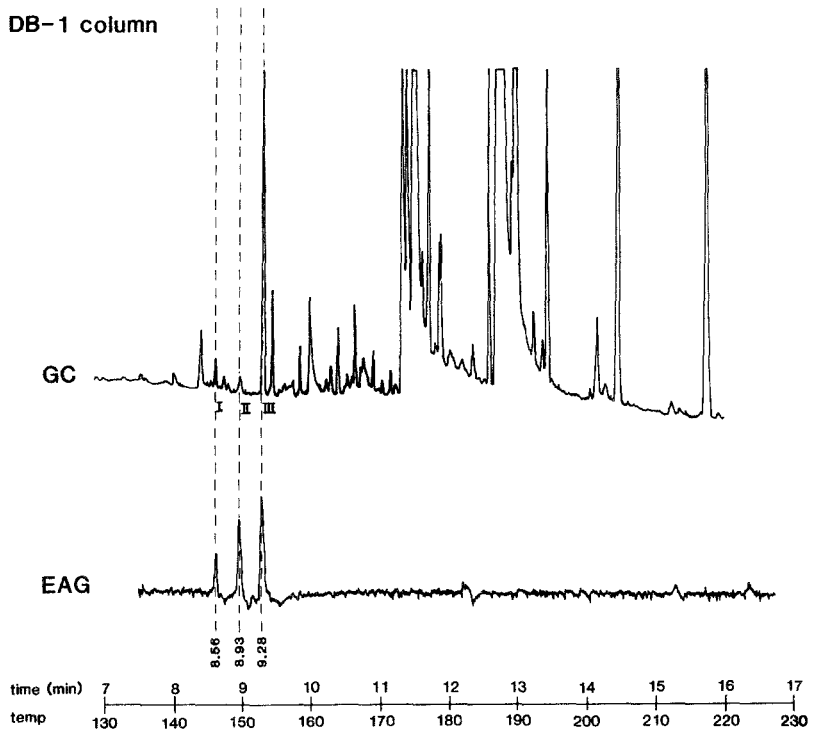


FIG. 2. GC-EAG response to 10 FE of female carob moth sex pheromone gland extract injected onto a 30-m DB-1 capillary GC column. The EAG-active peaks at 8.56, 8.93, and 9.28 min were labeled compounds, I, II, and III, respectively.

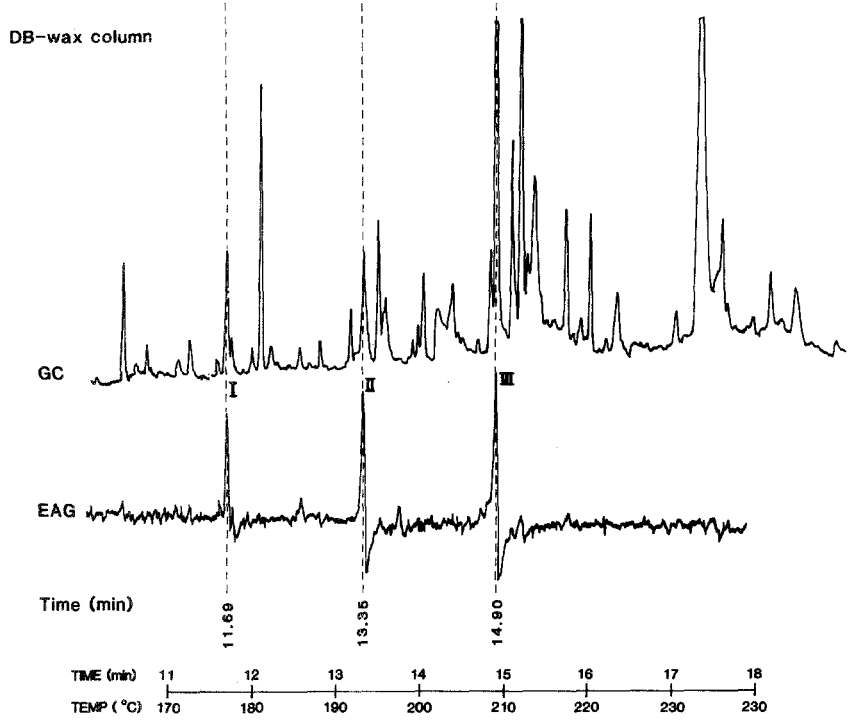


FIG. 3. GC-EAG response to 10 FE of female carob moth sex pheromone gland extract injected onto a 30-m DB-WAX capillary GC column. The EAG-active peaks at 11.69, 13.35, and 14.90 min were labeled compounds I, II, and III, respectively.

(III : II : I). The retention time of compound I corresponded precisely to that of (*Z*)-9-tetradecenal on both columns. The retention indices of compounds II and III relative to I were 1.043 and 1.048 on DB-1 and 1.142 and 1.274 on DB-WAX, respectively. The retention indices of II and III on the two columns relative to (*Z*)-9-tetradecenal and to the other compounds in our library indicated that compounds II and III possibly involved conjugated double bonds since nonconjugated systems elute very close to monounsaturated compounds (c.f., Heath and Tumlinson, 1984). Since the pattern of EAG responses to monounsaturated standards indicated that two configurations evoked high activity [(*Z*)-9 and (*E*)-11] and only one GC-EAG active peak was present in the monounsaturated region on GC in the extract, we reasoned that in at least one of the compounds, (either II or III), a (*Z*,*E*)-9,11 dienal would be involved.

GC-MS analysis of compound I revealed a mass spectrum identical to that of a synthetic standard of (*Z*)-9-tetradecenal. The mass spectrum of compound II showed a base peak at *m/z* 67 plus a significant signal at *m/z* 208, consistent

with the molecular ion of a tetradecadienal (Figure 4A). Conjugated double bonds stabilize the molecular ion in long-chain aliphatic compounds, whereas M+ is virtually missing in monounsaturated analogs or analogs with isolated double bonds (Löfstedt and Odham, 1984; Ando et al., 1988). Compound III had a mass spectrum characterized by a base peak at *m/z* 79 and an intense signal at *m/z* 206 (Figure 4B), suggesting a tetradecatrienal or a tetradecenynal with at least some conjugation.

Ozonolysis of the fraction of female-gland extract containing compound III, collected from the DB-1 column at 9.1–9.4 min, yielded a new peak at 6.70 min, which upon comparison with the bifunctional ozonolysis product of synthetic (Z)-9-tetradecenal proved to be 1,9-octanedial. This showed that the first double bond in compound III was at the C9 position and that any other double bonds must occur farther out on the alkyl chain. Excluding allenes and enynes, this meant that the other two double bonds could only be in the 11 and 13 positions. We thus suspected that compound III was (*Z,E*)-9,11,13-tetradecatrienal and that compound II was (*Z,E*)-9,11-tetradecadienal.

Synthetic II and III. The mass spectrum of synthetic (*Z,E*)-9,11-tetradecadienal was identical to that of the natural compound II from the extract (Figure 4A). Moreover, combined GC-EAG recordings on both the 30-m DB-WAX and DB-225 columns demonstrated that (*Z,E*)-9,11-tetradecadienal had a retention time identical to compound II from the extract and that was EAG active, while the *E,E* isomer eluted later and was relatively inactive. Likewise, the retention time of (*Z,E*)-9,11,13-tetradecatrienal was identical to that of nat-

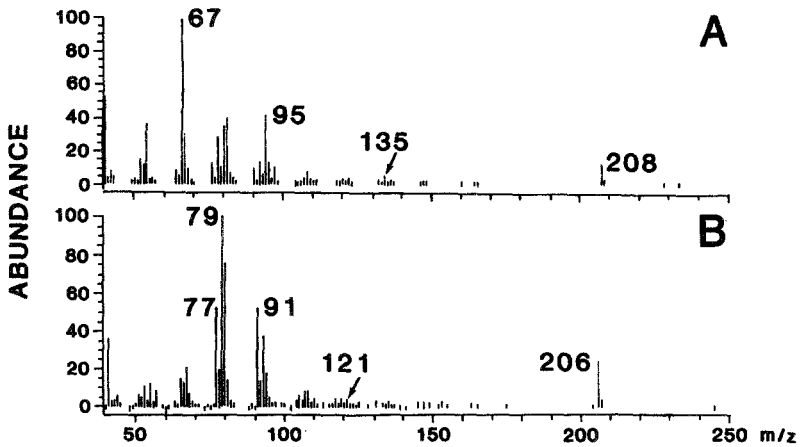


FIG. 4. (A) Mass spectrum (EI 70 eV) of compound II, identical to that of (*Z,E*)-9,11-tetradecatrienal. (B) Mass spectrum (EI 70 eV) of compound III, identical to that of (*Z,E*)-9,11,13-tetradecatrienal.

ural compound III, and it was EAG-active, whereas the *E,E* isomer eluted later and was relatively inactive on EAG. The mass spectrum of compound III was identical to that of synthetic (*Z,E*)-9,11,13-tetradecatrienal (Figure 4B), confirming the structure of III to be (*Z,E*)-9,11,13-tetradecatrienal.

Although the evidence strongly indicated that compound I was (*Z*)-9-tetradecenal, current knowledge of pheromonal biosynthetic pathways in the Lepidoptera allowed for the alternative possibility that compound I could be unsaturated in the 11 position. This possibility is enhanced when considering that the retention time of (*E*)-11-tetradecenal would likely be very similar to (*Z*)-9-tetradecenal on most columns, and it would exhibit a virtually identical mass spectrum. A small amount of (*E*)-11-tetradecenal was therefore synthesized from (*E*)-11-tetradecenol, and it consistently had a retention time of 9.77 min on the DB-225 column compared to 9.86 min for (*Z*)-9-tetradecenal. The natural extract exhibited no FID peak at 9.77 min and no EAG activity at that elution time in two different GC-EAG analyses on DB-225, whereas there was a small FID peak at 9.87 min plus strong EAG activity at 9.86 min during the same GC-EAG analyses of this extract. These results, coupled with the earlier GC-MS and GC-EAG analyses on the two other columns again strongly support (*Z*)-9-tetradecenal as the structure of compound I.

As was the case for compound I, for which no ozonolysis results could be obtained, possible double bond positions and geometries different from the indicated (*Z,E*)-9,11-tetradecadienal existed also for compound II. However, the same GC-EAG analyses of the natural extract on the DB-225 column revealed a small peak at 11.20 min with correspondingly strong EAG activity at 11.20 min and no other EAG activity except for the usual single peaks corresponding to compounds I and III. Synthetic (*Z,E*)-9,11-tetradecadienal exhibited a retention time of 11.19 min on this same column. These results, coupled with the earlier GC-MS and GC-EAG analyses on the two other columns again strongly support our assignment of (*Z,E*)-9,11-tetradecadienal as the structure of compound II.

GC–single-cell recordings using synthetic I, II, and III confirmed that the retention times and single-cell activity corresponded to (*Z*)-9-tetradecenal, (*Z,E*)-9,11-tetradecadienal, and (*Z,E*)-9,11,13-tetradecatrienal, respectively (Figure 5). Moreover, the monoenal and dienal both caused a large-spike-amplitude cell to fire in some of the sensilla, whereas the trienal caused a smaller-spike-amplitude cell in the same hair to fire. The fact that the trienal excites one kind of receptor cell and the monoenal and dienal excite a second type of cell is consistent with the behavioral data (below) that indicates that the trienal alone is not as active behaviorally as a blend of the trienal plus either I or II.

Wind-Tunnel and Field-Trapping Studies. The activity of synthetic compound III in evoking upwind flight by carob moth males was confirmed in wind-

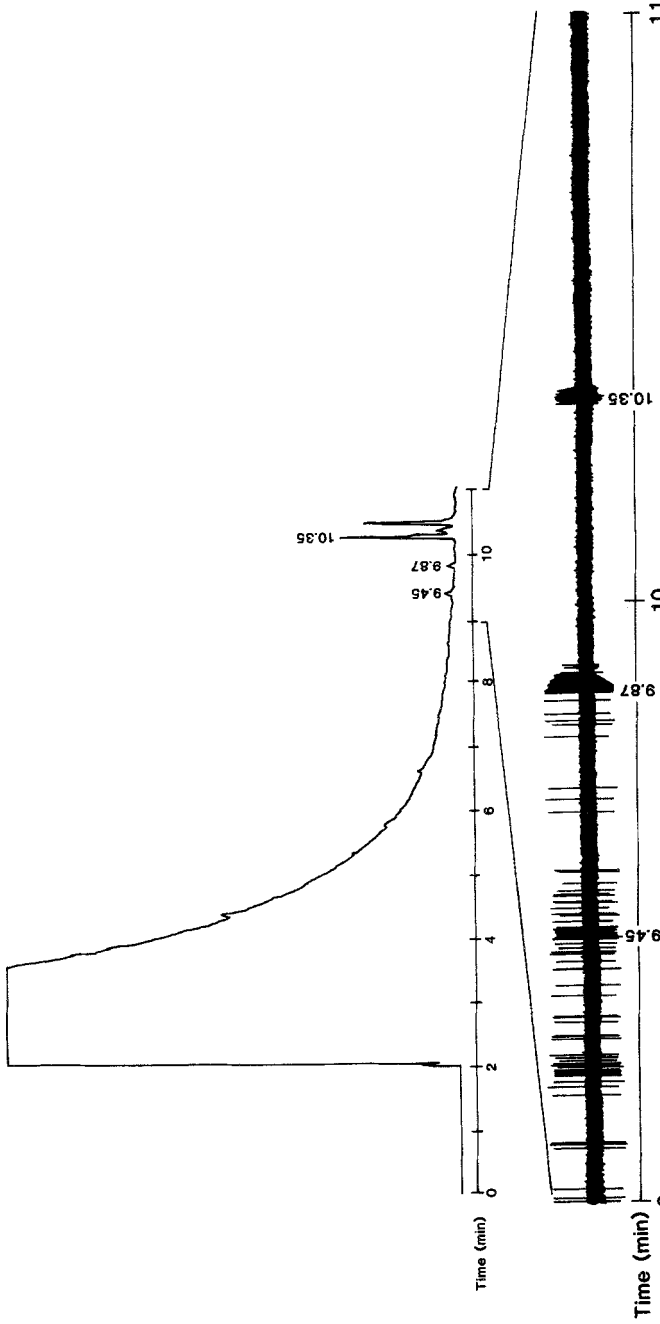


FIG. 5. GC–single-cell analysis of a 1:1:8 blend of synthetic (Z)-9-tetradecenal, (Z,E)-9,11-tetradecatrienal, and (Z,E)-9,11,13-tetradecatrienal, respectively, injected onto a 30-m DB-1 column in a Varian 3740 GC (column conditions 80°C, 2 min hold, 15°/min to 180°C; He carrier flow of 1.5 ml/min; injector temperature 200°C; FID detector temperature 250°C). The first two compounds, (monoenal and dienal), evoked action potentials from a large-spiking cell in the sensillum, whereas the trienal caused action potentials to be discharged from a smaller-spiking cell in the same sensillum.

tunnel studies. In an initial test, compounds I and II (0.5 ng each) were inactive by themselves in evoking upwind flight from 3 m downwind (0/18 and 0/19 males flew upwind, respectively), but 0.5 ng of the triene alone did cause upwind flight and source location (9/23 flew upwind, 4/23 touched the source). In further experiments, a blend of the three compounds (40 ng, 5 ng, 5 ng, respectively) approximating the 8 : 1 : 1 ratio found in gland extract was nearly as good as 5 FE of gland extract in eliciting upwind flight and source contact (Figure 6). The trienal by itself was poorer in causing source contact than blends that included either the monoenal, the dienal, or both (χ^2 2 × 2 test of independence, $P < 0.05$). Thus it appeared that either the monoenal or dienal or both were behaviorally important components of the sex pheromone blend, in which the trienal plays the primary role in attracting males.

Further wind-tunnel experiments with a wider variety of ratios (source loading, 40 ng of trienal) confirmed that the 8 : 1 : 1 ratio approximating that found in the female gland extract was significantly better at causing complete upwind flight and source contact than the 1 : 1 : 1 or 1 : 0 : 0 ratios (Figure 7; χ^2 2 × 2 test of independence, $P < 0.05$). Although there was a trend for the other ratios to be slightly poorer than 8 : 1 : 1, it appears that the trienal needs to exceed the amounts of the other two components by 2 : 1 : 1 or greater to be optimal. As in the first experiment, none of the synthetic blends was quite as

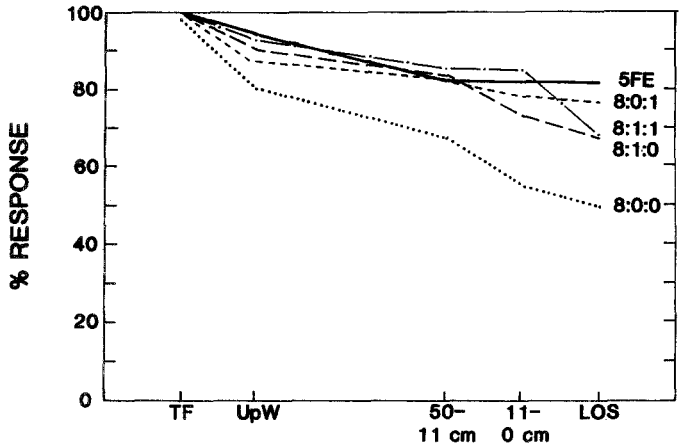


FIG. 6. Percentages of male carob moths flying in the wind tunnel in response to various blends of (*Z,E*)-9,11,13-tetradecadienal, (*Z,E*)-9,11-tetradecadienal, and (*Z*)-9-tetradecenal, or 5 FE of gland extract. Source loading on filter paper was 40 ng of the trienal applied in 10 μl of hexane. $N = 28$ males tested to each treatment. TF = take flight in plume, UpW = lock on and fly upwind in plume, 50–11 cm = flight to within between 11 and 50 cm of the source, 11–0 cm = flight to within between 0 and 11 cm of the source, and LOS = male lands on the source.

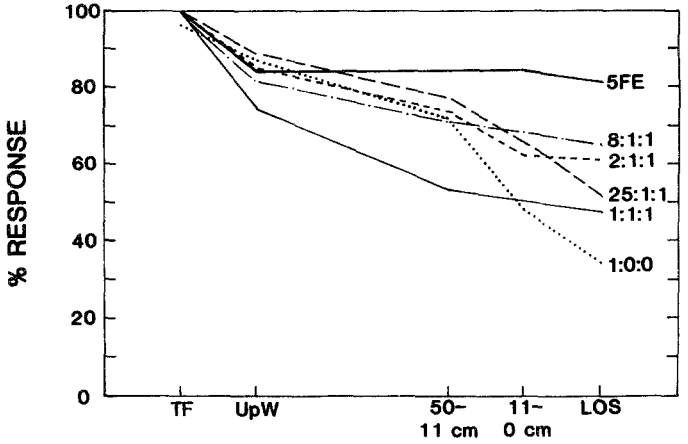


FIG. 7. Percentages of male carob moths flying in the wind tunnel in response to various blends of (*Z,E*)-9,11,13-tetradecatrienal, (*Z,E*)-9,11-tetradecadienal, and (*Z*)-9-tetradecenal, compared to 5 FE of gland extract. Source loading on filter paper was 40 ng of the trienal applied in 10 μl of hexane. Abbreviations are the same as in Figure 6. N = 35 males tested to each treatment.

effective as the natural extract in evoking source contact. Again the trienal alone evoked lower levels of source contact than blends containing the monoenal or dienal (Figure 7). A series of dosages of the 1 : 1 : 8 blend of I, II, and III was tested to determine if higher amounts of synthetic compounds could better mimic the natural extract. Although there was no dosage that was excessively high among the ones we tested, none of these source loadings caused levels of source contact as high as the natural extract (Figure 8) (χ^2 2 × 2 test of independence, $P < 0.05$).

In the field, the 8 : 1 : 1 mixture of the three components, dispensed at a loading of 333 μg of the major component in polyethylene Beem capsules, captured significant numbers of carob moth males. Traps containing five live females consistently captured greater numbers of males [18.6 ± 17.32 (SD) and 30.2 ± 34.1 (SD) N = 10 and 9, respectively] than the synthetics, which captured a mean of 9.9 ± 12.1 SD and 6.5 ± 9.9 SD males (N = 30 and 27, respectively). Blank traps captured 0 males in all tests.

The reduced attraction of males to the synthetic blend was not apparently due to contaminants in the synthetic compounds. In the wind tunnel six live calling females attracted and captured an average of 5.13 (±1.89 SD, N = 8) out of every 10 males that were released, whereas the addition of the synthetic blend on Beem capsules did not reduce the capture; rather this latter treatment increased the capture slightly, causing 5.85 (±2.17, N = 8) out of every 10 males released to become ensnared. In response to the females plus synthetics,

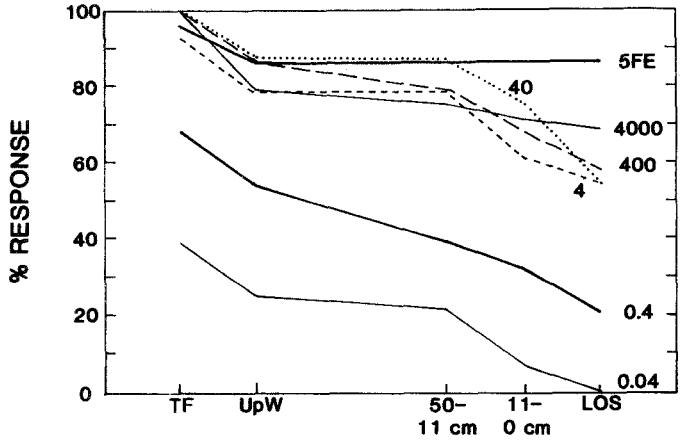


FIG. 8. Percentages of male carob moths flying in the wind tunnel in response to various source loadings of the 8 : 1 : 1 blend of (*Z*,*E*)-9,11,13-tetradecatrienal, (*Z*,*E*)-9,11-tetradecadienal, and (*Z*)-9-tetradecenal, compared to 5 FE of gland extract. Source loadings were 0.04, 0.4, 4, 40, 400, and 4000 ng of the trienal, applied in 10 μl of hexane. N = 60 males tested to each treatment. Abbreviations are the same as in Figure 6.

males only spent 7.96 sec (±6.90 SD) hovering in front of the trap, whereas they spent 11.47 sec (±13.71 SD) hovering in front of traps in response to live calling females. A mean of 6.25 (±1.83 SD, N = 8) males out of every 10 released was observed hovering in front of the trap containing synthetics plus females, whereas 4.75 (±2.12 SD, N = 8) out of 10 were seen hovering in front of traps containing females only.

DISCUSSION

The (*Z*,*E*)-9,11,13-tetradecatrienal identified as the major component of the carob moth sex pheromone in this study represents a novel structure with regard to previously identified lepidopteran sex pheromone components. Indeed, among the known acetate, alcohol, and aldehyde sex pheromone components of moths, only a few compounds possess more than two double bonds, and only recently were conjugated trienes reported. Tellier and Descoins (1990) confirmed our earlier findings (Baker et al., 1989) on *E. ceratoniae* and identified (*Z*,*E*)-9,11,13-tetradecatrienal and the corresponding acetate in the saturniid moth, *Stenoma cecropia*. (*E*,*E*,*E*)-10,12,14-Hexadecatrienyl acetate is a sex pheromone component of the mulberry pyralid, *Glyphodes pyloalis* (Ando et al., 1988; Honda et al., 1990) and (*E*,*E*,*Z*)-10,12,14-hexadecatrienal was iden-

tified as a component of the sex pheromone of *Manduca sexta* (Tumlinson, 1989; Tumlinson et al., 1990).

Although the activity of the 8 : 1 : 1 blend under field conditions appears to be low relative to calling females, we believe that this reduced activity can be explained in part by the lability of the trienal, which loses its integrity rapidly even in solution when stored in a freezer. The trienal appears to be the major component of the pheromone, without which attraction does not occur. The activity of freshly prepared lures in wind tunnel tests is nearly equal to that of female extract, and so it appears that in order for reliable field lures to be prepared for monitoring, the problem of decomposition of the trienal must be overcome. However, even with this limitation and the relatively low trap capture levels relative to females, the synthetic lures emitting the 8 : 1 : 1 blend of trienal, dienal, and monoenal have already produced useful seasonal data for use by date growers attempting to accurately time their single application of malathion dust to protect the date crop during ripening. Because of the labor-intensive nature of date-growing, development of a stable formulation holds promise for disruption of mating, because dispensers could easily be hand-placed and replaced during the season by workers who tend the crop, all with minimal extra labor costs.

Acknowledgments—We thank Wendy Villano for assisting in the rearing of the carob moth. This project was supported in part by the California Date Packers Council and by grants from the California Department of Food and Agriculture and the Swedish Natural Science Research Council (NFR).

REFERENCES

ANDO, T., OGURA, Y., KOJAMA, M., KURANE, M., UCHIYAMA, M., and SEOL, K.Y. 1988. Syntheses and NMR-analyses of eight geometrical isomers of 10,12,14-hexadecatrienyl acetate, sex pheromone candidates of the mulberry pyralid. *Agric. Biol. Chem.* 52:2459–2468.

ARN, H., STADLER, E., and RAUSCHER, S. 1975. The electroantennographic detector—a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. *Z. Naturforsch.* 30c:722–725.

BAKER, T.C., FRANCKE, W., LÖFSTEDT, C., HANSSON, B.S., DU, J.-W., PHELAN, P.L., VETTER, R.S., and YOUNGMAN, R. 1989. Isolation, identification and synthesis of sex pheromone components of the carob moth, *Ectomyelois ceratoniae*. *Tetrahedron Lett.* 30:2901–2902.

BEROZA, M. 1975. Microanalytical methodology relating to the identification of insect sex pheromones and related behavior-control chemicals. *J. Chromatogr. Sci.* 13:314–321.

GOTHILF, S. 1984. Biology of *Spectrobates ceratoniae* on almonds in Israel. *Phytoparasitica* 12(2):77–87.

HEATH, R.R., and TUMLINSON, J.H. 1984. Techniques for purifying, analyzing, and identifying pheromones, pp. 287–322, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, New York.

HERSCOVICI, J., EGRON, M.J., and ANTONAKIS, K. 1982. New oxidative systems for alcohols: Molecular sieves with chromium(VI) reagents. *J. Chem. Soc. Perkin I* 1982:1969–1973.

HONDA, H., ANDO, T., OGURA, Y., TERAMINE, T., and UEDA, R. 1990. Identification of the sex pheromone of the mulberry pyralid, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae). *Appl. Entomol. Zool.* 25:265–272.

KAISSLING, K.-E. 1974. Sensory transduction in insect olfactory receptors, pp. 243–273, *in* L. Jaenicke (ed.). Biochemistry of Sensory Functions. Springer-Verlag, Berlin.

KUENEN, L.P.S., and BAKER, T.C. 1982. Optomotor regulation of ground velocity in moths during flight to sex pheromone at different heights. *Physiol. Entomol.* 7:193–202.

LÖFSTEDT, C., and ODHAM, G. 1984. Analysis of moth pheromone acetates by selected ion monitoring using electron impact and chemical ionization mass spectrometry. *Biomed. Mass Spectrom.* 11:106–113.

MANCUSO, A., HUANG, S.-L., and SWERN, D. 1978. Oxidation of long-chain and related alcohols to carbonyls by dimethyl sulfoxide "activated" by oxalyl chloride. *J. Org. Chem.* 43:2480–2482.

MILLAR, J.G. 1989. Synthetic routes to the major component of the sex pheromone of the carob moth, *Ectomyelois ceratoniae*. Sixth Annual Meeting, International Society of Chemical Ecology, Göteburg, Sweden, August 7–11, 1989. Poster.

MILLAR, J.G. 1990. Synthesis of 9*Z*,11*E*,13-tetradecatrienal, the major component of the sex pheromone of the carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae). *Agric. Biol. Chem.* 54:2473–2476.

ROELOFS, W.L. 1984. Electroantennogram assays: rapid and convenient screening procedures for pheromones, pp. 130–159, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, New York.

TELLIER, F., and DESCOINS, C.L. 1990. Stereospecific synthesis of (*Z*,*E*)-9,11,13-tetradecatrien-1-yl acetate and aldehyde sex pheromone components of *Stenoma cecropia* and *Ectomyelois ceratoniae*. *Tetrahedron Lett.* 31:2295–2298.

TUMLINSON, J.H. 1989. Insect chemical communication systems. *Pure Appl. Chem.* 61:559–562.

TUMLINSON, J.H., BRENNAN, M.M., DOOLITTLE, R.E., MITCHELL, E.R., BRABHAM, A., MAZOMENOS, B.E., BAUMHOVER, A.H., and JACKSON, D.M. 1990. Identification of a pheromone blend attractive to *Manduca sexta* (L.) males in a wind tunnel. *Arch. Insect Biochem. Physiol.* 10(4):255–271.

VAN DER PERS, J.N.C., and DEN OTTER, C.J. 1978. Single cell responses from olfactory receptors of small ermine moths to sex-attractants. *J. Insect. Physiol.* 24:337–343.

VAN DER PERS, J.N.C., and LÖFSTEDT, C. 1983. Continuous single sensillum recordings as a detection method for moth pheromone components in the effluent of a gas chromatograph. *Physiol. Entomol.* 8:203–211.

WADHAMS, L.J. 1984. The coupled gas chromatography-single cell recording technique, pp. 179–189, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, New York.

WARNER, R.L. 1988. Contributions to the biology and management of the carob moth *Ectomyelois ceratoniae* (Zeller), in Deglet Noor date gardens in the Coachella Valley of California. PhD dissertation, University of California, Riverside. 260 pp.

Journal of Chemical Ecology, Vol. 17, No. 10, 1991

3-METHYL-3-BUTEN-1-OL: AN AGGREGATION PHEROMONE OF THE FOUR-EYED SPRUCE BARK BEETLE, *Polygraphus rufipennis* (KIRBY) (COLEOPTERA: SCOLYTIDAE)

W.W. BOWERS,[1,*] G. GRIES,[2] J.H. BORDEN,[2] and H.D. PIERCE, JR.[3]

[1]*Forestry Canada*
Newfoundland and Labrador Region
P.O. Box 6028
St. John's, Newfoundland Canada A1C 5X8

[2]*Centre for Pest Management*
Department of Biological Sciences

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

(Received November 29, 1990; accepted June 24, 1991)

Abstract—Porapak Q-captured volatiles of male *Polygraphus rufipennis* (Kirby) boring in black spruce and white spruce from Newfoundland and British Columbia, respectively, contained 3-methyl-3-buten-1-ol. Volatiles from logs in which the males had been joined by females contained the compound in reduced quantities. Hindgut extracts from male and female *P. rufipennis* disclosed no male-specific volatiles, but 3-methyl-3-buten-1-ol was detected in extracts of male-produced frass. The compound was not present in extracts from fresh phloem tissue. *P. rufipennis* of both sexes responded strongly in the field to traps baited with 3-methyl-3-buten-1-ol released at 4390 μg/day. There was little response to unbaited traps, fresh uninfested black spruce logs, or to 3-methyl-3-buten-1-ol released at lower rates. Combination of 3-methyl-3-buten-1-ol with either one of five terpenes prevalent in black spruce did not enhance beetle catch. Approximately half of 20 black spruce trees baited with 3-methyl-3-buten-1-ol were attacked, compared to 20.0% of 10 unbaited control trees. This new aggregation pheromone could be used to monitor or manage *P. rufipennis* populations.

*To whom correspondence should be addressed.

0098-0331/91/1000-1989$06.50/0

Key Words—*Polygraphus rufipennis*, Coleoptera, Scolytidae, aggregation pheromone, bark beetle, black spruce, 3-methyl-3-buten-1-ol.

INTRODUCTION

The four-eyed spruce bark beetle, *Polygraphus rufipennis* (Kirby), is a phloeophagous bark beetle restricted to Abietineae hosts in northern and mountainous areas of North America. It breeds principally in weakened and dying trees, but may be a major cause of tree death when it reaches outbreak levels in mature forests, especially in stands of black spruce, *Picea mariana* (Mill.) B.S.P. (Swaine, 1918). In Newfoundland, black spruce trees defoliated by eastern spruce budworm, *Choristoneura fumiferana* (Clem.), have been predisposed to attack by *P. rufipennis*, resulting in considerable mortality of these trees (Raske and Sutton, 1986).

As for other polygynous members of the tribe Polygraphini, *P. rufipennis* appears to have a Eurasian–African origin. It is closely allied to *Polygraphus poligraphus* (L.) of Europe and northern Asia, and reached North America in recent geologic time (Wood, 1982). *P. poligraphus* is reported to utilize three male-produced compounds, terpinene-4-ol and *cis*- and *trans*-4-thujanol, as aggregation pheromones (Leuze, 1977; Brümmer, 1981; Francke and Vité, 1983). Semiochemical-based communication in *P. rufipennis* has been demonstrated (Bowers and Borden, 1990) but its chemical basis is unknown. Semiochemicals could lead to the development of a valuable detection and monitoring tool for this pest. A potent aggregation pheromone was evident in the response of *P. rufipennis* to male-produced frass and male-infested logs (Bowers and Borden, 1990). We report the identification and field testing of 3-methyl-3-buten-1-ol, an aggregation pheromone of this species.

METHODS AND MATERIALS

Experimental Insects. P. rufipennis adults and brood for laboratory and field experiments in Newfoundland were collected and reared on black spruce from Powderhorn Lake and Miguels Lake in central Newfoundland and from Thorburn Lake and Conception Bay South in eastern Newfoundland. Adults and brood for laboratory experiments in British Columbia were collected from and reared on black spruce from Nazko and Cottonwood creek near Quesnel and from white spruce, *Picea glauca* (Moench) Voss, from Penticton and Bowron Lake. The cut ends of 40-cm-long infested logs were sealed with hot paraffin wax to delay desiccation, and the logs were placed in emergence cages

held at 23–28°C with a photoperiod of 16:8 (light–dark). Emergent beetles were collected every 24–48 hr, sexed (Bright, 1976), and used immediately in experiments. Logs not immediately used were maintained in cold chambers at 4°C for up to eight months.

Collection of Beetle and Host Volatiles. Volatiles from living adults boring in fresh host logs were collected by aeration and adsorption on Porapak Q (50–80 mesh, Applied Sciences Division, Milton Roy Laboratory Group, State College, Pennsylvania) (Byrne et al., 1975). The Porapak Q was thermally conditioned and packed into metal (15.5 cm × 6.4 mm OD) or glass traps (12.0 cm × 6.0 mm OD).

Aerations were carried out in Newfoundland in the laboratory at 21°C under full background lighting. Fresh logs were infested with newly emerged beetles and placed in a 120-liter container made from food-grade high-density polyethylene modified after that used by Peacock et al. (1975). Air was drawn with a vacuum pump at 1.8 liters/min through a charcoal filter, over a log, and through a Porapak Q-filled metal trap. Beetles were allowed to feed before aerations for 24–48 hr on logs to ensure gallery establishment and were checked for evidence of frass production; uninfested control logs received 50 holes made with a No. 5 cork borer to simulate beetle feeding. Eleven aerations of fresh spruce logs yielded a total of 792 lhr (1 lhr = amount of volatiles collected from one log for 1 hr); 10 aerations of male-infested logs yielded a total of 41,142 bhr (1 bhr = amount of volatiles collected from one beetle boring in a log for 1 hr); one aeration of a female-infested log yielded a total of 3750 bhr; six aerations of logs infested with one male and two female beetles per gallery yielded a total of 18,732 bhr from mated males.

Logs from the same tree were used for each aeration experiment to minimize possible effects of host variation. Within each experiment, uninfested control logs were aerated first, followed by male-infested logs and logs infested by males and females. The odor-laden Porapak Q was extracted after 72 hr in 5.0 ml of redistilled hexane or pentane and ethyl ether (95:5) and stored in a Teflon-lined vial at −40°C. Between treatments, the aeration container was wiped clean with acetone and the glass wool and the activated charcoal filter was replaced. Filters were flushed with double-distilled pentane and ethyl ether followed by a 2-hr nitrogen purge.

Aerations in British Columbia utilized a water aspirator with an airflow rate of 1–2 liters/min. Single beetle collections were made using an on-tree collection device (Gries et al., 1988), consisting of a Porapak Q trap connected to an expanded glass tube placed over the entrance hole of the beetle's gallery. Aerations were carried out in the laboratory at 21°C on a photoperiod of 16:8 (light–dark). Treatments included aeration of single males, one male and three females, a control vial filled with finely ground phloem tissue, and an empty control vial. Six males boring in white spruce were individually aerated for 60

hr in each of three experiments. Three females were added to each male gallery in experiments 1 and 2, and the 60-hr aeration was repeated. The third female refused to enter four male galleries in experiment 1. Beetle establishment and frass production were recorded for each experiment. Logs from experiment 1 were dissected three weeks following aeration to verify that females had established broods. Trapped volatiles were extracted in 0.5 ml pentane and ethyl ether (95:5), and stored at −40°C in a vial with a Teflon-lined cap.

Collection and Preparation of Frass and Abdominal Extracts. Phloem tissue was obtained by removing samples of bark from fresh black spruce with a No. 5 cork borer. Frass was collected after allowing newly emerged beetles to bore freely into fresh spruce logs or by confining them with gelatin capsules in preformed entrance holes. To prepare extracts, phloem and frass were pulverized in redistilled pentane or hexane and ethyl ether (95:5) (1.0 ml/0.1 g material) over Dry Ice. Abdominal extracts from 500 male and 250 female beetles were prepared by excising beetles from logs and crushing the abdomens in either chilled pentane or hexane and ethyl ether (95:5) (10 μl/abdomen) in a vial held on Dry Ice. The vials were sealed with a Teflon-lined cap and stored at −40°C.

Analysis of Trapped Volatiles. Volatiles were analyzed in a Hewlett-Packard 5830A gas chromatograph (GC) equipped with a 18835B capillary inlet system and a flame ionization detector (FID). Helium was the carrier gas, and the injection port and detector temperatures were 260 and 270°C, respectively. Mass spectroscopy (MS) was done with a Hewlett-Packard 5985 coupled GC-MS. Captured volatiles were concentrated to 5 μl by evaporation over gentle heat or with a stream of nitrogen at −10°C and analyzed on a 60-m × 0.32-mm-ID DB-1 fused silica column with temperature programming from 30 to 130°C at 1°C/min. Abdominal and frass extracts were analyzed on a 30-m × 0.25-mm-ID fused silica column coated with SP-1000 (Supelco, Inc., Bellafonte, Pennsylvania), with temperature programming from 70 to 180°C at 2°C/min. To identify beetle-produced compounds, chromatograms of volatiles produced by the attacking virgin males were compared with chromatograms of host volatiles and with those following the addition of the females.

Field Bioassays. The candidate pheromone was tested in 1987 and 1988 in black spruce stands near Powderhorn Lake and Crooked Bog 5.0 and 8.0 km, respectively, north of Badger, Newfoundland. Volatiles (Table 1) were released from 400 μl polypropylene Eppendorf tubes (Brinkman Instruments, Rexdale, Ontario) suspended in eight-unit multiple-funnel traps (Lindgren, 1983) or affixed at 1.3 m height to the north side of a black spruce bole. Open and capped tubes released 3-methyl-3-buten-1-ol at rates of 4390 and 200 μg/day, respectively. For an ultra-low rate of 6 μg/day, the candidate pheromone was released from 3-cm-long × 0.2-mm-ID, polyester Conrel fibers (Albany International Co., Needham, Massachusetts) placed inside a closed Eppendorf tube. Terpene hydrocarbons were released from an open Eppendorf tube.

TABLE 1. SOURCE, PURITY, AND RELEASE RATES OF VOLATILE MATERIALS USED IN *P. rufipennis* FIELD-TRAPPING AND TREE-BAITING EXPERIMENTS

Compound	Abbreviation	Source	Purity (%)	Approximate release rate (μg/day)[a]
3-Methyl-3-buten-1-ol	Mb	Aldrich Chem Co., Milwaukee, Wisconsin	97	4390 (open Eppendorf tube) 200 (capped Eppendorf tube) 6 (Conrel fiber)
α-Pinene	αP	Aldrich Chem Co., Milwaukee, Wisconsin	99	0.10
β-Pinene	βP	Aldrich Chem Co., Milwaukee, Wisconsin	98	0.10
3-Carene	C	Albany Int'l Co., Columbus, Ohio	95	0.15
Myrcene	M	Sigma Chem Co., St. Louis, Missouri	98	0.30
Bornyl acetate	Ba	Aldrich Chem Co., Milwaukee, Wisconsin	97	0.10

[a] Eppendorf tubes loaded with 200 μl of 3-methyl-3-buten-1-ol or terpene. For 3-methyl-3-buten-1-ol at lowest release rate, Conrel fibers were 3-cm long, sealed at one end, and placed inside a capped Eppendorf tube. Release rates determined in the laboratory at 20°C; values represent means for two measurements.

Vapona No-Pest Strip (Boyle-Midway Can. Ltd., Toronto, Ontario) was used to kill captured beetles in multiple-funnel traps. Trap baits were replaced at weekly intervals. Traps were monitored twice weekly and captured beetles were removed, placed in 75% ethanol, and separated by species and sex.

Six trapping experiments were conducted (Tables 2–5). Experiments 1 and 2 tested the candidate pheromone at two release rates, with treatments randomly assigned to 30 traps spaced at least 20 m apart. Experiment 1 was run during the beetle's spring flight and experiment 2 during the summer flight. Experiments 3 and 4 tested the candidate pheromone alone and in binary combination with each of the major terpenes identified in twigs of black spruce (von Rudloff, 1975) in a randomized complete block design. Because the Powderhorn stand was located on a gentle slope, blocks were laid on contours to minimize within-block variability. Blocks were 20 m apart and traps 15 m apart. Experiment 5 compared the candidate pheromone at two different release rates to a fresh spruce log infested with 50 newly emerged male beetles. The logs were wrapped with Saran screening to prevent further attack and tied to the side of multiple-funnel traps spaced 20 m apart in a linear sequence. Treatments were randomly assigned to traps at the beginning of each replicate. Experiment 6 was similar to exper-

TABLE 2. MEAN CATCHES OF *Polygraphus rufipennis* TO MULTIPLE-FUNNEL TRAPS BAITED WITH 3-METHYL-3-BUTEN-1-OL (M), CROOKED BOG, NEWFOUNDLAND, 5–30 JUNE (EXPERIMENT 1) AND JULY 1–AUGUST 15 (EXPERIMENT 2), 1987 (N = 10)

Treatment	Release rate (μg/24 hr)	Mean (±SE) number per trap[a]			
		Experiment 1		Experiment 2	
		Male	Female	Male	Female
Unbaited trap		0.6 ± 0.3a	0.7 ± 0.3a	0.3 ± 0.2ab	0.2 ± 0.1a
Mb	6	1.2 ± 1.0a	0.9 ± 0.4a	0a	0.4 ± 0.2a
Mb	200	13.8 ± 6.1b	8.8 ± 4.3b	1.2 ± 0.5b	2.7 ± 1.6a

[a] Means within columns for each experiment, followed by same letter not significantly different (P = 0.05, Duncan's multiple-range test).

TABLE 3. MEAN CATCHES OF *Polygraphus rufipennis* TO MULTIPLE-FUNNEL TRAPS BAITED WITH 3-METHYL-3-BUTEN-1-OL ALONE AND IN COMBINATION WITH TERPENES OF BLACK SPRUCE, POWDERHORN LAKE, NEWFOUNDLAND, JUNE 5–30 (EXPERIMENT 3) AND JUNE 30–AUGUST 13 (EXPERIMENT 4), 1987 (N = 5)

Treatment[a]	Mean (±SE) number per trap[ab]			
	Experiment 3		Experiment 4	
	Male	Female	Male	Female
Unbaited trap	0.2 ± 0.2a	0.6 ± 0.4a	0a	0.2 ± 0.2a
Terpene mixture	0.2 ± 0.2a	0.6 ± 0.4a	0.2 ± 0.2a	0.2 ± 0.2a
Mb, C	1.8 ± 1.2ab	2.0 ± 1.0a	0.4 ± 0.2a	1.0 ± 0.3a
Mb, BA	9.8 ± 4.5b	5.4 ± 1.6a	0.2 ± 0.2a	0.8 ± 0.4a
Mb, M	16.0 ± 6.8b	10.2 ± 4.8a	0.8 ± 0.6a	1.0 ± 0.3a
Mb, αP	20.4 ± 10.2b	12.2 ± 5.7a	5.0 ± 3.1a	7.4 ± 4.6a
Mb	28.4 ± 17.5b	14.8 ± 9.3a	1.6 ± 1.4a	3.8 ± 3.1a
Mb, βP	29.2 ± 15.2b	17.8 ± 11.2a	0.2 ± 0.2a	1.0 ± 0.6a

[a] Abbreviations and release rates for terpenes as in Table 1. Mb released at 200 μg/day.
[b] Means within columns for each experiment, followed by the same letter are not significantly different (P = 0.05, Duncan's multiple-range test).

iment 5, but was laid out in a randomized complete block design and replaced the low-dose pheromone stimulus with an uninfested log.

The effectiveness of the candidate pheromone in inducing attack on black spruce was evaluated in two tree-baiting experiments (experiments 7 and 8). Experiment 7 was a 10-replicate experiment laid out in a completely random-

TABLE 4. MEAN CATCHES OF *Polygraphus rufipennis* TO MULTIPLE-FUNNEL TRAPS BAITED WITH SYNTHETIC 3-METHYL-3-BUTEN-1-OL (MB), TO MALE-INFESTED LOGS AND UNBAITED TRAPS, CROOKED BOG, NEWFOUNDLAND, JUNE 30–AUGUST 13, (EXPERIMENT 5), 1987 ($N = 6$)

Treatment	Release rate (μg/24 hr)	Mean ($\pm$SE) number per trap[a]	
		Males	Females
Unbaited trap		0a	0a
Mb	200	0a	0a
Mb	4390	332.5 $\pm$ 94.7c	435.7 $\pm$ 121.1c
Log + 50 males		12.2 $\pm$ 2.6b	13.7 $\pm$ 3.7b

[a]Means within columns followed by the same letter are not significantly different ($P = 0.05$, Duncan's multiple-range test).

TABLE 5. MEAN CATCHES OF *Polygraphus rufipennis* TO MULTIPLE-FUNNEL TRAPS BAITED WITH SYNTHETIC 3-METHYL-3-BUTEN-1-OL, UNINFESTED HOST, AND MALE-INFESTED LOG, CROOKED BOG, NEWFOUNDLAND, MAY 27–JULY 4 (EXPERIMENT 6), 1988 ($N = 5$)

Treatment	Release rate (μg/24 hr)	Mean ($\pm$SE) number per trap[a]	
		Males	Female
Unbaited trap		0.8 $\pm$ 0.4a	1.2 $\pm$ 0.1a
Uninfested log		0.4 $\pm$ 0.1a	0.6 $\pm$ 0.2a
Mb	4390	685.6 $\pm$ 174.5c	879.2 $\pm$ 178.4c
Log + 50 males		34.4 $\pm$ 9.2b	38.6 $\pm$ 12.5b

[a]Means within columns followed by the same letter are not significantly different ($P = 0.05$, Duncan's multiple-range test).

ized design with at least 20 m between trees. Treatments included an empty (control) Eppendorf tube, and the candidate pheromone released at a rate of 6 μg/day from a 0.2-mm-ID Conrel fiber inside a tube, or released at 200 μg/day from a closed tube.

Treatments for experiment 8 included: an empty (control) tube, and the candidate pheromone at 200 μg/day alone or with either α-pinene, bornyl acetate, β-pinene, 3-carene, myrcene, or a mixture of the above five terpenes released from separate tubes at rates given in Table 1. Treatments were assigned at random to five blocks spaced 20 m apart and with baited trees spaced 15 m apart.

All baited trees were codominants ≥ 10 cm diameter at breast height (dbh) and had light damage (≤30% cumulative defoliation) from *C. fumiferana* in the previous 10 years. Baits were placed on trees at breast height on June 3 at the beginning of the spring flight; they were replaced on July 1 at the beginning of the summer flight. The incidence of attack on baited trees and the nearest four trees ≥ 10 cm dbh surrounding each baited tree was assessed on September 2 by counting attacks, represented by frass piles and resin flow, on areas of bark surface within two 16.7 × 30-cm open frames 1.3 m high on the north and south aspects of the tree. Attacks with fresh frass were considered successful and attacks with copious resin flow in the entrance holes were considered unsuccessful. The number of successful and unsuccessful attacks on the north and south aspects of attacked trees were combined and expressed as attack density per square meter of bark surface.

Data were analyzed using the SAS statistical package version 6.0 (SAS Institute Inc., Cary, North Carolina). Data were transformed with a $\log_{10}(X + 1)$ transformation to meet the assumptions of homoscedasticity and normality prior to analysis of variance and Duncan's multiple-range test. The chi-square statistic was used to examine differences in the attack frequencies of unbaited and baited trees. Differences in the mean attack density on unbaited and baited trees was tested using a two-sample *t* test. In all tests the maximum probability of a type-I error was set at 0.05.

RESULTS

Analyses of volatiles from *P. rufipennis* males, females, and hosts disclosed an unknown compound produced by males; this compound was produced in a lesser amount after males were joined by females (Figure 1). The unknown volatile was produced by males in both black and white spruce. Comparison of host volatiles with volatiles from female-infested logs did not disclose any female-specific compounds. There was no chromatographic evidence for more than one male-produced compound.

The mass spectrum of the unknown compound indicated major fragments of mass *m/z* 41(100), 56(88), 68(69), 31(65), and 86(20). This fragmentation pattern matched the mass spectrum of the primary alcohol, 3-methyl-3-buten-1-ol (Heller and Milne, 1978). GC-MS comparison of the beetle-produced compound with an authentic sample of 3-methyl-3-buten-1-ol indicated identical retention and mass spectral characteristics.

Chromatograms of hindgut extracts from male and female *P. rufipennis* disclosed no sex-specific volatiles. However, 3-methyl-3-buten-1-ol was detected by GC-MS in extracts of frass from males but not females, boring in

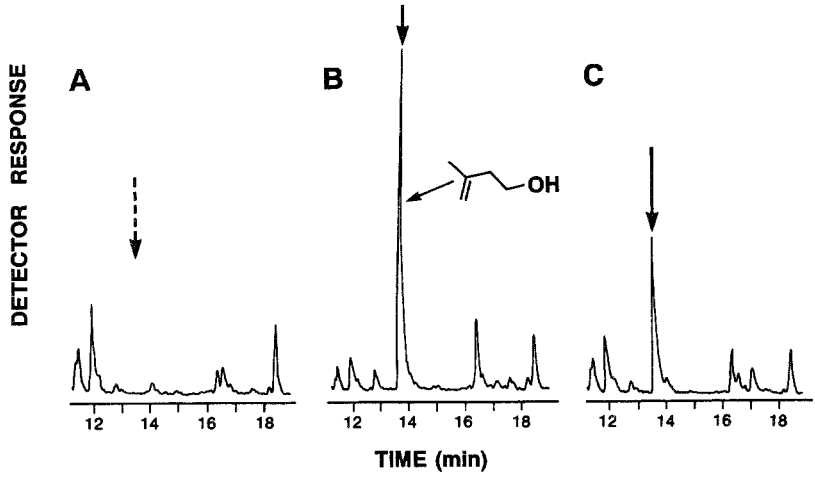


FIG. 1. Representative chromatograms of airborne volatiles collected from (A) a black spruce log, (B) from an individual *P. rufipennis* male boring in black spruce, and (C) the same male following addition of three females. Arrow denotes retention time of 3-methyl-3-buten-1-ol. Temperature programming: 30–130°C at 1°C/min.

black spruce (Figure 2). The compound was not present in extracts from phloem tissue.

Bioactivity of 3-methyl-3-buten-1-ol was demonstrated in field-trapping and tree-baiting experiments. Traps baited with 3-methyl-3-buten-1-ol released at 200 μg/day in the spring caught significantly higher numbers of both male and female beetles than unbaited traps or those baited with 3-methyl-3-buten-1-ol released at 6 μg/day (Table 2, experiment 1). Attraction of males and females to 3-methyl-3-buten-1-ol released at 6 μg/day did not differ significantly from unbaited traps. Catches during the summer were low, and there was no significant difference between treatments (Table 2, experiment 2). Release of 3-methyl-3-buten-1-ol at 200 μg/day in combination with terpenes of black spruce did not improve beetle catch (Table 3 , experiments 3 and 4). A significant increase in male and female response occurred when the release rate of 3-methyl-3-buten-1-ol was increased to 4390 μg/day (Tables 4 and 5). Responses by beetles of both sexes in the summer to the male-infested logs were significantly lower than to 3-methyl-3-buten-1-ol at high release rates but significantly higher than to 3-methyl-3-buten-1-ol at low release rates (Table 4, experiment 5). Similarly, in spring, responses by male and female beetles to male-infested logs were significantly higher than to unbaited traps and uninfested logs, but lower than to 3-methyl-3-buten-1-ol released at 4390 μg/day (Table 5, experiment 6).

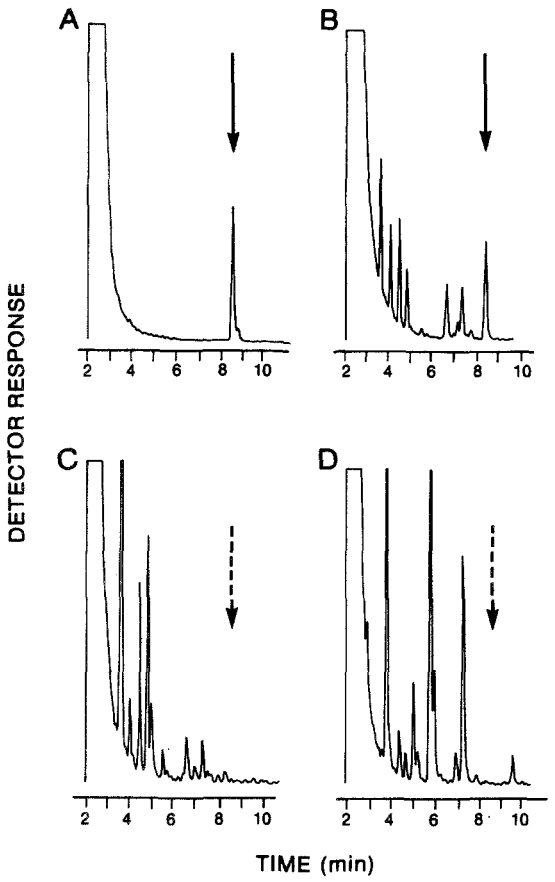


FIG. 2. Gas chromatograms of (A) synthetic 3-methyl-3-buten-1-ol, compared with (B) male frass extract, (C) female frass extract, and (D) extract of fresh phloem tissue. Arrow denotes retention time of 3-methyl-3-buten-1-ol. Temperature programming: 70–180°C at 2°C/min.

Forty-five percent of the black spruce trees baited with 3-methyl-3-buten-1-ol were attacked by *P. rufipennis* compared to 20% of the unbaited trees, but the difference was not significant. In contrast, significantly more trees surrounding baited trees were attacked than those surrounding unbaited trees. The rate of attack success was low for both control trees (10%) and baited trees (20%). No trees surrounding control trees were successfully attacked, but 52.8% of the trees surrounding baited trees were successfully attacked. Analysis of mean attack density on unbaited and baited trees, or those surrounding them, disclosed no evident trend. Similarly, there was no indication that the major ter-

penes of black spruce significantly enhanced beetle response to baited trees or to trees surrounding them. Very few trees in this experiment were attacked, precluding detection of differences in attack density.

DISCUSSION

Our results demonstrate that the isoprene alcohol, 3-methyl-3-buten-1-ol, is produced by male *P. rufipennis* and is an aggregation pheromone for this species. These findings are consistent with previously reported pheromones for polygynous bark beetles where pioneer males produce and release aggregation pheromones (Wood et al., 1966; Bakke, 1975, 1976; Harring and Mori, 1977; Francke et al., 1977; Stoakley et al., 1978; Baader, 1989). Although we have no direct evidence for more than one pheromone, the high release rate of 3-methyl-3-buten-1-ol required to elicit attraction in the field suggests other pheromones may be necessary to effect a maximum response. Knowledge that insects generally use multicomponent pheromones (Silverstein and Young, 1976) and recent advances on the pheromone system of the six-spined spruce bark beetle, *Pityogenes chalcographus* (L.), (Byers et al., 1989, 1990) provide impetus to hypothesize the existence of additional pheromones. The presence of 3-methyl-3-buten-1-ol as a pheromone in *P. rufipennis* is of ecological interest. Isoprene alcohols have been reported in only two bark beetle species of the Scolytinae: 2-methyl-3-buten-2-ol in males of the spruce bark beetle, *Ips typographus* L. (Bakke et al., 1977), and 3-methyl-3-buten-1-ol in males of the larch engraver, *Ips cembrae* Heer (Stoakley et al., 1978). Like *P. rufipennis*, both European species feed mainly on weakened trees but will attack healthy standing trees during epidemics (Postner, 1974). Although phylogenetically different, *P. rufipennis* exploits the same highly volatile methylbutenol as *I. cembrae* to further its individual reproductive success. Use of such a compound may be of selective advantage to individuals in northern habitats where midsummer temperatures are often low. Dickens (1981) and Birgersson et al. (1984) suggested that methylbutenol compounds may be short-range orientation or arrestment substances that act to concentrate pioneer beetles on a resistant host tree. Similarly, Schlyter et al. (1987) inferred from field trials that 2-methyl-3-buten-2-ol functions primarily as a landing stimulus for *I. typographus*. We have no definite knowledge as to the effective range of 3-methyl-3-buten-1-ol.

The pheromone system of *P. rufipennis* shows no apparent relationship to that of the closely related European species, *Polygraphus poligraphus* (L.), that also attacks spruce. Male *P. poligraphus* produce three sex-specific compounds, *trans*-4-thujanol, *cis*-4-thujanol, and terpinen-4-ol (Francke and Vité, 1983). *P. rufipennis* previously was treated as a synonym of *P. poligraphus* (Schedl, 1957); however, our findings support Wood (1982), who separated the

two species on the basis of statistical differences in pronotal punctures and tubercles at the bases of the elytra.

The presence of 3-methyl-3-buten-1-ol in male frass is consistent with earlier studies that demonstrated that both males and females respond positively to male-produced frass in laboratory bioassays (Bowers and Borden, 1990). The absence of pheromone in the hindgut is surprising. It is possible that another biosynthetic site may be responsible for its production. For example, (*E*,*Z*)-MD, a major component of the pheromone of *Pityogenes chalcographus*, is found predominantly in the head and thorax of the male beetle. Furthermore, contents of hindguts and the composition of the released pheromone are known to differ (Peacock et al., 1975; Silk et al., 1980). For these reasons it cannot be assumed that a released pheromone will always be detected in the hindgut.

The magnitude of the response to traps releasing 3-methyl-3-buten-1-ol at 4390 μg/day suggests that the 200 μg/day release rate used in tree-baiting experiments may not have been optimal and that a stronger response to baited trees would occur with higher release rates. It is also possible, as hypothesized above, that an additional pheromone may be required to effect an optimal response.

Although host terpenes often enhance response to beetle-produced pheromones (Hughes et al., 1976; Lanier et al., 1977), no such enhancement was evident for *P. rufipennis*, and 3-carene even appeared to inhibit aggregation (Table 3, experiment 3). The low response to fresh uninfested logs suggests that *P. rufipennis* does not rely exclusively on major host terpenes in searching for and exploiting quality resources. However, it is possible that minor host constituents may be involved in *P. rufipennis* host selection. Tømmerås and Mustaparta (1987) reported that minor host constituents stimulated single olfactory receptor cells of *I. typographus* and probably reveal qualitative differences between individual host trees. Moreover, computer-simulated searching strategies (Gries et al., 1989) suggested that random search alone is unlikely to maintain an *I. typographus* population.

Virtually nothing is known about the biogenesis of 3-methyl-3-buten-1-ol in *P. rufipennis*. Production probably occurs from acetate via the mevalonic acid pathway. Hackstein and Vité (1978) and Renwick and Dickens (1979) suggested that in *I. typographus* and *I. cembrae* the compound occurs de novo and is under hormonal control.

The results of this study have implications for management of *P. rufipennis*. Identification of the *P. rufipennis* aggregation pheromone could lead to the development of a monitoring or management tool for use in future pest management operations.

Acknowledgments—We thank J.W. Marshall, L.J. Chong, and G. Owen for their technical assistance and D.R. Miller for field assistance. We appreciate reviews of the manuscript by M. Winston, R. Alfaro, and D. Langor. The research was supported in part by The Natural Sciences and Engineering Research Council, Canada, grants A3881 and G1611; a postdoctoral fellowship

of the Deutsche Forschungsgemeinschaft (DFG) to G. Gries; and by a MacMillian Family Fund Fellowship to W.W. Bowers.

REFERENCES

BAADER, V.E.J. 1989. *Pityogenes* spp. (Col., Scolytidae): Untersuchungen über verhaltenssteuernde Duftstoffe und deren Anwendung im Waldschutz. *Z. Angew. Entomol.* 107:1–31.

BAKKE, A. 1975. Aggregation pheromone in the bark beetle *Ips duplicatus* (Schlberg). *Norw. J. Entomol.* 22:67–69.

BAKKE, A. 1976. Spruce bark beetle, *Ips typographus*: Pheromone production and field response to synthetic pheromone. *Naturwissenschaften* 63:92.

BAKKE, A., FROYEN, P., and SKATTEBOL, L. 1977. Field response to a new pheromonal compound isolated from *Ips typographus*. *Naturwissenschaften* 64:98.

BIRGERSSON, G., SCHLYTER, F., LÖFQVIST, J., and BERGSTROÖM, G. 1984. Quantitative variation of pheromone components in the spruce bark beetle *Ips typographus* from different attack phases. *J. Chem. Ecol.* 10:1029–1055.

BOWERS, W.W., and BORDEN, J.H. 1990. Evidence for a male-produced aggregation pheromone in the four-eyed spruce bark beetle, *Polygraphus rufipennis* (Kirby) (Coleoptera: Scolytidae). *Z. Angew. Entomol.* 110:292–299.

BRIGHT, D.E. 1976. The insects and arachnids of Canada. Part 2. The bark beetles of Canada and Alaska (Coleoptera: Scolytidae). Biosystematics Research Institute. Canadian Department of Agriculture, Ottawa, Ontario, Pub. No. 1576.

BRÜMMER, B. 1981. Identifizierung geschlechtsspezifischer Monoterpenalkohole aus dem Borkenkäfer *Polygraphus poligraphus* L. und systematische Untersuchungen zu deren massenspektrometrischem Verhalten. Dissertation. University of Hamburg, Hamburg, Germany.

BYERS, J.A., HÖGBERG, H.E., UNELIUS, C.R., BIRGERSSON, G., and LÖFQVIST, J. 1989. Structure-activity studies on aggregation pheromone components of *Pityogenes chalcographus* (Coleoptera: Scolytidae): All stereoisomers of chalcogran and methyl 2,4-decadienoate. *J. Chem. Ecol.* 15:685–695.

BYERS, J.A., BIRGERSSON, G., LÖFQVIST, J., APPELGREN, M., and BERGSTRÖM, G. 1990. Isolation of pheromone synergists of bark beetle, *Pityogenes chalcographus*, from complex insect-plant odors by fractionation and subtractive-combination bioassay. *J. Chem. Ecol.* 16:861–876.

BYRNE, K.E., GORE, W.E., PEARCE, G.T., and SILVERSTEIN, R.M. 1975. Porapak Q collection of airborne organic compounds serving as models for insect pheromones. *J. Chem. Ecol.* 1:1–7.

DICKENS, J.C. 1981. Behavioural and electrophysiological responses of the bark beetle, *Ips typographus*, to potential pheromone components. *Physiol. Entomol.* 6:251–261.

FRANCKE, W., and VITÉ, J.P. 1983. Oxygenated terpenes in pheromone systems of bark beetles. *Z. Angew. Entomol.* 96:146–156.

FRANCKE, W., HEEMANN, V., GERKEN, B., RENWICK, J.A.A. and VITÉ, J.P. 1977. 2-Ethyl-1,6-dioxaspiro[4.4]nonane, principal aggregation pheromone of *Pityogenes chalcographus* (L.). *Naturwissenschaften* 64:590–591.

GRIES, G., PIERCE, H.D., JR., LINDGREN, B.S., and BORDEN, J.H. 1988. New techniques for capturing and analyzing semiochemicals for scolytid beetles (Coleoptera: Scolytidae). *J. Econ. Entomol.* 81:1715–1720.

GRIES, G., NOLTE, R., and SANDERS, W. 1989. Computer simulated host selection in *Ips typographus*. *Entomol. Exp. Appl.* 53:211–217.

HACKSTEIN, E., and VITÉ, J.P. 1978. Pheromonbiosynthese und Reizkette in der Besiedlung von Fichten durch den Buchdrucker *Ips typographus*. *Mitt. Dtsch. Ges. Allg. Angew. Entomol.* 1:185–188.

HARRING, C.M., and MORI, K. 1977. *Pityokteines curvidens* Germ. (Coleoptera: Scolytidae): Aggregation in response to optically pure ipsenol. *Z. Angew. Entomol.* 82:327–329.

HELLER, S.R., and MILNE, G.W.A. 1978. EPA/NIH Mass Spectra Data Base. U.S. Government Printing Office, Washington, D.C.

HUGHES, P., RENWICK, J.A.A., and J.P. VITÉ. 1976. The identification and field bioassay of chemical attractants in the roundheaded pine beetle. *Environ. Entomol.* 5:1165–1168.

LANIER, G.N., GORE, W.E., PEARCE, G.T., PEACOCK, J.W., and SILVERSTEIN, R.M. 1977. Response of the European elm bark beetle, *Scolytus multistriatus* (Coleoptera: Scolytidae), to isomers and components of its pheromone. *J. Chem. Ecol.* 3:1–8.

LEUZE, C. 1977. Untersuchungen über Duftstoffe des doppeläugigen Fichtenbastkäfers *Polygraphus poligraphus* L. (Coleoptera: Scolytidae). Diplomarbeit Univ. Freiburg.

LINDGREN, B.S. 1983. A multiple funnel trap for scolytid beetles (Coleoptera). *Can. Entomol.* 115:299–302.

PEACOCK, J.W., CUTHBERT, R.A., GORE, W.E., LANIER, G.N., PEACE, G.T., and R.M. SILVERSTEIN. 1975. Collection on Porapak Q of the aggregation pheromone of *Scolytus multistriatus* (Coleoptera: Scolytidae). *J. Chem. Ecol.* 1:149–160.

POSTNER, M. 1974. Scolytidae, Borkenkäfer, pp. 334–482, *in* W. Schwencke (ed.). Die Forstschädlinge Europas, 2. Paul Parey, Hamburg.

RASKE, A.G., and SUTTON, W.J. 1986. Decline and mortality of black spruce caused by spruce budworm defoliation and secondary organisms. Canadian Forestry Service, Newfoundland Forestry Centre, Inf. Rep. N-X-236.

RENWICK, J.A.A., and DICKENS, J.C. 1979. Control of pheromone production in the bark beetle, *Ips cembrae*. *Physiol. Entomol.* 4:377–381.

SCHEDL, K.E. 1957. Bark- and timber-beetles from South Africa. *Ann. Mag. Nat. Hist.* 10:149–159.

SCHLYTER, F., LÖFQVIST, J. and BYERS, J.A. 1987. Behavioural sequence in the attraction of the bark beetle *Ips typographus* to pheromone sources. *Physiol. Entomol.* 12:185–196.

SILK, P.J., TAN, S.H., WEISNER, C.J., ROSS, R.J., and GRANT, G.G. 1980. Sex pheromone chemistry of the eastern spruce budworm *Choristoneura occidentalis* Free. *J. Chem. Ecol.* 8:351–362.

SILVERSTEIN, R.M., and YOUNG, J.C. 1976. Insects generally use multi-component pheromones, pp. 1–29, *in* M. Beroza (ed.). Pest Management with Insect Sex Attractants and Other Behavior-Controlling Chemicals. American Chemical Society Symposium Series 23, Washington, D.C.

STOAKLEY, J.T., BAKKE, A., RENWICK, J.A.A., and VITÉ, J.P. 1978. The aggregation pheromone system of the larch beetle, *Ips cembrae* Heer. *Z. Angew. Entomol.* 86:174–177.

SWAINE, J.M. 1918. Canadian bark beetles. Part 2. Dominion of Canada, Department of Agriculture Entomology Branch, Bulletin No. 14.

TØMMERÅS, B.Å., and MUSTAPARTA, H. 1987. Chemoreception of host volatiles in the bark beetle *Ips typographus*. *Comp. Physiol. A* 161:705–710.

VON RUDLOFF, E. 1975. Seasonal variation in the terpenes of the foliage of black spruce. *Phytochemistry* 14:1695–1699.

WOOD, S.L., 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae): A taxonomic monograph. *Great Basin Nat. Mem.* 6:1–1326.

WOOD, D.L., BROWNE, L.E., SILVERSTEIN, R.M., and RODIN, J.O. 1966. Sex pheromone of bark beetles-I. Mass production, bioassay, source, and isolation of the sex pheromone of *Ips confuses* (LeC.). *J. Insect Physiol.* 12:523–536.

Journal of Chemical Ecology, Vol. 17, No. 10, 1991

VOLATILE COMPOUNDS IN THE LARVAL FRASS OF *Dendroctonus valens* AND *Dendroctonus micans* (COLEOPTERA: SCOLYTIDAE) IN RELATION TO OVIPOSITION BY THE PREDATOR, *Rhizophagus grandis* (COLEOPTERA: RHIZOPHAGIDAE)

J.-C. GRÉGOIRE,[1,2,*] M. BAISIER,[1] A. DRUMONT,[1] D.L. DAHLSTEN,[3] H. MEYER,[4] and W. FRANCKE[4]

In memoriam, Prof. Dr. Helene Francke-Grosmann

[1]*Laboratoire de Biologie animale et cellulaire CP 160*
Université Libre de Bruxelles
50 av. F.D. Roosevelt
B-1050 Bruxelles, Belgium

[2]*Belgian Fund for Scientific Research*
Bruxelles, Belgium

[3]*University of California, Berkeley*
Division of Biological Control
1050 San Pablo Avenue
Albany, California 94706

[4]*Institut für Organische Chemie*
Universität Hamburg
Martin-Luther-King-Platz 6
D-2000 Hamburg 13, Germany

(Received April 17, 1991; accepted June 19, 1991)

Abstract—During a laboratory study evaluating *Rhizophagus grandis* (a specific native predator of the Eurasian bark beetle, *Dendroctonus micans*), as a potential biocontrol agent against the North American bark beetle, *Dendroctonus valens*, it was found that feeding larvae and laboratory-produced frass of the potential prey elicited very high oviposition responses in the predator. Comparative chemical analysis of this laboratory-produced larval frass revealed that one major volatile compound, (−)-fenchone, is associated with the larvae of both *Dendroctonus* species. *D. micans* also generated pinocamphone while oxygenated monoterpenes in the frass of *D. valens* were camphor, *cis*-4-thujanol, fenchol, terpinen-4-ol, myrtenal, pinocarvone, borneol,

*To whom correspondence should be addressed.

0098-0331/91/1000-2003$06.50/0

verbenone, piperitone, campholenaldehyde, *trans*-myrtanol, *cis*-myrtanol, *p*-cymen-8-ol and 5-oxo-camphor. This range of prey-produced compounds with a possible biological effect on *R. grandis* was narrowed down subsequent to comparative analysis of field-collected larval frass. (−)-Fenchone, pinocamphone, camphor, terpinen-4-ol, borneol, fenchol, and verbenone were found to be common to both prey species. A mixture of these seven components was tested in a bioassay, where it elicited as much oviposition as did larval frass of *D. micans*. The oviposition stimulants for *R. grandis* are thus clearly among the mixture's constituents.

Key Words—Dendroctonus micans, Dendroctonus valens, Rhizophagus grandis, Coleoptera, Rhizophagidae, Scolytidae, oviposition stimuli, biological control, monoterpenes.

INTRODUCTION

The highly specific predator *Rhizophagus grandis* Gyllenhal is very often found in the brood chambers of the greater European spruce beetle, *Dendroctonus micans* (Kugelann) (Bergmiller, 1903; Francke-Grosmann, 1954; Grégoire, 1988). Since the mid-1960s, *R. grandis* has been used for the biological control of *D. micans* in Soviet Georgia (Kobakhidze, 1965), France (Grégoire et al., 1984), United Kingdom (King and Evans, 1984), and Turkey (Serez, personal communication).

The red turpentine beetle *Dendroctonus valens* LeConte, which attacks several species of pine in North America (Smith, 1971), is considered phylogenetically very close to *D. micans* (Stock et al., 1987). It also has a very similar life history: both species are solitary colonizers of healthy trees, have a relatively long life cycle as compared to other *Dendroctonus* species, and spend their whole larval stage gregariously feeding on the living phloem of the host.

It has been found that the oviposition of *Rhizophagus grandis* is dependent on the presence of prey larvae and that the stimuli responsible for oviposition in *R. grandis* are volatile compounds present in the larval frass of *D. micans* (Baisier and Grégoire, 1988; Baisier et al., 1988). *R. grandis* does not occur in North America, but its introduction could be useful for the biological control of bark beetle species close to *D. micans*, such as *D. terebrans* (Miller et al., 1987; Moser, 1989) and *D. valens*. This latter species is locally considered as a pest and is not known to be associated with effective natural enemies (Smith, 1971). In laboratory experiments aimed at testing the potential of *Rhizophagus grandis* against *D. valens*, larval frass of *D. valens* elicited very high oviposition responses among the predators.

The objective of the present study was to carry out a comparative analysis of the larval frass of *D. micans* and *D. valens* in order to identify common compounds involved in eliciting predator oviposition. Using laboratory-produced larval frass, comprehensive chemical analyses were carried out by com-

bined gas chromatography–mass spectrometry. The volatiles produced by bark beetle larvae were assigned by differential analyses of frass extracts and extracts of bark and bark powder prepared under similar conditions. The volatiles common to both *Dendroctonus* species were then found by comparing the frass extracts from the two species. These results were completed by comparative analysis of field-collected larval frass of both species. Finally, the biological activity of the selected compounds selected was confirmed in oviposition bioassays.

METHODS AND MATERIALS

Extracts of Laboratory-Produced Frass

Laboratory-produced larval frass of both species was obtained from "oviposition boxes" (Baisier et al., 1988), which are 43-ml clear polystyrene containers filled with either fresh phloem of spruce (*D. micans*) or pine (*D. valens*), phloem powder, live larvae of either *D. micans* or *D. valens*, and are suitable for *R. grandis* oviposition. The control boxes contained fresh phloem and phloem powder, but no larvae. The extracts from *D. micans* were prepared in Brussels, Belgium, and those from *D. valens* were made in Berkeley, California. For each bark beetle species, three series of boxes were prepared:

Series 1 (control boxes) contained fresh host phloem and phloem powder (*Picea abies* for *D. micans*, *Pinus lambertiana* for *D. valens*). Care was taken to match the weights of the fresh phloem pieces in each box [average fresh weight $\pm$ SD = 19.0 g $\pm$ 0.93 of *P. abies* for *D. micans* ($N = 2$), 19.6 g $\pm$ 1.21 of *P. lambertiana* for *D. valens* ($N = 4$)].

Series 2 (oviposition boxes) was prepared like the first series, but also contained 25 live bark beetle larvae. Phloem fresh weight was 18.0 g $\pm$ 0.16 of *P. abies* for *D. micans* ($N = 2$) and 19.0 g $\pm$ 2.27 of *P. lambertiana* for *D. valens* ($N = 4$). Total mean larval biomass was 944.0 mg $\pm$ 22.6 mg for *D. micans* and 709.5 mg $\pm$ 65.9 mg for *D. valens*.

Series 3 was the same as series 2, except that two pairs of *R. grandis* were also included as an oviposition control test, to check that oviposition by the predators occurred in the experimental material ($N = 5$ boxes for both species).

When the first young *R. grandis* larvae started to appear in series 3 (after 19 days for *D. valens* and 10 days for *D. micans*), the boxes of series 1 and 2 were opened. The *Dendroctonus* larvae in series 2 were removed, and the content of the boxes (phloem, phloem powder, and, in addition, frass in series 2) was immersed in pentane (Merck, 95% pure for *D. micans*; Aldrich, 99% pure for *D. valens*).

Extracts of Field-Collected Larval Frass

The samples were collected on standing trees from brood chambers containing live larvae and immediately put into pentane. For *D. valens*, samples were collected in California on either *P. lambertiana* or *P. ponderosa*. For *D. micans*, samples were collected on *P. abies* in Belgium and in Great Britain.

Chemical Analysis

Gas chromatographic analysis of the extracts was carried out on a Carlo Erba Fractovap Series 2150 gas chromatograph with split injector and flame ionization detector. For separation, a fused silica column with FFAP as the stationary phase (50 m, 0.25 mm ID) was used under a temperature program of 60°C to 220°C at a rate of 3°/min. Helium served as the carrier gas with a flow rate of 1.8 ml/min. Mass spectra (EI^+, 70 eV) were recorded on a Varian MAT 311A and a VG 70-250 S instrument in a GC-MS coupling mode under the above gas chromatographic conditions.

Identifications were based on comparison of gas chromatographic retention times and mass spectra of natural compounds and authentic reference samples.

The absolute configurations of natural fenchone and isopinocamphone were determined by gas chromatographic enantiomer separation on a chiral stationary phase: octakis(3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-cyclodextrin (König et al., 1989).

Synthetic Compounds

Compounds used in bioassays were either purchased or synthesized in our labs. The racemate of camphor (Aldrich) and terpinen-4-ol (Merck) showed purities of 99% and 90%, respectively; (+)/(−)-borneol (Fluka) was 85% chemically pure and had a rotation value of $[\alpha]_D^{20} = -18°$ (c = 2.43-EtOH). The chemical purity of the ketones (−)-fenchone (Fluka) and (−)-verbenone (Bedoukian) was 97%; fenchone showed an enantiomeric excess of 98% and verbenone 80% (by gas chromatography). (−)-Fenchol was synthesized from (+)-fenchone according to Beckmann and Metzger (1956); chemical purity of the product proved to be 99%, rotation value $[\alpha]_D^{20} = 12.9°$ (c = 1.3 pentane). (−)-α-Pinene (Aldrich) served as starting material for the synthesis of (−)-pinocamphone (Zweifel and Brown, 1964). Our product was 99% chemically pure and showed a rotation value of $[\alpha]_D^{20} = (-23.8°$ (c = 0.47, EtOH).

Bioassay

Three series of 20 oviposition boxes were prepared: stimuli, frass, and control blank. Each box contained a pair of *R. grandis*, which were fed daily half a larva of *Calliphora erythrocephala*. *R. grandis* larvae produced in the

boxes and gathered on these prey were collected and counted daily. Adult predators were regularly checked; dead males were replaced, and when dead females were observed (one occurrence only) the corresponding box was removed from the test.

For the control blank series, the boxes were filled each with two disks of fresh spruce bark (4.1 cm in diameter) and bark powder.

For the frass series, the boxes each were filled with two disks of fresh spruce bark and with bark powder mixed with 8 g of *D. micans* larval frass collected in France (Gard) in September 1991.

For the stimuli series, the boxes each were filled with two disks of fresh spruce bark and bark powder. Each box also received 220 mg of a mixture of: pinocamphone, camphor (racemic), (−)-fenchone, terpinen-4-ol (racemic), (−)-α-fenchol, borneol, (*S*.vt)-(−)-verbenone, and pentane, in the ratios 4:14:14:14:14:14:15. For (−)-fenchone, this quantity corresponds approximately to the amount found in 56 g of frass (Francke and Meyer, unpublished). The quantity of pinocamphone used was determined by its availability. Pentane was added to the mixture in order to dissolve the camphor, borneol, and fenchol and 37 mg of this mixture (i.e., 50 μl) was deposited on the surface of the bark powder in each box on the first day of the experiment. Later, a new dose was incorporated into each box every fourth day during 20 days (corresponding thus to a total of six incorporations, amounting to a total of 220 mg/box). After each new incorporation, the boxes were left open for 1 min to allow evaporation of the pentane.

Statistical Analysis

Chemical Analysis. For each compound and for each bark beetle species, the percentages of total amount of volatiles in the control boxes and the oviposition boxes were compared using one-way analyses of variances after arcsine transformation of the data (Dagnelie, 1970).

Bioassay. The numbers of larvae collected in each series at the end of the bioassay were compared using one-way analysis of variance after square root transformation of the data, and a Newman-Keuls multiple comparison of means (Dagnelie, 1970). Only the boxes where oviposition had occurred were used in this analysis. The proportions of "sterile" boxes in each series were analyzed using binomial tests (Dagnelie, 1970) based upon a 0.033 individual probability for a box to be accidentally "sterile;" this 0.033 probability was calculated from previous rearings in normal oviposition boxes (i.e., containing one pair of predators and *D. micans* larvae) and where four boxes out of 120 were found to produce no larvae (Baisier, unpublished).

RESULTS

Oviposition control tests (series 3) showed perfect suitability of the oviposition boxes for oviposition:(1) *D. micans*: an average (±SD) of 138.8 ± 30.52 *R. grandis* larvae were produced; (2) *D. valens*: 219.2 ± 87.02 *R. grandis* larvae were produced. There were significantly more larvae in the *D. valens* oviposition boxes than in those with *D. micans* (*t* test: $t = 1.95$; $P > 0.05$), which is consistant with earlier results (unpublished).

Oviposition was not tested in control boxes, since these latter had never shown biological activity in previous studies.

Extracts of Laboratory-Produced Frass. The concentrations of monoterpene hydrocarbons and oxidized monoterpenes found in the material of the oviposition boxes containing *Dendroctonus* larvae (i.e., series 2) and control boxes containing no larvae (i.e., series 1) are given in Tables 1 and 2 (expressed as percentages of the total amount of volatiles) and in Figure 1A and 1B for *D. valens*. One-way analysis of variance for each oxidized monoterpene indicated

TABLE 1. EXTRACTS OF LABORATORY-PRODUCED FRASS PRODUCED BY *Dendroctonus micans*[a]

Compounds	Percentages of total amounts of volatiles	
	Control boxes (mean ± SD) ($N = 2$)	Oviposition boxes (mean ± SD) ($N = 2$)
Tricyclene	0.1 ± 0.005	0.1 + 0.003
α-Pinene	13.2 ± 0.98	9.3 ± 1.55
Camphene	0.2 ± 0.01	0.2 ± 0.03
β-Pinene	15.3 ± 1.22	12.02 ± 0.54
3-Carene	0.01 ± 0.01	0.01 ± 0.01
Myrcene	2.7 ± 0.20	1.8 ± 0.15
Limonene	2.3 ± 0.21	3.00 ± 0.31
β-Phellandrene	9.8 ± 0.76	10.8 ± 0.97
p-Cymene	0.1 ± 0.001	0.1 ± 0.03
Terpinolene	0.2 ± 0.02	0.1 ± 0.03
(*E*)-2-Methyl-6-methylen-1,3,7-octatriene	0.2 ± 0.03	0.1 ± 0.02
(−)-Fenchone	**0.1 ± 0.002**	**0.3 ± 0.03**[b]
Pinocamphone	**0.1 ± 0.003**	**0.5 ± 0.003**[c]
Camphor	0.1 ± 0.003	0.3 ± 0.19
(−)-Isopinocamphone	0.1 + 0.01	0.7 + 0.55
Terpinen-4-ol	0.03 ± 0.01	0.1 ± 0.03
trans-Pinocarveol	0.1 ± 0.01	0.1 ± 0.03
Piperitone	0.1 ± 0.001	0.1 ± 0.02
Thymyl methyl ether	0.3 ± 0.03	0.3 ± 0.09

[a] Boldface indicates components that have been found in significantly greater amounts in the laboratory-produced frass than in the control boxes.
[b] Highly significantly different from controls (*F* test, $\alpha = 0.01$).
[c] Very highly significantly different from controls (*F* test, $\alpha = 0.001$).

TABLE 2. EXTRACTS OF LABORATORY-PRODUCED FRASS PRODUCED BY *Dendroctonus valens*

Compounds	Percentages of total amount of volatiles	
	Control boxes (mean ± SD) ($N = 4$)	Oviposition boxes (mean ± SD) ($N = 4$)
n-Decane	0.01 ± 0.02	0.2 ± 0.03
Tricyclene	0.2 ± 0.02	0.3 ± 0.31
α-Pinene	16.6 ± 1.47	6.9 ± 3.87
Camphene	0.8 ± 0.04	0.6 ± 0.19
β-Pinene	40.0 ± 4.37	17.2 + 9.82
3-Carene	0.4 ± 0.07	0.3 ± 0.21
Myrcene	1.4 ± 0.23	0.8 ± 0.36
Limonene	4.3 ± 0.37	2.6 ± 1.90
β-Phellandrene	5.1 ± 0.85	3.4 ± 1.58
γ-Terpinene	0.1 ± 0.003	0.1 ± 0.05
p-cymene	0.2 ± 0.05	0.4 ± 0.17
Terpinolene	1.3 ± 0.20	1.1 ± 0.57
(−)-Fenchone	**0.1 ± 0.03**	**3.9 ± 1.87**[c]
Dimethylstyrene	**0.1 ± 0.02**	**0.5 ± 0.21**[d]
Camphor	**0.4 ± 0.10**	**5.7 ± 2.42**[c]
(−)-Isopinocamphone	0.8 ± 0.35	2.4 ± 1.38
***cis*-4-Thujanol**	**trace**[b]	**0.2 ± 0.16**[d]
Fenchol	**trace**	**0.5 ± 0.36**[d]
Terpinen-4-ol	**0.03 ± 0.06**	**0.7 ± 0.44**[d]
Myrtenal	**trace**	**0.4 ± 0.22**[c]
Acetophenone	0.1 + 0.03	0.2 + 0.13
trans-Pinocarveol	0.2 ± 0.11	0.2 ± 0.04
Pinocarvone	**0.1 ± 0.01**	**0.8 ± 0.43**[c]
α-Terpineol	0.8 ± 0.76	0.8 ± 0.85
Borneol	**trace**	**0.7 ± 0.44**[c]
Verbenone	**0.1 ± 0.04**	**4.2 ± 1.67**[e]
Piperitone	**trace**	**0.1 ± 0.03**[e]
Campholenaldehyde	**trace**	**0.2 ± 0.05**[e]
Myrtenol	0.1 ± 0.11	0.4 ± 0.12
***trans*-Myrtanol**	**0.0 ± 0.03**	**1.3 ± 0.33**[e]
***Cis*-Myrtanol**	**trace**	**0.8 ± 0.49**[c]
Thymyl methyl ether	0.0 ± 0.03	0.1 ± 0.05
***p*-Cymen-8-ol**	**0.0 ± 0.02**	**0.7 ± 0.39**[c]
5-Oxo-camphor	**0.0 ± 0.04**	**0.2 ± 0.03**[c]

[a] Boldface indicates components that have been found in significantly greater amounts in the laboratory-produced frass than in the control boxes.
[b] Trace: below 0.01.
[c] Highly significantly different from controls (F test, $\alpha = 0.01$).
[d] Significantly different from controls (F test, $\alpha = 0.05$).
[e] Very highly significantly different from controls (F test, $\alpha = 0.001$).

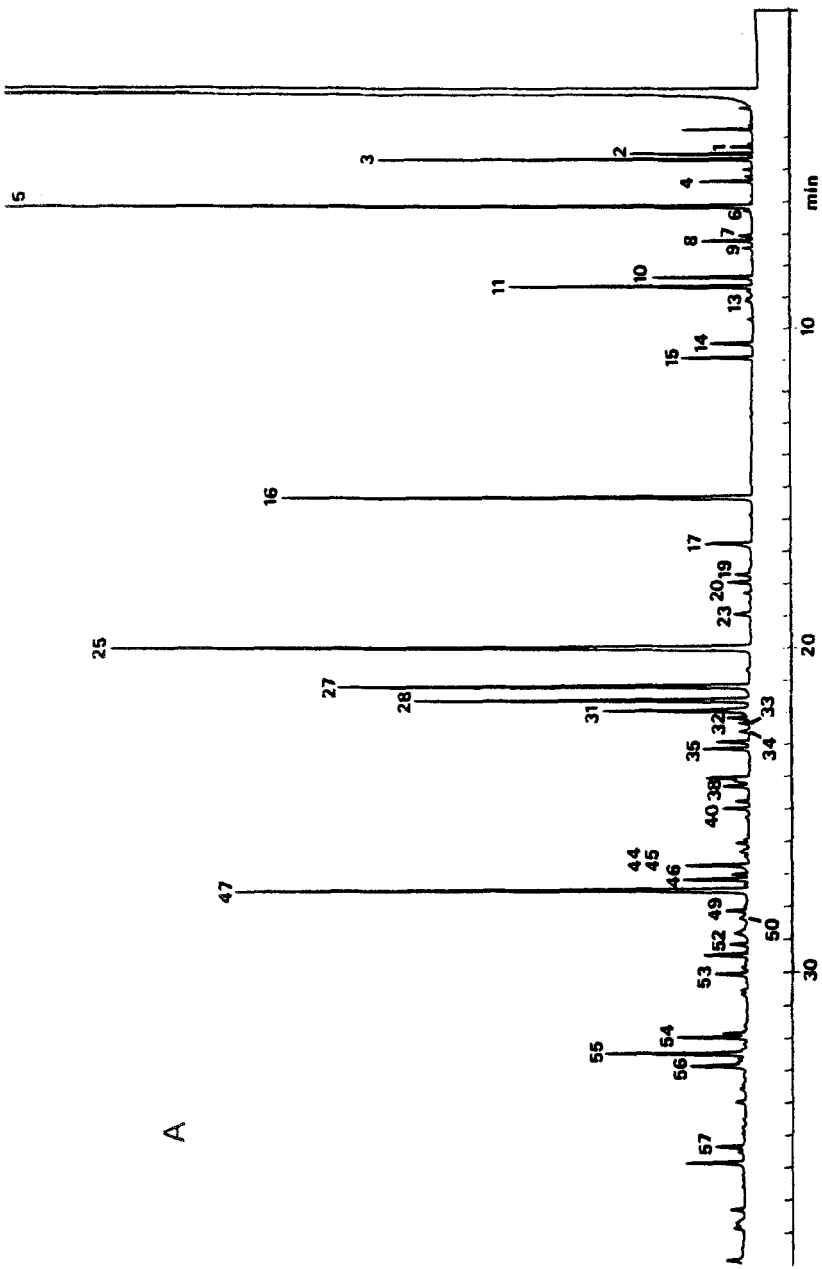
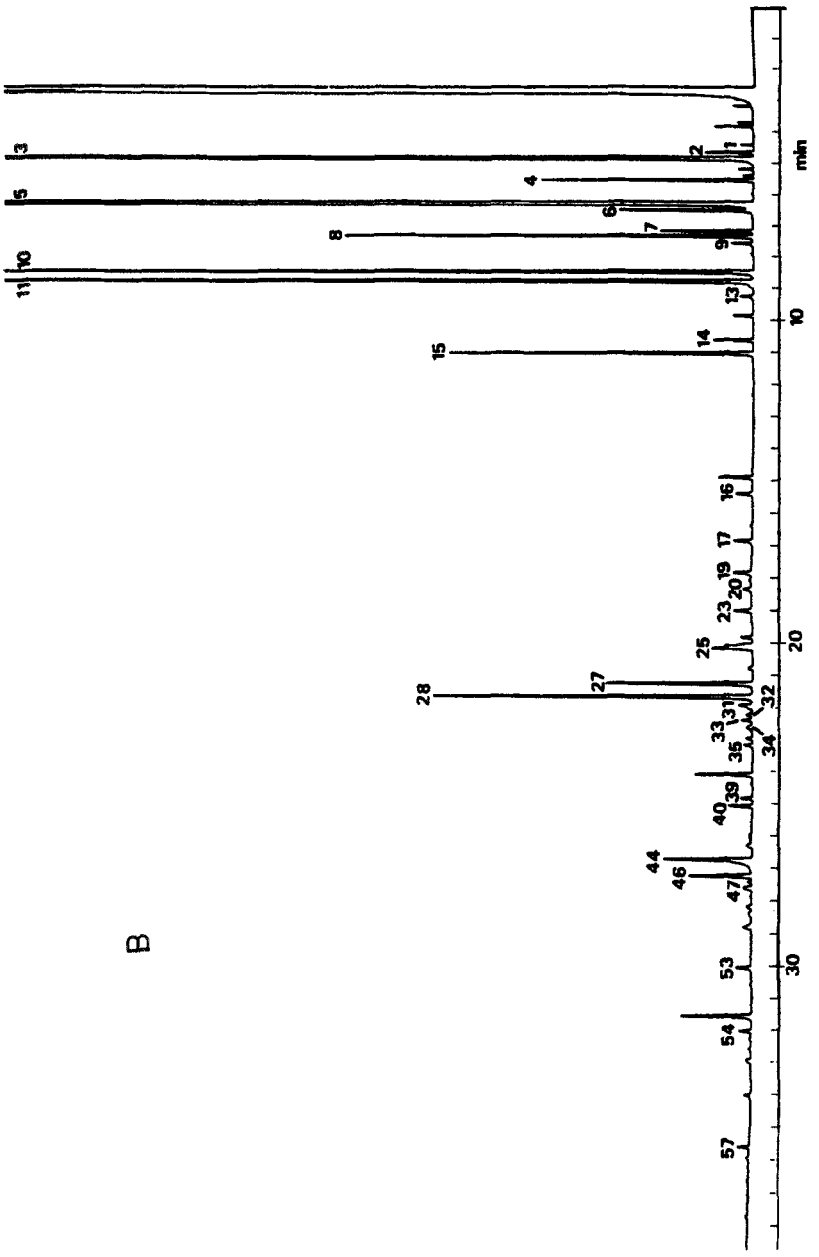

A
min
10
20
30
1
2
3
4
5
6
7
8
9
10
11
13
14
15
16
17
19
20
23
25
27
28
31
32
33
34
35
38
40
44
45
46
47
49
50
52
53
54
55
56
57

FIG. 1. (A) Chromatogram of laboratory-produced larval frass of *Dendroctonus valens* feeding on *Pinus lambertiana* bark. Peak numbers refer to the list below. (B) Chromatogram of the *Pinus lambertiana* bark extract used as control for analyzing the *D. valens* laboratory-produced frass. Peak numbers refer to the list below. (C) Chromatogram of field-collected larval frass of *D. micans* feeding on *Picea abies* bark. Peak numbers refer to the list below.

1. *n*-decane
2. tricyclene
3. α-pinene
4. camphene
5. β-pinene
6. sabinene
7. 3-carene
8. myrcene
9. α-terpinene
10. limonene
11. β-phellandrene
12. 1,8-cineol
13. γ-terpinene
14. *p*-cymene
15. terpinolene
16. (−)-fenchone
17. dimethylstyrene
18. unknown sesquiterpene
19. unknown sesquiterpene
20. unknown sesquiterpene
21. *trans*-4-thujanol
22. unknown sesquiterpene
23. unknown sesquiterpene
24. pinocamphone
25. camphor
26. unknown sesquiterpene
27. (−)-isopinocamphone
28. longifolene
29. unknown sesquiterpene
30. bornyl acetate
31. pinocarvone
32. fenchol
33. thymyl methyl ether
34. *cis*-4-thujanol
35. terpinen-4-ol
36. unknown sesquiterpene
37. unknown sesquiterpene
38. myrtenal
39. acetophenone
40. *trans*-pinocarveol
41. isoborneol
42. 1-allyl-2-methoxy-benzene
43. α-terpinyl acetate
44. α-terpineol
45. borneol
46. unknown sesquiterpene
47. verbenone
48. unknown sesquiterpene
49. piperitone
50. perillaldehyde
51. unknown sesquiterpene
52. α-campholene aldehyde
53. myrtenol
54. p-cymen-8-ol
55. *trans*-myrtanol
56. *cis*-myrtanol
57. 5-oxo-camphor


B
min
10
20
30
1
2
3
4
5
6
7
8
9
10
11
13
14
15
16
17
19
20
23
25
27
28
30
32
33
34
35
39
40
44
46
47
53
54
57

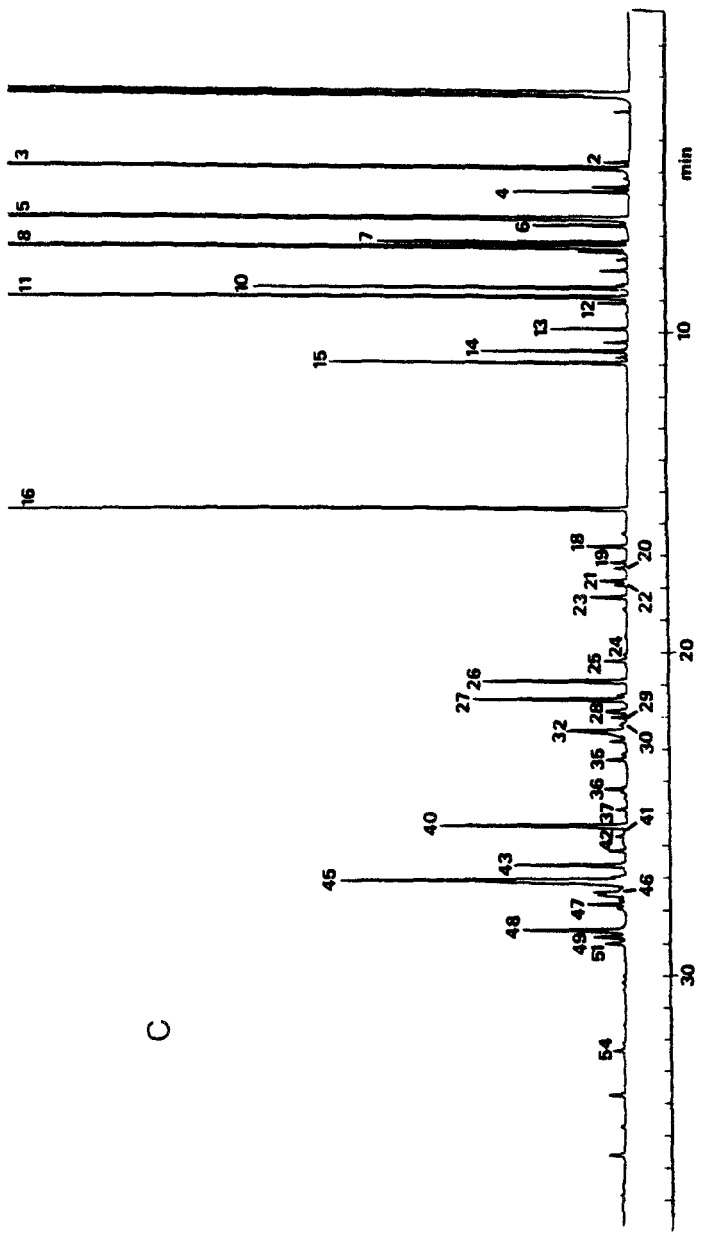


FIG. 1. Continued.

which ones were present in significantly greater amounts in the oviposition boxes, i.e., were generated by the *Dendroctonus* larvae (boldface type in Table 1 and 2).

One major volatile compound, (−)-fenchone, was produced by the larvae of both *Dendroctonus* species or possibly by associated microorganisms. In addition, *D. micans* produced significant amounts of pinocamphone (Table 1), while *D. valens* produced significant amounts of camphor and verbenone, as well as smaller amounts of dimethylstyrene, *cis*-4-thujanol, fenchol, terpinene-4-ol, myrtenal, pinocarvone, borneol, piperitone, campholenaldehyde, *trans*-myrtanol, *cis*-myrtanol, *p*-cymen-8-ol and 5-oxo-camphor (Table 2).

A comparison of the results obtained for both bark beetle species is given

TABLE 3. COMPARISON OF RELATIVE CONCENTRATIONS OF LABORATORY-PRODUCED FRASS *Dendroctonus micans* AND *D. valens*[a]

Compounds	Percentages of total amount of oxidized monoterpenes	
	D. micans (mean ± SD) (n = 2)	*D. valens* (mean ± SD) (n = 4)
(−)-Fenchone	**9.6 ± 1.02**	**15.3 ± 3.20**
Pinocamphone	**15.4 ± 0.24**	0
Camphor	15.4 ± 0.24	**23.1 ± 3.69**
(−)-Isopinocamphone	35.3 ± 3.41	9.7 ± 4.59
cis-4-Thujanol	0	**0.7 ± 0.50**
Fenchol	0	**1.8 ± 1.27**
Terpinen-4-ol	2.7 ± 0.46	**2.7 ± 1.15**
Myrtenal	0	**1.5 ± 0.86**
trans-Pinocarveol	3.00 ± 0.22	1.0 ± 0.22
Pinocarvone	0	**3.5 ± 1.86**
α-Terpineol	0	3.4 ± 3.72
Borneol	0	**2.7 ± 1.44**
Verbenone	0	**18.7 ± 6.42**
Piperitone	4.5 ± 0.29	**0.6 ± 0.10**
Campholenaldehyde	0	**0.6 ± 0.12**
Myrtenol	0	1.6 ± 0.59
trans-Myrtanol	0	**5.6 ± 1.59**
cis-Myrtanol	0	**3.4 ± 1.10**
Thymylmethylether	14.2 ± 1.40	0.2 ± 0.19
p-Cymen-8-ol	0	**2.8 ± 0.66**
5-Oxo-Camphor	0	**1.1 ± 0.39**

[a]Boldface indicates components that have been found in significantly greater amounts in the laboratory-produced frass of either species than in the control boxes.

in Table 3 (expressed as parts of total amounts of oxidized monoterpenes to compensate for differences in the total amounts of volatiles between the samples from both *Dendroctonus* species). In addition to (−)-fenchone, there were only three compounds produced by *D. valens* (camphor, terpinene-4-ol and piperitone) that were also present in the material from *D. micans* oviposition boxes, albeit they were in amounts not significantly greater than in the corresponding control boxes.

Extracts of Field-Collected Larval Frass. The results of GC-MS analyses of field-collected larval frass are given in Table 4 and Figure 1C for *D. micans*. Thirteen compounds (boldface type in Table 4) were also generated by the larvae of either or both species in the oviposition boxes. Among them, only seven

TABLE 4. COMPARATIVE ANALYSIS OF *D. valens* AND *D. micans* FIELD-COLLECTED FRASS

	Percentage of total amount of oxidized monoterpenes		
Compounds	*D. micans* (mean ± SD) (N = 5)	*D. valens* (*P. lambertiana*) (N = 1)	*D. valens* (*P. ponderosa*) (N = 1)
(−)-Fenchone	**37.1 ± 25.20**	**9.5**	**1.09**
Dimethylstyrene	**0**	**trace**[b]	**trace**
Pinocamphone	**0.4 ± 0.38**	**1.3**	**1.1**
trans-4-Thujanol	3.2 ± 3.96	0	0
Camphor	**1.6 ± 1.34**	**13.7**	**1.1**
(−)-Isopinocamphone	5.9 + 2.99	3.0	1.1
Bornylacetate	3.2 ± 5.40	0	0
***cis*-4-Thujanol**	**0**	**0**	**trace**
Fenchol	**4.7 ± 3.72**	**4.3**	**0**
Terpinen-4-ol	**7.8 + 8.94**	**43.5**	**9.8**
Myrtenal	**0**	**trace**	**8.7**
trans-Pinocarveol	4.7 ± 3.55	1.3	0
Pinocarvone	**0**	**1.5**	**4.4**
α-Terpinylacetate	2.4 ± 2.87	0	17.4
α-Terpineol	7.6 ± 5.86	trace	0
Borneol	**18.1 ± 11.25**	**16.7**	**0**
Verbenone	**1.1 ± 0.81**	**0**	**18.5**
Perillaldehyde	0	1.8	26.1
Piperitone	**1.9 ± 1.64**	**0**	**0**
Myrtenol	0	3.3	10.9
***p*-Cymen-8-ol**	**0.3 ± 0.39**	**trace**	**0**

[a] Boldface indicates components that have been found in significantly greater amounts in the laboratory-produced frass of either species than in the control boxes.
[b] Trace: below 0.01.

[(−)-fenchone, pinocamphone, camphor, fenchol, terpinen-4-ol, borneol, and verbenone] are present in the field-collected frass of both species (although verbenone is lacking from the *D. valens* frass from *P. lambertiana* and fenchol and borneol are lacking in the *D. valens* extract from *P. ponderosa*).

Bioassay. The candidate oviposition stimuli proved as effective as *D. micans* larval frass in eliciting oviposition (Figure 2). At the end of the experiment, the stimuli series produced an average (±SE) 26.3 ± 2.58 larvae while the frass series produced 35.1 ± 3.82 larvae, and the control blank series produced 9.6 ± 1.55 larvae. According to a Newman-Keuls multiple range test, oviposition in the stimuli and frass series were not significantly different from each other, but differed from the control blank series ($\alpha = 0.05$). These features appeared early in the process of egg production: daily range tests on the larvae collected in the three series showed a consistantly significant difference between the frass and stimuli series on the one hand, and the control blank series on the other hand, from day 12 onwards ($\alpha = 0.05$).

A binomial test showed an abnormal proportion of sterile boxes in the control blank series ($P = 0.0008$), while there was no significant sterility among the boxes in the two other series (frass: $P = 1.000$; stimuli: $P = 0.9429$).

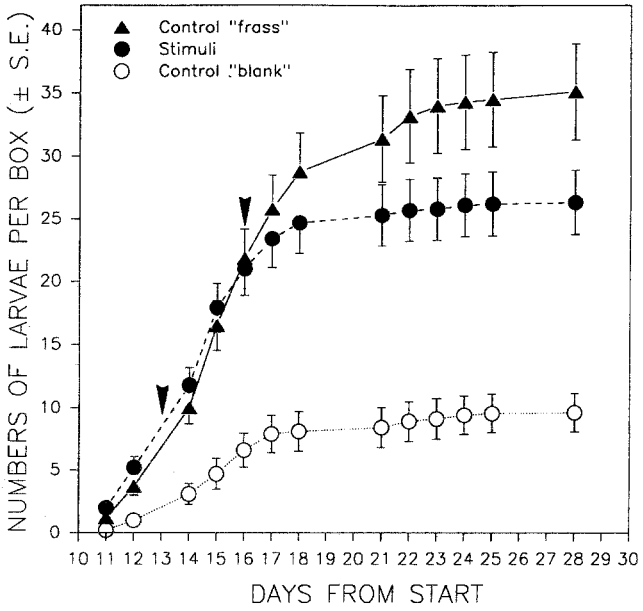


FIG. 2. Oviposition in the bioassay, as reflected by the daily collection of young larvae appearing in three series. Black arrows indicate when the last two fresh 37-mg doses of stimuli have been incorporated into each box of the stimuli series.

DISCUSSION

The different levels of comparisons made during this study allowed us to focus on a small number of candidates likely to act as oviposition stimuli for *R. grandis*.

1. As a first step, the compounds produced by the larvae of both bark beetle species were identified from biologically active material, i.e., laboratory-produced larval frass. Since earlier analyses of volatiles contained in the bark of *Picea abies* had demonstrated that a number of oxidized monoterpenes are already present in the bark of unattacked *Picea abies*, e.g., pinocamphone, isopinocamphone, *trans*- and *cis*-verbenol, verbenone, myrtenol (Heemann and Francke, 1977), it was necessary to distinguish between host components and compounds generated from bark beetle activity. This was achieved by comparing volatiles from oviposition boxes (containing either *D. micans* or *D. valens* larvae) with those from control boxes (containing only vegetal material).

2. By comparing the volatiles from oviposition boxes of both species, some compounds produced by the larvae of both species were shown to be possible oviposition stimuli for *R. grandis*. These compounds were (−)-fenchone, camphor, terpinen-4-ol, and piperitone.

3. By comparing the natural larval frass of both bark beetles, borneol, verbenone, fenchol, and pinocamphone also were taken into account, while the absence of piperitone in the field-collected frass suggested that this component could be excluded.

The results of the bioassays confirm the conclusions drawn from the differential method developed in this work. The synthetic stimuli elicited as much oviposition as the frass, and significantly more than the blank boxes. During a large part of the experiment, the oviposition rates as reflected by the numbers of larvae collected daily in the stimuli and the frass series were also similar (around 3 eggs/female/day, see Figure 2). However, there are important differences in the numbers of eggs laid in these series as compared to more normal situations, i.e., boxes containing also live *D. micans* larvae. Unfortunately no such control could be added to the present experiment, but in earlier experiments involving oviposition boxes with a pair of predators and live larvae of *D. micans* (Baisier, unpublished), we found an average 107.0 larvae collected per box (min. = 70.4; max. = 130.1; $N = 73$), which suggests that frass or synthetic stimuli elicit only about one third of the oviposition expected from more natural situations. One aspect of these differences lies in higher oviposition rates in boxes containing prey larvae: up to 7 or 8 eggs/female/day (Baisier, unpublished), instead of about three with frass or stimuli. It has been argued (Baisier et al., 1988) that the differences between frass and live larvae as sources of stimuli might be attributed to faster depletion of the stimuli from the frass. This argument does not seem to hold in the case of the synthetic chemicals,

which were introduced gradually into the boxes, even at periods when the numbers of larvae collected had started to decline (see Figure 2). There is a possibility that some active components are still missing from the mixture or that the stimuli were too concentrated in the experiment, inhibiting further oviposition. Finally, it is possible that the mixture tested includes too many candidate stimuli, among which are some antagonist or indifferent compounds. Focusing on sorting out the respective activities of these seven components will be the subject of further experiments.

Acknowledgments—Sincere thanks are due to J.M. Pasteels (Université Libre de Bruxelles) and to A. Foggo and T. Wyatt (University of Oxford) for their comments on the manuscript, to K. Hobson and M. Whitmore (University of California at Berkeley) for their help in the field and for fruitful discussions. This work was partly supported by EEC grant MA1B-0019-B (Programme "Wood as a Renewable Raw Material").

REFERENCES

BAISIER, M., and GRÉGOIRE, J.-C. 1988. Factors influencing oviposition in *Rhizophagus grandis* (Coleoptera: Rhizophagidae), specific predator of the bark beetle *Dendroctonus micans* (Coleoptera: Scolytidae). *Med. Fac. Landbouww. Rijksuniv. Gent* 53(3a):1159–1167.

BAISIER, M., GRÉGOIRE, J.-C., DELINTE, K., and BONNARD, O. 1988. The role of spruce monoterpene derivatives as oviposition stimuli for *Rhizophagus grandis*, a predator of the bark beetle, *Dendroctonus micans*, pp. 359–368, *in* W.J. Mattson, J. Lévieux, and C. Bernard-Dagan (eds.). Mechanisms of Woody Plant Defense Against Insects, Springer-Verlag, New York.

BECKMANN, S., and METZGER, R. 1956. Über sterisch selektive Reduktion von Ketonen der Campherreihe mit Lithiumaluminiumhydrid. *Chem. Ber.* 89:2738–2742.

BERGMILLER, F. 1903. *Dendroctonus micans* und *Rhizophagus grandis*. *Zentralbl. Ges. Forstw.*, 29:527–534.

DAGNELIE, P. 1970. Théorie et méthodes statistiques, Vol. II. Duculot, Gembloux.

FRANCKE-GROSMANN, H. 1954. Population-dynamische Faktoren bei der Massenvermehrung des *Dendroctonus micans* Kug. an der Sitkafichte in Schleswig-Holstein. *Verh. Dtsch. Ges. Angew. Entomol.* 00:108–117.

GRÉGOIRE, J.-C. 1988. The greater European spruce beetle, *Dendroctonus micans* (Kug.), pp. 455–478, *in* A.A. Berryman (ed.). Population Dynamics of Forest Insects, Plenum Press, New York.

GRÉGOIRE, J.-C., MERLIN, J., PASTEELS, J.M., JAFFUEL, R., VOULAND, G., and SCHVESTER, D. 1984. Mass-rearings and releases of *Rhizophagus grandis*, pp. 122–128, *in* J.-C. Grégoire and J.M. Pasteels (eds.). Proceedings of the EEC Seminar on the Biological Control of Bark Beetles (*Dendroctonus micans*), Brussels, October 3–4, 1984.

HEEMANN, V., and FRANCKE, W. 1977. Gaschromatographisch-massenspectrometrische Untersuchungen der flüchtigen Rindeninhaltsstoffe von *Picea abies* (L.) Karst. *Planta Med.* 32(4):342–346.

KING, C.J., and EVANS, H.F. 1984. The rearing of *Rhizophagus grandis* and its release against *Dendroctonus micans* in the United Kingdom, pp. 87–97, *in* J.-C. Grégoire and J.M. Pasteels (eds.). Proceedings of the EEC Seminar on the Biological Control of Bark Beetles (*Dendroctonus micans*), Brussels, October 3–4, 1984.

KOBAKHIDZE, D.N. 1965. Some results and prospects of the utilization of beneficial entomophagous insects in the control of insect pests in Georgian SSR (USSR). *Entomophaga* 10(4):323–330.

KÖNIG, W.A., KREBBER, R., and MISCHNICK, P. 1989. Cyclodextrines as chiral stationary phases in capillary gas chromatography. J. HRC 12:732–738.

MILLER, M.C., MOSER, J.C., MCGREGOR, M., GRÉGOIRE , J.-C., BAISIER, M., DAHLSTEN, D.L., and WERNER, R.A. 1987. The potential for biological control of native North American bark beetles (Coleoptera: Scolytidae). *Ann. Entomol. Soc. Am.* 80(3):417–428.

MOSER, J.C. 1989. Inoculative release of an exotic predator for the biological control of the black turpentine beetle, pp. 189–200, *in* D.L. Kulhavy and M.C. Miller (eds.). Potential for Biological Control of Dendroctonus and Ips Bark Beetles. Stephen F. Austin State University, Nacogdoches, Texas, 255 pp.

SMITH, R.H. 1971. Red turpentine Beetle. USDA Forest Service Forest Pest Leaflet.

STOCK, M.W., GRÉGOIRE, J.-C., and FURNISS, M.M. 1987. Electrophoretic comparison of European *Dendroctonus micans* and ten North American *Dendroctonus* species (Coleoptera: Scolytidae). *Pan-Pac. Entomol.* 63(4): 353–357.

ZWEIFEL, G., and BROWN, H.C. 1964. Hydroboration of terpenes. II. The hydroboration of α- and β-pinene. The absolute configuration of the dialkylborane from the hydroboration of α-pinene. *J. Am. Chem. Soc.* 86:393–397.

Journal of Chemical Ecology, Vol. 17, No. 10, 1991

ALLELOCHEMICALS PRODUCED DURING GLUCOSINOLATE DEGRADATION IN SOIL

PAUL D. BROWN, MATTHEW J. MORRA,*
JOSEPH P. McCAFFREY, DICK L. AULD,
and LIVY WILLIAMS, III

Department of Plant, Soil, and Entomological Sciences
University of Idaho
Moscow, Idaho 83843

(Received March 25, 1991; accepted June 25, 1991)

Abstract—A variety of plant pests are suppressed by the incorporation of cruciferous plant material into soil. Although this effect is attributed to decomposition of glucosinolates into toxic products, little is known concerning glucosinolate degradation in the soil environment. Arenas (30 × 18 × 8 cm) that contained soil amended with 30 g defatted winter rapeseed meal (*Brassica napus* L.)/kg soil on one half and unamended soil on the other were constructed. Isothiocyanate concentrations in the soil were measured using infrared analysis of CCl_4 extracts, and ionic thiocyanate (SCN^-) using ion chromatography on aqueous extracts. Quantities were monitored during a 100-hr time period in conjunction with a wireworm bioassay. Isothiocyanate production reached a maximum of 301 nmol/g soil at 2 hr, but decreased by 90% within 24 hr. Production of SCN^- reached a maximum of 180 nmol/g soil at 8 hr but persisted longer than isothiocyanate. Separate late instar wireworms (*Limonius infuscatus* Mots.) were repelled by the presence of rapeseed meal in less than 24 hr even though the meal was shown in separate experiments not to be toxic. We propose that rapidly produced isothiocyanates are responsible for this repellency, but other products such as SCN^- may play a role.

Key Words—Rapeseed, *Brassica* spp., isothiocyanate, thiocyanate, defatted

*To whom correspondence should be addressed.

0098-0331/91/1000-2021$06.50/0

seed meal, allelochemicals, *Limonius infuscatus*, wireworms, Coleoptera, Elateridae.

INTRODUCTION

Glucosinolates are organic anions possessing a β-D-thioglucose moiety, a sulfonated oxime, and any one of a variety of aliphatic or aromatic R groups (Figure 1). Nearly 100 different glucosinolates have been isolated or identified from degradative products (Chew, 1988). They are produced exclusively in dicotyledonous plants and are most common in members of the order Capparales. Those plants with the highest concentrations are in the families Resedaceae, Capparidaceae, and Brassicaceae (Fenwick et al., 1983).

Glucosinolates themselves possess limited biological activity. However, enzymatic degradation by thioglucoside glucohydrolase (EC 3.2.3.1) results in the formation of a number of allelochemicals (Figure 1). Glucosinolate chemistry and degradation pathways in plant tissues have been reviewed (Chew, 1988; Röbbelen and Thies, 1980; Larsen, 1981; Björkman, 1976; Kjaer, 1976).

Interest in glucosinolates and the associated degradation products has been generated because of the possibility of using plant residues as a substitute for synthetic organic pesticides. Cruciferous plant tissues or tissue extracts have

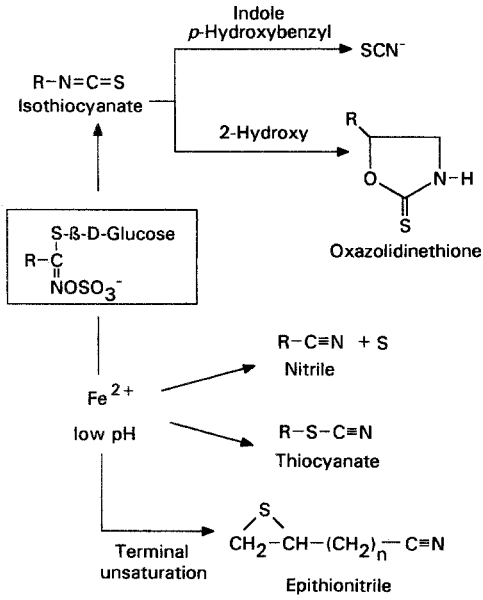


FIG. 1. Glucosinolate degradation products.

been reported to be phytotoxic (Jiménez-Osornio and Gliessman, 1987; Fenwick et al., 1983) and insecticidal (Lichtenstein et al., 1964). The most thoroughly described control of a plant pathogen is for *Aphanomyces* root rot of peas, in which a variety of cruciferous plant amendments applied to infested soil reduced either the level of *Aphanomyces euteiches* or the severity of the disease symptoms (Papavizas, 1966; Papavizas and Lewis, 1971; Lewis and Papavizas, 1971; Chan and Close, 1987). These effects are generally thought to result from glucosinolate degradation products. However, conflicting or inconsistent results with respect to pest inhibition have been obtained as a result of what we believe is an incomplete understanding of glucosinolate degradation pathways in soil (Parke and Rand, 1989; Davis, 1988; Waddington, 1978; Papavizas and Lewis, 1971).

No studies identifying products specific to glucosinolate degradation in soils have been reported. The role of glucosinolate degradation products and allelochemic suppression of pathogens or other soil-borne plant pests has, therefore, not been established. Our objective was to quantify glucosinolate degradation products occurring in soil amended with defatted rapeseed meal (RSM) in combination with a wireworm bioassay.

METHODS AND MATERIALS

Defatted Rapeseed Meal. Mature seed was harvested from the Dwarf Essex cultivar of winter rapeseed (*Brassica napus* L.) and the oil extracted using a CeCoCo experimental press, the design of which has been reported (Peterson et al., 1983). Glucosinolate concentrations in the RSM (Table 1) were determined using a modified procedure of Daun and McGregor (1983). Samples of

TABLE 1. GLUCOSINOLATE CONCENTRATIONS IN DEFATTED *B. napus* MEAL USED IN SOIL AMENDMENT EXPERIMENTS

Glucosinolate		Amount
Semisystematic name	Trivial name	(μmol/g meal)
2-Hydroxy-3-butenyl	Progoitrin	87.8
3-Butenyl	Gluconapin	41.8
4-Pentenyl	Glucobrassicanapin	11.7
Indole[a]	Glucobrassicin Neoglucobrassicin	7.6
2-Hydroxy-4-pentenyl	Napoleiferin	6.5

[a] Concentration determined from SCN^- formed by enzymatic degradation. Several different indole glucosinolates have been identified in rapeseed meal (Daun, 1986; Sang et al., 1984).

RSM (100 mg) were placed in 15 × 100-mm test tubes and heated to 100°C. After temperature equilibration, 6 ml of boiling water was added to denature endogenous thioglucoside hydrolase and extract glucosinolates. One micromole of benzyl glucosinolate was added to each sample as an internal standard. The samples were vortexed, 200 μl of a 1:1 (v/v) mixture of 0.6 M lead acetate and 0.6 M barium acetate was added, and the samples were vortexed again. The samples were centrifuged at 2000 *g* for 10 min, and the supernatant was poured onto diethylaminoethyl (DEAE) Sephadex A-25 anion exchange columns. The columns were washed with 3 ml of 4 M acetic acid and twice with 3 ml of distilled water and 3 ml of 0.02 M pyridine–acetate (1:3.2, v/v pyridine to glacial acetic acid) to remove neutral compounds. Purified sulfatase (1 ml) was added, and the columns were allowed to stand overnight. The resulting desulfoglucosinolates were eluted into 1.8-ml autosampler vials with 1.5 ml of distilled water, dried at 60°C under a stream of air, and derivatized with trimethylsilane. Samples of 1.5 μl were injected with a Varian model 8000 autosampler into a Varian 3700 gas chromatograph equipped with a FID detector and a 2.4-m × 6.4-mm-OD × 2-mm-ID glass column packed with OV-7 on Chromosorb W (AW-DMCS). Helium was used as a carrier gas at a flow rate of 30 cm/min. Injection port temperature was maintained at 280°C and detector temperature at 310°C. An initial oven temperature of 235°C was held for 5 min, and the temperature was increased at a rate of 3°C/min until a final oven temperature of 265°C was reached. This final temperature was held for 3 min. The glucosinolate peaks were quantified with a Hewlett-Packard 3390A integrator using the peak area of benzyl glucosinolate as the internal standard.

Indole glucosinolates were estimated by measuring SCN^- production in a water–RSM slurry. Indole and *p*-hydroxybenzyl glucosinolates yield inherently unstable isothiocyanates that spontaneously degrade to form SCN^-, thus providing the basis for the assay (McGregor, 1991). Our assay included only indole glucosinolates, because *p*-hydroxybenzyl glucosinolates require a basic pH for SCN^- formation and are not known to be present in RSM (Fenwick et al., 1983). A RSM sample (0.150 g) was placed in a 50-ml flask to which was added 20 ml of 0.0035 M $CaCl_2$ solution. The sample was incubated at 27°C for 20 min (with enzyme) or 1 hr (without enzyme), filtered through Whatman No. 42 filter paper, a 0.45-μm Supor-450 membrane (Gelman Sciences, Ann Arbor, Michigan), and a Maxi-Clean IC-RP cartridge (Alltech Associates, Deerfield, Illinois), and measured for SCN^- by ion chromatography. Omission of the Maxi-Clean filtration step, incubation for longer time periods, elevated pH values, and treatment with and without enzyme resulted in similar amounts of SCN^- production.

Glucosinolate Degradation Products in Soil. Isothiocyanates and ionic thiocyanate (SCN^-) were quantified in RSM-amended soil. We have adapted the method of Ashley and Leigh (1963) for measuring methyl isothiocyanate in

soils to the determination of isothiocyanates produced as a result of glucosinolate degradation. This method was used in combination with the ion chromatographic determination of SCN^- in the following manner. To a polypropylene centrifuge tube containing 6–8 g of soil, 10 ml of 0.005 M $CaCl_2$ and 20 ml of CCl_4 were added. The tube was capped, shaken for 1–2 min, and centrifuged for 10–12 min at 630*g*. The aqueous layer was removed with a syringe and analyzed for SCN^- using ion chromatography (Brown and Morra, 1991). A Dionex 4000i ion chromatograph equipped with an AS5 column, anion micromembrane suppressor, and conductivity detector was used in combination with an eluent containing 4.3 mM $NaHCO_3$, 3.4 mM Na_2CO_3, and 0.8 mM 4-cyanophenol. A second glass syringe was used to remove the bottom layer of CCl_4, and the extract was placed in a 25-ml flask along with 0.5–1.0 g of Na_2SO_4 to dry the sample. The sample was dried for 2–24 hr, filtered through a 0.45-μm syringe filter, and stored at 4°C until analysis by FT-IR. Sample extracts could be stored at least four days before analysis without a loss of isothiocyanates. Infrared analysis was conducted using a Digilab FTS-80 FT-IR spectrometer (Bio-Rad, Cambridge, Massachusetts) equipped with a TGS detector and a 3-mm NaCl cell (Spectra Tech, Stamford, Connecticut). Spectra were recorded in the absorbance mode and signal-averaged for 80 scans. Peak height for isothiocyanate in the soil extracts was compared to a standard curve produced using CCl_4 solutions of allyl isothiocyanate (AITC) (Aldrich, Milwaukee, Wisconsin).

Wireworm Bioassays. Wireworms were field collected in the spring of 1989 from sites in Walla Walla County, southeast Washington, and Umatilla County, northeast Oregon, under nonirrigated spring pea (*Pisum sativum* L.) and fall wheat (*Triticum aestivum* L.) rotations. Holes 6 cm diam. × 15 cm deep were dug at each collection site into which 50 ml of a 1:1 (v/v) mixture of wheat and corn seed was placed to act as a bait. The seed had been soaked in water for 36 hr prior to placement. The hole was then filled with soil to a height approximately 2 cm above the surface of the surrounding soil and covered with a "solar heater" (Toba and Turner, 1983) consisting of a 40-cm^2 black polyethylene sheet stapled to the center of a 1-m^2 transparent polyethylene sheet. A quantity of soil 15 cm diam. × 35 cm deep surrounding the bait was dug after three to five weeks. Soil was sieved through 6.5- and 3.5-mm wire mesh screens to collect the wireworms. The wireworms were late-instar *Limonius infuscatus* Mots. (Coleoptera: Elateridae). Voucher specimens are deposited in the W.F. Barr Entomological Museum, University of Idaho, Moscow, Idaho.

The effects of soil-incorporated RSM on wireworm mortality and behavior were tested in a Latahco silt loam soil (Argiaquic Xeric Argialbolls). The top 25 cm of soil was field-collected, air-dried, and crushed to pass a 2.0-mm sieve. The collected soil contained 17 g total C, 1.5 g total N, 185 g clay, and 745 g silt/kg soil, and had a pH of 6.0. Total C and N were determined by Dumas

combustion (CHN-600 Determinator, Leco Corp., St. Joseph, Michigan), particle-size distribution by the hydrometer method (Gee and Bauder, 1986), and pH by glass electrode (1 : 1 soil to water).

The effects of soil-incorporated RSM on wireworm larval mortality were studied in the laboratory using the following bioassay procedures. Air-dried Latahco soil was mixed with the RSM at a rate of 30 g meal/kg soil. Sufficient water was added to the soil–RSM mixture to bring it to an average moisture content of 16% (w/w), an amount of moisture equivalent to 56% of that contained by the soil at −0.033 MPa. The mixture was placed into 473-ml glass jars, and one wireworm was placed on the soil surface in each of 20 replicates. Twenty wireworms were also tested in the control, which contained unamended soil. The jars were incubated uncovered at 27°C under a 12-hr photoperiod. Each jar was weighed daily and sufficient water to replace evaporative losses was added. Larvae were checked after 17 days and were recorded as alive or dead.

Wireworm behavior in response to the RSM was tested individually in plastic arenas (30 × 18 × 8 cm). One side of the arena was filled with the soil–RSM mixture (30 g meal/kg soil) and the other side with Latahco soil only. The soil in the arenas was adjusted to an average moisture content (w/w) of 18%. A potato section of uniform size was placed at each end of the container at a depth of 2.5 cm. An individual wireworm was placed head downward in the soil at the center of each of 30 replicates, and the containers were covered with Clysar EHC 60 (DuPont) medium-gauge film (moderate O_2 and CO_2 permeability) for moisture retention. The arenas were maintained in the dark at 23°C for 24 or 100 hr at which time the x–y coordinates of each wireworm with respect to the interface of the unamended to the amended soil were determined by destructive sampling.

Soil was sampled from trays prepared identical to those above, but lacking wireworms. After a specific incubation time, one soil sample was removed from the amended and one from the unamended side of each of four replicates. Samples were obtained from the entire vertical profile of the soil using a hollow tube having a 1.6-cm ID. They were immediately placed in a centrifuge tube containing CCl_4 and analyzed for isothiocyanate and SCN^- as previously described. Duplicate soil samples were also removed and soil moisture content was determined by weight loss on drying at 105°C for 24 hr. Soil moisture content values were used to convert isothiocyanate and SCN^- concentrations in wet soil to concentration per unit weight of dry soil.

RESULTS AND DISCUSSION

Isothiocyanate Analysis. Although a method for the indirect determination of allyl isothiocyanate (AITC) in soil has been suggested (Chae and Tabatabai, 1983), it has not been tested with actual soil extracts or plant tissues. We have

instead chosen to use an infrared method originally proposed by Ashley and Leigh (1963) for the measurement of methyl isothiocyanate produced in dithiocarbamate-amended soils. In our experiments a similar infrared analysis technique proved to be a convenient method to identify and quantify total isothiocyanate content. Infrared spectroscopy can be used to measure asymmetrical stretching of the $-N=C=S$ functional group in the 2174–2041 cm^{-1} region (Silverstein and Bassler, 1967) and has the advantage of being both qualitative as well as quantitative. The $-C\equiv N$ functional group of nitriles, epithionitriles, and thiocyanates also absorbs at this frequency; however, confusion between group identification is unlikely because the sharp nitrile peak shape is easily distinguished from that of the strong, broader isothiocyanate peak. Characteristic isothiocyanate peaks were indeed observed for RSM-amended soils, while evidence for nitrile production was not observed under the present conditions (Figures 2 and 3). Preliminary experiments indicate that nitrile production may occur if the soil is waterlogged (unpublished data). Carbon tetrachloride soil extracts from unamended soil exhibited virtually no background absorbance in the desired range (Figures 2 and 3).

Quantification of isothiocyanate concentrations was performed using AITC as a standard to represent the anticipated 3-butenyl and 4-pentenyl isothiocyanates. Additional glucosinolates present in the meal are expected to produce unstable isothiocyanates leading to products other than isothiocyanate (Figure 1).

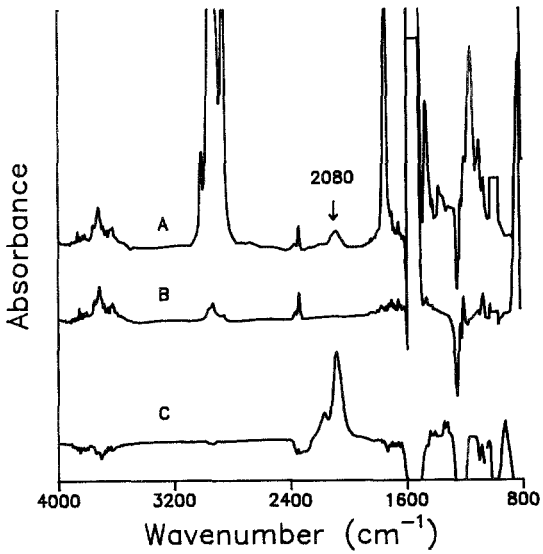


FIG. 2. Infrared spectra of (A) a soil extract from soil amended with defatted seed meal of *B. napus*, (B) a soil extract from unamended soil, and (C) an allyl isothiocyanate solution.

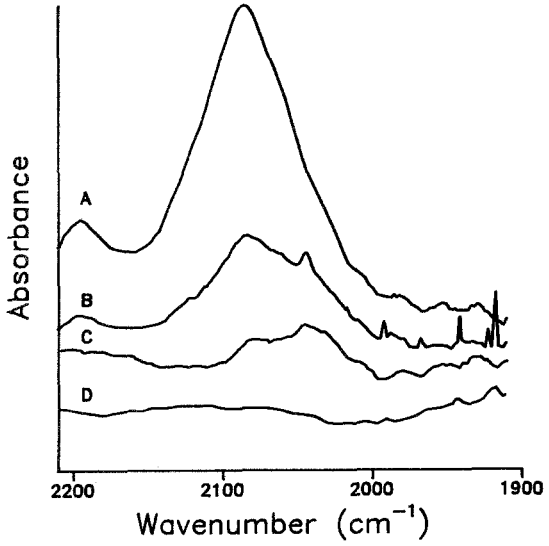


FIG. 3. Infrared spectra of soil extracts from soil amended with *B. napus* defatted seed meal at (A) 2, (B) 6, and (C) 12 hr after amendment. Spectrum D is a soil extract from an unamended soil.

AITC is similar to both 3-butenyl and 4-pentenyl isothiocyanates in that all are aliphatic with terminal unsaturation. Calibration curves were linear ($R^2 > 0.99$) over the concentration range of 1.4–18.0 μg/ml with an estimated limit of detection of 1.0 μg/ml (peak height for 1.4 μg/ml was 3.5X background noise). Various standard isothiocyanate solutions were examined for peak position and absorbance intensity. Intensity varied somewhat with the molecular character of the specific compound (Table 2); however, these effects are insignificant in the present case since aliphatic isothiocyanate intensities were all very similar.

Wireworm Bioassays. Defatted rapeseed meal amended to soil at the rate of 30 g/kg had little effect on wireworm survival as determined 17 days after wireworm introduction. Larval survival in the amended soil was 95% and in the unamended soil treatments 100%. Larval burrows were apparent in all containers except for one amended replicate in which the larva died, suggesting that death occurred soon after introduction into the jar.

In the behavioral test, wireworms responded negatively (G test, $df = 1$, $P < 0.01$) (Sokal and Rohlf, 1969) to defatted rapeseed meal when amended to soil at the same rate used in mortality studies (Figure 4). The response was rapid, with no difference in wireworm behavior between the 24- and 100-hr incubations. This is consistent with the time course for the production of isothiocyanates and SCN^- in which both compounds were produced immediately

TABLE 2. PEAK POSITION AND RELATIVE HEIGHT OF REPRESENTATIVE ISOTHIOCYANATES

Isothiocyanate	Peak position (wavenumber, cm^{-1})	Relative height[a]
Methyl	2105	0.99
Allyl	2088	1.00
Benzyl	2082	1.20
Propyl	2076, 2095[b]	0.98
Phenyl	2055	1.24
RSM	2080, 2046[c]	

[a] Peak heights relative to allyl isothiocyanate equal 1.00.
[b] Peak at 2076 cm^{-1} is dominant and was used for peak height comparison.
[c] Peak at 2080 cm^{-1} is dominant.

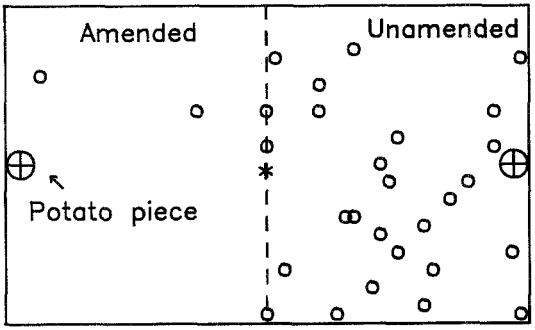


FIG. 4. Composited top view of wireworm locations in arenas 24 hr after amendment of soil with defatted seed meal of *B. napus*. Asterisk represents wireworm placement at bioassay initiation, open circles *x*–*y* coordinates of wireworms at bioassay termination, and dotted line the interface of amended and unamended soil.

upon introduction of water to the system (Figure 5). Carbon tetrachloride extracts of RSM showed no absorbance peaks corresponding to those produced in the presence of moisture. These data indicate that thioglucoside glucohydrolase was not inactivated by the pressing procedure used for oil removal. Isothiocyanates were not present in the RSM, and glucosinolates remained intact because water was absent and thus unavailable for glucosinolate hydrolysis. This is confirmed by the fact that addition of enzyme was unnecessary for maximum SCN^- production during the measurement of indole glucosinolates.

Maximum isothiocyanate concentrations of 301 nmol/g soil were measured at 2 hr (Figures 3 and 5). In RSM, substrates for isothiocyanate production

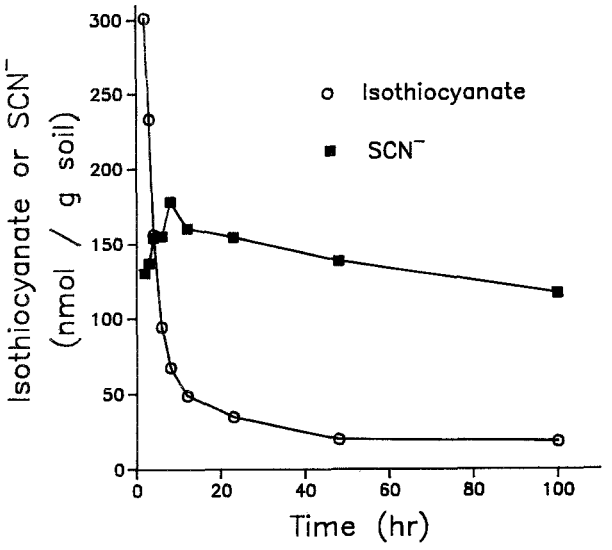


FIG. 5. Isothiocyanate and SCN^- in extracts from soil amended with defatted seed meal of *B. napus*.

include only aliphatic glucosinolates lacking a hydroxyl group at the β position (Figure 1). The amount of potentially available substrate for isothiocyanate production was equivalent to the total concentration of 3-butenyl and 4-pentenyl glucosinolate present in the RSM-amended soil or 1600 nmol/g soil (Table 1). The amount of isothiocyanate actually recovered in soil extracts was 19% of this total, assuming that all production took place in the first 2 hr of incubation.

Our method for isothiocyanate analysis does not measure total production, only the net extractable amount. Losses of isothiocyanate through volatilization and sorption processes are likely. Volatile losses for methyl isothiocyanate have been reported, and an increase in clay, organic matter, pH, or soil water content has been shown to reduce such losses (Ashley et al., 1963; Munnecke and Martin, 1964). Although the exact fate of isothiocyanates in soil is unknown, Lewis and Papavizas (1970) suggested that failure to detect isothiocyanates in headspace samples of soil amended with *Brassica* tissues may have resulted from an interaction with soil constituents. The likelihood of such reactions is increased by the fact that RSM contains high concentrations of protein. Isothiocyanates will react irreversibly with amine and sulfhydryl groups in this protein (Wood, 1975), thus reducing the amount available for extraction from the amended soils. More recently, it has also been shown that allyl isothiocyanate will react irreversibly with and oxidatively cleave disulfide bonds under

relatively mild conditions as occurred in our RSM–soil system (Kawakishi and Kaneko, 1985; Kawakishi et al., 1983).

It has been assumed that isothiocyanates play a key role in the inhibition of plant pests by cruciferous tissues. Pest inhibition by organic isothiocyanates, exclusive of glucosinolates, has been demonstrated in soil. For example, methyl isothiocyanate (MITC) is used directly as a soil fumigant or generated in situ from sodium *N*-methyldithiocarbamate (Miller, 1988). The amount of MITC recommended for soil sterilization can be calculated by assuming a 15.2-cm depth of incorporation, soil bulk density of 1.4 g/cm^3, and 100% conversion of dithiocarbamate to MITC. The calculated values range from 517 to 1294 nmol MITC/g soil depending upon the specific crop and type of control required. Although recommended rates exceed the amount of isothiocyanate measured from RSM degradation (301 nmol/g soil), it is likely that wireworms responded negatively to the concentrations produced in our RSM-amended soils.

Formation of SCN^- was rapid, but production lagged somewhat behind that of isothiocyanate with the maximum of 180 nmol/g soil measured at 8 hr (Figure 5). This amount of SCN^- is equivalent to 73% of the total potentially produced from indole glucosinolate present in the RSM. SCN^- was more persistent than isothiocyanate and 108 nmol/g soil remained after 100 hr. Longer residence times than isothiocyanate are expected, even though the biological decomposition of SCN^- has been documented (Beekhuis, 1975).

Although lower in toxicity than isothiocyanate, SCN^- destabilizes the tertiary structure of proteins through an electrostatic interaction (Voet and Voet, 1990; Wood, 1975). Thiocyanate has insecticidal properties when used in combination with other chemicals, exhibits herbicidal properties such as germination/growth inhibition (Stiehl and Bible, 1985; Ju et al., 1983), defoliant activity, and at higher rates acts as a soil sterilant (Beekhuis, 1975). The impact of SCN^- on wireworm behavior is unknown, but its production in RSM may contribute to repellency.

Other products potentially produced from the degradation of glucosinolates in RSM may also have allelochemic effects. It is possible that volatile products such as carbonyl sulfide (COS), carbon disulfide (CS_2), and hydrogen sulfide (H_2S) may be produced from glucosinolate degradation by way of an organic isothiocyanate intermediate (Bailey et al., 1961; Challenger, 1959). However, these volatiles were not detected in headspace samples above soils amended with *Brassica* tissues (Lewis and Papavizas, 1970). An additional product that would be expected from the degradation of glucosinolates containing a β-OH group is oxazolidinethione (Figure 1). Biological activity appears to be relatively low or unknown, other than well-known goitrogenic effects, but may be a factor in that glucosinolates producing these compounds comprise a major portion of the total glucosinolate content in RSM (Table 1).

These experiments showed that rapeseed meal was not toxic to wireworms

at the rate tested. However, wireworms were repelled by the presence of rapeseed meal in less than 24 hr. Rapidly produced isothiocyanates are probably responsible for this repellency, but other products such as SCN^- may also play a role. In order to fully understand the allelochemic behavior of glucosinolates in soil, it will be necessary to evaluate the biological activity of specific degradation products.

Acknowledgments—Funding for this research was provided by the Institute for Molecular and Agricultural Genetic Engineering and the Idaho Agricultural Experiment Station. Our identification of the wireworms was verified by P.J. Johnson, Department of Entomology, University of Wisconsin.

REFERENCES

ASHLEY, M.G., and LEIGH, B.L. 1963. The action of metham-sodium in soil. I. Development of an analytical method for the determination of methyl isothiocyanate residues in soil. *J. Sci. Food Agric.* 14:148–153.

ASHLEY, M.G., LEIGH, B.L., and LLOYD, L.S. 1963. The action of metham-sodium in soil. II. Factors affecting the removal of methyl isothiocyanate residues. *J. Sci. Food Agric.* 14:153–161.

BAILEY, S.D., BAZINET, M.L., DRISCOLL, J.L., and MCCARTHY, A.I. 1961. The volatile sulfur components of cabbage. *J. Food Sci.* 26:163–170.

BEEKHUIS, H.A. 1975. Technology and industrial applications, pp. 222–255, *in* A.A. Newman (ed.). Chemistry and Biochemistry of Thiocyanic Acid and its Derivatives. Academic Press, London.

BJÖRKMAN, R. 1976. Properties and function of plant myrosinases, pp. 191–205, *in* J.G. Vaughan, A.J. Macleod, and B.M.G. Jones (eds.). The Biology and Chemistry of the Cruciferae. Academic Press, London.

BROWN, P.D., and MORRA, M.J. 1991. Ion chromatographic determination of SCN^- in soils. *J. Agric. Food Chem.* 39:1226–1228.

CHAE, Y.M., and TABATABAI, M.A. 1983. Colorimetric determination of allyl isothiocyanate. *Anal. Lett.* 16:1197–1206.

CHALLENGER, F. 1959. Aspects of the Organic Chemistry of Sulphur. Academic Press, New York.

CHAN, M.K.Y., and CLOSE, R.C. 1987. *Aphanomyces* root rot of peas 3. Control by the use of cruciferous amendments. *N.Z.J. Agric. Res.* 30:225–233.

CHEW, F.S. 1988. Biological effects of glucosinolates, pp. 155–181, *in* H.G. Cutler (ed.). Biologically Active Natural Products: Potential Use in Agriculture. American Chemical Society, Washington, D.C.

DAUN, J.K. 1986. Glucosinolate analysis in rapeseed and canola—an update. *J. Jpn. Oil Chem. Soc.* (*Yakugaku*) 35:426–434.

DAUN, J.K., and MCGREGOR, D.I. 1983. Glucosinolate Analysis of Rapeseed (Canola), Method of the Canadian Grain Commission Grain Research Laboratory. Canadian Grain Commission, Agriculture Canada, Winnipeg.

DAVIS, J.B. 1988. Winter rapeseed (*Brassica napus* L.) with differential levels of glucosinolates evaluated as a green manure crop to suppress *Aphanomyces* root rot of peas (*Pisum sativum* L.). Master's thesis. University of Idaho, Moscow, Idaho.

FENWICK, G.R., HEANEY, R.K., and MULLIN, W.J. 1983. Glucosinolates and their breakdown products in food and food plants. *Crit. Rev. Food Sci. Nutr.* 18:123–201.

GEE, G.W., and BAUDER, J.W. 1986. Particle-size analysis, pp. 383–411, *in* A. Klute (ed.). Methods of Soil Analysis, Agron. Monogr. 9, 2nd ed., Part 1. ASA and SSSA, Madison, Wisconsin.

JIMÉNEZ-OSORNIO, J.J., and GLIESSMAN, S.R. 1987. Allelopathic interference in a wild mustard (*Brassica campestris* L.) and broccoli (*Brassica oleracea* L. var. *italica*) intercrop agroecosystem, pp. 262–288, *in* G.R. Waller (ed.). Allelochemicals: Role in Agriculture and Forestry. American Chemical Society, Washington, D.C.

JU, H.-Y., BIBLE, B.B., and CHONG, C. 1983. Influence of ionic thiocyanate on growth of cabbage, bean, and tobacco. *J. Chem. Ecol.* 9:1255–1262.

KAWAKISHI, S., and KANEKO, T. 1985. Interaction of oxidized glutathione with allyl isothiocyanate. *Phytochemistry* 24:715–718.

KAWAKISHI, S., GOTO, T., and NAMIKI, M. 1983. Oxidative scission of the disulfide bond of cystine and polypeptides by the action of allyl isothiocyanate. *Agric. Biol. Chem.* 47:2071–2076.

KJAER, A. 1976. Glucosinolates in the Cruciferae, pp. 207–219, *in* J.G. Vaughan, A.J. Macleod, and B.M.G. Jones (eds.). The Biology and Chemistry of the Cruciferae. Academic Press, London.

LARSEN, P.O. 1981. Glucosinolates, pp. 501–525, *in* E.E. Conn (ed.). The Biochemistry of Plants, Vol. 7, Secondary Plant Products. Academic Press, New York.

LEWIS, J.A., and PAPAVIZAS, G.C. 1970. Evolution of volatile sulfur-containing compounds from decomposition of crucifers in soil. *Soil Biol. Biochem.* 2:239–246.

LEWIS, J.A., and PAPAVIZAS, G.C. 1971. Effect of sulfur-containing volatile compounds and vapors from cabbage decomposition on *Aphanomyces euteiches*. *Phytopathology* 61:208–214.

LICHTENSTEIN, E.P., MORGAN, D.G., and MUELLER, C.H. 1964. Naturally occurring insecticides in cruciferous crops. *J. Agric. Food Chem.* 12:158–161.

MCGREGOR, D.I. 1991. Determination of total glucosinolate and total indole glucosinolate content of rapeseed/canola using glucose oxidase to measure glucose and ferric nitrate to measure free thiocyanate ion, *in* D.I. McGregor (ed.). Selected Methods for Glucosinolate Analysis, Proceedings of the Oil Crops Network, *Brassica* Sub-Network Workshop held in Shanghai, China, April 21–23, 1990, Agriculture Canada Research Station, Saskatoon, Saskatchewan. In press.

MILLER, D.B. 1988. Determination of methylisothiocyanate by an HPLC trapping and derivatization procedure. *LC-GC* 6:842–846.

MUNNECKE, D.E., and MARTIN, J.P. 1964. Release of methylisothiocyanate from soils treated with Mylone (3,5-dimethyl-tetrahydro-1,3,5,2H-thiadiazine-2-thione). *Phytopathology* 54:941–945.

PAPAVIZAS, G.C. 1966. Suppression of *Aphanomyces* root rot of peas by cruciferous soil amendments. *Phytopathology* 56:1071–1075.

PAPAVIZAS, G.C., and LEWIS, J.A. 1971. Effect of amendments and fungicides on *Aphanomyces* root rot of peas. *Phytopathology* 61:215–220.

PARKE, J.L., and RAND, R.E. 1989. Incorporation of crucifer green manures to reduce *Aphanomyces* root rot of snap beans. *Annu. Rep. Bean Improv. Coop.* 32:105–106.

PETERSON, C.L., AULD, D.L., and THOMPSON, J.C. 1983. Experiments with vegetable oil expression. *Trans. ASAE* 26:1298–1302.

RÖBBELEN, G., and THIES, W. 1980. Variation in rapeseed glucosinolates and breeding for improved meal quality, pp. 285–299, *in* S. Tsunoda, K. Hinata, and C. Gómez-Campo (eds.). Brassica Crops and Wild Allies Biology and Breeding. Japan Scientific Sciences Press, Tokyo.

SANG, J.P., MINCHINTON, I.R., JOHNSTONE, P.K., and TRUSCOTT, R.J.W. 1984. Glucosinolate profiles in the seed, root and leaf tissue of cabbage, mustard, rapeseed, radish and swede. *Can. J. Plant Sci.* 64:77–93.

SILVERSTEIN, R.M., and BASSLER, G.C. 1967. Spectrometric Identification of Organic Compounds. John Wiley & Sons, New York.

SOKAL, R.R., and ROHLF, F.J. 1969. Biometry. W.H. Freeman, San Francisco.

STIEHL, B., and BIBLE, B.B. 1985. Effect of crucifer toxin on seedling growth and germination of crop species. *HortScience* 20:185.

TOBA, H.H., and TURNER, J.E. 1983. Evaluation of baiting techniques for sampling wireworms (Coleoptera: Elateridae) infesting wheat in Washington. *J. Econ. Entomol.* 76:850–855.

VOET, D., and VOET, J.G. 1990. Biochemistry. John Wiley & Sons, New York.

WADDINGTON, J. 1978. Growth of barley, bromegrass and alfalfa in the greenhouse in soil containing rapeseed and wheat residues. *Can. J. Plant Sci.* 58:241–248.

WOOD, J.L. 1975. Biochemistry, pp. 156–221, *in* A.A. Newman (ed.). Chemistry and Biochemistry of Thiocyanic Acid and its Derivatives. Academic Press, London.

Journal of Chemical Ecology, Vol. 17, No. 10, 1991

EVIDENCE FOR PRESENCE AND NATURE OF FEMALE SEX PHEROMONE OF *Brithys crini* Fabricius (LEPIDOPTERA: NOCTUIDAE)

R. KOU,[1,*] Y.S. CHOW,[1] S. TAKAHASHI,[2] and R. YAMAOKA[3]

[1]*Institute of Zoology*
Academia Sinica
Nankang 11529, Taipei, Taiwan

[2]*Pesticide Research Institute*
Faculty of Agriculture, Kyoto University
Kyoto, Japan

[3]*Department of Applied Biology*
Kyoto Institute of Technology
Kyoto, Japan

(Received April 1, 1991; accepted June 26, 1991)

Abstract—The existence of a female sex pheromone of the noctuid moth *Brithys crini* Fabricius was confirmed in both laboratory bioassay and field tests. Crude extracts and airborne volatiles from females were analyzed by gas chromatography and mass spectrometry and the data compared with authentic compounds. The primary sex pheromonal compound was Z11-16:Ald. Scanning electron microscopy showed that the external surface of the sex pheromone gland was covered with folds that might increase the sex pheromone evaporation area.

Key Words—Sex pheromone; (Z)-11-hexadecenal; *Brithys crini*, Lepidoptera, Noctuidae.

INTRODUCTION

The noctuid moth, *Brithys crini* Fabricius, is a serious pest of the white amaryllis (*Zephyranthes candida* Herb.) during winter in the northern part of Taiwan. In nature, there are three to four generations during the year. This insect can have 10–11 generations a year in the laboratory at 27–28°C and a light-

*To whom correspondence should be addressed.

0098-0331/91/1000-2035$06.50/0

dark ratio of 16 : 8 hr. The current method of controlling this pest with pesticides is not always effective. The use of sex pheromones has had some success in combating insect pests (Mitchell, 1981) and may be an attractive supplement to existing control practices. Because the sex pheromone had not been identified, we conducted chemical identification using gland volatiles and extracts. Efficacy of the pheromone was tested both by laboratory bioassays and field tests. External morphology of the gland-bearing ovipositor was studied by scanning electron microscopy.

METHODS AND MATERIALS

Insects. The insects were reared on white amaryllis plants, and females were separated from males at the pupal stage. Adults were kept in separate rooms under a light–dark cycle of 16 : 8 hr.

Scanning Electron Microscopy. The abdominal tips of 3-day-old virgin females were cut off during the calling period. The scanning electron technique (fixation and dehydration) employed was as described by Chow et al. (1976). The dried abdominal tips were coated with gold in a vacuum evaporator (Eiko Engineering IB-2 Ion-coater). Micrographs were taken with a JEOL JSE-15 scanning electron microscope at an acceleration voltage of 15 kV.

Pheromone Extract. Pheromone glands were dissected from the ovipositors of 40 2- to 3-day-old females and immersed in hexane. After 15 min, the supernatant solution was transferred to a small vial and concentrated to 120 μl by letting the samples stand in a fume hood. Gland volatiles were obtained from calling females by putting one end of an open glass tube, 7 mm OD $\times$ 60 mm long and filled with 3 mg conditioned Porapak Q, near the female's sex pheromone gland and aspirating air for approximately 1 hr with a suction machine. The trapped volatiles were rinsed from the Porapak with 300 μl hexane and then concentrated to the desired volume.

Bioassay of Crude Extract. For aggregating effect, 10 3-day-old males, which had been conditioned under continuous light since emergence, were put into a glass jar 2 hr before the light was turned off. The crude extract was serially diluted with hexane to make the amount of the extract in 10 μl of solution 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, and 10^{0} female equivalents (FE). Each solution was applied to the inside wall of a pipet tip, and air was blown out through the pipet with a rubber bulb toward each test individual. Vibration of wings, exposure of the genital claspers, and hovering flight were the criteria of the male's positive sexual response. This experiment had five replicates.

Preliminary Field Trapping of Released Males. Preliminary field trapping with female crude extract was carried out during October 14–16, 1988, in two white amaryllis fields near the Institute of Zoology, Academia Sinica, Taipei.

For comparing the attractive efficacy of different amounts of FE, extracts of 2 FE, 6 FE, 10 FE, and 15 FE were applied on 1-cm × 1-cm filter paper. The filter paper then was hung on the inside and at the top of a wing-shaped sticky trap (sticky surface on inside trap bottom). Blank traps and traps baited with one virgin female per trap were used for comparison. Traps were randomly placed and rotated 5 m apart and 1.5 m above ground. A total of 85 laboratory-reared 3- to 4-day-old unmated males were released at dusk of the first test day.

Duncan's new multiple-range test (Steel and Torrie, 1980) was used to analyze the bioassay and field test results.

Chemical Analysis. Gas chromatography (GC) of extract was performed on three capillary columns: a Varian 3700 GC equipped with flame ionization detector, with a 30-m fused-silica capillary column coated with a 0.25-μm layer of DB-1 phase, a 35-m vitreous silica capillary column coated with a 0.25-μm film of Carbowax 20 M phase, and a 30-m fused-silica capillary column coated with a 0.25-μm film of SP-2340 phase. Helium at a flow rate of 30 ml/min was the carrier gas. Gas chromatographic–mass spectrometric (GC-MS) analysis was conducted as described previously (Kou et al., 1990).

The sex pheromone components were identified by comparison of their mass spectra with that of authentic standards. Subsequently, the identifications were verified by comparison of the GC retention time of the natural product to that of authentic standards on the three capillary columns under the same GC conditions as described above. Standards of (*Z*)-11-hexadecenal (Z11-16:Ald) were purchased from Sigma Chemical Co. (*Z*,*Z*)-7,11-hexadecadien-1-ol (*Z*7,Z11-16:OH) and (*Z*,*E*)-7,11-hexadecadien-1-ol (*Z*7,*E*11-16:OH) were produced by hydrolysis of hexalure (*Z*,*Z*)-7,11- and (*Z*,*E*)-7,11-hexadecadienyl acetate at the Pesticide Research Institute, Faculty of Agriculture, Kyoto University, Kyoto, Japan.

RESULTS AND DISCUSSION

Scanning Electron Microscopy. Scanning electron micrographs of the *B. crini* sex pheromone gland are shown in Figure 1; the whole abdominal tip is puffed like a round ball (Figure 1A). Further investigation showed that the external surface of the sex pheromone gland was covered with folds (Figure 1B), which might function to increase the evaporation area.

Laboratory Bioassay. The results of bioassay with female crude extract are shown in Table 1. Male sexual responses were significantly ($P < 0.05$) increased with the increasing amount of FE. The percentage of sexual response was 0% for the control and 10^{-5} FE, with an average of 4.0%, 34.0%, 62.0%, 82.0%, and 94.0% at 10^{-4} FE, 10^{-3} FE, 10^{-2} FE, 10^{-1} FE, and 1 FE, respectively.

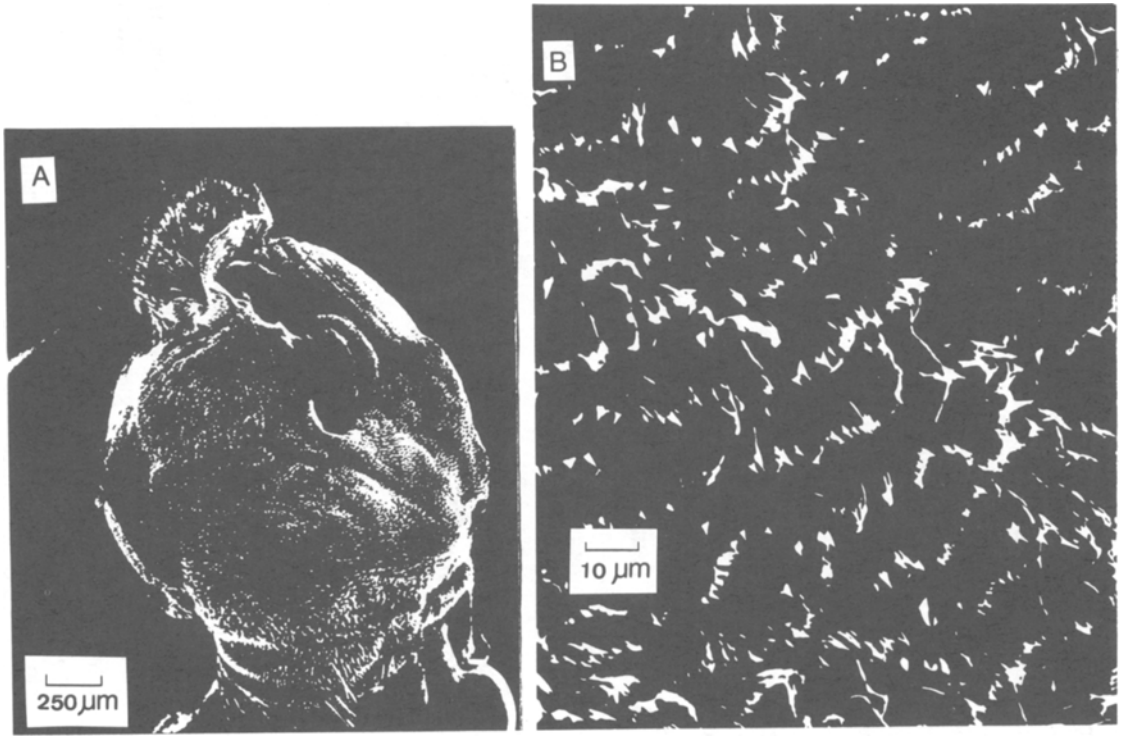


FIG. 1. (A) Scanning electron microscope view of the female *Brithys crini* sex pheromone gland (37X, 15 kV). (B) Scanning electron microscope view of the surface folding of female *Brithys crini* sex pheromone gland (720X, 15 kV).

Preliminary Field Trapping. The results of the field test are shown in Table 2. Totals of 11, 8, 21, and 17 males were trapped at dosages of 2 FE, 6 FE, 10 FE, and 15 FE, respectively. However, no significant difference was found between treatment and control; this might be attributed to the short experimental period and only two replicates being made.

Chemical Analysis. The gas chromatograms of the female crude extract (0.3 FE) and airborne collection are shown in Figure 2. A peak with an R_t value of 17.02 min was produced by both crude extract (Figure 2A) and airborne material (Figure 2B) from calling females (indicated by arrows), so this peak was regarded as the main sex pheromone component. The compound causing this peak was further isolated as shown in Figure 3. Comparison of the R_t values of the female extract, *Z*7,*Z*11-16:OH, *Z*7,*E*11-16:OH, and *Z*11-16:Ald are

TABLE 1. SEXUAL RESPONSE OF MALE *Brithys crini* TO CRUDE FEMALE EXTRACT

Pheromone source	Male response (%)
Control (CH_2Cl_2)	0a[a]
10^{-5} FE	0a
10^{-4} FE	4.0 ± 8.0a
10^{-3} FE	34.0 ± 19.5b
10^{-2} FE	62.0 ± 7.4c
10^{-1} FE	82.0 ± 14.6d
10^{0} FE	94.0 ± 12.0d

[a] Means followed by different letters are significantly different ($P < 0.05$, Duncan's new multiple-range test).

TABLE 2. NUMBER OF MALE *Brithys crini* TRAPPED IN WHITE AMARYLLIS FIELDS DURING OCTOBER 14-16, 1988

	Males trapped	
Pheromone source	Total	Mean (males/trap)
2 FE	11	5.5 ± 2.5a[a]
6 FE	8	4.0 ± 2.0a
10 FE	21	10.5 ± 4.5a
15 FE	17	8.5 ± 2.5a
1 Female	1	0.5 ± 0.5a
Control	0	0.0 ± 0.0a

[a] Means followed by same letters are not significantly different ($P = 0.05$, Duncan's new multiple-age test).

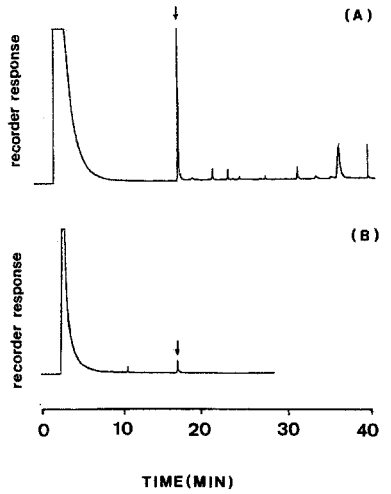


FIG. 2. Gas chromatograms of 0.3 FE gland extract (A) and an airborne collection (B) from calling *Brithys crini* females. The gas chromatography was performed on a 30-m × 0.25-mm DB-1 column that was temperature programmed from 100 to 250°C at 5°C/min.

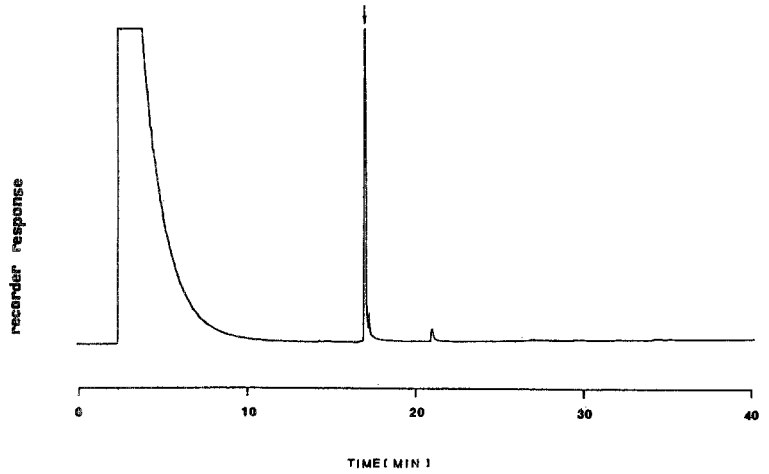


FIG. 3. Gas chromatograms of the supposed female sex pheromone fraction, which had a major peak as indicated by arrow, after elution from the silicic acid column. The gas chromatography was conducted as described for Figure 2.

shown in Table 3. The R_t value of Z11-16:Ald is quite close to that of the isolated peak in the female crude extract in all three columns used. The mass spectrum of the compound corresponding to the above isolated peak was also essentially identical to that of known Z11-16:Ald, as shown in Figures 4 and 5. Z11-16:Ald had been reported to be a sex pheromone in many noctuid moths and some other lepidopterous moths (Takahashi et al., 1979; Tamaki, 1988), so it is not surprising to find it in *B. crini*. With the external standard method

TABLE 3. COMPARISON OF RETENTION TIMES OF *Brithys crini* FEMALE CRUDE EXTRACT AND SOME AUTHENTIC COMPOUNDS

	Column (time in min)		
	DB-1	Carbowax 20 M	SP-2340
Female extract	16.98	8.01	11.26
Z7,Z11-16:OH	17.98	12.42	[a]
Z7,E11-16:OH	17.90	12.32	[a]
Z11-16:Ald	16.97	8.00	11.28

[a] Z7,Z11-16:OH and Z7,E11-16:OH cannot be analyzed by SP-2340 column.

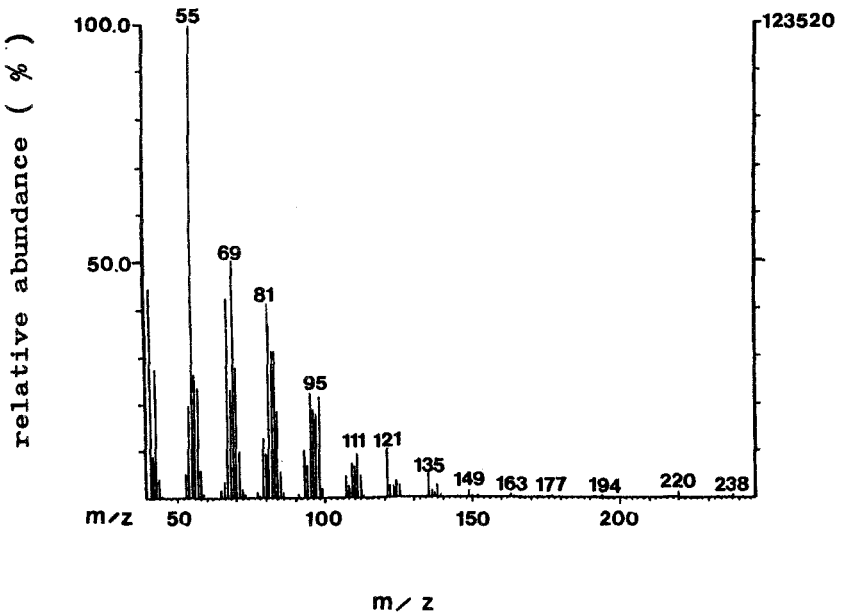


FIG. 4. Electron impact mass spectrum of authentic Z11-16:Ald.

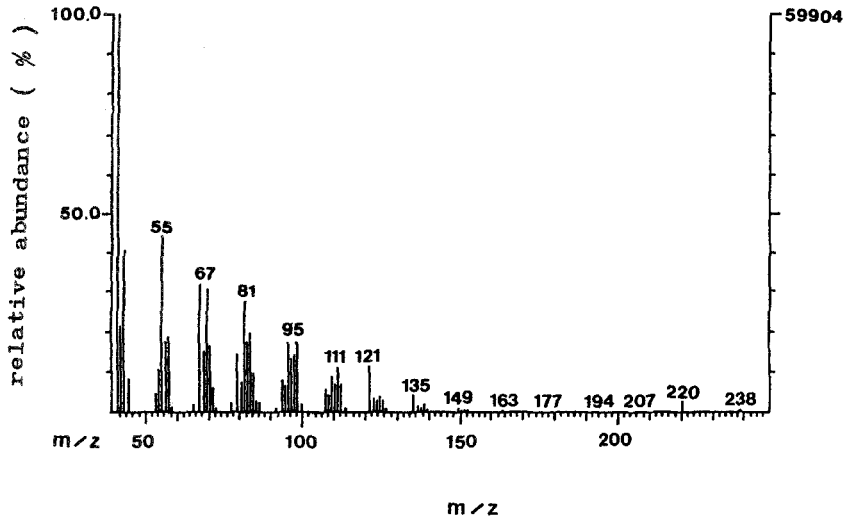


FIG. 5. Electron impact mass spectrum of the major peak in the supposed sex pheromone fraction (Figure 3) of the female extract.

of quantitation, the amount of Z11-16:Ald in the *B. crini* is about 0.6 μg per female. Further study will be conducted to determine the existence of other minor components in the sex pheromone system of this moth.

Acknowledgments—We thank the National Science Council, Taiwan, for financial support and Miss Y.H. Chen for help in typing this manuscript.

REFERENCES

CHOW, Y.S., CHEN, J., and CHOW, L.S.H. 1976. Anatomy of the female sex pheromone gland of the diamondback moth *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). *Int. J. Insect Morphol. Embryol.* 5(3):197-203.

KOU, R., TANG, D.S., CHOW, Y.S., and TSENG, H.K. 1990. Sex pheromone components of female smaller tea tortrix moth, *Adoxophyes* sp. (Lepidoptera: Tortricidae), in Taiwan. *J. Chem. Ecol.* 16(4):1409-1415.

MITCHELL, E.R. (ed.). 1981. Management of Insect Pests with Semiochemicals. Plenum Press, New York.

STEEL, R.G.D., and TORRIE, J.H. 1980. Principles and Procedures of Statistics, 2nd ed. McGraw-Hill, New York.

TAKAHASHI, S., KAWARADANI, M., SATO, Y., and SAKAI, M. 1979. Sex pheromone components of *Leucania separata* Walker and *Leucania loreyi* Duponchel. *Jpn. J. Appl. Entomol. Zool.* 23:78-81.

TAMAKI, Y. 1988. Pheromones of the Lepidoptera, pp. 35-94, *in* E.D. Morgan, and N.B. Mandava (eds.). Handbook of Natural Pesticides, Vol. IV, Pheromones, Part A. CRC Press, Boca Raton, Florida.

Journal of Chemical Ecology, Vol. 17, No. 10, 1991

Announcement

The Natural Products Network for Eastern and Central Africa (NAPRECA) announces

The Fourth NAPRECA Symposium
on Natural Products, December 16–21, 1991
in Addis Ababa, Ethiopia

It is hoped that this symposium will have a strong representation from Africa and also draw renowned scientists from all over the world. A number of leading scientists from many parts of the world have already expressed interest to participate in this symposium. The scientific program is intended to cover all aspects of natural products chemistry, pharmacology, and botany.

The following distinguished scientists have been invited and agreed to deliver lectures. We do hope and expect that this list will grow:

Prof. E. Wenkert (University of California, San Diego, USA), Prof. Bert Fraser-Reid (Duke University, USA), Prof. Atta-Ur-Rahman (HEJ Research Institute of Chemistry, Pakistan), Prof. L. Skattebøl (University of Oslo, Norway), Prof. P.G. Waterman (Strathclyde University, UK), Prof. B. Sener (Turkey), Dr. D.M. Vyas (Squib Co., USA), Prof. Y. Kashman (Tel Aviv University, Israel), Prof. J. Connolly (Glasgow University, UK), Prof. J.R. Cannon (University of Western Australia), Prof. N. Saleh (National Research Center, Egypt).

Oral and poster presentations are invited. The official language will be English.

For registration information please contact: Dr. Ermias Dagne, NAPRECA, Department of Chemistry, Addis Ababa University, P.O. Box 1176, Addis Ababa, Ethiopia.

0098-0331/91/1000-2043$06.50/0

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

EARTHWORM MUCUS STIMULATES OVIPOSITION IN A PREDATORY FLY (DIPTERA: ANTHOMYIIDAE)

DAEL E. MORRIS[1] and KENNETH A. PIVNICK[2,*]

[1]*Department of Entomology*
Royal Ontario Museum
Toronto, Ontario M5S 2C6, Canada

[2]*Agriculture Canada Research Station*
107 Science Place
Saskatoon, Saskatchewan S7N 0X2, Canada

(Received March 4, 1991; accepted July 1, 1991)

Abstract—*Coenosia tigrina* larvae feed on earthworms. We hypothesized that earthworm mucus contains a kairomone that stimulates oviposition behavior in adult *C. tigrina* females, thus minimizing the search area in the soil required for newly eclosed larvae to find earthworms. In bioassays, adult females responded with extension of the ovipositor 25–43% of the time to earthworm-mucus-soaked filter paper disks compared to 6–7% in response to water-soaked disks. Ovipositor extension on mucus-soaked disks was followed by egg-laying 29% of the time and 0% of the time on water-soaked disks. Egg-laying by *C. tigrina* followed a diurnal periodicity, with most eggs laid in the latter half of the photophase even in the absence of earthworm mucus. More eggs were deposited from 1600 to 1800 hr by females given access to earthworm mucus during that period than were deposited by females not given access. There was no difference in the number of eggs deposited from 0600 to 0800 hr, by females given access to earthworm mucus or not. This is a time of day when few eggs are normally laid. This paper is the first report of an earthworm-produced kairomone in an insect-earthworm interaction. The kairomone may have potential for enhancing biological control of the onion maggot, *Delia antiqua*, which is a prey of adult *C. tigrina*.

Key Words—*Coenosia tigrina*, *Eisenia foetida*, *Eisenia rosea*, Lumbricidae, kairomone, oviposition stimulant, diurnal periodicity, oviposition bioassay, onion maggot, *Delia antiqua*, Anthomyiidae, Diptera.

*To whom correspondence should be addressed.

INTRODUCTION

Coenosia tigrina (F.) (Diptera: Anthomyiidae) adults are predators of other flies (Hobby, 1931), including the onion maggot fly, *Delia antiqua* (Meig.) (Anthomyiidae), of which it may have potential as a biological control agent (Perron and LaFrance, 1952; LeRoux and Perron, 1960). The soil-dwelling *C. tigrina* larvae preferentially feed on lumbricid earthworms and require several worms to complete development (Morris and Cloutier, 1987). To date their only recorded natural prey is *Eisenia rosea* (Sav.) (Yahnke and George, 1972). However, they will develop normally in the laboratory while feeding on five different lumbricid species including *E. rosea* and the congeneric, *E. foetida* (Yahnke and George, 1972; Morris and Cloutier, 1987). Larvae hatch several days after eggs are deposited on the soil surface, and although they may survive for up to 12 days in the soil without food (Yahnke and George, 1972), they have limited mobility. Earthworms are also rather sedentary, generally migrating distances on the order of only a few meters each year (Bouché, 1983; Hoogerkamp et al., 1983). Thus, a dense earthworm population is normally a reliable food source for *C. tigrina* larvae.

Typically, female Diptera oviposit near the food of their larvae, thus increasing the probability that the larvae will survive. However, earthworms are negatively phototactic (Edwards and Lofty, 1977), and our observations have indicated that *C. tigrina* oviposits primarily during the day. Therefore, we hypothesized that *C. tigrina* females would oviposit in response to a cue indicating the presence of earthworms. Host-produced chemicals that stimulate increased search and oviposition have been documented in the host-selection process with respect to hymenopteran and dipteran parasitoids (Arthur et al., 1972; Nettles and Burke, 1975; Vinson, 1976; Bouchard and Cloutier, 1984; Nordlund and Lewis, 1985; Vinson, 1986). These chemical stimuli are referred to as kairomones because they mediate an interspecific interaction that benefits the receiver (Nordlund, 1981). An oviposition-enhancing kairomone for *C. tigrina* could improve the biological control potential of this insect.

The hypothesis that a kairomone(s) in earthworm mucus stimulates oviposition in *C. tigrina* was tested in the laboratory. We also documented the daily rhythm of oviposition and determined to what extent the rhythm was influenced by the putative kairomone.

METHODS AND MATERIALS

Effect of Earthworm Mucus on Oviposition Behavior. A laboratory culture of *C. tigrina* was established using eggs from field-collected females. Eggs were placed on wet strips of filter paper and placed in loosely capped glass vials at 25°C. After eclosion, larvae were kept at 20°C and were fed sections of the

earthworm, *E. foetida* once every two to five days. When flies emerged, they were placed individually (to prevent cannibalism) in clear plastic 250-ml vials screened with muslin at one end and placed over a plastic cup containing wet tissue paper, which acted both as a water source and an oviposition substrate. Flies were fed daily with 5–10 pomace flies, *Drosophila melanogaster* L., introduced into each cage and a sucrose solution dabbed on the muslin. Cages were kept in an environmental chamber at 25 ± 1°C with a relative humidity of 65 ± 5% and a 16:8 light–dark photoperiod. To ensure mating, each female was given access to a recently emerged male for three to four days during the preoviposition period (Morris and Cloutier, 1987). To ensure that emerging adults did not come into contact with earthworm products, pupae were placed on moist sterile soil. Outside of the tests, flies were not given access to earthworms and earthworm products.

To obtain earthworm mucus for the bioassay, we placed dry, 6-mm-diam. filter paper disks in clean plastic Petri dishes each containing five *E. foetida* earthworms. Copious amounts of mucus were produced under these conditions. Each *C. tigrina* female was introduced individually into an 8.8-cm-diam. plastic Petri-dish arena at room temperature (20–25°C) containing a mucus-saturated disk. Control females were given a distilled-water-moistened paper disk. Test flies were run simultaneously with control flies, one pair at a time. A total of 18 flies were used in this experiment, and all were used in either the experimental or control trials, never both. A fly was recorded as responding if there was at least one extension of the ovipositor within a 10-min test period. This was often followed by egg deposition, which was also recorded. Defecation by females, which also involves ovipositor extension, was recorded but not counted as a positive response. The bioassay was carried out during the last 3 hr of the photophase as preliminary observations indicated that most egg-laying occurred during this period. The 10-min bioassay period allowed time for seven to nine replicates every day for five consecutive days. Flies were used once every 24 hr, and fresh kairomone sources were used for each fly tested. This experiment was repeated two years later, with a fresh laboratory culture of flies and earthworms; 24 flies were tested.

Influence of Time of Day on Oviposition Response to Earthworm Mucus. Once it was determined that oviposition was affected by a kairomone from earthworms, the influence of the kairomone on the daily rhythm of egg-laying was tested by making earthworm mucus available during either the late afternoon when oviposition normally occurs or early in the morning when oviposition is rare. This experiment was designed to determine the degree to which oviposition is dependent on the earthworm kairomone. Field-collected *C. tigrina* females were kept at 20 ± 1°C, 65 ± 5% relative humidity, at a photoperiod of 16:8 light–dark (0400–2000 hr). For the first five days, July 19–23, 1985, females were maintained in cages as above in the absence of earthworm prod-

ucts [females lived 48 days on average at 20°C (Morris and Cloutier, 1987)]. Following this, two groups were exposed to earthworm mucus within their cages at different times of day by placing mucus-soaked filter paper disks in the oviposition cups during specific periods: one group from 0600 to 0800 hr when there was a minimum of egg-laying (Mucus-AM), and the other from 1600 to 1800 hr when a high frequency of oviposition was expected (Mucus-PM). The control group received no earthworm mucus in their oviposition cups. Oviposition cups were replaced every 2 hr, and the eggs, which were laid between the folds of the wet tissue paper, were counted.

RESULTS

Effect of Earthworm Mucus on Oviposition Behavior. In 18 of 42 trials (43%), flies extended the ovipositor in response to the mucus-saturated disks versus only 3 of 43 (7%) doing so in response to the water-saturated disks. These data indicate that earthworm mucus stimulates ovipositor extension [$\chi^2 = 12.8$, $P < 0.001$, all χ^2 values were calculated using Yates' correction (Wardlaw, 1985)]. Of the 18 trials in which flies extended the ovipositor, oviposition took place in six (33%) within the 10-min test period compared to none among the control flies. The incidence of defecation during the bioassays was not significantly different between the test and control flies ($\chi^2 = 0.36$, $P > 0.05$). When this experiment was repeated, flies extended their ovipositor in 17 of 67 trials (25%) in response to mucus but only in 4 of 67 trials (6%) in response to distilled water ($\chi^2 = 9.54$, $P < 0.005$). Eggs were laid in four of the trials with ovipositor extension in the test group and none in the control. In the two bioassay experiments together, egg deposition was significantly greater in the presence of mucus than in the controls ($\chi^2 = 8.29$, $P < 0.01$). In a concurrent test, we found that mucus from sexually mature, starved *E. foetida*, filtered through a 0.20-μm-pore diameter cellulose membrane filter and diluted to 1.7% by weight in water, also elicited an ovipositor extension response significantly more frequently (25%, $N = 69$) than the control (6%, $N = 67$; $\chi^2 = 8.29$, $P < 0.01$). In four of the trials that elicited ovipositor extension, eggs were also laid.

Influence of Time of Day on Oviposition Response to Earthworm Mucus. This experiment was started with 44 insects, but due to mortality, only 33 insects were used in the data analysis. The majority of eggs were laid in the last 8 hr of the photophase regardless of whether earthworm mucus was present or not: 91, 83, and 66% were laid during this period in the Mucus-PM, control, and Mucus-AM groups, respectively (Figure 1). The increased number of eggs laid during the last 8 hr of the photophase (bar graphs in Figure 1) was due to an increased percentage of females laying eggs (line graphs in Figure 1) and to

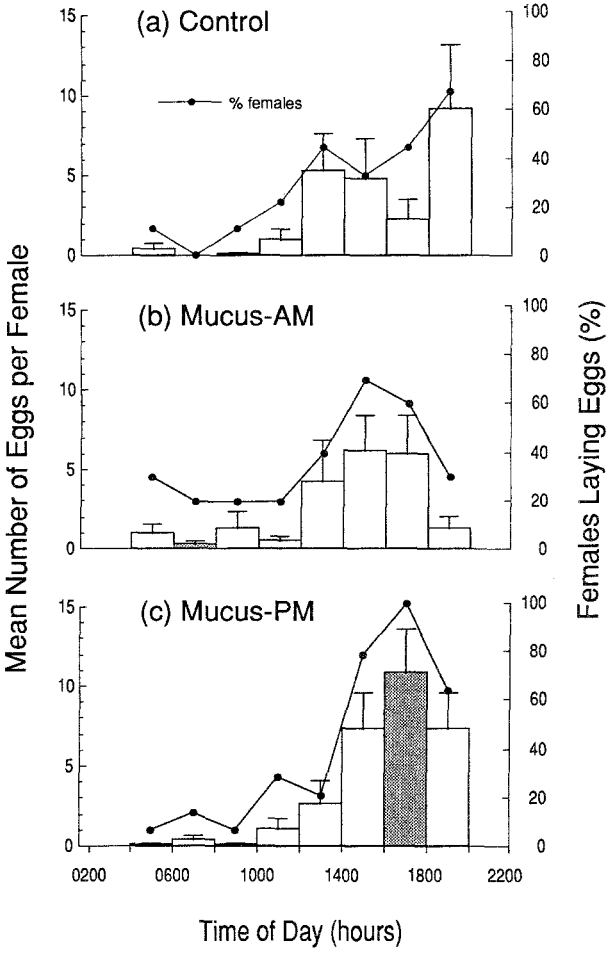


FIG. 1. The mean number of eggs (±SE; bar graphs) laid per female over five days during each 2-hr period of the photophase (0400–2000 hr) and the percentage of females that laid eggs at least once during each 2-hr period over the five days. Earthworm mucus was: (a) not present (control, $N = 9$); (b) present from 0600 to 0800 hr (Mucus-AM, $N = 10$), or (c) present from 1600 to 1800 hr (Mucus-PM, $N = 14$). Shaded bars indicate the period when earthworm mucus was present in (b) and (c). Eggs laid during the scotophase are indicated in the text.

females laying more eggs per bout of oviposition and laying on more days during this period.

The presence of earthworm mucus resulted in a significant increase in the mean number of eggs laid per female over the five days between 1600 and 1800

hr [for Mucus-PM, 10.9 ± 2.7 (±SE), $N = 14$, compared to 4.3 ± 1.4, $N = 19$ for the other groups; $t = 2.33$, $P < 0.05$]. This also was the only 2-hr period when 100% of females in a treatment laid eggs (Figure 1). No such increase was seen when earthworm mucus was presented in the morning (0.3 ± .2, $N = 10$ for Mucus-AM, compared to 0.2 ± .2, $N = 23$ for the others; $t = 0.30$, $P > 0.05$).

There were no significant differences in the mean number of eggs laid per female over five days in the different groups (30.5 ± 4.3, $N = 14$ for Mucus-PM; 26.8 ± 7.0, $N = 10$ for the Mucus-AM; and 27.3 ± 4.6, $N = 9$ for the control), indicating that the presence of earthworm mucus had little effect on the long-term rate of oviposition. Some eggs were laid during the scotophase: 1.1 ± .6, 6.0 ± 2.6 and 3.0 ± 1.9 for Mucus-PM, Mucus-AM, and control groups, respectively.

DISCUSSION

This is the first demonstration of an earthworm-produced kairomone in an insect–earthworm interaction. *C. tigrina* will oviposit without the kairomone. However, its presence increases the likelihood of oviposition and the number of eggs laid at the site and time of contact. Thus, the kairomone should act to concentrate oviposition in proximity to high earthworm populations. Presumably in nature, earthworm mucus is deposited by earthworm species that are periodically active at the soil surface, including the one recorded natural prey, *E. rosea*, and the species we used in our tests, *E. foetida* (Reynolds, 1977).

Egg-laying by both laboratory-reared and field-caught *C. tigrina* occurs primarily in the latter half of the photophase, and it is only during this period that *C. tigrina* is responsive to the earthworm kairomone. Oviposition periodicity is at least partly under endogenous control as it continues unchanged for at least 24 hr under continuous light (Morris, unpublished). Some insect rhythms have been shown to be synchronized with periods of high probability of exposure to a biologically active stimulant (Danislevsky et al., 1970). Oviposition by *C. tigrina* in the late afternoon and evening may be related to favorable environmental conditions, to the periodicity of activity of certain earthworm species, or to other factors.

Many arthropods are predators and parasites of earthworms (Reynolds, 1977). Some of these can be expected to use a similar kairomone. Kairomones from earthworms that mediate prey-finding or recognition by their predators have been demonstrated previously for garter snakes. Nonvolatile components of warm water washes of the earthworm *Lumbricus terrestris* elicit prey attack in newborn garter snakes after contact with the snakes' tongues (Sheffield et al., 1968). Garter snakes are also attracted to volatile components of water

washes of earthworms (Kirschenbaum et al., 1985), and some progress has been made toward the identification of both volatile and nonvolatile biologically active materials (Burghardt et al., 1988).

C. tigrina larvae develop normally when feeding on any of five lumbricid species tested in the laboratory, although at least one of these species was only used as prey if bisected (Yahnke and George, 1972; Morris and Cloutier, 1987). Thus, it is likely that a number of lumbricid species are prey whose mucus contains an oviposition kairomone. If true, this would be advantageous, as different earthworm species occur sympatrically (Satchell, 1980). Therefore, the presence of the mucus of one species at the soil surface could also signal the presence of other earthworm species that are prey for *C. tigrina*.

Management to encourage high earthworm populations in and around onion fields should increase *C. tigrina* densities and hence predation on the onion maggot fly. The kairomone itself, if identified and produced cheaply, could be used to manipulate *C. tigrina* behavior and possibly to achieve similar ends.

Acknowledgments—Thanks are due to J.N. McNeil, Université Laval, Québec, Canada, for advice on experimental methods, and to C. Cloutier, P. Harris, J.J. Soroka, and C.F. Hinks for comments on earlier versions of this manuscript. C. Cloutier, département de biologie, Université Laval, and A.E.R. Downe, Department of Biology, Queen's University, Kingston, Ontario, Canada, provided laboratory facilities for portions of this study. R. Underwood prepared the figure.

REFERENCES

ARTHUR, A.P., HEGDEKAR, B.M., and BATSCH, W.W. 1972. A chemically defined synthetic medium that induces oviposition in the parasite, *Itoplectis conquisitor* (Hymenoptera: Ichneumonidae). *Can. Entomol.* 104:1251–1258.

BOUCHARD, Y., and CLOUTIER, C. 1984. Honeydew as a source of host-searching kairomones for the aphid parasitoid *Aphidius nigripes* (Hymenoptera: Aphidiidae). *Can. J. Zool.* 62:1513–1520.

BOUCHÉ, M.B. 1983. The establishment of earthworm communities, Chapter 38, *in* J.E. Satchell (ed.). Earthworm Ecology. Institute of Terrestrial Ecology, Grange-over-Sands, Cumbria, U.K., 495 pp.

BURGHARDT, G.M., GOSS, S.E., and SCHELL, F.M. 1988. Comparison of earthworm- and fish-derived chemicals eliciting prey attack by garter snakes (*Thamnophis*). *J. Chem. Ecol.* 14:855–881.

DANISLEVSKY, A.S., GORYSHIN, N.I., and TYSHCHENKO, V.P. 1970. Biological rhythms in terrestrial arthropods. *Annu. Rev. Entomol.* 15:201–244.

EDWARDS, C.A., and LOFTY, J.R. 1977. Biology of Earthworms. Chapman and Hall, London, 333 pp.

HOBBY, B.M. 1931. The prey of *Coenosia tigrina* (Fab.). *Proc. Entomol. Soc. London.* 6:13–15.

HOOGERKAMP, M., ROGAAR, H., and EIJSACKERS, H.J.P. 1983. Effect of earthworms on grassland on recently reclaimed polder soils in the Netherlands, Chapter 8, *in* J.E. Satchell (ed.). Earthworm Ecology. Institute of Terrestrial Ecology, Grange-over-Sands, Cumbria, U.K., 495 pp.

KIRSCHENBAUM, D.M. SCHULMAN, N., YAO, P., and HALPERN, N. 1985. Chemoattractant for the

garter snake: Characterization of vomeronasally-mediated response-eliciting components of earthworm wash. III. *Comp. Biochem. Physiol.* 82B:447–453.

LEROUX, E.J., and PERRON, J.P. 1960. Descriptions of immature stages of *Coenosia tigrina* (F.) (Diptera: Anthomyiidae), with notes on hibernation of larvae and predation by adults. *Can. Entomol.* 92:284–296.

MORRIS, D.E., and CLOUTIER, C. 1987. Biology of the predatory fly *Coenosia tigrina* (Fab.) (Diptera: Anthomyiidae): Reproduction, development, and larval feeding on earthworms in the laboratory. *Can. Entomol.* 119:381–393.

NETTLES, W.C., and BURKE, M.L. 1975. A substance of *Heliothis virescens* larvae stimulating larviposition by females of the tachinid, *Archytas marmoratus. J. Insect Physiol.* 21:965–978.

NORDLUND, D.A. 1981. Semiochemicals: a review of the terminology, pp. 13–28, *in* D.A. Nordlund, R.L. Jones, and W.J. Lewis (eds.). Semiochemicals—Their Role in Pest Control. Wiley, New York.

NORDLUND, D.A., and LEWIS W.J. 1985. Response of females of the braconid parasite *Microplitis demolitor* to frass of larvae of the noctuids, *Heliothis zea* and *Trichoplusia ni* and to 13-methylhentriacontane. *Entomol. Exp. Appl.* 38:109–112.

PERRON, J.P., and LAFRANCE, E.J. 1952. A note on a dipterous predator of the onion maggot, *Hylemya antiqua* (Meig.). *Can. Entomol.* 84:112.

REYNOLDS, J.W. 1977. The Earthworms (Lumbricidae and Sparganophilidae) of Ontario. Life Science Miscellaneous Publication, Royal Ontario Museum, Toronto, Canada. 141 pp.

SATCHELL, J.E. 1980. r and K worms: A basis for classifying lumbricid earthworm strategies, pp. 848–864, *in* D.L. Dindal (ed.). Soil Biology as Related to Land Use Practices, Proc. 7th International Soil Zoology Colloquium, EPA, Washington, D.C.

SHEFFIELD, L.P., LAW, J.L., and BURGHARDT, G.M. 1968. On the nature of chemical food sign stimuli for newborn garter snakes. *Commun. Behav. Biol.* 2A:7–12.

VISON, S.B. 1976. Host selection by insect parasitoids. *Annu. Rev. Entomol.* 21:109–132.

VINSON, S.B. 1986. The role of behavioral chemicals for biological control. *Fortschr. Zool.* 32:75–87.

WARDLAW, A.C. 1985. Practical Statistics for Experimental Biologists. Wiley-Interscience, Toronto, Canada, 290 pp.

YAHNKE, W.E., and GEORGE, J.A. 1972. Earthworms as prey for larvae of *Coenosia tigrina* (Fab.) (Diptera: Anthomyiidae). *J. Econ. Entomol.* 65:1476–1477.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

INFLUENCE OF SAGEBRUSH TERPENOIDS ON MULE DEER PREFERENCE

ROBERT O. BRAY,[1] CARL L. WAMBOLT,[1,]* and RICK G. KELSEY[2]

[1]*Animal and Range Sciences Department*
Montana State University
Bozeman, Montana 59717

[2]*Forest Service*
Pacific Northwest Research Station
Corvallis, Oregon 97331

(Received March 25, 1991; accepted July 1, 1991)

Abstract—The effect on mule deer (*Odocoileus hemionus hemionus* Rafinesque) preference of compounds in mountain big sagebrush [*Artemisia tridentata* Nutt. ssp. *vaseyana* (Rydb.) Beetle], Wyoming big sagebrush (*A. t.* ssp. *wyomingensis* Beetle and Young), basin big sagebrush (*A. t.* ssp. *tridentata*), and black sagebrush (*A. nova* Nels.) was compared using a two-choice preference test. Compounds tested included: *p*-cymene, 1,8-cineole, methacrolein (two concentrations), and the nonvolatile crude terpenoid fraction (NVCTF) from each taxon. The compounds were tested by applying them to chopped alfalfa hay at concentrations similar to those found in nature. The intake of the treated hay was compared with that of an untreated control. Eight deer were used as test animals in an 8 × 8 Latin-square design. All compounds tested significantly deterred ingestion ($P < 0.05$). Compound influence on preference, in order of increasing deterrence, was as follows: 50% methacrolein, mountain big sagebrush NVCTF, methacrolein, basin big sagebrush NVCTF, *p*-cymene, Wyoming big sagebrush NVCTF, black sagebrush NVCTF, and 1,8-cineole. Methacrolein appears to be an important preference determinant among big sagebrush subspecies, and *p*-cymene between black sagebrush and big sagebrush. The NVCTFs containing sesquiterpene lactones as one of their constituents were closely related to the preference of all four taxa. Future studies of animal preference for sagebrush should consider all of the potential defensive chemicals in the foliage.

*To whom correspondence should be addressed.

0098-0331/91/1100-2053$06.50/0

Key Words—*Artemisia tridentata*, *Artemisia nova*, *Odocoileus hemionus*, allelochemicals, terpenoids, diet selection, preference test.

INTRODUCTION

The relative palatabilities of various sagebrush (*Artemisia* L.) taxa have been measured in numerous studies (Scholl et al., 1977; Sheehy and Winward, 1981; Welch et al., 1981, 1983; Behan and Welch, 1985; Welch and McArthur, 1986; Personius et al., 1987; Wambolt et al., 1991). Although these studies agreed that a taxon was a desired indicator of preference, they offered no clear explanation of causes for differences in taxon preference. It is desirable for management purposes to know which sagebrush taxa will be preferred by herbivores such as mule deer (*Odocoileus hemionus hemionus* Rafinesque). Therefore, accurate predictions require a better understanding of which plant characteristics influence preference.

Presumably, plant chemistry plays a major role in determining sagebrush palatability and thus preference by herbivores (Rosenthal and Janzen, 1979). Identification of compounds or groups of compounds that influence mule deer preference for sagebrush has not been accomplished. Many studies (Sheehy, 1975; Scholl et al., 1977; Narjisse, 1981; Welch et al., 1983; Behan and Welch, 1985; Personius et al., 1987) have attempted to correlate allelochemicals in sagebrush to palatability, but results have been inconsistent. Most studies have focused on monoterpenes (Welch et al., 1982), which are just one of several groups of compounds occurring in sagebrush foliage. We believe other allelochemicals in sagebrush, alone or in combination with monoterpenes, may determine herbivore preference.

Personius et al. (1987) isolated 31 chemical constituents from forage samples of four sagebrush taxa, mountain big sagebrush [*Artemisia tridentata* ssp. *vaseyana* (Rydb.) Beetle], Wyoming big sagebrush (*A. t.* ssp. *wyomingensis* Beetle and Young), basin big sagebrush (*A. t.* Nutt. ssp. *tridentata*), and black sagebrush (*A. nova* Nels.). Plants were growing together under natural conditions in southwestern Montana and preferentially browsed during winter by mule deer. Personius et al. (1987) related compound concentrations to mule deer preference for the four taxa using a stepwise discriminate analysis. Although they identified probable preference determinants, the deterring effects of the compounds remained conjecture as their individual effects on deer were not tested.

The objective of this study was to determine the individual effect of sagebrush allelochemicals on mule deer forage preference during controlled feeding

trials. The compounds tested were those previously found to be preference determinants (Personius et al., 1987).

MATERIALS AND METHODS

Sample Collection. Sagebrush foliage used to extract nonvolatile crude terpenoid fractions (NVCTFs) was collected between August 13 and September 19, 1986 at the site near Gardiner, Montana, described in detail by McNeal (1984), Personius et al. (1987), Striby et al. (1987), and Wambolt and McNeal (1987). The current year's vegetative leaders were clipped in the manner used by Personius et al. (1987) and Striby et al. (1987), to closely approximate the foliage used by browsing mule deer. Due to the extremely short annual leader growth of black sagebrush, some older growth and reproductive structures were unavoidably collected with the current year's material. Clippings were placed on ice in airtight plastic bags, transported daily to the laboratory, and stored at −23°C until extraction.

Extraction Procedure. Chloroform extractions of NVCTFs were conducted using a procedure similar to Kelsey et al. (1982) and Personius et al. (1987). Modifications were necessary to accommodate larger quantities of material required for a feeding trial. Batches of sagebrush foliage (250 g) were extracted with chloroform (3000 ml) by gently stirring for 5 min. After filtering through a screen and Whatman No. 4 paper, the chloroform was evaporated with vacuum and a 50–55°C water bath.

Volatile compounds were removed from the extract by steam distillation. Approximately 250 ml of extract was redissolved in 250–300 ml of chloroform. Steam was passed through the solution until 1000 ml of water had evaporated from the flask, generating the steam. This left a suspension of nonvolatile compounds in water that settled out upon cooling. The water was poured off and the extract spread in a glass dish (5–7 mm layer) to dry at 60°C for 72 hr. The water layer was washed with chloroform (3 × 100 ml) and discarded. The chloroform was used to redissolve the next batch of extract, from the same taxon, to be distilled.

The mountain big sagebrush nonvolatile extract was solid, whereas the extracts from the other taxa were tarlike. The solid extract was ground to a powder with a mortar and pestle to facilitate weighing and handling. All batches within a taxon were placed in a single container and mixed to ensure uniformity. Volatile compounds tested (1,8-cineole, methacrolein, and *p*-cymene) were purchased commercially, making their extraction unnecessary.

Compound Application. Compounds selected for testing among those identified by Personius et al. (1987) included: 1,8-cineole, methacrolein, *p*-cymene, and the NVCTF (containing sesquiterpene lactones) of each taxon. Volatile

terpenoids were tested at the same concentrations Personius et al. (1987) found in the taxon having the greatest quantity (percent extracted-tissue dry weight) of each compound. The highest concentration of 1,8-cineole (1.10%) was found in mountain big sagebrush. The greatest concentrations of methacrolein (0.24%) and *p*-cymene (0.24%) were found in basin big sagebrush and black sagebrush, respectively.

To understand the effect of compound concentration on preference, we also tested methacrolein at one half its greatest observed concentration. This lower concentration (0.12%) of methacrolein (50% of greatest methacrolein concentration) equaled levels Personius et al. (1987) found in Wyoming big sagebrush.

Nonvolatile extracts were applied to the feed to achieve a 7% concentration. Seventy-five grams of extract was dissolved in 1000 ml of chloroform and poured over 1000 g of chopped alfalfa hay. After thoroughly mixing by hand, the treated hay was spread under a fume hood to dry for 90 min at 22°C and stirred at 30-min intervals. The hay was then spread out, air-dried 12 hr at 22°C, and stored in plastic bags until needed.

Volatile compounds were applied to the feed with a water carrier immediately prior to the start of the preference tests to minimize concentration changes due to evaporation. Twenty milliliters of a water-compound suspension, calculated to deliver the desired concentration, was sprayed onto 250 g of feed. Similarly, 20 ml of untreated water was applied to the control feed so preference would not be biased by water content.

Test Facilities and Animals. The preference tests were conducted between November 1986 and January 1987 at the E.H. Steffen Center, Department of Forestry and Range Management, Washington State University, Pullman, Washington. During the preference tests and the pretest adjustment period, the deer were kept in individual wire mesh pens void of any other forage. One end of each pen opened into the rear of an adjacent wire mesh metabolism cage.

At the front of each cage, a feed and water bunk was divided into thirds with plywood sheets. During a trial, an 8-liter rubber water bucket was placed in one end section, and the treated and control feeds placed in the remaining two sections.

Eight captive deer, five females and three castrated males, were used in the preference tests. Animals included six Rocky Mountain mule deer, one Columbian black-tailed deer (*Odocoileus hemionus columbianus* Richardson), and one hybrid of the two subspecies. Deer were captured as young fawns, bottle-raised at Washington State University, and essentially treated the same since shortly after birth. They were maintained on free-choice diets of alfalfa hay, alfalfa–grain concentrate pellets, and grass growing in the pastures. Free access to water and trace-mineralized salt was provided. The three oldest deer (6.5 years and two at 2.5 years) had been exposed to a variety of diets during previous feeding trials. Some of these trials included terpenoid-containing con-

ifers, but no sagebrush. The remaining deer were 1.5 years old and had no such previous experience. All deer were accustomed to feeding trial protocols.

Experimental Design and Statistical Analysis. An 8 × 8 Latin square design was employed. Seven compounds (methacrolein at two concentrations) were rotated among eight deer during eight time periods. A two-choice preference test (Bell, 1959; Goatcher and Church, 1970) was conducted during each five-day test period. This was followed by a two-day rest when deer were fed only untreated feed to reduce any possible carryover effect of a compound during the next test period.

Due to the volatile nature of 1,8-cineole, *p*-cymene, and especially the highly volatile methacrolein, the daily preference tests were kept to 20 min to minimize any concentration changes. One group of four deer began the test at 1:00 PM, the second group started at 1:30 PM, each following a 5-hr fast. The fast was used because a pretrial test with domestic sheep showed it was necessary to stimulate animal intake immediately upon presentation of the feed. The fast was kept short to ensure selectivity would be expressed by unstarved animals. Preference was expressed as:

$$\text{Preference} = \frac{\text{intake treated feed (g)}}{\text{intake treated feed (g)} + \text{intake control feed (g)}} \times 100$$

A 12-day adaption period preceded the preference tests. The deer were placed in their individual pens and cared for as they were throughout the preference tests. This included a fast period, free access to water and trace-mineralized salt, and high-quality chopped alfalfa hay.

Each deer was given the simultaneous choice between 270 g treated and untreated feed on each day of the preference test. Positions of treated and control feeds in the bunk were switched daily to minimize the effect of habit or position preference. Immediately following each preference test, the deer were given their normal daily ration, at 15% higher than the previous day's intake. The deer had access to their ration until 8:00 or 8:30 AM the following day, i.e., 5 hr prior to the next test.

The black-tailed male was brought into the experiment prior to preference test three to replace a mule deer that became sick and had to be removed. The replacement animal had an 11-day adaption period, but was not tested with two compounds. Therefore, two of 64 cells in the Latin square were lost, and the NVCTF of mountain big sagebrush and the 50% methacrolein were tested with seven of the eight deer.

We analyzed differences in preference among compounds, animals, and test periods using the General Linear Model of SAS (Statistical Analysis Systems, 1985). Significant differences were found when least-squares mean separation was at ($P < 0.05$). We totaled the intakes of treated and control feed for the entire five-day test period to analyze for preference ratios. We used the

preference ratio derived from the square of the sums, rather than the sum of the squares to avoid giving too much weight to days with abnormal intake. This method also accommodated days when neither treated nor control feed was eaten, as happened on two of 310 possible deer test days. On days when neither feed was eaten, the sum of squares method gave a 0:0 preference ratio, which could not be defined. Preference ratios were determined to be different from the theoretical "no-preference" index of 50 with a Student's t test.

RESULTS AND DISCUSSION

Deer preference was significantly different ($P < 0.05$) among the compounds tested. Preference did not differ ($P < 0.05$) among test periods nor among test animals.

All of the preference ratio means were significantly different ($P < 0.05$) from the no-preference index of 50 (Table 1). The larger the mean, or the closer to the no-preference index of 50, the less the compound was discriminated against. All compounds except methacrolein, 50% methacrolein, and the NVCTF of mountain big sagebrush were below Bell's (1959) "rejection threshold" of 20% of intake, and fell within Goatcher and Church's (1970) "strong rejection zone." These three compounds fell within Goatcher and Church's (1970) "moderate rejection zone." Apparently all compounds tested acted as preference deterrents.

TABLE 1. MEAN MULE DEER PREFERENCE[a] FOR CHOPPED ALFALFA HAY TREATED WITH EIGHT COMPOUNDS FOUND IN FOUR SAGEBRUSH TAXA[b]

Compound[c]	Preference
1,8-Cineole	1.60A[d]
Arno NVCTF	11.06B
Artrwy NVCTF	13.21B
p-Cymene	15.09B
Artrtr NVCTF	15.83B
Methacrolein	23.00C
Artrva NVCTF	24.85CD
50% Methacrolein	29.87D

[a] Preference = [intake treated feed (g)/intake treated feed (g) + intake control feed (g)] × 100. An index of 50 would indicate an equal quantity of treated and untreated feed was consumed, or no preference was shown.

[b] Arno: *A. nova*, Artrwy: *A. t.* ssp. *wyomingensis*, Artrtr: *A. t.* ssp. *tridentata*, Artrva: *A. t.* ssp. *vaseyana*.

[c] 1,8-Cineole, *p*-cymene, and methacrolein are volatiles.

[d] Means followed by the same letter were not significantly different ($P < 0.05$).

Volatility of compounds had little influence on preference rankings at the concentrations tested (Table 1). Note that volatile and nonvolatile compounds were interspersed throughout the list.

1,8-Cineole ($P < 0.05$) was the strongest preference deterrent. Of the four volatile compounds tested, 1,8-cineole was the most abundant in sagebrush tissue and was applied to the test feed at the highest concentration. It is less volatile than methacrolein, so its concentration on the treated feed should have remained higher during the trial period. However, its vapor concentration immediately above the feed was likely lower than the more volatile compounds. Stepwise discriminate analysis (Personius et al., 1987) had shown it to be a possible preference "attractant," as its concentration among taxa increased with increasing preference by mule deer. Sheehy (1975) also indicated 1,8-cineole may be an attractant. Our study challenges those observations, although we cannot say conclusively it is a deciding preference determinant. Any effects it may have on preference may be masked by one or more of the other compounds in sagebrush.

p-Cymene was applied at the same concentration as methacrolein, yet it had a greater deterrent effect. Methacrolein was a better predictor of preference than *p*-cymene. These statements are not necessarily contradictory. A stronger deterrent may not be a better predictor of preference. Data from Personius (1985) indicated that *p*-cymene was most important as a preference indicator between black sagebrush and the big sagebrush complex. Our results concur that *p*-cymene may be an important preference determinate between the two species.

Methacrolein may be more important as a preference indicator among the three big sagebrush subspecies (Personius et al., 1987). These differences make a direct comparison of methacrolein and *p*-cymene, as preference determinants, difficult. Methacrolein is more volatile than *p*-cymene and 1,8-cineole. This would reduce its effectiveness as a preference deterrent and would reduce its concentration significantly more than the others by the end of each preference test.

Methacrolein is also a severe mucosal tissue irritant (Tatken and Lewis, 1983). When deer stuck their heads into a feed bunk containing feed freshly treated with methacrolein, they typically coughed. Yet, methacrolein and 50% methacrolein were the least deterring of all volatile compounds tested. One deer preferred methacrolein to the control feed on some days and would eat it while coughing.

Methacrolein was significantly ($P < 0.05$) more of a deterrent than was 50% methacrolein, indicating that compound concentration had an effect on preference. Inexperienced red deer calves (*Cervus elaphus*) rejected monoterpene odors by concentration rather than by quality (Elliott and Loudon, 1987). The highest methacrolein concentration tested was similar to the concentration found in basin big sagebrush, the least preferred taxon. The 50% methacrolein

was a concentration similar to that found in Wyoming big sagebrush, which is intermediately preferred. Mountain big sagebrush, the most preferred taxon, contains only a trace of methacrolein (Personius et al., 1987).

The NVCTF is a mixture of many compounds including: bitter tasting sesquiterpene lactones, cuticular waxes, coumarins, and flavonoids (Kelsey et al., 1982; Personius et al., 1987). This mixture varies in concentration and compound composition among the sagebrush taxa, making it necessary to test the extracts from all four taxa. To avoid confounding the effects of compound composition with concentration on preference, the NVCTFs from all taxa were tested at the same concentration.

Only the NVCTF from mountain big sagebrush was significantly different ($P < 0.05$) from the same fraction in the other taxa. As expected, it was the least deterrent NVCTF tested. Mountain big sagebrush naturally has the lowest NVCTF concentration (13.87% extracted tissue dry weight ETDW). If the other taxa had been tested at higher concentrations as they occur in nature, the difference probably would have been even greater.

Preference for the NVCTF of basin big sagebrush was not significantly different ($P < 0.05$) from the NVCTF of Wyoming big sagebrush. This was not surprising because the two taxa are preferred to nearly the same degree and have about the same quantity of NVCTF (16.98 and 15.55% ETDW, respectively) in nature (Personius et al., 1987). Although the NVCTFs from basin and Wyoming big sagebrush appear to be preferred to the NVCTF from black sagebrush, the difference was not significant ($P < 0.05$). Here again concentration could have an effect. The NVCTF of black sagebrush naturally occurs at higher concentrations (20.12% ETDW) than do those of basin big sagebrush or Wyoming big sagebrush (Personius et al., 1987). If the black sagebrush NVCTF had been tested at the higher concentration found in nature, the difference may have been significant. In addition, the extracted black sagebrush tissue had more stems and woody material than did the other taxa.

The epidermal extract of sagebrush stems and branches contains fewer terpenes than extracts of leaves (Kelsey, 1986). Consequently, the more woody tissue extracted, the lower the sesquiterpene lactone concentration in the final NVCTF. If sesquiterpene lactones are preference determinants, then this woody tissue could reduce the deterrent properties of the extract. This could partly explain why the preference for feed treated with the NVCTF of black sagebrush was not significantly lower ($P < 0.05$) than the preference for feed treated with the NVCTF of basin or Wyoming big sagebrush. Possibly the contents of NVCTF, *p*-cymene, or the combination of the two in black sagebrush may separate it from big sagebrush in terms of palatability.

The NVCTFs are likely important preference determinants. The results of this study concur with the findings of Personius et al. (1987), who found sesquiterpene lactones in the NVCTF were good indicators of preference. Burnett

et al. (1977) also found sesquiterpene lactones to deter herbivory by eastern cottontail rabbits (*Sylvilagus floridanus* Allen) and white-tailed deer (*Odocoileus virginianus* Zimmermann).

In preference tests it is often difficult to design experiments that accurately represent natural conditions. Although care was taken to approximate the concentrations found naturally in the taxa studied, the compounds were applied artificially. All sesquiterpene lactones and probably most of the monoterpenes in sagebrush occur in glandular trichomes on the epidermal surface (Kelsey and Shafizadeh, 1980; Kelsey et al., 1984). Under field conditions, volatile components, such as methacrolein and the monterpenes, may be released slowly through the trichome membrane and surrounding cuticular waxes of undisturbed or undamaged leaves, but released rapidly when trichomes are burst. Undoubtedly, volatile compounds sprayed onto the surface of chopped alfalfa were released more rapidly and in higher concentrations than occurred in nature. Volatility was of minor consequence to the application of the NVCTFs, which probably were more accurately simulated than were the volatile compounds.

Of all the studies attempting to correlate mule deer preference with sagebrush chemistry, the experiments of Personius et al. (1987) used the most natural conditions and the most comprehensive chemical analyses. Discriminate analysis determined that in addition to some monoterpenes, nonterpene volatiles and nonvolatile compounds such as sesquiterpene lactones could also be influencing preference. Our choice tests provide further evidence that a variety of allelochemicals, located in trichomes on the epidermal surface of sagebrush foliage, could be affecting deer selection. Consequently, future studies of animal preference for sagebrush should take into consideration all of the potential defensive chemicals associated with these shrubs.

Acknowledgments—Authors acknowledge the assistance of Dr. Richard Lund with statistical procedures and Bruce Davitt and Dr. Jack Nelson for the use of the facilities and deer. Published with the approval of the Montana Agriculture Experiment Station as journal article J-2619.

REFERENCES

BEHAN, B., and WELCH, B.L. 1985. Black sagebrush: Mule deer winter preference and monoterpenoid content. *J. Range Manage.* 38:278–280.

BELL, F.R. 1959. The sense of taste in domesticated animals. *Vet. Rec.* 71:1071–1081.

BURNETT, W.C., JONES, S.B., and MABRY, T.J. 1977. Evolutionary implications of sesquiterpene lactones in *Vernonia* (Compositae) and mammalian herbivores. *Taxon* 26:203–207.

ELLIOTT, S., and LOUDON, A. 1987. Effects of monoterpene odor on food selection by red deer calves (*Cervus elaphus*). *J. Chem. Ecol.* 13:1343–1349.

GOATCHER, W.D., and CHURCH, D.C. 1970. Taste responses in ruminants I. Reactions of sheep to sugars, saccharin, ethanol and salts. *J. Anim. Sci.* 30:777–783.

KELSEY, R.G. 1986. Foliage biomass and crude terpenoid productivity of big sagebrush (*Artemisia*

tridenta), pp. 375–388, *in* Proceedings, Symposium on the biology of *Artemisia* and *Chrysothamnus*. USDA, Forest Service General Technical Report INT-200, Ogden, Utah.

KELSEY, R.G., and SHAFIZADEH, F. 1980. Glandular trichomes and sesquiterpene lactones of *Artemisia nova* (Asteraceae). *Biochem. Syst. Ecol.* 8:371–377.

KELSEY, R.G., STEPHENS, J.R., and SHAFIZADEH, F. 1982. The chemical constituents of sagebrush foliage and their isolation. *J. Range Manage.* 35:617–622.

KELSEY, R.G., RAYNOLDS, G.W., and RODRIQUEZ, E. 1984. The chemistry of biologically active constituents secreted and stored in plant glandular trichomes, pp. 187–241, *in* E. Rodriquez, P.L. Healey, and I. Mehta (eds.). Biology and Chemistry of Plant Trichomes. Plenum Press, New York.

MCNEAL, A.F. 1984. Site characteristics and effect on elk and mule deer use of the Gardiner winter range, Montana. MS thesis. Montana State University, Bozeman, 133 pp.

NARJISSE, H. 1981. Acceptability of big sagebrush to sheep and goats: Role of monoterpenes. PhD dissertation. Utah State University, Logan, 124 pp.

PERSONIUS, T.L. 1985. The influence of crude terpenoid constituents on mule deer preference for big sagebrush and black sagebrush. MS thesis. Montana State University , Bozeman, 46 pp.

PERSONIUS, T.L., WAMBOLT, C.L., STEPHENS, J.R., and KELSEY, R.G. 1987. Crude terpenoid influence on mule deer preference for sagebrush. *J. Range Manage.* 40:84–88.

ROSENTHAL, G.A., and JANZEN, D.H. (eds.). 1979. Herbivores: Their Interaction with Secondary Plant Metabolities. Academic Press, New York.

SCHOLL, J.P., KELSEY, R.G., and SHAFIZADEH, F. 1977. Involvement of volatile compounds of *Artemisia* in browse preference by mule deer. *Biochem. Syst. Ecol.* 5:291–295.

SHEEHY, D.P. 1975. Relative palatability of seven *Artemisia* taxa to mule deer and sheep. MS thesis. Oregon State University, Corvallis, 147 pp.

SHEEHY, D.P., and WINWARD, A.H. 1981. Relative palatability of seven *Artemisia* taxa to mule deer and sheep. *J. Range Manage.* 34:397–399.

STATISTICAL ANALYSIS SYSTEMS. 1985. Proprietary Software release 6.02. SAS Institute, Cary, North Carolina.

STRIBY, K.D., WAMBOLT, C.L., KELSEY, R.G., and HAVSTAD, K.M. 1987. Crude terpenoid influence on in vitro digestibility of sagebrush. *J. Range Manage.* 40:244–248.

TATKEN, R.L., and LEWIS, R.J., SR. 1983. Registry of toxic effects of chemical substances (1981–82 ed.), Vols. 1, 2, and 3. U.S. Department of Health and Human Services Publication No. 83-107. Washington, D.C.

WAMBOLT, C.L., and MCNEAL, A.F. 1987. Selection of winter foraging sites by elk and mule deer. *J. Environ. Manage.* 25:285–291.

WAMBOLT, C.L., KELSEY, R.G., BRAY, R.O., PERSONIUS, T.L., and STRIBY, K.D. 1991. Wildlife reactions to sagebrush crude terpenoids, pp. 37–40, *in* H.G. Fisser (ed.). Proceedings of the seventeenth Wyoming shrub ecology workshop, 1988, Jackson, Wyoming. University of Wyoming, College of Agriculture, Department of Range Management, Laramie, Wyoming.

WELCH, B.L., and MCARTHUR, E.D. 1986. Wintering mule deer preference for 21 accessions of big sagebrush. *Great Basin Nat.* 46:281–286.

WELCH, B.L., MCARTHUR, E.D., and DAVIS, J.N. 1981. Differential preference of wintering mule deer for accessions of big sagebrush and for black sagebrush. *J. Range Manage.* 34:409–411.

WELCH, B.L., NARJISSE, H., and MCARTHUR, E.D. 1982. *Artemisia tridentata* monoterpenoid effect on ruminant digestion and forage selection, pp. 73–86, *in* N. Margaris, A. Koedam, and D. Vokou (eds.). Aromatic Plants: Basic and Applied Aspects. Martinus Nijhoff Publishers, The Hague.

WELCH, B.L., MCARTHUR, E.D., and DAVIS, J.N. 1983. Mule deer preference and monoterpenoids (essential oils). *J. Range Manage.* 36:485–487.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

CHEMICAL BASIS OF EGG CANNIBALISM IN A CATERPILLAR (*Utetheisa ornatrix*)[1]

FRANZ BOGNER and THOMAS EISNER*

Cornell University, Section of Neurobiology and Behavior
W347 Mudd Hall, Ithaca, New York 14853-2702

(Received March 27, 1991; accepted July 1, 1991)

Abstract—Larvae of the moth *Utetheisa ornatrix* are shown to cannibalize eggs in the laboratory. They proved most cannibalistic if they were systemically deficient in pyrrolizidine alkaloid (PA), the defensive agent that protects *Utetheisa* at all stages of development against predation, and which *Utetheisa* acquire as larvae from their food plant. In exercising cannibalistic choice, *Utetheisa* larvae feed preferentially on eggs that are PA-laden rather than PA-free. Egg cannibalism can therefore provide *Utetheisa* with a supplemental means of PA procurement. Moreover, presence of PA in the egg, while providing the egg with defense against predation, can increase its vulnerability to cannibalism. Although evidence is presented that *Utetheisa* larvae cannibalize eggs in nature, it is argued that such feeding may occur only opportunistically in the wild, rather than as a matter of course.

Key Words—*Utetheisa ornatrix*, Lepidoptera, Arctiidae, pyrrolizidine alkaloid, egg cannibalism, acquired defense, phagostimulant, specific hunger.

INTRODUCTION

The larvae of the moth *Utetheisa ornatrix* feed on plants of the genus *Crotalaria* (Leguminosae), containing toxic pyrrolizidine alkaloids (PAs). The larvae tolerate the PAs and store them systemically, retaining them through development into the adult stage. Adults transmit PAs to the eggs (Eisner and Meinwald, 1987; Dussourd et al., 1988). The sequestered PAs protect eggs against coccinellid beetles (Dussourd et al., 1988), larvae against wolf spiders (Eisner and

*To whom correspondence should be addressed.

[1] Paper No. 104 of the series Defense Mechanisms of Arthropods, No. 103 in Eisner and Eisner, 1991. *Psyche* 98:111–118.

0098-0331/91/1100-2063$06.50/0

Eisner, 1991), and adults against orb-weaving spiders (Eisner and Meinwald, 1987).

Utetheisa in nature contain variable quantities of PA. Existing evidence suggests that this can be a consequence of feeding on *Crotalaria* species of different PA content or of having varying access to the seeds of *Crotalaria*, where the PAs are concentrated (Conner et al., 1990). PAs, therefore, may be a limited resource, which *Utetheisa* may not always acquire at optimal levels, sufficient for defense.

PAs are strongly phagostimulatory to *Utetheisa* larvae. In concentrations as low as 10^{-4}% they can render an inert substrate such as agar edible to larvae (Blankespoor, Firlik, Pressman, Conner, and Eisner, unpublished). Moreover, PAs are phagostimulatory in the two chemical forms (free base, *N*-oxide) in which they occur in nature (Bogner and Eisner, 1991). Monocrotaline, for instance, one of the principal PAs available to *Utetheisa* from its food plants, when added as free base or *N*-oxide to filter paper, will induce larvae to chew holes into the paper (Bogner and Eisner, 1991).

Given this proven larval avidity for PAs, we postulated that the compounds might provide a basis for cannibalistic drive, and specifically for directing cannibalistic choice in *Utetheisa* larvae. Larvae deficient in PAs, able to recognize PA-laden conspecifics, might turn cannibalistically upon these as a way to make up for their chemical deficiency. Laboratory data that we obtained with PA-free larvae that were given access to pupae, showed that such larvae are in fact cannibalistic, and that they direct their attacks preferentially on pupae that are PA-laden (Bogner and Eisner, 1991). However, we discovered that pupal cannibalism is not likely to be a major factor in *Utetheisa*, since the larvae pupate at sites away from their food plants. The larvae themselves appeared not to be at high risk, since under most conditions they seemed able to take quick evasive action when contacted by other larvae. The truly vulnerable stage appeared to be the egg.

We here present evidence that under certain test conditions *Utetheisa* eggs are cannibalized by larvae. We demonstrate that larvae are particularly prone to be cannibalistic when they are PA-deficient, and that if given a choice, they show high preference for PA-containing eggs. We also present field data indicating that *Utetheisa* eggs may be cannibalized in nature.

METHODS AND MATERIALS

Experimental Animals. Except where otherwise specified, the *Utetheisa* were from a laboratory culture that we have maintained for over a decade, established with stock collected in the environs of Lake Placid, Florida (voucher specimens in Cornell insect collection, lot 1154).

We reared the moths on two diets: PB diet, a semisynthetic mixture (Miller et al., 1976) based on pinto beans and free of PAs, and CS diet, identical to the PB mixture, but with 10% (by weight) of its pinto bean fraction replaced by mature *Crotalaria spectabilis* seeds. *C. spectabilis* is one of the two major food plants of *Utetheisa* in the southeastern United States. Its principal PA is monocrotaline, present as both free base and *N*-oxide (Johnson et al., 1985).

Utetheisa raised on CS diet contain substantial levels of monocrotaline. Analyses of two samples taken years apart from our colony showed a microgram per moth content of 628 ± 48 (SEM) ($N = 31$) (B. Roach, unpublished) and 782 ± 141 (SEM) ($N = 4$) (J. Kopecky, unpublished). Moths raised in the field on seed-bearing *C. spectabilis* contain comparable levels of monocrotaline [701 ± 59 (SEM) μg/moth ($N = 22$); Conner et al., 1990]. *Utetheisa* raised on PB diet are PA-free.

Eggs from *Utetheisa* raised on CS diet contain monocrotaline [1.8 ± 0.1 (SEM) μg/egg, based on analyses of 10 egg clusters; J. Kopecky, unpublished], while those from PB-raised moths are PA-free. Chemical data for eggs in the wild are available only for *Utetheisa* associated with *Crotalaria mucronata*, another major food plant, which contains usaramine as its principal PA, rather than monocrotaline. Eggs from such moths contain on the order of half the PA present in eggs from CS-raised parents [0.8 ± 0.1 (SEM) μg usaramine/egg, based on analyses of seven egg clusters; B. Roach, cited in Dussourd et al., 1988]. The PAs in *Utetheisa* eggs, as in all developmental stages, are stored primarily as *N*-oxide rather than free base.

In what follows, we use the prefix (+) or (−) to denote *Utetheisa* eggs, larvae, or adults from our colony that are either monocrotaline-laden (CS-diet culture) or monocrotaline-free (PB-diet culture).

Collection of *Utetheisa* eggs was effected by providing cages housing (+) or (−) adults with linings of wax paper, upon which the females readily oviposited, clustering their eggs in batches as they do in nature [cluster size on *C. mucronata* leaves in the field = 20.2 ± 2.3 (SEM) eggs/cluster ($N = 45$) (range 6–100)]. Egg batches for testing were prepared by trimming the wax paper (1–2 cm^2) around the clusters, and adjusting egg number to 8–12/batch by removing excess eggs.

The larvae of the cabbage butterfly, *Pieris rapae*, used for comparative purposes in some of the tests, were from a laboratory colony raised on artificial diet (Bell et al., 1981). *Pieris* eggs were obtained by placing cups lined with Parafilm in cages housing *Pieris* adults. *Pieris* ordinarily lay their eggs singly, but they laid them in quantity in the cups, so that experimental batches comparable (8–12 eggs) to those of *Utetheisa* could be readily prepared by trimming of the Parafilm (due to the greater spacing of the *Pieris* eggs, the Parafilm pieces were somewhat larger than the wax paper pieces used with *Utetheisa* eggs).

In the laboratory experiments, egg age at time of testing (for both *Utetheisa* and *Pieris* eggs) was usually 24 hr (maximum 60 hr).

Chemical Samples. Monocrotaline-free base was obtained from a commercial source; the *N*-oxide was prepared in Professor Jerrold Meinwald's Cornell laboratories by oxidation of the free base with hydrogen peroxide (Mattocks, 1969).

Experimental Protocol. This was similar to the one we used in tests for pupal cannibalism (Bogner and Eisner, 1991). Mid-size *Utetheisa* or *Pieris* larvae were used. These were tested individually in cylindrical plastic chambers (85 mm diameter; 35 mm height), in which they were offered a choice of two egg batches, positioned on opposite quadrants of a circular piece of filter paper that fit the bottom of the chamber. The batches were anchored in place with small squares of double-sided sticky tape affixed to the underside of the wax paper or Parafilm backing of the eggs. The larvae were placed directly into the chamber without prestarving.

Fate of eggs was checked at 24 hr, and scored as consumption of one batch or the other, of both batches, or of neither. The criterion for consumption was injury or disappearance of a fixed minimum of eggs (three in batches of 8–9; four in batches of 10–12). This minimum applied to each of the two batches, when the result was scored as "both consumed." As a rule, consumptions were in excess of the minimum; (+) eggs, for instance, particularly in tests with (−) larvae, were usually eaten in their entirety.

All tests were repeated 25 times, using previously untested larvae for all trials.

Test Series 1. The intent here was to test for differences in acceptability of (+) and (−) eggs, in other words, of eggs naturally endowed with PA and of those free of PA. The test larvae were of three kinds: (+) larvae, (−) larvae, and field-collected larvae [(F) larvae]. The latter (also in the mid-size range) were taken on a *Crotalaria mucronata* stand outdoors (same stand as in field test C, below), in the vicinity of Lake Placid, Florida, and were tested immediately after capture (no prestarving).

Test Series 2. The aim was to see whether PA in the form of a topical additive to (−) eggs could render these as acceptable as (+) eggs. *Utetheisa* larvae were offered a choice of two (−) batches, of which one was topically treated with PA and the other was presented as control. The PA was monocrotaline, and it was tested separately as free base and *N*-oxide. The chemical was added to the egg batches in methanol solution (10 μl, providing about 1 μg PA/egg). Control batches were treated by addition of 10 μl methanol only. The tests were undertaken in parallel with (+) and (−) larvae.

Test Series 3. In tests 3A and 3B, *Utetheisa* larvae were offered a choice between *Utetheisa* eggs [either (+) or (−)] and *Pieris* eggs. In test 3C, they

were offered a choice of only *Pieris* eggs, with one batch treated by addition of about 1 μg/egg monocrotaline free base (administered in methanol solution as in test series 2), and the other (control) treated by addition of methanol only. The tests were done in parallel with (+) and (−) larvae.

Test Series 4. The test larvae here were of *Pieris* only, and they were given choices of *Pieris* egg batches as controls, and experimental batches consisting of either (+) or (−) *Utetheisa* eggs (tests 4A, 4B) or of *Pieris* eggs treated by topical addition of about 1 μg/egg monocrotaline free base (test 4C). Topical addition of the PA (and of the methanol solvent to the control batches) was effected as in test series 2.

Field Tests. In order to test for egg cannibalism under field conditions, *Utetheisa* egg clusters were staked out on *Crotalaria mucronata* plants outdoors and visually checked for fate at intervals after exposure. Batches of both (+) and (−) eggs, prepared as they were for the laboratory tests (8–12 eggs/batch, on wax paper backing), were affixed to the upper surfaces of *C. mucronata* leaves with small pieces of two-sided sticky tape. Batches of the two types were exposed in approximately equal numbers, in three separate tests. An effort was made not to group the batches by type, and to space them at minimal distances of about 0.5–1.5 m. Fate was recorded descriptively for each batch.

Tests A and B. The site for both these tests was an area (≈ 20 m^2) on the MacArthur Agro-ecology Research Center, Lake Placid, Florida, where both *C. mucronata* and *Utetheisa* occur in the wild. The *C. mucronata* on the site, estimated at some 50 plants, were mature and bore seed pods at all developmental stages. They carried a moderate infestation of *Utetheisa*, judged to be at a level of no more than several larvae per plant. The two tests were carried out a few days apart, and were each of 66 hr duration: (A) 27 (+) batches/26 (−) batches (December 25–28, 1990); (B) 18 (+) batches/14 (−) batches (January 3–6, 1991)

The egg batches in these tests were less than 24 hr old when exposed, so there were no hatchings during the experimental period (*Utetheisa* egg incubation time is four to five days).

Test C. The site for this test was on the grounds of the Archbold Biological Station, several miles West from the site used in the previous two tests. It consisted of an area (≈ 100 m^2) with approximately 500 *C. mucronata* grown from planted seeds. The plants were as mature as those in tests A and B and were also beset (visual estimate) with only moderate numbers of *Utetheisa* larvae.

The single test at this site involved staggered exposure, during the period of September 1–8, 1990, of 26 (+) batches and 26 (−) batches, for 40 hr/batch. Since the batches were of unknown mixed ages, there were some hatchings during the test. Visual checks were at intervals of 2 to several hours, between 8 AM and 7 PM.

RESULTS

Test Series 1 (Figure 1). Of the two categories of laboratory-reared larvae, only the (−) larvae proved strongly cannibalistic. Moreover, these larvae showed a clear preference for (+) eggs (see Figure 5). The field-collected larvae were also cannibalistic and shared the predilection for (+) eggs. What little feeding the (+) larvae did was also mostly on (+) eggs.

Test Series 2 (Figure 2). The (−) larvae, again, did most of the feeding, and they fed preferentially on the eggs bearing the monocrotaline additive, irrespective of whether this additive was in form of free base (Figure 2A) or *N*-oxide (Figure 2B).

Test Series 3 (Figure 3). The feeding was once again primarily by (−) larvae, and it was restricted largely to the two situations (3A, 3C) in which monocrotaline-bearing samples were available as choices. These samples were either (+) eggs or *Pieris* eggs topically laden with monocrotaline, and they were both strongly favored in their respective tests. Untreated *Pieris* eggs were largely shunned.

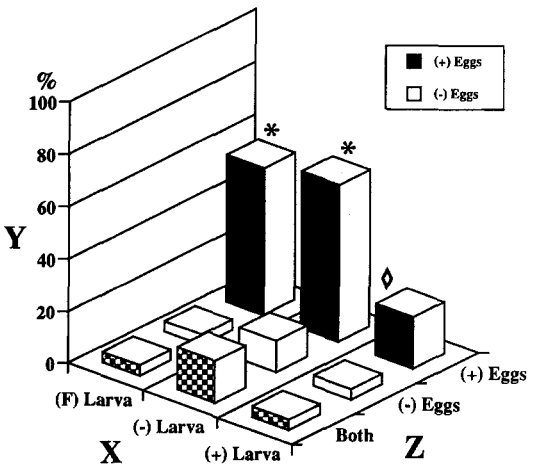


FIG. 1. Test series 1. Choice preference of *Utetheisa* larvae for (+) or (−) *Utetheisa* egg batches (choices are given in rectangles). The X axis gives the type of larva [(F) Larva = field-collected], Z axis the breakdown of choices made (nonfeeders are omitted), and Y axis the percent distribution of choices. $N = 25$ replications/test. Statistical conventions (applicable to Figure 1–4): asterisk(s) indicate level of significance of difference from alternative choice (and in most cases also from the "both" choice) in same test (* $P < 0.01$; ** $P < 0.001$); diamond(s) indicate level of difference from same choice in neighboring test ($^{\diamond}P < 0.025$; $^{\blacklozenge}P < 0.01$, $^{\blacklozenge\blacklozenge}P < 0.001$) [$G$ test, with P-value conversions (Sokal and Rohlf, 1981)].

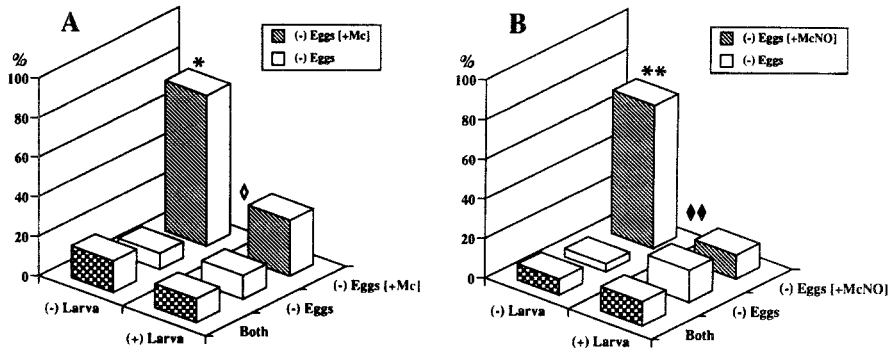


FIG. 2. Test series 2. Choice preference of (+) and (−) *Utetheisa* larvae for (−) *Utetheisa* eggs treated or untreated by addition of either monocrotaline free base [+Mc] or monocrotaline *N*-oxide [+McNO]. Conventions as in Figure 1.

Test Series 4 (Figure 4). *Pieris* larvae proved readily prone to feed on conspecific eggs. They essentially shunned *Utetheisa* eggs of either type, sampling these only in trials where they fed on the *Pieris* choice as well (Figure 4A, 4B). They also discriminated against *Pieris* eggs if these were treated with monocrotaline (Figure 4C).

Field Test A. This was the only test in which the majority of surviving batches were (−) rather than (+) eggs. Actual tallies at the end of the 66-hr exposure period were: 19 of the 27 (+) batches intact; 24 of the 26 (−) batches intact. Two cases of cannibalism were witnessed, both by nearly full-grown larvae (fourth instar, by estimate), which were discovered while still positioned over the site of the batch they had consumed (such lingering behavior was also noted in the laboratory tests). Nothing definitive can be said about the agents responsible for the fate of the other eight batches that we scored as "nonintact" in this test. Some of these batches had entirely disappeared, while others had disappeared in part or showed injury to some of the eggs.

Field Test B. The results here were similar, except that batch survival was skewed in favor of the (+) eggs. The tallies were: 13 of 18 (+) batches intact; 2 of 14 (−) batches intact. We again remained ignorant in most cases of the cause of injury or disappearance of batches, but we did witness two cases of cannibalism, and these again involved nearly grown larvae and (+) eggs. While in one of these cases the caterpillar was again found lingering over the batch site (the eggs had been consumed), in the other it was discovered in the act of eating.

Field Test C. No instances of cannibalism were witnessed in this test, which differed from the preceding two in that we were able to identify the primary predator responsible for batch mortality. The results were: 23 of 26 (+) batches

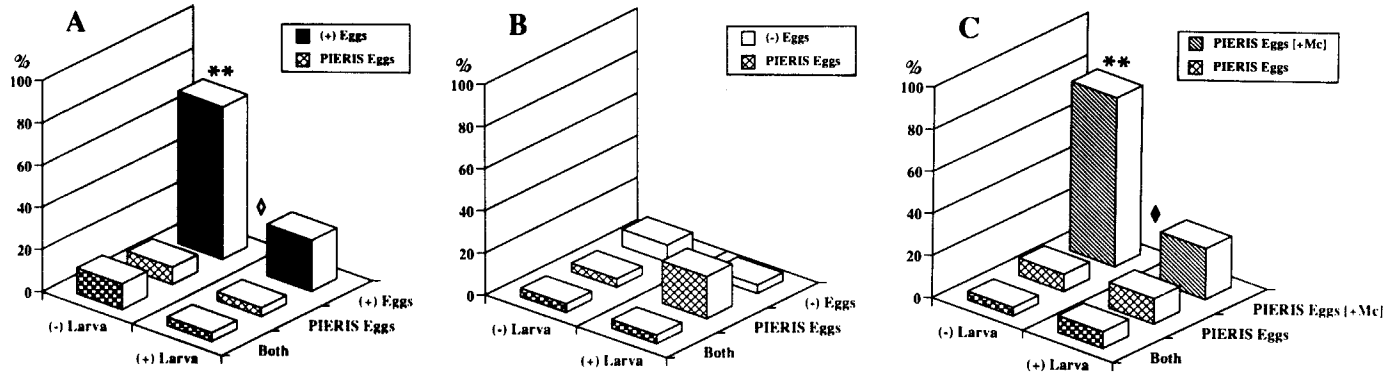


FIG. 3. Test series 3. Choice preferences of (+) and (−) *Utetheisa* larvae for *Utetheisa* eggs and *Pieris* eggs offered in various paired combinations. Mc = monocrotaline free base. Conventions as in Figure 1.

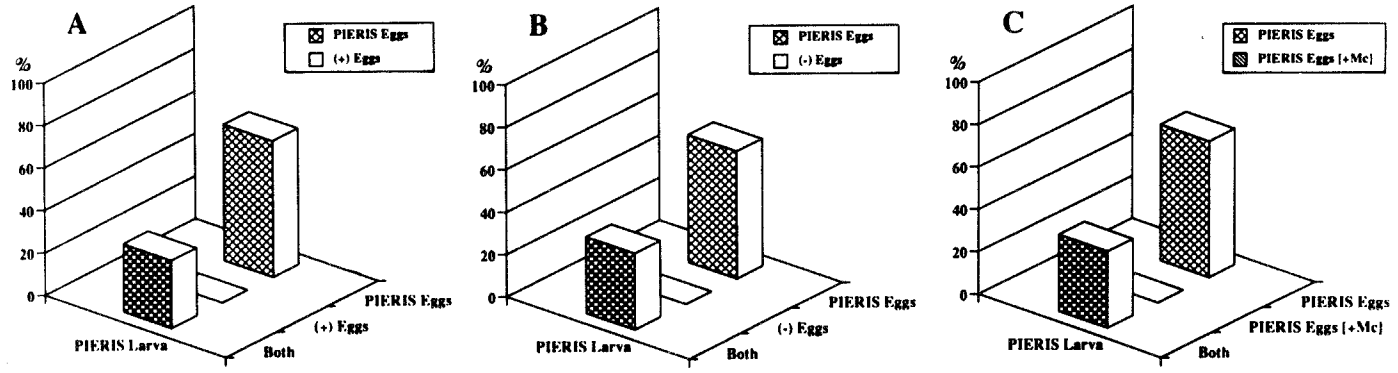


FIG. 4. Test series 4. Choice preference of *Pieris* larvae for *Utetheisa* eggs and *Pieris* eggs offered in various paired combinations. Mc = monocrotaline free base. Rectangles flush with base plane are 0 values. Conventions as in Figure 1.

intact; 14 of 26 (−) batches intact [about an equal fraction (13% and 14%) of intact batches of each category hatched during the experiment]. The predator responsible for consumption of batches that we scored as "nonintact" was a green lacewing larva (*Ceraeochrysa cubana*), which characteristically hollowed out the eggs by sucking up their contents. This predator [which was witnessed in the act of attacking (−) batches on two occasions] accounted for the death of 10 (−) batches and only one (+) batch. No causative agent was discovered to account for the fate of the other two (+) batches and two (−) batches that were scored as "nonintact" and that had actually disappeared or partly disappeared.

DISCUSSION

Utetheisa larvae are evidently prone to cannibalize eggs, certainly in the laboratory. Moreover, they are particularly inclined to consume eggs if they themselves are PA-deficient and the eggs PA-endowed. In other words, they appear to be driven to cannibalism by systemic PA-deficiency and are selective in their cannibalistic choice of items that contain the missing chemical. As we demonstrated in a previous study (Bogner and Eisner, 1991), the same caveats apply to pupal cannibalism in *Utetheisa*. Hunger alone cannot be the motivating force. The larvae used in both this and the pupal study were not starved prior to testing.

The specificity with which exogenous PA can trigger larval feeding in *Utetheisa* is noteworthy. The larvae showed no predisposition to cannibalize (−) eggs or to ingest *Pieris* eggs, unless these items were topically "labeled" by addition of monocrotaline. Moreover, as was clear from the tests with the (−) eggs, the free base and *N*-oxide forms of monocrotaline were equally effective as labels. In *Utetheisa* eggs, PA occurs primarily (>95%) as *N*-oxide (Dussourd et al., 1988).

The results with *Pieris*, although not bearing directly on the issue of *Utetheisa* cannibalism, raise points of interest. First, *Pieris* larvae themselves proved prone to cannibalize eggs. Moreover, they essentially ignored *Utetheisa* eggs, whether these contained PA or not. However, they were not unresponsive to PA. If monocrotaline was added topically to *Pieris* eggs, *Pieris* larvae decidedly avoided these. Vis à vis a caterpillar other than *Utetheisa*, therefore, monocrotaline can act as a deterrent rather than attractant. Conceivably, this could play a role in the natural protection of *Utetheisa* eggs against such caterpillars of other species as might show facultative tendencies to ingest insect eggs.

The field tests, while not as extensive or revealing as one might wish, did demonstrate that *Utetheisa* egg cannibalism can occur in nature. While the conditions under which we witnessed the event—and we did see four manifestations

of it—were not strictly natural, they did involve exposure of egg batches on food-plant leaves, where eggs are normally laid. The point worth noting is that all four instances involved larval consumption of (+) rather than (−) eggs, which is in line with what was to be predicted from the laboratory findings. The laboratory data had also told us that field-collected larvae can behave as if systemically deficient in PA and hungry for the compounds: the field larvae in our choice tests (Figure 5), which stemmed from the same *C. mucronata* stand on which we had exposed the egg batches (test C), were distinctly cannibalistic toward (+) eggs. Previous chemical work (Conner et al., 1990) had shown that *Utetheisa* field-reared on *C. mucronata* have lower systemic PA levels than those raised on *C. spectabilis*, the source of the seeds in the laboratory diet of our (+) larvae. It could be, therefore, that larvae raised on *C. mucronata*, unlike our (+) larvae, are somewhat underendowed with PA, and for that reason cannibalistically inclined.

The field data point further to the likelihood that cannibalism is only a lesser threat to *Utetheisa* eggs in nature. Predation, in our judgment, must be the greater hazard by far, and it is of course primarily as defensive agents against predators that PAs are acknowledged to function. Indeed, (+) egg batches in our field tests fared better on the whole than did (−) batches, and in one test, heavy predation by one predator (a chrysopid larva) accounted for most of the mortality of (−) eggs. In an earlier field test, in which 100 (+) and 100 (−)

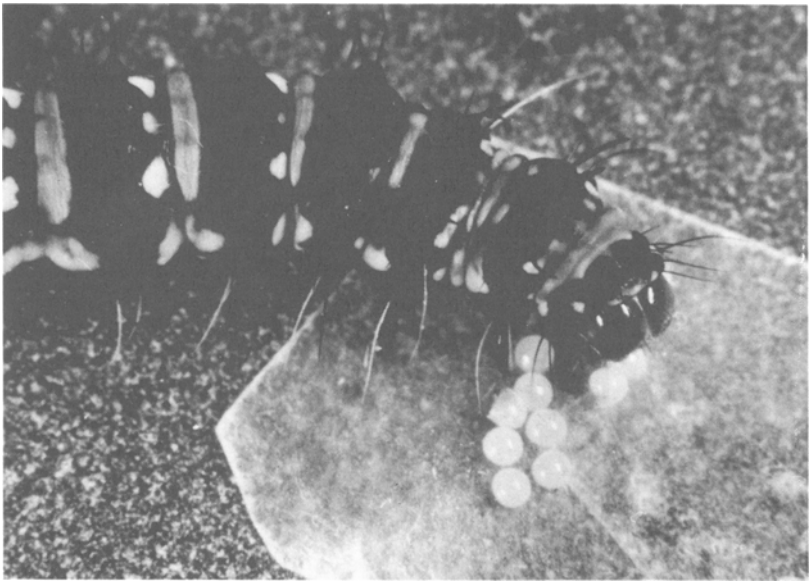


FIG. 5. Medium-sized (−) *Utetheisa* larvae in process of cannibalizing (+) eggs.

batches were staked out, the (−) eggs also endured higher mortality than the (+) eggs (Dussourd et al., 1988).

The fact that PAs can play conflicting roles in the eggs of *Utetheisa* raises questions. Are there times when the threat from cannibalism outweighs that from predation? In our earlier paper (Bogner and Eisner, 1991), we postulated that there might be such times and that they might be coincident with *Crotalaria* seeds being in short supply and *Utetheisa* larvae, consequently, PA-deficient. Such times could prevail when *Crotalaria* are immature or when their seeds are overgrazed by *Utetheisa* and other seed-eaters (for instance, caterpillars of the pyralid moth, *Etiella zinckenella*). *Utetheisa* feeding on immature *Crotalaria* are known to accrue sharply lessened systemic loads of PA (Conner et al., 1990). One could thus imagine dietary PA availability playing an important role in setting cannibalistic drive levels in *Utetheisa* populations in nature.

Egg cannibalism can provide *Utetheisa* larvae with only relatively modest quantities of PA. Eggs on average contain 1–2 μg PA, so an average-sized cluster of 20 eggs could supply no more than a small fraction of the several hundred micrograms of PA accrued by the adult moth. Egg cannibalism in *Utetheisa* might therefore occur only opportunistically, rather than as a matter of course, and serve only as a subsidiary means of PA procurement. One wonders, however, whether egg cannibalism might not be of greater significance in younger, perhaps first or second instar larvae, such as one does not generally find feeding on the PA-rich seeds of *Crotalaria*. We have no data on such young larvae and are also ignorant of whether these might early in life, as perhaps shortly after emergence, turn upon conspecific eggs that they encounter. It would be intriguing if, in the selection of cannibalistic targets by such young larvae, kin recognition were to enter into play.

Other cases have been documented (probably far fewer than actually exist) of insects bestowing defensive metabolites upon their eggs (McCormick and Carrel, 1987; Eisner, 1988; Dussourd et al., 1989). It would be interesting if the phenomenon here demonstrated, of usurpation of such chemicals by cannibalization of eggs, were to occur in other insects as well. We suggest that it does.

Acknowledgments—Study supported by NIH grant AI02908, Hatch funds (191-7402), and the Deutsche Forschungsgemeinschaft (Bo 944/1-2). We thank Patricia Bogner, Glen Scoles, and Maria Eisner for much help; B. Roach and J. Kopecky for unpublished analytical data; and B. Roach for preparing monocrotaline *N*-oxide. The field tests were carried out at the Archbold Biological Station and the MacArthur Agro-ecology Research Center, Lake Placid, Florida; Mark Deyrup kindly planted the *Crotalaria* stand.

REFERENCES

BELL, R.A., OWENS, C.D., SHAPIRO, M., and TARDIF, J.R. 1981. Development of mass-rearing technology, pp. 599–655, *in* C.C. Doane and M.L. McManus (eds.). The Gypsy Moth: Research Toward Integrated Pest Managment. USDA Technical Bulletin 1584.

BOGNER, F., and EISNER, T. 1991. Chemical basis of pupal cannibalism in a caterpillar (*Utetheisa ornatrix*). *Experientia*. In press.

CONNER, W.E., ROACH, B., BENEDICT, E., MEINWALD, J., and EISNER. T. 1990. Courtship pheromone production and body size as correlates of larval diet in males of the arctiid moth, *Utetheisa ornatrix*. *J. Chem. Ecol.* 16:543.

DUSSOURD, D.E., UBIK, K., HARVIS, C., RESCH, J., MEINWALD, J., and EISNER, T. 1988. Biparental defensive endowment of eggs with acquired plant alkaloid in the moth *Utetheisa ornatrix*. *Proc. Natl. Acad. Sci. U.S.A.* 85:5992–5996.

DUSSOURD, D.E., HARVIS, C.A., MEINWALD, J., and EISNER, T. 1989. Paternal allocation of sequestered plant pyrrolizidine alkaloid to eggs in the danaine butterfly, *Danaus gilippus*. *Experientia*. 45:896–898.

EISNER, T. 1988. Insekten als fürsorgliche Eltern. *Verhandl. Dtsch. Zool. Gesellsch.* 81:9–17.

EISNER, T., and EISNER, M. 1991. Unpalatability of the pyrrolizidine alkaloid-containing moth *Utetheisa ornatrix*, and its larva, to wolf spiders. *Psyche* 98:111–118.

EISNER, T., and MEINWALD, J. 1987. Alkaloid-derived pheromones and sexual selection in Lepidoptera, pp. 251–269, *in* G.D. Prestwich and G.J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, New York.

JOHNSON, A.E., MOLYNEUX, R.J., and MERILL, G.B. 1985. Chemistry of toxic range plants. Variation in pyrrolizidine alkaloid content of *Senecio*, *Amisickia*, and *Crotalaria* species. *J. Agric. Food Chem.* 33:50–55.

MATTOCKS, A.R. 1969. Dihydropyrrolizine derivatives from unsaturated pyrrolizidine alkaloids. *J. Chem. Soc. C.* 1969:1155–1162.

MCCORMICK, J.P., and CARREL, J.E. 1987. Cantharidin biosynthesis and function in meloid beetles, pp. 307–350, *in* G.D. Prestwich and G.J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, New York.

MILLER, J.R., BAKER, T.C., CARDÉ, R.T., and ROELOFFS, W.L. 1976. Reinvestigation of oak leaf roller sex pheromone components and the hypothesis that they vary with diet. *Science* 192:140–143.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry. W.H. Freeman Co., San Francisco.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

SEX PHEROMONE EVIDENCE FOR TWO DISTINCT TAXA WITHIN *Graphania mutans* (WALKER)[1]

B. FRÉROT[2] and S.P. FOSTER[3,*]

[2]*I.N.R.A., Laboratoire des Médiateurs Chimiques*
Domaine de Brouessy
78114 Magny-les-Hameaux, France

[3]*D.S.I.R. Plant Protection*
Private Bag
Palmerston North, New Zealand

(Received April 9, 1991; accepted July 1, 1991)

Abstract—The sex pheromones of two populations of *Graphania mutans* (Walker) were analyzed. Females from an Auckland population produced (*Z*)-9-tetradecenol (*Z*9–14 : OH), (*Z*)-9-tetradecenyl acetate (*Z*9–14 : OAc), (*Z*)-7-tetradecenol (*Z*7–14 : OH) and (*Z*)-7-tetradecenyl acetate (*Z*7–14 : OAc), while females from a Lincoln population produced these four compounds and a large amount of (*Z*)-9-tetradecenal (*Z*9–14 : Ald). Significant differences, paralleling the difference between females, were observed when the responses of males of both populations to the above and other related compounds were tested by electroantennogram, field-trapping, and wind-tunnel bioassays. The most distinct difference was observed in the wind tunnel. Males from both taxa flew upwind and touched pheromone sources containing sex pheromone extract of females of their own taxon, but either did not initiate upwind flight or arrested upwind flight shortly after taking flight in response to extract from females of the other taxon. The difference between the pheromone systems of the two populations is probably due to the presence and importance of *Z*9–14 : Ald in the pheromone blend of the Lincoln population. Thus the addition of a relatively large amount of *Z*9–14 : Ald to a four-component pheromone blend (i.e., *Z*9–14 : OH, *Z*9–14 : OAc, *Z*7–14 : OH, and *Z*7–14 : OAc) attractive to Auckland males completely suppressed trap catches of male *G. mutans* in Auckland but large numbers of males were caught at both Lincoln and Nelson in traps baited with this five-component blend. In wind-tunnel studies, the addition of even small (1% of amount of *Z*9–14 : OH) amounts of *Z*9–14 : Ald to the four-component blend resulted in a significantly greater proportion of Auckland males arresting upwind flight than to the four-com-

[1] Lepidoptera: Noctuidae: Hadeninae.
*To whom correspondence should be addressed.

0098-0331/91/1100-2077$06.50/0

ponent blend. It is suggested that these two populations of *G. mutans* represent distinct sibling species within the described concept.

Key Words—*Graphania mutans*, Lepidoptera, Noctuidae, sex pheromones, (Z)-9-tetradecenyl acetate, (Z)-9-tetradecenol, (Z)-9-tetradecenal, sibling species, identification, wind tunnel.

INTRODUCTION

Graphania mutans (Walker), (Noctuidae: Hadeninae), an endemic species distributed throughout New Zealand, is a pest of some horticultural crops such as apples. The larvae of this species feed on and scar the surface of the fruit, thereby making it unsuitable for export (Suckling et al., 1990). This damage often occurs early in the season and is additive to the damage caused by other lepidopterous pests such as leafroller moths and codling moth (Wearing et al., 1991).

In New Zealand, the identification of sex pheromones of all the other major lepidopterous pests found on apples, and the more accurate monitoring of populations of these pests that this has allowed, has facilitated the development of reduced insecticidal spray programs (Suckling, 1989). In particular, a number of insecticidal sprays can be omitted in many apple-growing areas during the early part of the season. During this period, damage to fruit by other pests such as *Graphania mutans* can occur, and hence the ability to specifically monitor populations of this pest, using sex pheromones, would be of great value in reduced spray programs.

The genus *Graphania* contains a number of species morphologically very similar to, and sympatric with, *G. mutans*. The adults of *G. mutans* are highly variable in color pattern; seven names have been synonymized with *mutans* (Dugdale, 1988). With this variability in mind, we used the approach previously used in the analysis of the sex pheromones of sibling species complexes of various tortricid species in the genera *Planotortrix* and *Ctenopseustis*, i.e., analysis of the sex pheromones of distinct geographic populations and determination of whether differences exist between the sex pheromones of these populations (Foster et al., 1991). We report here the results of this study on *G. mutans*.

METHODS AND MATERIALS

Insects. Two laboratory cultures of *G. mutans* were established by collecting gravid females in light traps situated in Auckland and Lincoln (near Christchurch) and allowing these females to oviposit inside small glass vials (2.5 cm diam. × 10 cm).

Neonate larvae were allowed to feed on a semisynthetic corn diet (Poitout

and Bues, 1970) until they had reached the second instar, whereupon they were transferred into a small waxed cardboard ice cream cup (Lily Inc., No. 134, Auckland) (three larvae per container), containing more of the corn diet. The larvae were maintained inside the cup at 18–20°C, 16:8 hr light–dark photoperiod until they had pupated. The sexes were separated as pupae and placed in moist vermiculite. Adults were used either in the various experiments (see below) or to perpetuate the culture. For the latter, groups of males and females (one to three each) were placed inside a plastic bag, supported by an inner wire frame (35 × 25 × 13 cm), and allowed to mate. The resultant eggs were removed and placed inside a small, humid container until they hatched.

Determination of Sexual Activity. The beginning of sexual activity by female *G. mutans* was defined as when the female extruded the terminal segments of her abdomen, thereby exposing her pheromone gland (i.e., "calling"). In order to observe the periodicity of calling behavior, females from the Auckland colony were placed in small transparent containers (4 cm high × 40 cm diam.) that allowed observation of, but did not interfere with, this behavior. Females 1–5 days old were observed every 15 min, commencing from 2 hr preceding the scotophase through to 1 hr into the photophase.

Mating of single pairs of 3-day-old Auckland males and females was observed under the above conditions.

Pheromone Extraction and Chemical Analysis. The tip of the abdomen of a 3- to 4-day-old female was everted and dissected just prior to the commencement of the calling period, placed in 10–50 μl of distilled pentane, and extracted for 0.5–12 hr prior to analysis by gas chromatography (GC) or gas chromatography–mass spectrometry (GC-MS). One to 10 glands were extracted and analyzed at a time.

Extracts were analyzed by GC using two columns: a 50-m × 0.32-mm-ID Carbowax 20 M (Quadrex Corp., New Haven, Connecticut), temperature-programmed from 100 to 210°C at 4°C/min (initial delay of 1 min), and a 60-m × 0.25-mm-ID SP2340 (Supelco Inc., Bellefonte, Pennsylvania), temperature-programmed from 60 to 140°C at 8°C/min (initial delay of 1 min), then to 180°C at 2°C/min. The gas chromatographs used were, respectively, a Philips 4500 and a Varian 3500. Splitless injection, and nitrogen at a linear flow velocity of 10 cm/sec as carrier gas, were used for both columns. This combination of columns and GC conditions allowed the separation of all monounsaturated tetradecenyl acetate isomers and virtually all tetradecenal isomers from each other (Heath et al., 1980). Alcohols could not be analyzed adequately using the SP2340 column as they were degraded on this column. Therefore they were converted to acetates by reaction with acetyl chloride for further confirmation of their structure. Tridecanyl acetate was used as an internal standard.

The mass spectra of compounds in extracts of pheromone glands were

recorded using a Nermag 10-10C equipped with either a 25-m × 0.22-mm-ID CPSIL 5CB or a 25-m × 0.22-mm-ID CPSIL 88CB (Chrompack, Middelburg, Netherlands). The mass spectra of the various compounds were compared with the mass spectra of synthetic samples.

Electroantennograms. The electroantennogram (EAG) responses of isolated male antennae were recorded according to the method of Roelofs (1984). The various chemicals (monounsaturated, 12-, 14-, and 16-carbon acetates, alcohols, and aldehydes; 1-μg dose on filter paper) were presented into the airstream using a 5-ml syringe. Data were corrected according to Renou (1979). The responses of at least five different antennae were tested to each of the series of chemicals.

Field-Trapping. Field trapping was carried out at Mt Albert Research Centre (Auckland, north North Island), Appleby Research Orchard (Nelson, northern South Island), and at Lincoln (near Christchurch, mid-coastal South Island). The appropriate amount of chemical was loaded onto a rubber septum (Thomas Scientific) and placed inside a Pherocon 1C sticky trap (Zoecon Corp., Palo Alto, California). The traps were hung approximately 1.5 m above the ground on various fruit trees (apple, nectarine, peach) or on monkey apple (*Acmena smithii*) hedges surrounding the orchards. Traps were placed at least 10 m apart. Unless stated otherwise, traps were checked and rerandomized every one to two days.

For analysis of the field-trapping experiments, data were transformed by $\sqrt{(X + 0.5)}$, an ANOVA conducted, and the means of the treatments compared using Duncan's multiple range test. Comparisons between treatment means were made at the $P = 0.05$ level.

Wind Tunnel. Male moths were flown inside a 1.3-m-long wind tunnel based on the design of Miller and Roelofs (1978). The tunnel was illuminated using six 15-W incandescent red light bulbs (Osram Co., Auckland) situated above the tunnel. The temperature throughout the various experiments was controlled at 20 ± 1°C. The chemicals or pheromone gland extract were placed on a piece of filter paper (1.5 × 1.5 cm) seated on a thin metal rod stand approximately 15 cm above the floor of the tunnel. Just prior to the start of the scotophase, individual males were placed inside capped wire mesh cylinders (2.5 cm diam. × 5 cm) and transferred into the wind tunnel room. In a preliminary experiment, males were found to respond best between 5.5 hr and 6.5 hr after the onset of the scotophase; all subsequent tests were carried out during this period. Individual 3-day-old male moths were introduced into the mouth of the tunnel (approximately 1.2 m downwind of the chemical source), and their behavioral responses to the source recorded. The following behaviors were recorded: raising of the antennae and wing movement (activation), initiation of upwind flight (take flight), flight to beyond the midpoint of the tunnel (midpoint), flight to within 15 cm of the source (approach), touching of the source

(touch), and landing on the source (land). Males from the Auckland colony were used in all the wind-tunnel experiments testing the responses of males to synthetic chemicals. Each wind-tunnel experiment was run as a complete randomized block design. The distributions of the separate behavioral responses to the various blends tested within an experiment were compared using Kruskall and Wallis tests. Differences are reported at $P = 0.05$.

RESULTS

Sexual Activity. Virtually all of the insects used in these observations emerged towards the end of the scotophase. In the first night following emergence, only 4/18 of the females called. However, on the second night following emergence, 80% of the females called. In subsequent nights (up to the fifth night following emergence), the percentage of females that called declined gradually. Females of all ages began calling during the fourth hour of the scotophase through to the onset of the photophase; the greatest percentage of females called during the fifth and sixth hours of the scotophase. Following the commencement of calling by females, males became highly activated and copulation quickly followed.

Chemical Analysis. Extracts of the pheromone glands of females from the Auckland *G. mutans* colony were initially analyzed by GC using the Carbowax 20 M column. From these extracts, the following compounds were tentatively identified on the basis of retention times (relative amounts): tetradecyl acetate (14:OAc) (0.9), (Z)-7-tetradecenyl acetate (Z7–14:OAc) (2.7), (Z)-9-tetradecenyl acetate (Z9–14:OAc) (23.1), tetradecanol (14:OH) (8.6), (Z)-7-tetradecenol (Z7–14:OH) (14.5), and (Z)-9-tetradecenol (Z9–14:OH) (100). Analysis of the crude extract using the SP2340 column was consistent with the assignments of the above acetates. When the extract was acetylated and the reaction product mixture analyzed by GC (using the Carbowax 20 M column), the chromatogram showed the almost complete disappearance of the peaks tentatively identified as alcohols and much greater amounts (relative to the internal standard) of the three acetates. Mass spectral analysis of gland extract from the Auckland females, and comparison with spectra of authentic samples, confirmed the assignments of these compounds. Females contained on average, 3.3 ng of Z9–14:OH.

G. mutans from Lincoln were analyzed similarly. GC analysis using the Carbowax 20 M column yielded chromatograms that were very different from those obtained from the analysis of Auckland *G. mutans* females. The following tentatively identified compounds (relative amounts) were observed: (Z)-9-tetradecenal (Z9–14:Ald) (100), Z7–14:OAc (0.5), Z9–14:OAc (34.9), 14:OH (3.0), Z7–14:OH (15.4), and Z9–14:OH (40.9). The tentative identification of

these acetates and aldehyde was supported by GC analysis using the SP2340 column. Acetylation of the gland extract and subsequent analysis using the Carbowax 20 M column, as above, was also consistent with the assignment of the three alcohols. GC-MS analysis of gland extract confirmed the assignments of the above compounds. Females contained on average 3.5 ng of Z9–14 : Ald.

Electroantennograms. For both the Auckland and Lincoln *G. mutans* males, the greatest EAG response in each of the series (i.e., 12-, 14-, and 16-carbon monounsaturated acetates, alcohols, or aldehydes) tested, was always to the isomer with a (*Z*)-9 double bond. Between the series, the largest amplitude response for both types of males was to the 14-carbon compounds; for the Auckland males the largest response amplitudes were to the acetates, whereas for the Lincoln males they were to the aldehydes. Between the 14-carbon series the converse was true for the lowest amplitude responses, i.e., the aldehydes and the acetates elicited the lowest amplitude response from the Auckland and Lincoln males, respectively. Except for the relative amplitudes of response, the EAG profiles to each of the series tested were very similar for both populations of males.

Field-Trapping. Field trials 1–5 (Table 1) tested the effects of the various chemicals identified in females of the Auckland and Lincoln populations on trap catches of males of the Auckland population.

In the first field trial (Table 1), *G. mutans* males were caught in significant numbers in traps baited with the four monounsaturated chemicals found in the Auckland females (Table 1); traps baited with the higher dosage (1000 μg of Z9–14 : OH) of the four-component blend caught significantly greater numbers of males than traps baited with the lower dosage (100 μg). At the higher dosage (1000 μg of Z9–14 : OH), the addition of 1% of Z9–14 : Ald to the four-component blend resulted in a significant decrease in the number of males caught.

In trial 2 (Table 1), blends that lacked Z7–14 : OAc did not elicit trap catches of male *G. mutans* significantly greater than the number caught in the blank traps. Similarly, in trial 3 (Table 1), traps baited with the blend that lacked Z9–14 : OAc did not catch any males. Increasing the amount of Z9–14 : OAc in the blend from 0 to 500 μg increased trap capture. However, increasing the amount of Z9–14 : OAc in the blend still further to 1000 μg significantly decreased (relative to the blend containing 500 μg) the catch (trial 3).

Changing the amount of Z7–14 : OH in the blend over the range 0–1000 μg resulted in slight, but not significant changes in the numbers of males caught, relative to the standard blend containing 130 μg of Z7–14 : OH (trial 4, Table 1). In contrast, the removal of Z7–14 : OAc from the three-component blend tested in this trial (trial 4) significantly reduced the numbers of males caught to a level not significantly different from the blank, thereby confirming the result from trial 2.

In trial 5, traps baited with blends containing less than 1000 μg of Z9–

TABLE 1. TRAP CATCHES OF MALE *Graphania mutans* TO VARIOUS BLENDS

Dosages of chemicals (μg)					Trap catch
Z9–14 : OH	Z9–14 : OAc	Z7–14 : OH	Z7–14 : OAc	Z9–14 : Ald	(males/trap)[a]
Trial 1[b]					
100	23	13	3		5.5 c
100	23	13	3	1	3.3 cd
100	23	13			1.8 cd
100	23				2.5 cd
1000	230	130	30		35.5 a
1000	230	130	30	10	23.8 b
					0.0 d
Trial 2[c]					
1000	230	130	30		16.7 a
1000	230	130			0.3 b
1000	230				0.3 b
					0.0 b
Trial 3[d]					
1000	230	130	30		7.3 b
1000		130	30		0.0 c
1000	50	130	30		2.3 c
1000	100	130	30		4.7 c
1000	500	130	30		11.7 a
1000	1000	130	30		2.5 c
					0.0 c
Trial 4[e]					
1000	500	130	30		13.3 a
1000	500		30		10.0 a
1000	500	50	30		8.7 a
1000	500	500	30		8.0 a
1000	500	1000	30		5.7 a
1000	500				2.0 b
					0.0 b
Trial 5[f]					
1000	500	130	30		20.8 a
	500	130	30		7.8 c
100	500	130	30		14.8 abc
500	500	130	30		10.5 b
1000	500	130	100		17.2 ab
100	1000	130	30		13.0 abc
500	1000	130	30		13.8 ab
					0.0 d

[a] Means accompanied by the same letter are not significantly different at $P < 0.05$ (Duncan's multiple range test).

[b] Conducted at Mt Albert Research Centre (MARC), Auckland, between January 28 and February 15, 1988; four replicates of each treatment were tested.

[c] Conducted at MARC and Kumeu Research Orchard, between February 2 and 19, 1988; four replicates per treatment.

[d] Conducted at MARC between February 16 and March 6, 1988; three replicates per treatment.

[e] Conducted at MARC between April 18 and June 1, 1988; three replicates per treatment.

[f] Conducted at MARC between September 7 and November 14 , 1988; four replicates per treatment. Traps were examined and rerandomized every week.

14 : OH caught fewer males than traps baited with the blend containing 1000 μg of Z9–14 : OH; with the exception of the blend that contained 100 μg of Z9–14 : OH, these catches were significantly different from the number of males caught in the 1000 μg (of Z9–14 : OH) blend. Also in this trial, the amount of Z7–14 : OAc in the four-component blend was increased from 30 μg to 100 μg. There was no significant difference between the number of *G. mutans* caught in traps baited with either of these two blends.

The field trials conducted at Lincoln and Nelson tested various blends containing chemicals identified in the females from the Lincoln population against the four-component blend that was attractive to male *G. mutans* in Auckland (Table 2). In both Lincoln and Nelson, traps baited with the Auckland-type blend caught *G. mutans* males. However, the greatest catch (not significantly different from the number caught in traps baited with the Auckland-type blend) of males was in traps baited with the five-component blend containing 1000 μg of Z9–14 : Ald. Traps baited with the four-component blend lacking Z7–14 : OAc but containing Z9–14 : Ald caught significantly less males than traps baited with the five-component blend, but significantly more males than the blank traps.

A trial comparing the four-component blend developed for the Auckland population of *G. mutans* with the five-component blend used in the Lincoln and Nelson trial was conducted in Auckland. Another five-component blend, consisting of the Auckland-type blend plus 1000 μg of Z9–14 : Ald, was also included (Table 3). Over two months, male *G. mutans* were caught only in traps containing the Auckland-type blend; the blank traps and traps baited with either the Lincoln-type blend or the other five-component blend did not catch any males.

TABLE 2. TRAP CATCHES OF MALE *Graphania mutans* AT LINCOLN AND NELSON TO VARIOUS BLENDS[a]

Dosages of chemicals (μg)					Trap catch (males/trap)		
Z9–14 : OH	Z9–14 : OAc	Z7–14 : OH	Z7–14 : OAc	Z9–14 : Ald	L	N	Total
1000	230	130	30		8.5	103	40.0 a
150	350			1000	2.5	15	6.3 bc
150	350	140		1000	7.0	17	10.3 b
150	350	140	50	1000	19.5	112	46.3 a 0.3 c

[a]Trial conducted from February 1 to May 11, 1988, at Lincoln (L; two replicates) and Appleby Research Orchard, Nelson (N; one replicate). Traps were checked and rerandomized weekly. Means accompanied by the same letter are not significantly different at $P < 0.05$ (Duncan's multiple range test).

TABLE 3. TRAP CATCHES OF MALE *Graphania mutans* TO VARIOUS BLENDS[a]

Dosages of chemicals					Trap catch
Z9–14:OH	Z9–14:OAc	Z7–14:OH	Z7–14:OAc	Z9–14:Ald	(males/trap)
1000	230	130	30		7.4 a
150	350	140	50	1000	0.0 b
1000	230	130	30	1000	0.0 b

[a]Trial conducted from March 30 to June 1, 1988 at MARC, using five replicates. Traps were checked and rerandomized weekly. Means accompanied by the same letter are not significantly different at $P < 0.05$ (Duncan's multiple range test).

Wind Tunnel. Male *G. mutans* from the Auckland colony were flown initially to extract of sex pheromone glands of their own females. Males responded to as little as 0.1 female equivalents (FE) of extract, with 33% (6/18) of the males tested touching the source. The percentage of males touching the source containing 1 FE of extract was greater (53%; 15/28), but not significantly ($P > 0.05$) so. After being placed in the plume of the 1 FE source, males initially remained stationary, with a mean latency to activation of 54 sec (range 6–217.8 sec). Activation consisted of males raising their antennae and fanning their wings, with a mean duration from activation to initiation of flight of 88.8 sec (range 6.6–93.6 sec). Once males took flight, the time of flight (to landing on the source) was relatively short, with a mean of 58.8 sec (range, 42–108 sec).

Three wind-tunnel experiments investigating the effects on male flight responses of varying the amount or ratios of Z9–14:Ald, Z9–14:OH:Z7–14:OH, and Z9–14:OAc:Z7–14:OAc, respectively, were conducted. In each of these experiments, males were flown to a standard reference blend consisting of 10 ng Z9–14:OH, 3 ng Z9–14:OAc, 1.5 ng Z7–14:OH, and 0.5 ng Z7–14:OAc, in addition to the various other blends tested.

An increase in the amount of Z9–14:Ald in the blend had a significant effect on male responses (Figure 1). The addition of 1% (of amount of Z9–14:OH) of Z9–14:Ald to the four-component blend resulted in a significant ($P < 0.05$) decrease in the percentage of males touching the source (74% of males for the four-component blend as opposed to 33% of males for the blend with 1% Z9–14:Ald). Rather than being due solely to a decrease in the percentage of males activating to the respective sources, this difference was the result of arrestment of flight occurring throughout each stage of upwind flight. However, greater amounts of Z9–14:Ald added to the blend reduced the number of males activating and significantly ($P < 0.05$) increased the percentage of arrestment of flight before males had reached the midpoint of the tunnel. Only 5% (1/20)

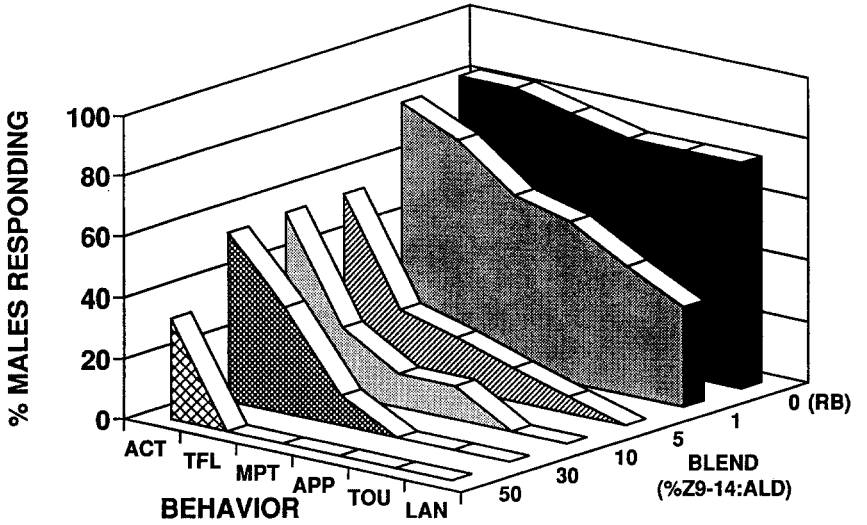


FIG. 1. Percentage of male *Graphania mutans* (Auckland population) responding to a four-component reference blend (RB) plus varying amounts of (*Z*)-9-tetradecenal (Z9–14:Ald), in a wind tunnel. The RB consisted of 10 ng (*Z*)-9-tetradecenol, 3 ng (*Z*)-9-tetradecenyl acetate, 1.5 ng (*Z*)-7-tetradecenol, and 0.5 ng (*Z*)-7-tetradecenyl acetate on filter paper. The responses measured were: activation (ACT), taking flight (TFL), midpoint (MPT), approach (APP), touch (TOU), and landing (LAN); for a detailed description of these responses refer to text.

of the males touched the source containing 5% Z9–14:Ald, and no males touched the sources with blends containing greater amounts of Z9–14:Ald.

Of all the ratios of Z9–14:OH:Z7–14:OH tested (Figure 2) the greatest percentage of males that touched the source did so to the reference blend; this percentage was significantly greater ($P < 0.05$) than the percentages of males that touched the sources containing any of the other blends tested (i.e., with higher or lower ratios of Z9–14:OH:Z7–14:OH). Blends with lower ratios of Z9–14:OH:Z7–14:OH (i.e., with lower amounts of Z9–14:OH) were characterized by greater numbers of males having arrested flight by each of the successive positions in the tunnel. However, to the blend lacking Z7–14:OH, once males had reached the midpoint of the tunnel they continued on to the source without any arrestment of flight.

To the different ratios of Z9–14:OAc:Z7–14:OAc, the greatest percentage of males that landed on the source was to the reference blend ratio (i.e., 3:0.5) (Figure 3). The percentage of males touching the source (64%) containing this blend was significantly greater ($P < 0.05$) than the percentages of males touching the source for all other blends except the 3:1.5 blend (41% of

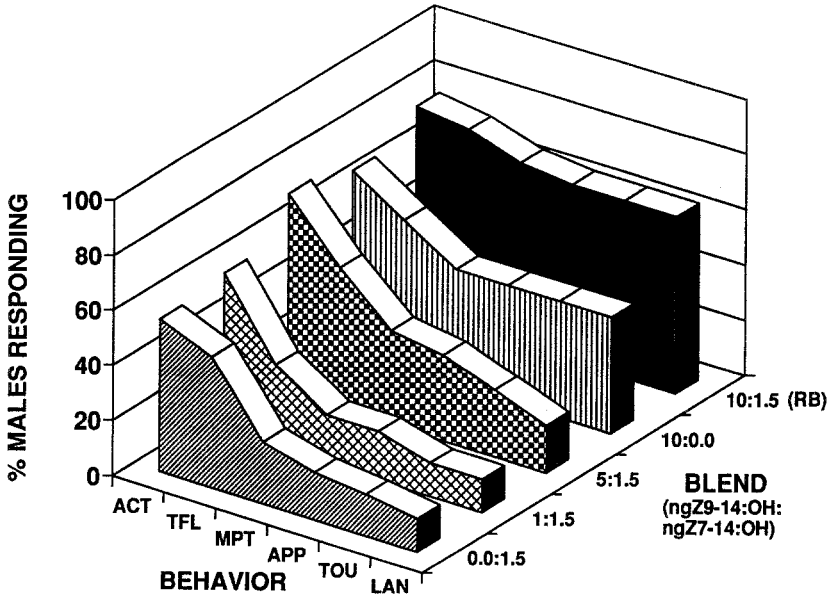


FIG. 2. Percentage of male *Graphania mutans* (Auckland population) responding to a four-component reference blend (RB) and four component blends with varying amounts of (Z)-9-tetradecenol (Z9–14 : OH) and (Z)-7-tetradecenol (Z7–14 : OH), in a wind tunnel. The RB and acronyms are as in Figure 1.

males). Consistent with the lack of catches in the field-trapping trials, blends lacking either Z9–14 : OAc or Z7–14 : OAc failed to elicit any males to touch the source. However, there was a significant ($P < 0.05$) difference between the responses of males to these two blends; the blend lacking Z9–14 : OAc did not activate males, but 50% of the males were activated by the blend lacking Z7–14 : OAc. Arrestment of flight to all blends (except the one lacking Z9–14 : OAc) tended to occur after males had activated and before they had reached the midpoint of the tunnel.

Males of the Auckland and Lincoln cultures were flown to pheromone extract from their own or the other females (Figure 4). Approximately 80% of both Auckland males and Lincoln males flew upwind and landed on the sources containing 1 FE of pheromone extract from their respective females. However, none of the Auckland or Lincoln males flew upwind and touched the source containing pheromone extract of the Lincoln or Auckland females, respectively. This failure to fly upwind and touch the source was generally characterized by low percentages of males activating (25–40%; c.f., 76% and 100% of males of both cultures were activated by extract from their own females). None of the nine (of 21) Lincoln males that were activated by Auckland female pheromone

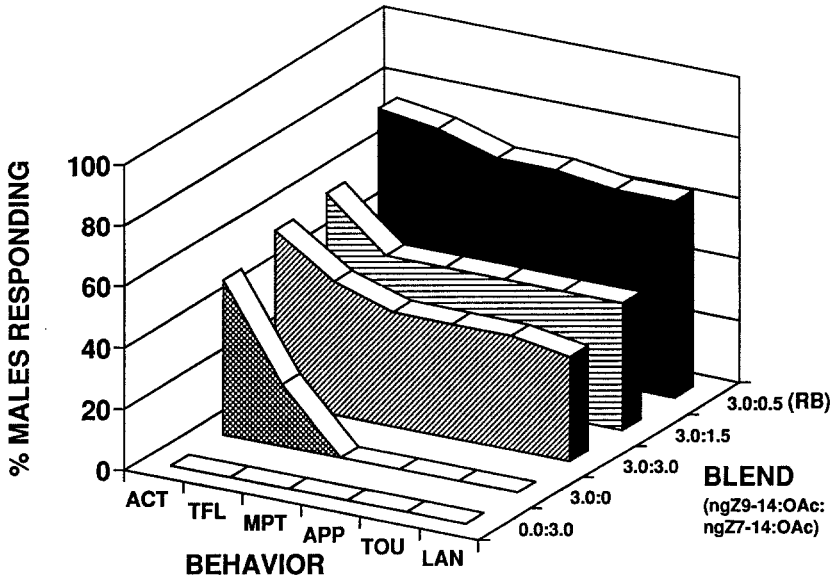


FIG. 3. Percentage of male *Graphania mutans* (Auckland population) responding to a four-component reference blend (RB) and four-component blends with varying amounts of (Z)-9-tetradecenyl acetate (Z9–14 : OAc) and (Z)-7-tetradecenyl acetate (Z7–14 : OAc), in a wind tunnel. The RB and acronyms are as in Figure 1.

extract flew upwind for greater than 20–30 cm, but all five (of 19) of the Auckland males that were activated by Lincoln pheromone extract flew upwind for approximately 30 cm; all of these males arrested upwind flight before the midpoint of the tunnel.

DISCUSSION

By analyzing the sex pheromones of two distinct populations of *G. mutans*, we have shown that this morphologically described species consists of (at least) two taxa that differ in their sex pheromones. In their respective pheromone blends, females of both taxa produce four components in common: Z9–14 : OH,Z7–14 : OH,Z9–14 : OAc, and Z7–14 : OAc, although in somewhat different ratios. However, females of the taxon from Lincoln also produce relatively large amounts of Z9–14 : Ald while females of the taxon from Auckland do not produce this compound. Paralleling pheromone production by the females, males of both taxa also show similarities and differences in responses to the chemicals found in females. Profiles of EAG responses to series of monounsaturated chemicals are quite similar for males of both taxa, suggesting

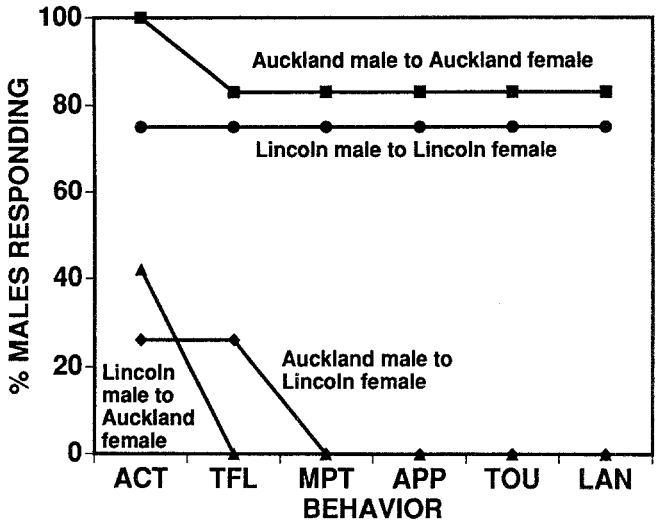


FIG. 4. The flight responses of male *Graphania mutans* from Auckland and Lincoln populations to pheromone extract from females of their own and the other population. The response acronyms are as in Figure 1.

that they share a similar olfactory receptor complement. However, the relative amplitudes of EAG responses to the different series by males of the two taxa suggest that the relative abundances of the different olfactory cells differ between the two types of males. These differences are further matched by the behavioral responses of males in a wind tunnel to pheromone extract from the respective females. Males of either taxon only respond by touching the filter paper source containing pheromone extract of their own females. In response to pheromone extract of the other females, males either did not activate or they arrested flight shortly after take off.

While there are probably other differences, such as the importance of ratios of the various compounds, we believe that a key difference between the sex pheromone systems of the two taxa is the presence and importance of Z9–14 : Ald in the blend of the Lincoln taxon. Although, due to a shortage of insects, we have not fully defined the sex pheromone blend of the Lincoln taxon and, in particular, unequivocally established the role of Z9–14 : Ald, we nevertheless find the evidence for Z9–14 : Ald being a critical element in the sexual communication system of this taxon to be compelling. This compound is the most abundant of the compounds identified in the gland of females of this taxon. Males of this taxon show a high-amplitude EAG response to this compound. In the field trials at Lincoln and Nelson, males were caught in high numbers in traps baited with the four common components plus 1000 μg of Z9–14 : Ald, as

well as in traps containing Z9–14 : Ald and lacking Z7–14 : OAc (in contrast, males of the Auckland taxon were not caught in significant numbers in traps baited with blends lacking Z7–14 : OAc). Finally, in the wind-tunnel experiment using extracts, the difference in response between males of the two taxa to their respective females' extract correlated with the major observed difference in extract from these females being Z9–14 : Ald content (i.e., either none or a relatively large amount). Therefore, the circumstantial evidence suggests that Z9–14 : Ald is a critical component in the sex pheromone of this taxon. If this is accepted, then it follows that the field-trapping results at Nelson and Lincoln, where males were caught in high numbers in traps baited with either the Lincoln-type blend or the Auckland-type blend is the result of both taxa being present in sympatry in these two locations. Further evidence in support of this comes from the field trial in Lincoln where traps baited with the Lincoln-type blend caught more than twice the number of males than traps baited with the Auckland type blend; unfortunately, only two replicates were used at Lincoln and so this difference was not statistically significant.

In contrast to the proposed effect of Z9–14 : Ald in the blend to Lincoln males, the presence of even small amounts of Z9–14 : Ald in the common four-component mix appears to inhibit the flight responses or field catches of *G. mutans* males from Auckland. Likewise, in field-trapping trials, the five-component blend that gave good catches of males in both Lincoln and Nelson did not catch any males in Auckland. Clearly, the very high relative proportion of Z9–14 : Ald produced by Lincoln females would result in the complete inhibition of upwind flight responses of the males of the Auckland taxon, as was observed in the wind tunnel using female extracts.

The effect of Z9–14 : Ald in blends on males of the two *G. mutans* taxa is contrasting; in one taxon (the Lincoln) it serves as a component (''major'') of the pheromone, in the other taxon (Auckland) it results in the arrestment of upwind flight responses when added to the pheromone blend. Compounds that are observed to have the latter function are usually viewed as ''reproductive isolating mechanisms'' between closely related species (in the sense outlined by Cardé 1986) and more recently as ''behavioral antagonists'' (Linn et al., 1988; Glover et al., 1989; Linn and Roelofs, 1989), i.e., their use in pheromone systems has evolved as a direct adaptation to ensure that so-called ''mating mistakes'' are reduced or eliminated. An alternative, more parsimonious explanation for the evolution of such compounds, based on the ''specific mate recognition'' theory of Paterson (1985), would be that Z9–14 : Ald has evolved in mate recognition in the Lincoln taxon. Therefore, the second function of this compound, as an intertaxon isolating mechanism or behavioral antagonist, would arise as an incident of recognition in the Lincoln taxon. Appealing as these possibilities are, neither parsimony nor the coincidence of the compound apparently eliciting different and exclusive behavioral responses from males of the

two taxa are evolutionary proof. Nevertheless, the two *G. mutans* taxa may offer an excellent example to study, and therefore test, specific mate recognition, reproductive isolation, or behavioral antagonism.

Given these differences between the pheromone systems of these two taxa, it appears that the two *G. mutans* taxa may in fact be distinct sibling species. This phenomenon in *G. mutans* is similar to that observed during similar studies on endemic New Zealand tortricid pests. These studies, led by sex pheromone analyses of different populations, showed that the previously described *Planotortrix excessana* and *Ctenopseustis obliquana* actually consist of two and three sibling species, respectively (Dugdale, 1990; Foster et al., 1991). These sibling species differ somewhat in their allozyme patterns and are morphologically very similar. However, there are major differences between their pheromone systems. The difference in the pheromonal communication systems of the two taxa of *G. mutans* is similarly striking, although, so far, no morphological difference between these two taxa has been detected (Dugdale, personal communication). More complete characterization of the sex pheromone of the Lincoln taxon, and further examination of the morphology, ecology, and allozyme patterns of these two taxa of *G. mutans* is planned and, it is hoped, will establish whether they should be considered as distinct sibling species.

In addition to elucidating the distinct pheromone taxa within *G. mutans*, we have identified and chemically and behaviorally characterized the sex pheromone of a *G. mutans* population from Auckland. The sex pheromone of this taxon consists of (at least) a four-component blend of two alcohols and two acetates. The most abundant compound identified in the gland was *Z*9–14 : OH; however, somewhat surprisingly, in our bioassays we found that this component could be removed from the blend without the complete suppression of either field catches or the upwind flight responses of males. In contrast, two of the less abundant components in the gland (*Z*9–14 : OAc and *Z*7–14 : OAc) could not be removed from the blend without complete suppression of field catches or arrestment of flight before the midpoint of the wind tunnel. At this time we do not have any information regarding the relative ratios of the compounds released in the effluvia of female *G. mutans*. However, it is interesting that the relative abundance of *Z*9–14 : OH in the gland does not correlate with a "major" behavioral function in the sense usually assigned to the most abundant component in a blend in pheromone studies. Rather this function is apparently served by *Z*7–14 : OAc and *Z*9–14 : OAc.

The fourth component in the blend of this taxon, *Z*7–14 : OH, when added in large amounts to the three-component blend significantly reduced trap catches. However, when *Z*7–14 : OH was completely removed from the blend, trap catches decreased slightly but not significantly. The effect of this component in the blend at relative ratios close to that found in the female was established in the wind tunnel. A comparative study using field-trapping techniques generally

observes differences in the end results of behavioral responses rather than differences in the actual behavioral responses themselves (Kennedy, 1977). Consequently, subtle effects in behavior elicited by chemicals are less likely to be detected by field-trapping techniques than by more direct, discriminating bioassays such as wind tunnels (Baker and Linn, 1984). By use of the wind tunnel we were able to establish the importance of *Z*7–14 : OH in the pheromone blend of *G. mutans*; the absence of this compound from the reference blend (i.e., four components) increased the percentage of males arresting flight before the midpoint of the tunnel and therefore resulted in a decrease in the percentage of males touching the source. The wind tunnel study also confirmed that the other three components elicited behavioral activity. Thus the absence of either *Z*9–14 : OAc or *Z*7–14 : OAc from the blend significantly decreased the percentages of males activating and taking flight relative to the four component blend, while significantly fewer males reached the midpoint of the tunnel when presented with the blend lacking *Z*9–14 : OH.

In many species of moths the ratios of pheromone components produced by females are regulated within fairly tight ranges that usually correspond with the ratios of these components to which males respond optimally (see for example Miller and Roelofs, 1980; Roelofs et al., 1975). Our data suggest that this is the case with *G. mutans*. Our initial formulation of a synthetic blend was based on the ratios observed in females. When various ratios in this blend were changed, generally the responses of males, as measured by trap catches and flight-tunnel behavior, decreased. In the flight tunnel, changes in the relative amounts of any of the four components were characterized by males arresting flight after activation and before reaching the midpoint of the tunnel rather than by arrestment at later stages (near the source) of upwind flight. A further study looking at more subtle changes in the various ratios along with examining the variability in production of the four components between individual females is planned.

The pheromone blends for both taxa of *G. mutans* can now be used for monitoring this pest in orchards. This should allow a better understanding of the pest status of both taxa and hence control programs can be modified for more effective control of these pests as necessary. The use of these two pheromone blends should also facilitate work examining the distribution of these two taxa around New Zealand.

Acknowledgments—We are very grateful to Mr. J.S. Dugdale for insects, advice, and assistance throughout the course of this work. Thanks are due to Dr. D.M. Suckling, Messrs W.P. Thomas, G. Burnip, D. Rogers, and Ms. A. Barrington, and Ms. D.C. Whiting for collecting or supplying insects used in this work; and also to Mr. P.W. Shaw and Dr. D.M. Suckling for running the field trails at Nelson and Lincoln. Thanks also to Mr. C. Malosse for recording the mass spectra. The visit of B.F. was supported by a grant from I.N.R.A.

REFERENCES

BAKER, T.C., and LINN, C.E. 1984. Wind tunnels in pheromone research, pp. 45–73, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research. Springer, New York.

CARDÉ, R.T. 1986. The role of pheromones in reproductive isolation and speciation in insects, pp. 303–317, *in* M.D. Huettel (ed.). Evolutionary Genetics of Invertebrate Behavior. Plenum Press, New York.

DUGDALE, J.S. 1988. Lepidoptera-annotated catalogue, and keys to family-group taxa. Fauna of New Zealand *14*. 262 pp., SIPC, DSIR, Wellington.

DUGDALE, J.S. 1990. Reassessment of *Ctenopseustis* Meyrick and *Planotortrix* Dugdale with descriptions of two new genera (Lepidoptera: Tortricidae). *N.Z. J. Zool.* 17:437–465.

FOSTER, S.P., DUGDALE, J.S., and WHITE, C.S. 1991. Sex pheromones and the status of the green-headed and brownheaded leafroller moths in New Zealand. *N.Z. J. Zool.* 18:63–74.

GLOVER, T.J., PEREZ, N., and ROELOFS, W.L. 1989. Comparative analysis of sex pheromone response antagonists in three races of European corn borer. *J. Chem. Ecol.* 15:863–873.

HEATH, R.R., BURNSED, G.E., TUMLINSON, J.H., and DOOLITTLE, R.E. 1980. Separation of a series of positional and geometrical isomers of olefinic aliphatic primary alcohols and acetates by capillary gas chromatography. *J. Chromatogr.* 189:199–208.

KENNEDY, J.S. 1977. Behaviorally discriminating assays of attractants and repellents, pp. 215–229, *in* H.H. Shorey, and J.J. McKelvey Jr. (eds.). Chemical Control of Insect Behavior: Theory and Application. Wiley, New York.

LINN, C.E., and ROELOFS, W.L. 1989. Response specificity of male moths to multicomponent pheromones. *Chem. Senses* 14:421–437.

LINN, C.E., HAMMOND, A., DU, J.W., and ROELOFS, W.L. 1988. Specificity of male response to multicomponent pheromones in noctuid moths *Trichoplusia ni* and *Pseudoplusia includens*. *J. Chem. Ecol.* 14:47–57.

MILLER, J.R., and ROELOFS, W.L. 1978. Sustained-flight tunnel for measuring insect responses to wind-borne sex pheromones. *J. Chem. Ecol.* 4:187–198.

MILLER, J.R., and ROELOFS, W.L. 1980. Individual variation in sex pheromone component ratios in two populations of the redbanded leafroller moth, *Argyrotaenia velutinana*. *Environ. Entomol.* 9:359–363.

PATERSON, H.E. 1985. The recognition concept of species, pp. 21–29, *in* E.S. Vrba (ed.). Species and Speciation. Transvaal Museum Monograph No. 4. Transvaal Museum, Pretoria.

POITOUT, S., and BUES, R. 1970. Elevage de plusieurs especes de Lépidopteres Noctuidae sur milieu artificiel riche et sur milieu artificiel simplifie. *Ann. Zool. Ecol. Anim.* 1:245–264.

RENOU, M. 1979. Contribution à l'étude de la comunication phéromonal chez trois tineeides ravageurs des culutures: *Acrolepiopsis assesctella* (Z.), *Scrobipalpa ocellatella* (Boyd), *Prays oleae* (Bern.). Thèse de 3ème cycle, Paris VI, 161 pp.

ROELOFS, W.L. 1984. Electroantennogram assays: rapid and convenient screening procedures for pheromones, pp. 131–159, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research. Springer, New York.

ROELOFS, W.L., HILL, A., and CARDÉ, R.T. 1975. Sex pheromone components of the redbanded leafroller, *Argyrotaenia velutinana* (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 1:83–89.

SUCKLING, D.M. 1989. Insect sex attractants keep pests at bay. *Orchardist N.Z.* 62:17–19.

SUCKLING, D.M., THOMAS, W.P., BURNIP, G.M., and ROBSON, A. 1990. Monitoring lepidopterous pests at two Canterbury orchards. Proceedings, 43rd New Zealand Weed and Pest Control Society Conference, pp. 322–327.

WEARING, C.H., THOMAS, W.P., DUGDALE, J.S., and DANTHANARAYANA, W. 1991. Australian and New Zealand species, pp. 453–472, in L. Van der Geest and H.H. Evenhuis (ed.). Tortricid Pests, Their Biology, Natural Enemies and Control. Elsevier, Amsterdam.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

SEX ATTRACTANTS AND SEX PHEROMONE COMPONENTS OF NOCTUID MOTHS *Euclidea cuspidea*, *Caenurgina distincta*, AND GEOMETRID MOTH *Eupithecia annulata*[1]

J.G. MILLAR,[2,*] M. GIBLIN,[3] D. BARTON,[3] J.W. WONG,[2,4] and E.W. UNDERHILL[3]

[2]*Department of Entomology*
University of California
Riverside, California 92521

[3]*Plant Biotechnology Institute*
110 Gymnasium Road
Saskatoon SASK S7N 0W9, Canada.

(Received April 26, 1991; accepted July 1, 1991)

Abstract—Pheromone components and sex attractant blends consisting of 3*Z*,6*Z*,9*Z*-triene hydrocarbons and racemic and chiral forms of 3*Z*,6*Z-cis*-9,10-epoxydienes have been elucidated for two noctuid and one geometrid moth species. Male *Euclidea cuspidea* moths were attracted to blends of 3*Z*,6*Z*,9*Z*-heneicosatriene (3*Z*,6*Z*,9*Z*-21 : H) with 3*Z*,6*Z-cis*-9,10-epoxyheneicosadiene (3*Z*,6*Z-cis*-9,10-epoxy-21 : H). In addition to these compounds, 3*Z*,6*Z*,9*Z*-20 : H, and two regioisomeric C_{21} epoxides were tentatively identified in pheromone gland extracts. *Caenurgina distincta* moths were attracted by an 8 : 1 blend of 3*Z*,6*Z*,9*Z*-20 : H with 3*Z*,6*Z-cis*-9,10-epoxy-20 : H. *Eupithecia annulata* moths were attracted by either 3*Z*,6*Z-cis*-9,10-epoxy-20 : H or 3*Z*,6*Z-cis*-9,10-epoxy-21 : H, and by the 9*S*,10*R* enantiomer of each epoxide. 3*Z*,6*Z*,9*Z*-21 : H and 3*Z*,6*Z-cis*-9,10-epoxy-21 : H were tentatively identified from pheromone glands. Pheromone components were identified by a combination of coupled gas chromatography–electroantennography, gas chromatography–mass spectrometry, and field bioassays.

Key Words—Pheromone, sex attractant, Noctuidae, Geometridae, epoxide,

*To whom correspondence should be addressed.

[1]Issued as NRCC #32477.

[4]Present address: Pharmaceutical Production Research Facility, 3553 31st Street N.W., Discovery Place 1, Calgary, ALTA T2L 2K7, Canada.

0098-0331/91/1100-2095$06.50/0

triene hydrocarbon, 3*Z*,6*Z*,9*Z*-eicosatriene, 3*Z*,6*Z*,9*Z*-heneicosatriene, 3*Z*,6*Z*-*cis*-9,10-epoxyeicosadiene, 3*Z*,6*Z*-*cis*-9,10-epoxyheneicosadiene.

INTRODUCTION

The first diene monoepoxide pheromone component, 3*Z*,6*Z*-*cis*-9,10-epoxyheneicosadiene (3*Z*,6*Z*-*cis*-9,10-epoxy-21:H; subsequent abbreviations follow this model), in combination with 9*Z*,12*Z*-octadecadienal (9*Z*,12*Z*-18:Al) and 9*Z*,12*Z*,15*Z*-18:Al, was identified in pheromone gland extracts and effluvia of the saltmarsh caterpillar moth, *Estigmene acrea* Drury (Lepidoptera: Arctiidae) (Hill and Roelofs, 1981). The biologically active epoxide enantiomer was subsequently determined to have the 9*S*,10*R* configuration (Mori and Ebata, 1981). Since that time, this compound, or homologs of it, have been identified as sex attractants or pheromone components for a number of lepidopteran species. For example, 3*Z*,6*Z*-*cis*-9,10-epoxy-21:H has been reported as a sex pheromone component of the arctiid species *Creatonotos transiens* L., *C. gangis* Walker (Bell and Meinwald, 1984), *Antichloris viridis* (Meyer, 1984), *Hyphantrea cunea* Drury (Hill et al., 1982; Einhorn et al., 1982), and *Phragmatobia fuliginosa* L. (Descoins and Frérot, 1984). This compound also has been identified in female pheromone gland extracts from the arctiids *Tyria jacobaeae* and *Cymbalophora pudica* Esper, and it was shown to elicit strong electroantennogram responses from male moth antenna, although no data on its attractiveness to male moths was reported (Frérot et al., 1988a,b). This C_{21} epoxide, or the C_{18-20} homologs, also have been reported as sex attractants for several geometrid and noctuid moth species (Wong et al., 1985; Kovalev and Nikolaeva, 1986).

Racemic and chiral regioisomers of the *cis*-9,10-epoxydienes, 3*Z*,9*Z*-*cis*-6,7- and 6*Z*,9*Z*-*cis*-3,4-epoxydienes, also have been reported as sex attractants and sex pheromone components for a number of geometrid and noctuid species (Becker et al., 1990; Millar et al., 1987, 1990 a–d; Hansson et al., 1990). The behavioral response to the compounds is often synergized or antagonized by the corresponding 3*Z*,6*Z*,9*Z*-trienes.

Preliminary data on the attraction of male moths of a number of noctuid and geometrid species to lures containing blends of 3*Z*,6*Z*,9*Z*-trienes, alone or in combination with mixtures of racemic *cis*-monoepoxydienes (combined monoepoxides, CME-3*Z*,6*Z*,9*Z*-X:H, X = 19–21), or racemic and enantiomeric forms of 19- to 21-carbon 3*Z*,6*Z*-*cis*-9,10-epoxydienes were published several years ago (Wong et al., 1985). Since that time, we have investigated the pheromone chemistry of several of these species in more detail. We report here: (1) the identification of sex pheromone components for the geometrid species *Eupithecia annulata* Hulst and the noctuid species *Euclidea cuspidea* Hübner, by a combination of coupled gas chromatography–electroantennogram detection (GC-EAD), gas chromatography–mass spectrometry (GC-MS), and

comparisons with synthetic standards; (2) the elucidation and field testing of sex pheromone and sex attractant blends for the above two species, and for *Caenurgina distincta* Cram; and (3) the determination of the chirality of the epoxydienes that are attractive to the above species.

METHODS AND MATERIALS

Insects, Electroantennography, and Pheromone Identification. Female moths of unknown mating status were collected from the field by sweep-netting or black-light trapping and used as a source of pheromone. Males were captured as described above or in sticky wing-traps (Phero Tech, Vancouver, British Columbia; traps are similar to Pherocon 1C traps from Scentry, Inc., Buckeye, Arizona). Preparation and analysis of pheromone gland extracts by coupled GC-EAD and coupled GC-MS was carried out as previously described (Millar et al., 1987, 1990d). GC-MS analyses were carried out with a Finnigan 4000E instrument interfaced to an INCOS 2300 data system, in electron impact (70 eV) or chemical ionization (isobutane) modes. DB-5 (50 m × 0.32 mm; J&W Scientific, Folsom, California) and Ultra-2 (Hewlett-Packard, Avondale, Pennsylvania) capillary columns were used with He carrier gas, programmed from 40 to 250°C. GC-EAD studies were carried out with a Hewlett-Packard 5910 GC equipped with either a DB-1701 or a DB-5 capillary column (30 m × 0.32 mm, J&W Scientific). The column effluent was split 7:3 in favor of the FID detector, and signals from the EAD and FID signals were recorded simultaneously with a matched pair of Hewlett-Packard 3392A integrators. Injections were made in splitless mode, programmed from 40 to 225°C.

Insect Trapping. Field experiments were carried out in a mixed forest area (black spruce, jack pine, willow, aspen) approx. 100 km northeast of Saskatoon, Saskatchewan. Phero Tech wing-traps were used. Details of lure preparation (red rubber septa), and the setup of field survey traps and field experiments were as previously described (Millar et al., 1990d). Trap captures were transformed $[(x + 1)^{1/2}]$ and subjected to analysis of variance. The homogeneity of the variances of the transformed means was confirmed with Bartlett's test (Sokal and Rohlf, 1981). Treatments with zero captures overall were not included in the ANOVA, as replicates within a treatment must have some variance to satisfy the assumptions of the ANOVA test. Significantly different means were separated by Duncan's (1955) multiple-range test.

Synthetic Chemicals. The syntheses of the 3*Z*,6*Z*,9*Z*-trienes (Underhill et al., 1983) and the enantiomerically enriched forms of the 3*Z*,6*Z*-*cis*-9,10-epoxydienes (Wong et al., 1985) have been reported previously. The chemical purity of all compounds used was >98% by capillary GC. The enantiomerically enriched 3*Z*,6*Z*-*cis*-9,10-epoxydienes had chiral purities of 93% ee (9*R*,10*S* series) and 92% ee (9*S*,10*R* series) respectively (Wong et al., 1985).

RESULTS

Euclidea cuspidea *Hübner*. The preliminary report by Wong et al. (1985) suggested that males of this species were attracted by blends of 3Z,6Z,9Z-21 : H in combination with one or more C_{21} monoepoxydienes. Analysis of a female pheromone gland extract by GC-EAD elicited three strong responses from a male moth antenna at retention times corresponding to 3Z,6Z,9Z-20 : H, 3Z,6Z,9Z-21 : H, and 3Z,6Z-*cis*-9,10-epoxy-21 : H (Figure 1). The concurrent flame ionization detection (FID) trace showed peaks at these retention times, and two further peaks at retention times corresponding to 3Z,9Z-*cis*-6,7-epoxy-21 : H and 3Z,9Z-*cis*-3,4-epoxy-21 : H. The five compounds were present in the ratio of 3Z,6Z,9Z-20 : H (1.6), 3Z,6Z,9Z-21 : H (100), 3Z,6Z-*cis*-9,10-epoxy-

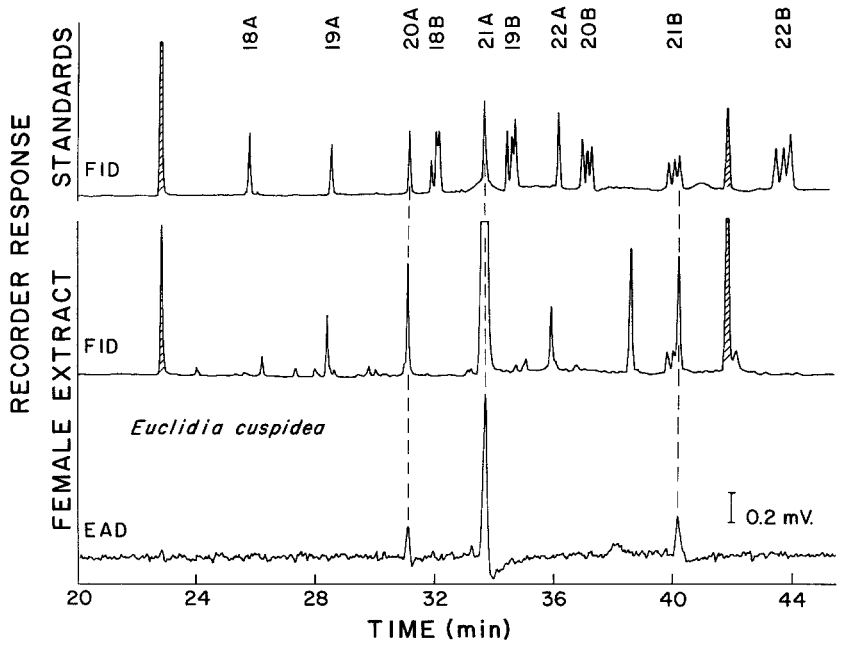


FIG. 1. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD) responses of *Euclidea cuspidea* male antennae in GC-EAD experiments. Upper trace is an FID trace of a mixture of C_{18-22} 3Z,6Z,9Z-trienes (designated by the letter A and preceded by the carbon chain length) and their monoepoxydiene analogs (designated with the letter B); the latter eluted from the 30-m DB-1701 column in the order 6,7, 3,4, and 9,10 monoepoxydiene. Internal standards (heptadecane and tetracosane) are shaded. Lower pair of traces are in response to a pheromone gland extract from conspecific females.

21:H (2), 3*Z*,9*Z*-*cis*-6,7-epoxy-21:H (0.4), and 6*Z*,9*Z*-*cis*-3,4-epoxy-21:H (0.4).

The presence of all five components in pheromone gland extracts was confirmed with both selected ion monitoring (SIM) and full-scan chemical ionization (isobutane) mass spectrometry. Exact retention time matches were obtained for all five compounds versus synthetic standards, and full-scan Cl mass spectral matches were obtained for 3*Z*,6*Z*,9*Z*-21:H and 3*Z*,6*Z*-*cis*-9,10-epoxy-21:H versus standards (Figures 2 and 3). The isobutane Cl mass spectrum of 3*Z*,6*Z*,9*Z*-21:H (Figure 2) was characterized by a base peak at *m/z* 289 $(M + H)^+$, and adducts at *m/z* 347 $(M + C_4H_9)^+$, and 291 $(M + H)^+$. Fragments at *m/z* 277, 263, 249, and 235 may be attributed to sequential losses of C_nH_{2n} (n = 5–8) from the *m/z* 347 adduct ion. Ions at *m/z* 123, 137, 151, 165, and 179 may be attributed to a series of homologous fragments ($C_nH_{2n-4} + H^+$, n = 10–14) containing the three unsaturations. Fragments at *m/z* 211 and 223 were not readily assignable.

The isobutane Cl spectrum of 3*Z*,6*Z*-*cis*-9,10-epoxy-21:H (Figure 3) was much simpler, with a base peak at *m/z* 307 $(M + H)^+$ and a large fragment at *m/z* 289 $(M + H - H_2O)^+$. Diagnostic ions arising from rearrangement and cleavage of the epoxide functionality were present at *m/z* 123 $(C_9H_{15})^+$ and 183 $(C_{12}H_{23}O)^+$, locating the epoxide at the 9,10 position. The potential M + 57 adduct at *m/z* 363 was beyond the scan range used (120–350 amu).

The full-scan isobutane Cl spectra of the other three components were not as good due to the low levels at which they were present, but the spectra clearly matched those of the synthetic standards. In particular, the peak corresponding to 3*Z*,6*Z*,9*Z*-20:H gave strong fragment ions at *m/z* 333 (M + 57), 277 (M+1), and 275 (M−1), and the peaks corresponding to 3*Z*,9*Z*-*cis*-6,7-epoxy-21:H and 6*Z*,9*Z*-*cis*-3,4-epoxy-21:H gave strong ions at *m/z* 307 (M+1) and *m/z* 289 (M+1−18).

Wong et al. (1985) had demonstrated that 3*Z*,6*Z*,9*Z*-21:H and CME-3*Z*,6*Z*,9*Z*-21:H were not active as single components, so our field tests began with blends of 3*Z*,6*Z*,9*Z*-21:H with epoxides. In the first experiment (replicated three times), lures containing 3*Z*,6*Z*,9*Z*-21:H + 3*Z*,6*Z*-*cis*-9,10-epoxy-21:H (450:20 μg) attracted 21 moths, while the corresponding blends with the *cis*-6,7- and *cis*-3,4-epoxide regioisomers caught none, demonstrating that the 9,10 epoxide was the attractive regioisomer. Furthermore, lures containing 3*Z*,6*Z*,9*Z*-21:H in combination with 3*Z*,6*Z*,9*S*,10*R*-epoxy-21:H (450:10 μg) attracted 42 moths, while the corresponding lure containing the 9*R*,10*S* enantiomer attracted no moths, indicating that the attraction was enantiospecific.

Experiments conducted in 1985 confirmed these data (Table 1). Mixtures of 3*Z*,6*Z*,9*Z*-21:H with the enantiomers of 3*Z*,6*Z*-*cis*-9,10-epoxy-21:H, in a series of ratios bracketing the blend ratio found in pheromone gland extracts were tested, and the blends with the 9*S*,10*R* enantiomer were significantly more

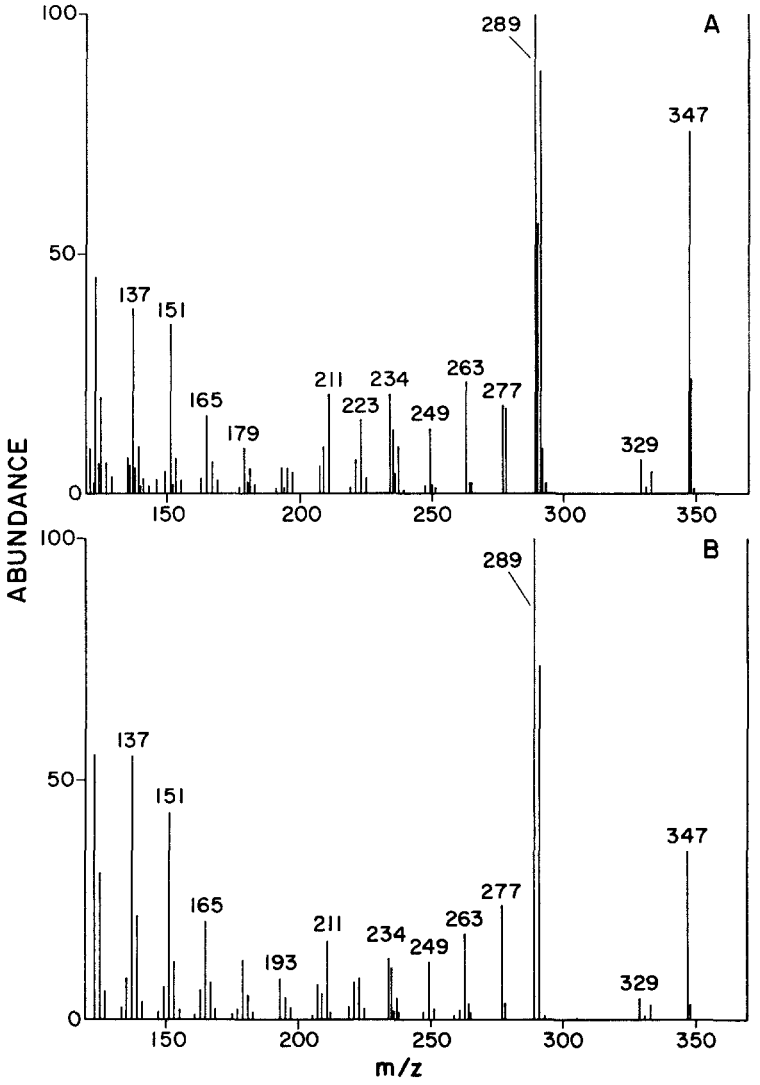


FIG. 2. Chemical ionization mass spectra (isobutane) of 3*Z*,6*Z*,9*Z*-heneicosatriene: (A) synthetic standard; (B) compound from *E. cuspidea* female pheromone gland extract.

attractive than those with the 9*R*,10*S* enantiomer. A blend of 3*Z*,6*Z*,9*Z*-21 : H with CME-21 : H (500 : 50 μg) was as attractive as the blend of 3*Z*,6*Z*,9*Z*-21 : H with 3*Z*,6*Z*-9*S*,10*R*-epoxy-21 : H, indicating that the other epoxide regioisomers were not antagonistic.

Further tests were conducted with a basic lure composed of 3*Z*,6*Z*,9*Z*-

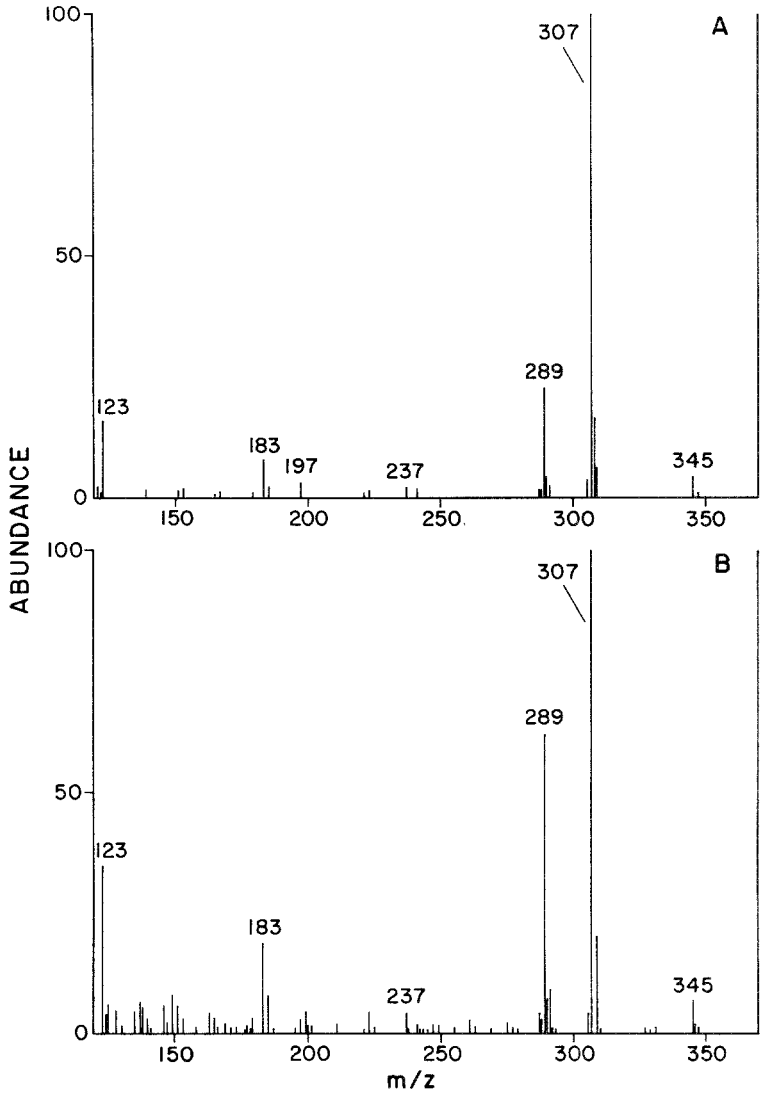


FIG. 3. Chemical ionization (isobutane) mass spectra of 3*Z*,6*Z*-*cis*-9,10-epoxyheneicosadiene: (A) synthetic standard; (B) compound from *E. cuspidea* pheromone gland extract.

TABLE 1. ATTRACTION OF *E. cuspidea* TO BLENDS OF 3Z,6Z,9Z-21 : H AND ENANTIOMERS OF 3Z,6Z-cis-9,10-EPOXY-21 : H[a]

Lure (μg)	Males captured
3Z,6Z,9Z-21 : H (500) + 3Z,6Z-9*R*,10*S*-epoxy-21 : H (1)	1a
3Z,6Z,9Z-21 : H (500) + 3Z,6Z-9*R*,10*S*-epoxy-21 : H (10)	1a
3Z,6Z,9Z-21 : H (500) + 3Z,6Z-9*R*,10*S*-epoxy-21 : H (50)	17b
3Z,6Z,9Z-21 : H (500) + 3Z,6Z-9*S*,10*R*-epoxy-21 : H (1)	2a
3Z,6Z,9Z-21 : H (500) + 3Z,6Z-9*S*,10*R*-epoxy-21 : H (10)	39c
3Z,6Z,9Z-21 : H (500) + 3Z,6Z-9*S*,10*R*-epoxy-21 : H (50)	49c
3Z,6Z,9Z-21 : H (500) + CME-3Z,6Z,9Z-21 : H (50)	45c

[a] Traps relicated three times and set out from June 1 to July 10, 1985. Values followed by the same letter are not significantly different ($P > 0.05$).

21 : H and 3Z,6Z,9*S*,10*R*-epoxy-21 : H (450 : 10 μg) spiked with varying amounts of 3Z,6Z,9Z-20 : H (1, 10, 50, or 100 μg), and the enantiomers of 3Z,9Z-*cis*-6,7-epoxy-21 : H (5 or 50 μg). There were no significant differences in trap captures between the basic lure and the adulterated lures; that is, the C_{20} triene and the 3Z,9Z-*cis*-6,7-epoxy-21 : H enantiomers have no apparent biological activity as attractants for this species despite being present in the pheromone gland.

Caenurgina distincta *Cram.* Blends of 3Z,6Z,9Z-20 : H with 3Z,6Z-*cis*-9,10-epoxy-20 : H have been implicated as potential sex pheromone components for this species (Wong et al., 1985). GC-EAD analysis of a pheromone gland extract from a field-collected female moth showed strong antennal responses from a male antenna at retention times corresponding to 3Z,6Z,9Z-19 : H, 3Z,6Z,9Z-20 : H, 3Z,6Z,9Z-21 : H, 3Z,6Z-*cis*-9,10-epoxy-20 : H, and 3Z,6Z-*cis*-9,10-epoxy-21 : H (Figure 4). The concurrent FID trace showed discernible peaks only at the retention times of the C_{20} and C_{21} trienes, indicating that the quantities of the other components in the extract eliciting antennal responses were very small. When challenged with synthetic standards, a male antenna demonstrated strong responses to C_{19}–C_{21} trienes, 3Z,6Z-*cis*-9,10-epoxydienes, and 3Z,9Z-*cis*-6,7-epoxydienes (Figure 4, upper traces).

A number of two-component blends of 3Z,6Z,9Z-20 : H with 3Z,6Z,9Z-21 : H were found to be minimally attractive and not significantly different from 3Z,6Z,9Z-20 : H alone. In like fashion, blends of 3Z,6Z,9Z-20 : H with the shorter-chain EAG-active homolog 3Z,6Z,9Z-19 : H had been demonstrated to have no synergistic effects (Wong et al., 1985).

A field test in 1985 (replicated four times) demonstrated that 3Z,6Z-9*S*,10*R*-epoxy-20 : H was the more attractive of the two enantiomers, as a 1 : 8 blend of epoxide–3Z,6Z,9Z-20 : H attracted 59 male moths, while the corresponding

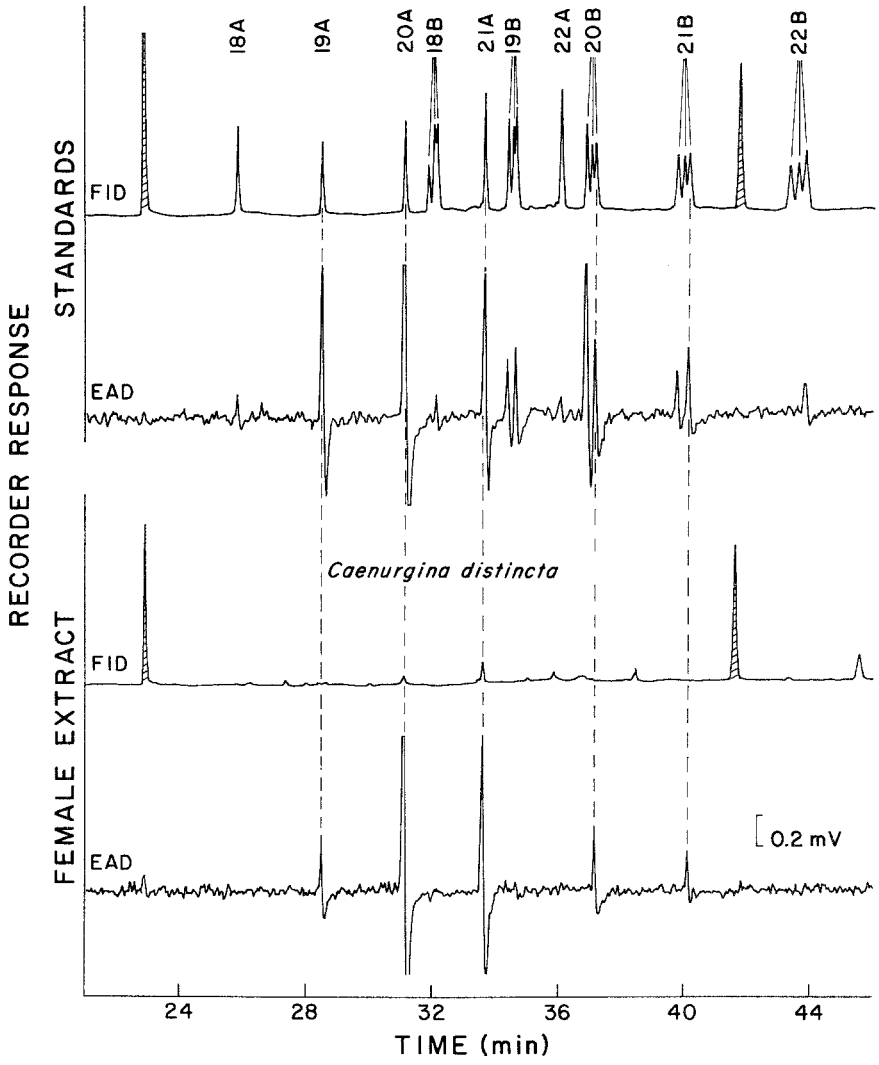


FIG. 4. Response of *Caenurgina distincta* male antennae in GC-EAD experiments using a DB-1701 column. Upper pair of traces are in response to a mixture of C_{18-22} synthetic standards; lower pair are in response to a pheromone gland extract from a conspecific female. Number and letter designations are identical to those of Figure 1. Internal standards (heptadecane and tetracosane) are shaded.

blend with the 9*R*,10*S* enantiomer attracted only five moths. Concurrently, lures containing racemic 3*Z*,6*Z*-*cis*-9,10-epoxy-20:H in combination with 3*Z*,6*Z*,9*Z*-20:H (1:4 blend) attracted 55 moths, suggesting that the 9*R*,10*S* enantiomer was not antagonistic.

Because a strong antennal response had been elicited by 3*Z*,9*Z*-*cis*-6,7-epoxy-20:H, the effect of adding the enantiomers of this compound to an attractive lure blend (3*Z*,6*Z*,9*Z*-20:H + 3*Z*,6*Z*-*cis*-9,10-epoxy-20:H, 400:100 μg) was tested. Additions of 5 or 50 μg of either 6,7-epoxide enantiomer had no discernible effect on the attractiveness of the basic blend.

Eupithecia annulata *Hulst (Geometridae).* The preliminary report of sex attractants for males of this species had implicated monoepoxydienes of chain lengths C_{19-21}, but moths were attracted by a variety of lures, and there was no clear indication of a specific attractant or attractants (Wong et al., 1985).

Further evidence for the role of C_{19-21} monoepoxydienes was obtained from electroantennogram studies with the enantiomers of the *cis*-3,4-, -6,7-, and -9,10-monoepoxydienes of chain length C_{18-22}. Only the *cis*-9,10 epoxides elicited responses greater than 1 mV. The C_{19-22} 3*Z*,6*Z*-9*R*,10*S*-monoepoxydienes elicited male antennal responses of 0.9 $\pm$ 0.1 mV (N = 2), 3.9 $\pm$ 1.7 mV (N = 8), 1.7 $\pm$ 0.6 mV (N = 8), and 0.6 $\pm$ 0.2 mV (N = 2) respectively. The corresponding 9*S*,10*R* series elicited responses of 2.1 $\pm$ 1.1 mV (N = 3), 3.0 $\pm$ 0.8 mV (N = 8), 3.6 $\pm$ 1.1 mV (N = 8), and 1.4 $\pm$ 0.6 mV (N = 3).

GC-EAD studies were conducted with pheromone gland extracts from three field-collected female moths of unknown mating status. The extract from the first female elicited a male antennal response at the retention time of 3*Z*,6*Z*-*cis*-9,10-epoxy-21:H. This was corroborated by analysis of the extracts from the second (Figure 5) and third females, and additional antennal responses were seen at the retention times of 3*Z*,6*Z*,9*Z*-21:H (both extracts) and 3*Z*,6*Z*-*cis*-9,10-epoxy-20:H (second extract only). An FID peak was detected concurrently for the triene in the second extract (Figure 5), but the region of the FID chromatogram where the epoxides eluted was obscured by late-eluting compounds from the previous extract. 3*Z*,6*Z*,9*Z*-20:H was not detected in the extracts either by the FID detector or the antennae; the small peak seen on the FID trace (Figure 5) in the vicinity of the 3*Z*,6*Z*,9*Z*-20:H had a retention time sufficiently different from that of 3*Z*,6*Z*,9*Z*-20:H to be discernible (0.13 min; run-to-run variability of retention times of standards was determined to be $\leq$0.02 min).

When a male antenna was challenged with synthetic standards, strong responses were elicited by the C_{19}–C_{21} *cis*-9,10 epoxides, with weaker responses to the C_{20} and C_{21} trienes and also to the 19–21 carbon 3*Z*,9*Z*-*cis*-6,7-monoepoxydienes (Figure 5, top traces).

Corroborating evidence for the presence of 3*Z*,6*Z*,9*Z*-21:H and 3*Z*,6*Z*-*cis*-9,10-epoxy-21:H in the pheromone gland extract from another field-collected

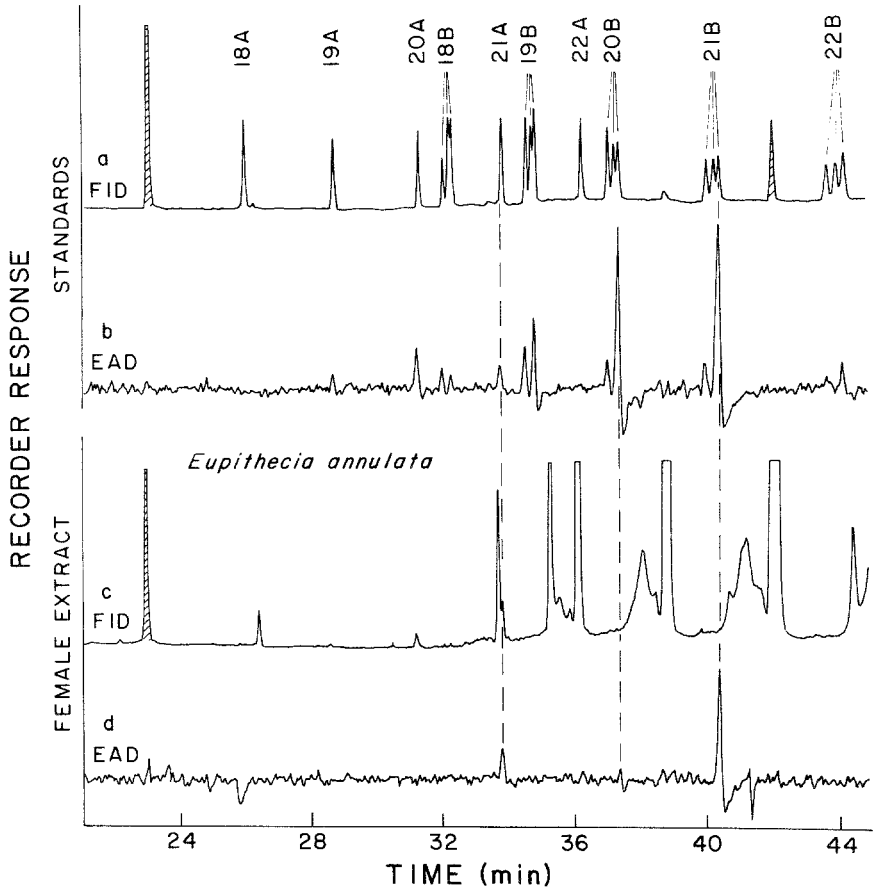


FIG. 5. Response of *Eupithecia annulata* male antennae in GC-EAD experiments using a DB-1701 column. Upper pair of traces are in response to a mixture of C_{18-22} synthetic standards; lower pair are in response to a pheromone gland extract from a conspecific female. Number and letter designations are identical to those of Figure 1. Internal standards (heptadecane and tetracosane) are shaded.

female moth was obtained from chemical ionization (isobutane) GC-MS analysis of the extract, with selected ion monitoring. Retention time matches (DB-5 column) and ion ratios matches versus synthetic standards were obtained for both compounds [3*Z*,6*Z*,9*Z*-21:H, 100 pg standard: *m/z* 291 (100, M+1), 289 (96, M−1); insect extract, *m/z* 291 (100), 289 (92); 3*Z*,6*Z*-*cis*-9,10-epoxy-21:H, 100 pg standard: *m/z* 307 (76, M+1), 289 (100, M+1−18), 275 (23); insect extract: *m/z* 307 (81), 289 (100), 275 (26)]. The homologous 3*Z*,6*Z*-*cis*-9,10-epoxy-20:H was not detected.

In preliminary field tests in 1984, 316 male moths were captured in total. Of these, only one moth was captured in a trap that did not contain either 3*Z*,6*Z*-*cis*-9,10-epoxy-20:H or 3*Z*,6*Z*-*cis*-9,10-epoxy-21:H as a major or single component. In addition, traps containing the regioisomeric 3*Z*,9*Z*-*cis*-6,7- or 6*Z*,9*Z*-*cis*-3,4-epoxydienes caught no moths, confirming that the 9,10 isomers were the attractive regioisomers in the CME-3*Z*,6*Z*,9*Z*-20 or -21:H mixtures.

When tested as single components, 3*Z*,6*Z*-*cis*-9,10-epoxy-21:H and the C_{20} and C_{19} homologs all attracted some moths, while the C_{22} homolog was not attractive at all (Table 2).

A further test demonstrated that 3*Z*,6*Z*-9*S*,10*R*-epoxy-21:H was the attractive C_{21} enantiomer, as three traps baited with this compound (100 μg) caught 60 moths, while traps with the 9*R*,10*S* enantiomer caught one moth. A consecutive experiment (replicated four times) with the C_{20} homologs demonstrated that the 9*S*,10*R* enantiomer of the C_{20} homolog was attractive, catching 119 moths, while the traps baited with the C_{20} 9*R*,10*S* enantiomer caught no moths.

Field tests with blends of the C_{20} and C_{21} 9*S*,10*R* epoxides were inconclusive, as captures in traps containing lures with a variety of blends (1:19 to 19:1) of the two homologs were the same or lower than to 3*Z*,6*Z*-9*S*,10*R*-epoxy-21:H alone, and there was no evident trend in the data as the ratio was changed (data not shown).

During the course of field tests with *E. annulata*, it was noticed that there was considerable variability in the trapping data. For example, in two instances in the field survey, one replicate of a lure caught >70 moths, while the other caught none, suggesting that moth populations may be localized or that the responses might depend on what type of tree a trap was hung on. An experiment was set out to try and explain the variability. One replicate of each lure in the experiment was placed at random in the lower canopy of each of four large black spruce trees (in previous experiments, traps in black spruce had caught moths). The trap captures in this experiment were still variable (Table 3). For

TABLE 2. ATTRACTION OF *E. annulata* TO RACEMIC 3*Z*,6*Z*-*cis*-9,10-EPOXYDIENES[a]

Lure (μg)	Males captured
3*Z*,6*Z*-*cis*-9,10-epoxy-19:H (200)	10a
3*Z*,6*Z*-*cis*-9,10-epoxy-20:H (200)	41ab
3*Z*,6*Z*-*cis*-9,10-epoxy-21:H (200)	119b
3*Z*,6*Z*-*cis*-9,10-epoxy-22:H (200)	0
Blank	0

[a]Traps replicated three times; insects caught from April 10 to May 10, 1985. Values followed by the same letter are not significantly different ($P > 0.05$).

TABLE 3. ATTRACTION OF *E. annulata* TO A VARIETY OF LURES PLACED WITHIN CANOPY OF SAME TREE[a]

Lure (100 lg)	Males captured				
	Tree 1	Tree 2	Tree 3	Tree 4	Total
3Z,6Z-*cis*-9,10-epoxy-20:H	236	11	25	20	292b
3Z,6Z-*cis*-9,10-epoxy-21:H	17	8	6	19	50ab
3Z,6Z-9*S*,10*R*-epoxy-20:H	227	19	78	244	568c
3Z,6Z-9*R*,10*S*-epoxy-20:H	0	1	1	0	2a
3Z,6Z-9*S*,10*R*-epoxy-21:H	28	4	19	13	64ab
3Z,6Z-9*R*,10*S*-epoxy-21:H	1	0	0	0	1a
3Z,6Z,9Z-20:H	0	1	0	0	1a
3Z,6Z,9Z-21:H	0	1	0	0	1a

[a]Traps replicated four times; insects captured from April 21 to May 20, 1986.

example, in tree 1, the trap catches with the 3Z,6Z-*cis*-9,10-epoxy-20:H and 3Z,6Z,9*S*,10*R*-epoxy-20:H lures were virtually identical (236 and 227 moths, respectively). However, the same lures in tree 4 produced quite different captures (20 and 240 moths, respectively). Similar amounts of variability can also be seen in comparisons of other lures.

DISCUSSION

3Z,6Z,9Z-21:H and 3Z,6Z-*cis*-9,10-epoxy-21:H have been identified as sex pheromone components of *E. cuspidea* by a combination of retention time matches with synthetic standards on two different capillary GC columns (DB-5 and DB-1701), by the EAD response elicited by these compounds in the pheromone gland extracts, and by full-scan Cl mass spectral matches with synthetic standards.

As further proof of the geometry of the double bonds in the triene, a mixture of all possible geometric isomers of 3Z,6Z,9Z-21:H was generated by scrambling the double bonds with nitrous acid (Sonnet, 1974), and GC retention times of all the isomers were compared. Only one isomer (an isomer with two *E* and one *Z* double bonds) of the seven other possible stereoisomers of 3Z,6Z,9Z-21:H had a retention time indistinguishable from 3Z,6Z,9Z-21:H with the GC conditions used.

The evidence for the identification of the other three components from the gland extracts is also strong, consisting of retention time matches on two GC columns, SIM mass spectral matches, and partial matches with full-scan Cl mass spectra of standards. The role of the components is unclear, as only one

compound (3Z,6Z,9Z-20:H) elicited an appreciable EAD response, and in field tests no biological activity could be attributed to either enantiomer of the 3Z,9Z-*cis*-6,7-epoxide component. These compounds may have a function as antagonists in interspecific communication.

In similar fashion, 3Z,6Z,9Z-21:H and 3Z,6Z-*cis*-9,10-epoxy-21:H were tentatively identified in *E. annulata* pheromone gland extracts by retention time and SIM mass spectral matches. The homologous 3Z,6Z-*cis*-9,10-epoxy-20:H was not detected by GC-MS, although an antennal stimulatory compound at this retention time had been detected in GC-EAD studies.

Pheromone gland extracts from *C. distincta* were also complex, with five antennal stimulatory compounds being detected in GC-EAD studies. Tentative evidence for the identifications of these compounds was provided by capillary GC (DB-1701) retention time matches with synthetic standards. We were unable to obtain sufficient numbers of female moths to analyze pheromone gland contents by GC-MS. It is also of interest to note that 3Z,6Z,9Z-20:H and 3Z,6Z,9Z-21:H, blends of which were minimally attractive to *C. distincta*, have been reported to be sex pheromone components of the sympatric congener, *C. erechtea* (Underhill et al., 1983).

It must be emphasized that the chiral compounds used in this study were highly enantiomerically enriched but not enantiomerically pure (approx. 96:4 ratio of enantiomers). The possibility that some of the results described were actually due to the blend of the major enantiomer admixed with the small amount of enantiomeric impurity, rather than to the major enantiomer alone, cannot be discounted.

Our objective in conducting the research described here and previously was to delineate the general types and patterns of pheromone components in the Geometridae, Noctuidae, and Arctiidae. We have not definitively elucidated the intricate details of the pheromone chemistry of one particular species, but rather have tried to define in general terms the types of compounds that may be active as pheromone components for a number of species, as demonstrated for the three species described here. In particular, the pheromone chemistry of the species reported here is complex, with extracts from pheromone glands containing as many as five components producing stimulation of male moth antennae in GC-EAD studies. In addition, the chirality of each of the epoxide compounds, which can exist in either of two enantiomeric forms, further complicates the elucidation of the correct pheromone blend. In several cases, it has been shown that moths produce and/or respond to blends of enantiomers (Hansen, 1984; Millar et al., 1990d, 1991a) and so the single peak seen for an epoxide in GC traces should realistically be considered as a possible blend of the two enantiomers until proven otherwise.

At this point, the significance of the components in the pheromone gland that elicit strong EAD responses but that have no apparent effect on the attrac-

tiveness of a blend to which they have been added is not clear. They may have some subtle function in courtship or close-range interactions, which may not be apparent from our relatively crude bioassay criterion of the number of insects caught in a trap. Alternatively, the presence of the compounds in the glands does not necessarily mean that they are actually released by calling females. For example, 3*Z*,6*Z*,9*Z*-19:H was identified several years ago from pheromone gland extracts of the giant looper *Boarmia selenaria* Schiffermüller and was reported as a pheromone component of this insect (Becker et al., 1983) based on EAG data. However, field tests later showed that this compound was not attractive in field or wind-tunnel tests and that the attractive pheromone component produced by calling female moths was actually 6*Z*,9*Z*-3*S*,4*R*-epoxy-19:H (Becker et al., 1990).

The strong antennal responses generated by challenging *C. distincta* antennae with 3*Z*,9*Z*-*cis*-6,7-epoxy-21:H are inexplicable at this point, because this compound was not found in pheromone gland extracts nor did it have any discernible effect on trap captures in field tests. A strong antennal response would suggest that there is a reason for the persistence of this detection capability in the insect. That is, one would not expect the capability to detect the 6,7-epoxides to persist unless these compounds were either conspecific pheromone compounds or behavioral antagonists produced by congenerics to prevent cross-attraction. It is also inexplicable that *E. annulata* antennae responded maximally to 3*Z*,6*Z*-9*R*,10*S*-epoxy-20:H of all the compounds tested in EAG studies, especially as this compound was not attractive in field trials nor did it appear to be strongly antagonistic, as numerous moths were caught over several field seasons in traps with lures containing 3*Z*,6*Z*-*cis*-9,10-epoxy-20:H, alone or in combination with other components (Table 2, for example).

In all cases reported here, the principal bioactive epoxides have the 9*S*,10*R* configuration. This configuration has been reported in the majority of *cis*-9,10 epoxide pheromone components and sex attractants for which the chirality of the attractive enantiomer has been investigated. For example, in EAG studies, the arctiid male moths *Estigmene acrea* Drury and *Hyphantria cunea* Drury (Mori and Ebata, 1981), *Cymbalophora pudica* Esper, and *Tyria jacobaeae* L. (Frérot et al., 1988a,b) all give stronger antennal responses to the 9*S*,10*R* enantiomer of their epoxide pheromone components than to the opposite enantiomer, and the configuration of the pheromone component in the first species has been proven by comparison of the circular dichroism and optical rotatory dispersion spectra with those of synthetic standards (Mori and Ebata, 1981). On the other hand, Wong et al. (1985) reported 9*S*,10*R* epoxide sex attractants for one geometrid and two noctuid species, and 9*R*,10*S* epoxide attractants for another two geometrid and one noctuid species. In addition, Wong et al. (1985) reported that the geometrid species *Cabera erythemaria* Guenée was attracted to a racemic blend of the enantiomers of 3*Z*,6*Z*-*cis*-9,10-epoxy-19:H, rather than

to either enantiomer alone. Thus, at this point it would appear that there is no clear biosynthetic bias for one epoxide enantiomer over the other.

The variability seen in the trap captures in the final experiment conducted with *E. annulata*, in which a set of one replicate of each treatment in the experiment was placed in each of four different trees, is inexplicable. Our hypothesis that the variability seen in previous field tests with this species would be decreased by standardizing trap placement, that is, by removing the effects of localized populations or host tree effects, was not supported. It is also unlikely that wind direction affected the trap captures, as the traps were set out for several days in an area of variable wind direction. In addition, the traps were rerandomized during the course of the experiment, to minimize effects due to wind direction and to nearest-neighbor traps. It is clear that populations were localized and nonrandomly distributed, as the overall trap captures per tree varied over an order of magnitude.

Acknowledgments—We gratefully acknowledge the assistance of Lawrence Hogge with mass spectral runs, and the assistance of Ian Warkentin, David Charest, and Leah Richardson with field testing.

REFERENCES

BECKER, D., KIMMEL, T., CYJON, R., MOORE, I., WYSOKI, M., BESTMANN, H.J., PLATZ, H., ROTH, K., and VOSTROWSKY, O. 1983. (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene—a component of the sex pheromonal system of the giant looper, *Boarmia* (*Ascotis*) *selenaria* Schiffermüller (Lepidoptera: Geometridae). *Tetrahedron Lett.* 24:5505–5508.

BECKER, D., CYJON, R., COSSE, A., KIMMEL, T., and WYSOCKI, M. 1990. Identification and enantioselective synthesis of (*Z*,*Z*)-6,9-*cis*-3*S*,4*R*-epoxynonadecadiene, the major sex pheromone component of *Boarmia selenaria*. *Tetrahedron Lett.* 31:4923–4926.

BELL, T.W., and MEINWALD, J. 1986. Pheromones of two arctiid moths (*Creatonotos transiens* and *C. gangis*): Chiral components from both sexes and achiral female components. *J. Chem. Ecol.* 12:385–409.

DESCOINS, C., and FRÉROT, B. 1984. XVIIth International Symposium of Entomology, Hamburg, August 20–26, 1984.

DUNCAN, D.B. 1955. Multiple range and multiple *F* tests. *Biometrics* 11:1–41.

EINHORN, J., LALLEMAND, J.-Y., ZAGATTI, P., GAULOIS, M., RIOM, J., and MENASSIEU, P. 1982. Isolément et identification de la pheromone sexuelle attractive de *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae). *C.R. Acad. Sci. Paris, Ser. C* 249:41–44.

FRÉROT, B., RENOU, M., MALOSSE, C., and DESCOINS, C. 1988a. Isolément et identification de composés a activité phéromonale chez les femelles de l'arctiide *Tyria jacobaeae*: Determination biologique de la configuration absolue du composé majoritaire. 1988. *Entomol. Exp. Appl.* 46:281–289.

FRÉROT, B., POUGNY, J.-R., MILAT, M.-L., ROLLIN, P., and MALOSSE, C. 1988b. Étude de la secretion phéromonale de *Cymbalophora pudica* (Esper) et de l'enantioselectivité de la perception phéromonale. *C.R. Acad. Sci. Paris, Ser. III* 306:157–160.

HANSEN, K. 1984. Discrimination and production of disparlure enantiomers by the gypsy moth and nun moth. *Physiol. Entomol.* 9:9–18.

HANSSON, B.S., SZÖCS, G., SCHMIDT, F., FRANCKE, W., LOFSTEDT, C., and TÖTH, M. 1990. Electrophysiological and chemical analysis of the sex pheromone communication system of the mottled umber, *Erranis defoliaria* (Lepidoptera: Geometridae). *J. Chem. Ecol.* 16:1887–1897.

HILL, A.S., and ROELOFS, W.L. 1981. Sex pheromone of the saltmarsh caterpillar moth, *Estigmene acrea*. *J. Chem. Ecol.* 7:655–668.

HILL, A.S., KOVALEV, B.G., NIKOLAEVA, L.N., and ROELOFS, W.L. 1982. Sex pheromone of the fall webworm moth, *Hyphantria cunea*. *J. Chem. Ecol.* 8:383–396.

KOVALEV, B.G., and NIKOLAEVA, L.A. 1986. Attractiveness of some epoxy compounds for males of a tiger moth, *Phragmatobia fuliginosa* (Arctiidae), and of a geometrid moth, *Caustoloma (Therapis) flavicaria* (Geometridae). *Zool. Zh.* 65:802–804.

MEYER, W. 1984. MS thesis. Cornell University, Ithica, New York.

MILLAR, J.G., UNDERHILL, E.W., GIBLIN, M., and BARTON, D. 1987. Sex pheromone components of three species of *Semiothisa* (Geometridae), (*Z*,*Z*,*Z*)-3,6,9-heptadecatriene and two monoepoxydiene analogs. *J. Chem. Ecol.* 13:1371–1383.

MILLAR, J.G., GIBLIN, M., BARTON, D., and UNDERHILL, E.W. 1990a. 3*Z*,6*Z*,9*Z*-Nonadecatriene and enantiomers of 3*Z*,9*Z*-*cis*-6,7-epoxy-nonadecadiene as sex attractants for two geometrid and one noctuid moth species. *J. Chem. Ecol.* 16:2153–2166.

MILLAR, J.G., GIBLIN, M., BARTON, D., and UNDERHILL, E.W. 1990b. 3*Z*,6*Z*,9*Z*-Trienes and unsaturated epoxides as sex attractants for geometrid moths. *J. Chem. Ecol.* 16:2307–2316.

MILLAR, J.G., GIBLIN, M., BARTON, D., MORRISON, A., and UNDERHILL, E.W. 1990c. Synthesis and field testing of enantiomers of 6*Z*,9*Z*-*cis*-3,4-epoxydienes as sex attractants for geometrid moths. Interactions of enantiomers and regioisomers. *J. Chem. Ecol.* 16:2317–2339.

MILLAR, J.G., GIBLIN, M., BARTON, D., REYNARD, D.A., NEILL, G.B., and UNDERHILL, E.W. 1990d. Identification and field testing of female-produced sex pheromone components of the spring cankerworm, *Paleacrita vernata* Peck (Lepidoptera: Geometridae). *J. Chem. Ecol.* 16:3393–3409.

MILLAR, J.G., GIBLIN, M., BARTON, D., and UNDERHILL, E.W. 1991a. Chiral lepidopteran sex attractants: Blends of optically active C_{20} and C_{21} diene epoxides as sex attractants for geometrid and noctuid moths. *Env. Entomol.* 20:450–457.

MILLAR, J.G., GIBLIN, M., BARTON, D., and UNDERHILL, E.W. 1991b. Synthesis and field screening of chiral monounsaturated epoxides as lepidopteran sex attractants and sex pheromone components. *J. Chem. Ecol.* 17:911–929.

MORI, K., and EBATA, T. 1981. Synthesis of optically active pheromones with an epoxy ring, (+)-disparlure and the saltmarsh caterpillar moth pheromone, [(*Z*,*Z*)-3,6-*cis*-9,10-epoxyheneicosadiene]. *Tetrahedron Lett.* 22:4281–4282.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry, 2nd ed. W.H. Freeman, San Francisco.

SONNET, P.E. 1974. A practical synthesis of the sex pheromone of the pink bollworm. *J. Org. Chem.* 39:3793–3794.

UNDERHILL, E.W., PALANISWAMY, P., ABRAMS, S.R., BAILEY, B.K., STECK, W.F., and CHISHOLM, M.D. 1983. Triunsaturated hydrocarbons, sex pheromone components of *Caenurgina erechtea*. *J. Chem. Ecol.* 9:1413–1423.

WONG, J.W., UNDERHILL, E.W., MACKENZIE, S.L., and CHISHOLM, M.D. 1985. Field trapping and electroantennographic responses to triene hydrocarbons and monoepoxydiene derivatives. *J. Chem. Ecol.* 11:727–756.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

SELECTIVE ODOR PERCEPTION IN THE SOIL COLLEMBOLA *Onychiurus armatus*

GÖRAN BENGTSSON, KATARINA HEDLUND,* and STEN RUNDGREN

Department of Ecology
University of Lund,
S-223 62 Lund, Sweden

(Received February 27, 1991; accepted July 3, 1991)

Abstract—The olfactorial response of the fungivorous soil collembolan *Onychiurus armatus* was examined in a bioassay covering volatile compounds identified in the odor blends of two of its preferred fungal species *Mortierella isabellina* and *Verticillium bulbillosum*. The odor of the fungi was trapped using activated carbon filters, extracted with diethyl ether, and subjected to GC-MS analysis. About 50% of the compounds resolved by GC were identified by a combination of electron impact and chemical ionization mass spectrometry. In a Y-shaped olfactometer the collembolans were attracted to a variety of common odors, such as CO_2 and 2-methyl-1-propanol, and a species-specific odor, such as 1-heptene, and arrested by, for example, decanal and 2-octene. The response was not improved by pairwise combinations of common and specific odors. An amount of 0.5 ng of ethyl acetate or 3 pg of 1-pentanol was sufficient to attract the collembolans. The specific compounds of *V. bulbillosum*, 1-heptene and 1-octen-3-ol, may be key stimuli explaining why *O. armatus* prefers *V. bulbillosum*.

Key Words—Fungal volatiles, GC-MS, *Mortierella*, *Verticillium*, olfactorial, attractants, fungivor, Collembola, *Onychiurus armatus*.

INTRODUCTION

The process of optimal foraging through habitat selection is catalyzed in insects by different perception mechanisms, enabling them to recognize an array of stimuli associated with specific food sources. Soil-living Collembola, are known to feed selectively on fungi (Leonard, 1984; Newell, 1984; Bengtsson et al.,

*To whom correspondence should be addressed.

0098-0331/91/1100-2113$06.50/0

1985), rank fungal species hierarchically in order of decreasing palatability (Shaw, 1988), and use odors to discriminate between species (Bengtsson et al., 1988). Mycelia produce volatile compounds formed via normal metabolic activities (Karahadian et al., 1985; Watson et al., 1986; Talou et al., 1987), but the odor blend differs with growth substrate (Bengtsson et al., 1988) and age of the colony (Karahadian et al., 1985).

The resolution power of the perception system of collembolans is not known. It is possible that the odor blend in general is used to distinguish between patches with relatively high and low microbial activity. In addition, animals may be attracted (sensu Barton Browne, 1977) to compounds that are species specific. A specificity may be achieved by single compounds that are only found in related species or by a specific ratio between compounds that are generally distributed among many species (Visser, 1986).

The collembolan *Onychiurus armatus* (Tullb. sensu Gisin, 1960) is a euedaphic species that is common in temperate soils. It is fungivorous and when given a choice among five species of soil fungi, it ranked *Verticillium bulbillosum* as the most palatable one followed by *Paeciliomyces farinosus* and *Mortierella isabellina* (Bengtsson et al., 1985). In an olfactometer test *O. armatus* preferred the odor of *V. bulbillosum* when the fungi grew on agar, but preferred that of *M. isabellina* when the growth substrate was soil (Bengtsson et al., 1988). The purpose of this work was to extend the examination of the odor blend of *M. isabellina* and *V. bulbillosum* by identifying individual odor components and evaluating their potential as olfactorial stimuli for *O. armatus*. The hypothesis was that the attraction to an odor source is triggered by a mixture of compounds associated with fungal metabolism in general and reinforced by some species specific substances that can be used for finer ranking.

METHODS AND MATERIAL

Collembolans and Fungal Cultures. Onychiurus armatus was extracted from a mor horizon of a spruce forest soil in southeastern Sweden. Cultures of the collembolans were kept in Petri dishes with a layer of plaster of Paris/charcoal (9:1, v/v) at 12°C and in darkness and fed hyphae of *Mortierella isabellina* (Oudem).

The fungi *M. isabellina* and *Verticillium bulbillosum* (W. Gams and Malla) were grown on a 5-mm layer of either soil or agar (2% agar, 2% malt extract, 30 μg/ml chlortetracycline) in 100-ml glass vials with a bottom area of 18 cm^2. The soil, a spruce forest mor, was sterilized in a 10 MeV electron accelerator with a dose of 45 kGy. Agar and soil were inoculated with a spore suspension, and the vials were sealed with a silicon septum and incubated at 15°C for one

week to ensure that the mycelium extended to the edge of the vials without apparent loss of activity in old parts.

Trapping of Volatiles. Two sterile syringes were pierced through the silicon septum and one of them (air outlet) was connected to a suction pump by Teflon tubing. Air was drawn into the vial through the other syringe (air inlet), that was connected by Teflon tubing to an activated carbon filter (4 cm OD, 12 cm long), to remove traces of volatiles in the incoming air. Fungal odor substances were trapped by sucking 100 ml of air from the culture vial through a 2-mm-thick layer of activated carbon that was sandwiched between disks of 300 mesh stainless steel mesh and fused into a 7-mm ID Pyrex tube. The activated carbon filter was placed between the air outlet and the suction pump, and the volatiles of 10 replicate vials were extracted with 3 × 20 μl diethyl ether (Merck). The extracts were kept in 3-mm OD glass capillaries at −20°C until analysis. Odors from sterile vials with agar and soil substrates, respectively, were trapped, extracted, and used as blanks. Aliquots of 10 μl were saved for GC-MS analysis without further purification.

Derivatization. Aliphatic alcohols in the extracts were derivatized with heptafluorobutyric anhydride (HFBA), as the resulting derivative is stable for molecular weight determination by GC-MS (Hogge and Olsson, 1982). HFBA (1 μl) was added to 10 μl of the extract and mixed in glass capillaries for 30 sec by repetitive refilling of a 10-μl Hamilton syringe. The reaction was completed after 5 min at room temperature. Excess reagent was removed by washing the derivative twice with 10 μl 0.1 M NaOH. The two phases were separated by centrifugation (500 *g*). The samples were kept in sealed glass capillaries at −20°C.

Gas Chromatography and Mass Spectrometry. A Varian 3700 GC with a flame ionization detector (FID) and a 25-m fused silica capillary column coated with SE-54 was used for analysis. The carrier gas, hydrogen, had a flow rate of 1.5 ml/min. The injector temperature was 250°C and the detector 300°C. The first 5 cm of the column was cooled 1 min before and after injection by sliding a solid aluminum cylinder (4 cm diam., 5 cm long) halfway onto the column by a vertical slit in the cylinder. The cylinder was kept at −20°C before the injection, which was made in splitless mode. Conditions for the oven were 45°C for 5 min and then programming at 2°C/min to 180°C.

The mass spectrometer was a Ribermag R10-10C quadropole instrument equipped with a Carlo Erba model 4160 GC. Helium was used as carrier gas at 40 ml/sec. Gas chromatographic conditions were as described for FID. The mass spectrometer was operated in the positive ion electron impact ionization mode (EI) at 75 eV. EI was used to get total ion spectra of underivatized odor extracts. Positive ion chemical ionization (pos. CI) was used to determine molecular weights of compounds from underivatized odor extracts that would

not produce stable molecular peaks by EI. The reagent gas, ammonia, was ionized with electrons at 45 eV. HFBA derivatives were analyzed by negative ion chemical ionization (neg. CI) with isobuthane as a reagent gas ionized with electrons at 90 eV.

Bioassay. Synthetic odor substances (pro analysis) of volatiles identified from *M. isabellina* and *V. bulbillosum* and some general microbial metabolites were assayed in a Y-shaped olfactometer made of a glass tube (10 mm ID) with 4-cm-long arms (angle of 45°) and a base of 2 cm. The glass surface was roughened on the inside to facilitate movements of collembolans and to hold water to prevent desiccation. A 2-cm-long glass tube, the release chamber, was connected at one end with clamps to the base of the Y and at the other end fitted to a 30-cm-long Teflon tubing connected to a flowmeter and a suction pump. The end of each arm was connected with clamps to two glass tubes in a series. The first one (2 cm long) held a 1-cm^2 piece of filter paper (Munktell, 1F) with the odor substance or a pure solvent, diethyl ether, as blank and the second one (3 cm long) was filled with activated carbon to provide clean air. An odor substance diluted in diethyl ether was deposited on a filter paper and the solvent was allowed to evaporate at room temperature for 1 min before the filter paper was placed in one side arm of the olfactometer and the blank, a filter paper with only solvent applied, in the other. Seven adult *O. armatus* were placed in a release chamber that was sealed with parafilm and were kept there for 15 min. Air was drawn through the Y-tube at a rate of 3 ml/min for 2 min using an empty release chamber, which was then replaced by the one with collembolans. Each group of *Onychiurus* was exposed to the odor for 10 min in darkness at 15°C in the horizontally positioned Y-tube. The numbers of collembolans present in the side arms or remaining in the release chamber were counted and compared with an expected distribution by a chi-square test, using the null hypothesis that animals were equally distributed in side arms and release chamber. All comparisons were made from six replicate observations. A substance was considered attractive if more than half the collembolans had left the release chamber and significantly more of them were present in the arm facing the odor than in the one with the solvent. When more than half of the collembolans left the release chamber and were found in the blank, the odor was regarded as a repellent. A substance was arresting to the collembolans when significantly more than half of them remained in the release chamber.

The amount of odor released from a filter paper during the olfactometry test was estimated by determining the evaporation rate of 50 μl of ethanol, ethyl acetate, and 1-pentanol, respectively, in 100-ml glass vials at room temperature. The vials were weighed repeatedly during 125 min, and the amount that evaporated during 10 min was calculated by linear regressions of logarithmic amounts of the odor versus time.

RESULTS

V. bulbillosum produced approximately equal numbers of odor substances regardless of the substrate, whereas *M. isabellina* emitted twice as many when grown in soil (Figure 1). Identified volatiles had a relatively low molecular weight ranging from 74 up to 152. Substances with molecular weights lower than 74 were not considered since they were obscured by the solvent peak. Aliphatic alcohols and alkenes were the most common volatiles, followed by ketones and aromatic substances (Table 1). The structure of the alcohols was suggested from the abundance of $C_nH_{2n-1}^+$ and C_n H_{2n}^+ ion series, although the EI spectra were inconclusive due to the decomposition of the molecular ion. As an example, the EI spectra of peak 11 in Figure 1 showed $C_nH_{2n}^+$ ion series at larger masses (Figure 2A), indicating a possible molecular ion at m/z 130. Analysis by neg. CI of HFBA derivative revealed the molecular weight of the alcohols based on the abundance of the fragment representing a loss of HF from the molecular ion (Figure 2B). More than 50% of the peaks remained unknown due to low ion abundance and poor spectral purity. Among the substances identified, 2-methyl-1-propanol, α-pinene, 1-phenylethanone, 2-octene, and decanal were present as volatiles from both species and independent of growth substrate (Table 1). Species-specific odors of *M. isabellina* were identified as pentanone, 3-methyl-1-butanol, γ-octalone, 1-methylphenylethanone and camphor, while *V. bulbillosum* emitted 1-heptene and 1-octen-3-ol.

Two thirds of the tested substances attracted *O. armatus* at one or more of the applied doses (Table 2). The collembolans were attracted to 2-methyl-1-propanol that was produced by both fungi as well as to other common fungal metabolites, e.g., CO_2 and 2-propanol (Table 2). Only one substance specific to *V. bulbillosum*, 1-heptene, attracted the collembolans. 2-Methyl-1-propanol and camphor attracted the collembolans at the highest dose applied and were the only tested substances with a negative correlation between dose and response. Half of the identified odors were neutral or arrested the collembolans in the olfactometer. All amounts of decanal and 2-octene tested arrested collembolans while 2-pentanone and α-pinene did not elicit any behavioral response at all.

Pairwise combinations of the general odors methanol and ethanol and the specific odors 1-octen-3-ol and camphor did not increase the numbers of attracted collembolans compared with the numbers attracted to a single odor (Table 3). A combination of 10 ng camphor and 10 ng methanol arrested the majority of the collembolans in the release chamber, and those leaving were repelled from the odor into the side arm with a blank. At the highest dose applied, 25 ng camphor and 25 ng methanol, the collembolans were repelled from the release chamber to the side arm with the blank.

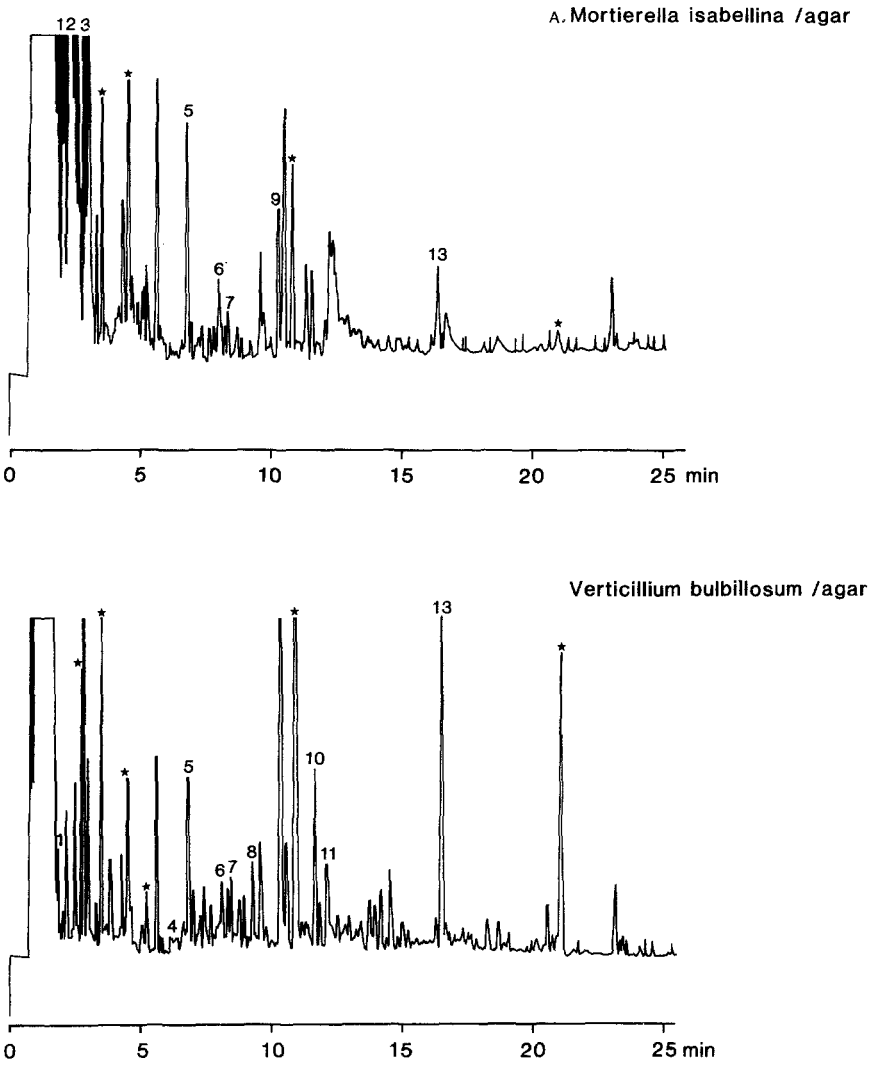
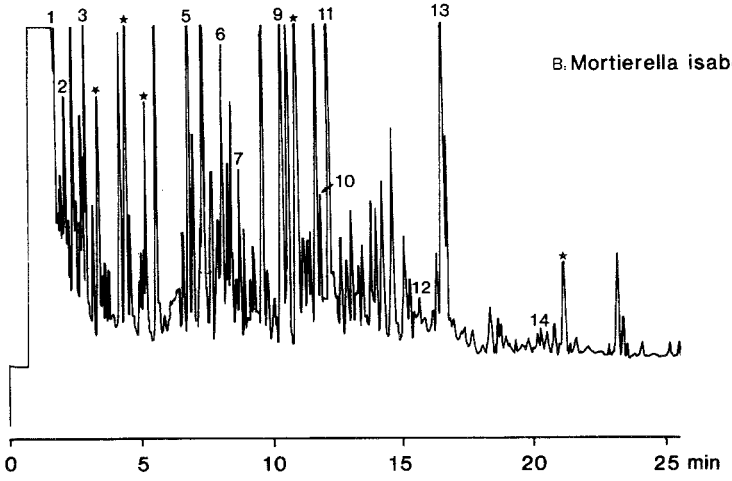


FIG. 1. Gas chromatograms of volatiles trapped from cultures of *Mortierella isabellina* and *Verticillium bulbillosum* (A) grown on 2% malt extract agar and (B) grown in soil. Identified compounds are numbered and listed in Table 1.* Volatiles present in vials with sterile agar or soil.

The actual amount of an odor reaching the collembolans was lower than the dose applied. About 0.5 ng evaporated from 1 ng of ethanol or ethyl acetate during 10 min, while only 3 pg evaporated from 1 ng of the less volatile substance pentanol.



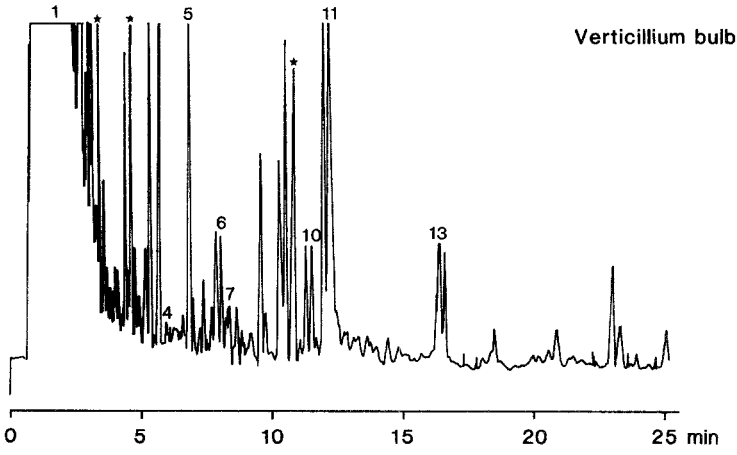


FIG. 1. *Continued*

DISCUSSION

The ability to perceive general compounds of microbial activity that are produced along common biosynthetic pathways, such as CO_2, ethanol, and 2-methyl-1-propanol (Williams et al., 1981; Zechmann et al., 1986; Talou et al., 1987), should help *O. armatus* in locating patches with high microbial activity. Collembolans are known to move towards sources of CO_2 (Moursi, 1962), which they locate in a similar way as plant parasitic nematodes find CO_2-

TABLE 1. IDENTIFIED SUBSTANCES (GC-MS ANALYSIS) OF VOLATILES OF *Mortierella isabellina* AND *Verticillium bulbillosum* GROWING IN SOIL AND ON AGAR, PRESENCE (+) AND ABSENCE (−)

Substance[a]	Molecular weight	*M. isabellina*		*V. bulbillosum*	
		Soil	Agar	Soil	Agar
1. 2-Methyl-1-propanol	74	+	+	+	+
2. 2-Pentanone	86	+	+	−	−
3. 3-Methyl-1-butanol	88	+	+	−	−
4. 1-Heptene	98	−	−	+	+
5. α-Pinene	104	+	+	+	+
6. 1-Phenylethanone	120	+	+	+	+
7. 2-Octene	112	+	+	+	+
8. 1-Octen-3-ol	128	−	−	−	+
9. γ-Octalone	142	+	+	−	−
10. Dipentene	136	+	−	+	+
11. 1-Octanol	130	+	−	+	+
12. 1-Methylphenylethanone	134	+	−	−	−
13. Decanal	152	+	+	+	+
14. Camphor	152	+	−	−	−

[a]The numbers refer to Figure 1.

emitting roots in soil (Klinger, 1965) or insect parasitoids locate the yeast in which their drosophilid hosts live (Vet et al., 1984). It is possible that a release of general fungal odors is perceived by collembolans and enables them to navigate among fungal patches in the habitat just as general plant odors, e.g. certain alcohols and aldehydes, are used in habitat selection by herbivorous insects (Visser, 1986). On the other hand, substances that characterize a specific fungus may be found among higher alcohols and less volatile substances, which can be synthesized by decomposition of the substrate (Kana et al., 1988) and be both species- and habitat-specific. These volatiles can aid the collembolans in their search for a palatable fungus within a patch of enhanced microbial activity. *O. armatus* preference for *V. bulbillosum* in comparison with *M. isabellina* (see Bengtsson et al., 1988), may be explained by the perception of 1-heptene and 1-octen-3-ol. The attraction to *M. isabellina* growing in soil may be associated with its production of camphor and 1-methylphenylethanone.

However, substances that in the present study appear to be species-specific also occur in odors of other microorganisms. 3-Methyl-1-butanol is produced by fungi (Kaminski et al., 1974, Sinha et al., 1988) but also by bacteria (Williams et al., 1981) and yeasts (Kana et al., 1988). 1-Octen-3-ol is produced by basidiocarps (Vanhaelen et al., 1980) and hyphae of other species (Kaminski

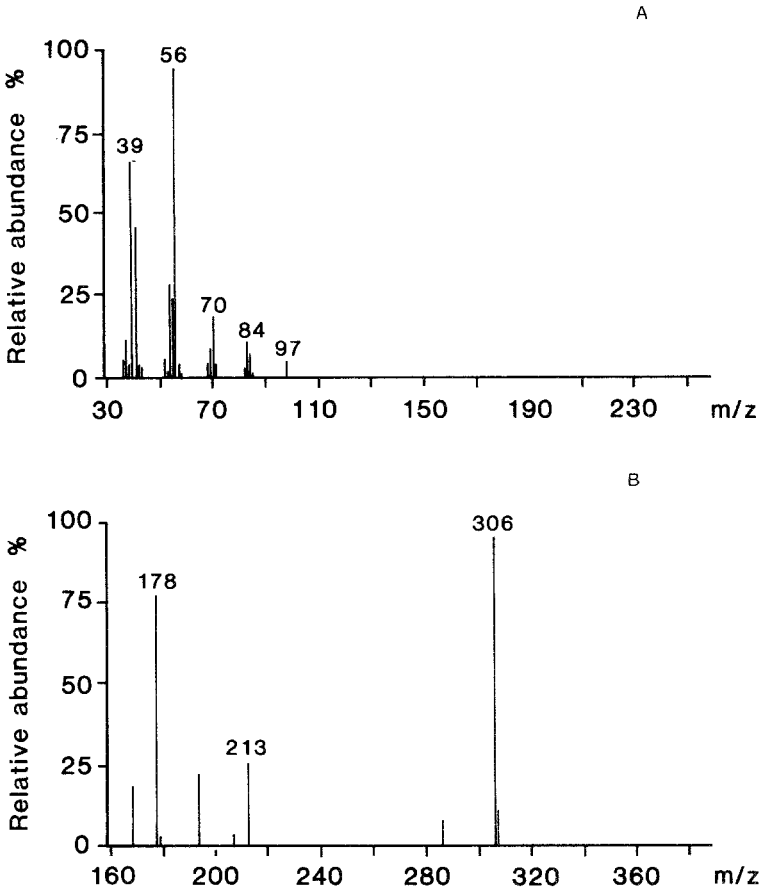


FIG. 2. (A) Electron impact mass spectra of 1-octanol (peak 11 in Figure 1) emitted by *Mortierella isabellina* grown in soil. (B) Negative ion (isobuthane) chemical ionization mass spectra of HFB *o*-acyl derivatives of 1-octanol from *M. isabellina*. The fragment 306 (*m/z*) is M-HF.

et al., 1974; Sinha et al., 1988). Camphor and γ-octalone are not reported from other fungi, but γ-octalone is produced by the yeast *Pityrosporum* (Labows et al., 1979). The specificity of the food choice of the collembolans may be further explained by some of the unknown odors, as 50% of the substances in the odor plumes are unidentified. However, a living fungus attracted about 50% of the collembolans (Bengtsson et al., 1988), which corresponds to their response to a single odor substance. Unless the specificity of the perception system increases, the response to the identified odors is sufficient to explain the response to the mycelium.

TABLE 2. PROPORTION OF *Onychiurus armatus* IN SIDE ARM OF Y-SHAPED OLFACTOMETER WITH ODOR SUBSTANCE AT 1, 10, OR 25 ng[a]

Substance	Proportions of collembolans in the side arm (%)		
	1 ng	10 ng	25 ng
Methanol	42.2[1b]	41.5[1]	39.5
Ethanol	18.6[3]	41.3[2]	4.9[3]
2-Propanol	34.1[1]	53.5[1,2]	21.4
2-Methyl-1-propanol	19.0[4c]	28.6	47.6[1,2]
2-Methyl-1-butanol	31.7	43.9[2]	17.1
1-Pentanol	55.9[1,2]	33.3	11.4[c]
1-Octen-3-ol	37.5[1]	2.9[c]	33.3
1-Octanol	38.1	7.1[c]	44.2[2]
Phenylethanol	7.0[c]	n.d.[d]	15.5[c]
Camphor	4.8[c]	10.0	39.0[2]
2-Pentanone	28.6	14.3	9.7
n-Decanal	26.2	21.4	16.7[c]
α-Pinene	33.3	35.7	26.2
1-Heptene	21.4	51.2[1,2]	9.8[c]
2-Octene	4.9[c]	2.4[3]	0.0[c]
Dipentene	7.1	14.3[3]	23.8
Ethyl acetate	43.6[1]	33.3	14.3
Carbon dioxide[e]	45.5[1,2]		

[a] The substances are given in order of chemical classes and molecular weights. The proportions were determined from the sum of six replicates with seven collembolans in each replicate.

[b] [1)]Significantly more ($P < 0.05$) than 50% of the collembolans moved out of the release chamber and [2)]were attracted to the arm with a substance or [3)]repelled to the side arm without odor substance.

[c] Significantly more ($P < 0.05$) than 50% of the collembolans were arrested in the release chamber.

[d] n.d. = not determined.

[e] Tested as 1% CO_2 in air.

Compounds that arrest the collembolans in the olfactometer, such as decanal and 2-octene, and those that attract at low and arrest at high concentrations, such as 1-pentanol and 1-heptene, can elicit a stop for searching and trigger feeding on a palatable fungus. Such mixed functions of odorous compounds are known from the host searching behavior of parasitoids (Waage, 1979), feeding of herbivorous insects (Miller and Strickler, 1984), and prey encounters by predatory mites (Sabelis et al., 1984).

The minimum amount of odor needed to elicit a response of collembolans in the olfactometer test was 1 ng. Other fungivores, for instance, nematodes were attracted to 0.1 ppm ethyl acetate in water agar (Balan, 1985) and *Drosophila* to 100 ppm of acetaldehyde in water (Hoffmann and Parsons, 1984). However, in a comparison with the total amounts of highly volatile odor sub-

TABLE 3. PROPORTION OF *Onychiurus armatus* IN SIDE ARM OF Y-SHAPED OLFACTOMETER WITH COMBINATION OF TWO ODOR SUBSTANCES AT 1 + 1, 10 + 10, AND 25 + 25 ng[a]

Substance	Proporation of collembolans in side arm (%)			
	1 + 1 ng	10 + 10 ng	25 + 25 ng	Other amounts
Camphor + methanol	26.2	2.4[b,c]	19.0[1,3b]	7.1[3] (25 + 1 ng)
Camphor + ethanol	19.0	33.0	14.3	14.3[3] (25 + 10 ng)
1-Octen-3-ol + methanol	11.9[3]	40.5	16.7[c]	
1-Octen-3-ol + ethanol	14.3[3]	19.0	19.0	40.5 (1 + 10 ng)

[a] The proportions were determined from the sum of six replicates with seven collembolans in each replicate.
[b] See footnote [b] of Table 2.
[c] See footnote [c] of Table 2.

stances applied in the tests, the collembolans have the lowest threshold for a response, as the dose was 1 ng, while that presented to the nematodes was about 20 ng (data estimated from Balan, 1985). The actual amount of odor reaching the collembolans was lower than the dose applied on the filter paper, as only 3 pg evaporated from 1 ng pentanol during 10 min. The minimum amount of odor needed to attract collembolans can be used to estimate the scale and distance on which collembolans find patches of food in natural soil.

Amounts as well as qualitative differences of volatiles can be used as keys of recognition by collembolans, as the proportions of the volatiles emitted from *V. bulbillosum* and *M. isabellina* differ between the species as well as between their growth substrates. Interspecific variation in amounts of fungal volatiles has been shown by Vanhaelen et al. (1980), who found that the proportion of 1-octen-3-ol from fruit bodies of 15 basidiomycete species varied from 0.4% to 22%. The amounts of volatiles emitted by a fungus also decrease with its age (Karahadian, et al. 1985), which may explain why collembolans discriminate old hyphae in favor of young ones (Leonard, 1984).

The number of volatiles that can be distinguished by a collembolan and the number of sensilla that are needed for food recognition are still unknown. Wright (1964) suggested that a bee could survive if it was able to recognize a minimum of six odors, but a rather immobile larva of the polyphagous caterpillar *Manduca sexta* can distinguish between 3^{23} combinations of odors (Schoonhoven, 1968). Whether a single key substance, a blend of substances, or the concentrations of fungal volatiles make *Onychiurus* able to choose a palatable fungus is unknown. The decisions are assumed to be based on the integration of inputs from a number of receptors with different sensitivity

(Chapman, 1988). It is unlikely, as remarked by Dethier (1947), that a single attractant guides insects to their food, but the location of food is achieved by a complex array of stimuli, involving soil humidity and temperature.

Acknowledgments—This work was supported by the Swedish Natural Science Research Council.

REFERENCES

BALAN, J. 1985. Measuring minimal concentrations of attractants detected by the nematode *Panagrellus redivivus*. *J. Chem. Ecol.* 11:105–111.

BARTON BROWNE, L. 1977. Host-related responses and their suppression: Some behavioural considerations, pp. 117–127, *in* H.H. Shorey, and J.J. McKelvey, Jr. (eds.). Chemical Control of Insect Behaviour: Theory and Application. Wiley-Interscience, London.

BENGTSSON, G., OHLSSON, L., and RUNDGREN, S. 1985. Influence of fungi on growth and survival of *Onychiurus armatus* (Collembola) in a metal polluted soil. *Oecologia (Berlin)* 68:63–68.

BENGTSSON, G., ERLANDSSON, A., and RUNDGREN, S. 1988. Fungal odour attracts soil Collembola. *Soil Biol. Biochem.* 20:25–30.

CHAPMAN, R.F. 1988. Sensory aspects of host-plant recognition by Acridoidea: Questions associated with the multiplicity of receptors and variability of response. *J. Insect Physiol.* 34:167–174.

DETHIER, V.G. 1947. Chemical Insect Attractants and Repellents. Lewis and Co. Ltd., London.

GISIN, H. 1960. Collembolenfauna Europas. Museum d'Histoire Naturelle, Geneva.

HOFFMANN, A.A., and PARSONS, P.A. 1984. Olfactory response and resource utilization in *Drosophila*: Interspecific comparisons. *Biol. J. Linn. Soc.* 22:43–53.

HOGGE, L.R., and OLSON, D.J.H. 1982. Detection of trace quantities of aliphatic alcohols using derivatization techniques suitable for positive and/or negative ion gas chromatography/chemical ionization mass spectrometry. *J. Chromatogr. Sci.* 20:109–113.

KAMINSKI, E., STAWICKI, S., and WASOWICZ, E. 1974. Volatile flavor compounds produced by molds of *Aspergillus, Penicillium* and *Fungi imperfecti*. *Appl. Microbiol.* 27:1001–1004.

KANA, K., KANELLAKI, M., KOUINIS, J., and KOUTINAS, A.A. 1988. Alcohol production from raisin extracts: volatile by-products. *J. Food Sci.* 53:1723–1724 and 1749.

KARAHADIAN, C., JOSEPHSON, D.B., and LINDSAY, R.C. 1985. Volatile compounds from *Penicillium* sp. contributing musty-earthy notes to Brie and Camembert cheese flavors. *J. Agric. Food. Chem.* 33:339–343.

KLINGER, J. 1965. On the orientation of plant nematodes and some other soil animals. *Nematologica* 11:4–18.

LABOWS, J.N., MCGINLEY, K.J., LEYDEN, J.J., and WEBSTER, G.F. 1979. Characteristic γ-lactone odor production of the genus *Pityrosporum*. *Appl. Environ. Microbiol.* 38:412–415.

LEONARD, M.A. 1984. Observations on the influence of culture conditions of the fungal feeding preferences of *Folsomia candida* (Collembola: Isotomidae). *Pedobiologia* 26:361–367.

MILLER, J.R. and STRICKLER, K.L. 1984. Finding and accepting host plants, pp. 127–157, *in* W.J. Bell, and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman & Hall, New York.

MOURSI, A.A. 1962. The attractiviness of CO_2 and N_2 to soil Arthropoda. *Pedobiologia* 1:299–302.

NEWELL, K. 1984. Interaction between two decomposer Basidiomycetes and a collembolan under Sitka spruce: Distribution, abundance and selective grazing. *Soil. Biol. Biochem.* 16:227–233.

SABELIS, M.W., VERMAAT, J.E., and GROENEVELD, A. 1984. Arrestment response of the predatory

mite, *Phytoseiulus persimilis*, to steep odour gradients of a kairomone. *Physiol. Entomol.* 9:437–446.

SCHOONHOVEN, L.M. 1968. Chemosensory bases of host plant selection. *Annu. Rev. Entomol.* 13:115–136.

SHAW, P.J.A. 1988. A consistent hierarchy in the fungal feeding preferences of the Collembola *Onychiurus armatus*. *Pedobiologia* 31:179–187.

SINHA, R.N., TUMA, D., ABRAMSON, D., and MUIR, W.E. 1988. Fungal volatiles associated with moldy grain in ventilated and non-ventilated bin-stored wheat. *Mycopathologia* 101:53–60.

TALOU, T., DELMAS, M., and GASET, A. 1987. Principal constituents of black truffle (*Tuber melanosporum*) aroma. *J. Agric. Food Chem.* 35:774–777.

VANHAELEN, M., VANHAELEN-FASTRÉ, R., and GEERAERTS, J. 1980. Occurrence in mushrooms (Homobasidiomycetes) of *cis*- and *trans*-octa-1,5-dien-3-ol, attractants to the cheese mite *Tyrophagus putrescentiae* (Schrank) (Acarina, Acaridae). *Experientia* 36:406–407.

VET, L.E.M., JANSE, C., VAN ACHTERBERG, C., and VAN ALPHEN, J.J.M. 1984. Microhabitat location and niche segregation in two sibling species of drosophilid parasitoids: *Asobara tabida* (Nees) and *A. rufescens* (Foerster) (Braconidae: Alysiinae). *Oecologia (Berlin)* 61:182–188.

VISSER, J.H. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol.* 31:121–144.

WAAGE, J.K. 1979. Foraging for patchily distributed hosts by the parasitoid, *Nemerotis canescens*. *J. Anim. Ecol.* 48:353–371.

WATSON, R.L., LARGENT, D.L., and WOOD, W.F. 1986. The "coal tar" odor of *Thricholoma inamoenum*. *Mycologia* 78:965–966.

WILLIAMS, A.A., HOLLANDS, T.A., and TUCKNOTT, O.G. 1981. The gas chromatographic–mass spectrometric examination of the volatiles produced by the fermentation of a sucrose solution. *Z. Lebensm. Unters. Forsch.* 172:377–381.

WRIGHT, R.H. 1964. The Science of Smell. George Allen and Unwin Ltd., London.

ZECHMAN, J.M., ALDINGER, S., and LABOWS, J.N., JR. 1986. Characterization of pathogenic bacteria by automated headspace concentration–gas chromatography. *J. Chromatogr.* 377:49–57.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

MALE-PRODUCED AGGREGATION PHEROMONE OF THE AMERICAN PALM WEEVIL, *Rhynchophorus palmarum* (L.) (COLEOPTERA, CURCULIONIDAE): COLLECTION, IDENTIFICATION, ELECTROPHYSIOGICAL ACTIVITY, AND LABORATORY BIOASSAY[1]

DIDIER ROCHAT,* CHRISTIAN MALOSSE, MARTINE LETTERE, PAUL-HENRI DUCROT, PIERRE ZAGATTI, MICHEL RENOU, and CHARLES DESCOINS

Laboratoire des Médiateurs Chimiques, INRA
Domaine de Brouessy
78114 Magny-les-Hameaux, France

(Received April 6, 1991; accepted July 3, 1991)

Abstract—Male American palm weevils (APWs), *Rhynchophorus palmarum* (L.) produced two sex-specific compounds, which were disclosed by volatile collections on Supelpak-2 and gas chromatography. One was a minor compound, not always detected. The major male-produced volatile was identified as (2*E*)-6-methyl-2-hepten-4-ol through coupled gas chromatography–mass spectrometry and gas chromatography–Fourier transform infrared spectrometry, proton nuclear magnetic resonance spectrometry, and rational synthesis. We propose the trivial name rhynchophorol for this new molecule, which proved to be the essential component of the APW aggregation pheromone by electroantennography, coupled gas chromatography–electroantennography and behavioral bioassays.

Key Words—Aggregation pheromone, Coleoptera, Curculionidae, *Rhynchophorus palmarum*, American palm weevil, (2*E*)-6-methyl-2-hepten-4-ol, synthesis.

*To whom correspondence should be addressed.
[1]Research supported in part by the IRHO-CIRAD, Avenue du Val de Montferrand, 34032 Montpellier CEDEX, France.

0098-0331/91/1100-2127$06.50/0

INTRODUCTION

The American palm weevil (APW), *Rhynchophorus palmarum* (L.), is a major pest of coconut and oil palm crops in tropical America and the West Indies (Lepesme et al., 1947; Genty et al., 1978). It is occasionally a pest on sugarcane (Restrepo et al., 1982). APW adults cause indirect damage to palm trees by vectoring the nematode responsible for red ring disease (Griffith, 1987). Direct damage is also caused by the larvae that develop in the stems, buds, and rachis of leaves and inflorescences of cultivated, ornamental, or wild palm trees (Lepesme et al., 1947; Wattanapongsiri, 1966). The only way to prevent red ring disease is to eliminate the adults and larvae of the APW since the difficulty and cost of nematicide treatments are extreme.

Despite its economic importance, few studies have been devoted to the chemical ecology of this weevil (Hagley, 1965). The APW is very large (35–50 mm long), hard to observe in the field, and difficult to manipulate in the laboratory. The endophytic development of the larvae (three to six months) in palm or sugarcane stems makes this species particularly difficult to rear in the laboratory, especially in temperate countries where natural food plants are not available.

Rochat et al. (1991) reported field and laboratory evidence documenting the existence of a male-produced aggregation pheromone in the APW. This species belongs to the second genus in the Rhynchophorinae for which pheromone-based communication has been reported. Pheromones in the Rhynchophorinae have been studied previously in the sole genus *Sitophilus* (Faustini et al., 1982; Phillips and Burkholder, 1981; Phillips et al., 1985, 1987, 1989; Walgenbach et al., 1983, 1987; Walgenbach and Burkholder, 1986).

This paper describes the collection, electrophysiological activity, and behavioral bioassays of the male-produced volatiles; and the identification, synthesis, and biological activity of the essential component of the APW aggregation pheromone.

METHODS AND MATERIALS

Insects and Volatile Collections. APWs were obtained from a laboratory colony reared on sugarcane. The colony originated from mixed strains from Colombia and Guadeloupe. Eclosed adults were separated by sex, kept individually (27 $\pm$ 2°C and 85 $\pm$ 10% relative humidity, in a 12:12 hr light–dark regime) in cylindrical plastic boxes (105 $\times$ 75 mm) with a wire mesh cover, and fed with sugarcane.

Five virgin APW males or females were placed in a cylindrical glass jar (6 $\times$ 22 cm) with three sugarcane pieces. Three pieces of the same size and from the same stem were placed in a similar jar as a control. The volatiles

emitted by APWs and/or sugarcane were simultaneously trapped in two glass cartridges connected downstream to the jars for two days (procedure 1) or one day (procedure 2). Each cartridge was filled with 0.5 g (procedure 1) or 0.2 g (procedure 2) of Supelpak-2 adsorbent (16/50 mesh; Supelco). The volatile collections were performed at 27 ± 2°C with a 12:12 hr light–dark regime. Air was passed at 500 ml/min through the glass systems. In procedure 1, the cartridges were eluted with 2.5 ml of methylene chloride (HPLC grade, Waters). The eluates were concentrated at 100 μl under nitrogen (150 ml/min) and used in pitfall bioassays. In procedure 2, the cartridges were eluted with 1 ml of methylene chloride and the extracts were used in electroantennography and for analytical investigations. Ten and 50 volatile collections were made using procedures 1 and 2, respectively. All extracts were stored at −30°C until use.

Analysis of Volatiles. Natural and synthetic volatiles were analyzed on a Carlo Erba Fractovap 2900 chromatograph equipped with 25-m × 0.32-mm-ID fused silica capillary columns coated with a polar phase (WCOT FFAP CB, Chrompack; temperature program: 35 to 55°C at 20°C/min, 8 min at 55°C and 55 to 220°C at 5°C/min) or a nonpolar phase (WCOT CPSil 8CB, Chrompack; temperature program: 35 to 60°C at 25°C/min, 5 min at 60°C, and 60 to 250°C at 5°C/min) with helium as carrier gas.

Gas chromatography–mass spectrometry (GC-MS) analyses were performed with a Girdel 32 chromatograph coupled to a R10-10C Nermag quadrupole mass spectrometer. The chromatograph was equipped with a fused silica capillary column (25 m × 0.32 mm ID; WCOT CPSil 5CB, Chrompack) and operated isothermally at 60°C. The electron impact (EI) was realized at 70 eV. The chemical ionization (CI) was obtained using ammonia as reactant gas at 92.5 eV.

Gas chromatography–Fourier transform infrared spectrometry (GC-FTIR) was carried out with a IFS-85 spectrometer coupled to a HRCG 5160 Carlo Erba chromatograph equipped with a fused silica capillary column (30 m × 0.32 mm ID; WCOT DB-5, J&W Scientific) heated from 30 to 170°C at 10°C/min. The light pipe was heated at 220°C.

Proton nuclear magnetic resonance spectrometry ([^{1}H]NMR) spectra of the natural major pheromone compound of APW males (R1) and synthetic compounds I–III (below) were recorded with a Brucker AC 400 spectrometer in C_6D_6 (99.93%, CEA, France). R1 (ca. 50 μg) was obtained by micropreparative capillary GC performed on a Girdel 300 chromatograph equipped with a 15-m × 0.53-mm-ID fused silica column (WCOT DB-5; J&W Scientific) and operated isothermally at 70°C. The splitting of crude extracts and the collections of R1 were performed with a Malosse (1990) fraction collector.

Chemical Synthesis. Racemic (2*E*)-6-methyl-2-hepten-4-ol (I), (2*E*)-5-methyl-2-hepten-4-ol (II), and (2*E*)-2-octen-4-ol (III) were synthesized by reacting crotonaldehyde at −10°C in THF with Grignard reagents of isobutyl

bromide, 2-bromobutane, and 1-bromobutane to give the corresponding alcohols I–III as confirmed by GC-MS and [^{1}H]NMR spectrometry (For proton number, see Figure 4).

Compound I showed EI ions at *m/z* (% relative abundance) 113 (2), 110 (3), 95 (8), 86 (8), 85 (6), 71 (100), 69 (8), 67 (11), 57 (10), 53 (8), 43 (20), 41 (21). I had [^{1}H]NMR δ (ppm) at 0.88 (d, 3H; H-8), 0.91 (d, 3H; H-7), 1.22 (ddd, 1H; H-5′) 1.44 (ddd, 1H; H-5), 1.5 (dd, 3H; H-1), 1.77 (m, 1H; H-6), 3.97 (m, 1H; H-4), 5.41 (m, 2H; H-2, H-3) with the following coupling constants (Hz): $J_{1-2} = 5$, $J_{1-3} = 0.8$, $J_{4-5} = J_{4-5'} = 8$, $J_{5-5'} = 13.5$, $J_{5-6} = J_{5'-6} = 6$, $J_{6-7} = J_{6-8} = 4.2$.

Compound II showed EI ions at *m/z* 110 (2), 100 (3), 95 (3), 81 (3), 71 (100), 69 (8), 57 (6), 53 (7), 43 (12), 41 (13). II had [^{1}H]NMR δ (ppm) at 0.89 (t, 3H; H-7), 0.95 (d, 3H; H-8), 1.25 (m, 2H; H-6), 1.55 (de, 3H; H-1), 3.75 (td, 1H; H-4), 5.45 (dd, 2H; H-2, H-3) with the following coupling constants (Hz): $J_{1-2} = 5.6$, $J_{1-3} = 2$, $J_{2-3} = 15$, $J_{3-4} = 5.4$, $J_{4-5} = 14.5$, $J_{4-OH} = 5.5$, $J_{5-8} = J_{6-7} = 6.8$.

Compound III showed EI ions at *m/z* 113 (2), 110 (3), 95 (2), 86 (12), 81 (8), 71 (100), 69 (9), 58 (8), 57 (7), 53 (9), 43 (14), 41 (17). III had [^{1}H]NMR δ (ppm) at 0.9 (t, 3H; H-8), 1.19–1.52 (m, 6H; H-5, H-6, H-7), 1.55 (de, 3H; H-1), 3.89 (m, 1H; H-4), 5.44 (dd, 2H; H-2, H-3) with the following coupling constants (Hz): $J_{1-2} = 5.9$, $J_{3-4} = 6.3$, $J_{4-5} = 6.4$, $J_{7-8} = 6.8$.

Compounds I–III were purified to >90% by distillation at 70°C and 10 mm Hg, as checked by GC-MS. I was further purified by HPLC using a Waters Associates model 510 chromatograph equipped with a 0.3 × 10-cm column (Silica normal phase, 8-μm particles; Chrompack). Compounds were detected with a differential refractometer R 401 (Waters Associates). The final purity of I was >99%. Racemic 6-methyl-4-heptanol (IV) was obtained by catalytic (PtO_2, methanol) hydrogenation of purified I.

Electroantennography. Electroantennograms (EAGs) were recorded from insects immobilized in a Plexiglas apparatus, using glass capillary electrodes filled with saline solution and connected to chloridized silver wires. The reference electrode was positioned at the base of the rostrum in a 0.5-mm-wide hole punched into the cuticle with an iron pin. The recording electrode was inserted into the antennal club (distal part and external area) on which the olfactory sensilla are located (Rochat, 1987). The electrical signal was fed into a 725-microprobe amplifier (WP Instruments inc.) and displayed on a Tektronix 5510 oscilloscope. Antennae of eight APWs of both sexes were subjected in randomized order to stimulation by air, solvent, six extracts containing APW and/or sugarcane volatiles, and eight synthetic chemicals (100 ng), including I tested also at 10 ng doses (Figure 2). All compounds except I–IV were commercial products of 99% purity. R1 was purified in an identical manner as I. One microliter of methylene chloride containing the natural or synthetic vola-

tiles was applied to a filter paper (17 × 17 mm) placed into a glass cartridge. Successive stimulations were separated by 2 min. EAG amplitudes were submitted to a two-way analysis of variance by stimulus (F1) and sex (F2). Interaction F1 vs. F2 would reveal sexual dimorphism in antennal responses to chemicals. Mean EAG amplitudes were compared using Newman-Keuls test ($\alpha = 0.01$).

Coupled gas chromatography–electroantennography (GC-EAD) was performed with a GIRA chromatograph modified according to Wadhams (1982). The chromatograph was equipped with a 25-m × 0.20-mm-ID fused silica column (WCOT HP-5, Hewlett Packard) heated from 60 to 220°C at 4°C/min. Helium was the carrier gas. The column effluents were split (50:50) between the FID and the antenna. A makeup flow of helium (20 ml/min) was added in the splitting device to accelerate the exit of the GC effluents towards the antenna. The effluents were driven from the chromatograph through a heated transfer line into a moistened airstream (1.4 l/min, 85% relative humidity, 26°C) focused onto the antennal preparation. The EAG signal was filtered (0.01–100 Hz bandpass) with an ERE amplifier (Ruy, France). GC and EAG signals were monitored on a two-channel chart recorder. Two APWs of both sexes were submitted, each one, to two extracts containing male (0.1 male-day-equivalent) + sugarcane volatiles.

Behavioral Bioassays. Insects were bioassayed using a two-choice pitfall olfactometer (Rochat et al., 1991) adapted from Pierce et al. (1981). The olfactometer consisted of a circular arena of 40 cm (diameter) × 14 cm (height) connected to two glass jars 9 cm (height) × 6.5 cm (diameter). According to preliminary investigations on the daily activity of the APW (Rochat, 1987), bioassays were run during the first half of the scotophase at 27 ± 2°C under red light at 40 lux. A single weevil was released at the center of the olfactometer arena and its position was recorded after 30 min. Weevils were classified as responding only if they fell into either the stimulus or the control jar or clung to the underside of the arena inside the jar.

Twenty to 32 weevils of both sexes were bioassayed in each of the following situations: (1) control treatment with two empty jars; (2) virgin male effluvia (0.1 male-day-equivalent) + sugarcane volatiles vs. sugarcane volatiles alone; and (3) 10 ng of (±)-I vs. solvent. One microliter of methylene chloride extract containing a natural or synthetic stimulus was applied to a filter paper (17 × 17 mm) placed into a glass jar just prior to the test. APW responses were quantified by the proportion of test weevils that responded after 30 min and the number of APWs choosing a stimulus jar. The first measure reflected the activity level of the population of test weevils (Walgenbach and Burkholder, 1986). The proportions of weevils that responded in situations 2 and 3 were compared with that obtained in absence of stimulus (situation 1) using the χ^2 test for two independent samples (Siegel, 1956). The numbers of weevils choosing a stim-

ulus were compared to the numbers of choices for the associated control jars using the nonparametric binomial test with the null hypothesis of an equal probability of choosing the control or the stimulus (Siegel, 1956).

RESULTS

Collection and Isolation of Male-Produced Pheromone. Comparisons of trapped volatiles from sugarcane, APW males + sugarcane, and APW females + sugarcane revealed the presence of two male-specific compounds: R1 and R2 (Figure 1). Females produced no detectible sex-specific volatiles. R1 was detected in 58 of 60 volatile collections analyzed by GC and was always the major of the two male-specific compounds. The mean release rate of R1 by APW males in 40 volatile collections was estimated by GC, using synthetic I as a standard, to be ca. 40 ng/male/hr. This rate varied between collections from ca. 0.5 to 140 ng/male/hr. R2 was detected in 48 of 60 extracts. The R1 : R2 ratio (based on GC peak areas) ranged most frequently between 100 : 15 and 100 : 5.

EAG Activity of Male-Produced Pheromone. Extracts containing male-produced + sugarcane volatiles gave very strong EAGs in both APW males and females (Figure 2). Extracts containing volatiles from sugarcane alone or from APW females + sugarcane induced EAGs as weak as those to air and solvent. Isolated R1 (40 ng) and a total extract containing R1 (15 ng) and R2 (R1 : R2 ratio of 100 : 18) induced EAGs of almost equal amplitudes.

All GC-EAD recordings from both male and female APWs showed high EAG responses to R1 (Figure 3). No EAGs were recorded in response to the sugarcane volatiles or to R2.

Pheromone Identification. The highest fragment ions observed in the EI mass spectrum of R1 were *m/z* 113 and *m/z* 110 (Figure 4). We assumed this compound to be an oxygenated aliphatic compound of mol wt 128. This assumption was confirmed by the CI $-$ NH_3 mass spectrum, which showed ions at *m/z* (% relative abundance): 146 ($M + NH_4^+$, 2), 129 ($M + H^+$, 9), 28 ($M^{+\cdot}$, 100), 111 ($M + H - H_2O^+$, 7), and 71 (1). Thus the *m/z* 110 ion was due to the loss of water and the *m/z* 113 ion to the loss of a methyl group from the molecule ion of a compound with $C_8H_{16}O$ formula. Absorption at 3649 cm^{-1} in the FTIR spectrum of R1 assigned the oxygen function to an hydroxyl group, and absorption at 966 cm^{-1} indicated a *trans* C—C double bond. Catalytic (PtO_2, methanol) microhydrogenation of R1 saturated the C–C double bond according to the CI mass spectrum, which showed ions at *m/z*: 148 ($M + NH_4^+$, 100), 130 ($M^{+\cdot}$, 5), 112 ($M - H_2O^+$, 11) and 87 (81). The couples of 18 amu-distant ions at *m/z* 73/55 and *m/z* 87/69 in the EI mass spectrum of the hydrogenated R1 (i) located the hydroxyl group in a C-4 position. (i), a couple

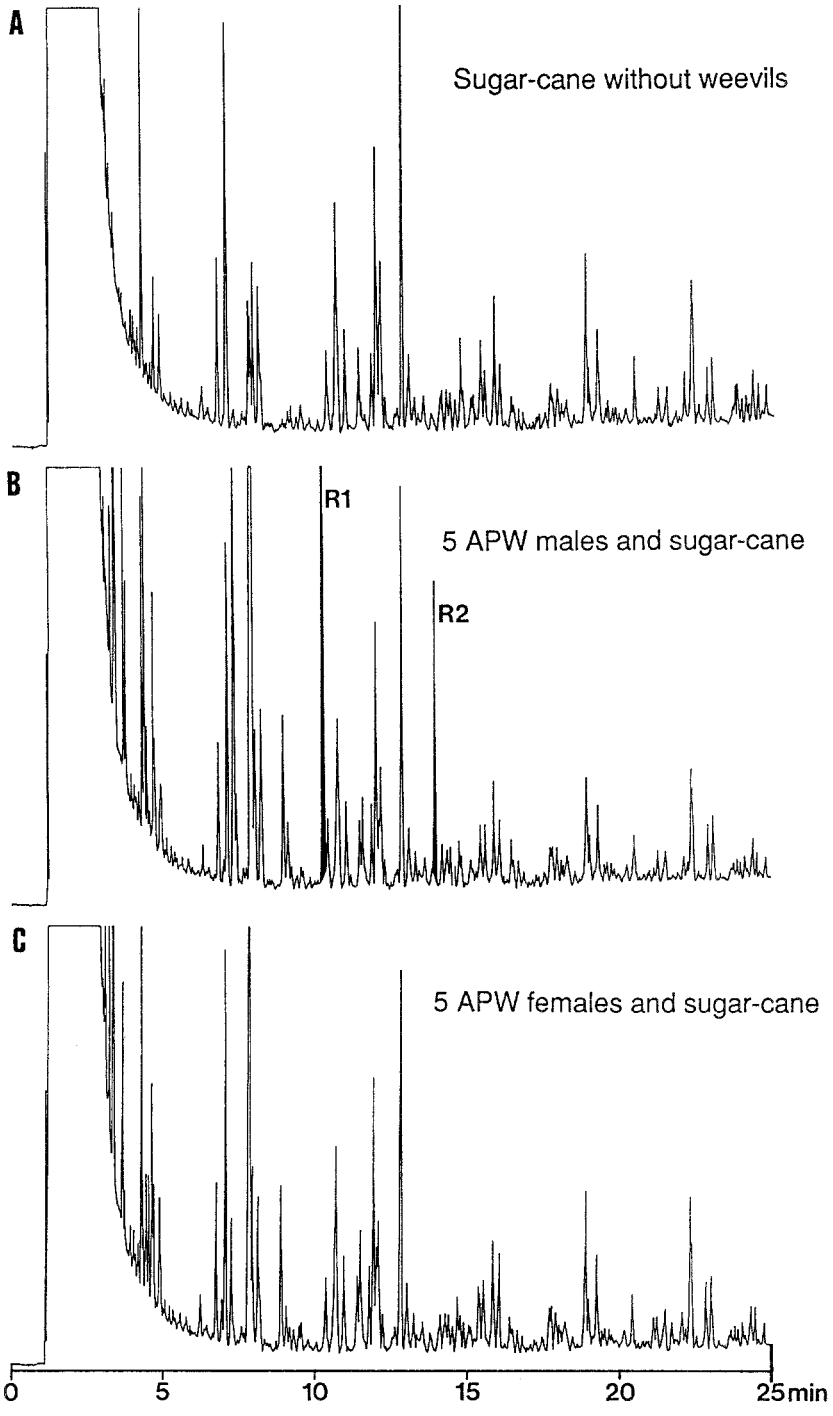


FIG. 1. Gas chromatograms of Supelpak-2-trapped volatiles from sugarcane (A), APW males and sugarcane (B), and APW females and sugarcane (C) showing two male-specific compounds: R1 and R2.

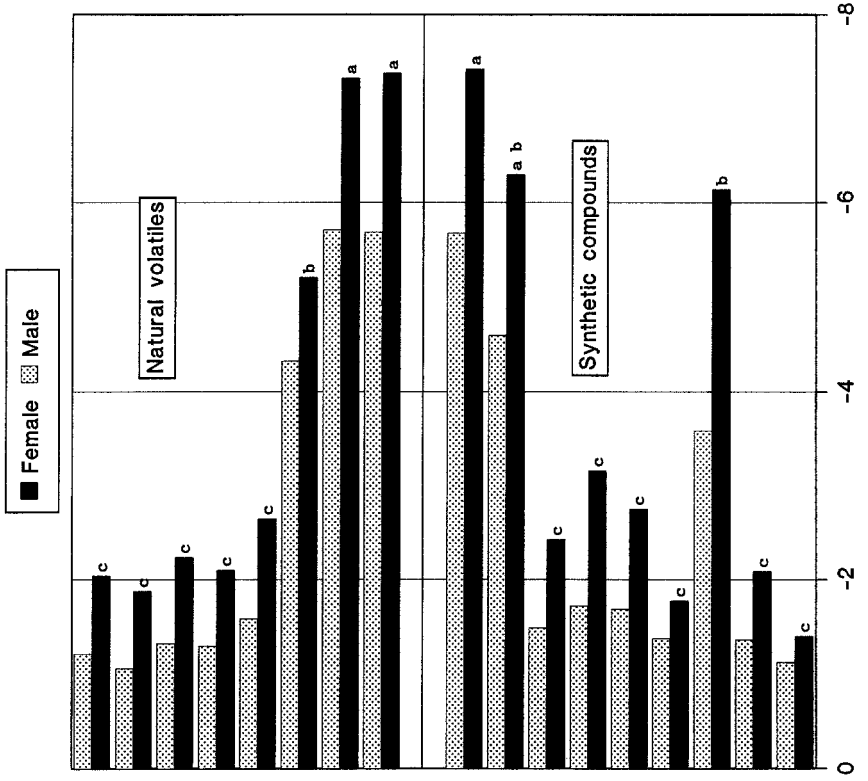


FIG. 2. Mean EAG responses from APW males and females ($N = 8$) to natural volatiles from APW and/or sugarcane and to synthetic pheromone-related compounds. Stimuli associated with the same letter do not differ significantly (Newman-Keuls test, $P < 0.01$) in terms of mean EAG amplitudes calculated on combined male and female responses.

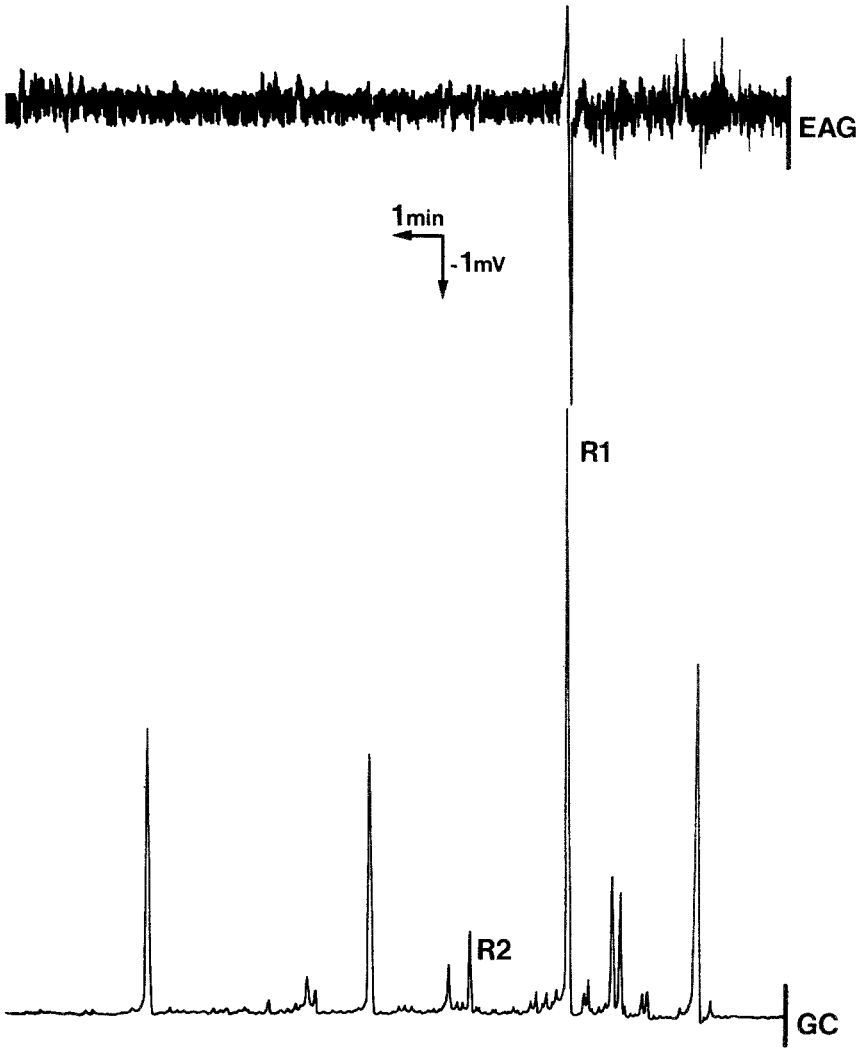


FIG. 3. GC-EAD recording from a female APW submitted to Supelpak-2-trapped volatiles from five APW males and sugarcane.

of 18 amu-distant ions at *m/z* 71/53 in the EI mass spectrum of R1 (ii) and GC-FTIR informations (iii) assigned the *trans* C–C double bond to be in C-2 position. The C-4 position of the hydroxyl group is in accordance with the presence of an even ion at *m/z* 86 resulting from a McLafferty rearrangement. Organization of the terminal four carbons of the chain to achieve complete structural identification of R1 could lead to three molecules: (2*E*)-6-methyl-2-hepten-4-

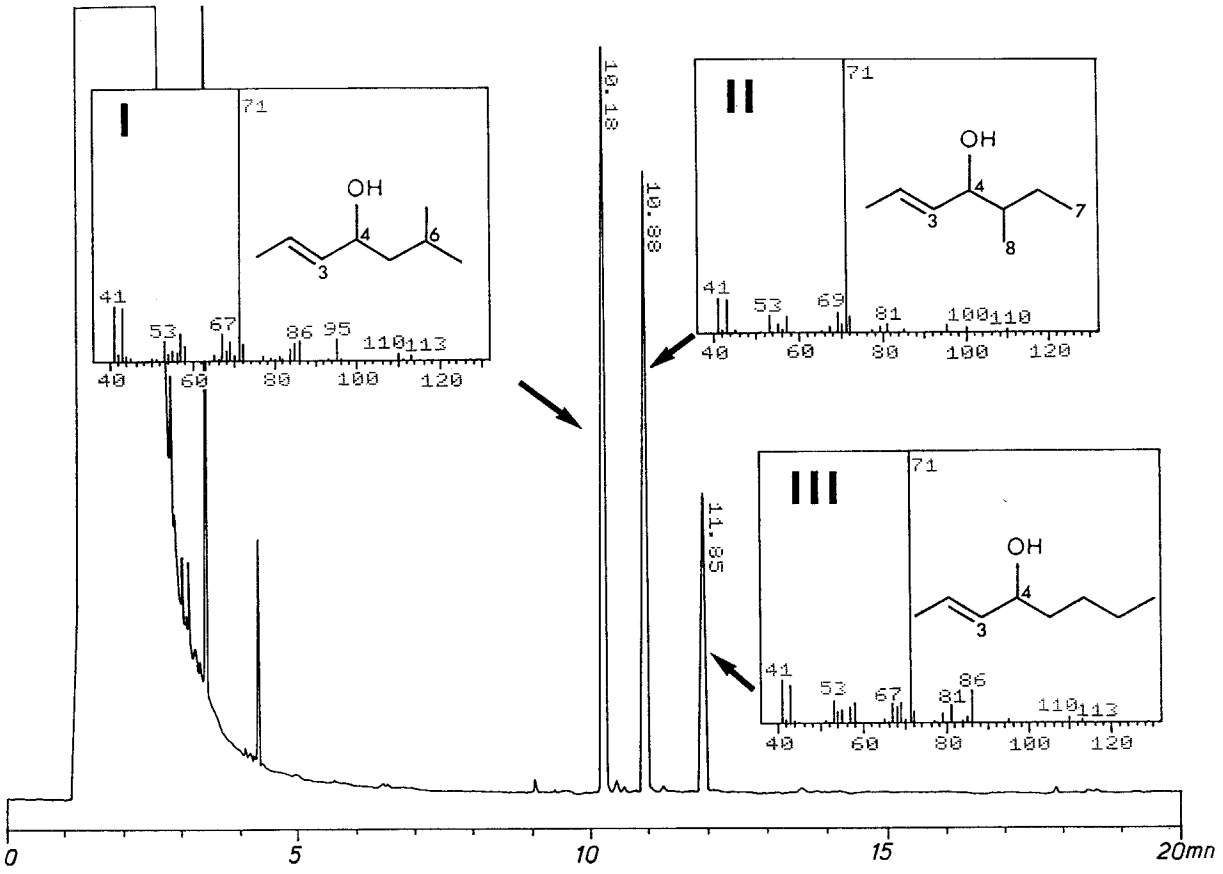


FIG. 4. Gas chromatogram (apolar column) and EI mass spectra of synthetic (2*E*)-5-methyl-2-hepten-4-ol (I), (2*E*)-5-methyl-2-hepten-4-ol (II), and (2*E*)-octen-4-ol (III).

ol (I), (2*E*)-5-methyl-2-hepten-4-ol (II), and (2*E*)-octen-4-ol (III). McLafferty rearrangement with II gave an even fragment ion at *m/z* 100 but not at *m/z* 86 as did R1, I and III. Comparisons of mass spectra; GC retention times of I, II, and III (Figure 4); and coinjection with R1, both on polar and apolar columns, led us to assign R1 to be (2*E*)-6-methyl-2-hepten-4-ol. The [^{1}H]NMR spectrum of R1 confirmed the identification of (2*E*)-6-methyl-2-hepten-4-ol. R1 shifts (ppm) were: 0.88 (d, 3H; H-8), 0.91 (d, 3H; H-7), 1.22 (ddd, 1H; H-5′), 1.44 (ddd, 1H; H-5), 1.5 (dd, 3H; H-1), 1.77 (m, 1H; H-6), 3.97 (m, 1H; H-4), 5.41 (m, 2H; H-2, H-3) with the following coupling constants (Hz): $J_{1\text{-}2} = 5$, $J_{1\text{-}3} = 0.8$, $J_{4\text{-}5} = J_{4\text{-}5'} = 8$, $J_{5\text{-}5'} = 13.5$, $J_{5\text{-}6} = J_{5'\text{-}6} = 6$, $J_{6\text{-}7} = J_{6\text{-}8} = 4.2$.

EAG Activity of Synthetic Pheromone and Analogs. Although the EAGs recorded from females were significantly greater in amplitude than those recorded from males ($F = 85.8$, $P < 0.001$), the EAG profiles disclosed no interaction stimulus by sex ($F = 1.27$, $P > 0.20$). Thus, there was no sexual dimorphism in the antennal responsness to the eight synthetic compounds tested. EAGs in response to I (10 and 100 ng) were significantly higher than to any octenol, including II and III (Figure 2) which in turn were not different from those to air and solvent. The mean EAG amplitudes obtained with 40 ng of isolated R1 and 100 ng of (±)-I were not statistically different. The mean EAG response to IV, a saturated analog of I, was significantly greater than to air and solvent and to any of the tested chemicals but I. However, IV had to be used at 10-fold higher doses than I to give EAGs of the same amplitude.

Behavioral Bioassay. In the two-choice pitfall olfactometer, (±)-I and volatiles from APW males induced similarly high numbers of weevils of both sexes to respond. Both males and females preferred significantly (±)-I to the solvent control (Table 1). Equivalent responses to (±)-I were obtained with wild APWs from Colombia and Guadeloupe (unreported data).

DISCUSSION

Rochat et al. (1991) showed that APW males produce a volatile aggregation pheromone. The strong EAGs in response to the male volatiles by APWs of both sexes corroborated the behavioral effect of the pheromone and showed that the response was due to a single major compound. The collection of variable amounts of this compound from male weevils may be due partly to the heterogeneity in age of the experimental weevils. Indeed, the age is a major factor influencing the production of aggregation pheromones in two *Oryzaephilus* species (Pierce et al., 1989).

Identification of (2*E*)-6-methyl-2-hepten-4-ol (I) as the essential pheromone compound was confirmed by both electrophysiological and behavioral data. We propose the trivial name rhynchophorol for this new pheromone.

TABLE 1. APW RESPONSES TO NATURAL AND SYNTHETIC MALE AGGREGATION PHEROMONE (RHYNCHOPHOROL) IN TWO-CHOICE PITFALL OLFACTOMETER AFTER 30 MIN

Treatment	Sex tested	Number tested	Number of responses[a]	Percent response		Binomial probability stimulus vs control[b]
				Stimulus	Control	
2 empty jars (control)	Male	30	8	50	50	0.637
	Female	20	2	50	50	—
Virgin male + food volatiles vs. food volatiles	Male	31	21**	71	29	0.021
	Female	21	14***	93	7	0.001
10 ng of (±)-I[c] vs solvent	Male	32	20**	85	15	0.001
	Female	31	20***	80	20	0.006

[a] Difference from the control (χ^2 test) indicated by:
n.s.: not significant, ** $P < 0.01$ and *** $P < 0.001$.
[b] Under the null hypothesis to respond to the stimulus and to the control with an equal probability of 0.5.
[c] (±)-I: racemic (2*E*)-6-methyl-2-hepten-4-ol (rhynchophorol).

(±)-Rhynchophorol was a potent lure for the APW in laboratory conditions without food volatiles or the minor male-specific compound. (±)-Rhynchophorol at 10-ng doses did not exhibit any inhibitory effect, and there was no evidence for a differential peripheral reception of (±)-rhynchophorol and natural rhynchophorol. Nevertheless, further investigations are underway to determine the enantiomeric composition of natural rhynchophorol since chirality in beetle communication is of major consequence (Mori, 1984; Borden, 1985; Walgenbach et al., 1987; Phillips et al., 1989).

Rhynchophorol is a new eight-carbon aliphatic and oxygenated pheromone. Such pheromones have been found in three coleopteran families: Cerambycidae (2,3-octanediol and 2-hydroxy-3-octanone; Sakai et al., 1984), Curculionidae (sitophilure, 5-hydroxy-4-methyl-3-heptanone; Phillips et al., 1985; 5-methyl-4,6-heptanedione; Blight et al., 1984) and Scolytidae (ipsenol, 2-methyl-6-methylene-7-octen-4-ol, and ipsdienol, 2-methyl-6-methylene-2,7-octadien-4-ol; Silverstein et al., 1966; sulcatol, 6-methyl-5-hepten-2-ol; Byrne et al., 1974; 4-methyl-3-heptanol; Pearce et al., 1975).

Although the genera *Rhynchophorus* and *Sitophilus* are very close, there is no obvious structural relationship between rhynchophorol and sitophilure. Because of its terpenoidic skeleton, rhynchophorol appears to be closely related to the scolytid pheromones ipsenol, ipsdienol, and sulcatol. This similarity suggests biogenesis of rhynchophorol from host-plant terpenes, as has been dem-

onstrated or assumed for many scolytid pheromones (Vanderwel and Oehlschager, 1987).

Field-trapping data (Rochat et al., 1991) and recent laboratory results (Rochat et al., unpublished data) support the hypothesis that APW males produce rhynchophorol only when they feed on the host plant. The biological significance of the male APW pheromone would thus be a signal to aggregate on a suitable food resource, as occurs in many other beetles (Walgenbach and Burkholder, 1986; Borden, 1985; Renwick, 1988).

The identification of the APW aggregation pheromone is a considerable step towards improving traditional methods used to control this major pest. APW adults have been caught in poisoned traps baited with palm stem pieces for many decades (Morin et al., 1988) without any attention paid to the role that the APWs themselves could play in attracting conspecifics. The use of synthetic rhynchophorol may lead, either alone or with host-plant kairomones, to a great improvement in the efficiency of APW trapping for survey and control of this major pest.

Acknowledgments—We thank IRHO and especially D. Mariau for financial and technical support, and Fedepalma and P. Genty (Colombia) for providing us with wild weevils. We are indebted to M. Leverve, F. Radnai, and G. Pype for insect rearing; I. de Cruz and J.L. LeQuéré for their assistance, respectively, in micropreparative HPLC and coupled GC-FTIR spectrometry; J. Einhorn for helpful discussions in mass spectrometry; and two anonymous reviewers for helpful comments on the manuscript.

REFERENCES

BLIGHT, M.M., PICKETT, J.A., SMITH, M.C., and WADHAMS, L.J. 1984. An aggregation pheromone of *Sitona lineatus*. *Naturwissenschaften* 71S:480–481.

BORDEN, J.H. 1985. Aggregation pheromones, pp. 257–285, *in* G.A. Kerkut and L.I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon press, Oxford.

BYRNE, K.J., SWIGAR, A.A., SILVERSTEIN, R.M., BORDEN, J.H., and STOKKINK, E. 1974. Sulcatol: Population aggregation pheromone in the scolytid beetle, *Gnathotrichus sulcatus*. *J. Insect Physiol.* 20:1895–1900.

FAUSTINI, D.L., GIESE, W.L., PHILLIPS, J.K., and BURKHOLDER, W.E. 1982. Aggregation pheromone of the male granary weevil, *Sitophilus granarius* (L.). *J. Chem. Ecol.* 8:679–687.

GENTY, P., DESMIER DE CHENON, R., and MORIN, J.P. 1978. Les ravageurs du palmier à huile en Amérique Latine. *Oléagineux* 33:325–419.

GRIFFITH, R. 1987. Red ring disease of coconut palm. *Plant Dis.* 71:193–196.

HAGLEY, E.A.C. 1965. Tests of attractants for the palm weevil. *J. Econ. Entomol.* 58:1002–1003.

LEPESME, P., GHESQUIERE, J., BOURGOGNE, J., CAIRASCHI, E., PAULIAN, R., and VILLIERS, A. 1947. Les insectes des palmiers, pp. 611–617. Paul Lechevalier, Paris.

MALOSSE, C. 1990. A simple and efficient fraction collector for micropreparative capillary gas chromatography. *J. High Resol. Chromatogr.* 13:784–785.

MORI, K. 1984. The significance of chirality: methods for determining absolute configuration and

optical purity of pheromones and related compounds, pp. 323–370, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research, Springer-Verlag, New York.

MORIN, J.P., LUCCHINI, F., ARAUJO, J.C., FERREIRA, J.M., and FRAGA, L.S. 1988. Le contrôle de *Rhynchophorus palmarum* par piégeage à l'aide de morceaux de palmier. *Oléagineux* 41:57–59.

PEARCE, G.T., GORE, W.E., SILVERSTEIN, R.M., PEACOCK, J.W., CUTHBERT, R.A., LANIER, G.N., and SIMEONE, J.B. 1975. Chemical attractants for the smaller european elm bark beetle *Scolytus multistriatus* (Coleoptera: Scolytidae). *J. Chem. Ecol.* 1:115–124.

PHILLIPS, J.K., and BURKHOLDER, W.E. 1981. Evidence for a male-produced aggregation pheromone in the rice weevil. *J. Econ. Entomol.* 74:539–542.

PHILLIPS, J.K., WALGENBACH, C.A., KLEIN, J.A., BURKHOLDER, W.E., and SCHMUFF, H.M., 1985. (*R**,*S**)-5-hydroxy-4-methyl-3-heptanone, male-produced aggregation pheromone of *Sitophilus oryzae* (L.) and *S. zeamais* Motsch. *J. Chem. Ecol.* 11:1263–1274.

PHILLIPS, J.K., MILLER, S.P., ANDERSEN, J.F., FALES, H.M., and BURKHOLDER, W.E. 1987. The chemical identification of the granary weevil aggregation pheromone. *Tetrahedron Lett.* 28:6145–6146.

PHILLIPS, J.K., CHONG, J.M., ANDERSEN, J.F., and BURKHOLDER, W.E. 1989. Determination of the enantiomeric composition of (*R**,*S**)-1-ethylpropyl 2-methyl-3-hydroxypentanoate, the male-produced aggregation pheromone of *Sitophilus granarius. Entomol. Exp. Appl.* 51:149–153.

PIERCE, A.M., BORDEN, J.H., and OEHLSCHLAGER, A.C. 1981. Olfactory response to beetle-produced volatiles and host-food attractants by *Oryzaephilus surinamensis* and *O. mercator. Can. J. Zool.* 59:1980–1990.

PIERCE, A.M., PIERCE, H.D., JR., BORDEN, J.H., and OEHLSCHLAGER, A.C. 1989. Production dynamics of Cucujolide pheromones and identification of 1-octen-3-ol as a new aggregation pheromone for *Oryzaephilus surinamensis* and *O. mercator* (Coleoptera: Cucujidae). *Environ. Entomol.* 18:747–755.

RENWICK, J.A.A. 1988. Comparative mechanisms of host selection by insects attacking pine trees and crucifers, pp. 303–316, *in* K.C. Spencer (ed.). Chemical Mediation of Coevolution. Academic Press, San Diego.

RESTREPO, G.L., RIVERA, A.F., and RAIGOSA, B.J. 1982. Ciclo de vida, habitos y morfometria de *Metamasius hemipterus* Olivier y *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae) en caña de azucar (*Saccharum officinarum* L.) *Acta Agron. Colomb.* 32:33–44.

ROCHAT, D. 1987. Etude de la communication chimique chez un Coléoptère Curculionidae: *Rhynchophorus palmarum* L. DEA report, Université Paris 6. 30 pp.

ROCHAT, D., GONZALEZ, V.A., MARIAU, D., VILLANUEVA, G.A., and ZAGATTI, P. 1991. Evidence for male-produced aggregation pheromone in American palm weevil, *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae). *J. Chem. Ecol.* 17:1221–1230.

SAKAI, T., NAKAGAWA, Y., TAKAHASHI, J., IWABUCHI, K., and ISHII, K. 1984. Isolation and identification of the male sex pheromone of the grape borer *Xylotrechus pyrrhoderus* Bates (Coleoptera: Cerambycidae). *Chem. Lett.* 263–264.

SIEGEL, S. 1956. Nonparametric Statistics for the Behavioral Sciences, McGraw-Hill, New York. 312 pp.

SILVERSTEIN, R.M., RODIN, J.O., and WOOD, D.L. 1966. Sex attractants in frass produced by male *Ips confusus* in ponderosa pine. *Science* 154:509–510.

VANDERWEL, D., and OEHLSCHLAGER, A.C. 1987. Biosynthesis of pheromones and endocrine regulation of pheromone production in Coleoptera, pp. 175–215, *in* G.D. Prestwich and G.J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, Orlando.

WADHAMS, L.J. 1982. Coupled gas chromatography-single cell recording: A new technique for use in the analysis of insect pheromones. *Z. Naturforsch.* 37c:947–952.

WALGENBACH, C.A., and BURKHOLDER, W.E. 1986. Factors affecting the response of the maize weevil, *Sitophilus zeamais* (Coleoptera: Curculionidae) to its aggregation pheromone. *Environ. Entomol.* 15:733–738.

WALGENBACH, C.A., PHILLIPS, J.K., FAUSTINI, D.L., and BURKHOLDER, W.E. 1983. Male-produced aggregation pheromone of the maize weevil, *Sitophilus zeamais*, and interspecific attraction between three *Sitophilus* species. *J. Chem. Ecol.* 13:831–841.

WALGENBACH, C.A., PHILLIPS, J.K., BURKHOLDER, W.E., KING, G.G.S., SLESSOR, K.N., and MORI, K. 1987. Determination of chirality in 5-hydroxy-4-methyl-3-heptanone, the aggregation pheromone of *Sitophilus oryzae* (L.) and *S. zeamais* Motschulsky. *J. Chem. Ecol.* 13:2159–2169.

WATTANAPONGSIRI, A. 1966. A revision of the genera *Rhynchophorus* and *Dynamis* (Coleoptera: Curculionidae). *Dep. Agric. Sci. Bull. (Bangkok)*. 1:1–328.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

SEX PHEROMONE COMMUNICATION IN THE SCREWWORM, *Cochliomyia hominivorax*: ONTOGENETIC AND STRAIN EFFECTS

L. HAMMACK[1]

Biosciences Research Laboratory
Agricultural Research Service, U.S. Department of Agriculture
Fargo, North Dakota 58105

(Received May 6, 1991; accepted July 3, 1991)

Abstract—A laboratory behavioral assay was used to examine ontogenetic and strain effects on sex pheromone production and reception in the screwworm, which employs a female-produced contact pheromone to stimulate male copulatory attempts. Pheromone levels peaked between three and six days after adult emergence in females of two strains that had been colonized fewer than 50 generations. Pheromone was detectable at low levels in newly emerged flies of both sexes but disappeared from males by two days of age, when males first became responsive to females. A similar decline in pheromone with ontogenetic age was previously described for females of a strain colonized over 100 generations. Interstrain tests between the two newer strains, which differed by 35 generations in duration of colonization, demonstrated lower female pheromone activity but higher male responsiveness to pheromone in the older strain, despite the similar shape within these strains of curves relating female age and pheromone activity. The data suggest that conditions of screwworm colonization result in gradual loss of the pheromone peak that develops three to six days after emergence in newly colonized females. Higher male responsiveness associated with longer colonization involved change in both thresholds and dose dependency and may be relevant to control of the screwworm via the sterile insect technique.

Key Words—Screwworm, *Cochliomyia hominivorax*, Calliphoridae, Diptera, blow fly, mating stimulant pheromone, contact sex pheromone, laboratory adaptation.

[1] Present address: Northern Grain Insects Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Rural Route 3, Brookings, South Dakota 57006.

0098-0331/91/1100-2143$06.50/0

INTRODUCTION

The screwworm, *Cochliomyia hominivorax* (Coquerel) has been eradicated from the United States and Mexico, where it has the potential to inflict losses exceeding $600,000,000 annually, primarily to livestock producers (Snow et al., 1985). It remains the most destructive agent of myiasis throughout tropical and subtropical areas of Central and South America (Guimarães et al., 1983; Dear, 1985; Snow et al., 1985; Krafsur et al., 1987) and threatens Old World livestock and wildlife after its recent discovery in Libya (El-Azazy, 1989; Gabaj and Beesley, 1989). The screwworm is well established in Libya and capable of spreading into sub-Saharan Africa and the Middle East, with seasonal incursions into southern Europe (Gabaj et al., 1989; Palca, 1990).

Eradication of the screwworm from North America was accomplished with the sterile insect technique (SIT), which is also planned for *C. hominivorax* populations in Central America and northern Africa (Snow et al., 1985, McGourty, 1989). SIT success is subject to factors influencing mating behaviors because sexually sterilized released males must mate with native females in order to render their eggs sterile and thereby drive the population toward extinction (Knipling, 1955).

Sex pheromones are important for mating in many species of insects (Tamaki, 1985). The screwworm is no exception. A contact pheromone in cuticular lipids of females stimulates copulatory attempts from males when applied to objects mimicking flies (Mackley and Broce, 1981; Hammack, 1986).

A distinctive asymmetry in mating behavior characterizes crosses between screwworm strains that differ in duration of colonization, i.e., mating attempts occur more frequently between old-strain males and new-strain females than in the reciprocal direction (Alley and Hightower, 1966; Spates and Hightower, 1967; Hammack, 1987; Mangan, 1988). Cuticular lipid profiles also change during colonization, but only in females (Pomonis and Mackley, 1985), suggesting colonization effects on sex pheromone communication. Reduced female pheromone activity and enhanced male responsiveness to pheromone associated with longer colonization account for at least one case of asymmetric mating isolation between old and new screwworm strains (Hammack, 1987). The dependence of mating on pheromonal cues indicates that the sex pheromone system can be used to assess the reproductive compatibility of screwworm populations.

The present study examines ontogenetic and strain effects on sex pheromone activity and male responsiveness in screwworm strains reared fewer than 50 generations in the laboratory and differing by 35 generations in duration of colonization. Such ontogenetic data are needed to ensure validity of interstrain comparisons, but detailed information is available only for a strain (009) colonized over 100 generations (Hammack, 1987). Age and strain data are also

needed to optimize pheromone recovery and detection for isolation and characterization of the screwworm sex pheromone(s).

METHODS AND MATERIALS

Insects. Two strains of screwworm were tested. The FC96 strain originated from a single egg mass collected in Chiapas, Mexico, in October 1983 and was used in generations 36–43, unless stated otherwise. FC96 had served as the newer strain in a previous study; it was maintained by single-pair matings from generation six to minimize sexual harassment of caged females by males and possibly reduce selection for pheromone changes (Hammack, 1987). A newer strain derived from multiple egg masses collected in Belize in October 1986 was tested after 11–19 generations in the laboratory. Belize colonies were maintained in mixed-sex groups. Belize is the strain currently being reared in Mexico for SIT, although its name was changed to OW87 upon introduction into mass production.

Larvae were reared on a beef-based diet; adults were maintained on corn syrup and water at 25 ± 1°C under a 12 : 12 hr light–dark photoperiod (Hammack, 1984). All test insects were virgin and, unless stated otherwise, were obtained by separating the sexes within 24 hr of emergence, using CO_2 anesthesia.

Pheromone Preparation and Bioassay. Pheromone was obtained from 4-day-old females, except as otherwise indicated. Insects supplying pheromone were killed by freezing, thawed, and either mounted on corks for testing or extracted for 15 sec at the rate of 20/ml of *n*-hexane (Hammack, 1987). Extracts were prepared from 200 females, unless stated otherwise, and were stored at −70°C.

The pheromone activity of killed and extracted insects was measured in bioassays that determined whether or not test males attempted copulation when paired with decoy insects for 1 min (Hammack, 1987). Each decoy was mounted on a cork that was fitted into a glass test tube (15 × 150 mm) containing one test male. Inversion of the tube forced the male to contact the decoy. Each decoy was exposed to a series of 10 test males and then discarded. Test males were used only once. Decoys were either whole dead screwworm adults (both sexes) or males washed three times in hexane (≥0.2 ml/wash) and then treated with female extract applied in 5 μl of hexane. Washed decoys treated only with hexane elicit no copulatory responses (Hammack, 1987).

Experiments. The first series of tests investigated the relationship within strains between age in days after adult emergence (±4 hr) and pheromone activity of females and males. Whole dead females of the FC96 strain were examined between 0 and 8 days old in one test and between 6 and 16 days in another.

Pheromone activity was also measured in extracts of FC96 females ranging from 2 to 16 days old and Belize females from 1 to 12 days old. Whole dead FC96 males 0–8 days old were examined in a fifth test. Within each test, flies supplying pheromone had emerged on the same day from a single larval rearing group. Pheromone activity was estimated from the responses of 100 5- to 8-day-old FC96 males or 50 10- to 12-day-old Belize males.

A second series of tests examined the effect of male age on responsiveness to dead female decoys in the FC96 strain. Different groups of males were tested daily 0–8 days after adult emergence in the first test of the series ($N = 100$) and at 7, 8, 10, 12, 14, and 16 days in the second one ($N = 50$). Males tested before 3 days of age had been separated from females without anesthesia.

An extract of each strain of females was prepared in six of eight successive generations beginning when Belize and FC96 strains were in generations 11 and 46, respectively. Each extract was applied at the rate of 0.5 female equivalent (FE) to separate hexane-washed decoys of each strain. Each extract and decoy combination was tested with 50 8- to 11-day-old test males of each strain. Percentages of males attempting copulation were transformed ($\arcsin\sqrt{Y}$) and examined by analysis of variance (ANOVA procedure, SAS Institute, 1988). The model was a factorial one having three classes (extract, test male, and decoy) each at two levels (FC96 and Belize strains).

The relationship between pheromone dose (X) plotted on a logarithmic scale and percent males attempting copulation (Y) was obtained within FC96 and Belize strains with 100 males per test dose. FC96 males were 6–8 days old; Belize males were 10–12 days old. The FC96 extract was prepared from 40 females. Linear regression was used to analyze each set of data (REG procedure, SAS Institute, 1988). Dose–response data were also acquired with extract of Belize females, but FC96 test males in generations 86–91. One hundred 6- to 8-day-old males were assayed at each of five doses between 0.064 and 0.25 FE. The procedure was repeated with 10- to 12-day-old males. Regression analysis was again used for data analysis, with the addition to the model of age (Z) as an independent variable (REG procedure, SAS Institute, 1988).

Within tests, responding males were matched with respect to larval rearing group of origin and time of day of testing. In addition, treatments within tests were bioassayed simultaneously with similarly aged males, except when age effects on male responsiveness to pheromone were examined.

RESULTS

The pheromone activity of FC96 female decoys increased from adult emergence until 2 days of age and then remained high enough through 16 days of age to stimulate copulatory attempts in more than 80% of test males (Figure

1). When the response frequency was reduced by diluting extracts to 0.5 FE, a peak in pheromone activity emerged between 4 and 6 days of age (Figure 1). Belize females were also maximally stimulatory when extracted between 3 and 6 days of age (Figure 2), although 1.0 FE was required to approach response levels obtained with 0.5 FE in the FC96 series.

Mature male decoys of the FC96 strain showed no pheromone activity, but newly emerged ones elicited copulatory attempts from nearly 30% of test males

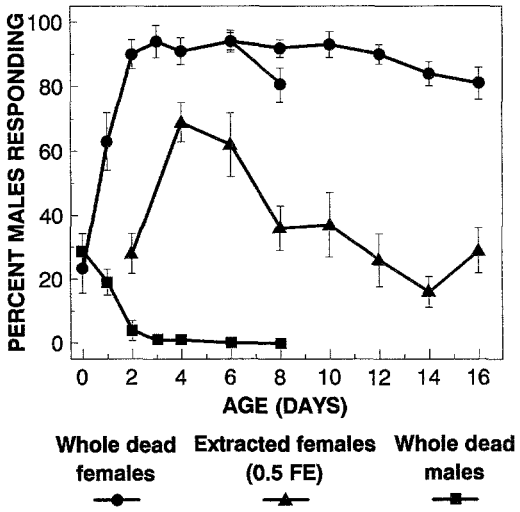


FIG. 1. Effect of age on sex pheromone activity in screwworm flies of the FC96 strain (mean ; pmSEM) pm SEM). Extract concentration is expressed in female equivalents (FE).

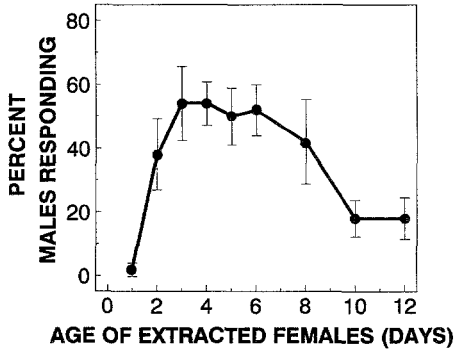


FIG. 2. Effect of age on sex pheromone activity in screwworm flies of the Belize strain. Each point is the mean response (±SEM) to 1.0 female equivalent of extract.

(Figure 1). The sharpest decline in pheromone activity of males occurred by 2 days of age (Figure 1).

Newly emerged and 1-day-old males failed to react to female decoys killed at 4 days of age (Figure 3), when female pheromone activity was highest. However, male responsiveness increased from 2 to 5 days of age and then remained high through 16 days (Figure 3).

Screwworm strain significantly affected the activity of female extracts and the responsiveness of males to those extracts; however, the strain used for decoys did not affect behavior when decoys were washed in hexane before treatment with extract (Table 1). None of the interaction terms was significant (Table 1). Belize females were more stimulatory than FC96 females to males of both strains, but Belize males were less responsive than FC96 males to females of both strains (Table 2).

Figure 4 shows that slope (b) of the regression line relating pheromone concentration plotted on a logarithmic scale and the percentage of males responding appeared steeper in the FC96 [b = 97.2, 95% confidence interval (CI) = 61.1–133.3] than in the Belize strain (b = 53.1, 95% CI = 41.1–65.2). Responses of FC96 males to extract of Belize females confirmed a strain difference in the slope of dose–response lines and indicated that the difference resulted from variation in male behavior rather than composition of female extracts. The regression model explained a significant amount of variation in the response of FC96 males to Belize extract ($F_{2,7} = 41.70$, $P < 0.0001$, $r^2 = 0.92$, $Y = 132.30 \log_{10} 100X - 0.50Z - 91.72$); however, only dose contributed significantly to the model ($t_1 = 9.13$, $P < 0.0001$), age of test males did not ($t_1 = -0.33$, $P = 0.75$). Deletion of the age variable from the model

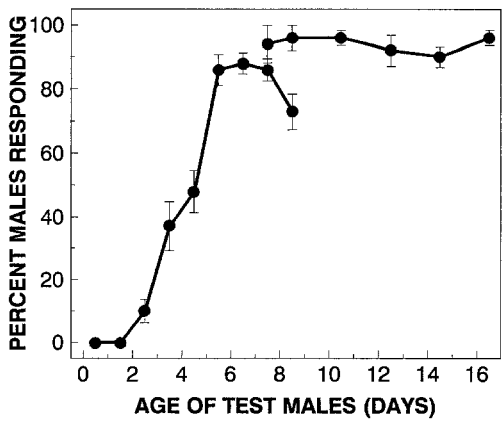


FIG. 3. Effect of age on male responsiveness to 4-day-old dead female decoys (mean ±SEM) within the FC96 screwworm strain.

TABLE 1. ANALYSIS OF VARIANCE OF EFFECTS OF STRAIN (FC96 AND BELIZE) OF FEMALES EXTRACTED, STRAIN OF TEST MALES, AND STRAIN OF DECOYS OF PERCENTAGE OF MALES ATTEMPTING COPULATION WITH HEXANE-WASHED DECOYS TREATED WITH 0.5 FEMALE EQUIVALENT OF EXTRACT

	Male response[a]				
Source	*df*	Sum of squares	Mean square	*F*	*P*
Model[b]	7	6.552	0.936	31.41	<0.0001
Extract (A)	1	1.391	1.391	46.69	<0.0001
Test male (B)	1	5.109	5.109	171.45	<0.0001
Decoy (C)	1	0.001	0.001	0.04	0.84
A * B	1	0.030	0.030	1.01	0.32
A * C	1	0.010	0.010	0.34	0.56
B * C	1	0.002	0.002	0.06	0.80
A * B * C	1	0.009	0.009	0.31	0.58
Error	40				

[a] Data transformed by $\arcsin\sqrt{Y}$.
[b] $r^2 = 0.85$

TABLE 2. MEAN PERCENTAGE OF MALES (± SEM) OF TWO SCREWWORM STRAINS ATTEMPTING COPULATION IN RESPONSE TO 0.5 FEMALE EQUIVALENT OF EXTRACT FROM EACH STRAIN[a]

Strain and sex	Belize females	FC96 females
Belize males	42.2 ± 2.5	12.0 ± 4.1
FC96 males	91.2 ± 2.9	70.8 ± 5.9

[a] Based on six replicates of 100 males per comparison.

simplified the equation to $Y = 132.30 \log_{10} 100X - 96.22$, with 95% CI about b ranging from 100.8 to 163.8. Thus, FC96 males generated a steep dose–response line whether tested with extract of FC96 or Belize females.

DISCUSSION

Sex pheromone activity peaked between 3 and 6 days of age in FC96 and Belize females that had been colonized fewer than 50 generations. In contrast, pheromone activity was greatest at emergence in 009 strain females that had

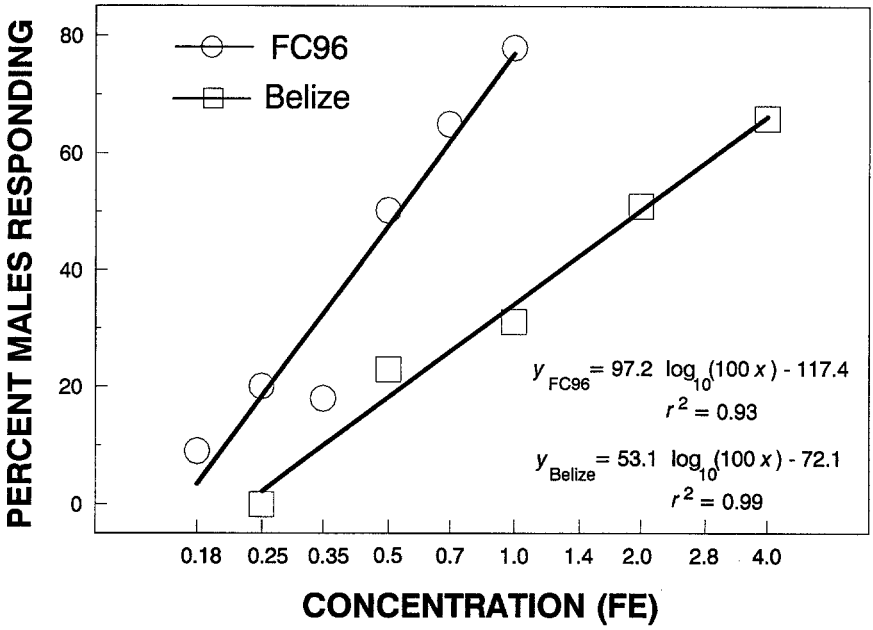


FIG. 4. Relationship between sex pheromone concentration expressed in female equivalents (FE) and male responses within FC96 and Belize screwworm strains.

been colonized more than 100 generations, presumably as a result of greater laboratory adaptation (Hammack, 1986, 1987; Mangan, 1988). Although the curves relating age and pheromone activity were similarly shaped within FC96 and Belize strains, the interstrain comparisons clearly demonstrated higher activity peaks in the Belize strain, but lower male responsiveness to pheromone. These differences followed expectation, i.e., lower pheromone activity in older strain females combined with greater willingness of older strain males to respond to pheromone and mate (Alley and Hightower, 1966; Spates and Hightower, 1967; Hammack, 1987; Mangan, 1988).

The data suggest that colonization leads not to a shift in the age when particular pheromone components are produced, but to gradual loss of the activity peak occurring three to six days after emergence in newer strain females. Screwworm females 3–6 days old are therefore best for determination of interstrain pheromone compatibilities and for recovery of maximum quantities of pheromone for chemical analysis.

It is conceivable that the strain differences in patterns relating ontogenetic age and female pheromone activity develop because different active chemicals occur at emergence and at sexual maturity, and only those present at maturity are affected by colonization. This scenario is consistent with the observations

that pheromone activity varies neither with strain nor sex of newly emerged fly (Pomonis and Mackley, 1985; Hammack, 1987) and is highest at emergence in 009 females colonized more than 100 generations (Hammack, 1987) and in males of a newer strain (FC96). Different active compounds are known to occur at emergence and sexual maturity in *Drosophila melanogaster*. Newly emerged flies of both sexes stimulate male courtship, albeit for reasons that are not entirely clear, but their activity derives from compounds other than the (*Z*,*Z*)-7,11-heptacosadiene that acts as the courtship-stimulating pheromone of mature *D. melanogaster* females (Pechine et al., 1988; Schaner et al., 1989).

Colonized females synthesizing little, if any, sex pheromone would be invaluable for screwworm behavioral studies and for deciphering hormonal, biochemical, and genetic mechanisms controlling pheromone production. A quantitative decrease in pheromone production with colonization is consistent with available data (Hammack, 1987), although qualitative changes such as altered pheromone blend or inhibitor cannot yet be ruled out. Male cuticular lipids potentially transferred at mating, as well as nonspecific paraffins absent from the cuticle, inhibit pheromonal responses of the tsetse fly, *Glossina morsitans* (Coates and Langley, 1982). In *D. melanogaster*, a courtship-inhibiting pheromone of males and mated females varies qualitatively with strain (Scott and Jackson, 1988). These examples illustrate that pheromone inhibitors occur in Diptera; however, any inhibitor in the screwworm likely functions in a different context because screwworm mating does not affect female pheromone activity (Hammack, 1987).

Whereas female screwworm flies colonized fewer than 50 generations attained peak activity by 3–4 days of age, male responsiveness to sex pheromone peaked only after four days. In nature, delayed maturation of males might deter mating among siblings that tend to emerge in spatial proximity to one another, especially when combined with the tendency for males of a cohort to emerge a day after females. Tests at reduced dosage would be necessary to establish that male responsiveness to pheromone is stable between five and 16 days; however, responses of FC96 males to dilutions of Belize extract provided no indication of response variation between six to eight and 10–12 days.

A stable response to pheromone between five and 16 days would correlate with a plateau in male mating frequency that lasts from 3–4 to 16 days of age (Crystal, 1967; Adams, 1979). Females become receptive to mating at 3 days of age (Crystal, 1967; Adams, 1979), when sex pheromone activity approached a peak in the FC96 and Belize strains. The decline in pheromone activity observed here after six days is not reflected in mating rates, but these rates are available only for a strain colonized more than 150 generations. Such colonized females maintain a stable mating level from 3 to 14 days of age (Crystal, 1967), which correlates with the absence of age-dependent changes in pheromone activity from three to eight days in the long-colonized 009 strain (Hammack,

1987). Discrepancies between pheromone and mating patterns may also result if females limit mating rates, which appears to be the case within screwworm strains (Adams, 1979; Hammack, 1987). Females are expected to mate less readily than males in a species with monogamous females and polygamous males displaying only the most cursory courtship before attempting copulation (Bushland and Hopkins, 1951; Thornhill and Alcock, 1983).

Differences were evident in sex pheromone dose–response patterns of FC96 and Belize males: thresholds were lower but dose dependency greater in the longer colonized FC96 strain. Greater dose dependency indicates less variation in response thresholds among individuals, which might be attributed in part to the single egg mass derivation of the FC96 strain. Inbreeding attending laboratory culture can also reduce genetic heterogeneity (see Delves and Barton Browne, 1989). Lower thresholds may simply reflect a greater willingness of colonized males to respond under suboptimal conditions. For example, screwworm mating in nature likely involves flight activity (Guillot et al., 1978), which is severely restricted in laboratory cages. The subsequent appearance in two FC96 sublines of further declines in female pheromone activity without immediate change in male responsiveness (unpublished data) suggests that pheromone loss by females drives male threshold changes. However, the apparent drop with colonization in the pheromone activity of FC96 females does not support the hypothesis that sexual harassment of caged females by males selects for pheromone loss (Hammack, 1987; Mangan, 1988) because breeding FC96 females had been isolated from males except for the few minutes needed for mating.

Changes in male mating behaviors with colonization are relevant to SIT because of its dependence on the mating success of sterilized mass-reared males with native females. Success rate may suffer because of detrimental effects on either sex or species discrimination. However, the cost to males of freely evaluating receptivity of potential mates is generally slight if it requires little time and energy (Thornhill and Alcock, 1983). Detrimental effects on discriminatory abilities may therefore only minimally affect mating success in an insect like the screwworm that lacks elaborate courtship. Mangan (1985) has even suggested that heightened mating aggressiveness may be beneficial because it could augment the transitory interference with host seeking by native females that follows sterile male releases. We clearly need a better understanding of colonization effects on mating behavior of screwworms reared for SIT. Knowledge that colonization modifies sex pheromone communication will assist in this endeavor.

Acknowledgments—I thank Patsy L. Evenson, Donna K. Galitz and Philip T. Meyer for insect rearing and technical assistance. Robert L. Mangan (USDA-ARS Subtropical Agriculture Research, Weslaco, Texas), Franklin D. Parker (USDA-ARS Screwworm Research, San Jose, Costa Rica), J. George Pomonis, and David B. Taylor kindly provided useful criticism of an earlier draft of the manuscript.

REFERENCES

ADAMS, T.S. 1979. The reproductive physiology of the screw-worm, *Cochliomyia hominivorax*, (Diptera: Calliphoridae). III. Mating. *J. Med. Entomol.* 15:488–493.

ALLEY, D.A., and HIGHTOWER, B.G. 1966. Mating behavior of the screw-worm fly as affected by differences in strain and size. *J. Econ. Entomol.* 59:1499–1502.

BUSHLAND, R.C., and HOPKINS, D.E. 1951. Experiments with screw-worm flies sterilized by X-rays. *J. Econ. Entomol.* 44:725–731.

COATES, T.W., and LANGLEY, P.A. 1982. The causes of mating abstention in male tsetse flies *Glossina morsitans*. *Physiol. Entomol.* 7:235–242.

CRYSTAL, M.M. 1967. Reproductive behavior of laboratory-reared screw-worm flies (Diptera: Calliphoridae). *J. Med. Entomol.* 4:443–450.

DEAR, J.P. 1985. A revision of the New World Chrysomyini (Diptera: Calliphoridae). *Rev. Bras. Zool.* 3:109–169.

DELVES, R.I., and BARTON BROWNE, L. 1989. Changes in reproductive performance of the Australian sheep blowfly, *Lucilia cuprina* (Wied.) (Dipt., Calliphoridae) following laboratory colonization. *J. Appl. Entomol.* 107:228–237.

EL-AZAZY, O.M.E. 1989. Wound myiasis caused by *Cochliomyia hominivorax* in Libya. *Vet. Rec.* 124:103.

GABAJ, M.M., and BEESLEY, W.N. 1989. American screwworm fly in Libya. *Vet. Rec.* 124:152.

GABAJ, M.M., WYATT, N.P., PONT, A.C., BEESLEY, W.N., AWAN, M.A.Q., GUSBI, A.M., and BENHAJ, K.M. 1989. The screwworm fly in Libya: A threat to the livestock industry of the Old World. *Vet. Rec.* 125:347–349.

GUILLOT, F.S., BROWN, H.E., and BROCE, A.B. 1978. Behavior of sexually active male screwworm flies. *Ann. Entomol. Soc. Am.* 71:199–201.

GUIMARÃES, J.H., PAPAVERA, N., and DO PRADO, A.P. 1983. As miíases na região neotropical (identifacação, biologia, bibliografia). *Rev. Bras. Zool.* 1:239–416.

HAMMACK, L. 1984. Relationships of larval rearing variables to fly attraction and oviposition responses in the screwworm, *Cochliomyia hominivorax* (Diptera: Calliphoridae) *J. Med. Entomol.* 21:351–356.

HAMMACK, L. 1986. Pheromone-mediated copulatory responses of the screwworm fly, *Cochliomyia hominivorax*. *J. Chem. Ecol.* 12:1623–1631.

HAMMACK, L. 1987. Chemical basis for asymmetric mating isolation between strains of screwworm, *Cochliomyia hominivorax*. *J. Chem. Ecol.* 13:1419–1430.

KNIPLING, E.F. 1955. Possibilities of insect control or eradication through the use of sexually sterile males. *J. Econ. Entomol.* 48:459–462.

KRAFSUR, E.S., WHITTEN, C.J., and NOVY, J.E. 1987. Screwworm eradication in North and Central America. *Parasitol. Today* 3:131–137.

MACKLEY, J.W., and BROCE, A.B. 1981. Evidence of a female sex recognition pheromone in the screwworm fly. *Environ. Entomol.* 10:406–408.

MANGAN, R.L. 1985. Reproductive response of native screwworm [*Cochliomyia hominivorax* (Coq.)] populations to sterile fly release. *Proc. Entomol. Soc. Wash.* 87:717–739.

MANGAN, R.L. 1988. Pedigree and heritability influences on mate selectivity and mating aggressiveness in the screwworm, *Cochliomyia hominivorax* (Diptera: Calliphoridae). *Ann. Entomol. Soc. Am.* 81:649–656.

MCGOURTY, C. 1989. African eradication plan threatened. *Nature* 340:422.

PALCA, J. 1990. Libya gets unwelcome visitor from the west. *Science* 249:117–118.

PECHINE, J.M., ANTONY, C., and JALLON, J.M. 1988. Precise characterization of cuticular compounds in young *Drosophila* by mass spectrometry. *J. Chem. Ecol.* 14:1071–1085.

POMONIS, J.G., and MACKLEY, J. 1985. Gas chromatographic composition profiles of surface lipid

extracts from screwworm compared by age, sex, colonization and geography. *Southwest. Entomol.* 10:65–76.

SCHANER, A.M., DIXON, P.D., GRAHAM, K.J., and JACKSON, L.L. 1989. Components of the courtship-stimulating pheromone blend of young male *Drosophila melanogaster*: (Z)-13-tritriacontene and (Z)-11-tritriacontene. *J. Insect Physiol.* 4:341–345.

SCOTT, D., and JACKSON, L.L. 1988. Interstrain comparison of male-predominant antiaphrodisiacs in *Drosophila melanogaster. J. Insect Physiol.* 34:863–871.

SNOW, J.W., WHITTEN, C.J., SALINAS, A., FERRER, J., and SUDLOW, W.H. 1985. The screwworm, *Cochliomyia hominivorax* (Diptera: Calliphoridae), in Central America and proposed plans for its eradication south to the Darien Gap in Panama. *J. Med. Entomol.* 22:353–360.

SAS Institute. 1988. SAS/STAT User's Guide, Release 6.03 Ed. Cary, North Carolina.

SPATES, G.E., and HIGHTOWER, B.G. 1967. Sexual aggressiveness of male screw-worm flies affected by laboratory rearing. *J. Econ. Entomol.* 60:752–755.

TAMAKI, Y. 1985. Sex pheromones, pp. 145–191, *in* G.A. Kerkut and L.I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 9. Pergamon, Oxford, U.K.

THORNHILL, R., and ALCOCK, J. 1983. The Evolution of Insect Mating Systems. Harvard University Press, Cambridge, Massachusetts.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

CORTICOSTERONE AND PROLACTIN DO NOT MEDIATE ALARM PHEROMONE EFFECT IN THE RAT

ERNEST L. ABEL* and MARAPPA G. SUBRAMANIAN

Wayne State University
Fetal Alcohol Research Center
Detroit, Michigan 48201

(Received May 13, 1991; accepted July 5, 1991)

Abstract—Corticosterone and prolactin do not reflect the alarm pheromone effect in the rat. Male rats were immersed in water previously swum in by 0, 1, or 2 other rats. Immobility decreased considerably after immersion in water previously swum in by one animal and did not decline further because of a "floor effect." Plasma corticosterone levels were increased by immersion in both fresh and soiled water. Prolactin levels were not increased significantly. Water condition did not significantly affect plasma corticosterone or prolactin levels when rats were sacrificed immediately after testing or 20 min after testing. These results indicate that neither corticosterone nor prolactin mediate the alarm pheromone phenomenon.

Key Words—Forced swim test, corticosterone, prolactin, rats, *Ratus ratus*.

INTRODUCTION

We have reported previously that when rats, *Ratus ratus*, are tested in fresh water in the forced swim test they exhibit considerable immobility, but if the water in this test has been soiled by another rat, they exhibit almost no immobility (Abel, 1991a,b; Abel and Bilitzke, 1990). We have interpreted this result as evidence that rats secrete an alarm pheromone in this test that affects the behavior of other rats subsequently exposed to it (Abel, 1991c).

There are several hypotheses concerning interpretation of the immobility response in the forced swim test. One of these hypotheses is that the test is stressful, and the immobility response during the test period represents an adaptive response to this stress (Hawkins et al., 1978). The argument that the test

*To whom correspondence should be addressed.

0098-0331/91/1100-2155$06.50/0

is stressful is supported by Satoh et al. (1985) and a recent study from our laboratory (Abel, 1991b) showing that animals that undergo the forced swim test exhibit significantly increased plasma corticosterone levels. Since both corticosterone and prolactin are secreted in response to a wide variety of stressors (Axelrod and Reisine, 1984; Gala, 1990), the first part of this hypothesis is supported. In a previous study, we have shown that foot shock prior to testing causes rats to exhibit less immobility in the forced swim test (Abel and Bilitzke, 1990), suggestive of an increased stress response. Other studies have shown that foot shock and other stressors increase plasma corticosterone and prolactin in rats (e.g., Kant et al., 1983; Amario et al., 1986; Brown and Martin, 1974). By extension, one might expect that plasma levels of corticosterone and/or prolactin ought to be higher in animals tested in soiled water since they exhibit much less immobility than rats tested in fresh water (Abel, 1991a,b; Abel and Bilitzke, 1990) since these hormones are both elevated in response to stressors. The purpose of this study was to determine if indeed testing rats in soiled water causes them to have higher corticosterone and prolactin levels than testing animals in fresh water. Since corticosterone levels may not attain their maximal levels until 15–30 min after arousal (Seggie and Brown, 1975), animals were sacrificed either immediately or 20 min after removal from the test.

METHODS AND MATERIALS

Male Sprague-Dawley rats (Charles River, Portage, Michigan) 90–100 days of age were housed in polycarbonate cages in a vivarium at a constant room temperature of 21 ± 1°C and humidity (40 ± 5%) and a 12-hr light–dark cycle. Food and water were available ad libitum. Animals were tested for floating immobility in a Plexiglas cylinder (Corning Glass, Corning, New York) (45.7 cm height, 22.2 cm inside diameter) filled to a height of 38 cm with tap water maintained at 28 ± 1°C. The criterion for immobility was floating vertically in the water making only those movements needed to keep the head above water (Borsini and Meli, 1988). Animals were immersed for a 11-min period.

Animals were tested in three squads (N = 8/squad). Animals in the first squad were tested in fresh tap water. Each animal in the second squad was tested immediately after an animal in the first squad had been removed. Animals in the third squad were tested in water previously swum in by an animal in the first and second squad. Thus, animals were immersed in water that had been previously swum in by either one or two animals.

Immediately after removal from the water, each animal was blotted dry and either sacrificed by decapitation within 15 sec of removal from the water or at 20 min after removal. However, immobility was scored only for the former group. An untreated group served as controls for the test procedure itself. Trunk

blood was collected for subsequent corticosterone and prolactin analysis. The data were analyzed by analysis of variance (ANOVA). Post-hoc testing was done by the Duncan multiple-range test.

Corticosterone and Prolactin Assay. Serum corticosterone levels were determined using a commercially available radioimmunoassay (RIA) kit (ICN Biomedicals Kit #07-120102). Serum prolactin was assayed by a double antibody RIA method as described previously (Subramanian and Abel, 1988) using the reagents provided by National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

RESULTS

The immobility data are shown in Figure 1. Animals immersed in soiled water were less immobile than those immersed in fresh water ($F = 92$, $df = 2,21$, $P < 0.001$). Post-hoc testing indicated that the two soiled conditions did not differ significantly.

Group differences in corticosterone levels were significant ($F = 41.5$, $df = 6,65$, $P < 0.001$) (Figure 2). Post-hoc testing indicated that all groups in the forced swim test had higher corticosterone levels than untreated controls ($P < 0.05$) and animals sacrificed 20 min after removal from the test had significantly higher levels than those sacrificed immediately after testing ($P < 0.05$).

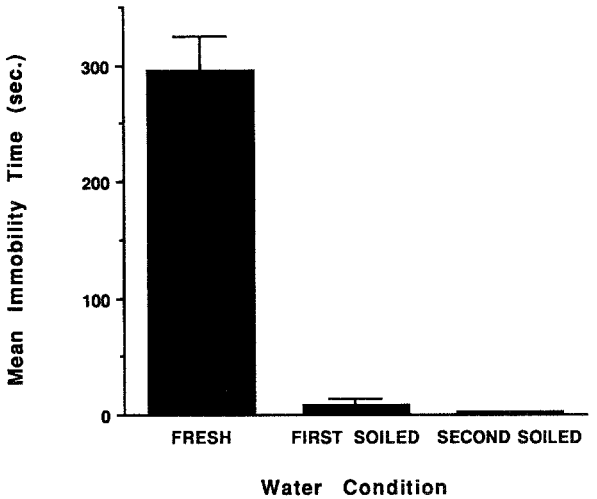


FIG. 1. Mean immobility times for rats in the forced swim test. Animals are tested in fresh water or water soiled by one or two other rats ($N = 8$/condition). Vertical lines indicate SEM.

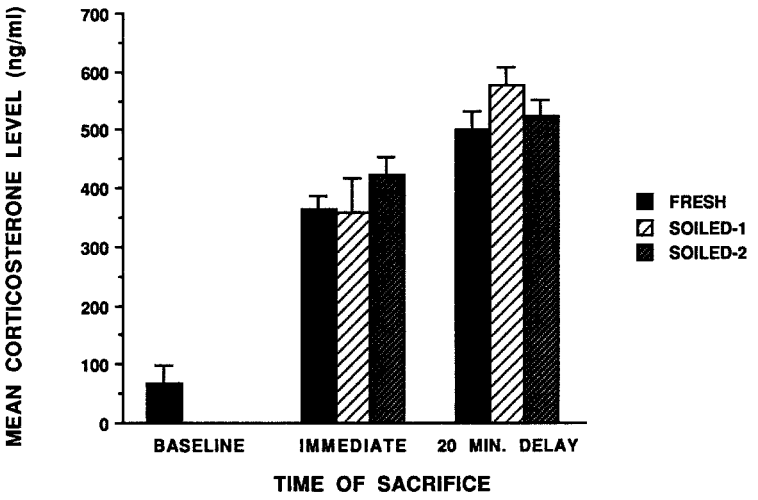


FIG. 2. Mean serum corticosterone levels in nontested rats (baseline), and rats tested in fresh water or water soiled by one or two rats. Animals were sacrificed immediately after an 11-min test or 20 min after the 11-min test (N = 8/condition). Vertical lines indicate SEM.

However, animals tested in water soiled by one or two animals did not differ from animals tested in fresh water.

Group differences in prolactin levels were not statistically significant (Figure 3).

DISCUSSION

As reported by Satoh et al. (1985), the forced swim test resulted in a relatively large increase in serum corticosterone levels relative to untreated control animals. The study also corroborated previous studies (e.g., Seggie and Brown, 1975) indicating that corticosterone levels in response to stress are likely to be higher if sampling occurs 15–30 min after testing than if immediately after testing. In contrast to these results, serum prolactin levels were not increased by the forced swim test. The study also indicated that while swimming in soiled water was more stressful than swimming in fresh water, as reflected in virtually no immobility (cf., Abel and Bilitzke, 1990; Abel, 1991a,b), swimming in soiled water did not result in significantly higher corticosterone levels than swimming in fresh water. Although plasma corticosterone levels rise in response to stress (Axelrod and Reisine, 1984; Hennessey et al., 1979; Amario et al., 1986), some studies have found that the increases do not reflect the intensity of

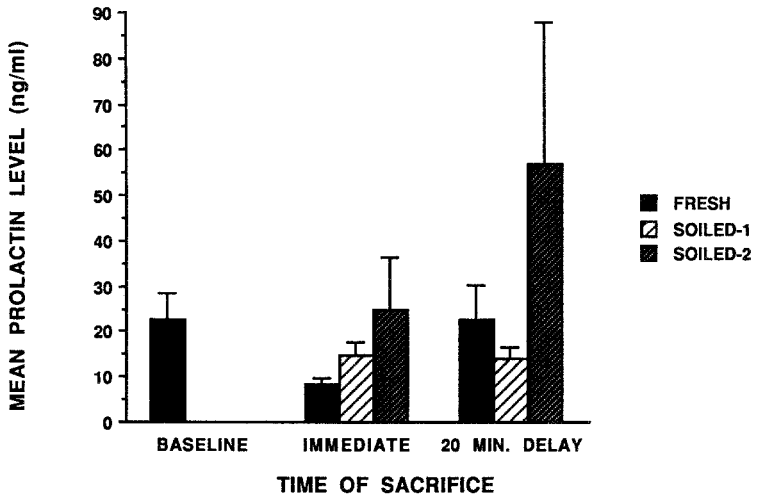


FIG. 3. Mean serum prolactin levels in nontested rats (baseline), and rats tested in fresh water or water soiled by one or two rats. Animals were sacrificed immediately after an 11-min test or 20 min after the 11-min test (N = 8/condition). Vertical lines indicate SEM.

the stressors experienced by animals (e.g., Ader, 1970). This insensitivity may be due to a "ceiling effect." In other words, some stressors may elicit a maximal response so that further increases cannot be seen. With less intense stressors, corresponding increases in corticosterone responses have been observed (Hennessey et al., 1979; Amario et al., 1986). A previous study from our laboratory supported this hypothesis by showing that the forced swimming test elicits a greater serum corticosterone response than being placed in an unfamiliar environment, which in turn produces a greater corticosterone response than baseline (Abel, 1991b). However, the higher corticosterone levels of animals sacrificed at 20 min after testing compared to immediately after testing showed that levels in the latter were not at their maximum, indicating that levels could still have increased if stress levels were different. Using a different paradigm, Mackay-Sim and Laing (1980) also reported that corticosterone levels were not increased in animals exposed to alarm pheromones (from shocked rats). Our results and Mackay-Sim and Laing's (1980) suggest that neither corticosterone nor prolactin mediate the behavioral response to alarm pheromone in rats.

The inescapable swim test used in this study is a variation of the original test described by Porsolt et al. (1977). In that test, an animal is given a 15 min preexposure to the test and 24 hr later, is tested again for 5 min. Duration of immobility is typically longer than during the first 5 min of the first immersion.

Increased immobility in this version of the test may reflect decreased stress due to habituation to the test condition (Borsini et al., 1989) or some other learning/memory function (West, 1990).

In the present version of the test, animals are tested on day one only, in either fresh water or water soiled by another animal. The diameter of the tank and the depth of the water are such that the animal is unable to float or touch the bottom of the tank. Since rats exposed to foot shock or loud noise prior to testing exhibit much less immobility in fresh water than controls, we have interpreted decreased immobility as reflecting a stress response (Abel and Bilitzke, 1990).

Although the condition of the water is not stated in studies using the Porsolt et al. (1977) procedure, we assume that in some cases, the water is not changed and is therefore soiled for some animals. However, immobility still occurs. On the surface, this would seem contrary to our findings. However, we have found that an animal must be immersed a minimum of 3.5 min for it to produce a detectable effect on subsequent animals in the Long-Evans strain. (Abel and Bilitzke, 1990). Other strains may require a longer period of soiling.

Acknowledgments—We thank D. Waselewsky, C. Zajac-Benitez, and B. Bergeski for technical assistance. This project was supported in part by grant P50 AA07606 from the National Institute on Alcohol Abuse and Alcoholism.

REFERENCES

ABEL, E.L. 1991a. Gradient of alarm substance in the forced swimming test. *Physiol. Behav.* 49:321–323.

ABEL, E.L. 1991b. Behavior and corticosteroid response of Maudsley reactive and nonreactive rats in the open field and forced swimming test. *Physiol. Behav.* 50:151–153.

ABEL, E.L. 1991c. Alarm substance emitted by rats in the forced swim test is a low volatile pheromone. *Physiol. Behav.* In press.

ABEL, E.L., and BILITZKE, P.J. 1990. A possible alarm substance in the forced swimming test. *Physiol. Behav.* 48:233–239.

ADER, R. 1970. The effects of early experience on the adrenocortical responses to different magnitudes of stimulation. *Physiol. Behav.* 5:837–839.

AMARIO, A., MONTERO, J.L., and BALASCH, J. 1986. Sensitivity of corticosterone and some metabolic variables to graded levels of low intensity stresses in adult males. *Physiol. Behav.* 37:559–561.

AXELROD, J., and REISINE, T.D. 1984. Stress hormones: Their interaction and regulation. *Science* 224:452–459.

BORSINI, F., and MELI, A. 1988. Is the forced swimming test a suitable model for revealing antidepressant activity? *Psychopharmacology* 94:147–160.

BORSINI, F., LECCI, A., SESSAREGO, A., FRASSINE, R. and MELI, A. 1989. Discovery of antidepressant activity by forced swimming test may depend on pre-exposure of rats to a stressful situation. *Psychopharmacology* 97:183–188.

BROWN, G.M., and MARTIN, J.B. 1974. Corticosterone, prolactin, and growth hormone responses to handling and new environment in the rat. *Psychosom. Med.* 36:241–247.

GALA, R.R. 1990. The physiology and mechanisms of the stress-induced changes in prolactin secretion in the rat. *Life Sci.* 46:1407–1420.

HAWKINS, J.D., HICKS, R.A., PHILLIPS, N., and MOORE, J.D. 1978. Swimming rats and human depression. *Nature*. 274:512.

HENNESSEY, M.B., HEYBACH, J.P., VERMIKOS, J., and LEVINE, S. 1979. Plasma corticosterone concentrations sensitivity reflect levels of stimulus intensity in the rat. *Physiol. Behav.* 22:821–825.

KANT, G.J., MOUGEY, E.H., PENNINGTON, L.L., and MEYERHOFF, J.L. 1983. Graded foot shock stress elevates pituitary cyclic AMP and plasma β-LPH, corticosterone and prolactin. *Life Sci.* 33:2657–2663.

MACKAY-SIM, A., and LAING, D.G. 1980. Discrimination of odors from stressed rats by non-stressed rats. *Physiol. Behav.* 24:699–704.

PORSOLT, R.D., LEPICHON, M. and JOLFE, M. 1977. Depression: A new animal model sensitive to antidepressant treatments. *Nature* 266:730–732.

SATOH, T., YAMADA, K. and TSUBOI, M. 1985. Effect of imipramine on serum corticosterone levels in the forced swimming rats. *Res. Commun. Psychol. Psychiatr. Behav.* 10:235–238.

SEGGIE, J.A., and BROWN, G.M. 1975. Stress response patterns of plasma corticosterone, prolactin, and growth hormone in the rat, following handling or exposure to novel environment. *Can. J. Physiol. Pharmacol.* 53:629–637.

SUBRAMANIAN, M.G., and ABEL, E.L. 1988. Alcohol inhibits suckling-induced prolactin release and milk yield. *Alcohol* 5:95–98.

WEST, A.P. 1990. Neurobehavioral studies of forced swimming: The role of learning and memory in the forced swim test. *Prog. Neurol. Psychopharmacol. Biol. Psychiatr.* 14:863–877.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

RESPONSE OF *Dendroctonus brevicomis* AND *Ips paraconfusus* (COLEOPTERA: SCOLYTIDAE) TO COMBINATIONS OF SYNTHETIC PHEROMONE ATTRACTANTS AND INHIBITORS VERBENONE AND IPSDIENOL

T.D. PAINE* and C.C. HANLON

Department of Entomology
University of California
Riverside, California 92521

(Received February 8, 1991; accepted July 8, 1991)

Abstract—*Dendroctonus brevicomis* and *Ips paraconfusus* are sympatric bark beetle species colonizing *Pinus ponderosa* in western North America. Interspecific and intraspecific competition for resources is, in part, mediated through semiochemicals. The response of *D. brevicomis* to its attractant pheromone was significantly reduced by simultaneous release of either verbenone or racemic ipsdienol. Trap catch was significantly further reduced by including both inhibitors with the attractant pheromones. However, although the response of *Ips paraconfusus* was significantly inhibited with the addition of either verbenone or racemic ipsdienol, both compounds together did not result in a significant further reduction in trap catch. There was a trend for greater reduction in response of *D. brevicomis* to attractant pheromones with increased release rates of either 69% (+)-/31% (−)-verbenone or 84% (−)-/16% (+)-verbenone. Response of associates to attractants and inhibitor combinations was also determined.

Key Words—*Dendroctonus brevicomis*, *Ips paraconfusus*, bark beetle, verbenone, ipsdienol, pheromones, inhibitors, Coleoptera, Scolytidae.

INTRODUCTION

The western pine beetle, *Dendroctonus brevicomis* LeConte, uses a combination of beetle-produced pheromones [(+)-*exo*-brevicomin {(+)-*exo*-7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane} and (−)-frontalin{(−)-1,5-dimethyl-6,8-dioxabiocyclo[3.2.1]octane}] (Silverstein et al., 1968; Kinzer et al., 1969;

*To whom correspondence should be addressed.

0098-0331/91/1100-2163$06.50/0

Wood et al., 1976) and host odors, primarily myrcene (Bedard et al., 1969), during the aggregation phase of tree colonization (Wood, 1972). The engraver beetles, *Ips paraconfusus* Lanier and *I. pini* (Say), are sympatric with *D. brevicomis* in California and may colonize the same host material. *Ips paraconfusus* pheromone consists of three components: (−)-ipsenol[(−)-2-methyl-6-methylene-7-octen-4-ol], (+)-*cis*-verbenol, and (+)-ipsdienol[(+)-2-methyl-6-methylene-2,7-octadien-4-ol] (Silverstein et al., 1966). The pheromone of *I. pini* in California was initially described as a single component pheromone, (−)-ipsdienol (Birch et al., 1980a, b); however, there is some suggestion that a second component may be important to complete the pheromone blend (S.A. Teale, personal communication).

The pheromones produced have interspecific effects in addition to the intraspecific effect. Attraction of *I. pini* to its pheromone is inhibited by racemic ipsenol (Birch and Light, 1977) and to (+)-ipsdienol (Birch et al., 1980a, b). These compounds are components of *I. paraconfusus* aggregation pheromone. Similarly, attraction of *I. paraconfusus* to its pheromone is inhibited by (−)-ipsdienol (Light and Birch, 1979), a component of the aggregation pheromone of *I. pini*. *Dendroctonus brevicomis* was inhibited by actively boring *I. paraconfusus* (Byers and Wood, 1980). Subsequent studies by Byers and Wood (1981a) demonstrated that at least two of the constituents of *I. paraconfusus* pheromone must be present for the inhibitory effect.

Although the constituents of *D. brevicomis* pheromone function to attract individuals, high release rates of *exo*-brevicomin or myrcene may also function to interrupt the response (Tilden et al., 1981). Oxidation of tree-produced α-pinene by either beetles or by microorganisms results in production of verbenone. This compound found in male *D. brevicomis* (Byers and Wood, 1980) interrupts the response of *D. brevicomis* (Bedard et al., 1980) to its attractant pheromone. In addition, verbenone similarly inhibits the attraction response of *I. paraconfusus* (Byers and Wood, 1980, 1981a).

Colonization of conifers usually occurs as a community process. Although there may be a temporal separation, it would be expected that each species in the community would produce the components of its pheromone with the resulting potential for interspecific and intraspecific interactions. The objective of the study presented here was to determine how the compounds associated with inhibition would effect aggregation of *D. brevicomis* and *I. paraconfusus* if presented singly or in combination.

METHODS AND MATERIALS

The behavioral responses of *D. brevicomis* and *I. paraconfusus* to interspecific and intraspecific chemical signals were examined at two locations during July and August 1990. Each site had obvious tree mortality, and examination

of the recently killed trees indicated that populations of both *D. brevicomis* and *I. paraconfusus* were high. While both sites were located within Yellow Pine Forest ecosystems (Munz and Keck, 1973), one site (Julian, San Diego County) was at 1300 m elevation, dominated by *Pinus coulteri* D. Don. and *Q. agrifolia* Nee near the interface with the Chaparral ecosystem, while the other site (Palomar Mountain, San Diego County) was at 1500 m elevation and dominated by mixed conifers.

One of 12 combinations of *D. brevicomis* attractant [Db = (+)-*exo*-brevicomin (released at 2.6 mg/24 hr at 25°C), (−)-frontalin (2.0 mg/24 hr), and myrcene (300 mg/24 hr)], *I. paraconfusus* pheromone [Ip = 50% (+)-/50% (−)-ipsenol (0.2 mg/24 hr), *cis*-verbenol (2 mg/24 hr), and 95% (+)-/5% (−)-ipsdienol (0.2 mg/24 hr)], 16% (+)-/84% (−)-verbenone (10 mg/24 hr), and/or 50% (+)-/50% (−)-ipsdienol (10 mg/24 hr) was placed on a Lindgren funnel trap (trap and all synthetic compounds from Phero Tech Inc., Delta, British Columbia, Canada). Inhibitors were always tested in combination with one or more of the attractant pheromones. An unbaited trap was also tested as a control. A 3-cm^2 piece of DDVP-impregnated plastic was placed in the collection cup of each trap to kill any captured beetles. A line of traps spaced at 20-m intervals containing one of each of the 13 treatments constituted a block. Three lines of traps were located in the Julian site and two lines of traps were located at Mount Palomar in two separate tests. Beetles were collected from the traps daily for 12 days, each day constituting a replicate. After each collection, the chemical baits were shifted in a predetermined sequence to a new position within each line. The trapped insects were brought into the laboratory and the numbers of *I. paraconfusus* and *D. brevicomis* were counted. We also counted *Dendroctonus valens* LeConte and the predators *Enocleris lecontii* (Wolcott) (Coleoptera: Cleridae) and *Temnochila chlorodia* (Mannerheim) (Coleoptera: Trogositidae).

Verbenone has been reported as an inhibitor of both *I. paraconfusus* and *D. brevicomis*. However it is not clear whether either or both enantiomers of verbenone are effective at inhibiting the response to the attractant pheromones. We tested a mixture of 16% (+)-/84% (−)-verbenone released at five elution rates at the Mount Palomar site during August 1990. Funnel traps were baited with one of six treatments: *D. brevicomis* pheromone [*exo*-brevicomin (released at 2.6 mg/24 hr at 24°C), frontalin (2.0 mg/24 hr), and myrcene (300 mg/24 hr)] alone and *D. brevicomis* pheromone plus the verbenone enantiomers eluted at 0.01 mg/24 hr (at 25°C), 0.18 mg/24 hr, 1.8 mg/24 hr, 5 mg/24 hr, and 20 mg/24 hr. As in the previous test, an unbaited trap served as a control. Each of the seven treatments was replicated 10 times. Beetles were collected from the traps three times at 72-hr intervals. An identical test was subsequently conducted using 69% (+)-/31% (−)-verbenone in the same location.

Differences in trap catches among the 13 treatments at each location for

each beetle species, as well as the differences in the response of *D. brevicomis* among release rate treatments for an enantiomeric blend were determined using General Linear Modeling procedures (PROC GLM) and Duncan's new multiple-range test on SAS for microcomputers (SAS Institute, 1988).

RESULTS

At both study sites, the traps baited with *D. brevicomis* pheromone (Db) alone caught significantly more of that species than any of the other treatments [Julian: $F = 18.41$, $df = 12$, $P = 0.0001$ (Table 1); Palomar: $F = 13.99$, $df = 12$, $P = 0.0001$ (Table 2)]. Four treatments [Db and verbenone, Db and ipsdienol, Db and *Ips paraconfusus* pheromone (Ip) and Db and Ip and ipsdienol] at both sites inhibited the response of *D. brevicomis* to the attractant pheromone, but still caught more beetles than the blank control traps. The three remaining treatments (Db and Ip and verbenone, Db and verbenone and ipsdienol, and Db and Ip and verbenone and ipsdienol) further reduced catches of *D. brevicomis* to levels not significantly different from either the unbaited control or to those treatments lacking the aggregation pheromone (Db) of this species.

The arrival of *I. paraconfusus* at traps at the Mount Palomar location was significantly inhibited ($F = 8.34$, $df = 12$, $P = 0.0001$) by the addition of verbenone and ipsdienol to treatments that included the aggregation pheromones (Table 2). The aggregation pheromones of this species alone and in combination with the aggregation pheromones from *D. brevicomis* were the only two treatments that were significantly different from the responses to the unbaited control. Although the response of *I. paraconfusus* was significantly greater ($F = 6.87$, $dd = 12$, $P = 0.0001$) to the aggregation pheromones of both *I. paraconfusus* and *D. brevicomis* together, the response to the aggregation pheromone of *I. paraconfusus* alone was not different from the unbaited control or any of the inhibitor treatments at the Julian location (Table 1).

While there were some beetles caught in the traps, there were no significant differences in the arrival of *E. lecontii* ($F = 0.76$, $df = 12$, $P = 0.2799$) to any of the 13 treatments tested in the first study at Julian, but the response of both *D. valens* ($F = 4.27$, $df = 12$, $P = 0.0001$) and *T. chlorodia* ($F = 3.25$, $df = 12$, $P = 0.0002$) was significantly greater to the E-M-F and [50% (+)-/50% (−)-ipsenol, *cis*-verbenol, and 95% (+)-/5% (−)-ipsdienol] and ipsdienol treatment (Table 1). The response of *D. valens* at the Mount Palomar location was not significantly different to any treatment ($F = 0.90$, $df = 12$, $P = 0.5519$). However, there were significant differences observed for the response of the other two associates to the same treatments at the Mount Palomar site [*E. lecontii* ($F = 2.06$, $df = 12$, $P = 0.0197$), *T. chlorodia* ($F = 2.12$, $df =$

TABLE 1. ARRIVAL OF BARK BEETLES AND ASSOCIATES AT LINDGREN FUNNEL TRAPS BAITED WITH SYNTHETIC ATTRACTANTS OR INTERRUPTANTS (JULY–AUGUST 1990, JULIAN, CALIFORNIA)

Treatment	N	Mean (±SD) number of beetles caught per 24-hr trapping periods[a]				
		D. brevicomis	*I. paraconfusus*	*D. valens*	*E. lecontei*	*T. chlorodia*
Db	36	32.86 ± 5.08 a	2.28 ± 2.16 bc	0.28 ± 0.62 b	0.00 ± 0.00 a	0.06 + 0.23 b
Db + ipsdienol	36	20.19 ± 3.60 b	0.22 ± 0.15 c	0.33 ± 0.59 b	0.06 ± 0.33 a	0.17 ± 0.45 b
Db + verbenone	36	16.78 ± 3.11 b	0.06 ± 0.06 c	0.11 ± 0.32 b	0.03 ± 0.17 a	0.03 ± 0.17 b
Db + verbenone + ipsdienol	36	6.89 + 2.65 c	0.06 ± 0.06 c	0.28 ± 0.74 b	0.06 ± 0.33 a	0.06 + 0.17 b
Db + Ip	36	16.42 ± 2.57 b	12.42 ± 2.73 a	0.28 ± 0.57 b	0.08 ± 0.37 a	0.19 ± 0.47 b
Db + Ip + ipsdienol	36	23.25 ± 4.84 b	3.25 ± 0.90 bc	0.97 ± 1.34 a	0.06 ± 0.33 a	0.39 ± 0.84 a
Db + Ip + verbenone	36	6.39 ± 1.47 c	5.92 ± 2.30 b	0.33 ± 0.59 b	0.00 + 0.00 a	0.06 ± 0.23 b
Db + Ip + verbenone + ipsdienol	36	4.83 ± 1.42 c	1.64 + 1.12 c	0.31 ± 0.62 b	0.03 ± 0.17 a	0.08 ± 0.28 b
Ip	36	0.36 ± 0.26 c	4.03 ± 1.10 b c	0.06 ± 0.23 b	0.14 ± 0.49 a	0.03 ± 0.17 b
Ip + ipsdienol	36	0.11 ± 0.09 c	0.50 ± 0.87 bc	0.33 ± 0.59 b	0.03 ± 0.17 a	0.06 ± 0.23 b
Ip + verbenone	36	0.00 ± 0.00	0.33 ± 0.12 c	0.22 ± 0.49 b	0.03 ± 0.17 a	0.06 ± 0.23 b
Ip + verbenone + ipsdienol	36	0.31 ± 0.16 c	0.25 ± 0.20 c	0.17 ± 0.38 b	0.00 ± 0.00 a	0.08 ± 0.28 b
Unbaited control	36	0.25 ± 0.13 c	0.42 ± 0.39 c	0.14 ± 0.35 b	0.06 ± 0.33 a	0.00 ± 0.00 b

[a]Insects removal and treatment position moved daily. Means within columns followed by the same letter are not significantly different ($P = 0.05$, analysis of variance and Duncan's new multiple-range test).

TABLE 2. ARRIVAL OF BARK BEETLES AND ASSOCIATES AT LINDGREN FUNNEL TRAPS BAITED WITH SYNTHETIC ATTRACTANTS OR INTERRUPTANTS (JULY–AUGUST 1990, MT. PALOMAR, CALIFORNIA)

Treatment	N	Mean (± SD) number of bettles caught per 24-h trapping period[a]				
		D. brevicomis	*I. paraconfusus*	*D. valens*	*E. lecontei*	*T. chlorodia*
Db	24	235.33 ± 43.58 a	0.00 ± 0.00 b	0.46 ± 0.93 a	0.00 ± 0.00 b	0.29 ± 0.75 a b c
Db + ipsdienol	24	72.21 ± 16.09 b c	0.25 ± 0.21 b	0.50 ± 1.29 a	0.08 ± 0.28 b	0.46 ± 0.83 a b
Db + verbenone	23	73.96 ± 34.14 bc	0.00 ± 0.00 b	0.18 ± 0.66 a	0.05 ± 0.21 b	0.09 ± 0.29 c
Db + verbenone + ipsdienol	22	16.09 ± 2.72 d	0.14 ± 0.14 b	0.27 ± 0.88 a	0.18 ± 0.59 b	0.41 ± 1.05 a b c
Db + Ip	24	106.42 ± 23.46 b	11.54 ± 3.62 a	0.38 ± 1.44 a	0.08 ± 0.28 b	0.83 ± 1.31 a
Db + Ip + ipsdienol	24	73.92 ± 12.52 b c	1.79 ± 0.54 b	0.79 ± 1.35 a	0.13 ± 0.34 b	0.67 ± 1.09 a b
Db + Ip + verbenone	24	24.17 ± 5.75 cd	3.50 ± 0.85 b	0.38 ± 1.44 a	0.04 ± 0.20 b	0.54 ± 0.93 a b c
Db + Ip + verbenone + ipsdienol	23	20.78 ± 5.14 cd	1.57 ± 0.62 b	0.57 ± 0.95 a	0.00 ± 0.00 b	0.30 ± 0.56 a b c
Ip	24	0.79 ± 0.31 d	12.50 ± 3.64 a	0.08 ± 0.28 a	0.00 ± 0.00 b	0.29 ± 0.86 a b c
Ip + ipsdienol	23	0.61 ± 0.26 d	1.13 ± 0.62 b	0.35 ± 1.78 a	0.22 ± 0.60 b	0.17 ± 0.39 bc
Ip + verbenone	20	1.05 ± 0.70 d	1.60 ± 0.74 b	0.70 ± 2.68 a	0.55 ± 1.43 a	0.50 ± 1.00 a b c
Ip + verbenone + ipsdienol	23	0.17 ± 0.10 d	0.17 ± 0.14 b	0.09 ± 0.29 a	0.22 ± 0.60 b	0.00 ± 0.00 c
Unbaited control	24	1.17 ± 0.32 d	0.08 ± 0.08 b	0.21 ± 0.51 a	0.04 ± 0.20 b	0.08 ± 0.28 c

[a] Insects removal and treatment position moved daily. Means within columns followed by the same letter are not significantly different ($P = 0.05$, analysis of variance and Duncan's new multiple-range test).

12, $P = 0.0159$), Table 2]. Individuals of these three species were also caught in traps baited with *D. brevicomis* aggregation pheromone and different release rates of verbenone. The response of *D. valens* was significantly less ($F = 3.60$, $df = 6$, $P = 0.0021$) when any 69% (+)-/31% (−)-verbenone treatment was included, but there were no significant differences among treatments for *E. lecontii* ($F = 0.96$, $df = 6$, $P = 0.4519$) or *T. chlorodia* ($F = 1.71$, $df = 6$, $P = 0.1199$) (Table 3). In contrast, when 16% (+)-/84% (−)-verbenone was tested, catch of *D. valens* was significantly greater than the unbaited control ($F = 2.37$, $df = 6$, $P = 0.0311$) for only the lowest release rate (Table 4). Response of *E. lecontii* was significantly different from the control ($F = 2.72$, $df = 6$, $P = 0.0147$) at the highest release rate, and response of *T. chlorodia* was not different to any verbenone treatment ($F = 1.37$, $df = 6$, $P = 0.2287$).

The response of *D. brevicomis* to the aggregation pheromone alone was significantly greater ($F = 15.06$, $df = 6$, $P = 0.0001$) than to any of the treatments containing the 69% (+)-/31% (−)-verbenone (Table 3). There was no clear dose–response relationship, but all treatments attracted more beetles than did the unbaited control. In contrast, the responses of *D. brevicomis* to the two lowest release rates of 16% (+)-/84% (−)-verbenone were not significantly different ($F = 17.57$, $df = 6$, $P = 0.0001$) than the response to the aggregation pheromone alone (Table 4). However, traps baited with the lowest release rate of this enantiomeric blend of verbenone plus aggregation pheromone caught significantly more beetles than did the treatment with the next lowest release rate. As in the previous test of enantiomers, all treatments attracted more beetles than did the unbaited control traps.

DISCUSSION

Our results confirm that the response of *D. brevicomis* to its aggregation pheromone is reduced by the presence of *I. paraconfusus* pheromone, racemic ipsdienol, or verbenone, but also demonstrate that the presence of multiple inhibitor compounds further reduces the response. While it is not clear why the response of *I. paraconfusus* to its aggregation pheromone was not different ($P = 0.05$) from the control treatment at the Julian site, the reduction in the response at the Mt. Palomar location was not significantly enhanced by the addition of more than one inhibitory compound. However, at the Julian location, there was a tendency for the treatments with either *D. brevicomis* or *I. paraconfusus* pheromones alone or both pheromones together plus only a single inhibitor to have higher trap catches. The response of the associates was generally very low to any of the treatments. However, Wood et al. (1968) demonstrated that *E. lecontii* responds to pheromone constituents of *I. paraconfusus*, while Vité and Pitman (1969) found that *T. chlorodia* responded to *D. brevicomis* pheromone.

TABLE 3. ARRIVAL OF BARK BEETLES AND ASSOCIATES AT LINDGREN FUNNEL TRAPS BAITED WITH FRONTALIN, *exo*-BREVICOMIN, AND MYRCENE, PLUS DIFFERENT RELEASE RATES OF 69% (+)-/31% (−)-VERBENONE (AUGUST–SEPTEMBER 1990, MT. PALOMAR, CALIFORNIA)

Treatment	*N*	Mean (± SD) number of beetles caught per 36-hr trapping period[a]				
		D. brevicomis	*I. paraconfusus*	*D. valens*	*E. lecontei*	*T. chlorodia*
Db	30	193.90 ± 29.43 a	0.03 ± 0.18 a	1.27 ± 2.75 a	0.47 ± 2.03 a	0.07 ± 0.25 a
Db + verbenone at 0.01 mg/24 hr	29	106.03 ± 15.12 b	0.07 ± 0.26 a	0.48 ± 0.95 b	0.17 ± 0.47 a	0.17 ± 0.47 a
Db + verbenone at 0.18 mg/24 hr	30	94.47 ± 15.58 bc	0.13 ± 0.73 a	0.37 ± 1.00 b	0.10 ± 0.31 a	0.07 ± 0.25 a
Db + verbenone at 1.8 mg/24 hr	30	120.10 ± 21.23 b	0.23 ± 1.10 a	0.10 ± 0.31 b	0.10 ± 0.31 a	0.03 ± 0.18 a
Db + verbenone at 5 mg/24 hrs	29	53.66 + 10.26 c	0.66 ± 1.72 a	0.31 ± 0.71 b	0.03 + 0.19 a	0.03 ± 0.19 a
Db + verbenone at 20 mg/24 hr	30	98.50 ± 19.35 bc	0.63 ± 1.96 a	0.07 ± 0.25 b	0.07 ± 0.25 a	0.00 ± 0.00 a
Unbainted control	28	0.39 ± 0.11 d	0.64 ± 1.97 a	0.07 ± 0.26 b	0.07 ± 0.26 a	0.00 ± 0.00 a

[a]Insects removed every three days from 10 locations per treatment. Numbers within columns followed by the same letters are not significantly different ($P = 0.05$, analysis of variance and Duncan's multiple-range test).

TABLE 4. ARRIVAL OF BARK BEETLES AND ASSOCIATES AT LINDGREN FUNNEL TRAPS BAITED WITH FRONTALIN, *exo*-BREVICOMIN, AND MYRCENE, PLUS DIFFERENT RELEASE RATES OF 16% (+)-/84% (−)-(AUGUST–SEPTEMBER 1990, MT. PALOMAR, CALIFORNIA)

Treatment	*N*	Mean (± SD) number of beetles caught per 36-hr trapping period[a]				
		D. brevicomis	*I. paraconfusus*	*D. valens*	*E.lecontei*	*T. chlorodia*
Db	30	278.27 ± 32.95 ab	0.03 ± 0.18 a	1.20 ± 1.81 ab	0.33 ± 0.61 ab	0.27 ± 0.45 a
Db + verbenone at 0.01 mg/24 hr	30	310.60 ± 37.15 a	0.00 ± 0.00 a	1.90 ± 3.54 a	0.30 ± 0.65 ab	0.33 ± 0.80 a
Db + verbenone at 0.18 mg/24 hr	30	210.73 ± 30.72 bc	0.00 ± 0.00 a	1.20 ± 2.73 ab	0.07 ± 0.25 b	0.23 ± 0.50 a
Db + verbenone at 1.8 mg/24 hr	30	148.07 ± 21.44 c	0.00 ± 0.00 a	0.87 ± 1.50 ab	0.33 ± 0.99 ab	0.17 ± 0.46 a
Db + verbenone at 5 mg/24 hr	30	171.30 ± 31.38 c	0.20 ± 1.10 a	0.63 ± 1.07 b	0.10 ± 0.31 b	0.17 ± 0.38 a
Db + verbenone at 20 mg/24 hr	30	163.53 ± 22.24 c	0.20 ± 0.92 a	0.83 ± 1.64 ab	0.60 ± 1.07 a	0.10 ± 0.31 a
Unbainted control	30	0.93 ± 0.37 d	0.00 ± 0.00 a	0.03 ± 0.18 b	0.03 ± 0.18 b	0.03 ± 0.18 a

[a] Insects removed every three days from 10 locations per treatment. Numbers within columns followed by the same letters are not significantly different (P 0.05, analysis of variance and Duncan's multiple-range test).

The process of tree colonization by *D. brevicomis* takes place within a very short time and is mediated by attractant semiochemicals (Wood, 1972). However, there is the potential for competition among the arriving beetles for the limited amount of available resource (Alcock, 1982; Berryman et al., 1985). One of the mechanisms used by the beetles appears to be a complex coordination of timing in release of attractants and production of inhibitory compounds (Byers et al., 1984). Consequently, the arriving beetles are spaced to maximize individual fitness (Schlyter et al., 1987; DeJong and Sabelis, 1988).

The recognition of verbenone as an inhibitor occurs in several species within the *Dendroctonus* and *Ips* genera. Pitman et al. (1969) identified verbenone from *D. brevicomis*, *D. frontalis* Zimmermann, and *D. ponderosae* Hopkins. Subsequent investigations demonstrated that the compound inhibits arrival of beetles to attractive sources (Bedard et al., 1980; Ryker and Yandell, 1983; Payne and Billings, 1988). *Ips paraconfusus* and *I. typographus* (L.) were also shown to be inhibited by verbenone (Byers and Wood, 1980, 1981a; Bakke, 1981).

It is not common to observe individuals of a single bark beetle species colonizing a tree. Rather it is usually a community of species (Paine et al., 1980). The interactions among the members of the community may be mediated by the pheromone communication system (Birch et al., 1980a, b; Smith et al., 1990). However, several beetle species may produce the same chemical compounds and exploit the inhibitor message to reduce competition. Male *D. brevicomis* and male *D. ponderosae* produce (+)-ipsdienol from myrcene (Renwick et al., 1976; Byers, 1982; Hunt et al., 1986). This enantiomer inhibits conspecifics (Byers, 1982; Hunt and Borden, 1988) as well as *I. pini* (Birch et al., 1980a, b), a potential competitor of either species. Thus, *D. brevicomis* and *D. ponderosae* release two compounds (verbenone and ipsdienol) that have interspecific and intraspecific inhibitory effects. The effect of releasing the inhibitors simultaneously has not been observed previously for a *Dendroctonus* species. There was a suggestion from the studies of Bakke (1981) that the release of two inhibitory compounds was more effective for inhibiting the response of *I. typographus* than the release of either compound alone, but subsequent work could not confirm those results (Schlyter et al., 1988).

The chemical components of the pheromone blends have multifunctional effects; they may function as attractant pheromones, inhibitory pheromones, or as synomones. The insects may produce the compounds, or production may be the result of microbial action on either ingested substrate or exposure to volatile substrates. Brand et al. (1975) isolated a bacterium from *I. paraconfusus* that utilized α-pinene to synthesize *cis*- and *trans*-verbenol. Byers and Wood (1981b) reported that streptomycin treatments of *I. paraconfusus* prevented the conversion of myrcene to ipsenol and ipsdienol. In contrast, *I. typographus* produces *cis*- and *trans*-verbenol but requires associated yeasts to convert these compounds to verbenone (Leufven et al., 1984; Leufven and Birgersson, 1987).

The yeasts were unable to produce either the verbenols or verbenone directly from α-pinene (Leufven et al., 1988). Similarly, *D. ponderosae* requires a gut microorganism to produce verbenone (Hunt and Borden, 1989).

There is an obvious ecological advantage to both arriving and colonizing beetles to avoid either marginal resources or intense competition. Verbenone, derived from α-pinene through the intermediate verbenols by microbial action in several systems, appears to be a common signal that the resource may be attacked and potentially unsuitable for further colonization (Schlyter et al., 1988). The terpene alcohols, ipsenol and ipsdienol, may also be interspecific and intraspecific inhibitors that serve to reduce detrimental competition. Light et al. (1983) demonstrated that interspecific competition between *I. paraconfusus* and *I. pini* produced greater negative effects than intraspecific competition and speculated that these competitive interactions may be the selective force that resulted in mutual pheromone inhibition between the species. The geographic range of *D. brevicomis* is within the range of *Pinus ponderosa* Lawson. *Ips pini* and *D. brevicomis* are sympatric in much of that range, and the enantiomer of ipsdienol produced by *D. brevicomis* is an effective inhibitor of *I. pini* where sympatry occurs (Birch et al., 1980a, b). It is, however, a constituent of the attractant pheromone of *I. paraconfusus* that overlaps with *D. brevicomis* in only a portion of the geographic range.

While parallel studies have not been completed for *I. paraconfusus* and *D. brevicomis*, the competitive interactions may be similarly detrimental. However, it is not clear why the presence of multiple inhibitors produced a greater effect on *D. brevicomis* than *I. paraconfusus*. It is possible that this *Ips* species is more sensitive to the presence of the inhibitors or the described pheromone blend for this species is incomplete, and as a result, the maximum reduction was observed to any single compound. There is a difference between *I. paraconfusus* and *I. typographus*. The results of Bakke (1981) suggest that attraction of *I. typographus* is inhibited more by a combination of ipsenol plus verbenone than by either compound alone. However, in the European spruce system, this species may be more of an ecological homologue to *D. brevicomis* than to *I. paraconfusus* in western North America.

Acknowledgments—We thank J. Trumble, R. Redak, P. Luft, T. Grasswitz, P. Leddy, S. Bertram, and M. Moratorio for helpful reviews of the manuscript; Phero Tech Inc. (Delta, British Columbia, Canada) for the release devices used in the enantiomeric dose test; and D. Messore, S. Gilbert, M. Goeden, and S. Bertram for data collection. This study was supported in part by a grant from the California Department of Forestry and Fire Protection.

REFERENCES

ALCOCK, J. 1982. Natural selection and communication among bark beetles. *Fla. Entomol.* 65:17–32.

BAKKE, A. 1981. Inhibition of the response in *Ips typographus* to the aggregation pheromone; field evaluation of verbenone and ipsenol. *Z. Angew. Entomol.* 92:172–177.

BEDARD, W.D., TILDEN, P.E., WOOD, D.L., SILVERSTEIN, R.M., BROWNLEE, R.G., and RODIN, J.O. 1969. Western pine beetle: Field responses to its sex pheromone and a synergistic host terpene, myrcene. *Science* 164:1284–1285.

BEDARD, W.D., TILDEN, P.E., LINDAHL, K.Q., JR., WOOD, D.L., and RAUCH, P.A. 1980. Effects of verbenone and *trans*-verbenol on the response of *Dendroctonus brevicomis* to natural and synthetic attractant in the field. *J. Chem. Ecol.* 6:997–1013.

BERRYMAN, A.A., DENNIS, B., RAFFA, K.F., and STENSETH, N.C. 1985. Evolution of optimal group attack with particular reference to bark beetles (Coleoptera: Scolytidae). *Ecology* 66:898–903.

BIRCH, M.C., and LIGHT, D.M. 1977. Inhibition of the attractant pheromone response in *Ips pini* and *I. paraconfusus* (Coleoptera: Scolytidae): Field evaluation of ipsenol and linalool. *J. Chem. Ecol.* 3:257–267.

BIRCH, M.C., LIGHT, D.M., WOOD, D.L., BROWNE, L.E., SILVERSTEIN, R.M., BERGOT, B.J., OHLOFF, G., WEST, J.R., and YOUNG, J.C. 1980a. Pheromonal attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. *J. Chem. Ecol.* 6:703–717.

BIRCH, M.C., SVIHRA, P., PAINE, T.D., and MILLER, J.C. 1980b. Influence of chemically mediated behavior on host tree colonization by four cohabiting species of bark beetles. *J. Chem. Ecol.* 6:395–414.

BRAND, J.M., BRACKE, J.W., MARKOVETZ, A.J., WOOD, D.L., and BROWNE, L.E. 1975. Production of verbenol pheromone by bacterium isolated from bark beetles. *Nature* 254:136–137.

BYERS, J.A. 1982. Male-specific conversion of the host plant compound, myrcene, to the pheromone, (+)-ipsdienol, in the bark beetle, *Dendroctonus brevicomis*. *J. Chem. Ecol.* 8:363–371.

BYERS, J.A., and WOOD, D.L. 1980. Interspecific inhibition of the response of the bark beetles, *Dendroctonus brevicomis* and *Ips paraconfusus*, to their pheromones in the field. *J. Chem. Ecol.* 6:149–163.

BYERS, J.A., and WOOD, D.L. 1981a. Interspecific effects of pheromones on the attraction of the bark beetles, *Dendroctonus brevicomis* and *Ips paraconfusus*, in the laboratory. *J. Chem. Ecol.* 7:9–18.

BYERS, J.A., and WOOD, D.L. 1981b. Antibiotic-induced inhibition of pheromone synthesis in a bark beetle. *Science* 213:763–764.

BYERS, J.A., WOOD, D.L., CRAIG, J., and HENDRY, L.B. 1984. Attractive and inhibitory pheromones produced in the bark beetle, *Dendroctonus brevicomis*, during host colonization: Regulation of inter- and intraspecific competition. *J. Chem. Ecol.* 10:861–877.

DEJONG, M.C.M., and SABELIS, M.W. 1988. How bark beetles avoid interference with squatters: an ESS for colonization by *Ips typographus*. *Oikos* 51:88–96.

HUNT, D.W.A., and BORDEN, J.H. 1988. Response of mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and the pine engraver, *Ips pini* (Say), to ipsdienol in southwestern British Columbia. *J. Chem. Ecol.* 14:277–293.

HUNT, D.W.A., and BORDEN, J.H. 1989. Terpene alcohol pheromone production by *Dendroctonus ponderosae* and *Ips paraconfusus* (Coleoptera: Scolytidae) in the absence of readily culturable microorganisms. *J. Chem. Ecol.* 15:1433–1463.

HUNT, D.W.A., BORDEN, J.H., PIERCE, H.D., JR., SLESSOR, K.N., KING, G.G.S., and CZYZEWSKA, E.K. 1986. Sex-specific production of ipsdienol and myrcenol by *Dendroctonus ponderosae* (Coleoptera: Scolytidae) exposed to myrcene vapors. *J. Chem. Ecol.* 12:1579–1586.

KINZER, G.W., FENTIMAN, A.F., PAGE, T.F., FOLTZ, R.L., VITÉ, J.P., and PITMAN, G.B. 1969. Bark beetle attractants: Identification, synthesis, and field bioassay of a new compound isolated from *Dendroctonus*. *Nature* 221:477–478.

LIGHT, D.M., and BIRCH, M.C. 1979. Inhibition of the attractant pheromone response in *Ips paraconfusus* by (*R*)-(−)-ipsdienol. *Naturwissenschaften* 66:159–160.

LIGHT, D.M., BIRCH, M.C., and PAINE, T.D. 1983. Laboratory study of intraspecific and interspecific competition within and between two sympatric bark beetle species, *Ips pini* and *I. paraconfusus*. *Z. Angew. Entomol.* 96:233–241.

LEUFVEN, A., and BIRGERSSON, G. 1987. Quantitative variation of different monoterpenes around galleries of *Ips typographus* (Coleoptera: Scolytidae) attacking Norway spruce. *Can. J. Bot.* 65:1038–1044.

LEUFVEN, A., BERGSTROM, G., and FALSEN, E. 1984. Interconversion of verbenols and verbenone by identified yeasts isolated from the spruce bark beetle *Ips typographus*. *J. Chem. Ecol.* 10:1349–1361.

LEUFVEN, A., BERGSTROM, G., and FALSEN, E. 1988. Oxygenated monoterpenes produced by yeasts, isolated from *Ips typographus* (Coleoptera: Scolytidae) and grown in phloem medium. *J. Chem. Ecol.* 14:353–362.

MUNZ, P.A., and KECK, D.A. 1973. A California Flora. University of California Press, Berkeley. 1681 pp.

PAINE, T.D., BIRCH, M.C., and SVIHRA, P. 1980. Niche breadth and resource partitioning by four sympatric species of bark beetles (Coleoptera: Scolytidae). *Oecologia* 48:1–6.

PAYNE, T.L., and BILLINGS, R.F. 1988. Effect of pheromone and pheromone plus tree cutting on suppression of southern pine beetle infestations, pp. 267–273, *in* T.L. Payne and H. Saarenmaa (eds.). Integrated Control of Scolytid Bark Beetles. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

PITMAN, G.B., VITÉ, J.P., KINZER, G.W., and FENTIMAN, A.F. 1969. Specificity of population-aggregating pheromones in *Dendroctonus*. *J. Insect Physiol.* 15:363–366.

RENWICK, J.A.A., HUGHES, P.R., PITMAN, G.B., and VITÉ, J.P. 1976. Oxidation products of terpenes identified from *Dendroctonus* and *Ips* bark beetles. *J. Insect Physiol.* 22:725–727.

RYKER, L.C., and YANDELL, K.L. 1983. Effect of verbenone on aggregation of *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae) to synthetic attractant. *Z. Angew. Entomol.* 96:452–459.

SAS Institute. 1988. SAS/STAT User's Guide, Release 6.03 Edition. Cary, North Carolina. 1028 pp.

SCHLYTER, F., BYERS, J.A., and LOFQVIST, J. 1987. Attraction to pheromone sources of different quantity, quality, and spacing: Density-regulation mechanisms in bark beetle *Ips typographus*. *J. Chem. Ecol.* 13:1503–1523.

SCHLYTER, F., BYERS, J.A., LOFQVIST, J., LEUFVEN, A., and BIRGERSSON, G. 1988. Reduction of attack density of bark beetles *Ips typographus* and *Tomicus piniperda* on host bark by verbenone inhibition of attraction to pheromone and host kairomone, pp. 53–68, *in* T.L. Payne and H. Saarenmaa (eds.). Integrated Control of Scolytid Bark Beetles. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

SILVERSTEIN, R.M., RODIN, J.O., and WOOD, D.L. 1966. Sex attractants in frass produced by male *Ips confusus* in ponderosa pine. *Science* 154:509–510.

SILVERSTEIN, R.M., BROWNLEE, R.G., BELLAS, T.E., WOOD, D.L., and BROWNE, L.E. 1968. Brevicomin: Principal sex attractant to the frass of the female western pine beetle. *Science* 159:889–890.

SMITH, M.T., PAYNE, T.L., and BIRCH, M.C. 1990. Olfactory-based behavioral interactions among five species in the southern pine bark beetle group. *J. Chem. Ecol.* 16:3317–3331.

TILDEN, P.E., BEDARD, W.D., WOOD, D.L., and STUBBS, H.A. 1981. Interruption of response of *Dendroctonus brevicomis* to its attractive pheromone by components of the pheromone. *J. Chem. Ecol.* 7:183–196.

VITÉ, J.P., and PITMAN, G.B. 1969. Insect and host odors in the aggregation of the western pine beetle. *Can. Entomol.* 101:113–117.

WOOD, D.L. 1972. Selection and colonization of ponderosa pine by bark beetles, pp. 101–117, *in* H.F. van Emden (ed.). Insect/Plant Relationships. Symposium, Royal Entomology Society, London. No. 6.

WOOD, D.L., BROWNE, L.E., BEDARD, W.D., TILDEN, P.E., SILVERSTEIN, R.M., and RODIN, J.O. 1968. Response of *Ips confusus* to synthetic sex pheromones in nature. *Science* 159:1373–1374.

WOOD, D.L., BROWNE, L.E., EWING, B., LINDAHL, K., BEDARD, W.D., TILDEN, P.E., MORI, K., PITMAN, G.B., and HUGHES, P.R. 1976. Western pine beetle: Specificity among enantiomers of male and female components of an attractant pheromone. *Science* 192:896–898.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

(3*Z*,6*Z*,8*E*)-3,6,8-DODECATRIEN-1-OL: SEX PHEROMONE IN A HIGHER FUNGUS-GROWING TERMITE, *Pseudacanthotermes spiniger* (ISOPTERA, MACROTERMITINAE)

CHRISTIAN BORDEREAU,[1,*] ALAIN ROBERT,[1,2] ODILE BONNARD,[1] and JEAN-LUC LE QUERE[3]

[1]*Université de Bourgogne, Zoologie*
UA CNRS 674, 6 Bd. Gabriel
21000 Dijon, France

[2]*Université des Sciences et Techniques de Masuku*
Département de Biologie
BP 943 Franceville, Gabon

[3]*INRA, Laboratoire de Recherches sur les Arômes*
17 rue de Sully, 21000 Dijon France

(Received March 25, 1991; accepted July 8, 1991)

Abstract—The female sex pheromone of the fungus-growing termite *Pseudacanthotermes spiniger* (Termitidae, Macrotermitinae) was isolated from sternal glands of alates. The compound inducing attraction and excitation in males was identified as the (3*Z*,6*Z*,8*E*)-3,6,8-dodecatrien-1-ol by GC-MS, microhydrogenation, GC-FTIR, and NMR. This unsaturated alcohol is present in both sexes but in much higher quantities in females than in males (about 10 times). The hypothesis is suggested that this alcohol, which is detected at extremely low concentrations by the workers of *P. spiniger*, may be used either as a trail-following pheromone or a sex pheromone according to concentrations and to target castes. The presence of this alcohol in Macrotermitinae reinforces the idea of a phylogenetic proximity between this subfamily of higher termites and the lower termites Rhinotermitidae, where the unsaturated alcohol was previously found. The sternal glands of alates of *P. spiniger* also contain a geometric isomer of (3*Z*,6*Z*,8*E*)-3,6,8-dodecatrien-1-ol.

Key Words—Termites, Isoptera, Macrotermitinae, *Pseudacanthotermes spiniger*, insect sex pheromone, (3*Z*,6*Z*,8*E*)-3,6,8-dodecatrien-1-ol.

*To whom correspondence should be addressed.

0098-0331/91/1100-2177$06.50/0

INTRODUCTION

Termite colonies generally reproduce by seasonal dispersal flights of winged males and females (alates). The meeting and the pairing of these alates is mediated through various factors, among which chemical stimuli from abdominal glands are of prime importance. However, sex pheromones have been demonstrated only in a few cases: *Kalotermes* (Wall, 1971), *Zootermopsis* (Pasteels, 1972; Stuart, 1975), *Hodotermes* (Leuthold, 1977), *Reticulitermes* (Buchli, 1960; Stuart, 1969, 1975; Clément, 1982), *Trinervitermes* (Leuthold and Lüscher, 1974; Leuthold, 1977), and only one publication has dealt with the chemical nature of termite sex pheromone (Clément et al., 1989).

In this paper, we report on the isolation and the identification of the female sex pheromone of a fungus-growing termite, *Pseudacanthotermes spiniger*. This species is one of the rare species of termites in which pairing occurs in the air during the swarming flight (Grassé and Noirot, 1951; Grassé, 1984). The nests of *P. spiniger* are entirely subterranean, but epigeous turrets (up to 1.5 m high) are built by the workers just before the swarming season. The alates are gathered in these "swarming turrets" some weeks before the flight. For some years, *P. spiniger* has been becoming more abundant in the sugarcane plantations of equatorial Africa and is becoming economically important.

METHODS AND MATERIALS

Alates of *P. spiniger* were collected in the savannah near Franceville in Gabon, where the colonies of *P. spiniger* swarm at the end of the rainy season (May 1988, 1989, 1990). One hundred fifty thousand alates were collected either directly from swarming turrets or during the flight by covering these turrets with mosquito nets.

In *P. spiniger* sex attraction is intense and can be observed in the laboratory for several days and even several weeks. In Petri dishes, the females take up a "calling" posture with their highly raised abdomen exposing the sternal gland. There is no differentiated tergal gland in the alates of *P. spiniger*. The males are attracted by these calling females. As soon as they touch the females, a tandem run is released. The female is the leader, the male follows, palpating with antennae and mouthparts the female abdominal extremity. As already mentioned (Stuart, 1969), this suggests that in the sexual behavior both olfactory and tactile stimuli are involved. Therefore, two males were used for each sex attraction bioassay since a single male is deprived of tactile communication and shows rather unstable behavior.

Laboratory Bioassays. Sex attraction bioassays were carried out by introducing two males into 15-cm-ID Petri dishes. These dishes contained small bent

pieces of Whatman No. 1 filter paper (1 cm^2) on which 20 μl pentanic extracts were applied with a microsyringe. The solvent was evaporated immediately before the bioassay. A similar piece of paper treated with 20 μl pentane and evaporated to dryness was used as control. The two males were introduced at the same time and at the same distance from the pieces of paper. During 300 sec, the time spent by one or two males licking or palpating the different pieces of paper was measured. A bioassay was noted as positive for an extract when the time was longer than 150 sec. After each bioassay, the Petri dish was cleaned with ethanol and pentane; males and pieces of paper were replaced.

Extracts. Two kinds of extracts were prepared: (1) Extracts from whole individuals were prepared by washing dealate males or females in doubly distilled pentane for 1 hr at 4°C, 250 ml solvent was used for 4000 individuals. Samples were stored at −20°C. (2) Extracts from sternal glands were prepared by removing the glands under a stereomicroscope with microscissors and forceps from cold anesthesized males or females. The glands [dimensions of the gland (μm)—females: 1800/550/150; males: 600/250/80] were removed from adjacent tissues (hemolymph, fat body) with a paper towel and transferred to a vial of doubly distilled pentane (200 glands/2 ml solvent) and allowed to soak for 1 hr at 4°C; then the glands were removed and the extracts were used immediately for bioassays or stored at −20°C for chemical analyses.

For all bioassays, the extracts were tested at a concentration of one individual equivalent.

Chemical Analyses. Gas chromatographic analyses (GC) were carried out with a Packard 429 gas chromatograph fitted with a split–splitless injector and a flame-ionization detector. The capillary column used was either a DB-5 fused silica column (30 m × 0.32 mm ID, J&W Scientific) or a CP-WAX 58 CB column (25 m × 0.25 ID, Chrompack) with temperature programming from 50°C to 220°C, at 2°C/min. Injector and detector temperatures were, respectively, 230°C and 250°C. Helium was used as the carrier gas at a flow rate of 5 ml/min. The GC traces were recorded and integrated by a CR3A Shimadzu integrator.

For preparative GC, a Girdel 30 chromatograph fitted with a Pyrex column (3 m × 3.2 mm) packed with 5% SE 30 on 100–120 mesh Chromosorb AW-DMCS, and a flame-ionization detector was used with helium as the carrier gas (flow rate 15 ml/min). Oven temperatures were programmed from 100°C to 200°C, at 2°C/min. An effluent splitter was used that allowed 50% of the effluent to flow to the detector. Fractions were collected in U-shaped glass tubes (1 mm ID) cooled to 0°C.

Gas chromatographic–mass spectrometric (GC-MS) analyses were carried out with a Nermag R 10-10-C quadrupole mass spectrometer coupled to a Girdel 31 gas chromatograph fitted with a split–splitless injector and a DB-5 fused

silica capillary column (60 m × 0.32 ID). The GC conditions described above were used. The column was connected directly to the ion source of the spectrometer through a heated transfer line maintained at 260°C.

Electron impact (EI) mass spectra were obtained at 70 eV on an 0.8-sec cycle, the instrument scanning from 25 to 300 amu with the ion source maintained at 150°C. Positive chemical ionization (CI) mass spectra were generated at 90 eV using methane or ammonia (and deuterated ammonia) at a source pressure of 0.2 and 0.3 torr, respectively, with a source temperature of 90°C, and the instrument scanning from 60 to 300 amu in 0.7 sec. Negative CI was obtained with the hydroxyl anion OH^- by ionizing a 5:1 mixture of methane and nitrous oxide (CH_4–N_2O) at a source pressure of 0.3 torr.

Microhydrogenation was performed on line in the GC-MS system by means of a fused silica capillary reactor. The reactor, consisting of a 60-cm piece of deactivated fused silica capillary column (6 m × 0.32 mm ID), statically coated with a 0.5% CH_2Cl_2 solution of palladium acetyl-acetonate (Fluka) was connected to the outlet of the analytical column with a zero-dead volume capillary butt-connector (Supelco) (Le Quéré et al., 1989). Palladium metal was precipitated at 220°C under hydrogen flow. Hydrogen was then used as the carrier gas at a flow rate of 1.5 ml/min, and the GC conditions were those previously described.

Fourier transform infrared (FTIR) spectra were taken in the gas phase on a Bruker IFS 85 FTIR spectrometer coupled, via a Bruker gold-coated lightpipe (20 cm × 1.0 mm ID) maintained at 200°C, to a Carlo-Erba 5160 gas chromatograph equipped with a DB-5 fused silica capillary column (30 m × 0.32 mm ID), an on-column injector, and a flame ionization detector. The carrier gas was helium at a flow rate of 3 ml/min and temperature programming from 30 to 60°C at 10°C/min and then to 200°C at 3°C/min.

For NMR analysis, the trapped component was rinsed from the tube into a Teflon-lined capped vial with distilled pentane, evaporated to dryness, and redissolved in deuterochloroform ($CDCl_3$, 99.96% D, CEN-CEA, Gif sur Yvette). The purity of collected fractions was monitored by capillary GC.

[^{1}H]NMR spectra were recorded on a Bruker WM400 instrument in 5-mm-ID NMR tubes. The signal due to the residual protons of the deuterated solvent was used as internal reference (δ = 7.24).

RESULTS

Origin Sex Pheromone. In *P. spiniger*, the males are attracted by the females; the females are never attracted by the males. In bioassays, the females are neither attracted by the male extracts nor by the female extracts (0 positive out of 30 bioassays performed).

The results obtained with the males (Table 1, A) show that the bioassays are not biased when using two males, since these two individuals never joined together for a long time on control papers in the Petri dish.

The males were strongly attracted by female extracts (Table 1, B). In most of the cases, the males started running rapidly toward the female extracts and met each other on the female extract in few seconds. Then, they could stay on the extract during the entire bioassay, while licking and palpating the piece of paper and each other alternately. In a few cases (3/20), males were attracted by the male extracts. This is not completely surprising since male–male homosexual tandems have been observed sometimes.

If given a choice between extracts from female sternal glands and extracts from whole females without sternal gland, the males always choose the sternal gland extract (Table 1, C). It could be concluded that the females of *P. spiniger* attract males by means of a sex pheromone that is secreted by their sternal gland.

Isolation of Sex Pheromone. The GC patterns of male and female washes were similar. However, a compound with a retention time of 57 min was about 10 times more abundant in females than in males. The same peak was observed in the sternal gland extracts of both sexes, and it was also about 10 times higher in female glands than in male glands (Figure 1, compound A). Compound B, with a retention time of 62 min, is also more abundant in females than in males.

The extracts from whole individual washes were fractionated by gas chro-

TABLE 1. SEX ATTRACTION BIOASSAYS: ORIGIN OF FEMALE SEX PHEROMONE OF *Pseudacanthotermes spiniger*[a]

Bioassay	Extract	Positive responses (%)	T (sec) ($\bar{X} \pm$ SD)
A	Control 1	0	0.8 ± 0.5
	Control 2	0	1 ± 1.1
B	Female wash	85	240.8 ± 45
	Male wash	15	40.5 ± 38
	Control 2	0	0
C	Female sternal gland	95	240.9 ± 24
	Female without sternal gland	0	0.7 ± 0.4
	Control 2	0	0.5 ± 0.4

[a] Choice tests for male dealates between filter paper impregnated with various extracts. All extracts were tested to one individual or one gland equivalent. A bioassay was considered as positive when males licked and palpated extracts for at least 150 sec. (Duration of the bioassay = 300 sec.) T: mean time ($\pm$SD) spent by males on extracts. Control 1: piece of filter paper. Control 2: filter paper previously impregnated with pentane. Number of replicates: 20.

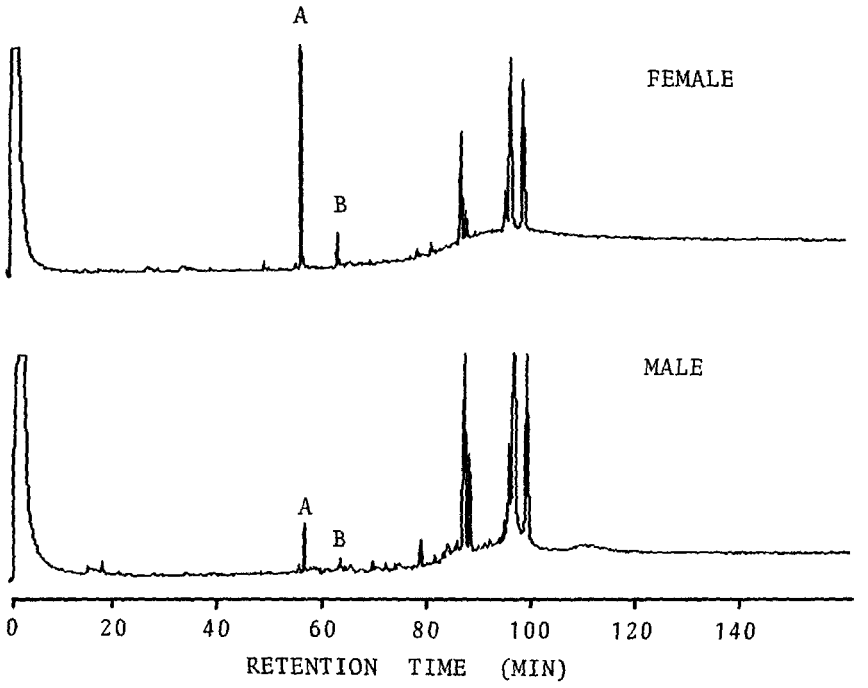


FIG. 1. Gas chromatograms of sternal gland extracts from female (upper) and male (lower) alates *Pseudacanthotermes spiniger*. Compounds A and B are much more abundant in females than in males. A is the female sex pheromone of *P. spiniger*.

matography, and the fractions were tested in bioassays at the concentration of one individual equivalent (Figure 2 and Table 2). Only fractions 2 and 4 were attractive for males and able to induce sexual behavior in these individuals (quick moving, licking, antennal palpating). Fraction 2, which only contained compounds A and B, was as active as the whole female wash. When only fractions 1 and 3 were tested, there was no positive response. Fractions 4 and 5 enabled us to separate compounds A and B: only fraction 4 with compound A was active. Compound A is the female sex pheromone of *P. spiniger*.

Identification of Sex Pheromone. The mass spectrum of compound A is presented in Figure 3. A spectral search in various MS data bases gave no plausible candidate. However, a fragment ion at *m/z* 31 suggested a primary aliphatic alcohol (Budzikiewicz et al., 1964), and the fragmentation pattern was found typical of a polyunsaturated compound. The molecular weight, suggested by an important ion at *m/z* 180, was confirmed by chemical ionization GC-MS, with methane and ammonia as reagent gases. The methane-CI mass spectrum displayed pseudomolecular ions at *m/z* 179 $(M - 1)^+$, 180 $(M)^+$ and 181 (M

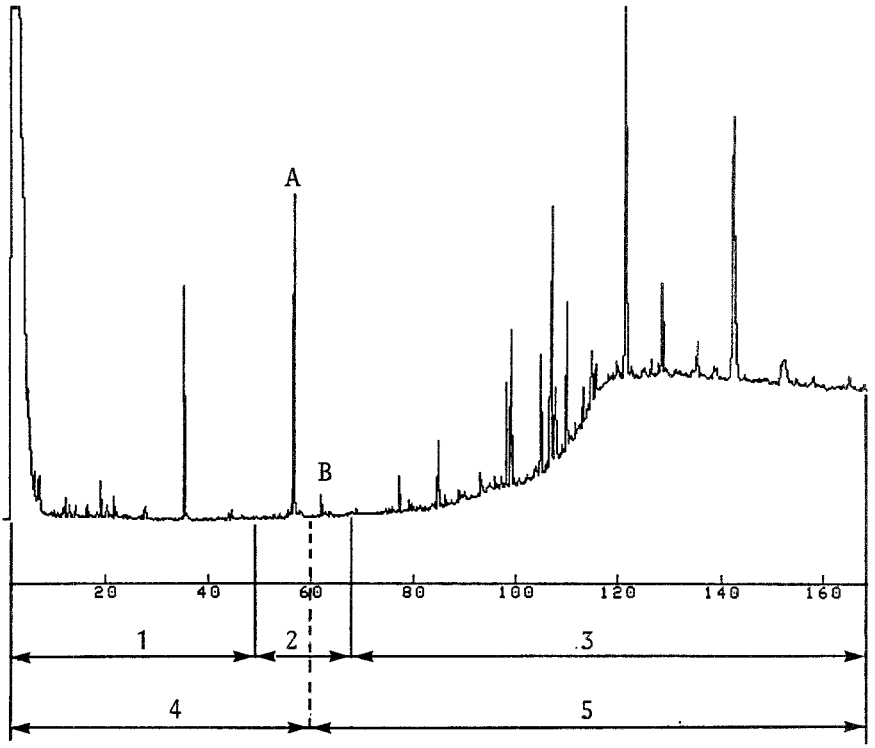


FIG. 2. Chromatographic fractions of washes of female alates *Pseudacanthotermes spiniger*. Fraction 2 contains compounds A and B, which are especially abundant in females. Fraction 4 contains A , fraction 5 contains B. Only fractions 2 and 4 induced attraction and excitation in male dealates.

$+ 1)^+$ and a prominent ion at m/z 163 $(M - 17)^+$, characteristic of an unsaturated alcohol of molecular weight 180 (Sarris et al., 1985). This was confirmed by the ammonia-CI mass spectrum, which showed intense pseudomolecular ions at m/z 181 $(M + 1)^+$ and 198 $(M + NH_4)^+$, and a weak ion at m/z 163 $(M - 17)^+$. These molecular ions and their isotopic contributions suggested a molecular formula $C_{12}H_{20}O$ and three sites of unsaturation.

The functionality was confirmed with a CI-MS experiment with deuterated ammonia (ND_3) as reagent gas. The pseudomolecular ions at m/z 183, $(M - H + D + D)^+$ and 203, $(M - H + D + ND_4)^+$ revealed one exchangeable proton (Lin and Smith, 1979).

Online microhydrogenation (Le Quéré et al., 1989) gave a mass spectrum typical of a straight-chain primary alkanol, with no molecular ion. However, an ion at m/z 168, tentatively attributed to $(M - H_2O)^+$, suggested a molecular

TABLE 2. SEX ATTRACTION BIOASSAYS: ATTRACTIVENESS OF CHROMATOGRAPHIC FRACTIONS DERIVED FROM FEMALE WASHES OF *Pseudacanthotermes spiniger*[a]

Bioassay	Extract	Positive responses (%)	T (sec) ($\bar{X} \pm$ SD)
A	Fraction 1	0	0.13 ± 0.15
	Fraction 2	100	280.2 ± 8.2
	Fraction 3	0	0.03 ± 0.06
	Control	0	0
B	Fraction 1	0	0.3 ± 0.3
	Fraction 3	0	1.1 ± 0.9
	Control	0	0.03 ± 0.06
C	Fraction 4	100	278.5 ± 10.9
	Fraction 5	0	1.4 ± 1.05
	Control	0	0.2 ± 0.17
D	Whole extract	46.6	131.4 ± 51.6
	Fraction 2	50	154.1 ± 50.9
	Control	0	0.9 ± 0.7

[a]Choice tests for male dealates between different chromatographic fractions of female pentanic washes. All fractions were tested to one individual equivalent. See table 1 for abbreviations. Number of replicates: 30, except for bioassay B where N = 10.

weight of 186, and thus, the structure 1-dodecanol. This was confirmed by spectral search in MS data bases and by OH^- CIMS [m/z 185 $(M - 1)^-$], revealing three carbon–carbon double bonds in the original compound, accounting for the three sites of unsaturation, and consequently, the structure dodecatrien-1-ol.

The GC-FTIR spectrum of A (Figure 4) confirmed the hydroxyl group (ν OH: 3657 cm^{-1}; ν C–O: 1049 cm^{-1}). The C–O stretching frequency was characteristic of a primary alcohol (Nyquist, 1984). The OH stretching frequency was rather low for a primary alcohol, suggesting an intramolecular hydrogen bonding, probably with one of the carbon–carbon double bonds, in a $(5 + \pi)$ system (Nyquist, 1984), revealing a 3-alken-1-ol subunit. Other important features of the vapor-phase infrared spectrum were the C=C stretching frequency at 1659 cm^{-1} and the CH out-of-plane deformation frequency (δ CH) at 719, 949, and 980 cm^{-1}. The former (719 cm^{-1}) was attributed to a *Z*-disubstituted ethylenic bond and the pair 949 and 980 cm^{-1} to *Z,E*-conjugated double bonds.

Finally, the structure of compound A, isolated by preparative GC, was established by [^{1}H]NMR, including two-dimensional chemical shifts correlation (COSY) and selective decoupling experiments. The 400-MHz [^{1}H]NMR spec-

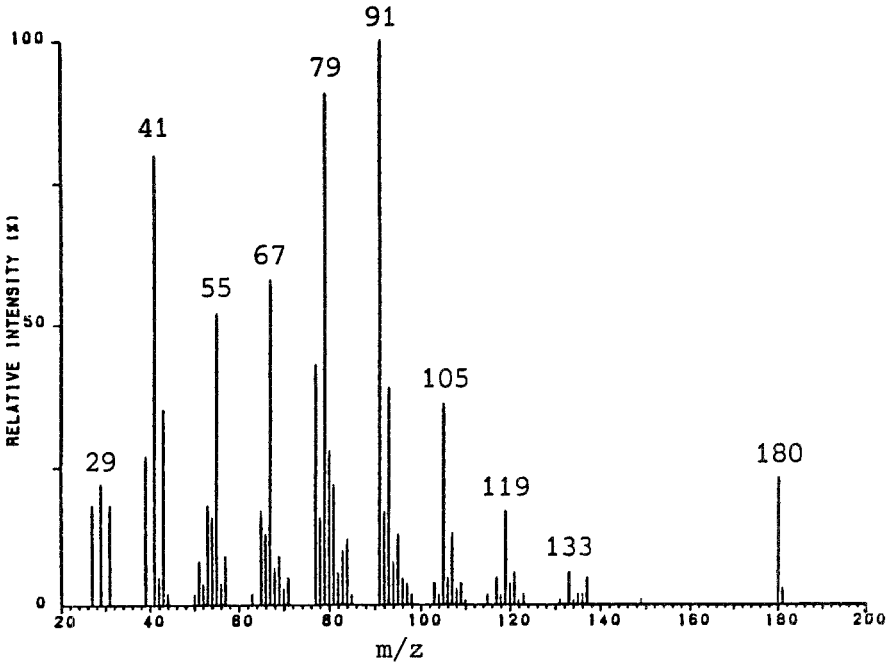


FIG. 3. Mass spectrum (70 eV) of compound A.

trum (Table 3) revealed well-resolved multiplets due to six olefinic hydrogens at δ 6.32, 5.98, 5.70, 5.58, 5.42, and 5.25 ppm.

The proton at δ 6.32 (dd) was coupled (J = 15 Hz) to a vinyl hydrogen (δ 5.70), confirming one *E*-double bond, and coupled (J = 10.9 Hz) to another olefinic proton (δ 5.98), confirming the conjugated double bonds. The *Z* geometry of this second double bond was confirmed by the coupling constant (J = 10.7 Hz) measured in the olefinic resonance at δ 5.98 (dd) which, itself, revealed a coupling with the vinyl hydrogen at δ 5.25.

The olefinic proton at δ 5.70 (dt) was also coupled (J = 6.9 Hz) to a methylene resonance at 2.09 (dt) which was connected (J = 7.0 Hz) to another methylene signal at δ 1.42 (tq), itself coupled to a methyl triplet (J = 7.4 Hz) at δ 0.92. Therefore, the partial structure $CH_3-CH_2-CH_2-CH=CH-CH=CH-$ could be deduced.

The olefinic multiplet at δ 5.25 (dt) was also coupled to a methylene group at δ 2.95 (dd, J = 7.4 Hz), itself coupled (J = 7.4 Hz) to another vinyl hydrogen at δ 5.58. The latter was coupled to the olefinic resonance at δ 5.42 with a coupling constant (J = 10.7 Hz) characteristic of a *Z*-ethylenic bond. This olefinic signal (dt) revealed a coupling (J = 7.3 Hz) with a methylene resonance

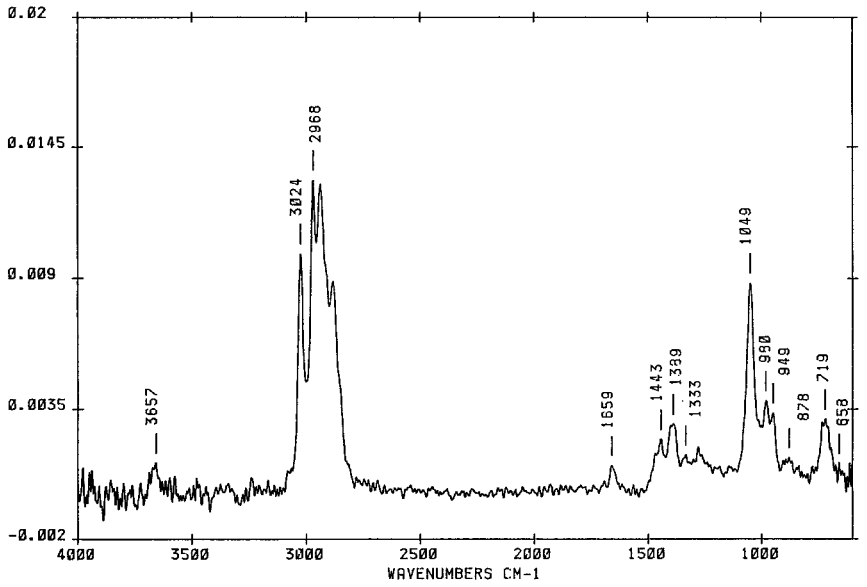
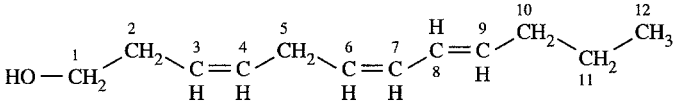


FIG. 4. Gas-phase infrared spectrum of compound A.

TABLE 3. [^{1}H]NMR SPECTRUM OF COMPOUND A IDENTIFIED AS (*Z,Z,E*)-3,6,8-DODECATRIEN-1-OL[a]



Compound A: (*Z,Z,E*)-3,6,8-Dodecatrien-1-ol

6.32 (1H, dd, J = 15 and 10.9 Hz, H_8)
5.98 (1H, dd, J = 10.9 and 10.7 Hz, H_7)
5.70 (1H, dt, J = 15 and 6.9 Hz, H_9)
5.58 (1H, dt, J = 10.7 and 7.4 Hz, H_4)
5.42 (1H, dt, J = 10.7 and 7.3 Hz, H_3)
5.25 (1H, dt, J = 10.7 and 7.4 Hz, H_6)
3.67 (2H, t, J = 6.4 Hz, H_1)
2.95 (2H, dd, J = 7.4 and 7.4 Hz, H_5)
2.37 (2H, dt, J = 7.3 and 6.4 Hz, H_2)
2.09 (2H, dt, J = 7 and 6.9 Hz, H_{10})
1.42 (2H, tq, J = 7 and 7.4 Hz, H_{11})
1.20 (1H, br s, —OH)
0.92 (3H, t, J = 7.4 Hz, H_{12})

[a] 400.13 MHz, δ ppm/TMS, $CDCl_3$. Chemical shift (integration, multiplicity, coupling constants, assignment).

at δ 2.37 (dt), which was connected (J = 6.4 Hz) to another methylene group at δ 3.67 (t). The chemical shift of the latter was characteristic of an oxygen-bearing methylene. Finally, a broad singlet at δ 1.20 was tentatively attributed to the hydroxyl proton resonance.

Thus, the structure of compound A was demonstrated to be (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol as depicted in Table 3.

Compound A was identical to the product identified as the trail-following pheromone of various subterranean termites (Matsumura et al., 1968; Tai et al., 1969; Tokoro et al., 1989, 1990). The spectroscopic data are in good agreement with those published for the trail-following pheromone. Particularly, the mass spectrum and the [^{1}H]NMR spectrum were identical (Yamaoka et al., 1987; Eya et al., 1990).

There is about 15–20 ng (3*Z*,6*Z*,8*E*)-3,6,8-dodecatrien per female and 1–3 ng per male. Until now, it was not possible to test the activity of the synthetic alcohol on male alates of *P. spiniger*.

The other product of fraction 2 (compound B), with a longer GC retention time, gave exactly the same EI and CI mass spectra; it was therefore identified as a geometric isomer of the major product. The complete assignment of its structure is in progress.

Attempts to Detect Dodecatrienol in Food. P. spiniger is a fungus-growing termite that eats dead plants. The debris of wood or leaves are first packed together in the nest in small sawdust heaps and then incorporated into fungus gardens before ingestion by the workers. Several samples of sawdust (10 g/25 ml pentane) and fungus combs (100 g/250 ml pentane) were extracted during 2 hr with distilled pentane and analyzed by gas chromatography. No trace of the dodecatrienol was found in either sawdust or fungus combs.

DISCUSSION

(3*Z*,6*Z*,8*E*)-3,6,8-dodecatrien-1-ol (dodecatrienol) was first isolated and identified by Matsumura et al. (1968, 1969) from total extracts of workers of *Reticulitermes virginicus* and from food of this termite, which was wood infected by the fungus *Gloephyllum trabeum*. As this alcohol was detected by the workers at extremely low concentrations, it was considered to be the trail pheromone. However, it was never identified in the sternal gland itself, which produces the trail pheromone in termite workers. Some authors considered that dodecatrienol could be either an exogenous diet-related pheromone or just an attractant (Stuart, 1970; Ritter and Coenen-Saraber, 1969; Tschinkel and Close, 1973). Nevertheless, dodecatrienol has been found recently in workers of *Reticulitermes speratus* (Tokoro et al., 1990) and *Coptotermes formosanus* (Tokoro et al., 1989) fed with filter paper and it actually seems to be a trail-following pheromone of termites.

Our results in *P. spiniger* show for the first time that dodecatrienol occurs in the sternal gland of termites. As there is no dodecatrienol in the food, this substance must be synthesized by the termites themselves. In *P. spiniger* dodecatrienol is found in the swarming imagoes and is about 10 times more abundant in the female sternal glands than in male glands. Dodecatrienol has an unquestionable power of attraction and excitation over the male alates. It therefore can be considered as the sex pheromone of *P. spiniger*. It is interesting to note that tetradecyl propionate, the ester identified as a sex pheromone in *Reticulitermes flavipes*, is also present in both sexes but in much larger quantities in female alates than in males (Clément et al., 1989).

In *P. spiniger* the role of dodecatrienol might be not restricted to the only pairing of the alates. Some observations (Bordereau et al., 1991) show that the dealate imagoes are able to follow artificial trails prepared with pentanic solutions of dodecatrienol at very low concentrations. This would reunite partners after an accidental separation during the nuptial promenade. Moreover, dodecatrienol is also detected at extremely low levels by the workers of *P. spiniger* (Bordereau et al., 1990). In *P. spiniger*, dodecatrienol could be so used either as a sex or as a trail-following pheromone. This double function would depend on a variation of the capacity of detection according to target caste. At high concentrations, it acts as a female sex pheromone, eliciting attraction and excitation of the males, whereas at low concentrations it would act as a trail-following pheromone for workers and swarming imagoes.

The observations of Quennedey and Leuthold (1978) in *Trinervitermes bettonianus* are in good agreement with this hypothesis of a functional duality, as the sternal gland extracts of the workers, when applied in adequate concentrations, have the same capacity for male attraction as the female sternal gland extracts. In this species, the cembrene A identified as a trail-following pheromone is present in workers and in very much larger quantities in alates; however, its biological activity in sex attraction is not known (McDowell and Oloo, 1984).

It may seem surprising that a compound can be used as both a trail-following pheromone, which is detected at a very short distance, and a sex pheromone, which is considered to act at a long distance. Dodecatrienol is not an highly volatile component and cannot be detected beyond some tens of centimeters. However, in termites, sexual attraction never occurs at great distance, the only exception being *Hodotermes mossambicus* (Leuthold, 1977). In most species and in *P. spiniger* in particular the conditions of the dispersal flight favor the meeting of sexes. Flights are usually synchronous, and the alates do not disperse very much. In *P. spiniger*, where pairing occurs in the air, the alates have a zigzag and whirling flight, which greatly favors meetings between individuals.

Therefore, it is suggested that *P. spiniger* represents a very parsimonious

pheromonal system in which a single compound can assume at least two functions. This agrees with the economy principle often observed in social insects (Blum and Brand, 1972; Pasteels, 1976), and this interpretation would explain the presence of dodecatrienol in male alates. In these individuals, it would function only as a trail pheromone. However, the presence of dodecatrienol in both sexes could be also a vestigial character. In some primitive species such as *Kalotermes flavicollis* (Wall, 1971) or *Zootermopsis* (Castle, 1934; Pasteels, 1972), males and females may attract reciprocally.

Finally, the presence of dodecatrienol both in Rhinotermitidae, which are lower termites, and in Macrotermitinae, which are higher termites, must be underlined. This reinforces the phylogenetic proximity of Rhinotermitidae and Macrotermitidae, which was previously suggested on the basis of morphological and anatomical characters (Emerson, 1955; Deligne, 1985). Moreover, a great number of species of lower and higher termites are very sensitive to dodecatrienol (Ritter and Coenen-Saraber, 1969; Matsumura et al., 1972; Howard et al., 1976; Kaib et al., 1982). This suggests that dodecatrienol could be a basic pheromonal component common to many termite species while additional components would provide the specificity. This could explain the anatomical diversity and the complexity of the termite sternal gland (Quennedey, 1977).

Acknowledgments—We thank Professor Ch. Noirot and R. Brossut for review of this manuscript; H. Nkombe, C. Moussavou, and P. Okouma for helpful technical assistance and collecting termite alates; and the University of Masuku, Gabon, for use of their facilities and financial support.

REFERENCES

BLUM, M.S., and BRAND, J.M. 1972. Social insect pheromones: Their chemistry and function. *Am. Zool.* 12:553–576.

BORDEREAU, C., ROBERT, A., BONNARD, O., and LE QUÉRÉ, J.L. 1990. *cis*-3, *cis*-6,*trans*-8-dodecatrien-1-ol: Sex and trail-following pheromone in a higher fungus-growing termite? Proceedings, 11th International Congress, IUSSI, Bangalore. pp. 39–40.

BORDEREAU, C., ROBERT, A., MARCEL, N., BONNARD, O., and LE QUÉRÉ, J.L. 1991. Detection of dodecatrienol in *Pseudacanthotermes spiniger* (Termitidae, Macrotermitinae) according to castes and concentrations. In preparation.

BUCHLI, H. 1960. Les tropismes lors de la pariade des imagos de *Reticulitermes lucifugus. Vie Milieu* 11:308–315.

BUDZIKIEWICZ, H., DJERASSI, C., and WILLIAMS, D.H. 1964. Interpretation of Mass Spectra of Organic Compounds. Holden Day, San Francisco.

CASTLE, G.B. 1934. The damp-wood termites of western United States, genus *Zootermopsis* (formerly *Termopsis*), pp. 273–310, *in* C.A. Kofoid (ed.). Termites and Termite Control. University of California Press, Berkeley.

CLÉMENT, J.L. 1982. Phéromones d'attraction sexuelle des termites européens du genre *Reticulitermes*. Mécanismes comportementaux et isolements spécifiques. *Biol. Behav.* 7:55–68.

CLÉMENT, J.L., LLYOD, H., NAGNAN, P., and BLUM, M.S. 1989. *n*-Tetradecyl propionate: Iden-

tification as a sex pheromone of the eastern subterranean termite *Reticulitermes flavipes*. *Sociobiology* 15:19–24.

DELIGNE, J. 1985. Apport de la micromorphologie du labre à la compréhension de la phylogenèse des termites (Isoptères). *Actes Coll. Insectes Soc.* 2:35–42.

EMERSON, A.E. 1955. Geographical origins and dispersions of termite genera. *Fieldiana, Zool.* 37:465–521.

EYA, B.K., OTSUKA, T., KUBO, I., and WOOD, D.L. 1990. Syntheses and NMR analyses of the eight geometric isomers of 3,6,8-dodecatrienol-1-ol, subterranean termite trail pheromone. *Tetrahedron* 46:2695–2706.

GRASSÉ, P.P. 1984. Termitologia, tome II. Masson, Paris.

GRASSÉ, P.P., and NOIROT, Ch. 1951. Nouvelles recherches sur la biologie de divers termites champignonnistes. *Ann. Sci. Nat., Zool.* 13:291–342.

HOWARD, R., MATSUMURA, F., and COPPEL, H.C. 1976. Trail following pheromones of the Rhinotermitidae: Approaches to their authentication and specificity. *J. Chem. Ecol.* 2:147–166.

KAIB, M., BRUINSMA, O., and LEUTHOLD, R.H. 1982. Trail following in termites: Evidence for a multicomponent system. *J. Chem. Ecol.* 8:1193–1205.

LE QUÉRÉ, J.L., SEMON, E., LANHER, B., and SEBEDIO, J.L. 1989. On-line hydrogenation in GC-MS analyses of cyclic fatty acid monomers isolated from heated linseed oil. *Lipids* 24:347–350.

LEUTHOLD, R.H. 1977. Post-flight communication in two termite species, *Trinervitermes bettonianus* and *Hodotermes mossambicus*. Proceedings, 7th International Congress, IUSSI, Wageningen. pp. 62–64.

LEUTHOLD, R.H., and LÜSCHER, M. 1974. An unusual caste polymorphism of the sternal gland and its trail pheromone production in the termite *Trinervitermes bettonianus*. *Insectes Soc.* 21:335–341.

LIN, Y.Y., and SMITH, L.L. 1979. Active hydrogen by chemical ionization mass spectrometry. *Biomed. Mass Spectrom.* 6:15–18.

MATSUMURA, F., COPPEL, H.C., and TAI, A. 1968. Isolation and identification of termite trail following pheromone. *Nature* 219:963–964.

MATSUMURA, F., TAI, A., and COPPEL, H.C. 1969. Termite trail following substance, isolation, and purification from *Reticulitermes* and fungus infected wood. *J. Econ. Entomol.* 62:599–603.

MATSUMURA, F., JEWETT, D.M., and COPPEL, H.C. 1972. Interspecific response to synthetic trail following substances. *J. Econ. Entomol.* 65:600–602.

MCDOWELL, P.G., and OLOO, G.W. 1984. Isolation, identification, and biological activity of trail-following pheromone of termite *Trinervitermes bettonianus* (Sjôstedt) (Termitidae: Nasutitermitinae). *J. Chem. Ecol.* 10:835–851.

NYQUIST, R.A. 1984. The Interpretation of Vapor-Phase Infrared Spectra, Vol. 1. Sadtler-Heyden, Philadelphia.

PASTEELS, J.M. 1972. Sex specific pheromones in a termite. *Experientia* 28:105–106.

PASTEELS, J.M. 1976. Evolutionary aspects in chemical ecology and chemical communication. Proceedings, 15th International Congress on Entomology, Washington. pp. 281–293.

QUENNEDEY, A. 1977. An ultrastructural study of the polymorphic sternal gland in *Reticulitermes santonensis* (Isoptera, Rhinotermitidae), another way of looking at the true termite trail pheromone. Proceedings, 7th. International Congress, IUSSI, Wageningen. pp. 48–51.

QUENNEDEY, A., and LEUTHOLD, R.H. 1978. Fine structure and pheromonal properties of the polymorphic sternal gland in *Trinervitermes bettonianus* (Isoptera, Termitidae). *Insectes Soc.* 25:153–162.

RITTER, F.J., and COENEN-SARABER, C.M.A. 1969. Food attractants and a pheromone as trail-

following substances for the saintonge termite. Multiplicity of the trail-following substances in *L. trabea*-infected wood. *Entomol. Exp. Appl.* 12:611–622.

SARRIS, J., ETIEVANT, P.X., LE QUERE, J.L., and ADDA, J. 1985. The chemical ionization mass spectra of alcohols, *in* J. Adda (ed.). Progress in Flavour Research. Elsevier, Amsterdam, pp. 591–601.

STUART, A.M. 1969. Social behavior and communication, pp. 193–232, *in* K. Krishna and F.M. Weesner (eds.). Biology of Termites. Academic Press, New York.

STUART, A.M. 1970. The role of chemicals in termite communication, pp. 79–105, *in* J.W. Johnson, D.G. Moulton and A. Turk (eds.). Advances in Chemoreception, Vol. 1, Communication by Chemicals signals. Appleton-Century-Crofts, New York.

STUART, A.M. 1975. Some aspects of pheromone involvement in the post-flight behaviour of the termites *Zootermopsis angusticollis* (Hagen) and *Reticulitermes flavipes* (Kollar), pp. 219–223, *in* Ch. Noirot, P.E. Howse, and G. Le Masne (eds.). Proceedings IUSSI, Symposium., Dijon, France.

TAI, A., MATSUMURA, F., and COPPEL, H.C. 1969. Chemical identification of the trail following pheromone for a southern subterranean termite. *J. Org. Chem.* 34:2180–2182.

TOKORO, M., TAKAHASHI, M., TSUNODA, K., and YAMAOKA, R. 1989. Isolation and primary structure of trail pheromone of the termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). *Wood Res.* 76:29–38.

TOKORO, M., YAMAOKA, R., HAYASHIYA, K., TAKAHASHI, M., and NISHIMOTO, K. 1990. Evidence for trail pheromone precursor in termite *Reticulitermes speratus* (Kolbe) (Rhinotermitidae: Isoptera). *J. Chem. Ecol.* 16:2549–2557.

TSCHINKEL, W.R., and CLOSE, P.G. 1973. The trail pheromone of the termite, *Trinervitermes trinervoides*. *J. Insect Physiol.* 19:707–721.

WALL, M. 1971. Zur Geschlechtsbiologie der Termite *Kalotermes flavicollis* (Fabr.) (Isoptera). *Acta Trop.* 28:17–60.

YAMAOKA, R., TOKORO, M. and HAYASHIYA, K. 1987. Determination of geometric configuration in minute amounts of highly unsaturated termite trail pheromone by capillary gas chromatography in combination with mass spectrometry and Fourier-transform infrared spectroscopy. *J. Chromatogr.* 399:259–267.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

RELATIONSHIPS BETWEEN CHEMICAL STRUCTURE AND INHIBITORY ACTIVITY OF C_6 THROUGH C_9 VOLATILES EMITTED BY PLANT RESIDUES[1]

JUDITH M. BRADOW

USDA, ARS, Southern Regional Research Center
P.O. Box 19687
New Orleans, Louisiana 70179

(Received April 26, 1991; accepted July 8, 1991)

Abstract—Leaf, stem, flower, fruit, and root residues of a wide variety of plants have been reported to emit bioactive *n*-alkanes, 2-alkanols, *n*-alkanals, 2-alkenals, 2-alkanones, and *n*-alkanoic acids containing from six to nine carbon atoms. During a 72-hr exposure to the vapor phase of these compounds (6.9, 20.8 or 34.4 μM/liter), germination of onion, carrot, and tomato seeds was inhibited to varying degrees. The extent of inhibition caused by these plant residue volatiles depended upon the compound type and concentration, carbon-chain length, and the seed species tested. Tomato seeds recovered more fully from exposure to these volatile inhibitors than did those of carrot and onion. Degree of recovery in all three species depended on the type and concentration of volatile present. The C_7 and C_8 compounds were the most inhibitory among these 24 volatiles. Of the six classes of chemicals examined, the 2-alkenals were the most inhibitory, followed by the 2-alkanols, *n*-alkanals, and 2-alkanones, which were equally effective as seed germination inhibitors. The straight-chain alkanes and alkanoic acids were relatively noninhibitory. Tests of a C_7 and C_9 alkadienal indicated that the C_7 compound was the more inhibitory.

Key Words—Allelopathy, allelochemicals, *Allium cepa*, *Daucus carota*, *Lycopersicon esculentum*, cover crops, insect attractants, conservation tillage, hydrocarbons, alcohols, ketones, aldehydes, carboxylic acids.

INTRODUCTION

Residues of both aerial and root tissues of many plant species emit mixtures of structurally simple volatile organic compounds containing six to nine carbon

[1] Mention of a commercial or proprietary product does not constitute an endorsement by the U.S. Department of Agriculture.

0098-0331/91/1100-2193$06.50/0

atoms in unbranched chains (Johnson et al., 1971a,b; Connick et al., 1987, 1989; Bradow and Connick, 1990). Many of the alcohols, aldehydes, and ketones from these volatile mixtures are well-known flavor components (Johnson et al., 1971a,b; French, 1985), have insect semiochemical activity (Visser, 1979, 1983; Light et al., 1988; Ramachandran et al., 1990), and also affect fungal growth and seed germination (French et al., 1977; French and Leather, 1979; French, 1985). Furthermore, these compounds are highly inhibitory of seed germination (Bradow and Connick, 1988a,b) and seedling growth (Bradow and Connick, 1988c) in several crop species. These volatiles also have been implicated in reductions in seedling survival and growth (Buntley, 1986; Dabney et al., 1986; Menges, 1987) and cotton yield (Rickerl et al., 1988) related to the presence in the soil of cover crop residues and other plant debris (Bradow and Connick, 1990).

Recent investigations of the activities of these compounds as seed germination inhibitors (Bradow and Connick, 1988a,b) and insect semiochemicals (Light et al., 1988; Ramachandran et al., 1990) have suggested that there are strong relationships between chemical structure, particularly carbon-chain length, and biological activity. Therefore, a comparative study was undertaken in which vapor-phase *n*-alkanes, 2-alkanols, 2-alkanals, *n*-alkanals, 2-alkenals, 2-alkanones, and *n*-alkanoic acids containing six, seven, eight, or nine carbon atoms were assayed for inhibitory effect on seed germination of onion, carrot, and tomato. The three seed species were exposed separately for 72 hr to three concentrations, either 6.9, 20.8, or 34.4 μM/liter, of the 24 individual compounds. The effects of a continuous seven-day exposure and a three-day exposure followed by removal of the test volatile and a 96-hr recovery period were also determined.

A C_7 and C_9 alkadienal were included in the assays on the basis of appropriate chain length and reported occurrence in volatile emissions from plant residues (Buttery et al., 1980, 1982a; Flath et al., 1984; Hernandez et al., 1989).

METHODS AND MATERIALS

Chemicals and assay seeds used in the bioassays were purchased from commercial sources. The assay technique, using desiccators as seed germination containers, was the protocol previously described (Bradow and Connick, 1987, 1988a,b; Connick et al., 1987). Briefly, seeds of onion (*Allium cepa* L., cv. Texas Early Grano 502), carrot (*Daucus carota* L., cv. Danvers Half-Long) or tomato (*Lycopersicon esculentum* Mill. cv. Homestead) were spread on double sheets of deionized water-saturated Whatman No. 1 filter paper placed on the porcelain plate of a 2.5-liter glass desiccator. Central circles (22 mm diam.)

had been removed from the filter paper to facilitate diffusion of the volatiles, and the filter-paper circles were divided into eight equal segments (replicates) containing 20 seeds each. Each desiccator well contained a 10-ml glass beaker (volatile source) resting on 50 g of pure sand in a glass crystallizing dish (100 × 50 mm). The sand was moistened with 10 ml of deionized water. The source beaker was left empty in the controls.

In the time-dependent studies, a volume equivalent to 34.4 μM/liter (full volatilization assumed) of the test compound was placed undiluted in the source beaker, and the separate seed species were incubated under a 10-hr/31°C day/21°C night temperature cycle for 72 hr before germination evaluation (three-day data). Radicle protrusion was the germination criterion for all seed species. The source beaker was then removed (three- + four-day data) or the test volatile was renewed by the addition of a second 34.4 μM equivalent (seven-day data). Each assay was performed twice (16 replicates, total), and controls were repeated at three-month intervals during the study. Germination of the control assays did not change significantly during the course of the experiment, and the germination rates of the seed lots used were not statistically different.

Concentration-dependent seed germination bioassays were performed using the same desiccator apparatus with the source beaker containing the appropriate volume of a single test compound to give a nominal volatile concentration of either 6.9, 20.8, or 34.4 μM/liter, assuming total volatilization and negligible adsorption (Bradow and Connick, 1988a). These concentrations were chosen on the basis of active concentration ranges determined earlier (Bradow and Connick, 1988a–c, 1990). With the control, the treatment concentration ratio was 0:1:3:5. Seed germination was evaluated after a three-day exposure to a test volatile. The volatile source was then removed, and the seeds were incubated for an additional four days. In both types of assays, the humidity within the desiccators was maintained by the addition of 10 ml deionized water to the sand after the three-day germination evaluation.

After normalization by the transformation $(x + 0.5)^{0.5}$, germination count data from the three-day and three- + four-day concentration-dependent assays were compared separately for each seed species, using three-way analyses of variance (six compound types × three concentrations × four carbon-chain lengths). For each seed species, two-way analyses of variance were used to examine the effects of chain length and concentration within compound type. Within the three-day (all three concentrations), seven-day, and three- + four-day data (34.4 μM/liter data only), one-way analyses of variance were used to determine significant differences between the mean effects of the test compounds and the appropriate deionized water controls, using Tukey's Honestly Significant Difference procedure (significance level, $P = 0.01$). The concentration-dependent data were also examined by regression analysis (Sokal and Rohlf, 1981).

RESULTS

Compound Type, Concentration, and Carbon-Chain-Length Effects on Seed Germination. When the seed germination rates of onion, carrot, or tomato seeds exposed for 72 hr to the C_6–C_9 plant residue volatiles (omitting the two alkadienals) were compared using separate three-way analyses of variance, compound type (*n*-alkane, 2-alkanol, etc.), volatile concentration (6.9, 20.8, or 34.4 μM), and carbon-chain length (six, seven, eight, or nine carbon atoms) had significant effects on all three seed species (Table 1). Significant interactions between compound type and concentration and between compound type and chain length were also observed. There were no significant interactions between chain length and concentration or between all three factors. The absence of an interaction between chain length and concentration may arise from the choice of only volatiles in the C_6–C_9 chain-length range, the range that had been found to be most active at low concentrations in earlier investigations (Bradow and Connick, 1988a,b; 1990).

When the volatile source was removed after the 72-hr exposure and the seeds were incubated an additional 96 hr before germination evaluation, three-way analyses of variance of the three-day + four-day data showed that the effects of compound type and concentration upon the "recovering" seeds remained highly significant (Table 2). Carbon-chain length continued to be a significant factor only in onion seed response. The interactions between compound type and concentration and chain length observed after the initial 72-hr

TABLE 1. MEAN SQUARES FROM ANALYSES OF VARIANCE OF EFFECTS OF COMPOUND TYPE, CONCENTRATION, AND CHAIN LENGTH ON GERMINATION OF ONION, CARROT, AND TOMATO SEEDS EXPOSED TO 6.9, 20.8, OR 34.4 μM C_6–C_9 BIOACTIVE PLANT RESIDUE VOLATILES FOR 72 HOURS

Source	*dF*	Mean squares		
		Onion	Carrot	Tomato
Compound type (*T*)	5	2.263***[a]	4.103***	13.327***
Concentration (*C*)	2	2.251***	3.815***	7.764***
Chain length (*L*)	3	0.579***	0.579***	0.908***
$T \times C$	10	0.191***	0.360***	1.037***
$T \times L$	15	0.139***	0.315***	0.347***
$C \times L$	6	0.076 NS	0.133 NS	0.146 NS
$T \times C \times L$	30	0.003 NS	0.006 NS	0.007 NS
Within	1080	0.055	0.095	0.116
Total	1151			

[a] *, **, *** = significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

TABLE 2. MEAN SQUARES FROM ANALYSES OF VARIANCE OF EFFECTS OF COMPOUND TYPE, CONCENTRATION, AND CHAIN LENGTH ON GERMINATION OF ONION, CARROT, AND TOMATO SEEDS EXPOSED TO 6.9, 20.8, OR 34.4 μM C_6–C_9 BIOACTIVE PLANT RESIDUE VOLATILES FOR 72 HOURS, FOLLOWED BY 96-HOUR RECOVERY PERIOD

Source	*dF*	Mean squares		
		Onion	Carrot	Tomato
Compound type (*T*)	5	2.724***[a]	4.331***	0.791***
Concentration (*C*)	2	2.381***	1.780***	1.461***
Chain length (*L*)	3	0.407**	0.296 NS	0.157 NS
$T \times C$	10	0.601***	0.482***	0.293***
$T \times L$	15	0.441***	0.712***	0.125 NS
$C \times L$	6	0.100 NS	0.081 NS	0.105 NS
$T \times C \times L$	30	0.007 NS	0.009 NS	0.005 NS
Within	1080	0.105	0.140	0.082
Total	1151			

[a] *, **, *** = significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

exposure persisted in the responses of onion and carrot, but the interaction between compound type and chain length was not significant in "recovering" tomato seeds.

Seed Germination Effects of Chain Length and Concentration within Compound Type. When the effects upon each seed species of a 72-hr exposure to 6.9, 20.8, or 34.4 μM of a specific type of compound were examined, no significant interactions were observed between concentration and chain length (Table 3). The seed species responded to different degrees to some compound types, e.g., there was no significant concentration effect when carrot seeds were exposed to 2-alkenals because all three concentrations of (*E*)-2-hexenal, (*E*)-2-heptenal, and (*E*)-2-octenal prevented germination (see Table 6 below). The chain length of the *n*-alkanes and 2-alkanols had no significant effect on carrot seeds, nor did chain length of 2-alkanols and alkanals alter the effect of those compound types on tomato germination (Table 3). In assays using onion seeds, chain length of the 2-alkanones was not important. In the case of the *n*-alkanoic acids in the volatile phase, neither increased concentration nor higher molecular weight affected onion or carrot response.

When the assay seeds were allowed to recover for 96 hr after a 72-hr exposure to one of the three concentrations of the test volatiles, no significant interactions were observed between carbon-chain length and concentration (Table 4). These two experimental factors had no significant effect on onion seeds exposed to *n*-alkanes, 2-alkanols, or *n*-alkanoic acids. Tomato seeds allowed to recover from volatile exposure showed no effects of concentration or chain

TABLE 3. MEAN SQUARES FROM ANALYSES OF VARIANCE OF CONCENTRATION AND CHAIN-LENGTH EFFECTS ON GERMINATION OF ONION, CARROT, AND TOMATO SEEDS EXPOSED (72 HOURS) TO 6.9, 20.8, OR 34.4 μM C_6–C_9 ALKANES, ALKANOLS, ALKANALS, ALKENALS, ALKANONES, AND ALKANOIC ACIDS

Source	dF	Mean squares		
		Onion	Carrot	Tomato
Alkanes				
Concentration (C)	2	0.099*[a]	0.265***	0.439*
Chain length (L)	3	0.191***	0.055 NS	0.816***
$C \times L$	6	0.002 NS	0.001 NS	0.008 NS
Within	180	0.030	0.023	0.128
Total	191			
2-Alkanols				
Concentration (C)	2	0.208**	0.685**	0.076***
Chain length (L)	3	0.311***	0.268 NS	0.009 NS
$C \times L$	6	0.002 NS	0.008 NS	0.001 NS
Within	180	0.039	0.136	0.009
Total	191			
Alkanals				
Concentration (C)	2	1.325***	2.012***	6.151***
Chain length (L)	3	0.206***	0.357***	0.748 NS
$C \times L$	6	0.002 NS	0.004 NS	0.018 NS
Within	180	0.032	0.059	0.290
Total	191			
2-Alkenals				
Concentration (C)	2	0.600*	0.445 NS	5.302***
Chain length (L)	3	0.480*	0.757**	0.895*
$C \times L$	6	0.011 NS	0.010 NS	0.016 NS
Within	180	0.176	0.167	0.257
Total	191			
2-Alkanones				
Concentration (C)	2	0.928***	2.012***	0.171***
Chain length (L)	3	0.074 NS	0.387***	0.016*
$C \times L$	6	0.003 NS	0.004 NS	0.0002 NS
Within	180	0.056	0.059	0.004
Total	191			
Alkanoic acids				
Concentration (C)	2	0.043 NS	0.173 NS	0.811***
Chain length (L)	3	0.013 NS	0.134 NS	0.156***
$C \times L$	6	0.006 NS	0.012 NS	0.001 NS
Within	180	0.019	0.191	0.015
Total	191			

[a] *, **, *** = significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

TABLE 4. MEAN SQUARES FROM ANALYSES OF VARIANCE OF CONCENTRATION AND CHAIN-LENGTH EFFECTS ON GERMINATION OF ONION, CARROT, AND TOMATO SEEDS EXPOSED (72 HOURS) PLUS 96-HOUR RECOVERY) TO 6.9, 20.8, OR 34.4 μM C_6–C_9 ALKANES, ALKANOLS, ALKANALS, ALKENALS, ALKANONES, AND ALKANOIC ACIDS

Source	*dF*	Mean squares		
		Onion	Carrot	Tomato
Alkanes				
Concentration (*C*)	2	0.051 NS[a]	0.007 NS	0.005 NS
Chain length (*L*)	3	0.019 NS	0.022***	0.0008 NS
C × *L*	6	0.002 NS	0.002 NS	0.0003 NS
Within	180	0.197	0.003	0.0005
Total	191			
2-Alkanols				
Concentration (*C*)	2	0.037 NS	0.170*	0.071*
Chain length (*L*)	3	0.037 NS	0.165*	0.061*
C × *L*	6	0.002 NS	0.003 NS	0.001 NS
Within	180	0.031	0.049	0.022
Total	191			
Alkanals				
Concentration (*C*)	2	0.254*	0.829***	0.043*
Chain length (*L*)	3	0.062 NS	0.089 NS	0.015 NS
C × *L*	6	0.005 NS	0.005 NS	0.001 NS
Within	180	0.080	0.084	0.011
Total	191			
2-Alkenals				
Concentration (*C*)	2	2.772***	2.914***	1.935**
Chain length (*L*)	3	2.282***	3.432***	0.585 NS
C × *L*	6	0.017 NS	0.035 NS	0.018 NS
Within	180	0.267	0.563	0.289
Total	191			
2-Alkanones				
Concentration (*C*)	2	2.237***	0.263*	0.847**
Chain length (*L*)	3	0.195 NS	0.145 NS	0.116 NS
C × *L*	6	0.012 NS	0.005 NS	0.011 NS
Within	180	0.197	0.081	0.182
Total	191			
Alkanoic acids				
Concentration (*C*)	2	0.034 NS	0.006 NS	0.028 NS
Chain length (*L*)	3	0.016 NS	0.003 NS	0.003 NS
C × *L*	6	0.001 NS	0.001 NS	0.001 NS
Within	180	0.012	0.003	0.013
Total	191			

[a] *, **, *** = significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

length when the test volatile type had been *n*-alkane or *n*-alkanoic acid. Volatile concentration effects persisted in carrot seeds exposed to 2-alkanols, alkanals, 2-alkenals, and 2-alkanones. Residual chain-length effects were observed in carrot responses after treatment with *n*-alkanes, 2-alkanols, and 2-alkenals.

Effects of Volatile Exposure Duration and Concentration on Onion Seeds. After an exposure of 72 hr, all concentrations of all the test compounds inhibited onion germination, compared to the deionized water control (Table 5). The two unsaturated C_7 aldehydes were the most inhibitory, followed by (*E*)-2-octenal. Regression analyses of these three-day data showed that, when the data for the water control were included, the concentration dependence of the effects of each of the test volatiles had a significant linear component. However, flat responses to many of the compounds over the assay concentration range used contributed to significant regression curve deviations from the linear. Simple linear concentration responses were observed only in onion seeds exposed to *n*-hexane, *n*-heptane, 2-hexanone, 2-heptanone, 2-octanone, and *n*-heptanoic acid. The steepest linear regression slopes were observed when the C_6, C_7, and C_8 2-alkenals, (*E*,*E*)-2,4-heptadienal, and *n*-heptanal were the test compounds. Significant concentration effects were observed in the three- + four-day data only when (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*,*E*)-2,4-heptadienal, 2-heptanone, 2-octanone, and 2-nonanone were the test volatiles. Compared to the three-day concentration-dependent data, there were no significant changes in slope in the regression curves obtained with these compounds and this "recovery" treatment protocol (data not shown).

When the volatile source was removed after the three-day exposure and seed germination evaluated under an exhausting safety hood, an additional four-day incubation allowed onion seeds exposed to 2-nonanol and 2-heptanone to germinate as fully as the water controls. Seeds exposed to (*E*)-2-hexenal, (*E*)-2-heptenal, and (*E*,*E*)-2,4-heptadienal did not germinate further after the volatile source was removed. Germination of onion seeds initially treated with 34.4 μM 2-hexanone was significantly higher than that of the controls. Furthermore, onion seeds exposed continuously to 34.4 μM *n*-hexane, *n*-nonane, 2-nonanol, and the *n*-alkanoic acids, with the exception of *n*-nonanoic, germinated as well as the controls. Over all time and concentration treatments of onion seeds, (*E*)-2-heptenal and (*E*,*E*)-2,4-heptadienal were the most inhibitory, *n*-hexane the least.

Effects of Volatile Exposure Duration and Concentration on Carrot Seed Germination. Carrot seeds were less sensitive than those of onion to low concentrations of the test volatiles (Table 6). At 6.9 μM, the four *n*-alkanes, the C_6–C_8 2-alkanals, (*E*)-2-nonenal, (*E*,*E*)-2,4-nonadienal, 2-hexanone, 2-octanone, and all *n*-alkanoic acids, except *n*-hexanoic, had no significant effect on carrot seed germination after a 72-hr incubation. When the concentrations of the individual test compounds were increased to 34.4 μM, only *n*-octane,

TABLE 5. EFFECTS OF VOLATILE EXPOSURE DURATION AND CONCENTRATION ON ONION SEED GERMINATION

	Exposure time				
	3 days			3 + 4 days	7 days
	Germination (%)[a]				
	6.9 μM	20.8 μM	34.4 μM	34.4 μM	34.4 μM
n-Alkanes					
Hexane	39.1 g	34.9 j	28.9 j	57.7 d	57.1 i
Heptane	33.0 fg	27.6 ij	15.8 fg	45.2 c	51.0 gh
Octane	26.2 d–f	27.3 ij	26.3 ij	57.2 d	57.3 i
Nonane	20.1 b–e	20.1 f–i	18.1 gh	52.9 d	40.1 e
2-Alkanols					
2-Hexanol	16.5 bc	14.8 d–h	10.3 d–f	48.1 c	53.0 h
2-Heptanol	14.2 b	4.8 ab	4.2 bc	59.9 d	40.7 ef
2-Octanol	16.4 bc	6.8 bc	4.8 bc	43.1 c	18.7 bc
2-Nonanol	19.8 b–e	19.9 f–i	19.8 g–i	62.6 de	63.1 i
n-Alkanals					
Hexanal	25.2 d–f	22.3 g–i	5.9 cd	26.5 b	18.5 bc
Heptanal	16.4 bc	4.1 ab	1.4 ab	41.1 c	34.9 de
Octanal	20.0 b–e	14.1 d–g	1.4 ab	47.6 c	24.9 c
Nonanal	19.5 b–e	11.3 c–e	1.7 ab	50.1 c	42.0 e–g
2-Alkenals					
(*E*)-2-Hexenal	16.5 bc	4.8 ab	0.0 a	0.2 a	0.0 a
(*E*)-2-Heptenal	2.5 a	1.0 a	0.0 a	0.5 a	0.9 a
(*E*)-2-Octenal	6.7 a	1.3 a	0.5 a	29.6 b	23.9 bc
(*E*)-2-Nonenal	25.1 d–f	19.1 e–i	12.1 gh	54.1 d	53.7 h
(*E*, *E*)-2,4-Heptadienal	2.8 a	0.7 a	0.2 a	0.9 a	0.9 a
(*E*, *E*)-2,4-Nonadienal	9.5 b	14.3 b–d	7.5 c–e	50.0 c	50.5 f–h
2-Alkanones					
2-Hexanone	28.6 f	13.6 c–g	11.4 ef	78.1 f	30.6 cd
2-Heptanone	24.2 c–f	9.4 b–d	3.0 bc	74.1 ef	39.1 de
2-Octanone	27.8 ef	8.4 b–d	4.1 bc	61.4 d	17.9 b
2-Nonanone	18.3 b–d	13.1 c–f	12.1 ef	48.1 c	34.8 de
n-Alkanoic acids					
Hexanoic	24.5 d–f	20.8 ij	24.5 h–j	52.3 d	63.4 i
Heptanoic	29.4 fg	20.6 f–i	16.7 g	53.7 d	66.7 i
Octanoic	24.1 c–f	23.5 h–i	19.0 g–i	54.4 d	61.7 i
Nonanoic	25.3 d–f	23.9 h–j	24.5 h–j	50.9 c	55.8 h
Control	53.4 h	53.4 k	53.4 k	67.8 e	67.8 i

[a] Percentages are the means of 16 replications. Values in columns followed by the same letter and associated with a given exposure time are not significantly different ($P = 0.01$). Standard errors of means were $<2.5\%$.

TABLE 6. EFFECTS OF VOLATILE EXPOSURE DURATION AND CONCENTRATION ON CARROT SEEDS GERMINATION

	Exposure time				
	3 days			3 + 4 days	7 days
	Germination (%)[a]				
	6.9 μM	20.8 μM	34.4 μM	34.4 μM	34.4 μM
n-Alkanes					
Hexane	39.5 h	40.0 h	19.9 e-g	81.8 jk	76.4 g
Heptane	35.2 gh	38.3 gh	16.9 de	74.2 g-k	75.1 fg
Octane	38.5 h	34.8 f-h	26.4 g-i	75.7 g-k	79.5 g
Nonane	32.3 gh	29.5 e-h	27.3 hi	81.3 j-k	83.5 g
2-Alkanols					
2-Hexanol	15.8 b-d	15.1 cd	3.9 c	78.9 i-k	58.5 cd
2-Heptanol	14.9 bc	0.0 a	0.2 a	61.5 ef	1.4 a
2-Octanol	15.5 b-d	0.2 a	0.2 a	40.8 c	0.0 a
2-Nonanol	11.0 b	11.2 bc	8.3 c	68.6 f-i	51.9 c
n-Alkanals					
Hexanal	37.8 h	18.1 c-e	5.2 c	66.6 f-h	76.3 g
Heptanal	21.6 c-f	10.7 bc	0.7 ab	48.8 cd	0.2 a
Octanal	26.2 e-g	1.1 a	0.2 ab	49.7 cd	0.0 a
Nonanal	16.0 b-d	4.4 ab	1.2 a-c	28.8 b	1.5 a
2-Alkenals					
(*E*)-2Hexenal	3.6 a	0.0 a	0.0 a	0.0 a	0.0 a
(*E*)-2-Heptenal	3.1 a	0.0 a	0.0 a	0.0 a	0.9 a
(*E*)-2-Octenal	2.0 a	1.0 a	0.5 a	28.6 b	1.5 a
(*E*)-2-Nonenal	32.3 gh	21.7 c-f	18.5 ef	75.3 g-k	79.4 g
(*E*, *E*)-2,4-Heptadienal	2.3 a	0.2 a	0.0 a	0.2 a	0.9 a
(*E*, *E*)-2,4-Nonadienal	29.6 e-h	24.5 d-g	5.6 c	70.6 f-j	67.4 ef
2-Alkanones					
2-Hexanone	33.6 gh	14.6 cd	12.0 cd	77.3 h-k	66.2 de
2-Heptanone	19.7 c-e	1.0 a	0.4 a	54.1 de	8.9 b
2-Octanone	24.2 d-g	0.0 a	0.0 a	42.5 c	0.0 a
2-Nonanone	15.9 b-d	0.5 a	0.7 ab	61.5 ef	1.2 a
n-Alkanoic acids					
Hexanoic	14.6 bc	19.4 c-e	23.5 e-i	86.6 k	75.8 fg
Heptanoic	25.7 e-g	24.3 d-g	21.3 e-h	81.6 jk	75.7 fg
Octanoic	28.9 e-hf	29.4 e-h	3.5 bc	79.5 i-k	81.6 g
Nonanoic	30.8 f-h	30.3 e-h	24.3 f-i	81.4 jk	76.2 g
Control	30.6 f-h	30.6 e-g	30.6 i	64.4 e-g	64.4 de

[a] Percentages are the means of 16 replications. Values in columns followed by the same letter and associated with a given exposure time are not significantly different ($P = 0.01$). Standard errors of means were $< 1.5\%$.

n-nonane, *n*-hexanoic acid, and *n*-nonanoic acid had no significant inhibitory effect. An increase in germination, compared to that of the water control, was observed in carrot seeds exposed to 20.8 μM *n*-hexane. At 34.4 μM, 2-heptanol, 2-octanol, *n*-heptanal, *n*-octanal, *n*-nonanal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*,*E*)-2,4-heptadienal, 2-heptanone, 2-octanone, and 2-nonanone prevented carrot seed germination. After 72 hr, (*E*,*E*)-2,4-heptadienal, (*E*)-2-heptenal, (*E*)-2-octenal, and (*E*)-2-hexenal were the most inhibitory of carrot seed germination, based on data from all three test concentrations. The three-day concentration-dependent responses of carrot seeds to all test compounds, except *n*-hexane, *n*-octane, *n*-nonane, *n*-hexanoic acid, *n*-heptanoic acid, and *n*-nonanoic acid, had significant linear components. As in the case of onion, flat concentration responses caused significant deviations from linear in the regression analyses. This deviation was nonsignificant in carrot seed assays of *n*-nonane, nonanal, 2-nonenal, 2-nonanol, *n*-nonanoic acid, and *n*-heptanoic acid.

After the volatile source was removed and the carrot seeds were incubated an additional four days, the initial three-day exposure to 34.4 μM (*E*)-2-hexenal, (*E*)-2-heptenal, and (*E*,*E*)-2,4-heptadienal proved sufficient to prevent germination. Residual, but lesser, inhibition was also observed in seeds exposed to 2-octanol, *n*-heptanal, *n*-octanal, *n*-nonanal, (*E*)-2-octenal, and 2-octanone. Initial exposure to 34.4 μM *n*-hexane, *n*-nonane, 2-hexanol, 2-hexanone, and all four *n*-alkanoic acids increased carrot seed germination over that observed in the water controls. Significant concentration-dependent effects persisted in carrot seeds exposed to (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*,*E*)-2,4-heptadienal, and 2-octanone (data not shown). A seven-day continuous exposure to 34.4 μM 2-hepatanol, 2-octanol, *n*-heptanal, *n*-octanal, *n*-nonanal, the C_6–C_8 2-alkenals, (*E*,*E*)-2,4-heptadienal, 2-heptanone, 2-octanone, and 2-nonanone suppressed germination to less than 10% of that of the water controls. The same treatment with the four *n*-alkanes, *n*-hexanal, 2-nonenal, and the four *n*-alkanoic acids increased carrot seed germination significantly in comparison to the controls. Overall, (*E*,*E*)-2,4-heptadienal, (*E*)-2-heptenal, and (*E*)-2-hexenal were the most inhibitory of carrot seed germination.

Effects of Volatile Exposure Duration and Concentration on Tomato Seed Germination. Tomato seed germination was very sensitive to a 72-hr exposure to low concentrations (6.9 μM) of 2-alkanols and 2-alkanones (Table 7). At higher concentration, *n*-octanal, *n*-nonanal, (*E*)-2-hexenal, (*E*)-2-heptenal, and (*E*,*E*)-2,4-heptadienal were also inhibitory. When the volatile concentration was 34.4 μM, the four 2-alkanols, alkanals, 2-alkanones, the C_6–C_8 2-alkenals, and (*E*,*E*)-2,4-heptadienal all prevented tomato seed germination. With exception of *n*-nonane, all the volatiles tested inhibited tomato seed germination at the end of the three-day exposure. The three-day concentration-dependent responses of tomato seed had significant linear components in all cases, except when

TABLE 7. EFFECTS OF VOLATILE EXPOSURE DURATION AND CONCENTRATION ON TOMATO SEED GERMINATION

	Exposure time				
	3 days			3 + 4 days	7 days
	Germination (%)[a]				
	6.9 μM	20.8 μM	34.4 μM	34.4 μM	34.4 μM
n-Alkanes					
Hexane	59.2 f–i	43.4 fg	37.7 f	95.5 m	95.6 g
Heptane	39.6 c–g	38.4 e–g	24.6 cd	93.1 k–m	95.2 gh
Octane	48.2 d–i	23.8 d–f	11.1 b	95.9 m	96.5 g
Nonane	60.3 f–i	58.8 g	65.2 h	96.8 m	94.3 g
2-Alkanols					
2-Hexanol	3.6 ac	0.0 a	0.0 a	95.9 m	4.7 b
2-Heptanol	2.3 a	0.0 a	0.0 a	68.5 fg	0.2 a
2-Octanol	2.5 ac	0.0 a	0.0 a	80.3 hi	0.0 ac
2-Nonanol	0.0 a	0.0 a	0.0 a	93.4 lm	86.9 g
n-Alkanals					
Hexanal	73.7 i	45.1 fg	0.0 a	94.9 lm	74.9 f
Heptanal	37.7 c–f	11.9 cd	0.0 a	85.8 i–l	27.8 e
Octanal	39.3 c–g	0.0 a	0.0 a	83.7 h–k	11.8 c
Nonanal	43.4 c–h	3.7 a–c	0.0 a	82.7 h–j	0.5 a
2-Alkenals					
(*E*)-2-Hexenal	67.0 g–i	0.0 a	0.0 a	25.1 bc	13.1 cd
(*E*)-2-Heptenal	21.3 bc	0.0 a	0.0 a	20.3 ab	0.2 a
(*E*)-2-Octenal	36.1 c–f	10.7 b–da	0.2 a	55.8 de	0.5 a
(*E*)-2-Nonenal	68.2 hi	50.7 g	21.1 c	93.7 lm	94.3 g
(*E*, *E*)-2,4-Heptadienal	28.1 c–e	0.2 ab	0.0 a	20.1 a	0.0 a
(*E*, *E*)-2,4-Nonadienal	57.1 f–i	18.5 de	8.7 b	96.8 m	85.9 fg
2-Alkanones					
2-Hexanone	6.5 ab	0.7 a–c	0.5 a	75.0 gh	19.1 d
2-Heptanone	2.8 a	0.0 a	0.0 a	62.1 ef	5.0 b
2-Octanone	3.2 a	0.0 a	0.2 a	27.9 c	0.0 a
2-Nonanone	2.1 a	0.0 a	0.0 a	49.7 d	0.0 a
n-Alkanoic acids					
Hexanoic	70.5 hi	65.8 g	45.3 fg	94.0 lm	95.6 g
Heptanoic	52.4 e–i	51.3 g	28.7 de	93.0 k–m	96.8 g
Octanoic	69.7 hi	55.6 g	37.1 ef	79.9 hi	94.0 g
Nonanoic	77.6 i	57.1 g	52.9 g	90.5 i–m	93.0 g
Control	65.5 g–i	65.5 g	65.5 h	97.8 m	91.1 g

[a] Percentages are the means of 16 replications. Values in columns followed by the same letter and associated with a given exposure time are not significantly different ($P = 0.01$). Standard errors of means were $< 1.5\%$.

n-nonane was the test substance. Significant deviations from linear were observed in the data obtained in assays of all four 2-alkanols, *n*-alkanals, 2-alkanones, (*E*)-2-hexenal, (*E*)-2-heptenal, and (*E*)-2-nonenal, both dienals, and *n*-hexanoic, *n*-heptanoic, and *n*-nonanoic acids. Unlike the germination of onion and carrot, tomato seeds were inhibited by the highest concentration of the *n*-alkanoic acids. Over all treatment concentrations, 2-nonanol, 2-nonanone, 2-heptanol, 2-octanol, 2-heptanone, 2-octanone, 2-hexanol, and 2-hexanone were the most inhibitory of tomato seed germination (in decreasing order of inhibitory activity).

Tomato seeds exposed for three days to the four *n*-alkanes, 2-hexanol, 2-nonanol, *n*-hexanal, (*E*)-2-nonenal, (*E*,*E*)-2,4-nonadienal, and the *n*-alkanoic acids, with the exception of *n*-octanoic, all germinated to the same degree as the controls when the volatile source was removed and the seeds incubated an additional four days. After the four-day recovery period, the only residual concentration-dependent effects were observed in tomato seeds initially exposed to (*E*)-2-heptenal, (*E*,*E*)-2,4-heptadienal, and 2-octanone (data not shown). Tomato seeds differed from the other assay seeds in "recovery" capacity, e.g., seeds completely inhibited after a three-day exposure to the *n*-alkanals germinated almost as fully as the water controls following the four-day "recovery" period. Continuous exposure for seven days to 2-heptanol, 2-octanol, *n*-nonanal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*,*E*)-2,4-heptadienal, 2-heptanone, 2-octanone, and 2-nonanone prevented tomato seed germination. Over all concentration and time treatments, the greatest inhibition responses were observed in tomato seeds exposed to 2-octanone, (*E*)-2-heptenal, 2-nonanone, (*E*,*E*)-2,4-heptadienal, 2-heptanone, 2-heptanol, 2-octanol, 2-hexanone, and 2-hexanol.

DISCUSSION

The C_6–C_9 volatiles examined in this study are released by the residues of roots, leaves, stems, flowers, and fruits of a large variety of plant species (Table 8). All 26 compounds have been reported to occur in several plants, with the exception of *n*-octanoic acid (caprylic acid). Both monocots (e.g., rice, maize, and oat) and dicots (e.g., *Amaranthus* spp., leguminous cover crops, and tomato) release these compounds when subjected to extraction and isolation procedures ranging from passive adsorption of headspace components to steam and vacuum distillation (references cited in Table 8).

The most commonly reported classes of compounds were the alkanals, followed by the alkenals and 2-alkanones. The *n*-alkanes and 2-alkanols were less commonly reported, and the *n*-alkanoic acids were the least widely distributed. Compounds containing six and seven carbon atoms were the most frequently observed and those containing eight carbons, the least. Two alkanals,

TABLE 8. C_6–C_9 BIOACTIVE VOLATILES RELEASED BY PLANT RESIDUES

Compound	Source residue	Reference[a]
n-Alkanes		
n-Hexane	Leaf, stem	4
	Flower	26
n-Heptane	Root	18
	Leaf, stem	1, 4, 18, 19
	Flower	18
n-Octane	Root	18
	Leaf, stem	1, 4, 18, 19
	Fruit	30
n-Nonane	Leaf, stem	1, 4, 19
2-Alkanols		
2-Hexanol	Leaf, stem	4
	Flower	11, 21
2-Heptanol	Root	18
	Leaf, stem	3, 18, 22
	Flower	3, 11, 18, 19, 21
	Fruit	9, 18
2-Octanol	Flower	11, 21
	Fruit	9
2-Nonanol	Leaf, stem	32
	Flower	11, 21
	Fruit	9
n-Alkanals		
n Hexanal	Root	18
	Leaf, stem	3, 10, 14, 18, 22, 25, 27, 28,
	Flower	29, 31, 33
	Fruit	3, 11, 18, 19, 20, 24, 26, 34
		5, 7, 8, 9, 13, 29, 30
n-Heptanal	Root	6
	Leaf, stem	10, 13, 19, 22, 25
	Flower	11, 24, 34
	Fruit	5, 8, 9, 23
n-Octanal	Root	6
	Leaf, stem	10, 13
	Flower	11, 34
	Fruit	5, 9
n-Nonanal	Root	6
	Leaf, stem	1, 4, 10, 12, 16, 22, 25, 28
	Flower	11, 19, 24, 35
	Fruit	5, 9, 13
2-Alkenals		
(*E*)-2-Hexenal	Leaf, stem	4, 10, 14, 15, 16, 22, 25, 27
	Flower	4, 12, 15, 17, 20, 21, 24, 26
	Fruit	5, 7, 8, 9, 13, 29, 33
(*E*)-2-Heptenal	Flower	11, 21
	Fruit	5, 8, 9, 33

TABLE 8. *Continued*

Compound	Source residue	Reference[a]
2-Alkenals (*continued*)		
(*E*)-2-Octenal	Leaf, stem	1, 25
	Flower	11, 19, 24
	Fruit	5, 8, 9, 12
(*E*)-2-Nonenal	Root	6
	Leaf, stem	1, 8, 12, 14
	Flower	11, 13, 19, 24
	Fruit	5, 8, 9, 12
Alkadienals		
(*E*, *E*)-2,4-Heptadienal	Leaf, stem	22, 25
	Flower	11, 12, 19
	Fruit	5, 8, 9
(*E*, *E*)-2,4-Nonadienal	Leaf, stem	22, 25
	Flower	11, 19, 25
	Fruit	5, 8, 9
	Fruit	7, 34
2-Alkanones		
2-Hexanone	Leaf, stem	2, 15
	Flower	2, 15
	Fruit	2, 5, 8, 9
2-Heptanone	Root	4, 18
	Leaf, stem	2, 4, 14, 15, 18, 19
	Flowers	2, 15, 18
	Fruit	2, 5, 7, 9, 18, 23, 33
2-Octanone	Root	18
	Leaf, stem	2
	Fruit	2, 5, 9
2-Nonanone	Root	18
	Leaf, stem	2, 4, 19
	Flower	2, 11, 21
	Fruit	2, 5, 9
Alkanoic acids		
n-Hexanoic acid	Flower	34
n-Heptanoic-acid	Flower	34
	Fruit	23
n-Octanoic acid	None reported	
n-Nonanoic acid	Flower	34

[a]Reference key: (1) Bestmann et al., 1988; (2) Bradow and Connick, 1988a; (3) Bradow and Connick, 1988b; (4) Bradow and Connick, 1990; (5) Bullard and Holguin, 1977; (6) Buttery et al., 1968; (7) Buttery et al., 1969; (8) Buttery et al., 1971; (9) Buttery et al., 1978; (10) Buttery and Kamm, 1980; (11) Buttery et al., 1980; (12) Buttery et al., 1982a; (13) Buttery et al., 1982b; (14) Buttery and Ling, 1984; (15) Buttery et al., 1984; (16) Buttery et al., 1985; (17) Buttery, et al., 1986; (18) Connick et al., 1987; (19) Connick et al., 1989; (20) Etievant et al., 1984; (21) Flath et al., 1978; (22) Flath et al., 1984; (23) Girardon et al., 1985; (24) Hedin et al, 1975; (25) Hernandez et al., 1989; (26) Kumar and Motts, 1986; (27) Lundgren et al., 1985; (28) Lwande and Bentley, 1987; (29) MacLeod and Nussbaum, 1977; (30) MacLeod and de Tronconis, 1982; (31) MacLeod et al., 1985; (32) Morrison, et al., 1986; (33) Wu and Liou, 1988; (34) Yamaguchi and Shibamoto, 1980.

n-hexanal and *n*-nonanal, and (*E*)-2-hexenal were the most common volatile components listed in the literature cited in Table 8. The next most common compounds were 2-heptanone and (*E*)-2-nonenal. Distributions of the C_7 and C_9 alkadienals were similar to those of the corresponding 2-alkenals and considerably smaller than those of the analogous *n*-alkanals.

Techniques used for sampling, identification, and assay of bioactive volatiles associated with various plant tissues and residues differ according to the purpose of the study and the interests of the researchers. Therefore, the compounds listed in Table 8 cannot be categorized simply as plant residue emissions, food flavor components, or insect semiochemicals. Many of the compounds in the compilation were identified in steam and vacuum distillates (e.g., Bestmann et al., 1988; Buttery et al., 1971, 1978; Buttery and Kamm, 1980; Flath et al., 1984) or in extracts of headspace gases trapped in liquid nitrogen (Bullard and Holguin, 1977; Morrison et al., 1986). Vacuum steam distillation and Tenax solid adsorbent trapping methods have been compared in studies of plant volatile profiles (Buttery et al., 1982b, 1983). More recently, adsorption on Tenax has become the method of choice for trapping volatile emissions in studies of insect attractants (Buttery et al., 1983, 1984, 1985, 1986; Buttery and Ling, 1985; Lundgren et al., 1985; Lwande and Bentley, 1987; Hernandez et al., 1989; Connick and French, 1990) and allelochemicals and aroma components emitted by plant tissue (Connick et al., 1987, 1989; Lundgren et al., 1985; Bradow and Connick, 1990; Connick and French, 1990).

If the list of C_6–C_9 compounds in Table 8 were limited to only those volatiles identified after desorption from Tenax traps, it would include the four *n*-alkanes, 2-heptanol, *n*-hexanal, *n*-heptanal, *n*-nonanal, the four (*E*)-2-alkenals, (*E*,*E*)-2,4-heptadienal, and the four 2-alkanones (Buttery et al., 1982a,b, 1984, 1985, 1986; Buttery and Ling, 1984, 1985; Lundgren et al., 1985; Connick et al., 1987, 1989; Lwande and Bentley, 1987; Hernandez et al., 1989; Bradow and Connick, 1990). (*Z*,*Z*)-2,4-Nonadienal (but not the *E* geometric isomer tested here) was identified by headspace sampling and Tenax trapping (Hernandez et al., 1989). The relative gentleness of the Tenax trap procedure suggests that volatile profiles determined using this method most closely approximate the natural emissions of the source plant material. Just as the bioassay species responded differently to natural plant residue emissions (Bradow and Connick, 1987), onion, carrot, and tomato seeds differed in sensitivity to those C_6–C_9 volatile compounds. The observed species differences depended on incubation duration and recovery capacity following removal of the volatile. The degree of germination inhibition, compared to the deionized water controls, summed across concentrations for each assay seed, allows comparisons of both species sensitivity and volatile activity. After a 72-hr incubation, (*E*)-2-heptenal and (*E*,*E*)-2,4-heptadienal were the most inhibitory of onion and carrot

seed germination. In contrast, after the same exposure period, tomato seeds were more strongly inhibited by 2-hexanol, 2-heptanol, 2-heptanone, 2-octanol, 2-octanone, 2-nonanol, and 2-nonanone, these compounds having approximately the same inhibitory activities.

Similar examinations of the cumulative inhibition of germination in the three assay species subjected to the three- + four-day "recovery" exposure protocol indicated that (*E*)-2-hexenal, (*E*)-2-heptenal, and (*E*,*E*)-2,4-heptadienal had the greatest residual effect on onion and carrot seed germination after the 96-hr recovery period. Much less residual inhibition was observed in tomato seeds, and these three compounds, plus 2-octanone, most effectively prevented further germination. These three aldehydes were also most effective in preventing onion seed germination during the seven-day incubation. After the seven-day exposure, (*E*)-2-hexenal, 2-heptanol, and three C_7 aldehydes, 2-octanol, *n*-octanal, (*E*)-2-octenal, 2-octanone, 2-nonanone, and *n*-nonanal caused the most inhibition of carrot seed germination. A seven-day exposure to 2-hexanol, 2-heptanol, (*E*)-heptenal, (*E*,*E*)-2,4-heptadienal, 2-heptanone, 2-octanol, (*E*)-octenal, 2-octanone, *n*-nonanal, and 2-nonanone essentially prevented tomato seed germination.

Over all three seed species and exposure periods, (*E*)-2-heptenal and (*E*,*E*)-2,4-heptadienal were the most inhibitory compounds tested. Based on germination inhibition in onion, carrot, and tomato, the activity series of the six compound classes was: (*E*)-2-alkenals > 2-alkanones ≥ *n*-alkanals ≥ 2-alkanols >> *n*-alkanoic acids ≥ *n*-alkanes. The overall inhibitory activity of C_7 and C_8 compounds was approximately equal. The C_6 compounds were less inhibitory than compounds with seven and eight carbons, but more inhibitory than those with nine, despite the marked sensitivity of tomato seeds to several C_9 compounds.

Similar decreases in bioactivity with increasing or decreasing chain length have been observed in electroantennogram (EAG) responses of insects to plant volatiles (Light et al., 1988; Ramachandran et al., 1990). The greatest response amplitude of Mediterranean fruit fly was observed when the plant volatile (e.g., 1-alkanol, *n*-alkanal, *n*-alkanoic acid) contained six carbons atoms (Light et al., 1988). Rice leaf folder EAG responses were greatest to C_7 *n*-alkanals, and C_8 alkenals (Ramachandran et al., 1990).

The C_6–C_9 compounds examined here have wide ecological significance, retarding seed germination, attracting insects (Visser, 1979, 1983; Light et al., 1988; Ramachandran et al., 1990), and retarding seedling root elongation (Bradow and Connick, 1988c). These biologically active compounds can have very significant effects on crop yield and quality whenever tillage practices that incorporate large amounts of green cover crop or other plant residues into the root zone are employed.

Acknowledgments—The author wishes to thank Mary P. Lovisa for her invaluable technical assistance and Mr. William J. Connick, Jr., and Dr. Richard C. French for their excellent suggestions.

REFERENCES

BESTMANN, H.-J., CLASSEN, B., KOBOLD, U., VOSTORWSKY, O., KLINGAUF, F., and STEIN, U. 1988. Steam volatile constituents from leaves of *Rhus typhina*. *Phytochemistry* 27:85–90.

BRADOW, J.M., and CONNICK, W.J., JR. 1987. Allelochemicals from palmer amaranth, *Amaranthus palmeri* S. Wats. *J. Chem. Ecol.* 13:185–202.

BRADOW, J.M., and CONNICK, W.J., JR. 1988a. Volatile methyl ketone seed-germination inhibitors from *Amaranthus palmeri* S. Wats. residues. *J. Chem. Ecol.* 14:1617–1631.

BRADOW, J.M., and CONNICK, W.J., JR. 1988b. Seed-germination inhibition by volatile alcohols and other compounds associated with *Amaranthus palmeri* residues. *J. Chem. Ecol.* 14:1633–1648.

BRADOW, J.M., and CONNICK, W.J., JR. 1988c. Inhibition of cotton seedling root growth by rhizosphere volatiles Proceedings, Beltwide Cotton Production Research Conference, pp. 90–91.

BRADOW, J.M., and CONNICK, W.J., JR. 1990. Volatile seed germination inhibitors from plants residue. *J. Chem. Ecol.* 16:645–666.

BULLARD, R.W., and HOLGUIN, G. 1977. Volatile components of unprocessed rice (*Oryza sativa* L.). *J. Agric. Food Chem.* 25:99–103.

BUNTLEY, G.J. 1986. Tennessee no-tillage update, pp. 100–102, *in* R.E. Phillips (ed.). Proceedings, Southern Region No-Tillage Conference, Southern Region Series Bulletin 319. University of Kentucky, Lexington, Kentucky.

BUTTERY, R.G., and KAMM, J.A. 1980. Volatile components of alfalfa: Possible insect host plant attractants. *J. Agric. Food Chem.* 28:978–981.

BUTTERY, R.G., and LING, L.C. 1984. Corn leaf volatiles: Identification using Tenax trapping for possible insect attractants. *J. Agric. Food Chem.* 32:1104–1106.

BUTTERY, R.G., and LING, L.C. 1985. Volatile components of corn roots: Possible insect attractants. *J. Agric. Food Chem.* 33:772–774.

BUTTERY, R.G., SEIFERT, R.M., GUADAGNI, D.G., BLACK, D.R., and LING, L.C. 1968. Characterization of some volatile constituents of carrots. *J. Agric. Food Chem.* 16:1009–1015.

BUTTERY, R.G., SEIFERT, R.M., GUADAGNI, D.G., and LING, L.C. 1969. Characterization of some volatile constituents of bell peppers. *J. Agric. Food Chem.* 17:1322–1327.

BUTTERY, R.G., SEIFERT, R.M., GUADAGNI, D.G., and LING, L.C. 1971. Characterization of additional volatile components of tomato. *J. Agric. Food Chem.* 19:1322–1327.

BUTTERY, R.G., LING, L.C., and CHAN, B.G. 1978. Volatiles of corn kernels and husks: Possible corn ear worm attractants. *J. Agric. Food Chem.* 26:866–869.

BUTTERY, R.G., LING, L., and TERANISHI, R. 1980. Volatiles from corn tassels: Possible corn earworm attractants. *J. Agric. Food Chem.* 28:771–774.

BUTTERY, R.G., LING, L.C., and WELLSO, S.G. 1982a. Oat leaf volatiles: Possible insect attractants. *J. Agric. Food Chem.* 30:791–792.

BUTTERY, R.G., KAMM, J.A., and LING, L.C. 1982b. Volatile components of alfalfa flowers and pods. *J. Agric. Food Chem.* 30:739–742.

BUTTERY, R.G., LING, L.C., TERANISHI, R., and MON, T.R. 1983. Insect attractants: Volatiles of hydrolyzed protein insect baits. *J. Agric. Food Chem.* 31:689–692.

BUTTERY, R.G., KAMM, J.A., and LING, L.C. 1984. Volatile components of red clover leaves, flowers, and seed pods: Possible insect attractants. *J. Agric. Food Chem.* 32:254–256.

BUTTERY, R.G., XU, C.-J., and LING, L.C. 1985. Volatile components of wheat leaves (and stems): Possible insect attractants. *J. Agric. Food Chem.* 33:115–117.

BUTTERY, R.G., MADDOX, D.M., LIGHT, D.M., and LING, L.C. 1986. Volatile components of yellow starthistle. *J. Agric. Food Chem.* 34:786–787.

CONNICK, W.J., JR., and FRENCH, R.C. 1990. Volatiles emitted during the sexual stage of the Canada thistle rust fungus and by the thistle flower. *J. Agric. Food Chem.* 39:185–188.

CONNICK, W.J., JR., BRADOW, J.M., LEGENDRE, M.G., VAIL, S.L., and MENGES, R.M. 1987. Identification of volatile allelochemicals from *Amaranthus palmeri* S. Wats. *J. Chem. Ecol.* 13:463–472.

CONNICK, W.J., JR., BRADOW, J.M., and LEGENDRE, M.G. 1989. Identification and bioactivity of volatile allelochemicals from amaranth residues. *J. Agric. Food Chem.* 37:792–796.

DABNEY, S.M., BOETHAL, D.J., BOQUET, D.J., GRIFFIN, J.L., HALLMARK, W.B., HUTCHINSON, R.L., MASON, L.F., and RABB, J.L. 1986. Update of no-tillage in Louisiana, pp. 67–72, *in* R.E. Phillips (ed.). Proceedings, Southern Region No-Tillage Conference, Southern Region Series Bulletin 319. University of Kentucky, Lexington, Kentucky.

ETIEVANT, P.X., AZAR, M., PHAM-DELEGUE, M.H., and MASSON, C.J. 1984. Isolation and identification of volatile constituents of sunflowers (*Helianthus annuus* L.). *J. Agric. Food Chem.* 32:503–509.

FLATH, R.A., FORREY, R.R., JOHN, J.O. and CHAN, B.G. 1978. Volatile components of corn silk (*Zea mays* L.): Possible *Heliothis zea* (Boddie) attractants. *J. Agric. Food Chem.* 26:1290–1293.

FLATH, R.A., ALTIERI, M.A., and MON, T.R. 1984. Volatile constituents of *Amaranthus retroflexus* L. *J. Agric. Food Chem.* 32:92–94.

FRENCH, R.C. 1985. The bioregulatory action of flavor compounds on fungal spores and other propagules. *Annu. Rev. Phytopathol.* 23:173–179.

FRENCH, R.C., and LEATHER, G.R. 1979. Screening of nonanal and related volatile flavor compounds on the germination of 18 species of weed seed. *J. Agric. Food Chem.* 27:828–832.

FRENCH, R.C., GRAHAM, C.L., GALE, A.W., and LONG, R.K. 1977. Structural and exposure time requirements for chemical stimulation of germination of uredospores of *Uromyces phaseoli*. *J. Agric. Food Chem.* 25:84–88.

GIRARDON, P., BESSIERE, J.M., BACCOU, J.C., and SAUVAIRE, Y. 1985. Volatile constituents of fenugreek seeds. *Planta Med. J. Med. Plant Res.* 00:533–534.

HEDIN, P.A., THOMPSON, A.C., and GUELDNER, R.C. 1975. A survey of the volatile constituents of cotton lint and waste with regard to byssinosis. *J. Agric. Food Chem.* 23:698–703.

HERNANDEZ, H.P., HSIEH, T.C.-Y., SMITH, C.M., and FISCHER, N.H. 1989. Foliage volatiles of two rice cultivars. *Phytochemistry* 28:2959–2962.

JOHNSON, A.E., NURSTEN, H.E., and WILLIAMS, A.A. 1971a. Vegetable volatiles: A survey of components identified—Part I. *Chem. Ind.* 00:556–565.

JOHNSON, A.E., NURSTEN, H.E., and WILLIAMS, A.A. 1971b. Vegetable volatiles: A survey of components identified—Part II. *Chem. Ind.* 00:1212–1265.

KUMAR, N., and MOTTS, M.G. 1986. Volatile constituents of peony flowers. *Phytochemistry* 25:250–253.

LIGHT, D.M., JANG, E.B., and DICKENS, J.C. 1988. Electroantennogram responses of the Mediterranean fruit fly, *Certitis capitata*, to a spectrum of plant volatiles. *J. Chem. Ecol.* 14:159–180.

LUNDGREN, L., NORELIUS, G., and STENHAGEN, G. 1985. Leaf volatiles from some wild tomato species. *Nord. J. Bot.* 5:315–320.

LWANDE, W., and BENTLEY, M.D. 1987. Volatiles of *Sorghum bicolor* seedlings. *J. Nat. Prod.* 50:950–952.

MACLEOD, A.J., and NUSSBAUM, M.L. 1977. The effects of different horticultural practices on the chemical flavor components of some cabbage cultivars. *Phytochemistry* 16:861–865.

MACLEOD, A.J., and DE TRONCONIS, N.G. 1982. Volatile flavor components of guava. *Phytochemistry* 21:1339–1342.

MACLEOD, A.J., SNYDER, C.H., and SUBRAMANIAN, G. 1985. Volatile aroma constituents of parsley leaves. *Phytochemistry* 24:2623–2627.

MENGES, R.M. 1987. Allelopathic effects of Palmer amaranth (*Amaranthus palmeri*) and other plant residues in soil. *Weed Sci.* 35:339–347.

MORRISON, W.H., III, HORVAT, R.J., and BURNS, J.C. 1986. GLC-MS analysis of the volatile constituents of *Panicum* sp. *J. Agric. Food Chem.* 34:788–791.

RAMACHANDRAN, R., KHAN, Z.R., CABALLERO, P., and JULIANO, B.O. 1990. Olfactory sensitivity of two sympatric species of rice leaf folders (Lepidoptera: Pyralidae) to plant volatiles. *J. Chem. Ecol.* 16:2647–2666.

RICKERL, D.H., GORDON, W.B., CURL, E.A., and TOUCHTON, J.T. 1988. Winter legume and tillage effects on cotton growth and soil ecology. *Soil Tillage Res.* 11:63–72.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry, 2nd ed. W.H. Freeman, San Francisco.

VISSER, J.H. 1979. Electroantennographic responses of the Colorado beetle, *Leptinotarsa decemlineata*, to plant volatiles. *Entomol. Exp. Appl.* 25:89–97.

VISSER, J.H. 1983. Differential sensory perceptions of plant compounds by insects, pp. 215–230, *in* P.A. Hedin (ed.). Plant Resistance to Insects, American Chemical Society Symposium Series 208. American Chemical Society, Washington, D.C.

WU, C.-M., and LIOU, S.-E. 1988. Effect of tissue disruption on volatile constituents of bell peppers. *J. Agric. Food Chem.* 34:770–772.

YAMAGUCHI, K., and SHIBAMOTO, T. 1980. Volatile constituents of the chestnut flower. *J. Agric. Food Chem.* 28:82–84.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

ROLE OF AVIAN TRIGEMINAL SENSORY SYSTEM IN DETECTING CONIFERYL BENZOATE, A PLANT ALLELOCHEMICAL

WALTER J. JAKUBAS[1,2,*] and J. RUSSELL MASON[3]

[1]*Monell Chemical Senses Center*
3500 Market Street, Philadelphia, Pennsylvania 19104
[3]*U.S. Department of Agriculture, Animal and Plant Health Inspection Service*
Denver Wildlife Research Center
c/o Monell Chemical Senses Center
3500 Market Street, Philadelphia, Pennsylvania 19104

(Received May 6, 1991; accepted July 8, 1991)

Abstract—Coniferyl benzoate, a secondary metabolite found in quaking aspen (*Populus tremuloides*) and other plants, is an avian feeding deterrent of ecological and potential commercial importance. This study was conducted to determine if coniferyl benzoate is a trigeminal stimulant for birds and to ascertain if trigeminal chemoreception of coniferyl benzoate can mediate avian feeding behavior. Five European starlings (*Sturnus vulgaris*) with bilateral nerve cuts (ophthalmic branch of the trigeminal nerve) and four starlings that had sham surgeries were fed a commercial diet treated with coniferyl benzoate. Birds receiving bilateral nerve cuts ate significantly more feed than intact birds, indicating trigeminal detection of coniferyl benzoate and trigeminal mediation of feeding behavior. In the past, trigeminal chemoreception has not been recognized as important in the detection of plant secondary metabolites despite the irritant or astringent properties of a number of them.

Key Words—European starlings, *Sturnus vulgaris*, ruffed grouse, *Bonasa umbellus*, phenylpropanoid, feeding deterrent, chemical senses.

INTRODUCTION

Anatomical and behavioral evidence that the chemical senses (i.e., taste, smell, and trigeminal) are sufficiently evolved in birds to permit discrimination among

*To whom correspondence should be addressed.
[2]Present address: Department of Wildlife Ecology, University of Wisconsin, Madison, Wisconsin 53706.

0098-0331/91/1100-2213$06.50/0

chemical cues has existed for some time (Wenzel, 1973). However, research on the diverse ways birds make use of their chemical senses (e.g., Baltharzart and Schoffeniels, 1979; Clark and Mason, 1985, 1988; Wenzel, 1986; Waldvogel, 1987) and the importance of avian chemoreception in the management of pest species (e.g., Mason and Silver, 1983; Mason et al., 1989; Mason, 1989) are just coming to the forefront.

Understanding how birds detect plant secondary metabolites could provide additional insights into avian ecology and the development of avian repellents. Plant secondary metabolites can influence avian food selection (Cook et al., 1971; Remington and Braun, 1985; Greig-Smith and Wilson, 1985; Jakubas et al., 1989), nestling parasite loads (Clark and Mason, 1988), and possibly, population densities (Jakubas and Gullion, 1991). In an applied context, the chemical structures of aversive plant secondary metabolites can serve as chemical models for developing avian repellents (Crocker and Perry, 1990; Jakubas et al., 1991). In this regard, phenylpropanoids related to cinnamic acid and coniferyl alcohol may be both ecologically and commercially important (Jakubas et al., 1989; Crocker and Perry, 1990; Jakubas et al., 1991). For example, coniferyl benzoate, an ester of coniferyl alcohol, is a feeding deterrent for ruffed grouse (*Bonasa umbellus*) (Jakubas and Gullion, 1990), European starlings (*Sturnus vulgaris*) (Jakubas et al., 1991), and possibly other passerines (Jakubas et al., 1989). Coniferyl benzoate naturally occurs in quaking aspen (*Populus tremuloides*); in gum benzoin Siam, from *Styrax tonkinensis*; and in jasmine oil (Freudenberg and Bittern, 1950; Adamson, 1972; Kato, 1984; Jakubas et al., 1989). Ecologically, it appears to be important in mediating the feeding behavior of ruffed grouse when they select quaking aspen flower buds (Jakubas et al., 1989; Jakubas and Gullion, 1990, 1991). It has been proposed that the irritant property of coniferyl benzoate may be one of the primary sensory cues used by ruffed grouse when selecting aspen buds (Jakubas, 1989).

Chemical irritation, such as from spicy foods or noxious fumes (e.g., ammonia) are sensations commonly attributed to trigeminal chemoreception (Silver, 1987). Trigeminal sensations (the common chemical sense) occur from stimulation of free nerve endings in the epithelium or mucosa and include the sensations of touch, pain, temperature, and proprioception (movement) (Silver, 1987). In most terrestrial vertebrates, the trigeminal (Vth) nerve innervates the epithelia of the head region, including intranasal, intraoral, and corneal surfaces (Silver, 1987). In the birds, the trigeminal nerve is principally divided into three branches, the ophthalmic (upper mandible and eye), maxillary (above eye and between mandibles), and mandibular (lower jaw and muscles) (Breazile and Yasuda, 1979).

Coniferyl benzoate was proposed to be a trigeminal stimulant because of the burning sensation it produces when ingested or rubbed on the eyelids (Jakubas, 1989, and unpublished data). In addition, coniferyl benzoate often will

cause an eczematous reaction when applied to the skin (Hjorth, 1961; Kato, 1984). Although coniferyl benzoate is a human irritant, this may not mean that birds perceive it similarly. For example, capsaicin, the pungent chemical found in peppers of the Capsicum family, is a strong trigeminal irritant to mammals but not to birds (Mason and Maruniak, 1983; Szolcsanyi et al., 1986). Confirmation that birds use trigeminal chemoreception to sense coniferyl benzoate, may help explain the repellency of analogous compounds (see Jakubas et al., 1991).

The objectives of this study were to (1) determine if coniferyl benzoate is a trigeminal stimulant for birds and (2) ascertain if trigeminal chemoreception of coniferyl benzoate can mediate avian feeding behavior. European starlings were chosen for this study because they have good chemosensory abilities (Clark and Mason, 1987; Espaillat and Mason, 1990) and are similar to ruffed grouse in their sensitivity to dietary coniferyl benzoate (see Jakubas et al., 1991; Jakubas and Gullion, 1990). In addition, anesthesia and surgical techniques for denervation of chemosensory systems in starlings are well documented (Mason and Silver, 1983; Clark and Mason, 1987; Mason et al., 1989).

METHODS AND MATERIALS

Diet Preparation. Coniferyl benzoate was obtained by continuous liquid–liquid extraction of benzoin Siam tears #3 (Alfred L. Wolff, Paris, France) and purified by crystallization following the procedures in Jakubas et al. (1991). Coniferyl benzoate was added to the test feed (5:1 mixture of Chick Starter and AVN Canary/Finch diet; Purina Mills Inc., St. Louis, Missouri) by dissolving a known quantity of crystalline material in diethyl ether, thoroughly mixing the solution with the feed, and evaporating the ether under a hood. The level of coniferyl benzoate applied to the feed (3.2% w/w) corresponds to levels in aspen flower buds that are generally not fed on by ruffed grouse (Jakubas and Gullion, 1990, 1991). Treated feed was stored under nitrogen, at −17°C, to prevent decomposition of coniferyl benzoate. The control feed was prepared by mixing the test feed with diethyl ether and evaporating the ether as described above.

Feeding Trials. Twelve European starlings were captured using a funnel trap in Philadelphia, Pennsylvania, during early April and transferred to indoor facilities three weeks prior to testing. The birds were individually caged (61 × 36 × 41-cm cages) and housed under constant temperature conditions (approx. 22°C), with a 11:13 hr light–dark cycle. Water and feed were provided ad libitum. The maintenance diet was a 5:1 mixture of Chick Starter and AVN Canary/Finch diet, to which oyster shell grit (United Volunteer Aviaries, Nashville, Tennessee) was added.

Seven days before surgery, birds were conditioned for five days to a food-deprivation routine. During this time, birds were deprived of their normal diet overnight. The following morning they were given 10 g of control feed approximately 2 hr after light onset. After 2 hr, consumption was measured and the maintenance diet returned to the cages for the remaining hours of light. Tap water was available ad libitum throughout the conditioning period and subsequent feeding trials.

On day 6 (one day prior to surgery), starlings were randomly assigned to receive either bilateral trigeminal nerve cuts (TNC) or sham surgeries and were food-deprived overnight. For TNC, birds were anesthetized by intraperitoneal injection of chloropent (4 ml/kg body wt) and placed in a head holder. To avoid disturbing the olfactory nerve, the ophthalmic branches of the trigeminal nerve were exposed and cut at the junction of the nervus ophthalmicus: ramus medialis and ramus nasales (see Breazile and Yasuda, 1979). Cut nerve ends were reflected to impede regeneration. The resulting cavity was packed with gelfoam and the skin closed with cyanoacrylate glue. Identical procedures were followed for sham surgeries except the trigeminal nerve was not cut. Five of six birds survived the TNC surgery, and four of six the sham surgery.

Starlings were allowed to recover from surgery for two days, after which they were given 2-hr feeding trials on each of seven days. Starlings were presented control feed on days 3–5 to evaluate their postsurgery condition. On days 6–9, birds were given the treatment diet containing 3.2% coniferyl benzoate.

Consumption of the control diet during the three days preceding and following surgery was analyzed in a three-factor analysis of variance (ANOVA) with repeated measures over days. Similarly, consumption of the treated diet was assessed in a two-factor ANOVA with repeated measures over days. Tukey's Honestly Significant Difference tests (Winer, 1962, p. 198) were used to isolate significant ($P < 0.05$) differences among means. Birds were euthanized by an overdose, via intraperitoneal injection, of sodium pentobarbitol. Nerve cuts were confirmed by necropsy.

RESULTS

Two-hour consumption of the control diet did not differ before or after surgery ($P > 0.25$). Prior to surgery, birds that were subsequently given TNC consumed 5.31 $\pm$ 0.25 g of control feed, while sham-operated birds ate 5.21 $\pm$ 0.31 g of feed. Following surgery, TNC birds consumed 5.59 $\pm$ 0.29 g of control feed and sham-operated birds ate 5.27 $\pm$ 0.34 g. However, TNC birds consumed significantly more of the 3.2% coniferyl benzoate diet than sham birds ($F = 22.8$; 1,7 *df*; $P < 0.01$) (Figure 1). Mean consumption of the 3.2% coniferyl benzoate diet over the four days of the trial was 0.99 $\pm$ 0.14 g for

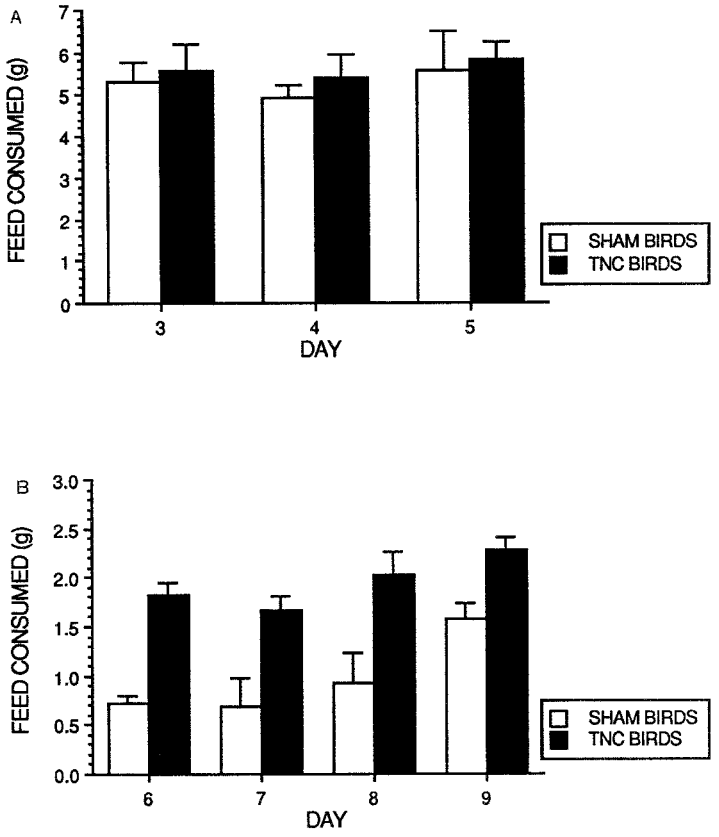


FIG. 1. Daily mean consumption (with standard error bars) during 2-hr feeding trials of (A) control feed and (B) feed treated with coniferyl benzoate (3.2% w/w) for starlings that received bilateral trigeminal nerve cuts (TNC) or sham surgeries.

sham-operated birds and 1.95 ± 0.09 g for the TNC birds. Over days, consumption differed ($F = 10.3$; 3,21 *df*; $P < 0.01$), and post-hoc tests revealed that for both TNC and sham birds, consumption on day 9 was significantly higher than consumption on day 6 (Figure 1). Necropsies on the TNC starlings did not reveal any regrowth of the sectioned trigeminal nerves.

DISCUSSION

Birds with bilateral sections of the ophthalmic branch of the trigeminal nerve (TNC) ate more feed treated with coniferyl benzoate than did sham birds. However, the groups did not differ in consumption of the control feed, sug-

gesting that birds receiving TNC were less sensitive to coniferyl benzoate than intact birds and that trigeminal chemoreception was involved in detection of this compound. These findings agree with other studies indicating that nasal–trigeminal chemoreception can mediate avian feeding behavior and that chemical repellency in birds, in some cases, may be associated with compounds that elicit a trigeminal response (Mason and Silver, 1983; Mason et al., 1989; but see Mason and Maruniak, 1983).

Trigeminal chemoreception has not been recognized in the past as being important to the perception of plant secondary metabolites by animals. For example, Chapman and Blaney (1979) recognized that the chemical senses are commonly used to perceive plant secondary metabolites; however, they incorrectly assumed that chemical irritation was not mediated by the chemical senses and virtually disregarded the importance of trigeminal chemoreception in this early review article.

An example of how trigeminal chemoreception may affect the feeding behavior of animals in the field can be illustrated with ruffed grouse and their selection of aspen buds. Typical avian trigeminal innervation (see Breazile and Yasuda, 1979) should enable ruffed grouse to sense coniferyl benzoate as it manipulates aspen buds with its beak when feeding. Consequently, the level of coniferyl benzoate in the copious external resin of the bud might serve as the initial cue to the bud's suitability. Trigeminal chemoreception (and other chemical senses) may be especially important to ruffed grouse when selecting aspen buds, due to a lack of visible patterns (e.g., color, size, outer resin, and UV absorbance) that could be used to distinguish buds having high levels of coniferyl benzoate or nutrients (Jakubas, 1989, and unpublished data). Indeed, preliminary data indicate that grouse do not feed in trees having buds that produced an oral burning sensation (human perception) (Jakubas, 1989). Given the unspecialized nature of trigeminal receptors (i.e., free nerve endings), the use of trigeminal chemoreception by starlings to detect coniferyl benzoate, by inference, supports the concept that ruffed grouse may use trigeminal chemoreception to detect differential levels of coniferyl benzoate. Differences in trigeminal chemoreception do exist among species; however, many irritants are perceived similarly (Szolcsanyi et al., 1986; Harti et al., 1989). Since trigeminal chemoreception is apparently involved in detecting coniferyl benzoate in species (i.e., humans and starlings) from two animal classes, it seems unlikely that this compound would not elicit a similar response in grouse.

Although ophthalmic nerve sections in starlings appear to have decreased their aversion to coniferyl benzoate-treated feed, it is obvious that TNC birds still found it less suitable than control feed (Figure 1). One explanation for the difference in consumption of control (5.59 ± 0.29 g) and treated feed (1.95 ± 0.09 g) among TNC birds may be the incomplete denervation of the trigeminal (Vth) nerve. Both the mandibular and maxillary branches of the nerve were left

intact. Complete denervation was not attempted because it would likely have resulted in prolonged aphagia and lack of responsiveness to food (see Zeigler and Karten, 1973a,b). Alternatively, the data suggest that taste and/or olfaction may have been involved in detection of coniferyl benzoate. Passerine species possess functional olfactory (Clark and Mason, 1989) and taste (Espaillat and Mason, 1990) capabilities, and either or both may have influenced the starling's response.

The increase in consumption of feed treated with coniferyl benzoate over time (Figure 1) may indicate that the birds habituated to the effects (sensory or postingestional) of coniferyl benzoate. Whether habituation to this compound occurs in field situations remains open to question. Factors such as increased toxicity, due to higher consumption of coniferyl benzoate or synergistic reactions with other phenolic compounds, may reinforce avoidance of coniferyl benzoate in field situations. We speculate that toxicity was not a significant factor in the laboratory, since the quantity of coniferyl benzoate consumed during the 2-hr feeding trials was relatively small and may have not been sufficient to invoke a toxic response.

Taste and olfaction are frequently cited as important in animal food selection (e.g., Arnold and Hill, 1972) and in the detection of plant secondary metabolites (Chapman and Blaney, 1979). However, based on our study and earlier work indicating that nasal–trigeminal chemoreception can mediate feeding behavior (e.g., Mason and Silver, 1983; Mason et al., 1989), we propose that trigeminal chemoreception may be important in the detection of many of the common secondary metabolites that have irritant or astringent properties. Compounds with these properties include: tannins (astringent), diphenols (irritants), phenylpropanoids (irritants), and diterpenoid esters (irritants) among others (Robinson, 1983). Trigeminal sensations elicited by these compounds may simply serve as a cue for avoidance or may significantly contribute to the compound's repellency.

Acknowledgments—We would like to thank Larry Clark and Florence Caputo for their advice on surgical procedures, Don Norman for his technical assistance, Pankaj Shah for his assistance in the laboratory, and the two anonymous reviewers for their comments on the manuscript. Funding for this project was provided by NIDCD postdoctoral training grant DC0014-12 and by U.S. Department of Agriculture, Animal and Plant Health Inspection Service Cooperative Agreement No. 12-34-41-0040-CA between the Monell Chemical Senses Center and the Denver Wildlife Research Center.

REFERENCES

ADAMSON, A.D. 1972. Oleoresins production and markets with particular reference to the United Kingdom. *Rep. Trop. Prod. Inst. Ser. G* 56:1–46.

ARNOLD, G.W., and HILL, J.L. 1972. Chemical factors affecting food plants by ruminants, pp.

71–101, *in* J.B. Harborne (ed.). Phytochemical Ecology: Proceedings of the Phytochemical Society Symposium. Academic Press, New York.

BALTHARZART, J., and SCHOFFENIELS, E. 1979. Pheromones are involved in the control of sexual behaviour in birds. *Naturwissenschaften* 66:55–56.

BREAZILE, J.E., and YASUDA, M. 1979. Systema nervosum peripheriale, pp. 473–503, *in* J.J. Baumel (ed.). Nomina anatomica avium—an annotated anatomical dictionary of birds. Academic Press, London.

CHAPMAN, R.F., and BLANEY, W.M. 1979. How animals perceive secondary compounds, pp. 161–198, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores—Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

CLARK, L., and MASON, J.R. 1985. Use of nest material as insecticidal and anti-pathogenic agents by the European starling. *Oecologia* 67:169–176.

CLARK, L., and MASON, J.R. 1987. Olfactory discrimination of plant volatiles by the European starling. *Anim. Behav.* 35:227–235.

CLARK, L., and MASON, J.R. 1988. Effect of biologically active plants used as nest material and the derived benefit to starling nestlings. *Oecologia* 77:174–180.

CLARK, L., and MASON, J.R. 1989. Sensitivity of brown-headed cowbirds to volatiles. *Condor* 19:922–932.

COOK, A.D., ATSTATT, P.R., and SIMON, C.A. 1971. Doves and dove weed: Multiple defenses against avian predation. *Bioscience* 21:277–281.

CROCKER, D.R., and PERRY, S.M. 1990. Plant chemistry and bird repellents. *Ibis* 132:300–308.

ESPAILLAT, J.E., and MASON, J.R. 1990. Differences in taste preference between red-winged blackbirds and European starlings. *Wilson Bull.* 102:292–299.

FREUDENBERG, K., and BITTERN, F. 1950. Coniferyl alcohol from Siam benzoin gum. *Chem. Ber.* 83:600–604 (taken from *Chem. Abstr.* 45:3359; 1951).

GREIG-SMITH, P.W., and WILSON, M.F. 1985. Influences of seed size, nutrient composition, and phenolic content of the preferences of bullfinches feeding in ash trees. *Oikos* 44:47–54.

HARTI, G., SHARKEY, K.A., and PIERAU, Fr-.K. 1989. Effects of capsaicin in rat and pigeon on peripheral nerves containing substance P and calcitonin gene-related peptide. *Cell Tissue Res.* 256:465–474.

HJORTH, N. 1961. Eczematous allergy to balsams, allied perfumes and flavoring agents. *Acta Dermato-Venereol. Suppl.* 46:1–216.

JAKUBAS, W.J. 1989. Ruffed grouse feeding behavior and ecology: Its relationship to the chemical composition of quaking aspen flower buds. PhD. thesis. University of Minnesota, St. Paul.

JAKUBAS, W.J., and GULLION, G.W. 1990. Coniferyl benzoate in quaking aspen—a ruffed grouse feeding deterrent. *J. Chem. Ecol.* 16:1077–1087.

JAKUBAS, W.J., and GULLION, G.W. 1991. Use of quaking aspen flower buds by ruffed grouse: Its relationship to grouse densities and bud chemical composition. *Condor.* 93:473–485.

JAKUBAS, W.J., GULLION, G.W., and CLAUSEN, T.P. 1989. Ruffed grouse feeding behavior and its relationship to the secondary metabolites of quaking aspen flower buds. *J. Chem. Ecol.* 15:1899–1917.

JAKUBAS, W.J., SHAH, P.S., MASON, J.R., and NORMAN, D.M. 1991. Avian repellency of coniferyl and cinnamyl derivatives. *Ecol. Appl.* In press.

KATO, S. 1984. Process to formulate hypoallergenic jasmine oil. *Perfum. Flavor.* 9:137–140, 143–145.

MASON, J.R. 1989. Avoidance of methocarb-poisoned apples by red-winged blackbirds. *J. Wildl. Manage.* 53:836–840.

MASON, J.R., and MARUNIAK, J.A. 1983. Behavioral and physiological effects of capsaicin in red-winged blackbirds. *Pharmcol. Biochem. Behav.* 19:857–862.

MASON, J.R., and SILVER, W.L. 1983. Trigeminally mediated odor aversions in starlings. *Brain Res.* 269:196–199.

MASON, J.R., ADAMS, M.A., and CLARK, L. 1989. Anthranilate repellency to starlings: chemical correlates and sensory perception. *J. Wildl. Manage.* 53:55–64.

REMINGTON, T.E., and BRAUN, C.E. 1985. Sage grouse food selection in winter, North Park, Colorado. *J. Wildl. Manage* 49:1055–1061.

ROBINSON, T. 1983. The organic constituents of higher plants their chemistry and interrelationships, 5th ed. Cordus Press, North Amherst, Massachusetts.

SILVER, W.L. 1987. The common chemical sense, pp. 65–87, *in* T.E. Finger and W.L. Silver (eds.). Neurobiology of Taste and Smell. John Wiley & Sons Inc., New York.

SZOLCSANYI, J., SANN, H., and PIERAU, Fr-.K. 1986. Nociception in pigeons is not impaired by capsaicin. *Pain* 27:247–260.

WALDVOGEL, J.A. 1987. Olfactory navigation in homing pigeons: Are the current models atmospherically realistic? *Auk* 104:369–379.

WENZEL, B.M. 1973. Chemoreception, pp. 389–415, *in* D.S. Farner, J.R. King, and K.C. Parkes (eds.). Avian Biology, Vol. 3. Academic Press, New York.

WENZEL, B.M. 1986. The ecological and evolutionary challenges of procellariiform olfaction, pp. 357–368, *in* D. Duvall, D. Müller-Schwarze, and R.M. Silverstein (eds.). Chemical Signals in Vertebrates, IV: Ecology, Evolution, and Comparative biology. Plenum Press, New York.

WINER, B.J. 1962. Statistical Principles in Experimental Design. McGraw-Hill Book Co., New York.

ZEIGLER, H.P., and KARTEN, H.J. 1973a. Brain mechanisms and feeding behavior in the pigeon (*Columbia livia*). I. Quinto-frontal structures. *J. Comp. Neurol.* 152:59–82.

ZEIGLER, H.P., and KARTEN, H.J. 1973b. Brain mechanisms and feeding behavior in the pigeon (*Columbia livia*). II. Analysis of feeding behavior deficits after lesions of quinto-frontal structures. *J. Comp. Neurol.* 152:83–102.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

POTENTIAL ALLELOCHEMICALS FROM *Pistia stratiotes* L.

G. ALIOTTA,[1,*] P. MONACO,[2] G. PINTO,[1] A. POLLIO,[1] and L. PREVITERA[2]

[1]*Dipartimento di Biologia Vegetale*
via Foria 223, I-80139, Naples, Italy.

[2]*Dipartimento di Chimica Organica e Biologica*
via Mezzocannone 16, I-80134, Naples, Italy
Universitá degli Studi Federico II
Naples, Italy

(Received April 9, 1991; accepted July 12, 1991)

Abstract—Among the substances isolated from ethyl ether extract of *Pistia stratiotes* L., linoleic acid, γ-linolenic acid, (12*R*,9*Z*,13*E*,15*Z*)-12-hydroxy-9,13,15-octadecatrienoic acid, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroxy-10,12,15-octadecatrienoic acid, α-asarone, and 24*S*-ethyl-4,22-cholestadiene-3,6-dione were found to inhibit the growth of some microalgae in solid medium. Toxicity of α-asarone on the growth of sensitive algal strains in liquid medium is discussed.

Key Words—*Pistia stratiotes*, allelochemicals, microalgae, unsaturated C_{18} fatty acids, α-asarone, sterols, growth inhibition.

INTRODUCTION

Pistia stratiotes L. (Araceae), commonly known as water lettuce, is a floating-leaved macrophyte occurring in tropical waters (Holm et al., 1977). In slow-flowing rivers and in the littoral regions of lakes, *P. stratiotes* can form a dense aggregation of floating rosettes, which create compact, thick mats, disturbing navigation and fisheries. Moreover, water lattuce can invade the rice fields, seriously limiting the growth of this crop (Sculthorpe, 1985).

Apart from the mechanical effects, floating mats of *P. stratiotes* can have a strong impact on its habitat. In fact, the large and extensive cover of the plant reduces gaseous exchanges between the water and the atmosphere and curtails

*To whom correspondence should be addressed.

0098-0331/91/1100-2223$06.50/0

the penetration of light (Attionu, 1976), limiting the growth of submerged hydrophytes and phytoplankton (Chokder, 1968).

At the present time, it has not been ascertained if the interference of *P. stratiotes* on other organisms living in its habitat involves exclusively competition, through removal or reduction of resources from the environment, or if it can also be caused by allelopathic effects. In a series of studies concerning in vitro interactions between aquatic plants and microalgae, we have now considered the isolation and the characterization of potential allelochemicals from *P. stratiotes*.

METHODS AND MATERIALS

Plant Collection and Extraction. Plants of *P. stratiotes* were cultured outdoors in Naples Botanical Garden during spring and summer of 1989 in 1000-liter cement tanks. Fresh, whole plants were collected in September and dried at room temperature. Dried plants (5 kg) were sequentially extracted with ethyl ether, chloroform, and methanol in a Soxhlet apparatus. All the extracts were frozen and stored at −20°C until used.

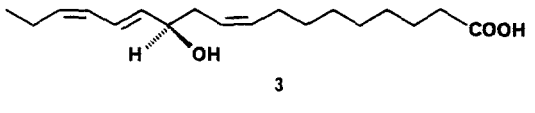
General Experimental Procedures. The gas–liquid chromatoghraphic apparatus consisted of a capillary-column chromatograph (FRACTOVAP 4160, Carlo Erba) equipped with a Varian 4270 integrator. An OV-1 column (25 m × 0.32 mm ID, film thickness 0.1–0.15 μm) with a column temperature of 180°C was used for analysis of fatty acid methyl esters with authentic samples purchased from Alltech Associates. The HPLC apparatus consisted of a pump module (Varian Vista 5500) and a refractive index detector (Varian RI 3). A Hibar-LiChrosorb RP8 column (Merck, 7 μm, 250 mm × 10 mm ID, MeOH–H_2O 4:1) was used for fatty acids separation. A LiChrosorb SI 60 (Merck, 7 μm, 250 mm × 7 mm ID, hexane–isopropyl ether 4:1) was used for sterols separations. A LiChrosorb-NH_2 column (Merck, 7 μm, 250 mm × 10 mm ID, $CHCl_3$–MeOH 41:4) was used for sitosterol acylglycosides separation. [^{1}H]NMR spectra were recorded at 400 MHz on a Bruker AM 400 spectrometer in $CDCl_3$ solution. Chemical shifts (δ values) are relative to residual $CHCl_3$ (δ 7.270). [^{13}C]NMR spectra were recorded at 100 MHz in $CDCl_3$ solution and the chemical shifts (δ values) are relative to the central signal of the $CDCl_3$ triplet (δ 77.00). MS spectra were recorded at 70 eV on a Kratos MS 80 spectrometer.

Ethyl Ether Extract Fractionation. The extract (10 g) was redissolved in ethyl ether (500 ml) and shaken with 2 N aq. NaOH (2 × 250 ml). The organic layer was washed with water until neutral to give a mixture of neutral compounds (8 g). The aqueous layers were acidified with 2 N aq. H_2SO_4 and then extracted with ethyl ether to give a mixture (1.2 g) of acidic compounds.

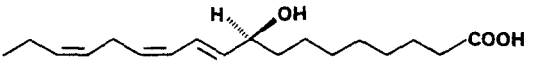
The acid mixture was chromatographed on HCl-washed silica gel, eluting with mixtures hexane–ethyl ether. The fractions eluted with a 19:1 mixture (370 mg) were separated through reverse-phase HPLC to give linoleic (**1,** 72 mg), γ-linolenic (**2,** 105 mg), oleic (47 mg), elaidic (7 mg), and stearic (125 mg) acids, identified as methyl ester derivatives by comparison with authentic samples (MS, GLC). The fractions eluted with a 4:1 mixture (150 mg) were rechromatographed on acid alumina (grade III) to give (12*R*,9*Z*,13*E*,15*Z*)-12-hydroxy-9,13,15-octadecatrienoic acid (**3,** 35 mg) (Figure 1), (9*S*,10*E*,12*Z*,15*Z*)-9-hydroxy-10,12,15-octadecatrienoic acid (**4,** 44 mg), and ricinoleic acid (60 mg). Aliquots of **3** and **4** were converted with ethereal CH_2N_2 into the corresponding methyl esters, which showed physical properties (MS, [^{1}H]NMR, [^{13}C]NMR) identical to those of authentic samples (Pollio et al., 1988).

The mixture of neutral compounds was column chromatographed on silica gel (82 g) eluting with hexane–ethyl ether mixtures to give fractions A–E. Fraction A (hexane, 900 mg) consisted of a complex mixture of triacylglycerols [^{1}H]NMR: 4.15 (dd), 4.30 (dd), 5.28 (m), which was not further investigated, and acetylphytol, identical to an authentic sample. Fraction B (hexane–ethyl ether 9:1, 2.5 g) was rechromatographed on silica gel. Hexane–ethyl ether (44:1) gave a mixture of polyprenols that was resolved through reverse-phase preparative TLC (acetone–water 9:1) into 9-prenol, 10-prenol, 11-prenol, and 12-prenol, which were identified on the basis of their spectroscopic features (Monaco et al., 1983). Elution with hexane–ethyl ether (43:2) gave α-asarone (**5,** 30 mg), which was compared with a commercially available sample. An aliquot of fraction C (hexane–ethyl ether 4:1, 1.6 g) was column rechromatographed on silica gel to give the fractions that were resolved through preparative TLC to give 24*S*-ethyl-4,22-cholestadiene-3,6-dione (**6**), 24*S*-ethyl-5,22-cholestadien-3β-ol (**7**), 24*S*-ethyl-8(14),22-cholestadien-3β-ol (**8**), and 24*S*-ethyl-22-cholestene-3,6-dione (**9**), identified on the basis of their IR, UV, [^{1}H]NMR (Table 1) and [^{13}C]NMR spectra (Table 2). Fraction D (hexane–ethyl ether 1:1, 170 mg) consisted of four sterols that were purified through preparative TLC ($CHCl_3$–acetone 21:1) and HPLC and identified as 5α,8α-epidioxy-24*S*-ethyl-6,22-cholestadien-3β-ol (**10**), 24*S*-ethyl-5,22-cholestadien-7-on-3β-ol (**11**), 24*S*-ethyl-4,22-cholestadien-6β-ol-3-one (**12**), and 24*S*-ethyl-22-cholesten-11β-ol-3,6-dione (**13**). Fraction E (hexane–ethyl ether 3:7, 120 mg) was column rechromatographed on silica gel (12 g) eluting with hexane–ethyl ether 3:7 to give four sterols that were separated through argentation preparative TLC ($CHCl_3$–acetone 9:1) and identified as 24*S*-ethyl-5,22-cholestadiene-3β,7α-diol (**14**), 24*R*-methyl-5-cholestene-3β,7α-diol (**15**), 24*S*-ethyl-5,22-cholestadiene-3β,7β-diol (**16**) and 24*R*-methyl-5-cholestene-3β,7β-diol (**17**).

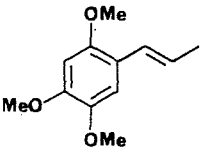
Chloroform Extract Fractionation. The extract (85 mg) was column rechromatographed on silica gel. Hexane–ethyl ether 3:2 gave sitosterol-3-*O*-



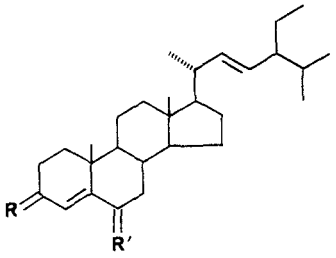
3



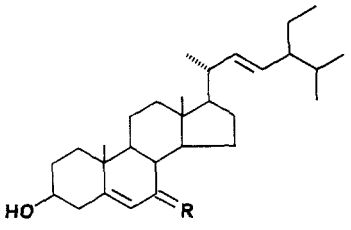
4



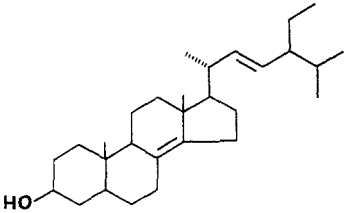
5



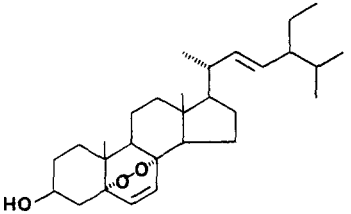
6 R = R' = O
12 R = O R' = H, βOH



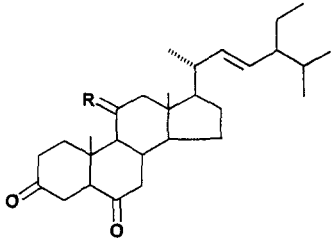
7 R = H_2 11 R = O
14 R = H, αOH 16 R = H, βOH



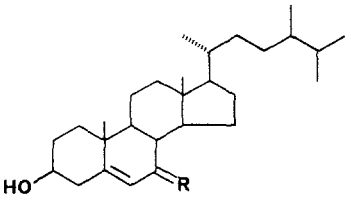
8



10



9 R = H_2
13 R = H, βOH



15 R = H, α OH
17 R = H, β OH

FIG. 1. Structures **3** to **17**.

TABLE 1. [^{1}H]NMR OF STEROLS **6–17** IN $CDCl_3$[a]

H	6	7	8	9	10	11	12	13	14	15	16	17
3		3.521 m	3.110m		3.978 m	3.692 m			3.595 m	3.596 m	3.554 m	3.556 m
4	6.178 s						5.817 s					
6		5.352 m			6.248 d (8.4)	5.702 d (1.7)	4.342 m		5.620 d (4.8)	5.620 d (4.9)	5.302 s	5.301 s
7					6.511 d (8.4)				3.854 m	3.855 m	3.850 d (4.3)	3.852 d (4.4)
11								4.010 m				
18	0.728 s	0.689 s	0.628 s	0.697 s	0.821 s	0.689 s	0.741 s	0.711 s	0.693 s	0.712 s	0.699 s	0.716 s
19	1.169 s	1.009 s	0.954 s	0.963 s	0.888 s	1.205 s	1.374 s	1.077 s	1.004 s	1.004 s	1.056 s	1.056 s
21	1.023 d (6.6)	1.012 d (6.5)	1.019 d (6.6)	1.022 d (6.6)	1.022 d (6.6)	1.025 d (6.5)	1.023 d (6.5)	1.023 d (6.6)	1.029 d (6.5)	0.893 d (6.4)	1.033 d (6.4)	1.033 d (6.4)
22	5.162 dd (8.5 15.1)	5.163 dd (8.6 15.0)	5.162 dd (8.5 15.0)	5.164 dd (8.6 15.1)	5.166 dd (8.5 15.0)	5.164 dd (8.4 15.2)	5.158 dd (8.4 15.0)	5.162 dd (8.5 15.1)	5.165 dd (8.5 15.1)		5.168 dd (8.4 15.0)	
23	5.020 dd (8.5 14.8)	5.022 dd (8.6 14.9)	5.021 dd (8.5 14.9)	5.021 dd (8.6 15.0)	5.022 dd (8.5 15.1)	5.024 dd (8.4 15.0)	5.022 dd (8.4 15.1)	5.020 dd (8.5 14.8)	5.023 dd (8.5 14.9)		5.030 dd (8.4 14.8)	
26	0.842 d (6.0)	0.840 d (6.3)	0.839 d (6.5)	0.841 d (6.4)	0.842 d (6.5)	0.846 d (6.5)	0.841 d (6.6)	0.842 d (6.5)	0.841 d (6.5)	0.848 d (6.5)	0.843 d (6.5)	0.846 d (6.4)
27	0.790 d (6.6)	0.787 d (6.5)	0.788 d (6.6)	0.790 d (6.6)	0.793 d (6.6)	0.794 d (6.6)	0.791 d (6.5)	0.790 d (6.6)	0.793 d (6.6)	0.801 d (6.6)	0.794 d (6.5)	0.805 d (6.5)
28										0.772 d (6.5)		0.773 d (6.4)
29	0.810 t (7.3)	0.807 t (7.8)	0.807 t (7.7)	0.809 t (7.5)	0.810 t (7.7)	0.811 t (7.7)	0.808 t (7.7)	0.810 t (7.3)	0.808 t (7.4)		0.810 t (7.5)	

[a]The couplings (Hz) are reported in parantheses.

TABLE 2. [^{13}C]NMR OF STEROLS **6–17** IN $CDCl_3$

C	6	7	8	9	10	11	12	13	14	15	16	17
1	35.51	37.35	35.11	38.05	34.69	36.58	37.09	37.61	36.90	36.84	36.90	36.88
2	33.95	31.68	31.55	39.35	30.11	31.24	34.30	39.87	31.36	31.42	31.61	31.80
3	199.48	71.75	70.98	209.18	66.48	70.45	200.23	208.33	71.33	71.54	71.45	70.84
4	125.42	42.18	38.22	37.10	36.88	41.70	126.12	37.33	41.70	41.60	41.82	42.00
5	161.05	140.77	40.75	57.32	82.20	169.22	168.50	57.86	143.70	143.48	143.46	143.78
6	202.31	121.64	25.44	211.18	135.32	126.04	73.29	211.24	123.80	123.57	125.48	124.95
7	46.79	31.92	27.19	46.51	130.72	204.15	38.48	46.08	65.30	65.90	73.30	73.54
8	39.11	31.79	128.02	37.32	79.44	45.22	29.64	36.44	37.48	36.88	40.88	41.06
9	50.94	51.10	134.89	53.74	51.02	50.18	53.70	59.17	42.20	41.90	48.28	48.57
10	34.21	36.39	35.61	41.32	36.81	38.12	37.90	42.87	37.42	37.84	36.39	36.78
11	20.77	21.12	22.78	21.72	20.64	21.28	21.04	69.01	20.75	21.04	21.12	21.32
12	39.88	39.76	36.87	38.01	39.28	39.74	39.68	51.68	39.10	39.88	39.55	39.85
13	42.52	42.34	42.02	42.86	44.49	41.84	42.50	43.07	42.18	41.75	42.92	42.70
14	55.81	56.78	51.81	55.83	51.61	49.87	55.77	55.88	49.39	48.64	55.40	55.72
15	23.90	24.15	23.96	24.04	23.54	26.38	24.29	23.93	25.86	26.35	26.38	25.95
16	27.93	28.30	28.75	28.12	28.54	28.46	28.24	28.00	28.17	28.50	28.61	28.32
17	56.55	56.05	54.72	56.49	56.21	54.55	56.18	56.06	55.78	56.30	55.96	56.02
18	11.92	11.88	11.25	12.72	12.80	11.96	11.91	12.80	11.63	11.91	11.92	11.85
19	17.43	19.40	17.89	12.12	18.08	17.28	19.58	12.95	18.21	19.30	19.20	19.54
20	40.54	40.50	40.37	40.68	39.78	40.33	40.59	40.53	40.56	35.95	40.66	35.46
21	21.09	21.04	21.02	21.23	21.00	21.29	21.25	21.10	21.21	18.74	21.54	18.54
22	138.34	138.25	138.27	138.44	137.96	138.26	138.54	138.35	138.49	33.87	138.07	33.77
23	129.28	129.47	129.38	128.98	129.11	129.30	130.00	129.12	129.87	30.30	129.75	30.44
24	51.33	51.03	51.15	50.99	50.89	51.21	51.55	51.30	51.48	38.88	51.80	38.75
25	31.87	31.80	31.87	31.65	31.87	31.80	31.19	31.90	31.92	32.52	30.84	32.32
26	21.22	21.09	21.01	21.00	21.11	21.16	21.00	21.23	21.15	18.41	20.98	18.38
27	19.08	19.00	19.09	19.26	19.29	19.20	19.11	18.98	19.16	20.18	19.51	20.36
28	25.40	25.23	25.14	25.62	25.40	25.58	25.40	25.40	25.48	15.40	25.84	15.32
29	12.29	12.11	12.06	12.01	12.15	12.36	12.26	12.21	12.22		12.20	

[2′,4′-diacetyl-6′-stearyl]-β-D-glucopyranoside, whereas ethyl ether gave a mixture of sitosterol-3-*O*-[2′-stearyl]-β-D-xylopyranoside and sitosterol-3-*O*-[4′-stearyl]-β-D-xylopyranoside, which were separated by HPLC LiChrosorb-NH_2. These glycosides were identified (Della Greca et al., 1991) on the basis of their spectroscopic properties and by chemical degradation.

Methanolic Extract Fractionation. The extract (70 g) was distributed between H_2O and AcOEt. The organic layer gave a residue (3.2 g) that was chromatographed on silica gel. $CHCl_3$–acetone (7:3) gave a mixture of acylglycosylsterols that was acetylated with acetic anhydride in dry pyridine. The mixture of acetyl derivatives was resolved through argentation preparative TLC (hexane–AcOEt 4:1) to give peracetyl derivatives of sitosterol-3-*O*-[6′-*O*-stearyl]-β-D-glucopyranoside, sitosterol-3-*O*-[6′-*O*-linaloyl]-β-D-glucopyranoside, and sitosterol-3-*O*-[6′-*O*-linalonyl]-β-D-glucopyranoside.

Bioassay. All the algal strains were cultivated on Bold basal medium (BBM) (Nichols, 1973). The growth of each alga was followed at 550 nm with a colorimeter (Bausch and Lomb Spectronic 20). Fractionated material and the purified compounds were assayed on algal strains as previously reported (Aliotta et al., 1990). For liquid tests, α-asarone was dissolved in EtOH. These solutions (20 μl each) were added to test tubes containing 6 ml of inoculated medium, giving final concentrations of 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, and 10^{-5} M, ranges generally used to evaluate a biological activity. A blank containing only EtOH (20 μl) in BBM was also tested.

The test tubes were incubated on a shaking apparatus which held them at a 71° angle and rocked them with an amplitude of 11 cm at the rate of 62 cycles per minute (Shihira and Krauss, 1965). The shaker was illuminated by fluorescent lamps Philips (TLD 30 w/55) (150 μE sec/m^2) under a photoperiod of 16 hr light–8 hr dark.

Growth of cultures was followed daily either by measuring the absorbance increase at 550 nm with a Bausch and Lomb Spectronic 20 Colorimeter or by counting the cell numbers with a Coulter counter Industrial Model D. The initial inocula corresponded to 0.05 units of absorbance. Cell numbers ranged from 5×10^4 (*Ankistrodesmus braunii* C202-7a) to 6×10^5 ml (*Synechococcus leopoliensis* T625), depending on the algal size.

Growth experiments were carried out in triplicate, and results were evaluated on the basis of the average of three tests. The different types of inhibition caused by α-asarone on the sensitive algae (reduced growth rate or lag-phase) were calculated, respectively, according to Blankley (1973) and to Pinto and Taddei (1986).

α-Asarone was assayed in the medium previously filtered on Whatman glass microfiber filters (diameter 1.6 μm) in order to remove the algae and subsequently lyophilized. The residue was redissolved in EtOH and α-asarone was

detected with a Perkin-Elmer LAMBDA 7 Spectrophotometer at λ 313 and 354 nm.

RESULTS AND DISCUSSION

The crude extracts of *P. stratiotes* were assayed for their biological activity on microalgae. The ethyl ether extract was effective in inhibiting the growth of 17 algal strains (Table 3), whereas both the chloroform and the methanol extracts as well as the pure compounds isolated from these sources were inactive (not shown). Sequential chromatographic processes (CC, TLC, and HPLC) afforded the pure compounds, which were identified on the basis of their spectroscopic properties as fatty acids, hydroxy fatty acids, α-asarone, polyprenols, sterols and glycosyl sterols.

Concerning the active compounds isolated from *P. stratiotes*, it must be emphasized that linoleic **(1)** and γ-linolenic **(2)** acids are widely distributed in the plant kingdom, and their activity may be attributed to the hydroperoxide derivatives formed in the bioassay conditions (Aliotta et al., 1990). By contrast, hydroxy fatty acids (**3** and **4**) are uncommon and have been isolated recently from *Dunaliella acidophila* (Pollio et al., 1988). These compounds, which showed an increased toxicity with respect to **1** and **2** on the same algal strains, are produced by lypoxygenase oxidation of α-linolenic acid (Funk et al., 1976).

The phenylpropanoid α-asarone **(5)** has been found in a few plants belonging to Aristolochiaceae and Araceae and seems to be a potential chemotaxonomic marker of these families.

Finally, among sterols isolated, 24*S*-ethyl-4,22-cholestadiene-3,6-dione **(6)** showed a slight growth-inhibitory activity on two algal strains (C249-1 *Muriella aurantiaca* and T1075 *Closterium acerosum*). The widely distributed 24*S*-ethyl-5-cholesten-3β-ol **(7)** had algistatic effects on some strains (not shown).

The toxicity of α-asarone, the most active compound isolated from *P. stratiotes*, was also tested in liquid cultures of the most sensitive strains. The growth of all the algae was severely inhibited by this compound at concentrations ranging between 5×10^{-5} M and 10^{-4} M (not shown). It is noteworthy that α-asarone caused two different types of inhibition. In fact, in the experiments carried out on the strains C202-7a (*Ankistrodesmus braunii*), T76 (*Scenedesmus quadricauda*), and T755 (*Porphyridium aerugineum*) we observed a lag phase, whose lengthening was dependent on the initial concentration of the phenylpropanoid. After the lag time, the algae resumed the same growth rates as the controls. By contrast, the growth rates of strains T625, T1444, T1816, and T1648 decreased with increasing α-asarone concentrations.

In Figure 2 are reported the growth curves of strains T76 *Scenedesmus quadricauda* (lag phase) and T1648 *Selenastrum capricornutum* (decreased

TABLE 3. INHIBITION OF ALGAL GROWTH BY *Pistia stratiotes*

Phylum	Strain	Species	Ethyl ether extract (1.0 mg)	Active compounds isolated from Et_2O extract[a]					
				1[b]	**2**[b]	**3**	**4**	**5**[b]	**6**
Cyanochloronta	T485	*Plectonema boryanum*	++[c]	−	−	−	−	+	−
	T625	*Synechococcus leopoliensis*	+++	+	++	+++	++	+++	−
	T1444	*Anabaena flos-aquae*	+++	+	+	+++	++	+++	−
	T1547	*Lyngbya kuetzingii*	−	−	−	−	−	−	−
	T1580	*Phormidium autumnale*	+	−	−	++	++	+	−
	T1816	*Porphyrosiphon notarisii*	+	+	++	−	−	+++	−
	T1824	*Aulosira terrestre*	+	−	−	+	+	++	−
	T2349	*Scytonema hofmanni*	++	−	++	+++	++	−	−
Rhodophycophyta	T755	*Porphyridium aerugineum*	++	−	+	++	+	+++	−
Chrysophycophyta	T671	*Navicula pelliculosa*	+	−	+	−	−	++	−
	T656	*Navicula minima*	+	−	+	−	−	++	−
Chlorophycophyta	C202-7a	*Ankistrodesmus braunii*	+	−	+	−	−	+++	−
	C249-1	*Muriella aurantiaca*	+	+	+	−	−	−	++
	C379-1c	*Stichococcus bacillaris*	++	−	+	−	−	−	−
	T76	*Scenedesmus quadricauda*	+++	−	−	+	+	+++	−
	T268	*Coccomyxa elongata*	+	−	−	−	−	−	−
	T1075	*Closterium acerosum*	++	−	−	+	−	++	++
	T1648	*Selenastrum capricornutum*	++	−	+	++	+	+++	−
	A489	*Chlorella saccharophila*	−	−	−	−	−	−	−

[a] 1, linoleic acid (1.0 μmol); 2, γ-linolenic acid (1.0 μmol); 3, (12*R*,9*Z*,13*E*,15*Z*)-12-hyroxy-9,13,15-octadecatrienoic acid (1.0 μmol); 4, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroxy-10,12,15-octadecatrienoic acid (1.0 μmol); 5, α-asarone (0.7 μmol); 6, 24*S*-ethyl-4,22-cholestadiene-3,6-dione (0.7 μmol).

[b] Commercially available products showed identical activity.

[c] +, diameter of inhibition 7–14 mm; ++, diameter of inhibition 15–23 mm; +++, diameter of inhibition > 23 mm; −, no inhibition.

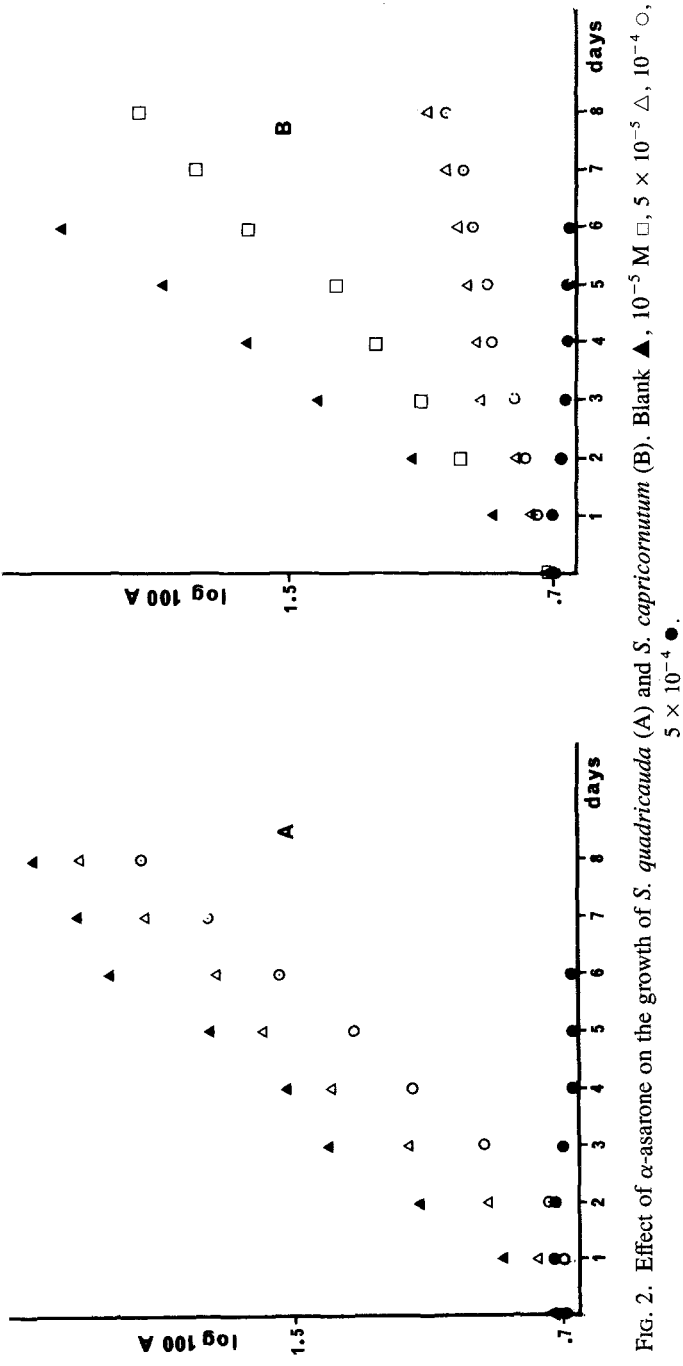


FIG. 2. Effect of α-asarone on the growth of *S. quadricauda* (A) and *S. capricornutum* (B). Blank ▲, 10^{-5} M □, 5×10^{-5} △, 10^{-4} ○, 5×10^{-4} ●.

TABLE 4. CONCENTRATIONS (μM) OF α-ASARONE IN CULTURE MEDIUM DURING FIRST FOUR DAYS OF *Scenedesmus quadricauda* (A) AND *Selenastrum capricornutum* (B)[a]

Days	A		B	
0	100	50	100	50
1	90	49	90	50
2	60	47	85	48
3	50	47	84	48
4	50	47	85	48

[a] No variation was observed in the blanks.

growth rate) at different concentrations of α-asarone. The concentration of α-asarone in the medium of the two cultures was also measured (Table 4). In the medium of strain T1648 with 5×10^{-5} M and 10^{-4} M solutions, the concentration remained fairly constant during the first four days of the growth, whereas a progressive reduction was found in the medium of strain T76. In the experimental conditions reported, the value 5×10^{-5} M of α-asarone represented the lowest toxic dose to this latter strain. These preliminary results seem to indicate that the algae in which α-asarone caused a lag phase are able to partially detoxify the medium, as already reported for other toxic substances (Blankley, 1973; Albertano et al., 1981).

The marked antialgal activity of α-asarone confirms that the phenylpropanoids may interfere with growth processes in plants. In this respect, we are investigating a possible relationship between structure and biological activity of these compounds.

Acknowledgments—This work was supported by C.N.R. (Progetto Chimica Fime II).

REFERENCES

ALBERTANO, P., PINTO, G., and TADDEI, R. 1981. Evaluation of toxic effects of heavy metals on unicellular algae. III Subtraction of the toxic element from the medium by the cells. *Delpinoa* 21-22:47-61.

ALIOTTA, G., DELLA GRECA, M., MONACO, P., PINTO, G., POLLIO, A., and PREVITERA, L. 1990. In vitro algal growth inhibition by phytotoxins of *Typha latifolia* L. *J. Chem. Ecol.* 16(9):2637-2646.

ATTIONU, R.M. 1976. Some effects of water lattuce (*Pistia stratiotes*, L.) on its habitat. *Hydrobiologia* 50(3):245-254.

BLANKLEY, W.F. 1973. Toxic and inhibitory materials associated with culturing, *in* J.R. Stein (ed.). Handbook of Phycological Methods 207–223. Cambridge University Press, Cambridge, U.K.

CHOKDER, A. 1968. Further investigation on control of aquatic vegetation in fisheries. *Agric. Pak.* 19:101–118.

DELLA GRECA, M., MOLINARO, A., MONACO, P., and PREVITERA, L. 1991. New acylglycosyl sterols from *Pistia stratiotes* L. *Phytochemistry*. 30(7):2422–2424.

FUNK, M.O., ISAAC, R., and PORTER, N.A. 1976. Preparation and purification of lipid hydroperoxides from arachidonic and γ-linolenic acids. *Lipids* 11(2):113–117.

HOLM, L.G., PLUCKNET, D.L., PANCHO, J.V., and HERBERGER, J.P. 1977. The World's Worst Weeds. The University Press of Hawaii, Honolulu.

MONACO, P., PREVITERA, L., and BELARDINI, M. 1983. Polyprenols from the leaves of *Quercus ilex* infected by *Microsphaera alphitoides*. *J. Nat. Products* 46:174–177.

NICHOLS, H.W. 1973. Growth media—freshwater, *in* J.R. Stein (ed.). Handbook of Phycological Methods 7–24. Cambridge University Press, Cambridge, U.K.

PINTO, G., and TADDEI, R. 1986. Evaluation of toxic effects of heavy metals on unicellular algae. VI Analysis of the inhibition manifesting itself with an increased lag phase. *Bull. Soc. Nat. Napoli* 95:303–316.

POLLIO, A., DELLA GRECA, M., MONACO, P., PINTO, G., and PREVITERA, L. 1988. Lipid composition of the acidophilic alga *Dunaliella acidophila* (Volvocales, Chlorophyta) I. Non-polar lipids. *Biochim. Biophys. Acta* 963:53–60.

SCULTHORPE, C. 1985. The Biology of Aquatic Vascular Plants. Edward Arnold, London.

SHIHIRA, I., and KRAUSS, R.W. 1965. *Chlorella*. Physiology and Taxonomy of Forty-one Isolates. Port City Press, University of Maryland, Baltimore.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

ISOLATION AND IDENTIFICATION OF ALLELOCHEMICALS THAT ATTRACT THE LARVAL PARASITOID, *Cotesia marginiventris* (CRESSON), TO THE MICROHABITAT OF ONE OF ITS HOSTS

TED C.J. TURLINGS,* JAMES H. TUMLINSON, ROBERT R. HEATH, ADRON T. PROVEAUX, and ROBERT E. DOOLITTLE

Insect Attractants, Behavior, and Basic Biology Research Laboratory
Agricultural Research Service, U.S. Department of Agriculture
Gainesville, Florida 32604

(Received March 22, 1991; accepted July 15, 1991)

Abstract—Volatiles released from corn seedlings on which beet armyworm larvae were feeding were attractive to females of the parasitoid, *Cotesia marginiventris* (Cresson), in flight tunnel bioassays. Analyses of the collected volatiles revealed the consistent presence of 11 compounds in significant amounts. They were: (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, α-*trans*-bergamotene, (*E*)-β-farnesene, (*E*)-nerolidol, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. A synthetic blend of all 11 compounds was slightly less attractive to parasitoid females than an equivalent natural blend. However, preflight experience with the synthetic blend instead of experience with a regular plant–host complex significantly improved the response to the synthetic blend. Our results suggest that *C. marginiventris* females, in their search for hosts, use a blend of airborne semiochemicals emitted by plants on which their hosts feed. The response to a particular odor blend dramatically increases after a parasitoid experiences it in association with contacting host by-products.

Key Words—Hymenoptera, Braconidae, *Cotesia marginiventris*, corn, parasitoid, host searching, semiochemicals, synomones.

*To whom correspondence should be addressed.

0098-0331/91/1100-2235$06.50/0

INTRODUCTION

Although many studies have demonstrated the attraction of parasitoids to volatile semiochemicals, only a few of these chemicals have been isolated and identified (Weseloh, 1981; Vinson, 1981; Eller, 1990). Recent findings that show that responses to semiochemicals by parasitoids are often flexible and can be modified by experience (e.g., Alphen and Vet, 1986; Lewis and Tumlinson, 1988; Vet and Groenewold, 1990) have led to increased interest in the subject from the biological control perspective. It appears possible to condition parasitoid females with specific chemicals so that their subsequent responses to these chemicals are intensified significantly (Lewis and Tumlinson, 1988). This may have positive consequences for mass release programs that so far have suffered from low searching efficiency and high departure frequencies from target areas by released parasitoids (Lewis and Nordlund, 1985). Prerelease experience with volatiles that will guide the wasps to larval pests in a target area is likely to increase the rate of parasitization and, thereby, the effectiveness of the wasps as control agents. To make this possible, identification and formulation of the essential semiochemicals will be necessary.

Cotesia marginiventris (Cresson) (Hymenoptera: Braconidae), a larval endoparasitoid, attacks many economically important Lepidopterous species such as *Spodoptera frugiperda*, *S. exigua*, *Pseudoplusia includens*, and *Heliothis* spp. (Lepidoptera: Noctuidae). The parasitoid frequently causes high mortality among these pests (e.g., Tingle et al., 1978; Pair et al., 1982; McCutcheon and Turnipseed, 1981), but might be much more effective after well-timed augmentative releases. Like other parasitoids, *C. marginiventris* increases its responses to volatile semiochemicals emitted by a plant–host complex after experiencing certain contact kairomones in association with these volatiles (Dmoch et al., 1985; Turlings et al., 1989, 1990a). Several host-derived cues stimulate close-range searching behavior in *C. marginiventris* (Loke et al., 1983; Loke and Ashley, 1984a–c). However, plants damaged by the host larvae are the main source of the volatiles (synomones) that attract the wasp in the vicinity of hosts (Turlings et al., 1991). Here we present the results of a study in which these synomones were collected, tested for their attractiveness to the parasitoid, and eventually identified. The activity of the identified compounds was confirmed by testing synthetic versions in flight-tunnel bioassays.

METHODS AND MATERIALS

The Insects. Cotesia marginiventris were reared and held as described by Turlings et al. (1989). All flight-tunnel tests were conducted with 3- to 5-day-old mated females 4–8 hr into the photophase. In all cases, late second- or early

third-instar beet armyworm (BAW), *Spodoptera exigua* (Hübner), larvae were used as hosts in the experiments. They were reared according to the procedure described by King and Leppla (1984).

Mass Collections of Volatiles for Bioassays and Identification. Volatiles released by BAW larvae feeding on corn seedlings were collected using an all glass push–pull odor collection system. Before entering the system, air was humidified in a gas dispersion tube and purified with in-line activated charcoal filters. The air then entered the first of three Pyrex glass tubes connected in series. The first tube contained a glass frit, which ensured that a laminar air flow would enter the second tube, which contained the odor source. The first tube ended in a 110- to 115-mm male ground-glass joint that was connected to a female counterpart of the second joint. The second tube was ~36 cm long (including joints) with an outside diameter of 11 cm. Its outlet was a 50- to 55-mm male ground-glass joint that fitted the inlet of the third tube, which ended in three collector ports. To optimize the collection, two ports were used simultaneously by attaching stainless-steel tubes (0.64 cm OD × 0.5 cm ID) to each port. The third port was not used and was sealed with a glass stopper. A collection trap was connected to the upwind end of each steel tube, while the downwind end was connected with Tygon tubing to Alborg flowmeters, which were connected to house vacuum.

Collection traps consisted of 3.7-mm-ID × 4-cm-long glass tubes with a 325-mesh stainless-steel frit sealed across the diameter of each tube about 4 mm from the upwind end. Approximately 50 mg of Super Q adsorbent (80–100 mesh) (Alltech, Deerfield, Illinois) was placed on top of the frit and held in place with a small plug of glass wool. Before each collection, the traps were rinsed with methylene chloride.

In the collection chamber, we placed 100 early third-instar BAW larvae on 40 10-day-old greenhouse-grown corn seedlings that were cut just before collection, and their stems were wrapped in wetted cotton. With the odor source and traps in place, a collection was started by balancing house air and house vacuum so that approximately 300 ml/min passed through each trap and the pressure was only slightly higher inside the system than outside. The pressure could be read with a user-designed glass pressure gauge that was connected to a side arm at the downwind part of the collection chamber.

Each collection was run for 24 hr. After 10–12 hr, traps were removed and extracted with 300 μl of pure methylene chloride, then connected back on to the system and extracted once more after another 12–14 hr. The amount of volatiles collected is expressed in collection minute equivalents (CME). Each collection contains 1440 CME (= 24 hr).

Collections for Quantifications. For more exact quantification of the volatiles released by a complete plant–host complex, a smaller system was used. The glass collection chambers consisted of two parts. Purified air entered the

first part through a 2-cm-long $\frac{1}{4}$-in.-OD inlet, which widened into a section (6 cm long; 3 cm ID) containing a glass frit. The second part, also 3 cm in diam, was 15 cm long with a 2-cm-long $\frac{1}{4}$-in. outlet. The second part contained the odor source. Both parts had fitting glass ball joints that were clamped together. One collection trap with 25 mg Super Q adsorbent was connected to the $\frac{1}{4}$-in. outlet with a brass Swagelock fitting containing Teflon ferules. Three corn seedlings that had been fed upon overnight were placed with 15 BAW in the chambers. Volatiles were collected for 1 hr (300 ml/min). Each trap was rinsed with 200 μl methylene chloride, and internal standards were added (1 μg each of octane and nonyl-acetate in 50 μl methylene chloride). Of each collection sample (total volume 250 μl), 1–2 μl was analyzed using capillary gas chromatography (GC) (see below). The collection was repeated on six different days with new plant and herbivore material. The same procedure was followed to collect volatiles from undamaged seedlings. Collections with only wetted cotton wool inside the chamber were performed to determine system impurities.

Collections of Different Components of Complete Plant–Host Complex. The above procedure also was used to collect volatiles of the three main components of a complete plant–host complex. A complex of three overnight damaged corn seedlings was divided into larvae, frass, and damaged leaves as described by Turlings et al. (1991). The larvae were starved for 2 hr and then washed with water. The frass was wiped off the leaves with two pieces of cotton wool (one wet and one dry), and the damaged leaves were washed with water to remove any remaining larval by-products. Volatiles of the three components and of a complete plant–host complex were collected simultaneously for 3 hr. Collections were repeated five times, and GC analyses were used to determine the volatiles present in each of the samples.

Chemical Analyses. Analyses were conducted on a Varian model 3700 GC and a Hewlett-Packard model 5890 GC, both equipped with split–splitless capillary injector systems and flame ionization detectors. Data collection, storage, and subsequent analysis was performed on a Perkin Elmer chromatographic data system. Helium at a linear flow velocity of 19 cm/sec was used as a carrier gas. Most of the initial analyses of the volatile collections were performed on two fused silica capillary columns. They were 50 m $\times$ 0.25 mm ID with a 0.25-μm-thick film of bonded methyl silicone (007) and 50 m $\times$ 0.25 mm ID with a 0.25-μm-thick film of bonded cyanopropyl methyl silicone (CPS-1). Both columns were obtained from Quadrex Corporation (New Haven, Connecticut) and were run at an initial temperature 50°C for 3 min, then temperature programmed at 5°C/min to 180°C. All injections of 1–3 μl were made in the splitless mode.

Samples were also analyzed by GC-mass spectroscopy (GC-MS) with a Nermag model R1010 mass spectrometer in both the electron impact and the

chemical ionization modes. The methyl silicone and CPS-1 columns used in the previous analyses were used in the GC-MS analyses with helium as carrier gas. Methane and isobutane were used as reagent gases for chemical ionization.

Vapor-phase infrared spectra were obtained from a Nicolet 20SXC GC-FTIR spectrometer interfaced to a Hewlett-Packard model 5890 GC. Samples were introduced to the FTIR via the methyl silicone column described above.

Several compounds that required [^{1}H]NMR analysis for full identification were analyzed with a Nicolet 300 MHz Fourier transform NMR spectrometer interfaced to a Nicolet model 1280 data system. The natural compounds as well as synthetic standards were purified by micropreparative GLC on a 30-m × 0.53-mm-ID SPB-1 (1.5-μm film thickness) column (Supelco) in a Hewlett-Packard model 5890 gas chromatograph. To allow injection of large volumes (up to 100 μl), a deactivated column (30 m × 0.53 mm, Quadrex Corporation) preceded the SPB-1 column [see Grob (1982) for details on this technique]. Just before entering the detector, the effluent from the column was split, one part going to the FID detector, the other exiting into a collector as originally designed by Brownlee and Silverstein (1968). The split ratio was manipulated so that >95% of the sample entered a glass capillary collection tube (35 cm long, 1.2 mm ID) in the collector. Dry Ice in acetone was used to cool the part of the capillary tube furthest away from the GC. Tubes were inserted into the collector just prior to elution of the compound to be collected and removed immediately after elution. For collection of the most volatile compounds, approximately 8 mg of Super Q adsorbent was packed between two glass wool plugs at the cold end of a collection tube. Temperature programs varied for the collection of different compounds. The collected material was transferred into an NMR tube by rinsing the glass capillary with approximately 25 μl of benzene-D6. The NMR tubes were 5 mm (OD) at the top, with a 50 × 2-mm (OD) coaxial extension at the bottom (Wilmad Glass Company, Buena, New Jersey). Data points were collected with a 6-μsec pulse (90° tip angle). Where necessary, proton decoupling was accomplished by standard decoupling techniques (decoupler power ca. 0.5 W).

All spectra of the natural products were compared with those of candidate synthetic compounds. (3*E*)-4,8-Dimethyl-1,3,7-nonatriene and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene were synthesized by the Wittig reaction of geranial and farnesal, respectively, with methylenetriphenyl phosphorane (analogous to Maurer et al., 1986). *α-trans*-Bergamotene was provided by Douglas B. McIlwaine at Brown University, Providence, Rhode Island. All other synthetic standards used in this study were obtained from commercial sources.

Flight-Tunnel Bioassays. The Plexiglas flight tunnel described by Turlings et al. (1991) was used to test the attractiveness of specific samples to females

of *C. marginiventris*. During each test, the following conditions were maintained inside the tunnel: 15 cm/sec airflow; 55–70% relative humidity; 27.5–29°C; and approximately 500 lux lumination. For each experiment, a sample dissolved in 200 μl methylene chloride was applied on a strip (1 × 5 cm) of green construction paper. The paper was pinned at the upwind end of the tunnel with an insect pin at a 45° angle from horizontal (highest point upwind), on top of a $\frac{1}{4}$-in. stainless-steel tube, centered 30 cm above the flight-tunnel floor. Four minutes were allowed for the solvent to evaporate before the first wasps were tested. The wasps were released from a glass funnel (Turlings et al., 1991) in groups of three (a number that easily allows the observer to recognize each individual throughout each trial), 80 cm downwind from the odor source. Unless stated otherwise, females were given a 20-sec contact experience with a complex of corn seedlings fed upon by BAW larvae, just before their introduction into the flight tunnel. This experience significantly increases their response to host-related odors (Turlings et al., 1989, 1990a). Here we report only on the percentage of females that exhibited a complete flight toward each odor source.

Dose–Response Tests. The volatiles collected using the above-described mass collection procedure were tested at five different doses. Equivalents of 10, 30, 100, 300, and 1000 collection minute equivalents (CME) were diluted with methylene chloride to a total volume of 200 μl/sample. Samples were labeled such that the experimenter did not know the concentration of each sample until after a complete trial. Six females (two groups of three) were tested to each concentration on a given day. This was repeated six times.

Flights to Synthetic Odor Blends. After all major compounds in the complete natural odor blend emitted by BAW larvae feeding on corn were identified, a synthetic blend of these compounds was made. First, all compounds were purified by preparative GLC as described above. After purification, the compounds were combined in a blend that closely mimicked a collected natural blend (Table 2 below). Both the synthetic and natural blends were tested for attractiveness to *C. marginiventris* in the flight tunnel at a concentration close to the optimal (approximately 250 CME) found in the dose–response tests. Five treatments were tested individually on a given day. Females that were experienced on a regular plant–host complex were tested to either a natural blend, a synthetic blend, or to solvent (methylene chloride) only. In addition, females that had a preflight experience with the synthetic blend were tested to either the natural or the synthetic blend. For the synthetic experience, a filter paper disk (diam. 5.5 cm) was treated with a synthetic sample (same as used for odor sources) and with five to seven fecal pellets of BAW fed on corn. Several minutes after the solvent had evaporated, wasps were introduced to the paper, which they would traverse and antennate vigorously. After 30 sec, the females were transferred to the flight tunnel to observe their long-range responses to one of the odor blends.

RESULTS

Dose–Response Tests. Female *C. marginiventris* exhibited complete flights to all doses of the collected volatiles. Responses, however, were clearly dose related, with an optimal response of 60–75% complete flights to dosages between 200 and 300 CME (Figure 1).

Surprisingly, the overall response dropped significantly at the highest dose (1000 CME) tested. The percentages of females flying to the optimal dose were similar to those that have been observed for females responding to volatiles of complete plant–host complexes (Turlings et al., 1991). This is strong evidence that the naturally active compounds were present in the tested extracts.

Analyses and Identifications of Collected Volatiles. The GC profiles of the collected volatiles revealed the consistent occurrence of 11 compounds in large amounts (Figure 2; Table 1). None of the compounds were found in the profiles from system blanks (= collections made with only wetted cotton present in the collection chamber), and only trace amounts of some volatiles were detected in collections from undamaged leaves (Figure 2).

Compounds 1–5 and compound 7 were identified by comparing their GC-MS spectra with those obtained from synthetic standards and reported spectra

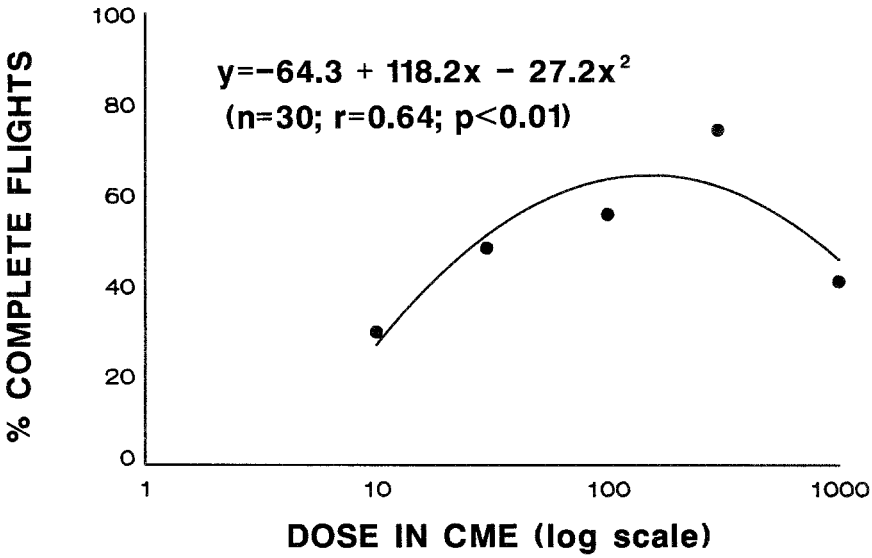


FIG. 1. Responses by (N = 36) *C. marginiventris* females to different doses of extracts of volatiles collected from BAW larvae feeding on corn seedlings. Units are collection minute equivalents (CME), representing the time during which the volatiles were collected from 100 second-instar BAW larvae feeding on 40 seedlings.

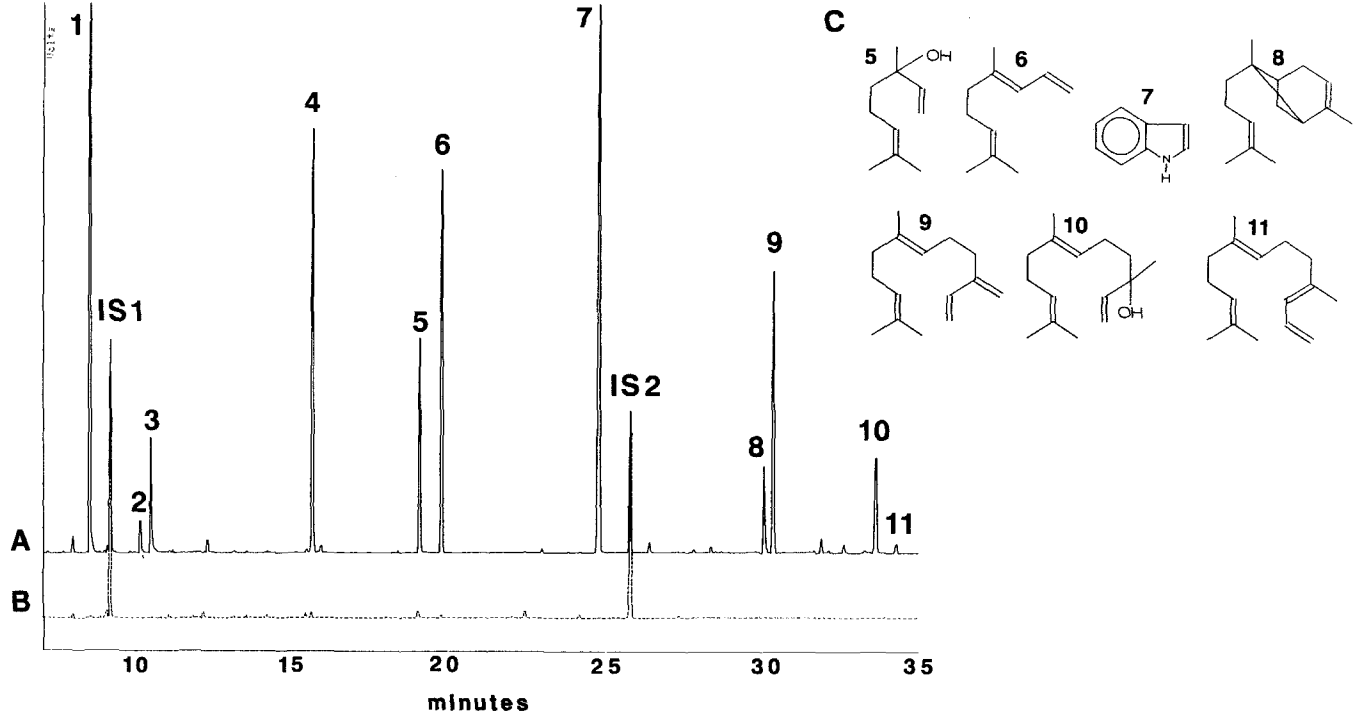


FIG. 2. (A) Chromatogram of volatiles released by a complex of corn seedlings damaged by BAW larvae. The 11 major compounds were identified as 1, (*Z*)-3-hexenal; 2, (*E*)-2-hexenal; 3, (*Z*)-3-hexen-1-ol; 4, (*Z*)-3-hexen-1-yl acetate; 5, linalool; 6, (3*E*)-4,8-dimethyl-1,3,7-nonatriene; 7, indole; 8, α-*trans*-bergamotene; 9, (*E*)-β-farnesene; 10, (*E*)-nerolidol; 11, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tri-decatetraene. (B) Chromatogram of volatiles released by undamaged corn seedlings. (C) Structures of the last seven compounds with corresponding peak numbers. Chromatograms were obtained by analysis of the volatiles on a 50-m 007 methyl silicone column (0.25 mm ID, 0.25 μm film thickness), after a 2-hr collection. IS1 and IS2 are the internal standards *n*-octane and *n*-nonyl-acetate.

TABLE 1. COMPOUNDS IDENTIFIED IN VOLATILES COLLECTED FROM CORN SEEDINGS FED UPON BY BAW LARVAE

Peak[a]	Compound	Kovats[b]	Amount (ng/hr[c])	Relative %
1	(*Z*)-3-hexenal	775	2015 (785)	5–21
2	(*E*)-2-hexenal	834	310 (108)	1–4
3	(*Z*)-3-hexen-1-ol	845	502 (118)	2–5
4	(*Z*)-3-hexen-1-yl acetate	991	2058 (286)	12–20
5	Linalool	1089	427 (40)	2–4
6	(3*E*)-4,8-dimethyl-1,3,7-nonatriene	1110	676 (140)	3–7
7	Indole	1266	2333 (617)	9–26
8	α-*trans*-bergamotene	1441	834 (503)	2–9
9	(*E*)-β-farnesene	1451	3153(1867)	8–35
10	(*E*)-nerolidol	1551	947 (329)	6–10
11	(3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tri-decatetraene	1569	61 (16)	0–1

[a] Correspond with peak numbers in Figure 2.
[b] Kovats GLC index (Kovats, 1965) for the 48–m methyl silicone capillary column.
[c] Average amounts released by three corn seedlings that had been fed upon overnight. During the 1-hr collections, 15 BAW larvae fed on the seedlings ($n = 6$). Standard deviations are shown in parentheses.

(Visser et al., 1979; Stenhagen et al., 1974). They were (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, linalool, and indole. All are commonly found in plants (e.g., Visser et al., 1979; Tollsten and Bergström, 1988). Compounds 6 and 8–11 are less common but have all been reported several times as plant-produced chemicals (see Discussion). Synthetic candidates for these compounds, when analyzed by GC, had the same retention times on the two different columns as their natural versions. Moreover, MS, IR and [^{1}H]NMR spectra were identical to the natural products. The [^{1}H]NMR spectra matched with the spectra reported by others: (3*E*)-4,8-dimethyl-1,3,7-nonatriene and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Maurer et al., 1986; Dicke et al., 1990a), α-*trans*-bergamotene (Corey et al., 1971), (*E*)-β-farnesene (Bowers et al., 1972), and (*E*)-nerolidol (Doskotch et al., 1980).

Sources of Collected Volatiles. The damaged plants released, by far, most of the volatiles (Figure 3). All compounds were collected from the damaged leaves in significant amounts, except for compounds 1–3. Preliminary data, however, indicated that when starved larvae are allowed to feed on the leaves, the three most volatile compounds are released in large amounts. Clearly, the highly volatile compounds are only released in the observed amounts as a result of active plant damage. No detectable amounts of the 11 volatiles were released

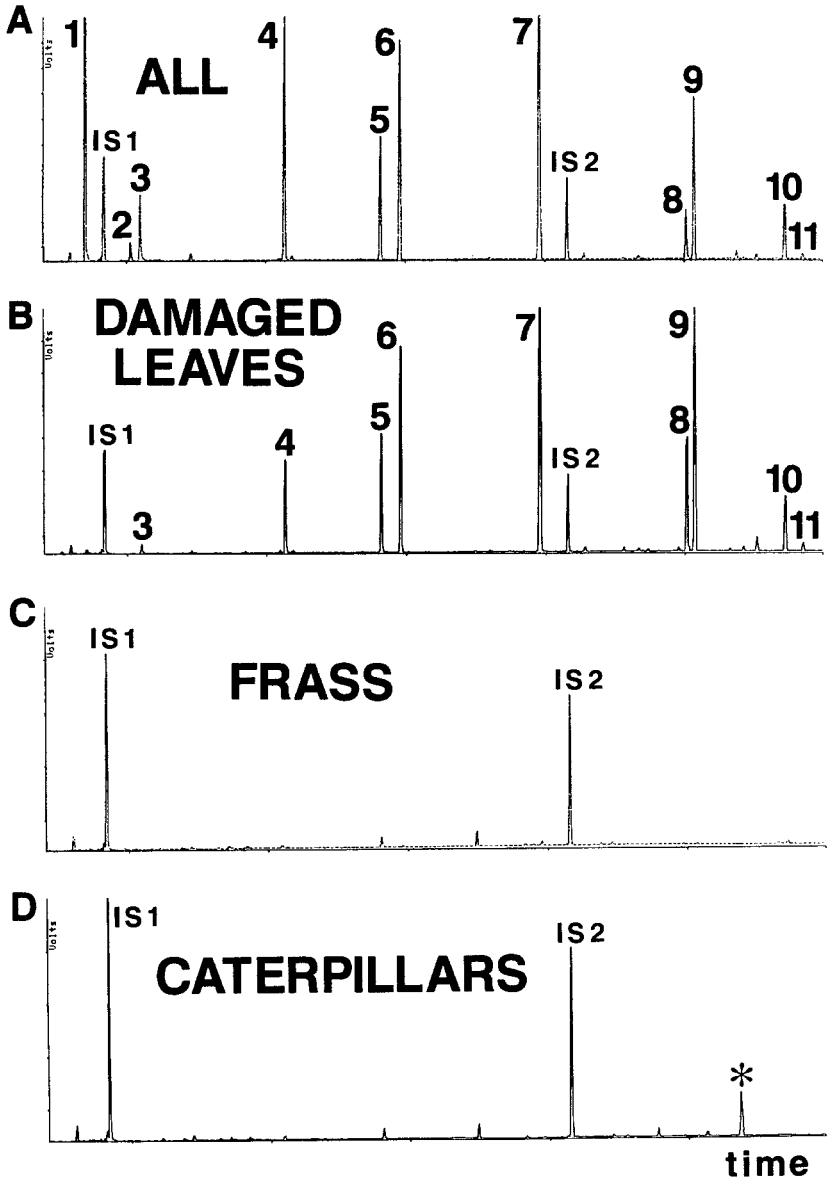


FIG. 3. Volatiles released by different components of a complete plant–host complex: (A) complete complex of BAW larvae feeding on corn seedlings; (B) water-washed corn seedlings that were damaged by BAW larvae; (C) BAW frass wiped from the damaged corn seedlings; and (D) starved water-washed BAW larvae. Peak numbers correspond with those in Figure 1. The asterisk marks pentadecane, a volatile emitted by the oral secretions from BAW larvae. Details on chromatography and internal standards are given with Figure 1.

by frass or larvae (Figure 3). In the collections of the starved larvae, however, an additional peak showed up. This compound was emitted by oral secretions that the larvae regurgitated while fighting among themselves. This was confirmed when collections from hexane extracted filter paper saturated with the oral secretion resulted in the same peak. The compound was identified as pentadecane by GC-MS.

Responses to Synthetic Odor Blend. A collected natural blend and a synthetic mimic (Table 2) were used in further bioassays. Collection procedures were different for Table 1 and Table 2. Volatiles in Table 1 were collected for only 2 hr the day after the larvae had started feeding on the corn seedlings. In contrast, the collections for Table 2 started with larvae on fresh leaves and were continued over a 24-hr period. Several compounds are not released when the larvae just start feeding (Turlings et al., 1990b). Hence, the large differences obtained for some of the major compounds.

A majority of the insects would fly to the extracts of volatiles collected from BAW feeding on corn seedlings (Table 3). Females that were experienced on the synthetic blend and those that had a natural experience responded equally well to the naturally derived volatiles. However, experience did make a difference for the females that were tested to a synthetic blend of the identified compounds. Significantly fewer wasps that had received a natural experience flew

TABLE 2. QUANTITATIVE COMPARISON OF NATURAL AND SYNTHETIC BLENDS USED FOR BIOASSAYS

		Relative %[b]	
Peak[a]	Compound	Natural	Synthetic
1	(*Z*)-3-hexenal	49.3	48.5
2	(*E*)-2-hexenal	1.7	1.8
3	(*Z*)-3-hexen-1-ol	10.4	10.3
4	(*Z*)-3-hexen-1-yl acetate	15.3	14.9
5	Linalool	3.2	2.8
6	(3*E*)-4,8-dimethyl-1,3,7-nonatriene	2.5	2.3
7	Indole	2.3	2.2
8	α-*trans*-bergamotene	3.2	3.4
9	(*E*)-β-farnesene	10.0	11.7
10	(*E*)-nerolidol	1.6	1.6
11	(3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tri-decatetraene	0.5	0.5

[a] Peak numbers in Figure 2.
[b] Percentage of total amount in blend (for each test sample 1% = 205 ng).

TABLE 3. FLIGHT-TUNNEL RESPONSES OF *C. marginiventris* FEMALES WITH DIFFERENT EXPERIENCES TO METHYLENE CHLORIDE EXTRACTS OF SUPER Q-TRAPPED VOLATILES COLLECTED FROM BAW LARVAE FEEDING ON CORN SEEDLINGS (NATURAL), SYNTHETIC MIMIC OF SAME VOLATILES CONTAINING 11 MAJOR COMPOUNDS IN METHYLENE CHLORIDE SOLUTION (SYNTHETIC), AND METHYLENE CHLORIDE ONLY (SOLVENT)

Odor source[a]	Experienced odor	*n*	Number of flights	Average % of flights[b]
Natural	NATURAL	36	24	66.7 (16.7) A
	SYNTHETIC	36	21	58.3 (16.0) A
Synthetic	NATURAL	36	11	30.6 (11.5) B
	SYNTHETIC	36	19	52.8 (15.0) A
Solvent	NATURAL	36	0	0.0 C

[a] Blends from Table 2 were used in amounts equivalent to 200–300 CME (1% = 25 ng).
[b] On six different days six females were tested for each treatment. Standard deviations in parenthesis. Analysis of variance was performed on the daily percentages after arcsin transformation, followed by Duncan's multiple-range test. Different letters indicate significant differences between treatments ($P < 0.05$)

to the synthetic blend than did females that had received a synthetic experience. The responsiveness exhibited by females with a synthetic experience to a synthetic odor blend did not differ from that of females that were tested to the natural extract. None of the tested females flew to the solvent alone.

DISCUSSION

C. marginiventris females responded in a dose-related manner to the collected volatiles (Figure 1). The reduced response at the highest dose tested indicated that for optimal bioassay results release rates should be balanced carefully. By applying the extracts to filter paper, the obtained release rates were obviously far from natural. Before the importance of each individual compound in the attraction of *C. marginiventris* can be determined, exact formulations resulting in release rates similar to a natural situation (Table 1) must be obtained.

The technique of collecting plant volatiles directly from an airstream passed over the odor source has some major advantages over the often applied harsh methods of extraction or steam distillation. The latter two techniques give no indication of how much of each identified compound is actually released into the environment and may result in destruction or isomerization of essential chemicals. This may account for our finding that (*Z*)-3-hexenal was one of the major components, whereas elsewhere it is seldom reported as a plant volatile. Due to its high volatility, (*Z*)-3-hexenal is much harder to collect than the other

green leaf volatiles, including (*E*)-2-hexenal. This is illustrated by our preliminary attempts to re-collect the compounds from the GC as described earlier. Initially, a (U-) tube immersed in liquid nitrogen was used; collection efficiency was extremely poor for (*Z*)-3-hexenal, while it was high for all other volatiles. Only when the collection tubes were used that contained a small amount of Super Q adsorbent was it possible to collect (*Z*)-3-hexenal effectively. High amounts of (*Z*)-3-hexenal are not only characteristic for BAW damage or corn. Preliminary results showed that artificial damage of corn and damage of other plants (i.e., cotton, tomato, and cowpea) resulted in the release of similar relative amounts of (*Z*)-3-hexenal (unpublished data). Buttery et al. (1987) reported on the fast isomerization of (*Z*)-3-hexenal [probably into (*E*)-2-hexenal] in crushed tomato leaves. Our respective results suggest that damaged green leaves release much more (*Z*)-3-hexenal than previously reported.

When Buttery and Ling (1984) collected the volatiles of corn plants that were cut at the stem, they also found (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, linalool, and (*E*)-β-farnesene. Thompson et al. (1974) extracted the essential oil from corn and identified 59 compounds, including indole and nerolidol. No previous reports on corn volatiles mention α-*trans*-bergamotene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, or (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. The latter two apparently related methylene terpenoids were recently reported by Maurer et al. (1986), who found them in the oil of *Elettaria cardamomum* (cardamom oil). Kaiser (1987) (as referenced by Dicke et al., 1990a) reported these compounds from night-scented flowers of different plant species that are pollinated by moths. These compounds are released by Lima bean leaves (Dicke et al., 1990a) and cucumber leaves (Dicke et al., 1990b) that have been subjected to spider mite infestation (see below).

The terpenoid α-*trans*-bergamotene appears to be rare. It has been reported from cotton (Minyard et al., 1966) and from buck's horn (staghorn sumac) (*Rhus typhina*) (Bestmann et al., 1988). (*E*)-β-Farnesene was the most predominant terpenoid in the collections. It has been identified previously from corn leaves by Buttery and Ling (1984), but they found it in much smaller amounts relative to the other compounds. (*E*)-β-Farnesene attracts a chalcid wasp (Kamm and Buttery, 1983) and serves as an alarm pheromone for aphids (Bowers et al., 1972; Edwards et al., 1973; Wohlers, 1981). The *E* isomer of nerolidol is present in the essential oil of several *Melaleuca* tree species (Jones and Harvey, 1936; Naves, 1960; Doskotch et al., 1980). Picker et al. (1976) found it also in the bark of the Australian tree *Flindersia laevicarpa*. Doskotch et al., (1980), who isolated (*E*,*S*)-nerolidol from *Melaleuca leucadendron* leaves, showed that it functions as a feeding deterrent for gypsy moth larvae.

We still need to establish which optical isomers of linalool, α-*trans*-bergamotene, and (*E*)-nerolidol are released by corn seedlings. We used racemic mixtures of the latter three in our synthetic blend, and this may explain why

females with a natural experience did not respond well to the synthetic blend (Table 3). An experience with the blend that contained the racemic mixtures, however, increased the responses to a level comparable to the responses to a natural blend. These results again illustrate the significant effects of experience on the responses to airborne semiochemicals in parasitoids. If, in the future, synthetic blends are used to condition parasitoids to perform more effectively when released in host-infested areas, the best results are likely to be obtained with the closest mimics of naturally released odors.

The damaged plants are clearly the main source of the identified compounds (Figure 3). This agrees with the responses of the parasitoids to the different components of a complete plant–host complex (Turlings et al., 1991). The wasps are significantly more attracted to the damaged plants than to frass or to larvae. Undamaged plants are far less attractive (Turlings et al., 1991), and here it is shown that undamaged plants release only minute amounts of some of the compounds (Figure 2). That undamaged plants are relatively odorless may be a trait that makes them inconspicuous to herbivores. When under attack by herbivores, however, a dramatic change occurs in the number and amounts of volatiles plants release. Results similar to ours were obtained by Dicke and coworkers, who found that spider mite-infested plants initiate the release of terpenoids (Dicke and Sabelis, 1988; Dicke et al., 1990a,b). The compounds were not released by undamaged plants, nor were they detected from artificially damaged leaves. Of the compounds that we identified as being released by BAW-damaged corn, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, linalool, and the two methylene terpenoids also were released by spider mite-infested Lima bean (Dicke et al., 1990a). At least one of the methylene terpenoids, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, is involved in the attraction of predatory mites that feed on spider mites (Dicke and Sabelis, 1988; Dicke et al., 1990a,b). Preliminary results suggest that these methylene terpenoids are released by several green plants that have been under attack by herbivores (unpublished data), and they may elicit behavioral responses in a variety of insects. These plant responses may be direct defenses against their attackers, as high terpenoid contents inhibit insect feeding in several cases (e.g., Doskotch et al., 1980; Mihaliak et al., 1987; Gunasena et al., 1988; Turlings and Tumlinson, 1991). In addition, however, the volatiles released by the injured plants may serve as signals to attract natural enemies of herbivores (Dicke and Sabelis, 1988; Dicke et al., 1990a,b; Turlings et al., 1990b; Turlings and Tumlinson, 1991).

Acknowledgments—We are particularly grateful to B.D. Dueben and R.E. Murphy for valuable advice and assistance. We thank Drs. R.J. Bartelt, L.B. Bjostad, M. Dicke, F.J. Eller, P. Greany, and R.L. Jones for their helpful comments on the initial manuscript. D.B. McIlwaine at Brown University provided us with α-*trans*-bergamotene. Dr. V. Chew provided us with valuable statistical advice.

REFERENCES

ALPHEN, J.J.M. VAN, and VET, L.E.M. 1986. An evolutionary approach to host finding and selection, pp. 23–61, *in* J.K. Waage, and D.J. Greathead (eds.). Insect Parasitoids. Academic Press, London.

BESTMANN, H.-J., CLASSEN, B., KOBOLD, U., VOSTROWSKY, O., KLINGAUF, F., and STEIN, U. 1988. Steam volatile constituents from leaves of *Rhus typhina*. *Phytochemistry* 27:85–90.

BOWERS, W.S., NAULT, L.R., WEBB, R.E., and DUTKY, S.R. 1972. Aphid alarm pheromone: Isolation, identification, synthesis. *Science* 177:1121–1122.

BROWNLEE, R.G., and SILVERSTEIN, R.M. 1968. A micro-preparative gas chromatograph and a modified carbon skeleton determinator. *Anal. Chem.* 40:2077–2079.

BUTTERY, R.G., and LING, L.C. 1984. Corn leaf volatiles: Identification using tenax trapping for possible insect attractants. *J. Agric. Food Chem.* 32:1104–1106.

BUTTERY, R.G., TERANISHI, R., and LING, L.C. 1987. Fresh tomato aroma volatiles: A quantitative study. *J. Agric. Food Chem.* 35:540–544.

COREY, E.J., CANE, D.E., and LIBIT, L. 1971. The synthesis of racemic α-*trans*- and β-*trans*-bergamotene. *J. Am. Chem. Soc.* 93:7016–7021.

DICKE, M., and SABELIS, M.W. 1988. How plants obtain predatory mites as bodyguards. *Neth. J. Zool.* 38:148–165.

DICKE, M., VAN BEEK, T.A., POSTHUMUS, M.A., BEN DOM, N., VAN BOKHOVEN, H., and DE GROOT, Æ. 1990a. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. *J. Chem. Ecol.* 16:381–396.

DICKE, M., SABELIS, M.W., TAKABAYASHI, J., BRUIN, J., and POSTHUMUS, M.A. 1990b. Plant strategies of manipulating predator-prey interactions through allelochemicals: Prospects for application in pest control. *J. Chem. Ecol.* 16:3091–3118.

DMOCH, J., LEWIS, W.J., MARTIN, P.B., and NORDLUND, D.A. 1985. Role of the host-produced stimuli and learning in host selection behavior of *Cotesia (=Apanteles) marginiventris* (Cresson). *J. Chem. Ecol.* 11:453–463.

DOSKOTCH, R.W., CHENC, H.-Y., ODELL, T.M., and GIRARD, L. 1980. Nerolidol: An antifeeding sesquiterpene alcohol for gypsy moth larvae from *Melaleuca leucadendron*. *J. Chem. Ecol.* 6:845–851.

EDWARDS, L.J., SIDDAIL, J.B., DUNHAM, L.L., UDEN, P., and KISLOW, C.J. 1973. *trans*-β-Farnesene alarm pheromone of the green peach aphid, *Myzus persicae* Sulzer. *Nature* 241:126–127.

ELLER, F.J. 1990. Foraging behavior of *Microplitis croceipes*, a parasitoid of *Heliothis* species. PhD dissertation. University of Florida, Gainesville. 221 pp.

GROB, K., JR. 1982. Partial solvent trapping in capillary gas chromatography. Description of a solvent effect. *J. Chromatogr.* 251:235–248.

GUNASENA, G.H., VINSON, S.B., WILLIAMS, H.J., and STIPANOVIC, R.D. 1988. Effects of caryophyllene, caryophyllene oxide, and their interaction with gossypol on the growth and development of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 81:93–97.

JONES, T.G.H., and HARVEY, J.M. 1936. Essential oils from the Queensland flora. Part VIII. The identity of melaleucol with nerolidol. *Proc. R. Soc. Queensl.* 47:92–93.

KAISER, R. 1987. Night-scented flowers, not only attractive to moths. Abstracts EUCHEM conference: Semiochemicals in the Plant and Animal Kingdoms. October 12–16, 1987, Angers, France.

KAMM, J.A., and BUTTERY, R.G. 1983. Response of the alfalfa seed chalcid, *Bruchophagus roddi*, to alfalfa volatiles. *Entomol. Exp. Appl.* 23:129–134.

KING, E.G., and LEPPLA, N.C. 1984. Advances and Challenges in Insect Rearing. Agricultural Research Service, USDA, U.S. Government Printing Office, Washington, D.C.

KOVATS, E. 1965. Retention index system. *Adv. Chromatogr.* 1:229–234.

LEWIS, W.J., and NORDLUND, D.A. 1985. Behavior-modifying chemicals to enhance natural enemy effectiveness, pp. 89–101, *in* M.A. Hoy, and D.C. Herzog (eds.). Biological Control in Agricultural IPM Systems. Academic Press, New York.

LEWIS, W.J., and TUMLINSON, J.H. 1988. Host detection by chemically mediated associative learning in a parasitic wasp. *Nature* 331:257–259.

LOKE, W.H., and ASHLEY, T.R. 1984a. Behavioral and biological responses of *Cotesia marginiventris* to kairomones of the fall armyworm, *Spodoptera frugiperda*. *J. Chem. Ecol.* 10:521–529.

LOKE, W.H., and ASHLEY, T.R. 1984b. Sources of fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), kairomones eliciting host-finding behavior in *Cotesia (=Apanteles) marginiventris* (Hymenoptera: Braconidae). *J. Chem. Ecol.* 10:1019–1027.

LOKE, W.H., and ASHLEY, T.R. 1984c. Potential uses of kairomones for behavioral manipulation of *Cotesia marginiventris* (Cresson). *J. Chem. Ecol.* 10:1377–1384.

LOKE, W.H., ASHLEY, T.R., and SAILER, R.I. 1983. Influence of fall armyworm. *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae and corn plant damage on host finding in *Apanteles marginiventris* (Hymenoptera: Braconidae). *Environ. Entomol.* 12:911–915.

MAURER, B., HAUSER, A., and FROIDEVAUX, J.C. 1986. (*E*)-4,8-Dimethyl-1,3,7-nonatriene and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, two unusual hydrocarbons from cardamom, oil. *Tetrahedron Lett.* 27:2111–2112.

MCCUTCHEON, G.S., and TURNIPSEED, S.G. 1981. Parasites of Lepidopterous larvae in insect resistant and susceptible soybeans in South Carolina. *Environ. Entomol.* 10:69–74.

MIHALIAK, C.A., COUVET, D., and LINCOLN, D.E. 1987. Inhibition of feeding by a generalist insect due to increased volatile leaf terpenes under nitrate-limiting conditions. *J. Chem. Ecol.* 13:2059–2067.

MINYARD, J.P., TUMLINSON, J.H., THOMPSON, A.C., and HEDIN, P.A. 1966. Constituents of the cotton bud. Sesquiterpene hydrocarbons. *J. Agr. Food Chem.* 14:332–336.

NAVES, Y.R. 1960. On the presence of geraniol, nerol, linalool, farnesols, and nerolidols in essential oils. *C.R. Acad. Sci. Ser. C* 251:900–902.

PAIR, S.D., LASTER, M.L., and MARTIN, D.F. 1982. Parasitoids of *Heliothis spp.* (Lepidoptera: Noctuidae) larvae in Mississippi associated with sesame interplantings in cotton, 1971–1974: Implications of host-habitat interaction. *Environ. Entomol.* 11:509–512.

PICKER, K., RITCHIE, E., and TAYLOR, W.C. 1976. The chemical constituents of Australian *Flindersia* species. XXI. An examination of the bark and the leaves of *F. laevicarpa*. *Aust. J. Chem.* 29: 2023–2026.

STENHAGEN, E., ABRAHAMSSON, S., and MCLAFFERTY, F.W. 1974. Registry of Mass Spectral Data. Wiley, New York. 1257 pp.

THOMPSON, A.C., HEDIN, P.A., and GUELDNER, R.C. 1974. Corn bud essential oil. *Phytochemistry* 13:2029–2032.

TINGLE, F.C., ASHLEY, T.R., and MITCHELL, E.R. 1978. Parasites of *Spodoptera exigua*, *S. eridania* (Lep.: Noctuidae) and *Herpetogramma bipunctalis* (Lep.: Pyralidae) collected from *Amaranthus hybridus* in field corn. *Entomophaga* 23:343–347.

TOLLSTEN, L., and BERGSTRÖM, G. 1988. Headspace volatiles of whole plants and macerated plant parts of *Brassica* and *Sinapis*. *Phytochemistry* 27:4013–4018.

TURLINGS, T.C.J., and TUMLINSON, J.H. 1991. Do parasitoids use herbivore-induced plant chemical defenses to locate hosts? *Fla. Entomol.* 74:42–50.

TURLINGS, T.C.J., TUMLINSON, J.H., LEWIS, W.J., and VET, L.E.M. 1989. Beneficial arthropod behavior mediated by airborne semiochemicals. VII. Learning of host-related odors induced

by a brief contact experience with host by-products in *Cotesia marginiventris* (Cresson), a generalist larval parasitoid. *J. Insect Behav.* 2:217–225.

TURLINGS, T.C.J., SCHEEPMAKER, J.W.A., VET, L.E.M., TUMLINSON, J.H., and LEWIS, W.J. 1990a. How contact foraging experiences affect the preferences for host-related odors in the larval parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae). *J. Chem. Ecol.* 16:1577–1589.

TURLINGS, T.C.J., TUMLINSON, J.H., and LEWIS, W.J. 1990b. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250: 1251–1253.

TURLINGS, T.C.J., TUMLINSON, J.H., ELLER, F.J., and LEWIS, W.J. 1991. Larval-damaged plants: Source of volatile synomones that guide the parasitoid *Cotesia marginiventris* to the microhabitat of its hosts. *Entomol. Exp. Appl.* 58:75–82.

VET, L.E.M., and GROENEWOLD, A.W. 1990. Semiochemicals and learning in parasitoids. *J. Chem. Ecol.* 16:3119–3135.

VINSON, S.B. 1981. Habitat location, pp. 51–77, *in* D.A. Nordlund, R.L. Jones, and W.J. Lewis (eds.). Semiochemicals: Their Role in Pest Control. John Wiley & Sons, New York.

VISSER, J.H., VAN STRATEN, S., and MAARSE, H. 1979. Isolation and identification of volatiles in the foliage of potato, *Solanum tuberosum*, a host plant of the colorado beetle, *Leptinotarsa decemlineata*. *J. Chem. Ecol.* 5:13–25.

WESELOH, R.M. 1981. Host location by parasitoids, pp. 79–95, *in* D.A. Nordlund, R.L. Jones, and W.J. Lewis (eds.). Semiochemicals: Their Role in Pest Control. John Wiley & Sons, New York.

WOHLERS, P. 1981. Effects of the alarm pheromone (*E*)-β-farnesene on dispersal behavior of the pea aphid *Acyrthosiphon pisum*. *Entomol. Exp. Appl.* 9:117–124.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

CROSS-REACTION TO SKIN EXTRACT BETWEEN TWO GOBIES, *Asterropteryx semipunctatus* AND *Brachygobius sabanus*

R.J.F. SMITH,* B.J. LAWRENCE, and M.J. SMITH

Biology Department
University of Saskatchewan
Saskatoon, Saskatchewan, Canada, S7N 0W0

(Received May 6, 1991; accepted July 15, 1991)

Abstract—Two gobies that possess alarm pheromones were tested for cross-reactions to water extracts of injured members of the other species in the first such cross-reaction test conducted in the Family Gobiidae. *A. semipunctatus* reacted to extract from injured *Brachygobius sabanus* with the same bobbing and reduction in activity as it shows in response to conspecific extract. However, *B. sabanus* showed a feeding response, including increased activity, to extract from injured *A. semipunctatus*. Even a one-way cross-reaction suggests some degree of homology between the alarm pheromone systems of the two species.

Key Words—Alarm pheromone, Schreckstoff, alarm substance, goby, *Asterropteryx semipunctatus*, *Brachygobius sabanus*, Gobiidae, fish behavior.

INTRODUCTION

In the alarm pheromone systems of ostariophysan fishes (Schutz, 1956; Pfeiffer, 1977; Smith, 1986) and percid darters (Smith, 1979, 1982), the members of the taxonomic group that share the chemical alarm system show some degree of cross-reaction between species or even between genera or families. Although there is often some reduction in response with increasing taxonomic distance, the presence of any cross-reaction probably indicates homology between the alarm systems of the members of a taxonomic group. Such homology of response to a common active compound occurs in the ostariophysan fishes (Schutz, 1956; Pfeiffer, 1982).

*To whom correspondence should be addressed.

0098-0331/91/1100-2253$06.50/0

Two species of gobiid fishes, *Asterropteryx semipunctatus* (Smith, 1989) and *Brachygobius sabanus* (Smith and Lawrence, 1992) have now been reported to show a defensive response (fright reaction) to chemicals from injured conspecifics. Six other gobiid species, *Gobius niger*, *G. paganellus*, *Pomatoschistus microps*, *P. minutus* (Pfeiffer, 1977), *Gnatholepis anjerensis* (Smith, 1989), and *Coryphopterus nicholsii* (Hugie, personal communication) have been tested and apparently do not show a fright reaction to chemicals from injured conspecifics. The question thus arises, do *A. semipunctatus* and *B. sabanus* react to each other's skin extract? If they do cross-react, it may indicate that there is a homologous component to goby alarm pheromones. If they do not cross react, it would support, but not confirm, the hypothesis that chemical alarm signaling has arisen independently in two gobiid species. We compared cross-reactions between the two species using the same observers and criteria as had been used in the original studies of each species.

METHODS AND MATERIALS

Asterropteryx semipunctatus were collected from patch reefs in Kaneohe Bay, Oahu, Hawaii, by scuba divers and snorkelers using plastic bags or hand nets. Those *A. semipunctatus* that were used as receivers for skin extract from *Brachygobius sabanus* were kept in running seawater in holding tanks at the Hawaii Institute of Marine Biology, then transferred to 38-liter observation aquaria in groups of three. They were allowed at least seven days of acclimation before observation. The holding and observation aquaria were maintained between 24° and 27°C under a 12:12 hr light–dark photoperiod. During this time, the fish were fed with live plankton collected in Kaneohe Bay. *A. semipunctatus* that were used as extract donors were transported by air to the Department of Biology, University of Saskatchewan, where they were maintained in artificial seawater at 24°C under a 12:12 hr light–dark photoperiod and fed live and frozen brine shrimp and commercial flake food. Extract receivers included 12 males (mean total length 34 mm), 13 females (mean total length 37 mm), and two small fish that could not be sexed (mean 22 mm). Extract donors were four males (mean 28 mm) and six females (mean 25 mm).

Brachygobius were purchased from commercial pet suppliers in Calgary, Alberta, and Kaneohe and Aiea, Hawaii. Our specimens (ROM 56628) were tentatively identified by R. Winterbottom of the Royal Ontario Museum as *Brachygobius sabanus* Inger, 1958. There are some inconsistencies involving the degree of scalation of the operculum and pelvic-fin length of our specimens and the description of the type specimens (collected in North Borneo). The original source of the fish is unknown. A sample of the receivers, eight males (mean total length 25 mm) and two females (mean 27 mm), was measured. The nine donors included four females (mean 27 mm) and five males (mean 24 mm).

B. sabanus were kept in brackish water prepared by mixing 37 liters of freshwater with 1 liter of seawater. At the Hawaii Institute of Marine Biology they were fed live brine shrimp and marine plankton and kept under a 12 : 12 hr light–dark photoperiod between 24° and 27°C. At the University of Saskatchewan they were kept at 27–29°C under a 12 : 12 hr light–dark photoperiod and fed live whiteworms and live brine shrimp. They were observed in groups of four and were allowed at least seven days of acclimation before being observed.

All observations were conducted using 38-liter tanks. The observer sat quietly in full view of the fish and recorded their behavior using a mechanical counter. The behavior of *B. sabanus* receivers was also recorded on videotape for later analysis of swimming time. The observation tanks in Hawaii, housing *A. semipunctatus* receivers, were equipped with seawater inlet tubes and the chemical stimuli were injected into the inlet. The observation tanks in Saskatoon, housing *B. sabanus* receivers, were equipped with power filters and the chemical stimuli were injected into the inlet tube running from the filter to the tank. The arrangement of sand and shelters was the same as in the original studies of alarm pheromone responses in the two species (Smith, 1989; Smith and Lawrence, 1992).

In the standard test procedure, the fish were observed for 15 min; then the chemical stimuli were injected into the inlet tube, and the fish were observed for an additional 15 min. The totals for the two 15-min observation periods were compared for each tank of fish. This procedure was repeated nine times for *A. semipunctatus* receivers and 10 times for *B. sabanus* receivers, each time with a different group of fish. Each group was used only once. Since the fish in a tank cannot be considered independent, comparisons were made on the basis of group totals. Following each test, food dye was injected into the system and the time from injection to dispersal through the tanks was recorded as an estimate of the time from stimulus injection to the reception of the stimulus by all fish in the tank. The mean time for the dye to enter the tank was 22 sec, with 181 sec for full dispersal in the *A. semipunctatus* tanks and 10 sec for entry and 32 sec for dispersal in the *B. sabanus* tanks.

A. semipunctatus are negatively buoyant, benthic fish. They move episodically rather than continuously, with movements separated by intervals when they remain stationary. Each time a fish changed position, it was counted as making one move. Bobbing behavior was also counted, in which the anterior part of the goby rose slowly off the bottom, apparently by extension of the pelvic fins, then settled to the bottom again. A fish could move without bobbing and bob without moving. In *A. semipunctatus*, bobbing occurs in response to visual and olfactory stimuli from predators (Smith, 1989; Smith and Smith, 1989). *B. sabanus* are often negatively buoyant and can move about episodically in much the same manner as described above, but they can also swim in

midwater, maintaining neutral buoyancy with their swim bladder. As well as counting moves, we recorded the total swimming time, the time in a 15-min observation period when one or more fish was swimming, for *B. sabanus*. Agonistic encounters were also counted, as in Smith and Lawrence (1992). *B. sabanus* was never observed to bob.

Chemical extracts of injured fish were prepared by killing the donor with a blow to the head, placing it in a clean disposable Petri dish, cutting it several times on each side with a scalpel, rinsing the fish in tank water and drawing up the extract into a clean syringe. *A. semipunctatus* donors were cut 25 times and rinsed with 7 ml of water, and the extract was drawn into a 5-ml syringe. *B. sabanus* donors were cut 50 times, rinsed in 10 ml, and the extract was drawn into a 10-ml syringe. These procedures mimic the original studies for each type of receiver. Extracts were used within 20 min of preparation. Control stimuli, water, and extracts of nongobiid fishes were tested in the original studies of each species and were not repeated here.

The results of each test were analyzed with a Wilcoxon matched-pairs, signed-rank test (Siegel, 1956) comparing the total number of acts, or minutes of swimming, before stimulus introduction with the total after introduction, for each group of fishes.

RESULTS

A. semipunctatus responded to skin extract from *B. sabanus* with decreased activity in eight of the nine trials; the mean change was −15% (Figure 1). There were no bobs during the prestimulus interval but one group performed 63 bobs and another 20 bobs in the poststimulus interval. These two groups showed substantially greater activity reduction than the other groups, −40% and −42%, respectively. Qualitatively, the response of the *A. semipunctatus* resembled the response shown to conspecific extract (Smith, 1989).

B. sabanus responded to skin extract from *A. semipunctatus* with increased moves in all 10 trials; the mean change was +86% (Figure 1). Swimming time did not change significantly, but the number of agonistic encounters increased in eight of 10 groups, by an average of 123%. This response was opposite to the response of *B. sabanus* to conspecific extract and more closely resembled a feeding response (Smith and Lawrence, 1992). Other, qualitative resemblances to a feeding response included increased biting at the substrate, moving out of shelters, and attacking snail shells.

DISCUSSION

A. semipunctatus responds to extract from injured *B. sabanus* with the same sort of antipredator response that it shows in response to extract from injured conspecifics or stimuli from predators (Smith, 1989). The response

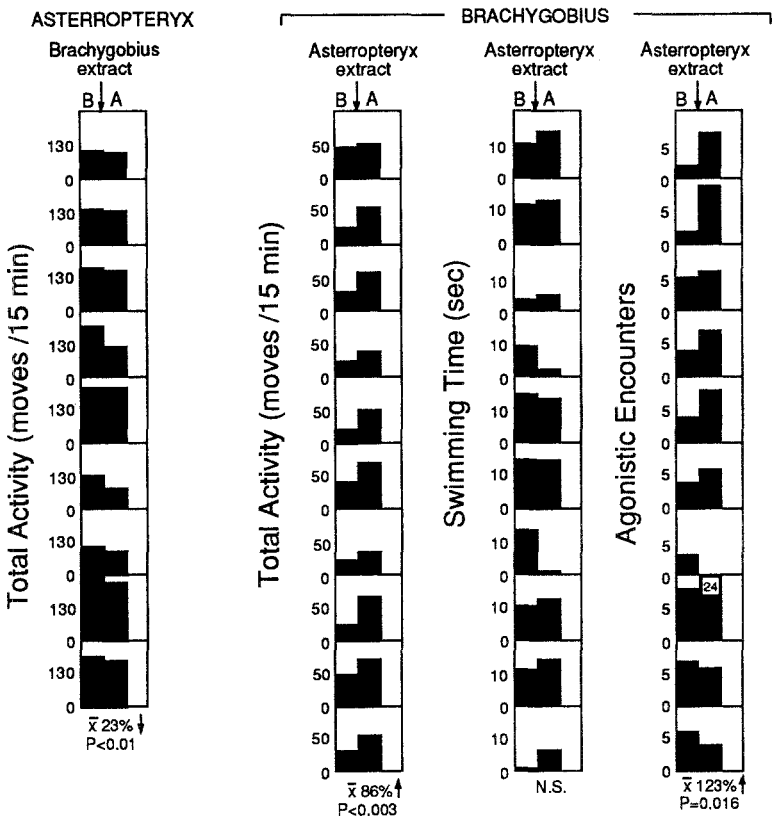


FIG. 1. Behavioral responses of gobies to skin extracts from heterospecific fishes. Values plotted are the number of moves by *Asterropteryx semipunctatus* in 15-min intervals before (B) and after (A) exposure to extract from injured *Brachygobius sabanus* and the number of moves, total swimming time, and number of agonistic encounters by *B. sabanus* in 15-min intervals before and after exposure to extract from injured *A. semipunctatus*. P = probability based on Wilcoxon matched-pairs, signed-rank test, N.S. = not significant.

seems slightly weaker than the response to conspecific extract—a mean decrease in moves of 23% with bobbing in six tanks of 10 (Smith, 1989)—but this is reasonable considering that the two species are in different genera. Schutz (1956) catalogued several examples of declining cross-reaction with phylogenetic separation in the alarm system of ostariophysan fishes. The presence of two very strong reactions to *B. sabanus* extract with approximately 40% activity reduction and frequent bobbing reinforces the conclusion that *A. semipunctatus* responds to *B. sabanus* extract with an antipredator response. In contrast to these results, control tests by Smith (1989) had demonstrated that *A. semipunc-*

tatus showed small, about 5%, nonsignificant increases in activity and no increase in bobbing when given control water or water that had contained uninjured conspecifics as a stimulus. Extract from a nongobiid fish, the Mexican molly *Poecilia mexicana*, induced an 18% increase in activity but no bobbing, and extract from *Gnatholepis anjerensis*, a goby that lacks alarm pheromone, led to a similar 18% increase in activity (Smith, 1989).

B. sabanus responds to extract from injured *A. semipunctatus* as if it were a feeding stimulus. In control tests conducted by Smith and Lawrence (1992), *B. sabanus* responded to extract of an injured nongobiid, the guppy *Poecilia reticulata*, with significant increases in moves, swimming, and agonistic encounters. This response was characterized as a feeding response because it resembled the response to brine shrimp extract. The response to conspecific extract was a reduction in moves, swimming, and agonistic activity, associated with moving to shelters. The response of *B. sabanus* to *A. semipunctatus* extract differed from the feeding responses described by Smith and Lawrence (1992); responses were directed more to the bottom of the tank, whereas the response to guppy extract and brine shrimp extract was directed more to the surface or midwater. The significance of this observation in not clear, although cyprinodonts are usually surface fish while gobies are often benthic.

A possible explanation for this one-way cross-reaction between two gobiid species that have been shown to possess alarm pheromones is that the two species share active components in their chemical alarm signal but that *B. sabanus* is more selective in avoiding "false alarms" from injured heterospecific fishes. This selectivity might be favored if *B. sabanus* shared its environment with other pheromone-bearing gobies that were subject to attack by predators that do not prey on *B. sabanus* or if *B. sabanus* preyed on other gobies, perhaps by fin nipping. *A. semipunctatus* does not respond indiscriminately to all goby skin extracts since it responds with feeding behavior to skin extract from injured *Gnatholepis anjerensis*, a sympatric goby (Smith, 1989). Our results suggest that there are homologies between the two gobiid alarm pheromone systems. The phylogeny of gobies is not clear, and it is therefore difficult to fit our results into any phylogenetic pattern.

Acknowledgments—This study was supported by facilities provided by the Hawaii Institute of Marine Biology and the University of Saskatchewan, and by funding from the Natural Science and Engineering Research Council of Canada and the Edwin W. Pauley Foundation grant to the Hawaii Institute of Marine Biology Summer Program. We thank Frank Stanton, Lisa Privitera, Jeff Mahon, and Steve Woytowich for collecting fish; Elizabeth Hinks for feeding and maintaining fish; and Alicia Mathis for reading the manuscript and offering helpful comments.

REFERENCES

PFEIFFER, W. 1977. The distribution of fright reaction and alarm substance cells in fishes. *Copeia* 1977:653-665.

PFEIFFER, W. 1982. Chemical signals in communication, pp. 306–326, *in* T.J. Hara (ed.). Chemocreception in Fishes. Amsterdam, Elsevier.

SCHUTZ, F. 1956. Vergleichende Untersuchungen über die Schreckreaktion bei Fischen und deren Verbreitung. *Z. Vergl. Physiol.* 38:84–135.

SIEGEL, S. 1956. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York. 312 pp.

SMITH, R.J.F. 1979. Alarm reaction of Iowa and johnny darters (*Etheostoma*, Percidae, Pisces) to chemicals from injured conspecifics. *Can. J. Zool.* 57:1278–1282.

SMITH, R.J.F. 1982. Reaction of *Percina nigrofasciata*, *Ammocrypta beani*, and *Etheostoma swaini* (Percidae, Pisces) to conspecific and intergeneric skin extracts. *Can. J. Zool.* 60:1067–1072.

SMITH, R.J.F. 1986. The evolution of chemical alarm signals in fishes, pp. 99–115, in D. Duvall, D. Müller-Schwarze, and R.M. Silverstein (eds.). Chemical Signals in Vertebrates, 4. Plenum Press, New York.

SMITH, R.J.F. 1989. The response of *Asterropteryx semipunctatus* and *Gnatholepis anjerensis* (Pisces, Gobiidae) to chemical stimuli from injured conspecifics, an alarm response in gobies. *Ethology*81:279–290.

SMITH, R.J.F. and LAWRENCE, B.J. 1992. The response of a bumblebee goby, *Brachygobius sabanus*, to chemical stimuli from injured conspecifics. *Environ. Biol. Fish.* In press.

SMITH, R.J.F., and SMITH, M.J. 1989. Predator-recognition behavior in two species of gobies, *Asterropteryx semipunctatus* and *Gnatholepis anjerensis*. *Ethology* 83:19–30.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

EFFECT OF APIFOROL AND APIGENINIDIN ON GROWTH OF SELECTED FUNGI

CINDY SCHUTT[1] and DAVID NETZLY*

Biology Department
Hope College
Holland, Michigan 49423-3698

(Received December 10, 1990; accepted July 16, 1991)

Abstract—Selected fungi were grown on agar plates in the presence of naringenin, apiforol, apiforol 7-*O*-rhamnoglucoside, or apigeninidin. Of the four compounds tested, only apigeninidin inhibited the growth of *Fusarium oxysporum*, *Gibberella zeae*, *Gliocladium roseum*, *Alternaria solani*, and *Phytophthora infestans*. In contrast, the growth of *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Rhizopus stolonifer* (− and +) was not effected by any compound. Since apigeninidin is present in seeds of *Sorghum* sp., we hypothesize that apigeninidin may play a role in mold resistance and that apiforol accumulates as a biosynthetic precursor of apigeninidin, not as a fungal defense compound.

Key Words—Fungi, mold resistance, apiforol, 3-deoxyanthocyanidin, apigeninidin, flavan-4-ol, allelopathy, *Sorghum bicolor*.

INTRODUCTION

In many arid regions of the world, sorghum is grown as the major food crop. However, much of the potential yield is lost due to preharvest fungal growth (molding) on seeds of certain *Sorghum bicolor* lines (Frederiksen, 1986). A positive correlation has been reported between seed extracts of sorghum that contain high amounts of flavan-4-ols and increased mold resistance from both greenhouse-grown (Jambunathan et al., 1986) and field-grown sorghum lines (Jambunathan et al., 1990). Recently, apigeninidin, a 3-deoxyanthocyanidin,

*To whom correspondence should be addressed.
[1] Present address: Department of Criminal Justice, Baker Hall, Michigan State University, East Lansing, Michigan 48824.

0098-0331/91/1100-2261$06.50/0

was reported to accumulate in response to *Helminthosporium maydis* and *Colletotrichum graminicola* infection in sorghum mesocotyls (Nicholson et al., 1989; Snyder and Nicholson, 1990). Both apiforol (flavan-4-ol) and apigeninidin are present in seeds of certain sorghum cultivars (Nip and Burns, 1969; Watterson and Butler, 1983). Apiforol is the presumed precursor of apigeninidin (see Stich and Forkman, 1988) (Figure 1). The purpose of this work was to determine (1) if apigeninidin is inhibitory to fungi other than members of the Deuteromycetes and (2) if apiforol (flavan-4-ol) or apiforol 7-*O*-rhamnoglucoside inhibit the growth of any fungal species.

METHODS AND MATERIALS

Flavan-4-ol and 3-Deoxyanthocyanidin Chemical Synthesis. Naringenin (100 mg, Sigma) or naringin (100 mg, Sigma) was dissolved in 2 ml of 100% methanol and reduced with sodium borohydride (50 mg). After 45 min, the reduction product was acidified with 10% (v/v) acetic acid in methanol to quench the borohydride and to lower the pH of the solution to between 6 and 7. This solution was evaporated to dryness at 35°C. Residue containing apiforol or apiforol 7-*O*-rhamnoglucoside was scraped off the flask and stored dry at room temperature in the dark. Presence of flavan-4-ol was confirmed by the formation of a red pigment in the presence of 30% (v/v) conc. HCl in isopropanol.

Apigeninidin was synthesized from the residue containing apiforol. Residue was reconstituted in a small amount of methanol and the solution was added to 7 ml of 2 N HCl and heated (100°C) for 1 hr. Apigeninidin was extracted from the acidic solution with ethyl acetate and taken to dryness at 35°C.

Fungal Bioassay. Residue containing apiforol, apigeninidin, and apiforol 7-*O* -rhamnoglucoside was dissolved in absolute methanol and applied to filter

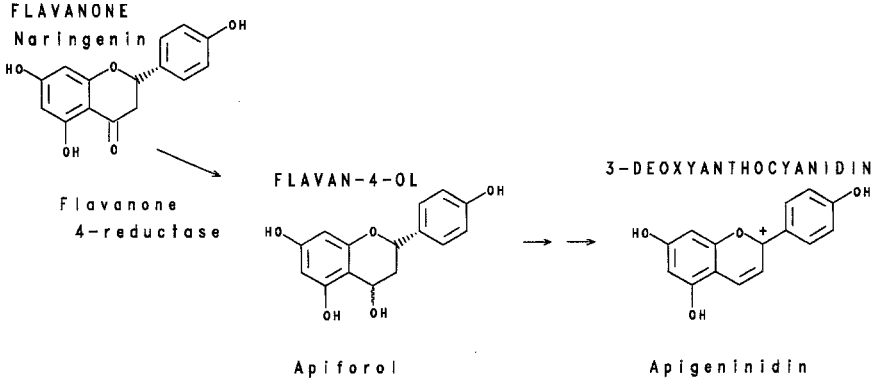


FIG. 1. Proposed biosynthetic pathway for apigeninidin (3-deoxyanthocyanidin).

paper disks (1.2 cm diameter). Control disks had only methanol applied. Petri plates containing potato dextrose agar (Difco) were inoculated with propagules of the following fungi obtained from Carolina Biological Supply Co.: *Fusarium oxysporum*, *Gibberella zeae*, *Gliocladium roseum*, *Rhizopus stolonifer* (+ and − strains), *Alternaria solani*, *Phytophthora infestans*, *Rhizoctonia solani*, or *Sclerotium rolfsii*. Filter disks containing the individual compounds were placed in the center of the agar plates after the methanol had evaporated. Petri plates were incubated in the dark at room temperature. The ring of growth inhibition around the filter disks was measured (average of three measurements) each day for three days, or, in the case of apigeninidin, for up to 10 days.

RESULTS

The growth of *Fusarium oxysporum*, *Gibberella zeae*, and *Gliocladium roseum* was inhibited by apigeninidin (3.6 μmol) in the three-day experiment (Table 1). In contrast, apiforol and apiforol 7-*O*-rhamnoglucoside were not effective in inhibiting the growth of any of the fungal species tested (Table 1). Naringenin, the biosynthetic precursor of apiforol, was also ineffective as a fungal growth inhibitor.

Based on these results, the effect of apigeninidin on the growth of five additional fungal species was tested (Figure 2). The length of this experiment was extended to 10 days.

The growth of *Alternaria solani* and *Phytophthora infestans* was inhibited by apigeninidin. The growth of *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Rhizopus stolonifer* (−) was not appreciably inhibited. Of the fungi tested, *R. stolonifer* (− and +) was the least sensitive to apigeninidin.

DISCUSSION

Apigeninidin inhibited the growth of members of the Deuteromycetes, Oomycetes, and Ascomycetes. Since apigeninidin is accumulated in sorghum seeds and it inhibits the growth of *Alternaria solani*, which is a common pathogen on sorghum grain (Frederiksen, 1986), apigeninidin may have a direct role in the resistance of *A. solani* on sorghum grain. However, there is evidence that mold resistance is more complex than can be explained by one chemical such as apigeninidin (see Jambunathan et al., 1990).

Apiforol has been detected in sorghum seeds (Bate-Smith and Rasper, 1969; Watterson and Butler, 1983; Jambunathan et al., 1990). Apiforol 7-*O*-rhamnoglucoside has not been found as a natural constituent of sorghum but was tested in order to determine what affect glycosylation might have an apiforol

TABLE 1. EFFECT OF APIGENINIDIN, APIFOROL, APIFOROL 7-*O*-RHAMNOGLUCOSIDE AND NARINGENIN ON GROWTH OF FOUR FUNGAL SPECIES

Fungi	Inhibition zone (mm)[a]			
	Apigeninidin (3.6 μmol)	Apiforol (3.6 μmol)	Apiforol glucoside (5.3 μmol)	Naringenin (3.6 μol)
Fusarium oxysporum				
Day 1	–	–	–	–
Day 2	7.0 ± 2[b]	0	0	0
Day 3	4.8 ± 1.2	0	0	0
Gibberella zeae				
Day 1	–	–	–	–
Day 2	6.8 ± 0.4	0	0	0
Day 3	5.4 ± 1.6	0	0	0
Gliocladium roseum				
Day 1	–	–	–	–
Day 2	4.9 ± 1.7	0	0	0
Day 3	2.6 ± 0.6	0	0	0
Rhizopus stolonifer (+)				
Day 1	5.9 ± 1.2	0	0	0
Day 2	0	0	0	0
Day 3	0	0	0	0

[a] Inhibition zones (width of growth inhibition ring) were measured each day for three days. A minus symbol indicates fungi had not grown sufficiently to obtain data.
[b] Values are an aveage ± SD of two to five experiments.

activity. Our results with these flavan-4-ols do not rule out the possibility that other flavan-4-ols and/or their glycosides may be biologically active.

In our method of apiforol synthesis, some naringenin was detected and presumably some self-polymerization products were present in the final apiforol preparation. Flavan-4-ols readily undergo acid-induced self-polymerization reactions (Stich and Forkmann, 1988). Therefore, we tested naringenin by itself and found it to be inactive. However, we do not know if a polymer of apiforol would have different biological activity than its monomer. Similarly, apigeninidin undergoes apparent self-polymerization reactions under acidic conditions after its synthesis, as determined by the difference in mobility between freshly prepared and "aged" apigeninidin on paper chromatography in 2 N HCl (Netzly, unpublished).

In summary, apigeninidin was found to inhibit the growth of various fungi. It is not known if this compound has other biological activities as well. Preliminary data indicate that its mode of action is not as a mutagen (Netzly, unpub-

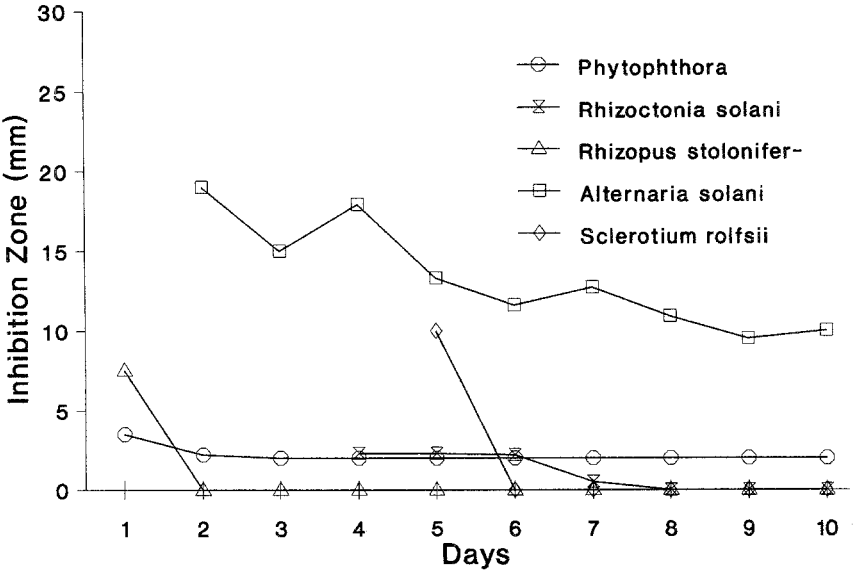


FIG. 2. Effect of apigeninidin (3.6 μmol) on the growth of additional fungal species. Values are an average of four to five experiments.

lished). Secondly, while there is a direct correlation between mold resistance and the presence of flavan-4-ols in sorghum seeds (Jambunathan et al., 1986), our data suggest that apiforol does not directly affect the growth of any fungal species tested. We hypothesize that the correlation between mold resistance and flavan-4-ol accumulation in seeds is because apiforol (flavan-4-ol) is the presumed precursor of apigeninidin, a fungal growth inhibitor.

Acknowledgments—This work was supported by NSF-REU grant 8804082-01 and Research Corporation grant C-2608.

REFERENCES

BATE-SMITH, E.C., and RASPER, V. 1969. Tannins of grain sorghum: Luteoforol (leucoluteolinidin), 3′,4,4′,5,7-pentahydroxyflavan. *J. Food Sci.* 34:203–209.

FREDERIKSEN, R.A. 1986. Compendium of Sorghum Diseases. American Phytophathological Society, St. Paul, Minnesota. 82 pp.

JAMBUNATHAN, R., BUTLER, L.G., BANDYOPADHYAY, R., and MUGHOGHO, L.K. 1986. Polyphenol concentrations in grain, leaf, and callus tissues of mold-susceptible and mold-resistant sorghum cultivars. *J. Agric. Food Chem.* 34:425–429.

JAMBUNATHAN, R., KHERDEKAR, M.S., and BANDYOPADHYAY, R. 1990. Flavan-4-ols concentration

in mold-susceptible and mold-resistant sorghum at different stages of grain development. *J. Agric. Food Chem.* 38:545–548.

NICHOLSON, R.L., KOLLIPARA, S.S., VINCENT, J.R., LYONS, P.C., and CADENA-GOMEZ, G. 1987. Phytoalexin synthesis by the sorghum mesocotyl in response to infection by pathogenic and nonpathogenic fungi. *Proc. Natl. Acad. Sci. U.S.A.* 84:5520–5524.

NIP, W.K., and BURNS, E.E. 1969. Pigment characterization in grain sorghum. I. Red varieties. *Cereal Chem.* 46:490–495.

SNYDER, B.A., and NICHOLSON, R.L. 1990. Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. *Science* 248:1637–1638.

STICH, K., and FORKMANN, G. 1988. Biosynthesis of 3-deoxyanthocyanins with flower extracts from *Sinningia cardinalis. Phytochemistry* 27:785–789.

WATTERSON, J.J., and BUTLER, L.G. 1983. Occurrence of an unusual leucoanthocyanidin and absence of proanthocyanidins in sorghum leaves. *J. Agric. Food Chem.* 31:41–45.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

ALLELOPATHIC DOMINANCE OF *Miscanthus transmorrisonensis* IN AN ALPINE GRASSLAND COMMUNITY IN TAIWAN[1]

CHANG-HUNG CHOU* and YI-FENG LEE

Institute of Botany
Academia Sinica
Taipei, Taiwan 11529
Republic of China

(Received March 6, 1991; accepted July 22, 1991)

Abstract—A study site located at 2600 m elevation in Tartarchia Anpu, Nantou county, Taiwan, exhibits a unique grassland community composed of two principal species, *Miscanthus transmorrisonensis* and *Yushinia niitakayamensis*, and 35 other species. The relative frequencies of the two species are 12% and 11%, while their relative coverages are 25% and 19.5%, respectively. The values for the remaining 35 species are lower than 4% each, while species diversity of the community is −3.04839, indicating great diversity. To elucidate the mechanism of dominance of *M. transmorrisonensis*, allelopathic evaluation of the plant was conducted. Aqueous extracts of *M. transmorrisonensis* plant parts with two ecotypes were bioassayed. The extracts showed significant phytotoxic effects on seed germination and radicle growth of four tested plants: rye grass, lettuce, and two varieties of Chinese cabbage. In addition, rhizosphere soils under *Miscanthus* also exhibited significant phytotoxicity, indicating that allelopathic interaction was involved. Some responsible phytotoxic phenolics, namely, *p*-coumaric, ferulic, vanillic, protocatechuic, *o*-hydroxyphenylacetic, and *m*-hydroxyphenylacetic acids, and 4-hydroxycoumarin and phloridzin were identified. Allelopathy thus can play an important role in regulating plant diversity in the field.

Key Words—*Miscanthus transmorrisonensis*, allelopathy, phytotoxin, population, phenolics, plant diversity, grassland community.

*To whom correspondence should be addressed.

[1] The study was supported by grants to C.H. Chou awarded by the Academia Sinica, Taipei and the National Science Council (NSC-80-0211-B001-25) of The Republic of China.

0098-0331/91/1100-2267$06.50/0

INTRODUCTION

Miscanthus floridulus (Labill.) Warb., a dominant endemic grass, is ubiquitously distributed in areas below 2000 m elevation in Taiwan. On the other hand, *M. transmorrisonensis* is distributed in mountainous areas above 2200 m. Chou et al. (1987) reported that four major clusters of ecotypic populations were found among 27 populations of *M. floridulus*. The unique ecotypes of the species were present in various habitats and adapted to different environmental regimes, such as those of high salinity, polluted soil, and moisture deficiency (Chou and Chang, 1988; Chou and Chen, 1990). On the other hand, the adaptive mechanism of *M. transmorrisonensis* to areas of high elevations in Taiwan depends primarily on temperature (Chou et al., 1991). A reciprocal transplanting experiment conducted on the two plants revealed that *M. transmorrisonensis* could survive in the high temperature of lower elevations but that *M. floridulus* did not survive two severe winters at the higher elevation. Both species displayed relatively pure vegetation, and the allelopathic potential of *M. floridulus* was demonstrated (Chou and Chung, 1974). The mechanism of dominance of *M. transmorrisonensis* has yet to be studied. The growth performance and ecological adaptation of the grass has resulted in excellent survival at low temperatures in Yushan National Park (Chou et al., 1991).

Allelopathy has been regarded as one of the important survival strategies and plays a significant role in the dominance of grassland communities (Rice, 1977, 1984; Muller, 1966; Tinnin and Muller, 1971; Newman and Miller, 1977; Gliessman, 1978). The allelopathic dominance of *M. transmorrisonensis* was evaluated by conducting a series of experiments in fields at Yushan National Park, in a Phytotron of the National Taiwan University, and in a greenhouse and laboratory of the Academia Sinica at Taipei.

METHODS AND MATERIALS

Study Site. The study site chosen for most field experiments in the present study is located at 2600 m elevation at Tartarchia Anpu of the Yushan National Park, Nantou County, Taiwan. The weather pattern of the study site during the period 1988–1990 was described by Chou et al. (1991). The temperature ranged from 4.5°C to 8°C in winter and from 16°C to 18°C in summer. The amount of annual precipitation was high, about 2000–2600 mm. The site at Tartarchia Anpu contains a unique grassland community, comprised of two dominant stands of *Miscanthus transmorrisonensis* and *Yushinia niitakayamensis* (Figure 1).

Field Experiments. The botanical composition of the study site was determined by the quadrat method, 1 × 1 m each, and the coverage and frequency of each species present in the community was measured. Sixty quadrats were randomly used in a 10-hectare section of grassland. Most of the species appear-

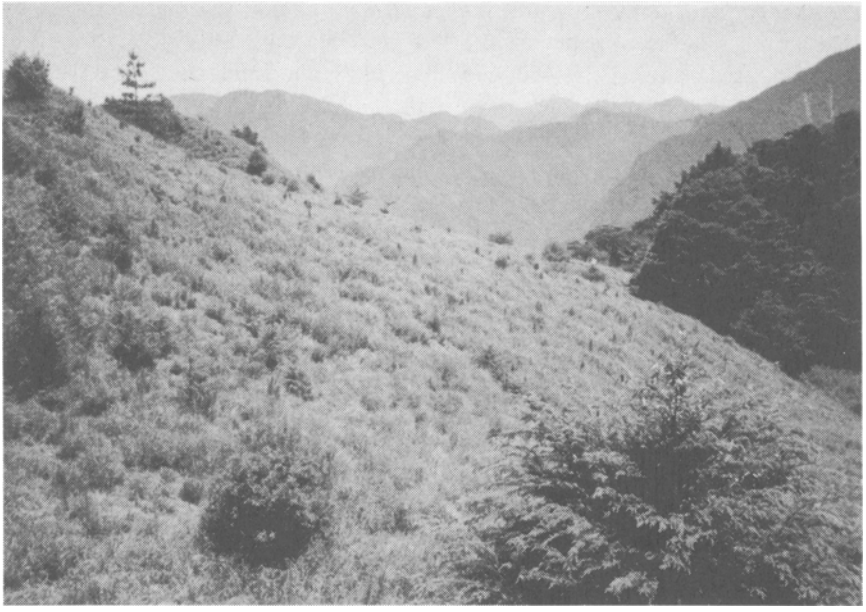


FIG. 1. A unique vegetation of alpine grass, *Miscanthus transmorrisonensis*, exhibits a relatively pure stand at Tartarchia Anpu, Nantou, Taiwan, at 2600 m elevation.

ing in the quadrats were identified, but some were identified only as to genus and need further study. In addition, species diversity of the grassland community was also computed by using the Shannon-Wienner index (Brewer, 1979), defined as follows:

$$H' = \sum P_i \times \ln P_i \quad (1)$$

where P_i is the number of individuals of each species over total number of individuals in the community, and $\ln P_i$ is the natural log of P_i.

Clearing Experiment. Four quadrats, 2 × 2 m each, in the Tartarchia Anpu grassland were selected for a ground-clearing experiment in order to learn the succession of vegetation in the area.

Materials. Plant materials were collected from the study site. Some of the samples were air-dried at room temperature, and the remainder were stored in a cold room prior to use. Samples collected from rhizosphere soil of *M. transmorrisonensis* at depths 0–10 cm and 10–20 cm from the soil surface were used for bioassays and for chemical extraction and identification of some components.

Seed Germination and Growth Performance between M. transmorrisonensis *and* Lolium multiflorum. According to Harper (1977), seeds of rye grass

(*Lolium multiflorum*) and *M. transmorrisonensis* were sown alone and sown by mixing seeds of the two species in a 1 : 1 ratio in a tray, 34 × 43 cm for each. Six planting densities, 225, 450, 675, 900, 1350, and 1800 seeds per tray, were employed. Each treatment was done in three replicates. The trays were placed in a greenhouse of the Academia Sinica, Taipei. The number of seeds germinated, number of leaves per seedling, and seedling height were recorded two, three, five, and six months after sowing.

Preparation of Aqueous Extracts of Plant Parts. A series concentration, 1%, 2%, 3%, 4%, and 5%, of aqueous extracts of *M. transmorrisonensis* plant parts and their rhizosphere soil was obtained by methods described by Chou (1989) and Chou and Young (1975). The extracts of leaves and soils were employed for bioassay of phytotoxicity and for further chemical identification of phytotoxins, by using techniques described by Chou and Muller (1972) and Chou and Kuo (1986).

Laboratory Assays. The grass leachates and extracts were bioassayed to determine phytotoxicity (Chou and Young, 1975). Seeds of lettuce (*Lactuca sativa* var. Great Lakes), Chinese cabbage (*Brassica oleracea*), tall fescue (*Festuca arundinacea*), and rye grass (*Lolium perenne* and *L. multiflorum*) were used for bioassay. The seed germination percentage and length of radicle growth of the test plants were measured 72 hr after incubation.

Quantitative Determination of Phytotoxic Phenolics. The phytotoxic phenolics present in the aqueous extract and leachate of the grass were obtained by using extraction and purification techniques as described by Chou and Muller (1972) and Chou (1989). The phytotoxins from the *Miscanthus* rhizosphere soil were obtained by using extraction techniques (Wang et al., 1967; Tang, 1986), and the compounds were identified by means of paper chromatography (Wang et al., 1967) and HPLC (Chou and Kuo, 1986).

RESULTS

Botanical Composition of Study Site. Relative frequency and relative coverage for each species in the 60 quadrats used are given in Table 1; 37 species appeared in the area. The relative frequencies of *Miscanthus transmorrisonensis* and *Yushinia niitakayamensis* were significantly higher than those of the other 35 species, being 11.76% and 10.95%, respectively. Besides these two species, *Baeothryon subcapitatum*, *Carex satsumensis*, *Curculigo orchioides*, *Gaultheriza itoata*, *Gentiana atkinsonii*, *Haloragis misrantha*, *Hypericum nagasawai*, *Rhododendron* spp., and *Swertia arisanensis* had relative frequencies above 4%, while those of the other 26 species were much below 4% (Table 1).

The relative coverages of *M. transmorrisonensis* and *Yushinia niitakayamensis* were 24.83% and 19.45%, respectively; these values are significantly

TABLE 1. VEGETATION COMPOSITION OF GRASSLAND COMMUNITY AT TARTARCHIA ANPU, NANTOU, TAIWAN

Species name	Frequency	Relative frequency (RF) (×100%)	Coverage (cm^2)	Relative coverage (RC) (×100%)	Sum RF + RC (%)
Agrostis spp.	10	2.208	10,200	0.86	2.888
Aletris formosana Sasaki	12	2.434	11,000	0.92	3.354
Astragalus sinicus Linn.	1	0.203	1,000	0.08	0.283
Baeothryon subcapitatum (Thwaites) T. Koyama	24	4.868	97,500	8.19	13.058
Carex satsumensis Franch. sav	32	6.491	72,700	6.11	12.601
Cirsium spp.	2	0.406	1,500	0.13	0.536
Caldonia spp.	5	1.014	6,500	0.55	1.564
Curculigo orchioides Gaertn.	21	4.260	50,500	4.24	8.5
Dicyanopteris linearis (Burm.f.) Under Var.	1	0.203	500	0.04	0.243
Eupatorium spp.	1	0.203	500	0.04	0.243
Eurya spp.	2	0.406	2,000	0.17	0.576
Galium spp.	1	0.203	500	0.04	0.243
Gaultheriza itoata Hay.	27	5.477	44,000	3.70	9.177
Gentiana arisanensis Hay.	5	1.014	2,500	0.21	1.224
Gentiana atkinsonii Burk. var. *formosana* (Hay.) Yamamota	32	6.491	62,000	5.21	11.701
Gentiana scabrida Hayata	2	0.406	3,200	0.27	0.676
Haloragis misrantha (Thunb.) R. Br.	24	4.868	38,000	3.19	8.058
Hemiphragma heterophyllum Wall. var. *dentatum*	2	0.406	3,000	0.25	0.656
Hydrocotyl spp.	2	0.406	1,000	0.08	0.486
Hypericum nagasawai Hay.	36	7.302	32,500	5.42	12,722

TABLE 1. *Continued*

Species name	Frequency	Relative frequency (RF) (×100%)	Coverage (cm^2)	Relative coverage (RC) (×100%)	Sum RF + RC (%)
Ilex spp.	1	0.203	500	0.08	0.283
Lycopodium complanatum Linn	10	2.028	18,000	3.00	5.028
Lycopodium veitchii Christ	20	4.057	43,500	1.51	5.576
Miscanthus transmorrisonensis Hay	58	11.764	295,500	24.83	36.594
Nertera nigricarpa Hayata	3	0.609	2,500	0.21	0.819
Pieris taiwanensis Hay.	17	3.448	31,000	2.61	6.058
Pityrogramma calomelanos (L.) Link	1	0.203	500	0.04	0.243
Rhododendron sppl.	24	4.868	39,800	3.34	8.208
Rosa transmorrisoninsis Hay.	3	0.609	6,000	0.50	1.109
Rubus spp.	4	0.811	10,000	0.84	1.651
Senecio spp.	1	0.203	1,000	0.08	0.283
Stravaesia niitakayamensis (Hay.) Hay.	5	1.014	5,500	0.462	1.476
Swertia arisanensis Hay.	30	6.058	30,000	2.52	8.605
Tripterospermum taiwanense (Masam.) Satake	2	0.406	1,000	0.08	0.486
Viola spp.	7	1.420	3,700	0.31	1.730
Usnea plicata	11	2.231	29,500	2.48	4.711
Yushania niitakayamensis (Hay.) Keng f.	54	10.953	231,500	19.45	30.403
Total	493	100	1,190,100	100	200

higher than those of the other 35 species. The values are well correlated to the relative frequencies mentioned above. Overall, *M. transmorrisonensis* and *Yushinia niitakayamensis* were dominant in the area (Table 1). To express the species diversity of this grassland community at Tartarchia Anpu, the Shannon-Wienner Index (H′) was used. The index of the community was −3.04839, indicating that the species diversity in the community is great.

Vegetation Succession of Grassland Community in Tartarchia Anpu. To understand the botanical succession in the Tartarchia grassland area, the response of plants after experimental clearing is given in Table 2. Fourteen species were found 18 months after clearing. The percent coverages of *M. transmorrisonensis* and *Yushinia niitakayamensis* were 17.4% (relative coverage is 29.06%) and 9.33% (relative coverage is 15.57%), respectively, indicating that these two species are dominant. Compared to the findings given in Table 1, the total number of species appearing in the cleared plots had not reached its maximum diversity, indicating that the succession was still proceeding.

Seed Germination and Seedling Growth of M. transmorrisonensis. Because the dominant species in the area is *M. transmorrisonensis*, 1000 seeds of the plant were sown in a 43 × 34-cm tray filled with soil collected from the study site of Tartarchia Anpu. The seeds germinated were counted two, three, five, and six months after sowing. The rate of seed germination per 1000 seeds was

TABLE 2. APPEARANCE OF SEED EMERGENCE IN SOIL AFTER EXPERIMENTAL CLEARING AT TARTARCHIA ANPU

Plant name	Mean coverage (%)	Relative coverage (%)
Aletris formosana Sasaki	2.210	3.69
Baeothryon subcapitatum (Thwaites) T. Koyama	0.545	0.90
Carex satsumesis Franch. sav	7.140	12.04
Gaultheria itoata Hay.	1.495	2.04
Gentiana arisanensis Hay.	6.008	10.03
Gramineae	6.273	10.47
Haloragis micrantha (Thunb.) R. Br.	1.508	2.50
Hypericum nagasawai Hay.	2.540	4.17
Ludwigia adscendens (L.) Hara.	4.960	8.28
Lycopodium veitchii Christ	0.120	2.00
Miscanthus transmorrisonensis Hay.	17.408	29.06
Swertia arisanensis Hay.	0.053	0.08
Viola spp.	0.300	0.50
Yushania niitakayamensis (Hay.) Keng f.	9.330	15.57
Total	59.897	100

extremely low, ranging from 8.00 to 8.50 (Figure 2). The findings are indicative of the growth performance of *M. transmorrisonensis*, reflecting the low survival rate and poor growth of the plant in a greenhouse of the Academia Sinica, Taipei.

Competitive Exclusion between Lolium multiflorum *and* M. transmorrisonensis. To compare the competitive exclusion between grasses of *L. multiflorum* and *M. transmorrisonensis*, experiments were performed by sowing seeds of each species alone as control and 1 : 1 mixtures of seeds in different densities. Results showed that, in both control and mixed experiments, the seed germination rate varied with densities. In general, in both separate and mixture experiments, seed germination rates of *L. multiflorum* were significantly higher than those of *M. transmorrisonensis*, regardless of planting density. Some recent field experiments also confirmed that the germination rate of *L. multiflorum* was significantly higher than that of *M. transmorrisonensis* (Chou et al., 1991, unpublished data). The germination rate in general was drastically decreased with time elapsed after sowing (Figure 3). There was no significant difference between treatments of seeds sowed alone and mixed, suggesting that no competition occurred between the grasses during the germination stage. Comparing the growth of these two plants based on seedling height, the growth of *M. transmorrisonensis* was better than that of *L. multiflorum* (Figure 4); this was particularly pronounced at the higher density of 900 seeds/0.15 m^2 (Figure 4). The growth of *Lolium multiflorum* was significantly poorer than that of *M.*

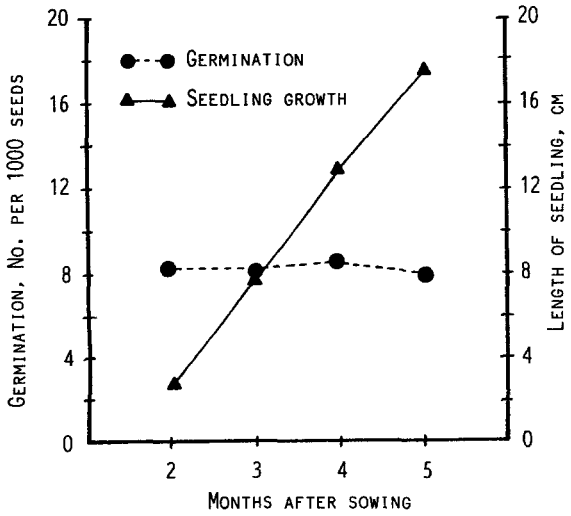


FIG. 2. The survival and growth performance of *M. transmorrisonensis* expressed by seed germination and growth in a greenhouse of the Academia Sinica, Taipei.

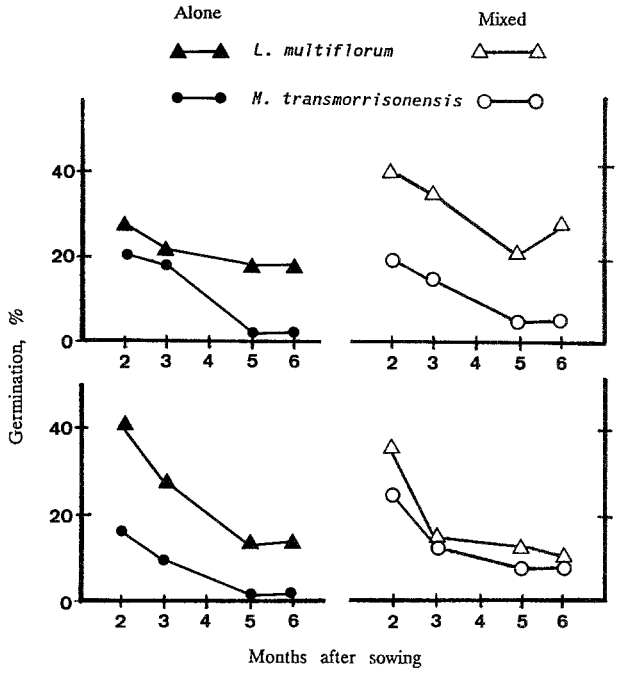


FIG. 3. A comparison of seed germination between *Lolium multiflorum* and *M. transmorrisonensis* by sowing seeds of each species alone or mixed seeds at a density of 450 seeds/0.15 m^2 (upper figures) and 1800 seeds/0.15 m^2 (bottom figures).

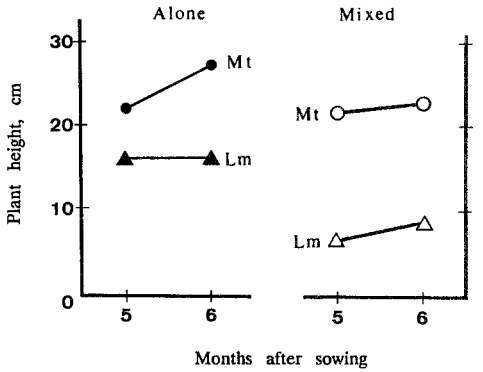


FIG. 4. A comparison of seedling growth between *M. transmorrisonensis* (Mt) and *Lolium multiflorum* (Lm) by sowing seeds of each species alone or mixed at a density of 1800 seeds/0.15 m^2.

transmorrisonensis after the *Miscanthus* seedlings were established in the field (the experiment is still continuing and data will be reported later). It could be suggested that *L. multiflorum* is not able to compete with *M. transmorrisonensis* in the field, so that the latter species becomes dominant.

Phytotoxic Effect of Aqueous Extracts on Radicle Growth of Tested Plants. The results of aqueous extracts of leaves of *M. transmorrisonensis* bioassays showed that the extracts significantly suppressed the radicle growth of plants, lettuce, rye grasses (both annual and perennial species), festuca, and cabbage, at the 1% concentration, in which the osmotic concentration was below 10 mosm. Among the test plants, lettuce (*Lactuca sativa*) was the most sensitive to the extract, and *Lolium perenne* was the least (Figure 5). The inhibition of radicle growth ranged from 13% to 35% for the 1% extract and from 42% to 90% for the 5% extract.

Leaf extracts of two major ecotypes, narrow leaf type (<1 cm in width) and wide-leaf type (>2 cm), of *M. transmorrisonensis* were bioassayed against lettuce and two varieties of Chinese cabbage. Bioassay results represented by

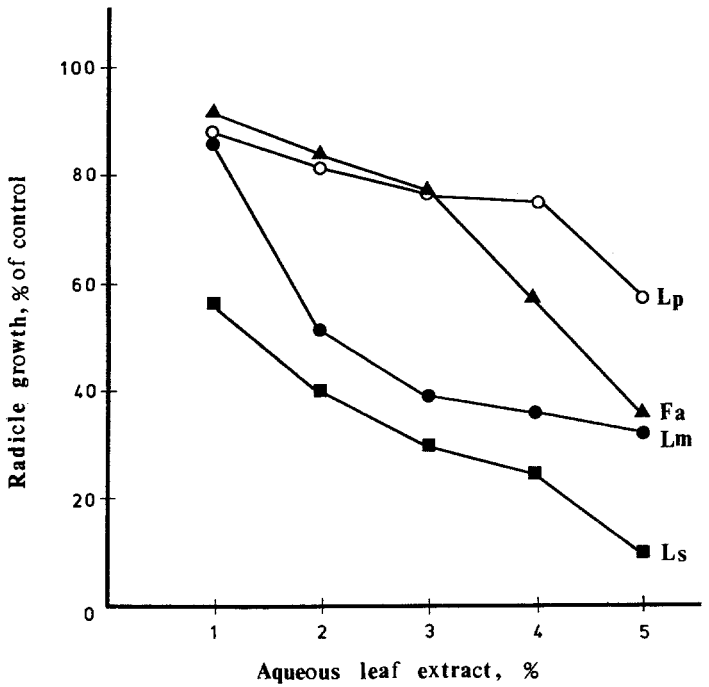


FIG. 5. The aqueous extracts of *M. transmorrisonensis* leaves in a series of concentrations on the radicle growth of four tested plants: Lp = *Lolium perenne*, Lm = *L. multiflorum*, Fa = *Festuca arundinacea*, and Ls = *Lactuca sativa*.

3% extracts exhibited remarkable phytotoxicity on the test plants; the phytotoxicities were significantly higher in the extract of narrow leaves than in that of wide leaves, reflecting that the allelopathic activity varied with different ecotypes of *Miscanthus* (Figure 6). Evidently, most leaves of *M. transmorrisonensis* found in the study site of Tartarchia Anpu were narrow ones, but those found in Hoshe were wide ones.

Bioassay of aqueous extracts of *Miscanthus* rhizosphere soils collected from two soil depths, 0–10 and 10–20 cm, was also conducted on the radicle growth of four test plants: rye grass, two varieties of Chinese cabbage, and lettuce. Results showed that the soil collected from the 10- to 20-cm layer exhibited significantly higher phytotoxicity than did that from the 0- to 10-cm layer. The inhibition of lettuce and Chinese cabbage ranged from 60% to 70%, while that of rye grass was insignificant, indicating that soil phytotoxicity varied with the plant tested (Figure 7). We conclude that both leaves and soils of *M. transmorrisonensis* contain phytotoxic substances, which suppress the growth of weeds in the field.

Identity of Phytotoxic Substances in Leaves and Soils of M. transmorriso-

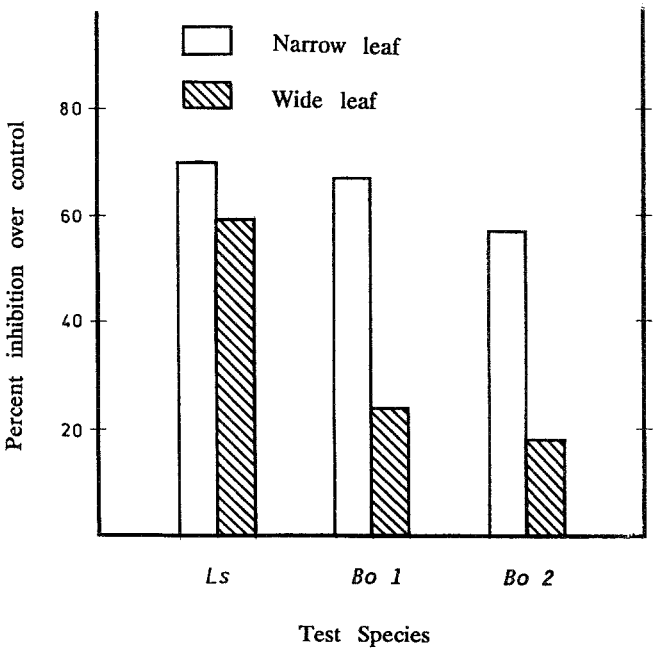


FIG. 6. The inhibitory effect of aqueous extracts of *M. transmorrisonensis* with two different ecotypes on the radicle growth of two species, *Ls* = *Lactuca sativa*, *Bo*1 = *Brassica oleracea* variety 1, and *Bo*2 = *B. oleracea* variety 2.

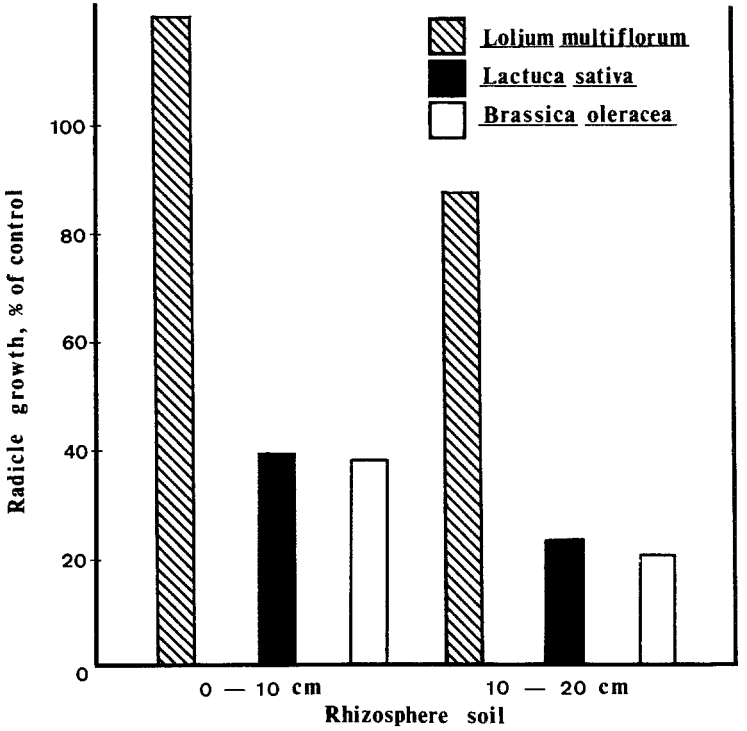


FIG. 7. The phytotoxic effects of the rhizosphere soils collected from the soil surface at two depths under a *M. transmorrisonensis* stand.

nensis. Some phytotoxic substances present in aqueous leachate and extracts of leaves of *M. transmorrisonensis* were identified by paper chromatography and HPLC. The compounds identified are caffeic, gallic, *p*-hydroxybenzoic, ferulic, *m*-hydroxybenzoic, and *o*-hydroxyphenylacetic acids, and 4-hydroxycoumarin and phloridzin (Table 3). The first five compounds were present in the leaf, and the first four compounds in the root of *Miscanthus*. Caffeic, gallic, ferulic, and *p*-hydroxybenzoic acids were found in the soil of *Miscanthus*. Relative amounts of phytotoxins in plants versus soil are shown in Table 3.

By using chromatography followed by bioassay as described by Chou and Chung (1974), spots appearing on paper chromatograms developed in 2% acetic acid were cut off and eluates bioassayed with lettuce seeds as test material. The same chromatographic paper without spotting with extract was also developed in 2% acetic acid and served as a control. The bioassay results for chromatographic segments according to R_f values showed significant inhibition. The inhibiting substances were isolated, and the structures were identified by PC

TABLE 3. INDENTITY OF PHYTOTOXIC SUBSTANCES IN LEAF AND SOIL EXTRACTS OF *M. transmorrisonensis*

Phytotoxic substances[a]	Leaves	Root	Rhizosphere soil
Caffeic acid	+	+	+
Gallic acid	+ + +	+ +	+ + +
p-Hydroxybenzoic acid	+ + +	+	+ + +
Ferulic acid	+	+	+
m-Hydroxybenzoic acid	+	−	−
o-Hydroxphenylacetic acid	+ +	−	−
4-Hydroxycoumain	−	+	−
Phloridzin	−	+	−

[a] Quantitative comparison among compounds was determined by HPLC, where the amounts of content were as follows: + + +: 10^{-3} mol/g sample; + +: 10^{-3}–10^{-4} mol/g sample; +: 10^{-4} mol/g sample.

and HPLC; the responsible phytotoxins were those given in Table 3. These compounds have been found to be allelopathic by many scientists (Chou and Lin, 1976; Chou and Muller, 1972; Chou and Waller, 1989; Rice, 1977, 1984; Tang, 1986; Waller, 1987).

DISCUSSION

Regarding the mechanism of dominance of *M. transmorrisonensis*, competition and allelopathy should both be considered and evaluated by experiments. So far as competition for light and soil moisture is concerned, the area is wide open, receiving sufficient solar radiation and substantial precipitation throughout the year (Chou et al., 1991). Soil nutrients are sufficient for the growth of *Miscanthus* and weeds (Chou and Chiu, unpublished data). Physical competition for light, soil moisture, and soil nutrients would not likely be limiting factors for permitting the dominance of *M. transmorrisonensis*.

On the other hand, the allelopathic potential of *M. transmorrisonensis* has been clearly demonstrated by bioassay results mentioned in Figure 5. The plants exhibited phytotoxic activity on the growth of many tested plants, and the rhizosphere soil of the plants also showed significant phytotoxicity (Figure 7). The same phytotoxins were found in both the plant parts and soil (Table 3). The data suggest that the dominance of *M. transmorrisonensis* may be due to allelopathic effect. Because the amounts of several phytotoxic substances in the plants and soil were so small, the identification of these compounds was difficult, and some remain unidentified.

Because *Miscanthus transmorrisonensis* and *Yushinia niitakayamensis* are

dominant in the grassland community, the competitive exclusion between the two species has to be taken into consideration. More than 15 years of observation by the senior author has shown that both species are aggressive and also resistant to the invasion of other species. One important cause of the aggressive nature of *Y. niitakayamensis* is its rhizome system, which leads to suppression of the growth of other weeds except *M. transmorrisonensis*. The growth performance of *M. transmorrisonensis* is unique, and its rhizome system is the same as that of *Yushinia*. Consequently, the potential of competitive exclusion between these two species is apparently the same, and it is difficult to draw conclusions as to which is superior.

Although it was impossible to collect seeds of associated weeds mentioned in Table 1 for competition study, we took a temperate grass species, *Lolium multiflorum*, for the bioassay study. Naqui and Muller (1975) reported that *L. multiflorum* exhibited a strong allelopathic potential and aggressiveness in fields. The present findings obtained from experiments of mixing seeds of *M. transmorrisonensis* and *L. multiflorum* indicated that the seed germination rate of the latter was significantly higher than that of the former but that the growth performance of the former was noticeably better; this results in better survival of the former species as far as adaptation is concerned. On the basis of previous studies, the authors have found that both *M. floridulus* and *M. transmorrisonensis* can adapt well to different habitat and, thus, form different ecotypes (Chou and Chang, 1988; Chou and Chen, 1990; Chou et al., 1991). As described in Figure 6, the narrow-leaf ecotype of *M. transmorrisonensis* found mostly in the area of Tartarchia Anpu showed significantly higher phytotoxicity than did the wide leaf ecotype found in the Hoshe area. This finding suggests that the allelopathic potential was more pronounced in the severe winter than that in the moderate environment.

Acknowledgments—The authors express their gratitude to Drs. C.Y. Chiu, F.S. Hsu, Y.C. Wang, and colleagues of the Forestry Experiment Station at Hoshe, National Taiwan University, for their field assistance. Acknowledgments are also due to Professors Otis Dermer and G.R. Waller for their critical review of the manuscript.

REFERENCES

BREWER, R. 1979. Principles of Ecology. W.B. Saunders Company, Philadelphia.

CHOU, C.H. 1989. Allelopathic research of subtropical vegetation in Taiwan IV. Comparative phytotoxic nature of leachate from four subtropical grasses. *J. Chem. Ecol.* 15:2149–2159.

CHOU, C.H., and CHANG, F.C. 1988. Population study of *Miscanthus floridulus* II. Ecotypic variation of *M. floridulus* and *M. transmorrisonensis* as affected by altitude in Nantou, Taiwan. *Bot. Bull. Acad. Sin.* 29:301–314.

CHOU, C.H., and CHEN, Y.Y. 1990. Population study of *Miscanthus floridulus* III. Population variation of *M. floridulus* in Green and Orchid islets of Taiwan. *Bot. Bull. Acad. Sin.* 31:223–233.

CHOU, C.H., and KUO, Y.L. 1986. Allelopathic research of subtropical vegetation in Taiwan III. Allelopathic exclusion of understory by *Leucaena luecocephala* (Lam) de Wit. *J. Chem. Ecol.* 12:1431–1448.

CHOU, C.H., and LIN, H.J. 1976. Autointoxication mechanism of *Oryza sativa* I. Phytotoxic effect of decomposing rice residues in soil. *J. Chem. Ecol.* 2:353–357.

CHOU, C.H., and MULLER, C.H. 1972. Allelopathic mechanism of *Arctostaphylos glandulosa* var. *zacaensis*. *Am. Midl. Nat.* 88:324–347.

CHOU, C.H., and WALLER, G.R. (eds.). 1989. Phytochemical Ecology: Allelochemicals, Mycotoxins, Pheromones and Allomones. Institute of Botany, Academia Sinica Monograph Series No. 9. 535 pp.

CHOU, C.H., and YOUNG, C.C. 1975. Phytotoxic substances from twelve subtropical grasses in Taiwan. *J. Chem. Ecol.* 1:183–193.

CHOU, C.H., HWANG, S.Y., and CHANG, F.C. 1987. Population study of *Miscanthus floridulus* (Labill.) Warb. I. Variation of peroxidase and esterase in 27 populations in Taiwan. *Bot. Bull. Acad. Sin.* 28:247–281.

CHOU, C.H., LEE, Y.F., CHIU, C.Y., WANG, Y.C., and HSU, F.H. 1991. Population study of *Miscanthus* IV. Growth performance of *M. floridulus* and *M. transmorrisonensis* and their acclimation to temperatures and water stress *Bot. Bull. Acad. Sin.* 32:87–96.

GLIESSMAN, S.R. 1978. The allelopathic mechanisms of dominance in bracken (*Pteridium aquilium*) in southern California. *J. Chem. Ecol.* 4:337–362.

HARPER, J.L. 1977. Population Biology of Plants. Academic Press, London.

MULLER, C.H. 1966. The role of chemical inhibition (allelopathy) in vegetational composition. *Bull. Torrey Bot. Club* 93:332–351.

NAQUI, H.H., and MULLER, C.H. 1975. Biochemical inhibition (allelopathy) exhibited by Italian ryegrass (*Lolium multiflorum* L.). *Pak. J. Bot.* 7:139–147.

NEWMAN, E.I., and MILLER, M.H. 1977. Allelopathy among some British grassland species II. Influence of root exudates on phosphorous uptake. *J. Ecol.* 65:399–411.

RICE, E.L. 1977. Some roles of allelopathic compounds in plant communities. *Biochem. Syst. Evol.* 5:201–106.

RICE, E.L. 1984. Allelopathy, 2nd Ed. Academic Press, New York. 422 pp.

TANG, C.S. 1986. Continuous trapping techniques for the study of allelochemicals from higher plants, pp. 113–131, *in* A.L. Putnam and C.S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

TINNIN, R.O., and MULLER, C.H. 1971. The allelopathic potential of *Avena fatua*: Influence on herb distribution. *Bull. Torrey Bot. Club* 98:243–250.

WANG, T.S.C., YANG, T.K., and CHUNG, T.T. 1967. Soil phenolic acids as plant growth inhibitors. *Soil Sci.* 103:239–246.

WALLER, G.R. (ed.). 1987. Allelochemicals: Role in Agriculture and Forestry. ACS Symposium Series 330. American Chemical Society, Washington, D.C. 606 pp.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

COMPARISON OF LANTADENES CONTENT AND TOXICITY OF DIFFERENT TAXA OF THE LANTANA PLANT

OM P. SHARMA,[1,*] JAGDISH VAID,[2] and PRITAM D. SHARMA[3]

[1]*Biochemistry and* [2]*Disease Investigation Laboratories*
Indian Veterinary Research Institute
Regional Station, Kangra Valley
Palampur 176 061, India

[3]*Department of Pharmaceutical Sciences*
Panjab University
Chandigarh 160 014, India

(Received May 7, 1991; accepted July 22, 1991)

Abstract—Three taxa of the lantana plant (*Lantana camara* var. *aculeata*)—I (white-pink), II (yellow-pink), and III (yellow-red)—differed in the content of lantadenes and compounds X, Y, and Z. Lantadene A, lantadene B, and lantadene C were the major lantadenes of taxon III. It contained small amounts of lantadene D. Taxa I and II contained very small amounts of lantadene A and lantadene B. Compounds X, Y, and Z were the major constituents of taxa I and II, and they constituted 59% and 92% of the total constituents in these taxa, respectively. Ingestion of lantana leaf powder of taxon III by male guinea pigs caused severe hepatotoxicity. Taxon I elicited mild hepatotoxicity in one out of six animals while taxon II was nontoxic. The biological and ecological significance of the presence of high amounts of compounds X, Y, and Z in taxa I and II is not known.

Key Words—Lantana, *Lantana camara* var. *aculeata*, lantadenes, hepatotoxicity, ecological aspects.

INTRODUCTION

Lantana (*Lantana camara* L.) is one of the ten most noxious weeds in the world (Holm et al., 1979). The plant causes animal toxicosis, resulting in hepatotoxicity and photosensitization, allelopathy, and has a number of other interactions with the biosphere (Sharma et al., 1988b). The generic epicenter of *L.*

*To whom correspondence should be addressed.

0098-0331/91/1100-2283$06.50/0

camara is regarded as Mexico and Central America (Spies, 1984) from where it has been introduced to the rest of the world via Europe (Stirton, 1977). At present, the lantana plant has wide distribution in nearly 50 countries spread over Oceania, Asia, Africa, South America, and North America (Sharma et al., 1988b). Furthermore, this plant is rapidly spreading to areas hitherto free of it and is turning into a great ecological threat in lantana-infested regions of the world (Sharma et al., 1988b). Varietal differences have been found in the chemical composition and toxicity of lantana plant in Australia (Hart et al., 1976a). However, no information is available on the comparative aspects of the chemistry and toxicosis of lantana in other parts of the world (Sharma and Sharma, 1989). There are some reports on the chemistry of lantana in India (Sharma and Sharma, 1989). These reports do not mention details of the variety and geographic location. The corresponding information on animal toxicosis is also lacking except for *L. camara* var. *aculeata* (Red) (Sharma, 1984). *L. camara* var. *aculeata* (Red) is the most wide spread and is very rich in lantadene A followed by lantadene B and C (Sharma and Sharma, 1989). One of the active principles in the lantana plant responsible for eliciting hepatotoxicity is lantadene A (Sharma et al., 1988a). Hybridization in the lantana plant has resulted in continuous variation in morphological characters (Spies, 1984). Numerous taxa of lantana plant differ in the characteristic of leaves, prickles, and flower color, and their nomenclature is not established so far (Yates, 1966, Mathur and Mohan Ram, 1978, 1986). *L. camara* plants bearing prickles have been placed in the variety *aculeata*. However, there are further flower color variations in the same variety. Thus, the different taxa in the variety examined in our study (*Lantana camara* var. *aculeata*) have been organized according to flower color, as done by the previous workers (Spies, 1984, Mathur and Mohan Ram, 1978). Our field observations on the three taxa used in our study over most of the last decade reveal that they are quite constant in their morphological characters, which precludes the possibility of free natural hybridization. Information on the chemistry of these toxic plants would be helpful in understanding the ecological aspects of their spread, in exploring the possibility of utilizing their biomass, and in developing a molecular rationale for control strategies through biological or chemical approaches (Hart et al., 1976a; Harborne, 1989; Sharma, 1988). Recently, we have emphasized the possibility of utilization of lantana as a resource for drug research and investigations into development of biocides (Sharma and Sharma, 1989; Sharma et al., 1988b). We report here the comparative lantadenes content and toxicity of three taxa of the lantana plant.

METHODS AND MATERIALS

Lantana Samples. The flower color in lantana changes after anthesis. So, for description of the taxa investigated, flower color just after anthesis and on

maturity has been given. Table 1 lists the taxa of the lantana plants (*Lantana camara* var. *aculeata*) studied.

Lantana leaf samples of taxa I and II were collected from the vicinity of Panjab University Chandigarh Campus [550 m above mean sea level (msl); latitude, 30°42′N; longitude, 76°48′E], India. Leaf samples of taxon III were collected from the vicinity of IVRI Regional Station, Palampur, Kangra Valley Laboratories (1290.8 m above msl; latitude, 32°60′N; longitude, 76°32′E), India. The samples were air-dried and finely ground in an electric grinder using a 1-mm sieve. The powdered samples were used for analysis of lantadenes and toxicity testing using a guinea pig as the laboratory animal model.

Analysis of Lantadenes. Lantana leaf powder (100 g) was extracted with methanol, decolorized, and purified further by solvent partition, acetone extraction, and column chromatography on silica gel (60–120 mesh) and neutral alumina as described previously (Sharma et al., 1987). The residue from the above step was subjected to isocratic column chromatography on silica gel (60–120 mesh). The elution was done with benzene–diethyl ether (80:20) as described previously (Sharma et al., 1990), and the volume of the partially purified extract was made to 50 ml. Aliquots (10 μl) of the above preparation were used for ascertaining the profile of lantadenes and related compounds by thin-layer chromatography using standard lantadene A, B, C, and D prepared in our laboratories (Sharma and Sharma, 1989; Sharma et al., 1990). Thin-layer chromatography was done on silica gel G plates (Stahl, 1969) using the solvent systems hexane–methanol–ethyl acetate (85:10:5) and benzene–methanol–ethyl acetate (85:10:5). Determination of the relative contents of the lantadenes was done by thin-layer chromatography in combination with dichromate oxidation (Skipski and Barclay, 1969). The relative amounts of different lantadenes and other constituents are expressed as percent of total content in each taxon.

Toxicity Studies. Twenty-four male guinea pigs (body weight 400–450 g) drawn from the stock colony of the Indian Veterinary Research Institute were fasted overnight with water ad libitum. They were divided at random into four groups (A, B, C, and D) of six animals each. Animals in groups A, B, and C were offered lantana leaf powder (10 g/kg body weight) of taxa I, II, and III, respectively, mixed in feed. Animals in group D served as controls. Animals

TABLE 1. LANTANA TAXA STUDIED

	Flower color on anthesis	Flower color on maturity
Taxon I	White	Pink
Taxon II	Yellow	Pink
Taxon III	Yellow	Red

in the experimental and control groups were monitored daily for appearance of ictericity by the microictericity test (Sharma et al., 1988a). Nearly 10 μl blood was drawn from the ear vein into heparinized capillaries. The capillaries were sealed at one end with modeling clay, centrifuged at 2000 rpm for 10 min, and the color of plasma layer was observed. The plasma layer in control animals was off-white in color and was graded negative. Appearance of any yellowness in the plasma layer indicated development of ictericity, jaundice, and toxicity and was graded positive qualitatively (Sharma et al., 1988a). Animals in group C became severely icteric in two to four days and were sacrificed along with animals in groups A, B, and D. Liver was collected for determination of hepatomegaly (Sharma et al., 1980) and histopathological examination (Culling, 1957). Blood was collected in EDTA-Na_2 and plasma prepared for bilirubin estimation (Malloy and Evelyn, 1937) and assay of acid phosphatase activity (EC 3.1.3.2) (Barret and Heath, 1977).

RESULTS AND DISCUSSION

The comparative thin-layer chromatographic profile of the extracts of three taxa of lantana is shown in Figure 1. Taxa I and II are similar with respect to their relative lack of lantadene A, C, and D. The special feature of both these taxa is the presence of compounds X, Y, and Z. Interestingly, compounds X, Y, and Z are absent from taxon III (Table 2). As reported earlier, lantadene A and B are the predominant lantadenes, followed by lantadene C and D in taxon III (Sharma and Sharma, 1989). Taxon I contained another unidentified compound (lantadene D'), which moved closer to lantadene D (Figure 1). Lanta-

TABLE 2. LANTADENES CONTENT OF TAXA I, II, AND III OF LANTANA PLANT[a]

	Taxon I	Taxon II	Taxon III
Total lantadenes (mg/g dry wt.)	11.33 ± 0.44	11.67 ± 0.42	14.15 ± 0.53
Lantadene A	8.83 ± 1.02	0.37 ± 0.12	37.50 ± 2.55
Lantadene B	11.43 ± 1.38	3.53 ± 0.61	32.33 ± 2.05
Lantadene C	6.00 ± 0.55	N.D.	19.33 ± 1.70
Lantadene D	0.40 ± 0.08	4.20 ± 0.65	8.30 ± 1.25
Lantadene D*	14.07 ± 0.82	N.D.	N.D.
Compound X	20.93 ± 1.12	32.56 ± 0.34	N.D.
Compound Y	13.95 ± 0.75	21.64 ± 0.25	N.D.
Compound Z	24.39 ± 1.31	37.70 ± 0.45	N.D.
Other minor components			2.54 ± 0.62

[a]The values are mean ±SD, $N = 3$. The data for individual constituents have been expressed as percent of the total lanatdenes content. N.D., not detectable.

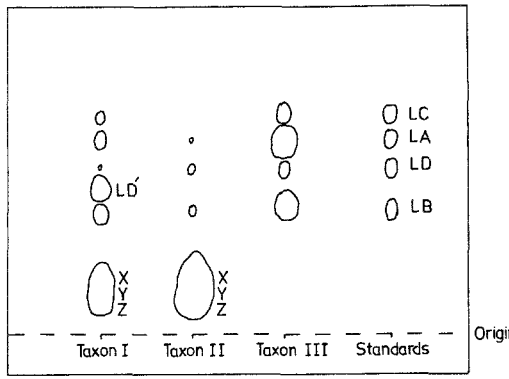


FIG. 1. Diagrammatic sketch of the thin-layer chromatographic profile of partially purified lantadenes from taxa I, II, and III of lantana. Solvent system: hexane–methanol–ethyl acetate (85:10:5). Amount of sample applied for extracts of taxa I, II, and III, 10 μl; amount of sample applied for standard lantadene A, B, C, and D, 40 μg. LA, lantadene A; LB, lantadene B; LC, lantadene C; LD, lantadene D; LD', lantadene D'.

dene A, B, C, and D are distinctly resolved in TLC using hexane–methanol–ethyl acetate (85:10:5) as the solvent system (Figure 1). Under these conditions, lantadene A, B, C, and D were detectable even in samples of taxon II, but the amount of sample was high enough to mask the resolution of compounds X, Y, and Z, which appeared as a single unresolved spot (Figure 1). Compounds X, Y, and Z could be better resolved in TLC using the solvent system benzene–methanol–ethyl acetate (85:10:5) and had R_f values of 0.34, 0.30, and 0.27, respectively. They differed in their color reaction as well, using acetic anhydride–sulfuric acid (9:1) as spray reagent. Lantadenes A, B, C, and D gave reddish brown spots, while compounds X and Y gave purple spots that had golden fluorescence at 366 nm. On the other hand, compound Z gave pinkish purple spots that had pinkish golden fluorescence at 366 nm. The separation profile on TLC indicated that compounds X, Y, and Z are much more polar than lantadene A, B, C, and D. Color reaction using Libermann-Burchard reagent indicated that compounds X, Y, and Z could be 3-β-hydroxy triterpenoids or sterols (Brieskorn and Herring, 1959). Hart et al. (1976a,b) observed that *L. camara* Common Pink did not contain lantadene A or B but contained lantic acid and lantanolic acid as the major constituents. Both lantic acid and lantanolic acid have a 3-β-hydroxyl group in their structure (Sharma and Sharma, 1989). The identity of compounds X, Y, and Z with the known triterpenoids of *L. camara* Common Pink has not been established so far. The molecular aspects of the impact of the relative differences in the content of lantadenes and other components in three taxa of lantana on interaction between insects used for biological control of lantana (Sharma, 1988) or herbivores are not known so

far. Taxon III is the most widespread (Sharma et al., 1988b); perhaps its high toxicity contributes to its better survival. Hart et al. (1976a) observed that lantadene A and B are the major triterpenoids in *L. camara* Common Pinkedged Red, Townsville Red-centered Pink, and Mackay Red-centered Pink. On the other hand, *L. camara* Common Pink was not found to contain either lantadene A or B (Hart et al., 1976a). The data of Hart et al. (1976a) are comparable to our observations (Figure 1) in that red flower color invariably appeared to be associated with a high content of lantadene A and B, and absence of red color in lantana flowers implied absence or relative lack of lantadene A and B. Investigations on the chemistry of plants are useful in resolving taxonomic problems (Tanowitz et al., 1987; Hegnauer, 1988). The phytochemical data on the lantana plant from the present studies and those reported earlier (Hart et al., 1976a; Sharma and Sharma, 1989) would also be helpful in ascertaining the interrelationship between different taxa and delineation of their exact taxonomic position.

The effect of ingestion of three taxa of lantana plant in guinea pigs is shown in Table 3. Five of six animals offered taxon III became severely icteric within two to three days. One animal in this group exhibited only transitory ictericity. Accidental ingestion of lantana foliage by the grazing animals or oral administration of partially purified lantana toxins is known to cause anorexia, constipation, hepatomegaly, obstructive jaundice, and photosensitization (Yadava and Verma, 1978; Sharma et al., 1981). There was a marked increase in the conjugated form of bilirubin in blood plasma in group C (Table 3), which is characteristic of obstructive jaundice (Cornelius, 1980). Similarly, there was a marked increase in liver weight in animals in group C (Table 3). Lantana poisoning is known to cause lysosomal injury and release of lysosomal acid phosphatase (Sharma et al., 1983). Animals in group C had significant increases in acid phosphatase activity (Table 3). Histopathological studies showed that all the animals in group C had liver lesions typical of lantana poisoning (Sharma et al., 1981). Only one animal in group A exhibited hyperbilirubinemia (plasma bilirubin content was 6.61 mg/100 ml). This animal had hepatomegaly as well, the liver weight being 1.3 times the normal value. The remaining five animals in group A had no symptoms of toxicity and were biochemically comparable to the controls (Table 3). Only one animal in group A that had exhibited clinical signs of toxicity, an increase in bilirubin level, and hepatomegaly exhibited hepatic lesions of lantana toxicity. The animals in group B had no cellular or subcellalar lesions representative of liver injury in lantana poisoning and were comparable to the control animals (Table 3). None of the animals in group B had ictericity, hepatomegaly, or a rise in acid phosphatase activity (Table 3). Seawright (1965) investigated the hepatotoxicity of a number of taxa of *Lantana* spp. in Queensland. It was observed that, except for flower color, no other morphological characteristics of the plant samples examined were related to

TABLE 3. EFFECT OF THREE TAXA OF LANTANA PLANT IN GUINEA PIGS[a]

Group	Lantana sample	Liver weight (g/100 g)	Bilirubin (mg/100 ml)			Acid phosphatase (μmol pNP/min/liter)
			Conjugated	Unconjugated	Total	
A	Taxon I	3.24 ± 0.13 (5), N.S.	0.13 ± 0.04 (5), N.S.	0.14 ± 0.09 (5), N.S.	0.28 ± 0.09 (5), N.S.	56.27 ± 13.00 (5), N.S.
B	Taxon II	3.30 ± 0.26 (6), N.S.	0.21 ± 0.04 (6), N.S.	0.13 ± 0.04 (6), N.S.	0.34 ± 0.05 (6), N.S.	47.10 ± 12.97 (6), N.S.
C	Taxon III	4.87 ± 0.95 (6), a	7.17 ± 1.31 (6), a	1.57 ± 0.85 (6), a	8.74 ± 1.92 (6), a	91.59 ± 23.06 (6), a
D	Control	3.14 ± 0.17 (6)	0.18 ± 0.06 (6)	0.18 ± 0.06 (6)	0.36 ± 0.08 (6)	48.04 ± 04.70 (6)

[a]Values are mean ±S.D. a, $P < 0.005$; N.S., nonsignificant. pNP, *p*-nitrophenyl phosphate. The intake of lantana leaf powder from the amount offered was: group A, 10 g/kg body wt; group B, 10 g/kg body wt; Group C, 7.8 g/kg body wt. The data for the sixth animal in group A, which got intoxicated are given in the text.

toxicity. The survey further indicated that pink lantana was either nontoxic or mildly toxic, while red-flowered lantana was consistently toxic (Seawright, 1965). The phytochemical basis of these observations was reported later (Hart et al., 1976a,b). These studies did not mention the flower color at anthesis. Perhaps the nontoxic pink taxon investigated by Seawright (1965) was comparable to taxon II in our study and the mildly toxic one could be taxon I of our study (Tables 2 and 3). In New Zealand, the lantana taxon most wide spread is the pink flower type, and it has been safely grazed by sheep and goats (Hill and Seawright, 1983). After the report of Hill and Seawright (1983), red-flowered lantana was spotted in New Zealand and resulted in the first incidence of lantana poisoning in that country (Black and Carter, 1985). Thus, data on morphological, toxicological, and phytochemical observations on different taxa of lantana in different parts of the world suggest that there could be a genetic linkage between flower color and triterpenoid profile, resulting in the evolution of taxa with varying degrees of hepatotoxicity (Seawright, 1965; Hill and Seawright, 1983; Black and Carter, 1985; Hart et al., 1976a,b; Sharma and Sharma, 1989). The full implications of the differences in chemical composition of different taxa of lantana plant on biological control measures, predator preferences, allelopathic aspects, spread potential, and overall interactions with the biosphere are not known at this stage. It would be important to further explore the possibility of utilization of nontoxic taxa of lantana, viz., taxon II in animal feeding systems, particularly in the ruminants. It is also germane to develop strategies to exploit abundant natural products such as lantadene A and B in taxon III and compounds X, Y, and Z in taxa I and II for drug research and development of value-added products.

Acknowledgments—We thank Prof. O.P. Sharma Sr., Department of Basic Sciences, Himachal Pradesh Krishi Vishvavidyalaya, Palampur, for help in identification of lantana specimens.

REFERENCES

BARRET, A.J., and HEATH, M.F. 1977. Lysosomal enzymes, pp. 19–127, *in* J.T. Dingle (ed.). Lysosomes, A Laboratory Handbook. Elsevier/North-Holand, Amsterdam.

BLACK, H., and CARTER, R.G. 1985. Lantana poisoning of cattle and sheep in New Zealand. *N.Z. Vet. J.* 33:136–137.

BRIESKORN, C.H., and HERRING, H. 1959. Chemistry of the Liebermann-Burchard color reaction for sterols and triterpenoids and their esters. *Arch. Pharm. Berlin.* 292:485–496.

CORNELIUS, C.E. 1980. Liver function, pp. 210–257, *in* J.J. Kaneko (ed.). Clinical Biochemistry of Domestic Animals. Academic Press, New York.

CULLING, C.F.A., 1957. Handbook of Histopathological Techniques, 1st ed. Butterworth and Company, London.

HARBORNE, J.B., 1989. Recent advances in chemical ecology. *Nat. Prod. Rep.* 6:86–109.

HART, N.K., LAMBERTON, J.A., SIOUMIS, A.A., and SAURES, H. 1976a. New triterpenoids of

Lantana camara, a comparative study of the constituents of several taxa. *Aust. J. Chem.* 29:655–671.

HART, N.K., LAMBERTON, J.A., SIOUMIS, A.A., SUARES, H., and SEAWRIGHT, A.A. 1976b. Triterpenes of toxic and nontoxic taxa of *Lantana camara*. *Experientia* 32:412–413.

HEGNAUER, R. 1988. Biochemistry, distribution and taxonomic relevance of higher plant alkaloids. *Phytochemistry* 27:2423–2427.

HILL, R.L., and SEAWRIGHT, A.A. 1983. The status of lantana in New Zealand. Proceedings of the 36th N.Z. Weed and Pest Control Conference. pp. 38–40.

HOLM, L., PANCHO, J.V., HERBERGER, J.P., and PLUCKNETT, D.L. 1979. A Geographical Atlas of Weeds. John Wiley, New York.

MALLOY, H.T., and EVELYN, K.A. 1937. The determination of bilirubin with the photoelectric colorimeter. *J. Biol. Chem.* 119:481–490.

MATHUR, G., and MOHAN RAM, H.Y. 1978. Significance of petal color in thrips-pollinated *Lantana camara* L. *Ann. Bot.* 42:1473–1476.

MATHUR, G., and MOHAN RAM, H.Y. 1986. Floral biology and pollination of *Lantana camara*. *Phytomorphology* 36:79–100.

SEAWRIGHT, A.A. 1965. Toxicity of *Lantana* spp. in Queensland. *Aust. Vet. J.* 41:235–238.

SHARMA, O.P. 1984. Review of the biochemical effects of *Lantana camara* toxicity. *Vet. Hum. Toxicol.* 26:488–493.

SHARMA, O.P. 1988. How to combat the lantana (*Lantana camara* L.) menace? A current perspective. *J. Sci. Ind. Res.* 47:611–616.

SHARMA, O.P., and SHARMA, P.D. 1989. Natural products of the lantana plant—the present and prospects. *J. Sci. Ind. Res.* 48:471–478.

SHARMA, O.P., MAKKAR, H.P.S., PAL, R.N., and NEGI, S.S. 1980. Lantadene A content and toxicity of lantana (*Lantana camara*) plant to guinea pigs. *Toxicon* 18:485–488.

SHARMA, O.P., MAKKAR, H.P.S., PAL, R.N., and NEGI, S.S. 1981. A review of the toxicity of *Lantana camara* (Linn.) in animals. *Clin. Toxicol.* 18:1077–1094.

SHARMA, O.P., MAKKAR, H.P.S., and DAWRA, R.K. 1983. Effect of lantana toxicity on lysosomal and cytosol enzymes in guinea pig liver. *Toxicol. Lett.* 16:41–45.

SHARMA, O.P., DAWRA, R.K., and MAKKAR, H.P.S. 1987. Isolation and partial purification of lantana (*Lantana camara* L.) toxins. *Toxicol. Lett.* 37:165–172.

SHARMA, O.P., DAWRA, R.K., and MAKKAR, H.P.S. 1988a. Effect of polymorphic crystal forms of lantana toxins on icterogenic action in guinea pigs. *Toxicol. Lett.* 42:29–47.

SHARMA, O.P., MAKKAR, H.P.S., and DAWRA, R.K. 1988b. A review of the noxious plant *Lantana camara*. *Toxicon* 26:975–987.

SHARMA, O.P., DAWRA, R.K., and RAMESH, D. 1990. A triterpenoid acid, lantadene D from *Lantana camara* var. *aculeata*. *Phytochemistry* 29:3961–3962.

SKIPSKI, V.P., and BARCLAY, M. 1969. Thin layer chromatography of lipids. *Methods Enzymol.* 14:530–598.

SPIES, J.J. 1984. A cytotaxanomic study of *Lantana camara* (Verbenaceae) from South Africa. *S. Afr. J. Bot.* 3:231–250.

STAHL, E. 1969. Thin Layer Chromatography, A Laboratory Handbook, Springer-Verlag, New York.

STIRTON, C.H. 1977. Some thoughts on the polyploid complex *Lantana camara* L. (Verbenaceae). Proc. 2nd Natl. Weeds Conf. Balkema pp. 321–340.

TANOWITZ, B.D., SMITH, D.M., and JUNAK, S.A. 1987. Triterpenoid constituents of three taxa of *Monardella*. *Phytochemistry* 26:2751–2752.

YADAVA, J.N.S., and VERMA, N.S. 1978. An outbreak of lantana poisoning in domesticated animals. *Indian Vet. Med. J.* 2:1–9.

YATES, G.J.E. 1966. Lantanas. *Gardens Chronicle*, 160:8–9.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

PERFORMANCE CRITERIA AND SPECIFICATIONS FOR LAMINATED PLASTIC SEX PHEROMONE DISPENSER FOR *Helicoverpa zea* (LEPIDOPTERA: NOCTUIDAE)[1]

J.D. LOPEZ, JR.,[2,*] B.A. LEONHARDT,[3] and T.N. SHAVER[2]

[2]*USDA, ARS, Crop Insect Pests Management Research Unit*
Rt. 5, Box 808, College Station, Texas 77845

[3]*Insect Chemical Ecology Laboratory, USDA, ARS*
Bldg. 011A, Rm 165A, BARC-WEST
Beltsville, Maryland 20705.

(Received March 13, 1991; accepted July 23, 1991)

Abstract—Biological and chemical evaluations of a plastic laminate sex pheromone dispenser for *Helicoverpa zea* (Boddie) were made in central Texas. Based on these evaluations, the following performance criteria and specifications are suggested for a dispenser to be effective for two weeks: (1) 1.27 × 2.54-cm size; (2) 400-μm thick orange PVC outer layers; (3) 1.25 mg of a blend of (*Z*)-11-hexadecenal, (*Z*)-9-hexadecenal, (*Z*)-7-hexadecenal, and hexadecanal in a ratio of 87:3:2:8 formulated in the adhesive reservoir layer; (4) release rate in the range of 0.12–0.33 μg/hr/dispenser, as measured in the laboratory at 35°C; and (5) residual pheromone content in the range of 0.55–1.25 mg/dispenser during the period of use.

Key Words—Bollworm, sex pheromone, trapping, corn earworm, *Helicoverpa zea*, Lepidoptera, Noctuidae.

INTRODUCTION

Sex pheromone dispensers for monitoring populations of numerous pest species are currently being used. However, established criteria or specifications for the field performance of these dispensers are limited (Leonhardt et al., 1990). These performance criteria or specifications are important in ensuring the availability of consistently effective sex pheromone dispensers. The parameters that are

*To whom correspondence should be addressed.

[1] This paper reports the results of research only. Mention of a proprietary product does not constitute an endorsement by the USDA.

important are: (1) content of active ingredient, (2) rate of emission, (3) ratio of components in pheromone blend, and (4) duration of effectiveness. For trapping the bollworm (BW), *Helicoverpa zea* (Boddie), a highly effective laminated plastic pheromone dispenser has been developed (Hartstack et al., 1980; Lopez et al., 1981, 1987; Zvirgzdins and Henneberry, 1983; Lopez and Shaver, 1990; Drapek et al., 1990); however, performance criteria or specifications are not available. Research reported here was aimed at the development of these. The variables evaluated were: (1) active ingredient—amount and ratio of components, (2) biological assay—effect of aging, and (3) release of active ingredient—rate, composition, and residual quantities with aging time.

METHODS AND MATERIALS

Source of Dispensers. The pheromone dispensers for BW were obtained from Hercon Environmental Co., Emigsville, Pennsylvania 17318. The initial dispensers came from lot V0283 and the subsequent supply from lot V0436. These dispensers, which were 1.27 × 2.54 cm in size, consisted of an adhesive reservoir layer containing the pheromone sandwiched between two layers of orange polyvinyl chloride (PVC) film (400 μm thick). This laminated dispenser system has been described by Quisumbing and Kydonieus (1982). According to information provided by the supplier, each dispenser contained 1.25 mg of the four-component pheromone blend in the ratios identified by Klun et al. (1980). These components were (*Z*)-7-hexadecenal (*Z*7–16:Al) (2%), (*Z*)-9-hexadecenal (*Z*9–16:Al) (3%), (*Z*)-11-hexadecenal (*Z*11–16:Al) (87%), and hexadecanal (16:Al) (8%). A small piece of copper wire, inserted through a small hole punched at one end of the dispenser, was used to attach the dispenser to the trap.

Field Bioassays. All field tests were conducted in the Brazos River Valley near College Station, Texas, in Burleson County. The primary agricultural products in the area include corn, sorghum, cotton, small grains, and cattle. Large populations of BW develop each year in this cropping system.

The pheromone trap used was the 75–50 wire cone trap (Hartstack et al., 1979). The traps were installed along fence lines or turn rows either adjacent to or just within the borders of corn and cotton fields.

The traps were generally installed so that the base of the traps was about 1 m above ground level, but the height was adjusted so that the bottom of the trap was at canopy level as the crops grew taller than 1 m. In tasseling corn, the height of the traps was maintained at tassel level. At each trap location, a 1.8-m length of 1.9-cm-diam. electrical conduit was driven into the ground so that the top of the conduit was about 1 m above ground level. The pipe base of the wire traps was inserted into the conduit. To adjust trap height, reinforcement

bars (1.3 cm diam.) of the proper height were inserted inside the conduit and the top of the reinforcement bar was inserted into the base pipe of the wire cone traps.

Conventional agricultural practices were conducted in the areas where traps were operated, including the application of insecticides as needed. In cotton fields, numerous insecticide applications were made during the period of evaluations. Traps were operated when the different crops were actively fruiting to have greater BW activity.

Unless otherwise specified, all evaluations were made using trap lines with traps spaced 50 m apart. Whenever possible, additional traps that were not included as part of the evaluations were operated at each end of a trap line to minimize edge effects. In all tests involving three or more treatments, a Latin-square in time design was used. Treatments were randomized initially so that each treatment occurred only once at each location in each replicate. They were rotated within each replicate after each daily service so that for each period of days equal to the number of treatments, each treatment was exposed for one night at each location. When only two treatments were evaluated, the two treatments were alternated in the trap line and were rotated nightly. The treatments were rotated by moving the dispensers and leaving the traps in place. This allowed each dispenser to be evaluated in each trap in each replicate during a period equivalent to the number of treatments. Dispensers were attached to the traps via pieces of thin wire and a paper clip to reduce contact of the dispensers with the trap and possible residual effects.

Evaluation of Pheromone Dispensers. Evaluations were conducted to determine whether dispensers from both lots V0283 and V0436 were equally effective as baits in the wire cone traps. Initial comparisons were made from April 24 to May 7 with five replicates in one trap line with traps spaced 38 m apart. In subsequent evaluations, dispensers of both lots aged under field conditions for 14 days were compared to unaged dispensers to determine the effect of aging.

To determine the effect of aging, dispensers (V0436) were exposed for 8, 16, and 24 days to ambient conditions from June 10 to July 8, 1986, by attaching them to a wire stretched between two poles in an outdoor grassy area. This exposure was similar to the exposure that the dispensers would receive in a trap. After exposure, the dispensers were wrapped in foil and stored in a freezer for about two to three weeks until evaluated. In an initial test, unaged and 8- and 16-day-old dispensers and five virgin females per trap were compared. The virgin females (1–2 days old) were reared in the laboratory and were replaced every two days. The females were confined in a small screen container and were fed a 5% sucrose solution in a small plastic cup with a cotton dental wick. The screen container was secured to the angle iron forming the base of the cone trap as close as possible to the same location as the pheromone dispensers. This

test was conducted for eight days (July 23–30). A second test was conducted by replacing the unaged dispensers in the first test, which were now eight days old, with unaged dispensers and comparing these with the 16- and 24-day-old dispensers. The 16- and 24-day-old dispensers were the ones that had been used in the initial test for eight days. Test III was a repeat of test II with the addition of unaged dispensers and use of the 16- and 24-day-old dispensers from the other tests.

Another evaluation of aged dispensers was conducted using six dispensers (lot V0436) for each age group exposed as previously described for 8, 16, and 24 days starting on August 14, 22, and 30 until September 6. After exposure, all the dispensers were removed from the field, wrapped in aluminum foil, sealed with transparent tape, and stored in a freezer. Starting on September 13, four of the dispensers from each age group were evaluated for eight days using the procedure previously described. The remaining two dispensers of each group also were aged in the field in the same area where all the dispensers were originally exposed to provide a total of six similarly aged dispensers.

To compare the effect of pheromone dosage on the attractiveness of the laminated plastic dispensers (lot V0436), the basic 1.27 × 2.54-cm dispenser containing 1.25 mg of the pheromone blend was either subdivided or used in multiples to obtain dosages of 0.625, 2.5, and 5.0 mg. The 0.625-mg treatment consisted of half of the standard dispenser (1.27 × 1.27 cm). The 2.5- and 5.0-mg treatments consisted of two and four of the basic dispensers, respectively, stacked broadside and exposed simultaneously. The same baits were evaluated for 16 days.

Chemical Analyses. Initial and residual amounts of pheromone components in unaged and aged dispensers were determined. Pheromone components were extracted from the dispensers for about 14 hr with a solvent mixture of 10–20 ml of 1 : 1 hexane–acetone.

Rates of emission from unaged and aged dispensers were measured using procedures reported by Leonhardt et al. (1988). Two dispensers at a time were placed in a glass tube housed in an oven at 35°C; a constant nitrogen flow (100 ml/min) was passed over the dispensers and through traps that were packed with glass beads and Tenax (ST-023; Envirochem., Kemblesville, Pennsylvania). After 6 hr, trapped volatiles were eluted with a 1 : 1 hexane–ether mixture for analyses. The emission rate per dispenser was calculated by dividing the amount of volatiles collected by six and two.

The combined amounts of *Z*7–16 : Al + *Z*9–16 : Al + *Z*11–16 : Al and 16 : Al were determined by automatic injection of 3 μl onto a 30-m × 0.75-mm wide-bore, capillary column, coated with nonpolar SPB-1 (160°C for 2 min; 2°C/min to 190°C final temperature). The amounts of the positional isomers, *Z*7–16 : Al, *Z*9–16 : Al, and *Z*11–16 : Al, were determined on a 60-m × 0.25-

mm capillary column coated with bonded polar Supelcowax 10 stationary phase (100°C for 2 min, 5°C/min to 175°C where it was held).

Chemical analyses were made of the initial and residual pheromone content of one unaged dispenser and one aged for 48 days and of the emission rate from two dispensers at one time for each age category for unaged and aged pheromone dispensers of lot V0283. The dispensers were aged for 7, 21, and 48 days in the laboratory at room temperature. This evaluation was conducted to determine the relative release rates of all four components in the dispensers.

Total combined amounts of unsaturated aldehydes for each of five dispensers from lots V0283 and V0436 were evaluated to determine differences among dispensers of the same lot and between lots. In these analyses, only the total of the unsaturated aldehydes was determined, as a combined value, without identification of the amounts of each positional isomer.

Chemical analyses were made of the unaged and 8-, 16-, 24-, and 32-day-old V0436 dispensers aged and evaluated in the field from the 1990 test. Six dispensers from each age group were analyzed; four dispensers evaluated two at a time were analyzed for emission rate, and all six (including the four dispensers evaluated for emission rate) were analyzed for residual content. The emission rate and residual content of dispensers from each age group were calculated based on mean amount per dispenser.

Statistical Analyses. Statistical analyses were made using the General Linear Models Procedure and means were separated using Duncan's (1955) multiple-range test (SAS, Institute 1985) with $\alpha = 0.05$ when three or more treatments were involved. Student's t tests ($\alpha = 0.05$) were used when the tests involved only two treatments. Regression analyses were made using Statgraphics (STSC Inc., 1987).

RESULTS AND DISCUSSION

Field Bioassays. No significant difference ($t = 0.5$; $df = 138$, $P > 0.05$) was observed between dispensers of lots V0283 and V0436. The mean numbers ($\pm$SD) of BW males captured per trap per night during a two-week period with each dispenser were 25.0 ($\pm$19.4) and 24.6 ($\pm$16.6), respectively.

Comparison of lots V0283 and V0436 over a period of 20 days indicated no significant differences between the unaged dispensers of each lot until the last four days of the evaluation period ($F = 39.3$, $df = 29$, $P < 0.05$) (Table 1). Comparison of dispensers from each lot aged for 14 days with unaged dispensers indicated a decline in capture due to aging. Significant differences for lot V0283 between the unaged and aged dispensers were observed on the ninth to sixteenth day but not the last four days of the evaluation. For lot V0436,

TABLE 1. MEAN NUMBERS OF *H. zea* MALES CAUGHT IN WIRE CONE TRAPS BAITED WITH UNAGED OR AGED LAMINATED PLASTIC DISPENSERS, LOTS V0283 AND V0436, 1986

Dates (May)	Mean no. (±SD) males captured/trap/night with indicated treatments[a]			
	V0283 (unaged)	V0283 (14 days old)	V0436 (unaged)	V0436 (14 days old)
8–11	16.7 ± 6.3 a	13.8 ± 5.0 ab	15.2 ± 7.3 ab	13.1 ± 4.1 b
12–15	22.2 ± 7.2 ab	20.7 ± 6.8 ab	23.7 ± 6.4 a	19.2 ± 7.2 b
16–21	21.0 ± 9.0 a	13.7 ± 6.2 b	16.7 ± 6.8 ab	13.9 ± 5.0 b
22–25	53.0 ± 29.8 a	40.6 ± 28.3 b	57.7 ± 32.9 a	40.4 ± 26.6 b
25–29	65.3 ± 19.5 b	63.8 ± 19.2 b	79.2 ± 19.8 a	48.4 ± 10.7 c
Overall	35.6 ± 25.8 a	30.5 ± 24.9 b	38.8 ± 31.2 a	27.0 ± 19.2 b

[a] Means in the same row followed by different letters are significantly different according to Duncan's (1955) multiple-range test with $\alpha = 0.05$. Values (F; df) associated with the statistical analyses for the different periods are: 8–11 (5.13; 12, 51), 12–15 (3.30; 12, 51), 16–21 (3.40; 12, 50), 22–25 (13.47; 12, 51), 26–29 (6.71; 12, 51) and overall (39.28; 28, 290), all with $P < 0.05$.

significant differences were observed between the unaged and aged dispensers on the fifth and eighth days and again on the thirteenth to the twentieth days of the evaluation.

Field aging for eight days of laminated plastic dispensers from lot V0436 in test I indicated no significant difference between the unaged and 8-day-old dispensers (Table 2). However, there was a significant reduction in captures with dispensers aged for 16 days as compared with either unaged or dispensers aged 8 days. Additional evaluations conducted in tests II and III further verified that the effectiveness of the 16- and 24-day-old dispensers had declined significantly compared to the unaged dispensers. The results of the 1990 test verified the significant reduction in efficacy of the dispensers after 16 days of aging (Table 3). The duplication of this test was necessary to show that storage of the dispensers for a long period of time did not reduce their efficacy. It also provided field-evaluated dispensers for use in the emission rate and residual chemical content analyses.

These findings indicate that the laminated plastic dispenser can be used for at least 12 days under central Texas conditions without a significant decline in efficacy. Although there were no unaged dispensers in these tests for comparison with the dispensers that were 5–8 and 13–16 days old, the fact that no significant differences were observed between the 1- to 4- and 9- to 12-day-old dispensers supported the conclusion of no significant decrease in efficacy over at least a 16-day period. One significant aspect of these evaluations is the com-

TABLE 2. MEAN NUMBER OF *H. zea* MALES CAUGHT IN WIRE CONE TRAPS BAITED WITH LOT V0436 PLASTIC LAMINATED DISPENSERS FIELD AGED FOR 0, 8, 16, AND 24 DAYS AND WITH FIVE VIRGIN FEMALES, 1986

Test and days[b]	Mean no. (±SD) males captured/trap/night with indicated bait[a]				
	Virgin females	Unaged	8 days old	16 days old	24 days old
I					
1–4	125.5 ± 33.8 ab	143.6 ± 47.6 a	139.3 ± 32.3 a	105.9 ± 28.0 b	
5–8	23.7 ± 18.7 b	55.6 ± 20.9 a	50.9 ± 19.0 a	32.3 ± 12.4 b	
II					
1–4[c,d]	32.9 ± 47.1 c	100.4 ± 55.3 a		64.7 ± 29.3 b	40.8 ± 25.9 c
5–8	49.6 ± 29.9 c	99.4 ± 35.0 a		68.9 ± 25.9 b	37.6 ± 14.2 c
III					
1–4[e]	50.8 ± 43.1 c	115.9 ± 46.9 a		74.5 ± 29.1 b	47.1 ± 23.7 c
5–8	68.4 ± 38.4 b	125.4 ± 36.2 a		76.4 ± 29.5 b	37.2 ± 13.6 c

[a] Means in the same row followed by different letters are significantly different according to Duncan's (1955) multiple-range test ($\alpha = 0.05$). Values (F) for the statistical analyses are: I(1–4) = 5.88, I(5–8) = 6.44, II(1–4) = 11.54, II(5–8) = 7.57, III(1–4) = 7.69, III(5–8) = 9.54, all with $df = 12, 51$ and $P \leq 0.05$.

[b] Test I was run July 23–30; test II was run July 31 to August 9; test III was run August 4–11.

[c] Traps were not operated on August 1–3, 1986, because of unavailability of virgin females of the right age. During this two-day period, the dispensers were placed in a freezer in the laboratory.

[d] Most of the virgin females died during the two-day period between July 30 and August 1, 1986, so the traps were operated an extra two days to make up for this effect.

[e] The dispensers evaluated were actually two days older than indicated because of the extra two days of exposure between July 30 and August 1, 1986.

parison of the captures in the unaged and 8-day-old dispensers to virgin females; the dispensers were consistently more effective than the virgin females. Zvirgzdins and Henneberry (1983) and Carpenter et al. (1984) reported similar results.

A definite dosage response for pheromone amounts above 2.5 mg was observed for BW males (Table 4). Equal or higher numbers of males were captured in traps baited with the standard 1.25-mg dispenser than the 0.625-mg dose in the plastic laminate; however, the differences were not significant. The 2.5-mg dosage consistently captured lower numbers of BW males; however, the differences were significant for only two of the four evaluation periods. Overall, for the 16-day evaluation period, captures with the 2.5-mg dose were significantly lower than for the 1.25- or 0.625-mg dosages. The 5.0-mg dosage captured significantly lower numbers of BW males when compared with the 0.625- and 1.25-mg dosage throughout the test period. These results indicate that a 1.25-mg dosage is optimum for field efficacy with some flexibility allowed for a lower or higher dosage. Similar trends were observed by Hartstack et al.

TABLE 3. MEAN NUMBER OF *H. zea* MALES CAUGHT IN WIRE CONE TRAPS BAITED WITH LOT V0436 PLASTIC LAMINATE DISPENSERS FIELD AGED FOR 0, 8, 16, AND 24 DAYS, 1990

Age (days)[b]	Mean no. (±SD) males captured/trap/night during indicated periods[a]		
	September 14–17	September 18–21	Overall
0	122.8 ± 60.6 a	64.3 ± 23.4 a	93.6 ± 54.1 a
9	107.6 ± 55.4 ab	57.4 ± 17.9 a	82.5 ± 47.9 a
16	83.6 ± 36.9 b	46.7 ± 12.0 b	65.2 ± 32.9 b
24	49.3 ± 16.6 c	32.9 ± 10.8 c	41.1 ± 16.1 c

[a]Means in the same row followed by different letters are significantly different according to Duncan's (1955) multiple-range test ($\alpha = 0.05$). Values (F; df) for the statistical analyses for the different periods are: September 14–17 (6.38; 12, 51), September 18–21 (6.13; 12, 51), and overall (12.62; 16, 111), all with $P < 0.05$.

[b]Dispensers were aged under field conditions starting on August 14, and a new set was added on August 22 and August 30 until September 6, when all the dispensers were placed in a freezer until the start of evaluation on September 13.

TABLE 4. MEAN NUMBER OF *H. zea* MALES CAPTURED IN WIRE CONE TRAPS BAITED WITH VARYING PHEROMONE QUANTITIES IN LAMINATED PLASTIC, 1987[a]

Dates (June)	Mean no. (±SD) males captured/trap/night with indicated dosage (mg)[b]			
	0.625	1.25	2.5	5.0
3–6	12.5 ± 5.1 a	12.4 ± 6.5 a	8.3 ± 5.8 b	5.4 ± 3.6 b
7–10	34.0 ± 15.9 a	36.7 ± 14.9 a	29.0 ± 13.5 ab	20.8 ± 14.5 b
11–14	71.4 ± 32.7 a	73.0 ± 26.2 a	52.4 ± 15.0 b	42.5 ± 19.4 b
15–18	51.1 ± 19.7 a	54.9 ± 23.4 a	45.1 ± 17.2 ab	34.8 ± 14.2 b
Overall	42.1 ± 30.0 a	44.1 ± 29.5 a	33.7 ± 21.6 b	25.9 ± 19.8 c

[a]The standard 1.25-mg (2.54 × 1.27 cm) dispenser was used. For the 0.625-mg treatment, the standard dispenser was cut in half and for the 2.5- and 5.0-mg treatments, two and four standard dispensers were used, respectively.

[b]Means in the same row followed by different letters are significantly different according to Duncan's (1955) multiple-range test ($\alpha = 0.05$). Values (F; df) associated with the statistical analyses for the different periods are: 8–11 (5.13; 12, 51), 12–15 (3.30; 12, 51), 16–21 (3.40; 12, 50), 22–25 (13.47; 12, 51), 26–29 (6.71; 12, 51) and overall (39.28; 28, 290), all with $P < 0.05$.

(1980); however, the differences they observed when comparing 5.0-, 2.5-, 1.25-, 0.63-, and 0.32-mg dosages cut from a 10-mg, 6.7 cm^2 dispenser were not statistically significant.

Chemical Analyses. Chemical analysis of one unaged plastic laminate dispenser from lot V0283 indicated a pheromone content of 1.46 mg, higher than

the nominal 1.25 mg (Table 5). The percentages of Z7–16:Al (2.7%) and Z9–16:Al (5.5%) were higher than the nominal 2.0 and 3.0%, respectively, while the measured amount of Z11–16:Al was somewhat lower (83.6% vs. 87%) and 16:Al was about the same (8.2 vs. 8.0%). Laboratory aging for 48 days reduced total pheromone content of one dispenser to 0.85 mg. The percentages of each component remained similar to those in the unaged dispenser, indicating that each component was released in proportion to its content. This was verified by collection and analysis of volatiles. Emission rates of total pheromone blend ranged from 0.31 μg/hr/dispenser for the unaged dispensers to 0.15 μg/hr/dispenser for the dispensers aged 48 days.

Chemical analyses of five dispensers each from lots V0436 and V0283 showed considerable variation in the pheromone content of the dispensers. Dispensers from lot V0436 contained a mean (±SD) of 1.29 (±0.21) mg of the pheromone blend with a range of 1.15–1.66 mg/dispenser. The pheromone content of dispensers from lot V0283 was 1.43 (±0.12) mg/dispenser; variation between dispensers ranged from 1.27 to 1.53 mg. Because no differences were observed in the performance of dispensers from these two lots in field bioassays, differences observed in the pheromone content of the dispensers from both lots were not sufficient to cause differences in efficacy. Also, the highest amounts of pheromone in these dispensers were not high enough to cause a decrease in efficacy.

Chemical composition of unaged and field-aged dispensers of lot V0436 evaluated in the 1990 tests indicated that the mean (±SD) total pheromone content of 1.23 (±0.08) mg in the unaged dispensers was essentially identical

TABLE 5. COMPOSITION (mg/DISPENSER) AND EMISSION RATES (μg/hr/DISPENSER) OF PHEROMONE CHEMICALS FROM UNAGED OR AGED LAMINATED PLASTIC DISPENSERS (LOT V0283) EXPOSED UNDER LABORATORY CONDITIONS

Days aged	Indicated component, mg (% of total)[a]				
	Z7–16:Al	Z9–16:Al	Z11–16:Al	16:Al	Total
Chemical composition					
0	0.04 (2.7)	0.08 (5.5)	1.22 (83.6)	0.12 (8.2)	1.46
48	0.02 (2.3)	0.03 (3.5)	0.73 (85.9)	0.07 (8.2)	0.85
Release rate					
0	0.006 (1.9)	0.008 (2.6)	0.28 (90.3)	0.02 (6.5)	0.31
7	0.006 (2.1)	0.008 (2.8)	0.26 (87.7)	0.02 (6.9)	0.29
21	0.005 (1.9)	0.008 (3.0)	0.24 (88.9)	0.02 (7.4)	0.27
48	0.003 (2.0)	0.004 (2.7)	0.14 (93.3)	0.01 (6.7)	0.15

[a] (Z)-7-Hexadecenal = Z7–16:Al, (Z)-9-Hexadecenal = Z9–16:Al, (Z)-11-Hexadecenal = Z11–16:Al, and Hexadecanal = 16:Al.

to the total pheromone dosage of 1.29 (±0.21) mg found for dispensers of lot V0436, which were compared with dispensers from lot V0283 (Table 6). There was a gradual decrease in the amount of pheromone in the dispensers to 0.89 (±0.09), 0.53 (±0.03), 0.26 (±0.02), and 0.17 (±0.02) mg after 8, 16, 24, and 32 days of field aging, respectively. A similar pattern was observed in emission with mean (±SD) rates of 0.33 (±0.03), 0.21 (±0.03), 0.10 (±0.02), 0.063 (±0.033), and 0.034 (±0.014) μg/hr/dispenser for dispensers aged 0, 8, 16, 24, and 32 days, respectively. The low emission rates at 16 and 24 days were probably the cause of the poor trap captures. Regression analyses of mean residual pheromone content and mean emission rate produced the equations: $y = 1.35664 \exp(-0.064856x)$ ($R^2 = 0.983$) and $y = 0.338363 \exp(-0.0721527)$ ($R^2 = 0.997$) for residual pheromone content in milligrams per dispenser and emission rate in micrograms per hour per dispenser both designated as y, respectively, as a function of aging time in days (x).

The relative percents of the 16:Al emitted and in the residual were very similar for the 0- and 8-day-old dispensers; however, the residual pheromone in the 16- and 24-day-old dispensers showed increasing percents of the somewhat less volatile 16:Al.

Calculation of the ratio(s) of the mean number of males captured with the

TABLE 6. COMPOSITION (mg/DISPENSER) AND EMISSION RATES (μg/hr/DISPENSER) OF PHEROMONE COMPONENTS FROM UNAGED OR AGED (8, 16, 24 OR 32 DAYS) LAMINATED PLASTIC DISPENSERS (LOT V0436) EXPOSED UNDER FIELD CONDITIONS, 1990

Days aged	Mean (±SD) amount of indicated components (% of total)		
	16^1:Al	16^0:Al	Total pheromone
Chemical composition[a]			
0	1.11 ± 0.07 (90.2)	0.12 ± 0.01 (9.8)	1.23 ± 0.08
8	0.80 ± 0.08 (89.9)	0.09 ± 0.01 (10.1)	0.89 ± 0.09
16	0.47 ± 0.03 (88.7)	0.06 ± 0.00 (11.3)	0.53 ± 0.03
24	0.22 ± 0.02 (84.6)	0.04 ± 0.01 (15.4)	0.26 ± 0.02
32	0.14 ± 0.02 (82.4)	0.02 ± 0.01 (11.8)	0.17 ± 0.02
Emission rate[b]			
0	0.2910 ± 0.0297 (89.5)	0.0340 ± 0.0028 (10.5)	0.3250 ± 0.0325
8	0.1815 ± 0.0276 (87.9)	0.0250 ± 0.0028 (12.1)	0.2065 ± 0.0304
16	0.0870 ± 0.0014 (85.7)	0.0145 ± 0.0007 (14.3)	0.1015 ± 0.0021
24	0.0505 ± 0.0290 (85.7)	0.0100 ± 0.0042 (16.5)	0.0605 ± 0.0332
32	0.0125 ± 0.0120 (79.1)	0.0070 ± 0.0028 (20.9)	0.0335 ± 0.0149

[a] Mean based on six dispensers.
[b] Mean based on the release rate from four dispensers evaluated two at a time in each emission tube.

aged dispensers as compared to the unaged dispensers for the two years (1987 and 1990) indicated a dramatic decrease in efficacy after approximately 14 days of use (Figure 1). These ratios were calculated by considering the period of evaluation in four-day intervals. The ratios were calculated by dividing the mean number of males captured by the aged dispensers (first or second four-day period) by the mean number of males captured with the unaged dispensers during each corresponding four-day period (0–4 and 5–8 days old) from Tables 2 and 3. Each value was plotted as the midpoint of each interval for age.

Criteria for satisfactory performance of a pheromone dispenser in terms of the ratio described above have not been established; however, the closer the ratio values are to 1 over the period of aging, the better the dispenser. With the laminated plastic dispenser, a 0.5 ratio was observed at approximately 24 days of aging. At 10, 14, 18, and 22 days of aging, the mean ratios were 0.93, 0.91, 0.68, and 0.65, respectively. Using a best-fit regression model ($R^2 = 0.970$) for the ratios indicated that the efficacy of the dispensers decreased by 10% in 12.0 days, by 20% in 15.5 days, and by 30% in 18.5 days. As such, a 14-day

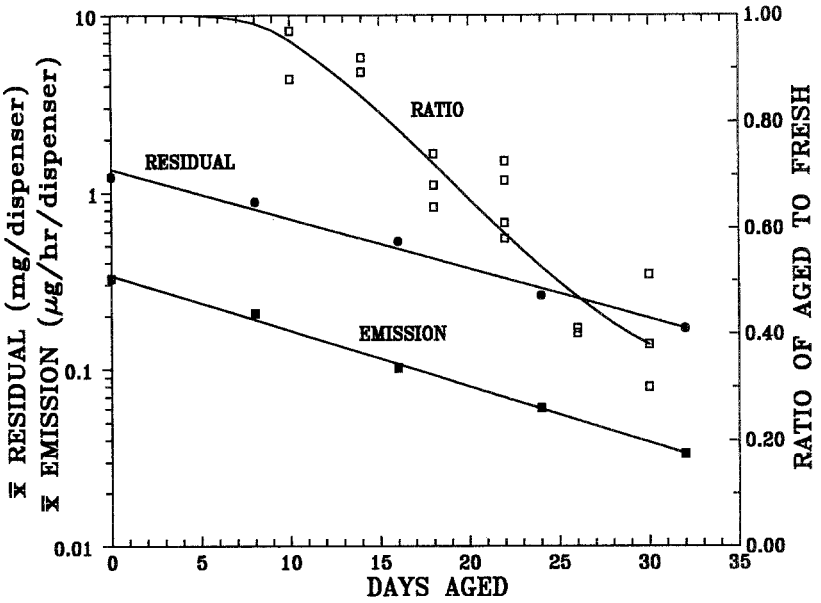


FIG. 1. Residual content, emission rate, and ratio of mean trap captures of aged to new plastic laminate dispensers for *H. zea* relative to the number of days of aging. Ratios were calculated from the values in Table 2 by dividing the mean number of males captured by the aged dispensers (first or second four-day period) by the mean number of males captured with the new dispensers during each corresponding four-day period (0–4 and 5–8 days old).

period of use would be satisfactory with less than a 20% reduction in efficacy. Corresponding criteria for the dispenser to have satisfactory performance (a ratio of 0.85 or more) over a 14-day period calculated from the regression equations would be a residual pheromone content equal to or greater than 0.55 mg/dispenser but not much greater than 1.25 mg/dispenser and an emission rate equal to or greater than 0.12 μg/hr/dispenser but not much greater than 0.33 μg/hr/dispenser.

In summary, performance criteria and specifications recommended for a plastic laminate pheromone dispenser expected to be effective for two weeks are: (1) 1.27 × 2.54 cm in size; (2) 400-μm thick orange PVC outer layers; (3) 1.25 mg of a blend of *Z*11–16:Al, *Z*9–16:Al, *Z*7–16:Al, and 16:Al in a ratio of 87:3:2:8 formulated in the adhesive reservoir layer; (4) release rate in the range of about 0.12–0.33 μg/hr/dispenser as measured in the laboratory at 35°C; and (5) residual pheromone content in the range of 0.55–1.25 mg/dispenser during the period of use. Although these criteria and specifications were evaluated for performance under central Texas conditions, it is likely that they will be broadly applicable because they are based on evaluations conducted under typical summer conditions.

Acknowledgments—This research was conducted in cooperation with the Texas Agricultural Experiment Station, Texas A&M University, College Station, Texas 77843.

REFERENCES

CARPENTER, J.E., PAIR, S.D., and SPARKS, A.N. 1984. Trapping of different noctuid moth species by one trap baited with two lures. *J. Ga. Entomol. Soc.* 19:120–124.

DRAPEK, R.J., COOP, L.B., CROFT, B.A., and FISHER, G.C. 1990. *Heliothis zea* pheromone trapping: Studies of trap and lure combinations and placement in sweet corn. *Southwest. Entomol.* 15:63–69.

DUNCAN, D.B. 1955. Multiple range and multiple F tests. *Biometrics* 11:1–41.

HARTSTACK, A.W., WITZ, J.A., and BUCK, D.R. 1979. Moth traps for the tobacco budworm. *J. Econ. Entomol.* 72:519–522.

HARTSTACK, A.W., LOPEZ, J.D., JR., KLUN, J.A., WITZ, J.A., SHAVER, T.N., and PLIMMER, J.R. 1980. New trap designs and pheromone bait formulations for *Heliothis*. Proceedings, Beltwide Cotton Production and Research Conferences, National Cotton Council, Memphis, Tennessee, pp. 132–160.

KLUN, J.A., PLIMMER, J.R., BIERL-LEONHARDT, B.A., SPARKS, A.N., PRIMIANI, M., CHAPMAN, O.L., LEE, G.A., and LEPONE, G. 1980. Sex pheromone chemistry of female corn earworm moth, *Heliothis zea*. *J. Chem. Ecol.* 6:165–175.

LEONHARDT, B.A., DICKERSON, W.A., RIDGWAY, R.L., and DEVILBLISS, E.D. 1988. Laboratory and field evaluation of controlled release dispensers containing grandlure, the pheromone of the boll weevil (Coleoptera: Curculionidae). *J. Econ. Entomol.* 81:937–943.

LEONHARDT, B.A., CUNNINGHAM, R.T., DICKERSON, W.A., MASTRO, V.C., RIDGWAY, R.L., and SCHWALBE, C.P. 1990. Dispenser design and performance criteria for insect attractants, pp. 113–129, *in* R.L. Ridgway, R.M. Silverstein, and M.N. Inscoe (eds.). Behavior-Modifying

Chemicals for Insect Management—Applications of Pheromones and Other Attractants, Marcel Dekker, New York.

LOPEZ, J.D., JR., and SHAVER, T.N. 1990. Plastic laminate dispenser for components of the *Heliothis zea* sex pheromone. *Southwest. Entomol.* 15:1–8.

LOPEZ, J.D., JR., SHAVER, T.N., and HARTSTACK, A.W. JR., 1981. Evaluation of dispensers for the pheromone of *Heliothis zea. Southwest. Entomol.* 6:117–122.

LOPEZ, J.D., JR., SHAVER, T.N., and GOODENOUGH, J.L. 1987. Research on various aspects of *Heliothis* spp. pheromone trapping. Proceedings, Beltwide Cotton Production and Research Conferences, National Cotton Council, Memphis, Tennessee, pp. 300–307.

QUISUMBING, A.R. and KYDONIEUS, A.F. 1982. Laminated structure dispensers, pp. 213–135, *in* A.F. Kydonieus and M. Buoys (eds.). Insect Suppression with Controlled Release Systems, Vol. 1. CRC Press, Boca Raton, Florida.

SAS Institute. 1985. SAS User's Guide: Statistics, 5th ed. SAS Institute, Inc., Cary, North Carolina.

STSC Inc. 1987. Statgraphics User's Guide. Rockville, Maryland.

ZVIRGZDINS, A., and HENNEBERRY, T.J. 1983. *Heliothis* spp.: Sex pheromone trap studies. Proceedings, Beltwide Cotton Production and Research Conferences, National Cotton Council, Memphis, Tennessee, pp. 176–180.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

CHEMISTRY *vis-à-vis* MATERNALISM IN LACE BUGS (HETEROPTERA: TINGIDAE): ALARM PHEROMONES AND EXUDATE DEFENSE IN *Corythucha* AND *Gargaphia* SPECIES

JEFFREY R. ALDRICH,[1,*] JOHN W. NEAL, Jr.,[2]
JAMES E. OLIVER,[1] and WILLIAM R. LUSBY[3]

[1]*Insect Chemical Ecology Laboratory, USDA-ARS, Bldg 467*
[2]*Florist and Nursery Crops Laboratory, USDA-ARS, Bldg 470*
[3]*Insect Neurobiology and Hormone Laboratory, USDA-ARS, Bldg 467*
Beltsville, Maryland 20705

(Received March 20, 1991; accepted July 23, 1991)

Abstract—The hawthorn lace bug, *Corythucha cydoniae*, and the eggplant lace bug, *Gargaphia solani*, possess alarm pheromones that are produced in dorsal abdominal glands (DAGs). When *G. solani* nymphs are grasped, they emit secretion from both DAGs; the posterior DAG secretion alone elicits alarm, but the anterior DAG secretion may hasten the response. In *C. cydoniae*, the response is due to a synergism between the anterior and posterior DAG secretions, and nymphs are apparently unable to voluntarily release their DAG secretions; both DAGs must be ruptured for the pheromone to escape. The alarm pheromones are interspecifically active in patterns matching the intraspecific activities. Compounds identified from tingid DAG secretions that are involved in the alarm messages are: (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, acetaldehyde, geraniol, and linalool. A new natural product of unknown function (designated nerolidol aldehyde) was identified from the anterior DAG secretions of both species.

Key Words—Hemiptera, Tingidae, *Corythucha*, *Gargaphia*, altruism, semiochemical, defensive behavior, maternalism, allomone, abdominal glands, (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, acetaldehyde, geraniol, linalool.

INTRODUCTION

Of the 1800 species of tingids worldwide, relatively few are gall-makers or myrmecophiles; the vast majority feed gregariously on mesophyll underneath

*To whom correspondence should be addressed.

0098-0331/91/1100-2307$06.50/0

leaves (Drake and Ruhoff, 1965). Exposed, sedentary lace bugs seem likely to have specialized defenses against predators and parasitoids. Indeed, two adaptations by which tingids blunt their potential attackers have now been extensively studied. Females of the eggplant lace bug, *Gargaphia solani*, and other *Gargaphia* species, assiduously guard their eggs and nymphs (Fink, 1915). Maternalism does effectively deter most predators, although a "cheating" counterstrategy has evolved in *G. solani* whereby some females furtively "dump" their eggs among those of a guarding female (Tallamy and Horton, 1990, and references therein). However, maternal care is not widespread among tingids, at least not to the extent observed for *Gargaphia* spp. (Faeth, 1989), but nymphs of species from many genera have secretory setae bearing fluid droplets scattered over their bodies (Livingston, 1978). In *Stephanitis* and *Corythucha* the droplets contain compounds (probably in aqueous solution) having an oxygenated six-member ring with a long side chain (acetogenins) (Oliver et al., 1990, and references therein). A biological role for these acetogenins has not been firmly established, yet it seems likely that the droplets fulfill a defensive role. Perhaps the acetogenins simply act as soaps to allow the droplets to wet small aggressors (Aldrich, 1988a).

Kearns and Yamamoto (1981) reported that aggregated nymphs of *G. solani* quickly become alarmed when a conspecific nymph is crushed nearby. Furthermore, they described similar alarm behavior toward crushed nymphs for the hawthorn lacebug, *Corythucha cydoniae*, as well as two other *Corythucha* spp., and found cross-activity between *G. solani* and all three *Corythucha* spp. To our knowledge, alarm pheromones in the Tingidae have not been explored further. Therefore, the present study of the alarm pheromone systems of *G. solani* and *C. cydoniae* was undertaken.

METHODS AND MATERIALS

Insects. The hawthorn lace bugs, *C. cydoniae*, used in the study were from a colony reared on cuttings of Washington hawthorn, *Crataegus phaenopyrum*, at 26 ± 1°C with a 16:8 hr light–dark photoperiod. The initial behavioral and chemical work with *G. solani* used nymphs collected in the field from horsenettle, *Solanum carolinense*, near the Beltsville laboratory during late summer, 1989. Later experiments with *G. solani* were performed using individuals from a laboratory colony maintained on greenhouse-grown eggplant, *Solanum melongena*, under insectary conditions as above.

Scanning Electron Microscopy. Lace bug exuviae were sputter coated with gold–palladium in a Technics Hummer 5 instrument and examined with a Hitachi S-530 scanning electron microscope.

Extractions. Immature tingids possess two dorsal abdominal glands (DAGs) attached to the 3–4 and 4–5 intersegmental membranes (Cobben, 1978), and the contents of the glands are shed at each molt with the exuviae (Aldrich, 1988b). For *G. solani*, the combined contents of the DAGs could be satisfactorily extracted by cutting out a small piece of cuticle containing the gland reservoirs under a dissecting microscope from fresh exuviae and extracting several such reservoir pairs with 25–100 μl CH_2Cl_2 or CS_2. 1-Nonanol was used as an internal standard in one extract of 20 *G. solani* fifth-instar exuviae (< 1-day-old) for quantitation of DAG volatiles. DAG samples from *G. solani* were also prepared by pressing small pieces of filter paper down on the abdominal tergum of live nymphs and then extracting the papers in ca. 100 μl of CH_2Cl_2. For *C. cydoniae*, these methods were unsatisfactory because the acetogenins from the setal droplets (Lusby et al., 1989) overwhelmingly contaminated the samples. The following sampling technique was devised to avoid this problem. Fresh exuviae were removed from leaves and placed ventral-side down on double-stick tape on a slide under the dissecting microscope. Using microscissors, a cut was made laterally along each side of the tergum; this flap of cuticle was peeled backwards with forceps and stuck to the tape, thus exposing the inner surface of the two DAG reservoirs. A capillary tube drawn to a very fine point was inserted in a Drummond Microcap (Broomall, Pennsylvania) holder and fastened in a micromanipulator next to the microscope stage. When the reservoir of a DAG was pierced with the micropipet, the secretion was captured by capillary action, and could then be expelled into solvent or used directly for bioassay. This method was subsequently used to separately sample the DAGs of *G. solani*.

Chemical Analysis. Samples were analyzed by gas chromatography (GC) on a bonded methyl silicone column (0.25 mm film, 14 m × 0.25 mm ID; DB-1, J&W Scientific, Folsom, California) using a Varian 3700 GC with helium as carrier (40 cm/sec), and a temperature program from 45°C for 2 min to 230°C at 15°/min. Electron impact mass spectra (EI-MS) were obtained at 70 eV using a Finnigan 4510 GC-MS, equipped with a 30-m DB-1 column. Volatiles from exuviae of *C. cydoniae* were derivatized with *o*-benzylhydroxylamine after the method of Ollett et al. (1986). Approximately 50 exuviae were crushed in a 250-μl gastight syringe equipped with a GTS Valve (Hamilton Company, Reno, Nevada) in the off position, and then the volatiles were bubbled through the derivatizing solution by opening the valve and slowly depressing the syringe plunger. The *o*-benzyloximes were also analyzed by chemical ionization mass spectrometry (CI-MS) using NH_3 and CH_4 as reagent gases. One underivatized sample of *C. cydoniae* exuviae crushed in a gastight syringe as described above was analyzed by GC-MS. The sample was injected under pressure using the GTS valve with the GC oven turned off and cooled to ca.

0°C with Dry Ice in order to obtain EI-MS of very volatile compounds normally inseparable from organic solvents. A head-space sample of acetaldehyde was analyzed in the same fashion.

The unknown compound eluting at a retention time (*RT*) of 11.0 min (Figures 2 and 3) was isolated from an exuvial extract of *G. solani* by applying the concentrated CH_2Cl_2 extract to a Baker 3-ml silica gel extraction cartridge, and eluting successively with hexane (5 ml), 10% ethyl acetate in hexane (5 ml), and 25% ethyl acetate in hexane (3 ml). The unknown reproducibly eluted in the last fraction. A small sample thus prepared was hydrogenated for 20 min (PtO_2, EtOH, 1 atm). For NMR, ca. 165 mg of *Gargaphia* exuviae were processed as described, and the unknown was further purified by preparative GC (15 m × 0.53 mm ID, DB-1). A [^{1}H]NMR spectrum for this compound was recorded in D_6-benzene at 300 MHz on a General Electric QE-300 instrument with TMS as an internal standard.

Standards. The following compounds were obtained commercially: acetaldehyde and 1-nonanol (Aldrich Chemical, Milwaukee, Wisconsin); geraniol and nerol (Bedoukian Research, Danbury, Connecticut); and linalool (Givaudan, Clifton, New Jersey). (*E*)-4-Oxo-2-hexenal was synthesized according to Ward and VanDorp (1969). Nerolidol aldehyde [(*E*,*E*)-2,6,10-trimethyl-10-hydroxy-2,6,11-dodecatrienal] was synthesized after the method of Demole and Enggist (1973).

Bioassays. Bioassays were begun with *C. cydoniae* using a procedure similar to that of Kearns and Yamamoto (1981). A feeding aggregation of >10 fifth-instar nymphs was chosen for observation, and a conspecific nymph from another aggregation (normally on a different cutting) was sacrificed for the test. A leg was grasped with forceps and the nymph was held 5–10 mm above the test aggregation for ca. 1 min; then the entire body of the test nymph was positioned between the prongs of the forceps, crushed, and immediately held over the test aggregation. A response was considered positive if most of the nymphs walked away within 1 min. Fifth-instar nymphs in the process of ecdysis (evident in aggregations by their unmelanized cuticle) were also bioassayed as above, after which the fresh exuviae were tested for alarm induction as follows: First the exuviae were lifted by an appendage and offered to the test aggregation for 1 min; then the DAG reservoirs of the exuviae were ruptured with forceps under a microscope and the macerated exuviae were again held over the same test aggregation.

Alarm pheromone tests were performed with the hawthorn lace bug by positioning a leaf aggregation of *C. cydoniae* fifth-instar nymphs about 1 cm in front of the exit port from the thermal conductivity detector of the GC equipped with a DB-1 megabore column. For GC-effluent testing, several aggregations (>200 individuals) of *C. cydoniae* would be quick-frozen by placing the leaves

on which they were aggregated onto Dry Ice. The frozen nymphs were brushed off the leaves into a 10-ml gastight syringe barrel, having a Teflon membrane (0.5-mm pore size; Millipore Corp., Bedford, Massachusetts) in the bottom, and equipped with a GTS valve. The barrel of the syringe was flushed with a stream of N_2, and the plunger was inserted as far as possible without crushing the nymphs. Then, with the GTS valve in the off position, the nymphs were crushed and the volatiles injected into the GC under pressure. Control experiments were performed in an analogous manner with an empty syringe. Responses were judged positive if most of the aggregated nymphs were dispersed by the effluent.

For bioassay of the DAG secretions (test I), the contents of the anterior and/or posterior gland exuvial reservoirs were extracted in a micropipet as previously described. A leaf with a nymphal aggregation to be tested (>10 third- to fifth-instar nymphs) was placed on the microscope stage with the illumination lowered so as not to warm the insects. A second micromanipulator was arranged on the opposite side of the microscope stage with a pair of forceps holding a small triangular piece of filter paper. The DAG secretion(s) to be tested were transferred to the point by bringing the pipet in contact with the paper and then the impregnated paper was quickly lowered to within about 0.5 cm of the aggregation. As part of this series, solutions (3 μg/μl CH_2Cl_2) of geraniol, linalool, (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, and combinations thereof, were tested by wetting the point tip with test solution and proceeding as for the gland extracts. Absence of a response was scored a (−), a moderate response was (±), and complete dispersal was a (+).

In an effort to fully mimic the natural response of the hawthorn lace bug with synthetic standards, a second bioassay series (test II) was performed using only *C. cydoniae* nymphs. The day before the testing session, five fifth-instar nymphs (or fourth instars if necessary) were placed on single hawthorn leaves with petioles in water vials; 25–50 such preparations were set up for each session. Test solutions of acetaldehyde, geraniol, linalool, nerol, (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, and combinations thereof, were prepared in CH_2Cl_2 at a range of concentrations, plus CH_2Cl_2 controls (see Table 2 below). Responses were scored by a "blind" observer with no prior knowledge of the material being tested. An aggregation to be tested was positioned by one person (J.W.N.) on a microscope stage with reduced illumination, while a second person (J.R.A.) applied 1 μl (Drummond Microcap) of test solution to a paper point on the end of an insect pin. The loaded filter paper was held about 0.5 cm above the aggregation and the response of the majority of nymphs was scored as follows: 0 = no response, 1 = agitation and jiggling in place, 2 = wandering, 3 = slowly moving away (after a delay of >20 sec), and 4 = quickly moving away (within 20 sec).

RESULTS

Gargaphia solani nymphs do not have setal droplets (Figure 1A). In *C. cydoniae* nymphs, setal droplets are abundant (Figure 1B), and after ecdysis or when droplets are brushed off due to overcrowding, they are regenerated. In *G. solani* the openings of the anterior and posterior DAGs are evident in SEMs, but in *C. cydoniae* the external openings of the DAGs are not apparent.

Preliminary experiments (J.W.N.) with the hawthorn lace bug confirmed that a potent alarm pheromone is released from crushed nymphs. Feeding aggregations of nymphs usually respond within 10–15 sec by removing their stylets from the leaf, jiggling and bobbing, and rapidly dispersing. If a *C. cydoniae* nymph is grasped without being crushed, aggregated nymphs are not alarmed. Occasionally, aggregated *C. cydoniae* nymphs failed to respond to crushed nymphs. Microscopic examination (J.R.A.) indicated that the alarm pheromone was released only if the abdomen was crushed, suggesting that the pheromone

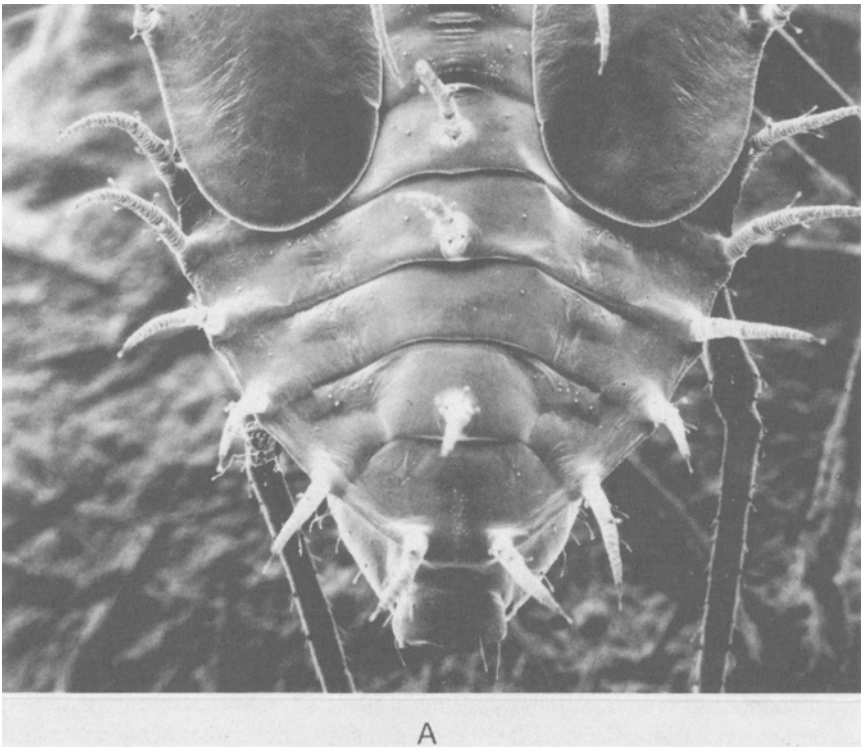


FIG. 1. (A) *Gargaphia solani* fifth-instar nymph with setae lacking droplets and, (B) *Corythucha cydoniae* fifth-instar replete with setae bearing droplets (SEMs: 80×).

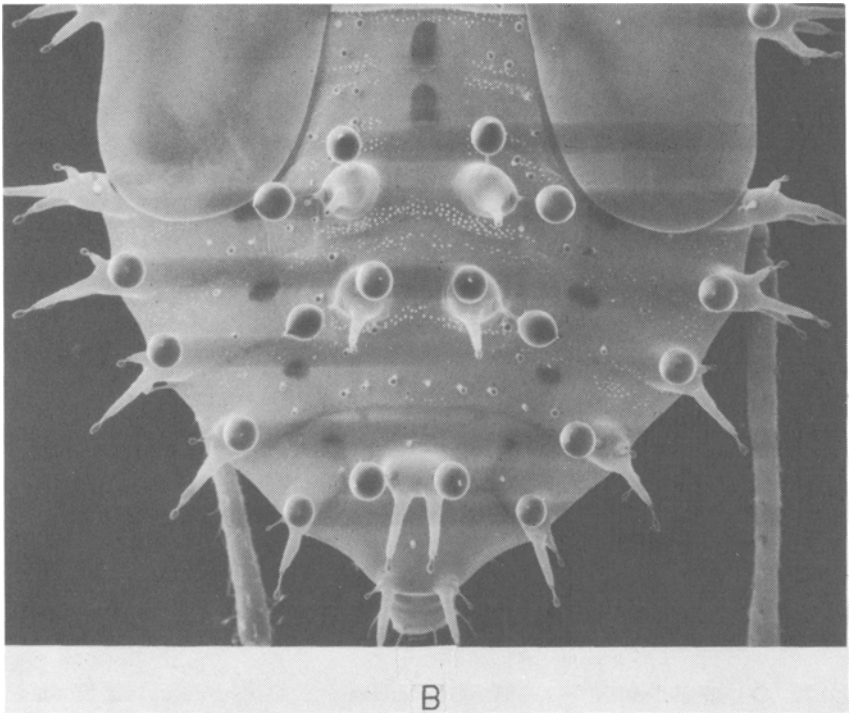


FIG. 1. Continued

is contained in the DAGs. Exuviae with intact DAGs did not elicit alarm, but when the exuvial DAG reservoirs were ruptured the full range of alarm behavior was elicited. When the abdomen of the newly ecdysed fifth instar (ca. 10 min after ecdysis) was crushed and the nymph was held near an aggregation, no alarm was induced. This experiment was repeated many times with the same result.

In contrast to *C. cydoniae*, it is not necessary to crush a nymph of *G. solani* in order to elicit alarm in aggregated conspecific nymphs. Merely grasping a nymph firmly by a leg is usually sufficient to elicit clear-cut alarm among clustered *G. solani* nymphs. Pinched *G. solani* nymphs that induced alarm had a distinctly terpenoid odor (J.R.A., personal observation).

Techniques for directly sampling DAG secretions were first perfected using *G. solani* fifth-instar nymphs. Extracts of exuviae with intact reservoirs exhibit four prominent peaks by GC, but late-eluting compounds (characteristic of tingids having setal droplets) are absent (Figure 2; bottom trace, compounds **1**–**4**). A fifth-instar *G. solani* nymph produces about 8 ng of **1**, 19 ng of **2**, 34 ng

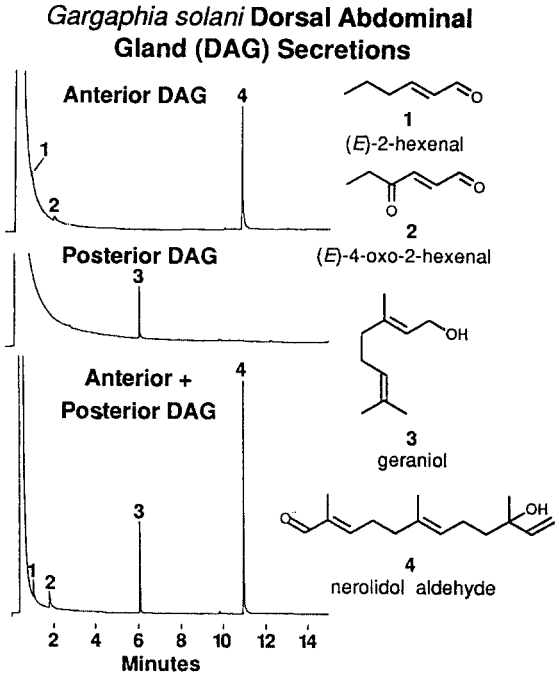


FIG. 2. Dorsal abdominal gland (DAG) secretions of *Gargaphia solani* fifth-instar nymphs: anterior DAG (one exuvial reservoir; splitless GC), posterior DAG (one exuvial reservoir; splitless GC), and anterior plus posterior DAGs (60 exuviae; 1:50 GC split).

of **3**, and 100 ng of **4**, based on quantitation of an extract of 20 exuviae. Extracts of filter paper pressed onto the tergum of *G. solani* nymphs produced GC traces (not shown) matching those of exuvial DAG reservoirs, verifying that these nymphs are able to voluntarily release secretion from both DAGs. GC analysis of secretion isolated from the anterior DAG reservoir of exuviae showed that this secretion contains compounds **1**, **2**, and **4** (Figure 2, top), whereas secretion isolated from the posterior DAG exuvial reservoir contains compound **3** (Figure 2, middle).

For *C. cydoniae*, separate extraction and GC analysis of the anterior and posterior DAG reservoirs also showed that the two glands produce qualitatively different secretions (Figure 3). Compound **4** is the predominant component in the anterior DAG secretion of the hawthorn lace bug, but compound **2** was not detected and compound **1** was barely detectable. In the posterior DAG secretion of *C. cydoniae*, compound **3** occurs with another component **(5)** not found in *G. solani* nymphs.

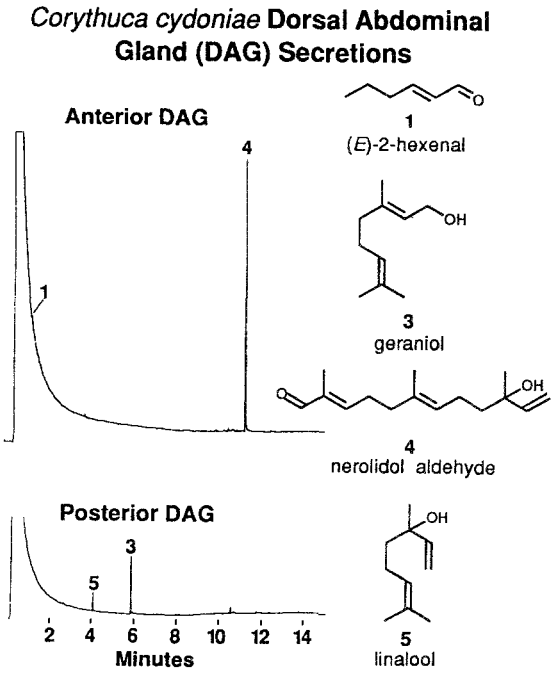


FIG. 3. Dorsal abdominal gland (DAG) secretions of *Corythucha cydoniae* fifth-instar nymphs: anterior DAG (10 exuvial reservoirs; splitless GC) and posterior DAG (two exuvial reservoirs; splitless GC).

The EI-MS of compounds **1, 2,** and **5** matched those for the following compounds commonly encountered in heteropteran exocrine secretions (Aldrich, 1988a,b): (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, and linalool, respectively. The EI-MS of compound **3** gave a near-perfect computer-matched fit to the MS of geraniol. The identities of these four compounds were verified by coinjection with authentic compounds.

A match for the EI-MS of compound **4** was not found in the computerized mass spectral library, but the pattern of lower mass (m/z < 140 amu) ions resembled that of nerolidol. The CI-MS with NH_3 indicated a molecular weight of 236, and ND_3 CI-MS showed that the molecule contains one exchangeable hydrogen. Hydrogenation produced a compound with a molecular weight of 244 and two exchangeable hydrogens, suggesting the initial presence of three C=C bonds and one C=O bond, the latter possibly as an aldehyde. The [^{1}H]NMR (C_6D_6) spectrum was as follows: δ 1.10 (s), 1.46 (s), 1.64 (s), 1.84 (t, J = 7.2), 2.03 (m), 4.96 (d, J = 11.7), 5.11 (t, J = 7), 5.19 (d, J = 16.8), 5.74 (dd, J = 18 and 11), 5.89 (t, J = 7), and 9.31 (s). The singlet at δ = 9.31 clearly indicates an aldehyde, and both the mass spectral and NMR data are

consistent with an oxygenated nerolidol, most likely with one of the distal methyls oxidized to —CHO. A search of the literature revealed that Demole and Enggist (1973) had synthesized (*E,E*)- and (*E,Z*)-2,6,10-trimethyl-10-hydroxy-2,6,11-dodecatrienal. Although different solvents precluded a direct comparison of our NMR data with those reported, the *E,E* isomer seemed most consistent with our data. Spectral comparisons and GC coinjection experiments of the synthetic standard of (*E,E*)-2,6,10-trimethyl-10-hydroxy-2,6,11-dodecatrienal with component **4** (designated nerolidol aldehyde) confirmed this identification for the natural product.

Bioassays testing the response of *C. cydoniae* nymphs to volatiles from crushed conspecifics eluting from the GC were performed before determining that the DAGs produce the alarm pheromone. Nymphs at the effluent were alarmed within 30–40 sec after injection, indicating that one or more extremely volatile compounds were involved. Once it was established that exuvial DAG reservoirs contain the alarm pheromone, the low temperature GC-MS experiment to identify the very volatile compound(s) was undertaken using *C. cydoniae* exuviae. A small peak occurred just after a scan time of 150 sec at ca. 0°C, and the EI-MS of this peak gave a high computer fit for the MS of acetaldehyde (Figure 4A). GC-MS of a head-space sample (ca. 90 ng) of authentic acetaldehyde under similar GC conditions (temperature was difficult to exactly duplicate) produced a peak having nearly the same retention time and an EI-MS virtually identical to that for the exuvial compound (Figure 4B). CI-MS of *C. cydoniae* exuvial volatiles derivatized with *o*-benzylhydroxylamine confirmed the presence of the benzyloxime of acetaldehyde ($CH_3CH{=}NOCH_2C_6H_5$; mol wt 149); NH_3 CI-MS *m/z* (%): 167 ($[M+NH_4]^+$, 100) and 184 ($[M+(NH_3)_2+H]^+$, 55); CH_4 CI-MS *m/z* (%): 91 (100) and 150 ($[M+H]^+$, 75).

The first intraspecific bioassays using DAG secretions showed that alarm behavior in *C. cydoniae* occurs only if nymphs are simultaneously exposed to anterior and posterior DAG secretions, whereas *G. solani* nymphs are alarmed by exposure to posterior DAG secretion alone (Table 1). Inclusion of the anterior DAG secretion with the posterior secretion caused *G. solani* nymphs to respond sooner. When *C. cydoniae* nymphs were tested using secretions from *G. solani* exuviae, the results were the same; neither secretion alone had significant activity, while the combined secretions were highly alarming. The result of the one reciprocal interspecific test performed was also consistent with the intraspecific tests; posterior DAG secretion from *C. cydoniae* elicited alarm in *G. solani* nymphs. Bioassays with synthetic compounds **1, 2, 3,** and **5** showed that for *G. solani* nymphs geraniol **(3)** by itself released typical alarm behavior (Table 1). Geraniol with (*E*)-2-hexenal **(1)** and/or (*E*)-4-oxo-2-hexenal **(2)** may have slightly hastened the response of *Gargaphia* nymphs, but this effect was not precisely assessed. Linalool **(5)** by itself or with **1** or **2** was inactive for *G.*

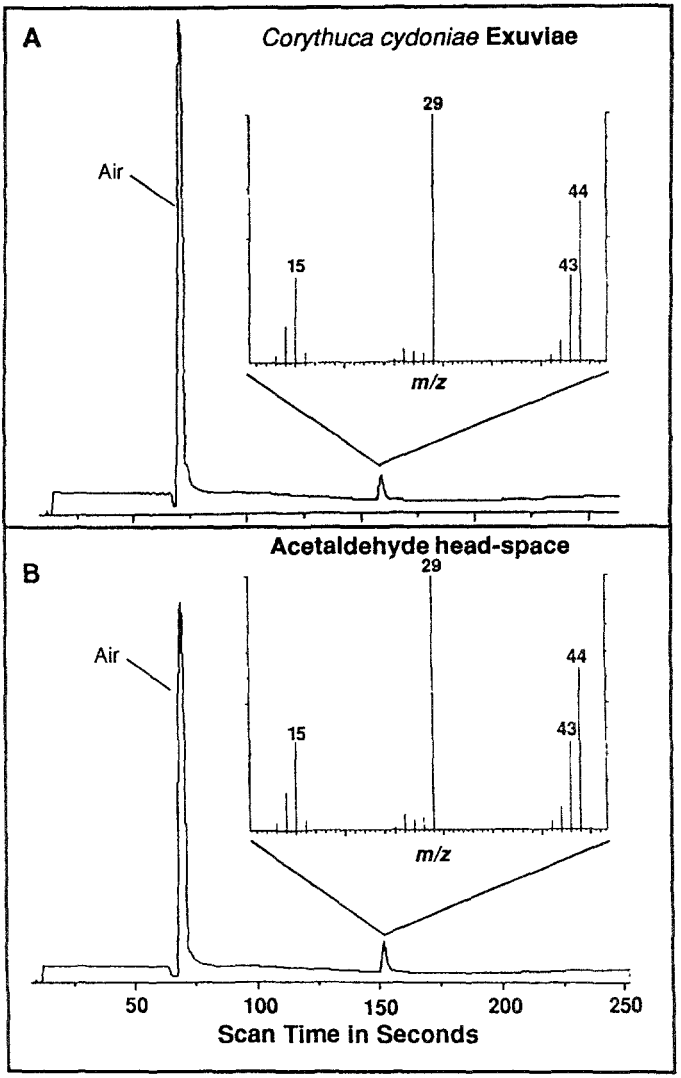


FIG. 4. Low-temperature gas chromatography-mass spectrometry (GC-MS) of (A) *Corythucha cydoniae* exuviae and (B) acetaldehyde head-space (reconstructed ion chromatograms and EI-MS).

solani. For *C. cydoniae* nymphs, the bioassay results using synthetics were less clear than for *G. solani*. Although (*E*)-4-oxo-2-hexenal (**2**) was not detected in the DAGs of *C. cydoniae* nymphs, this compound alone showed moderate alarm-inducing activity, and combinations **2** + **3**, **2** + **5**, and **1** + **2** + **3** yielded

TABLE 1. ALARM PHEROMONE TEST I USING DORSAL ABDOMINAL GLAND (DAG) SECRETION AND SYNTHETIC COMPOUNDS[a]

Test material	Response (# replicates)	
	G. solani	*C. cydoniae*
G. solani DAG secretion		
Anterior	− (3)	− (3)
Posterior	+ (8)	− (2)
Anterior and posterior	+ (1)	+ (4)
C. cydoniae DAG secretion		
Anterior	NT[d]	− (9)
Posterior	+ (2)	− (9)
Anterior and posterior	NT	+ (7)
Geraniol (**3**)	+ (3)	− (3)
Linalool[b] (**5**)	− (1)	− (2)
(*E*)-2-Hexenal (**1**)	− (2)	− (2)
4-Oxo-(*E*)-2-hexenal[c] (**2**)	− (2)	± (2)
1 + **3**	+ (1)	± (1)
1 + **5**[b]	− (1)	± (1)
2 + **3**[c]	± (2)	+ (1)
2 + **5**[cb]	− (1)	+ (1)
2 + **1** + **3**[c]	+ (5)	+ (3)

[a]No response = −; moderate response = ±; positive response = +. Secretion collected from exuviae (≤ 1 day after-ecdysis); synthetics total 3 $\mu g/\mu g$ CH_2Cl_2. Test material applied to filter paper point and held over aggregations (≥ 10 third-fifth instars) (details in text).
[b]Not found in *G. solani* DAGs.
[c]Not found in *C. cydoniae* DAGs.
[d]Not tested.

the highest activities. Despite the positive response rating for the latter three combinations, it must be pointed out that this chemically elicited alarm behavior did not fully mimic the natural response of *C. cydoniae* nymphs.

The second bioassay series with *C. cydoniae* nymphs used a more precise scoring system and included two additional compounds (acetaldehyde and nerol) (Table 2). The results are consistent in that (*E*)-4-oxo-2-hexenal **(2)** was more active than (*E*)-2-hexenal **(1)** and the monoterpenes. The combination of **2** + **3** was quite active (score = 3.33 ± 0.49), whereas combining nerol, which is a geometrical isomer of geraniol **(3),** with **2** greatly reduced activity. The role of acetaldehyde in the alarm pheromone remains questionable; the two highest concentrations tested induced responses interpreted as alarm, but the observer sometimes noted the responses "seemed different." An attempt to demonstrate a synergism between acetaldehyde and compounds **1–3** (concentration = 0.0065

TABLE 2. ALARM RESPONSES OF *Corythuca cydoniae* NYMPHS TO SYNTHETIC COMPOUNDS[a]

	Concentration[c]								
Compound(s)[b]	0.0125	0.125	0.25	0.50	0.625	0.0065 M	1.25	2.50	12.5
Acetaldehyde (CH_3CHO)	0	0.73 ± 0.16 (33)	0.13 ± 0.09 (15)	0.50 ± 0.27 (14)		1.00 ± 0.49 (15)	3.17 ± 0.54 (6)		3.00 ± 0.58 (3)
Geraniol (**3**)						0.33 ± 0.12 (15)		0.89 ± 0.20 (9)	
Linalool (**5**)								0.67 ± 0.67 (3)	
Nerol[d]								0.33 ± 0.33 (3)	
(*E*)-2-Hexenal (**1**)						1.58 ± 0.42 (12)		1.67 ± 0.33 (3)	
(*E*)-4-Oxo-2-Hexenal[e] (**2**)						1.73 ± 0.23 (15)		2.25 ± 0.25 (8)	
1 + **3**							0.33 ± 0.33 (3)	1.00 ± 0.31 (7)	
1 + **5**								2.00 ± 0.58 (4)	
1 + nerol[d]								0.67 ± 0.33 (3)	
2[e] + **3**					1.67 ± 0.33 (3)		2.33 ± 0.88 (3)	3.33 ± 0.49 (6)	
2[e] + **5**					2.25 ± 0.67 (4)		1.00 ± 0.41 (4)	1.33 ± 0.33 (3)	
2[e] + nerol[d]					1.00 ± 0.58 (3)		2.33 ± 0.88 (3)	1.33 ± 0.88 (3)	
1 + **2**[e] + **3** + CH_3CHO						1.00 ± 0.21 (4)			

[a] Responses were scored from 0 (no response) through 4 (full response) as described in the text. Scores listed are $X \pm \text{SEM}(N)$.
[b] Responses to CH_2Cl_2 alone = 0.60 ± 0.13 (40).
[c] Units are $\mu g/\mu l$ CH_2Cl_2 except for one solution tested on a molar basis (0.0065 M).
[d] Not found in *C. cydoniae* or *G. solani* DAGs.
[e] Occurs in *G. solani* DAGs, but not detected in *C. cydoniae* DAGs.

M) failed to show any appreciable synergism. None of the responses recorded in this bioassay series consistently equaled a natural response.

Nerolidol aldehyde **(4)** was synthesized after both bioassay series were completed, but when **4** was tested (2.5 $\mu g/\mu l$) against *C. cydoniae* nymphs it failed to elicit alarm behavior either alone or with **3** and/or **5.**

DISCUSSION

In *G. solani*, maternalism appears to substitute for the droplet chemical defense. A potent alarm pheromone is a second line of defense for *Gargaphia*, as well as for other tingids, and our discovery that tingid alarm pheromones emanate from the DAGs adds to the list of heteropterans having alarm pheromone activity associated with the secretions from these glands (Blum, 1985).

Gargaphia solani nymphs clearly are able to release DAG secretion. Notwithstanding the discussion of Kearns and Yamamoto (1981), the alarm pheromone system of the eggplant lace bug does not resemble that of various treehoppers (Homoptera: Membracidae), where the body wall of a nymph must be ruptured for the alarm pheromone to be released (Nault et al., 1974; Wood, 1976). The *Gargaphia* alarm-aggregation system seems much more like that of cotton stainer bugs (Pyrrhocoridae: *Dysdercus* spp.), where the posterior DAG secretion of nymphs triggers alarm and the two anterior DAGs produce an aggregation pheromone (Farine, 1987; Calam and Youdeowei, 1968). A diol corresponding to the tingid sesquiterpenoid is known from plants (Bohlman and Zdero, 1980), but nerolidol aldehyde is a new natural product. Whether this compound is part of an aggregation pheromone in *Gargaphia* remains to be determined.

While the alarm pheromone system of *Gargaphia* is not exceptional for Heteroptera, the system as it appears to function in *Corythucha* is extraordinary. Evidence that the alarm message in *Corythucha* is due to synergism between the two DAG secretions is, we believe, compelling. Yet all indications are that the nymphs are unable to voluntarily emit their DAG secretions; the glands must be physically ruptured for the pheromone to escape, a system truly analogous to that of treehoppers. Furthermore, many other tingid lineages having setal droplets might possess alarm pheromone systems as in *Corythucha*. This is apparently true for nymphs of the azalea lace bug *Stephanitis pyrioides* and the rhododendron lace bug *S. rhododendri* (J.W.N., personal observation).

The occurrence of sealed DAGs is not unprecedented in the Heteroptera; adults and nymphs of some mirids in the Bryocorinae specializing on toxic plants reportedly have one DAG with no external opening (Aryeetey and Kumar, 1973). When immatures of these mirids are disturbed, the DAG is vibrated, and drops of fluid simultaneously exude from setae over their bodies. It is pos-

sible that host-plant alkaloids are sequestered in the DAG and delivered when needed via the blood or an epidermal syncitium, as in milkweed bugs (Lygaeidae: *Oncopeltus* spp.) (Scudder et al., 1986), to setae or cuticular weak points (Aldrich, 1988a). However, none of the compounds identified in *Corythucha* DAG secretions have been detected in their setal droplets (Lusby et al., 1989).

Many questions remain unanswered in the present study, not the least of which is: what role, if any, does acetaldehyde play in the alarm behavior of *Corythucha*? For that matter, is acetaldehyde present in *Gargaphia*, and why do crushed adults cause delayed alarm among their nymphs (Kearns and Yamamoto, 1981)? (A single effort to directly observe acetaldehyde from exuviae of *G. solani* by low-temperature GC was inconclusive, and the metathoracic scent gland secretion has not been analyzed for any tingid adults.) Perhaps the most interesting question to emerge from this study is: has the alarm pheromone system of some nonmaternalistic tingids become totally altruistic?[4]

Acknowledgments—We wish to thank Rose Haldenmann for tireless efforts maintaining tingid colonies and preparing for bioassays, and Rolland Waters for the NMR spectrum. We are also grateful to Kim Kal for helping to collect *Gargaphia* and prepare extracts. Ken Wilzer helped collect tingids and provided invaluable computer assistance in the preparation of the manuscript, and Dawn Harrison performed many of the mass spectral analyses.

REFERENCES

ALDRICH, J.R. 1988a. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol.* 33:211–238.

ALDRICH, J.R. 1988b. Chemistry and biological activity of pentatomoid sex pheromones, pp. 417–431, *in* H.G. Cutler (ed.). Biologically Active Natural Products: Potential Use in Agriculture. ACS Symposium Series No. 380, Washington, D.C.

ARYEETEY, E.A., and KUMAR, R. 1973. Structure and function of the dorsal abdominal gland and defence mechanism in cocoa-capsids (Miridae: Heteroptera). *J. Entomol. Ser. A* 47:181–189.

BLUM, M.S. 1985. Alarm pheromones, pp. 193–224, *in* Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 9. Behaviour. G.A. Kerkut and L.I. Gilbert (eds.). Pergamon Press, Oxford, U.K.

BOHLMAN, F., and ZDERO, C. 1980. Neue Sesquiterpenlactone und Nerolidol-derivate aus Ursinia-Arten. *Phytochemistry* 19:587–591.

CALAM, D.H., and YOUDEOWEI, A. 1968. Identification and functions of secretion from the posterior scent gland of fifth instar larva of the bug *Dysdercus intermedius*. *J. Insect Physiol.* 14:1147–1158.

COBBEN, R.H. 1978. Evolutionary Trends in Heteroptera. Part II. Mouthpart Structures and Feeding Strategies. Veenman & Zonen, Wageningen, The Netherlands. 407 pp.

[4]Nymphs of the olive lace bug, *Froggattia olivinia*, collected in Queensland, Australia, since the submission of this manuscript were observed microscopically (J.R.A.) to possess short setae with conspicuous droplets over the entire body and to be able to excrete material from their DAGs. Mild alarm behavior was elicited from crushed nymphs and exuviae, and GC-MS analysis of an exuvial extract indicated the major DAG components are 2-phenethyl and benzyl esters of isovaleric and isobutyric acids.

DEMOLE, E., and ENGGIST, P. 1973. Applications synthétiques de la cyclisation d'alcools tertiaires γ-ethyléniques en α-bromotetrahydrofurannes sous l'action du *N*-bromosuccinimide. III. Synthèse du trimethyl-2,6,10-hydroxy-10-dodecatriene-2,6,11-al (*trans/trans* et *trans/cis*), hydroxy-aldehyde apparente aus sinensals. *Helv. Chim. Acta* 56:2053–2056.

DRAKE, C.J., and RUHOFF, F.A. 1965. Lacebugs of the World, A Catalogue (Hemiptera: Tingidae). U.S. National Museum Bulletin 243.

FARINE, J.-P. 1987. The exocrine glands of *Dysdercus cingulatus* (Heteroptera, Pyrrhocoridae): Morphology and function of nymphal glands. *J. Morphol.* 194:195–207.

FAETH, S.H. 1989. Maternal care in a lace bug, *Corythucha hewitti* (Hemiptera: Tingidae). *Psyche* 96:101–110.

FINK, D.E. 1915. The eggplant lace-bug. Bulletin U.S. Department of Agriculture 239, pp. 1–7.

KEARNS, R.S., and YAMAMOTO, R.T. 1981. Maternal behavior and alarm response in the eggplant lace bug, *Gargaphia solani* Heidemann (Tingidae: Heteroptera). *Psyche* 88:215–230.

LIVINGSTONE, D. 1978. On the body outgrowths and the phenomenon of "sweating" in the nymphal instars of Tingidae (Hemiptera: Heteroptera). *J. Nat. Hist.* 12:377–394.

LUSBY, W.R., OLIVER, J.E., NEAL, J.W., JR., and HEATH, R.R. 1989. Acylcyclohexanediones from setal exudate of hawthorn lace bug nymph *Corythucha cydoniae* (Hemiptera: Tingidae). *J. Chem. Ecol.* 15:2369–2378.

NAULT, L.R., WOOD, T.K., and GOFF, A.M. 1974. Treehopper (Membracidae) alarm pheromones. *Nature* 249:387–388.

OLIVER, J.E., LUSBY, W.R., and NEAL, J.W., JR. 1990. Exocrine secretions of the andromeda lace bug *Stephanitis takeyai* (Hemiptera: Tingidae). *J. Chem. Ecol.* 16: 2243–2252.

OLLETT, D.G., ATTYGALLE, A.B., and MORGAN, E.D. 1986. Microchemical method for determining formaldehyde, lower carbonyl compounds and alkylidene end groups in the nanogram range using the Keele micro-reactor. *J. Chromatogr.* 367:207–212.

SCUDDER, G.G.E., MOORE, L.V., and ISMAN, M.B. 1986. Sequestration of cardenolides in *Oncopeltus fasciatus*: Morphological and physiological adaptations. *J. Chem. Ecol.* 12: 1171–1187.

TALLAMY, D.W., and HORTON, L.A. 1990. Costs and benefits of the egg-dumping alternative in *Gargaphia* lace bugs (Hemiptera: Tingidae). *Anim. Behav.* 39:352–359.

WARD, J.P., and VANDORP, D.A. 1969. A stereospecific synthesis of 4-oxo-2-*trans*-hexenal. *Recueil* 88:989–993.

WOOD, T.K. 1976. Alarm behavior of brooding female *Umbonia crassicornis* (Homoptera: Membracidae). *Ann. Entomol. Soc. Amr.* 69:340–344.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

ANTHRAQUINONES IN DIFFERENT DEVELOPMENTAL STAGES OF *Galeruca tanaceti* (COLEOPTERA, CHRYSOMELIDAE)

M. HILKER[1,*] and S. SCHULZ[2]

[1]*Lehrstuhl für Tieroekologie II*
Universität Bayreuth
Postfach 101251, D-8580 Bayreuth, Germany

[2]*Institut für Organische Chemie*
Universität Hamburg
Martin-Luther-King Platz 6
D-2000 Hamburg 13, Germany

(Received May 23, 1991; accepted July 23, 1991)

Abstract—The overwintering eggs and the larvae of the leaf beetle *Galeruca tanaceti* (L.) contain hydroxylated anthraquinones. In both developmental stages, 1,8-dihydroxy-3-methylanthraquinone (= chrysophanol) and 1,8-dihydroxyanthraquinone (= chrysazin) were detected by GC-MS and GC-FTIR analyses. In the eggs, chrysazin was found only in traces. Anthraquinones were also present in ovaries and hemolymph of gravid females, which were investigated in order to examine the incorporation of these substances into the eggs. Neither in acidified nor in nonacidified extracts of the host plants *Tanacetum vulgare* L. and *Achillea millefolium* L. were anthraquinones found. The activity of these anthraquinones as chemical defense substances was proved in bioassays with the ant *Myrmica ruginodis* NYL. Further possible biological significances of anthraquinones are discussed.

Key Words—*Galeruca tanaceti*, Coleoptera, Chrysomelidae, anthraquinones, eggs, larvae, hemolymph, ovaries, *Tanacetum vulgare*, *Achillea millefolium*, feeding deterrence.

INTRODUCTION

Chrysomelids protect and defend their eggs against enemies by different strategies (Hinton, 1981; Lengerken, 1954). Females of *Phaedon cochleariae*, for

*To whom correspondence should be addressed.

0098-0331/91/1100-2323$06.50/0

example, hide their eggs in small cavities gnawed into the undersurface of the host-plant leaf. This oviposition behavior possibly serves not only as egg camouflage, but also as protection against desiccation of the eggs. Other chrysomelid species make their eggs almost inaccessible for predators and parasites by covering the eggs with feces and/or secretion. Eggs of *Timarcha* species, for example, are so firmly embedded in a "nest" consisting of plant material and feces that a single egg can hardly be loosened from the site. Eggs of Clytrinae are coated by a hardened scatoshell consisting of fecal material and gland secretions. Several chrysomelid species provide their eggs with chemicals that act as feeding deterrents against predators. Other chemicals are even toxic for the predators (overview: Pasteels et al., 1988a,b). The eggs of several *Chrysomela* species contain salicin in amounts that are toxic for ants (Pasteels et al., 1986). Other defensive egg compounds that cause feeding deterrence or rejection in predators are cardenolides in *Chrysolina* species (Pasteels and Daloze, 1977; Daloze and Pasteels, 1979), isoxazolinone derivatives in several *Chrysomela* and *Phratora* species, as well as in *Plagiodera versicolora* and *Gastrophysa viridula* (Pasteels et al., 1986), high amounts of oleic acid in *Gastrophysa cyanea* (Howard et al., 1982a), and cucurbitacins in the eggs of several Galerucinae that feed upon Cucurbitaceae (Ferguson and Metcalf, 1985).

Galeruca tanaceti L. (subfamily Galerucinae) is usually found on the Compositae *Tanacetum vulgare* L. and *Achillea millefolium* L. (Prevett, 1953). Eggs are deposited in September/October preferably at the tip of grass stalks; they overwinter until next April or May when the larvae hatch. The eggs are laid in batches that vary in size: usually about 20–30 eggs are deposited per batch. Each egg batch is cemented together and covered by a bright yellow secretion, which is produced by glandular cells of the oviduct (Scherf, 1956, 1966). Within half an hour after egg deposition, the liquid secretion is tanned by a dopa-oxidase and colored black peripherally (Messner, 1983). The hardened secretion, which is unsoluble in organic solvents, may serve as a mechanically protective device. The aims of this study were to examine whether the eggs of *G. tanaceti* are chemically defended and protected during the long period of overwintering, and to determine the origin of protective compounds. Furthermore, we investigated whether biologically active substances of the eggs are also present in the larvae of *G. tanaceti*.

METHODS AND MATERIALS

Larvae, eggs, and adults of *Galeruca tanaceti* (L.) were collected in 1989 and 1990 in the environs of Bayreuth and stored at $-40°C$ until preparation of extracts.

Chemical Analysis. The hardened secretion from 15 egg batches was

removed and the eggs were crushed in acetone, treated by ultrasound, centrifuged, and the clear supernatant fractionated by thin-layer chromatography (TLC) on silical gel 60 F_{254} (0.2 mm) with hexane–isopropyl acetate–1-butanol (6:2:1; v/v/v) as eluent. A parallel TLC run of an acetone extract, which was prepared from a single egg batch, showed, after spraying with 6% methanolic KOH, a bathochromic shift from yellow to purple in the fraction of the R_f value 0.86. The remaining nonsprayed fraction was scraped off the plate, dissolved in acetone, and repeatedly centrifuged for complete removal of the silicagel. The purified TLC fraction was analyzed by GC-MS and GC-FTIR. EI mass spectra (70 eV) were obtained using a Carlo Erba Vega Series 2 gas chromatograph with split injection (split 1:5; injector temperature: 220°C) coupled to a Finnigan Iontrap ITD 800. A 12 m × 0.32 mm FS-OV-1701 column was used, which was programmed from 100°C to 220°C at 20°C/min, from 220°C to 260°C at 5°C/min, and from 260°C to 280°C at 20°C/min (carrier: 50 kPa helium). Infrared spectra were obtained by GC-FTIR on a HP 5890 Series II gas chromatograph coupled to a HP 5965 infrared detector (temperature: 250°C) with splitless injection at 220°C. Samples were separated on a HP1 column (25 m × 0.31 mm) with an oven temperature program from 100°C to 240°C at 50°C/min (carrier: 65 kPa helium). Transmission spectra were recorded from 750 cm^{-1} to 4000 cm^{-1}.

A TLC fractionation of an acetone extract of 20 *G. tanaceti* larvae (third instar), conducted with the same method as used for the egg extract, showed, after spraying with 6% methanolic KOH, a bathochromic shift from yellow to purple in the fraction of the R_f value 0.83. The remaining nonsprayed TLC fraction of an acetone extract prepared from 20 *G. tanaceti* larvae (L3) was purified and analyzed as described before.

Hemolymph was obtained from 20 gravid *G. tanaceti* females by cutting the forelegs at the articulation between coxa and femur and soaking up the emerging hemolymph with small pieces of filter paper, which were extracted in acetone. Then, ovaries were dissected from these females (oviducts were cut off) and also dissolved in acetone. Detailed descriptions and drawings of galerucine genitalia have been presented by Silfverberg (1976) and Suzuki and Yamada (1976). Both hemolymph and ovary extracts were treated by ultrasound, repeatedly centrifuged, and the clear supernatants analyzed by GC-MS.

Extracts of host-plant leaves also were investigated. Three leaves each of *Tanacetum vulgare* L. and *Achillea millefolium* L. were ground, extracted in acetone, and examined for hydroxylated anthraquinones using the TLC method described above. These are hereafter called nonacidified extracts. The method of Wouters (1985) was modified for preparation of acidified extracts. Fragments of three leaves from each investigated host plant were hydrolyzed in unstoppered reaction tubes with 2 N HCl for 3 hr in a boiling water bath. The hydrolyzed solutions were cleared in a centrifuge and repeatedly extracted with

diethylether. The combined ether phases were evaporated under N_2 to a few milliliters and analyzed by GC-MS. The conditions for the GC-MS analyses of hemolymph, ovary, and acidified host-plant extracts were the same as for the investigation of the egg extracts.

Bioassay. A feeding bioassay was conducted with the ant *Myrmica ruginodis* NYL., in order to examine whether eggs of *G. tanaceti* were avoided by predators. Twenty ants were placed in a Petri dish (14 cm diam.) and starved for two days. After this starvation period, an aqueous test suspension was prepared by crushing two egg masses in 500 μl H_2O. A mealworm (last instar) was ground in 500 μl H_2O for the control suspension. Five microliters of each suspension was offered simultaneously to the ants. Every minute the number of feeding ants at the test and control suspension was recorded for a period of 10 min. After 20 replications, the bioassay was statistically evaluated by the Wilcoxon signed-rank test for paired differences (Sachs, 1984). The same method was used for testing an aqueous mealworm suspension plus synthetic chrysophanol (test) against a "pure" aqueous mealworm suspension without chrysophanol (control) to examine the effect of chrysophanol on the feeding behavior of *M. ruginodis*. The chrysophanol concentration of the test suspension was 1.4×10^{-2} M and 0.6% of the mealworm weight (0.3 g/500 μl H_2O).

RESULTS

The TLC of an acetone extract of *G. tanaceti* eggs revealed a fraction at R_f 0.86 with a bathochromic shift from yellow to purple when sprayed with 6% methanolic KOH. A bathochromic shift in alkaline solution is reported for a great many hydroxyquinones (Thomson, 1976). The total ion current chromatogram of this (nonsprayed) fraction revealed two peaks with mass spectra that show the typical fragmentation patterns of anthraquinones (Figure 1A). The EI mass spectrum of compound **1** had characteristic ions at *m/z* 240 (100%, M^+), 212 (36%, M − CO), 184 (47%, M − 2CO), 155 (9%), 138 (21%), 128 (30%), 92 (24%), indicating 1,8-dihydroxyanthraquinone (= chrysazin) (Howard et al., 1982b; McLafferty and Stauffer, 1989). A synthetic reference sample (Roth AG, Basel, Switzerland) showed an identical mass spectrum and the same retention time as compound **1**. The EI mass spectrum of compound **2** revealed fragments at *m/z* 254 (100%, M^+), 226 (26%, M − CO), 198 (18%, M − 2CO), 169 (7%), 152 (20%), 141 (10%), 115 (19%), suggesting 1,8-dihydroxy-3-methylanthraquinone (= chrysophanol), which was confirmed upon comparison with a synthetic reference sample (Aldrich Chemical Co., Steinheim, Germany) (Howard et al., 1982b; McLafferty and Stauffer, 1989). In addition, the FTIR spectrum of compound **2** conformed with that of synthetic chrysophanol (Figure 2).

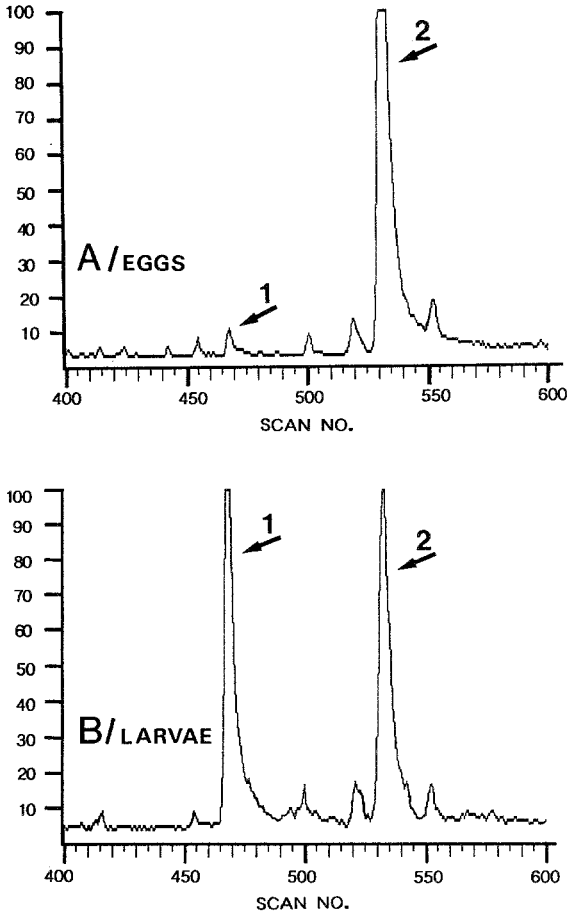


FIG. 1. Total ion current chromatogram of TLC fraction of 15 *Galeruca tanaceti* egg masses (A) and 20 larvae (B) from scan number 400 to 600. Characteristic fragments of the mass spectra of peak 1 and 2 are given in the text.

The site of production of the anthraquinones may be microorganisms living on deposited eggs, the embryo itself, or the female, which incorporates anthraquinones into the eggs. The GC-MS analysis of the hemolymph of *G. tanaceti* females revealed the presence of chrysophanol and traces of chrysazin. Chrysophanol was also detected in the ovaries; chrysazin, however, was not found. In the TLC of the nonacidified acetone extracts of *T. vulgare* and *A. millefolium*, no bathochromic shift occurred. GC-MS analyses of acidified extracts, which were examined in order to check for possible glycosidated chrysophanol and chrysazin derivatives, also showed no anthraquinones in the plant material.

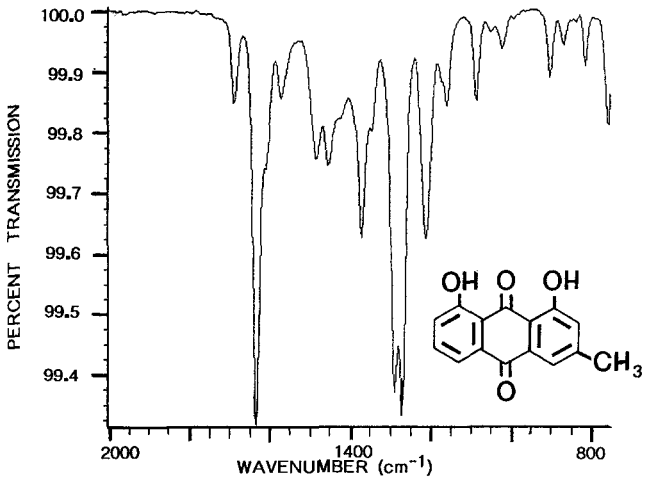


FIG. 2. FTIR spectrum of compound 2 from Figure 1A, in all respects identical with the spectrum of chrysophanol.

Therefore, the females do not acquire these substances from their host plants, since no hydroxylated anthraquinones were detected in the leaf extracts.

The TLC of an acetone extract of *G. tanaceti* larvae showed, after spraying, a bathochromic shift from bright yellow to purple in the fraction of R_f 0.83. A GC-MS analysis of the respective nonsprayed fraction revealed the presence of the same anthraquinones as in the eggs. The total ion current chromatogram of this TLC fraction is shown in Figure 1B. The mass spectra of these peaks were the same as those described above for the egg extract. A comparison of the FTIR spectrum of synthetic chrysazin (Roth AG) and of compound **1** from the total ion current chromatogram of the larval extract showed no differences (Figure 3).

The feeding bioassays with *M. ruginodis* revealed that the ants significantly preferred feeding upon mealworms compared to feeding upon the eggs of *G. tanaceti* (Figure 4). The highest difference between the number of feeding ants upon eggs and mealworms was reached after a test period of 10 min: only 27.5% of the feeding ants were recorded at the eggs of *G. tanaceti*. In order to examine whether this deterring activity of *G. tanaceti* eggs is due to the detected anthraquinones, a second feeding bioassay was conducted which tested feeding of *M. ruginodis* upon mealworms plus chrysophanol (test) compared to feeding upon "pure" mealworms (control). Figure 5 shows that after a test period of 8, 9, and 10 min, significantly fewer ants fed upon the test than upon the control suspension. The highest significant difference between the number of feeding

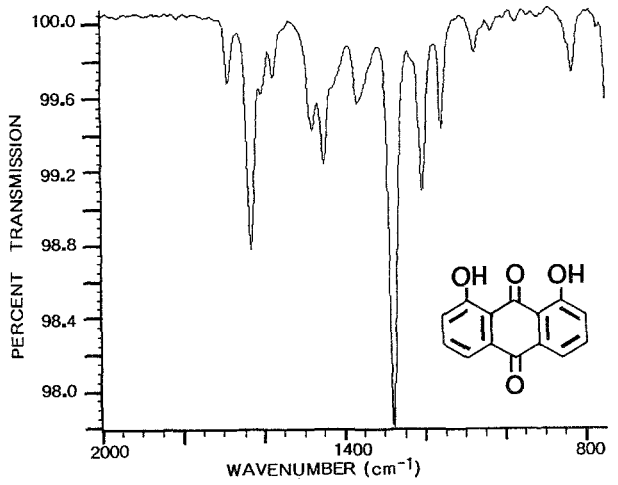


FIG. 3. FTIR spectrum of compound 1 from Figure 1B, in all respects identical with the spectrum of chrysazin.

ants upon test and control suspension was attained after 8 min: 35.8% of the feeding ants were recorded at the mealworm suspension plus chrysophanol.

DISCUSSION

Up to now, anthraquinones were unknown in eggs of Coleoptera; however, they have been detected in chrysomelid larvae. Howard et al. (1982b) found chrysophanol, chrysazin, and two anthrones in larvae of the elm leaf beetle, *Pyrrhalta luteola*, which, like *G. tanaceti*, belongs to the subfamily Galerucinae. Both chrysomelid species obviously do not acquire the anthraquinones from their host plants. As suggested for the production of anthraquinones in scale insects (Kayser, 1985), symbiontic microorganisms may also produce these compounds in both chrysomelid species. In *G. tanaceti*, the presence of anthraquinones in the hemolymph of females and in the ovaries indicates that females incorporate these compounds into the eggs, thus providing the eggs with protective compounds.

Eisner et al. (1980) reported on the feeding deterrence of the anthraquinone glucoside carminic acid against the ant *Monomorium destructor*, whereas the carnivorous caterpillar of a pyralid moth, *Laetilia coccidivora*, is not deterred by carminic acid; rather, it utilizes the ingested carminic acid from prey cochineals for defensive purposes of its own. As demonstrated by the bioassay described above, feeding of ants upon *G. tanaceti* eggs is significantly reduced compared to feeding upon normally accepted mealworms. An additional bioas-

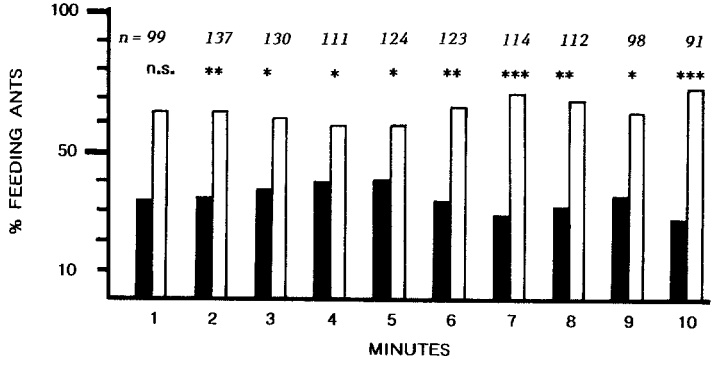


FIG. 4. Feeding bioassay with the ant *Myrmica ruginodis*. Black bars: feeding activity on an aqueous test suspension of *Galeruca tanaceti* eggs (2 egg masses/500 μl H_2O). White bars: feeding activity on simultaneously offered aqueous control suspension of mealworms (1 last-instar mealworm/500 μl H_2O). Registration of feeding ants during a test period of 10 min. Asterisks indicate level of significance: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; n.s. = not significant; Wilcoxon signed-rank test for paired differences.

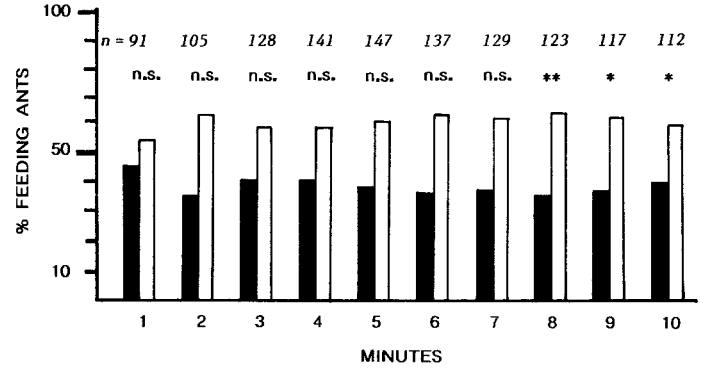


FIG. 5. Feeding bioassay with the ant *Myrmica ruginodis*. Black bars: feeding activity on an aqueous test suspension of mealworms plus added synthetic chrysophanol (1.4×10^{-2} M). White bars: feeding activity on a simultaneously offered aqueous control suspension of mealworms without chrysophanol. Registration of feeding ants during a test period of 10 min. Asterisks indicate level of significance: *, $P \leq 0.05$; **, $P \leq 0.01$; n.s. = not significant; Wilcoxon signed-rank test for paired differences.

say revealed that synthetic chrysophanol also causes a diminished feeding activity of the ants; however, the feeding reduction in the bioassay with eggs was stronger (see Figures 4 and 5). These results indicate that the main anthraquinone in the eggs of *G. tanaceti* contributes to the feeding deterrence of the eggs

against ants. Detailed knowledge on the anthraquinone concentrations in the eggs is essential to assess exactly the role of anthraquinones in the feeding-deterring activity of the eggs. In addition to the dosage, the mixture of the detected anthraquinones may also be relevant when bioassaying for feeding deterrence. Further quantitative investigations of the anthraquinones in *G. tanaceti* will be necessary to elucidate their biological significance. In this qualitative study, only a rough estimation of the chrysophanol concentrations was possible: the chrysophanol content is lowest in the hemolymph and ovaries of a single female, increases in the larva, and is highest in one egg mass.

When tasting an insect egg, a predator destroys the tiny prey. Therefore, the deposition of distasteful eggs in batches seems to be advantageous, since this oviposition behavior enhances the chance that only a single egg of an egg mass is damaged while the others remain untouched (Stamp, 1980). Anthraquinones from the above-mentioned chrysomelid larvae can only be released and display their activity against enemies by the emerging hemolymph from wounds. It remains to be proved whether the chrysomelid larvae are able to survive slight wounding. Wiklund and Järvi (1982, p. 1001) demonstrated that naive bird predators "often release distasteful prey insects unharmed after tasting them."

In addition to the feeding-deterring activity against ants, anthraquinones are also known as repellents against birds. For the protection of seeds, 9,10-anthraquinone is sold commercially as an avian repellent. Furthermore, hydroxylated anthraquinones have been demonstrated to act as antimicrobial agents (Cudlin et al., 1976) and might protect the eggs against microbial diseases. Adewunmi and Adesogan (1984) report on the molluscicidal activity of anthraquinones against snails. The ingestion of trematod eggs by herbivorous snails is a well-known phenomenon, whereas the coincident uptake of insect eggs and plant material, to our knowledge, has not been examined.

Acknowledgments—Many thanks are due to Konrad Dettner, Uwe Noldt and Regina Fettköther for valuable contributions and critical review of the manuscript.

REFERENCES

ADEWUNMI, C.O., and ADESOGAN, E.K. 1984. Anthraquinones and oruwacin from *Morinda lucida* as possible agents in fascioliasis and schistosomiasis control. *Fitoterapia* 55:259–263.

CUDLIN, J., BLUMAUEROVA, M., STEINEROVA, N., MATEJU, J., and ZALABAK, V. 1976. Biological activity of hydroxyanthraquinones and their glucosides toward microorganisms. *Folia Microbiol.* 21:54–57.

DALOZE, D., and PASTEELS, J.M. 1979. Production of cardiac glycosides by chrysomelid beetles and larvae. *J. Chem. Ecol.* 5:63–77.

EISNER, T., NOWICKI, S., GOETZ, M., and MEINWALD, J. 1980. Red cochineal dye (carminic acid): Its role in nature. *Science* 208:1039–1042.

FERGUSON, J.E., and METCALF, R.L. 1985. Cucurbitacins. Plant-derived defense compounds for diabroticites (Coleoptera: Chrysomelidae). *J. Chem. Ecol.* 11:311–317.

HINTON, H.E. 1981. Biology of Insect Eggs, Vol. II. Pergamon Press, Oxford.

HOWARD, D.F., BLUM, M.S., JONES, T.H., and PHILLIPS, D.W. 1982a. Defensive adaptations of eggs and adults of *Gastrophysa cyanea* (Coleoptera: Chrysomelidae). *J. Chem. Ecol.* 8:453–462.

HOWARD, D.F., PHILLIPS, D.W., JONES, T.H., and BLUM, M.S. 1982b. Anthraquinones and anthrones: Occurrence and defensive function in a chrysomelid beetle. *Naturwissenschaften* 69:91–92.

KAYSER, H. 1985. Pigments, pp. 367–415, *in* G.A. Kerkut and L.I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 10. Pergamon Press, Oxford.

LENGERKEN, H. VON. 1954. Die Brutfürsorge- und Brutpflegeinstinkte der Käfer. Geest & Portig K.-G., Leipzig.

MCLAFFERTY, F.W., and STAUFFER, D.B. 1989. The Wiley/NBS Registry of Mass Spectral Data. Wiley, New York.

MESSNER, B. 1983. Dopa-Oxidase-gehärtete Sekrete schützen das Eigelege von *Galeruca tanaceti* L. (Coleoptera, Chrysomelidae). *Entomol. Nachr. Berichte* 27:221–224.

PASTEELS, J.M., and DALOZE, D. 1977. Cardiac glycosides in the defensive secretion of chrysomelid beetles: Evidence for their production by the insects. *Science* 197:70–72.

PASTEELS, J.M., DALOZE, D., and ROWELL-RAHIER, M. 1986. Chemical defence in chrysomelid eggs and neonate larvae. *Physiol. Entomol.* 11:29–37.

PASTEELS, J.M., BRAEKMAN, J.-C., and DALOZE, D. 1988a. Chemical defense in the Chrysomelidae, pp. 233–252, *in* P. Jolivet, E. Petipierre, and T.H. Hsiao (eds.). Biology of Chrysomelidae. Kluwer Academic Publishers, Dordrecht.

PASTEELS, J.M., ROWELL-RAHIER, M., and RAUPP, M.J. 1988b. Plant-derived defense in chrysomelid beetles, pp. 235–272, *in* P. Barbosa and D. Letourneau (eds.). Novel Aspects of Insect–Plant Interactions. Wiley, New York.

PREVETT, P.F. 1953. Notes on the feeding habit and life-history of *Galeruca tanaceti* L. (Col., Chrysomelidae). *Entomol. Mon. Mag.* 89:292–293.

SACHS, L. 1984. Angewandte Statistik. Springer-Verlag, Berlin.

SCHERF, H. 1956. Zum feineren Bau der Eigelege von *Galeruca tanaceti* L. (Coleopt., Chrysom.). *Zool. Anz.* 157:124–130.

SCHERF, H. 1966. Beobachtungen an Ei und Gelege von *Galeruca tanaceti* L. (Coleoptera, Chrysomelidae). *Biol. Zentralbl.* 85:7–17.

SILFVERBERG, H. 1976. Studies on galerucine genitalia I (Coleoptera, Chrysomelidae). *Not. Entomol.* 56:1–9.

STAMP, N.E. 1980. Egg deposition patterns in butterflies: Why do some species cluster their eggs rather than deposit them singly? *Am. Nat.* 115:367–380.

SUZUKI, K., and YAMADA, K. 1976. Intraspecific variation of ovariole number in some chrysomelid species (Coleoptera, Chrysomelidae). *Kontyu* 44:77–84.

THOMSON, R.H. 1976. Isolation and identification of quinones, pp. 207–232, *in* T.W. Goodwin (ed.). Chemistry and Biochemistry of Plant Pigments, 2nd ed. Academic Press, London.

WIKLUND, C., and JÄRVI, T. 1982. Survival of distasteful insects after being attacked by naive birds: A reappraisal of the theory of aposematic coloration evolving through individual selection. *Evolution* 36:998–1002.

WOUTERS, J. 1985. High performance liquid chromatography of anthraquinones: Analysis of plant and insect extracts and dyed textiles. *Stud. Conserv.* 30:119–128.

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

ANNOUNCEMENT

The 9th annual meeting of the International Society of Chemical Ecology will take place in Kyoto, Japan, July 6–9, 1992.

Sessions will consist of 30 min. presentations by invited speakers and 12 min. presentations by contributing speakers, with 5 min. and 3 min. discussion periods, respectively. Thus far, organizers have planned sesssions on the chemical ecology of medicinal plants (K. Fuji), allelopathy (C.H. Chou), aqueous biosphere (D. Daloze), natural products (K. Mori, K. Nakanishi, and A.B. Smith, III), and fruit flies (J.L. Nation). Poster presentations on session themes, as well as general chemical ecology, are invited.

An abstract is required for oral and poster presentations, to include author's name, title, organization, and address, paper title, and subject matter. A form for this purpose should be requested. Program details, abstract forms, registration forms, and accommodation applications are also available from:

Dr. Shozo Takahashi
Pesticide Research Institute
Faculty of Agriculture
Kyoto University
Kyoto 606, Japan
Telephone +75-753-6306, FAX +75-753-6312

0098-0331/91/1100-2333$06.50/0

Journal of Chemical Ecology, Vol. 17, No. 11, 1991

ANNOUNCEMENT

SYMPOSIUM ON PHYTOCHEMISTRY AND AGRICULTURE

The Phytochemical Society of Europe and the Department of Organic Chemistry of the Wageningen Agricultural University are sponsoring an international symposium to be held at the International Agricultural Centre in Wageningen, The Netherlands on April 22–24, 1992.

Speakers will include W. Barz (Münster), A. Bruggink (Venlo), B.V. Charlwood (London), W.M.J. van Gelder (Wageningen), F.J. Gommers (Wageningen), E. Haslam (Sheffield), B.P.S. Khambay (Harpenden), W. Kraus (Stuttgart), E. Krebbers (Gent), G. Massiot (Reims), J.N.M. Mol (Amsterdam), R.J. Molyneux (Albany), A. Nahrstedt (Münster), J.A. Pickett (Harpenden), P.W. Price (Flagstaff), J. O'Reilly (Little Island), L.M. Schoonhoven (Wageningen), A. Sheak (Kathmandu), S. Stymne (Uppsala), D. Tepfer (Versailles), and M. Wink (Heidelberg).

Topics will include crop protection with natural products, antifeedants, pyrethroids, screening for new insecticides, interactions between plants and microorganisms, phytoalexins, tritrophic interactions, cyanogenesis and food plants, alfalfa saponins, alkaloids in potatoes, plants toxic for cattle, natural colors, production of aroma chemicals, plant cell cultures, biosynthesis of fatty acids, polyphenols, cultivation and use of *Ginkgo biloba*, setting up an essential oil producing firm in a developing country, and comparing production of chemicals by plants and synthesis.

Poster presentations are invited. Any enquiries regarding the conference should be addressed to:

Dr. T.A. van Beek
Department of Organic Chemistry
Phytochemical Section
Agricultural University
Dreijenplein 8, NL-6703 HB
Wageningen, The Netherlands

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

CONTROL OF CHEMICALLY STIMULATED FEEDING BEHAVIOR IN SAND FIDDLER CRABS *Uca pugilator*: EVIDENCE FOR HEMOLYMPH FEEDING INHIBITORY FACTOR

MARGARET A. SEARS and DAN RITTSCHOF*

Department of Zoology and Marine Laboratory
Duke University
Beaufort, North Carolina 28516

(Received April 18, 1991; accepted July 24, 1991)

Abstract—*Uca pugilator*, the sand fiddler crab, is a deposit-feeder. It feeds on exposed sand flats as the tide recedes. Feeding movements are evoked by stimulation of chemoreceptors on the dactyls. Previous studies have demonstrated that a proteinaceous factor associated with the sinus gland region inhibits chemically-stimulated feeding behavior. Here, that work is extended to show the existence of a similar inhibitory factor present in the hemolymph of fed crabs in the laboratory and in the hemolymph of crabs returning from feeding in the field. The factor is not detected in fasted laboratory crabs or in field crabs walking from burrow areas to feeding sites. Injection of glucose results in inhibition of feeding activity in intact crabs. Injection of glucose, but not galactose, stimulates feeding in eyestalk-ablated crabs. We suggest that neural responsiveness is stimulated by glucose, and that elevated glucose in intact crabs results in release of feeding inhibitory factor from the sinus gland. Release of feeding inhibitory factor into the blood enables crabs to return to burrow areas without stopping when they encounter food-laden sediments.

Key Words—*Uca pugilator*, crustacean feeding, endocrine factors, feeding behavior, daily movements, feeding ecology.

INTRODUCTION

The needs of organisms, and consequently their behavior, can be ordered into a hierarchy (c.f., Maslow, 1970), a prioritization of activities that has, through

*To whom correspondence should be addressed.

0098-0331/91/1200-2337$06.50/0

evolutionary time, proven successful. For example, organisms meet energetic needs and then perform activities that use energy to ensure survival and reproduction. We hypothesize that in crabs, behavioral prioritization can be hormonally controlled. That is, hormones play a direct role in determining the behavioral hierarchy and time budgeting of daily activities. This concept is supported for crustaceans by two reports in the literature (Gleeson et al., 1987; Sears et al., 1991). For the sand fiddler crab (*Uca pugilator* Bosc), daily activities include feeding (Robertson et al., 1980, 1981), burrow building and maintenance, sexual displays, and (at appropriate lunar phases) mating (Crane, 1975; Salmon and Hyatt, 1983). Here we provide evidence that hormones modulate chemosensory behavior in sand fiddlers.

Crab and shrimp eyestalks contain a proteinaceous factor that modulates chemically-stimulated feeding behavior in *U. pugilator* (Sears et al., 1991). This feeding inhibitory factor (FIF) is associated with the eyestalk (X-organ, sinus gland complex), a secretory neuroendocrine organ that has a major role in the regulation of moulting, ovulation, ion balance, and hemolymph glucose levels (for review see Beltz, 1988). FIF-like activity can be found in fed intact crabs and in intact crabs injected with glucose. Crabs captured while walking from burrow areas to feeding sites and fasted intact crabs do not contain detectable amounts of FIF activity in their hemolymph. Crabs returning from feeding, and fed intact crabs, contain high levels of FIF in their hemolymph. Eyestalk-ablated crabs are also devoid of FIF activity and do not produce FIF-like activity when fed or injected with glucose. The molecular size of circulating FIF is similar to that of FIF isolated from eyestalks.

Here, we show that the circulating levels of FIF are related to the behavioral and satiation state of the crabs.

METHODS AND EXPERIMENTS

Eyestalk-Ablated Crabs

Eyestalks were ablated with forceps. At least 12 hr elapsed after ablation and before testing (Rittschof and Buswell, 1989). This interval was sufficient for depletion of eyestalk hormones as indicated by chromatophore state (Brown, 1940). Eyestalk-ablated crabs were held overnight in finger bowls containing several mm of seawater at 23°C. Bowls were rinsed and seawater was replaced 15 min prior to feeding assays.

Assays of Feeding Inhibition

For assays, intact and eyestalk-ablated crabs were transferred to 20-cm finger bowls containing 20 ml of 0.0625 M glucose (the threshold concentration for a detectable response) in 100 kd filtered seawater. Feeding responses were

observed directly. Feeding assays tested glucose-stimulated feeding responses (Sears et al., 1991). Seawater (34–37 ppt), filtered to remove particles >100 kd, was used to make solutions for feeding assays in which 10–30 crabs were tested in groups of five and were observed for 30 sec. A positive feeding response was recorded for each crab that moved the minor chela at least twice in a feeding motion.

Hemolymph Glucose Assays

Hemolymph glucose concentrations were assayed enzymatically using the deproteinated blood protocol from a commercially available glucose oxidase reagent kit (Sigma Chemical, St. Louis, MO #510-DA). Hemolymph glucose levels were determined on intact, eyestalk-ablated, and ablated crabs injected with glucose 1 hr beforehand in isosmotic (900 mOsM/1) 100 kd filtered seawater.

Effects of Increased Hemolymph Glucose on Feeding Responses of Intact and Ablated Crabs

The effect of elevated glucose levels on feeding responses was determined using 30 intact and 30 eyestalk-ablated crabs. Each crab was injected with 25 μl of 4 g/l (100 μg) glucose in saline solution. Based on previous glucose assays, this amount of glucose was sufficient to bring the hemolymph glucose concentration of a 2.1 g ablated crab to levels (10 mg/dl) found in intact crabs. Feeding assays were conducted 1 hr after injection.

Effects of Increased Hemolymph Glucose or Galactose on Feeding in Ablated Crabs

Glucose can be metabolized directly by neural tissue, but galactose cannot (Lehninger, 1978). The effects of elevated glucose and galactose in eyestalk-ablated crabs were compared. Thirty ablated crabs were injected with saline, 25 μl of 4 g/l or 40 g/l glucose or galactose solution. Based on previous glucose assays, these concentrations were sufficient to bring hemolymph hexose concentrations to 10 mg/dl and 100 mg/dl, respectively. Sixty minutes after injection, feeding responses were measured and stimulation of feeding was calculated:

$$\frac{\text{Final \% response} - \text{Initial \% response}}{\text{Initial \% response}}$$

FIF Titres of Hemolymph from Unfed and Fed Crabs

FIF titres in fiddler crab hemolymph were estimated by injecting hemolymph into eyestalk-ablated crabs 1 hr prior to bioassay. A 25 μl volume of hemolymph was drawn from the sinus within the large cheliped of a male crab, using a Hamilton syringe. This sample of hemolymph was immediately injected into an eyestalk-ablated crab (technique described in Sears et al., 1991). Eyestalkless crabs were assayed immediately before injecting samples and then 1 hr after injection. Materials tested for inhibition of feeding activity were diluted with isosmotic (900 mOsM/l) 100 kd filtered seawater and 25 μl volumes injected into crab blood sinuses. Injection was either into the blood sinus within the large cheliped of eyestalkless males, or into the sinus at the proximal joint of the fifth walking leg (both sexes). Preliminary tests indicated equivalent response times for the two injection methods.

Hemolymph titres of FIF with respect to feeding were determined in both laboratory and field situations. In the laboratory, intact crabs were maintained for 3 days with either an unlimited supply of fish flake food (Tetramin) and cat food (Friskies Ocean Fish Flavor), or fasted in finger bowls containing 100 kd filtered seawater. Hemolymph was drawn from 30 crabs in each condition, injected into eyestalk-ablated crabs, and the injected crabs bioassayed for feeding inhibition.

In the field, 25 μl samples of hemolymph were taken from crabs on the way down the beach to feed as the tide ebbed, or 2 hr after low tide as crabs traveled back to their burrows after feeding. Samples were from crabs located at Bird Shoal, Beaufort, North Carolina, USA. Hemolymph from each sample of crabs was immediately injected into eyestalk-ablated crabs which had been brought into the field. Crabs were tested in the field in the feeding bioassay immediately before injection and in the field 1 hr after injection. When not in use, ablated crabs were stored in buckets containing 1 cm of seawater, covered with towels. Ambient temperature was 37°C.

Size Estimation of FIF from Hemolymph

Hemolymph containing FIF activity was subjected to size fractionation to estimate the molecular size of circulating FIF. Cascade pressure dialysis was used to estimate the molecular size of hemolymph FIF. A total of 2 ml of hemolymph was drawn in the field from 100 intact male crabs as they were returning to their burrows after feeding (approximately 2 hr after low tide). The pooled hemolymph was diluted as it was collected into 20 ml of isosmotic 100 kd filtered seawater stored on ice until it was returned to the laboratory.

In the laboratory, the diluted hemolymph was centrifuged at 10,000 rpm for 10 min to remove particulates. The supernatant was then separated into $<5{,}000$ d and $>5{,}000$ d size fractions by ultrafiltration through a 5000 d mem-

brane (Amicon YM series). The >5000 d fraction residue was washed twice with isosmotic saline and brought back to the original 20 ml volume. Both the filtrate and the residue were lyophilized and returned to 2 ml volumes. FIF titres in the two size fractions of hemolymph from fed crabs was determined. For assays, 25 μl of <5000 d and >5000 d size fractions of hemolymph were injected into 30 eyestalk-ablated crabs. Feeding responses were measured and percent inhibition of feeding was calculated.

Statistical Analysis

Levels of feeding responsiveness under different conditions were compared by determining a Z statistic for the difference between two proportions (Walpole, 1974). Significance was set at the $p < 0.05$ level.

RESULTS

Effects on Feeding of Elevated Hemolymph Glucose Concentrations in Intact and Ablated Crabs

Injection of glucose inhibited feeding in intact crabs but stimulated feeding in eyestalk-ablated crabs (Figure 1). A 10 mg/dl increase in hemolymph glucose concentration resulted in 45% inhibition of feeding by intact crabs ($Z = 3.15$, $P < 0.001$) compared to 112% stimulation in ablated crabs ($Z = 10.6$, $P < 0.001$).

Effects of Increased Glucose or Galactose on Feeding of Eyestalk-ablated Crabs

Glucose stimulated feeding to a much greater extent than galactose in ablated crabs (Figure 2). Hemolymph concentrations of glucose $\geq$ 10 mg/dl resulted in 100% stimulation of feeding ($Z = 10.6$, $P < 0.001$). In contrast, injection of 10 and 100 mg/dl galactose resulted in 17% and 22% stimulation, respectively, which failed to differ significantly from control (saline injected) crabs.

Effects of Hemolymph Taken from Unfed and Fed Crabs

Injection of 25 μl of hemolymph drawn from intact fed crabs in both the laboratory and the field significantly inhibited feeding ($Z = 7.46$, $P < 0.001$) in eyestalk-ablated crabs (Figure 3). Injection of hemolymph from fasted (unfed) laboratory crabs stimulated feeding ($Z = 1.79$, $P < 0.05$), while hemolymph from crabs moving toward feeding areas (field) had no effect.

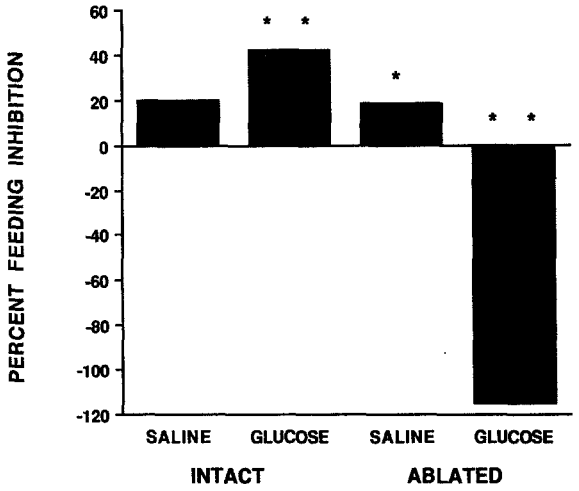


Fig. 1. Change in feeding activity of intact and eyestalk-ablated crabs following injection of saline or glucose. Feeding activity for each group of crabs was determined prior to injection. One hour after injection feeding activity was again assayed. Injection of glucose significantly inhibited feeding in intact crabs while injection of saline did not. Eyestalk-ablated crabs injected with saline were inhibited from feeding, but when injected with glucose, feeding activity increased (negative inhibition is stimulation of feeding). * = $p < 0.05$; ** = $p < 0.01$.

Comparison of Hemolymph Size Fractions

Feeding inhibition activity was found in the >5000 d fraction of hemolymph drawn from satiated intact crabs. Injection of the >5000 d fraction resulted in 80% inhibition of feeding responses by eyestalk-ablated crabs ($Z = 5.58$, $P < 0.001$), whereas the <5000 d fraction resulted in 0% inhibition.

Summary of Injection Experiments

Inhibition of feeding responses and corresponding eyestalk equivalents for injection experiments are summarized in Table 1. Levels of FIF were undetectable in ablated crabs injected with saline or with hemolymph from fasted or crabs in the field that were on their way to feeding areas.

DISCUSSION

Feeding inhibition might be the result of depressed glucose levels due to the absence of crustacean hyperglycemic hormone (CHH) (Kleinholz et al., 1967). Injection of glucose into eyestalk-ablated crabs stimulates feeding

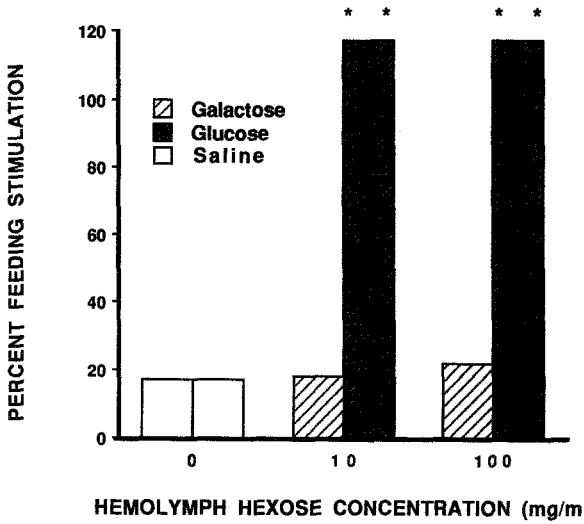


Fig. 2. Feeding activity after injection of hexose sugars into eyestalk ablated crabs. After determination of feeding activity, galactose or glucose was injected into eyestalk-ablated crabs. One hour later, crabs were assayed a second time. Glucose dramatically stimulated feeding. Galactose had no effect. ** = $p < 0.01$.

responsiveness to glucose. This result eliminates the possibility that feeding inhibition is mediated indirectly by CHH through elevation of glucose levels.

Since injection of glucose stimulates feeding in ablated crabs, we suggest it acts directly on the nervous system. The nervous system is an obligate glucose user (Lehninger, 1978). Ablated crabs which lack CHH have depressed glucose levels (Kleinholz et al., 1967). After injection of glucose, the nervous system may work more efficiently, thereby increasing feeding responsiveness. Thus, for ablated crabs, the more they eat, the more they are stimulated to feed. A common dietary sugar, galactose, cannot be used directly by nervous tissue. Injection of galactose resulted in only slight stimulation. This supports the hypothesis that glucose is acting directly on the nervous system.

Glucose injections result in inhibition of feeding in intact animals, but not in ablated animals. This suggests that the sinus gland secretes FIF in response to elevated hemolymph glucose levels. Injection of hemolymph from satiated, but not from hungry crabs, inhibits feeding in ablated crabs. The biological activity found in the hemolymph is similar in molecular size to FIF isolated from the eyestalk (Sears et al., 1991). These results suggest that after the crabs have fed for some time, increased glucose levels stimulate the release of FIF. FIF modulates and finally halts chemically-stimulated feeding responses.

We have constructed an algorithm of fiddler crab responsiveness. As fid-

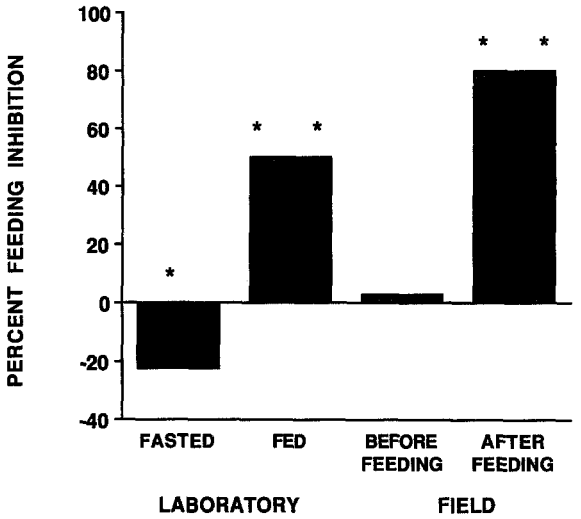


Fig. 3. Feeding activity of crabs following injection of hemolymph from crabs sampled in the laboratory and in the field (on their way to and returning from feeding areas). Hemolymph from unfed (fasted) crabs in the laboratory stimulated feeding in eyestalk-ablated crabs. Hemolymph from fed crabs from either the laboratory of the field dramatically inhibited feeding in ablated crabs. * = $p < 0.05$; ** = $p < 0.01$.

dler crabs feed, hemolymph glucose levels rise. Elevated glucose triggers the release of FIF from the sinus gland into the hemolymph. Circulating FIF acts on the central or peripheral nervous system to decrease responsiveness to external chemical stimuli, first restricting feeding to rich patches of food and finally halting feeding entirely. Once feeding has stopped, crabs return to burrow areas and continue with other activities. There are times when crabs are food-limited and forced to stop feeding by the tides (Salmon and Hyatt, 1983). It would be informative to titer FIF levels at these times. If normal FIF titres decline with fasting at a rate comparable to injections of FIF (Sears et al., 1991), then inhibitory effects of FIF could be expected to diminish prior to the time of the next declining tide.

Male fiddler crabs spend considerable time performing elaborate mating displays (Salmon and Hyatt, 1983). In addition, fiddler crabs are at risk of predation when they are away from their burrows on the sand flat feeding (see Salmon and Hyatt, 1983, p. 41). They must build burrows for predator avoidance and for use as incubation chambers for gravid females. By halting feeding once nutritional and energetic needs are met, FIF plays an important role in budgeting time crabs spend on non-feeding activities.

TABLE 1. SUMMARY OF INHIBITION OF FEEDING RESPONSES AND APPROXIMATE CORRESPONDING EYESTALK EQUIVALENTS OF FIF IN EYESTALK-ABLATED CRABS OF 0.1 ml HEMOLYMPH VOLUME AFTER INJECTION WITH SALINE, HEXOSES, OR HEMOLYMPH. NEGATIVE INHIBITION VALUES INDICATE STIMULATION

Material injected	Percent inhibition	FIF eyestalk equivalents per crab
Lab		
Saline	17	0
Glucose	112	0
Galactose	−22	0
Unfed hemolymph	−22	0
Fed hemolymph	43	0.32
< 5000 d fed hemolymph	0	0
> 5000 d	80	1.2
Field		
Unfed hemolymph	0	0
Fed	80	1.2

There are a variety of criteria for determining whether a specific factor is a hormone (Beltz, 1988): (1) presence in a neurosecretory organ: FIF has been localized to the region of the X-organ sinus gland complex in shrimp eyestalks. Immunocytochemistry would be required to demonstrate the existence of FIF in this neurosecretory organ. (2) Release into the circulation: FIF-like biological activity has been demonstrated in both the laboratory and the field in the hemolymph of fed, but not of unfed crabs. This circulating biological activity has the same approximate molecular size as FIF from the sinus gland region. Purification and characterization of FIF should establish whether or not eyestalk and hemolymph factors are actually the same molecules. (3) Effectors that respond to the factor at its circulating concentration: crabs show dramatic alteration in chemically-stimulated behavior when injected with FIF from eyestalk extracts or from hemolymph of fed crabs. Donor crabs with different FIF titres exhibit different behaviors. Eyestalk-ablated crabs do not respond by producing FIF activity as do intact crabs. (4) Sufficient stability in the hemolymph: although FIF is less solvent-, heat-, and trypsin-stable than many other sinus gland hormones (Sears et al., 1991), FIF is sufficiently stable in the hemolymph to be detected for changes in its titre before and after feeding. These results support the hypothesis that FIF is a hormone from the sinus gland. Its function appears to be to regulate behavior by modulating chemically-stimulated feeding.

REFERENCES

BELTZ, B.S. 1988. Crustacean neurohormones, pp. 235–258, *in* H. Lafer and G.H. Downer (eds.). Endocrinology of Selected Invertebrate Types. Alan R. Liss, New York.

BROWN, F.A. 1940. The crustacean sinus gland and chromatophore activation. *Physiol. Zool.* 19:343–355.

CRANE, J. 1975. Fiddler Crabs of the World (Oncypode, genus Uca). Princeton University Press, Princeton, New Jersey.

GLEESON, R.A., ADAMS, M.A., and SMITH, A.B., III. 1987. Hormonal modulation of pheromone mediated behavior in a crustacean. *Biol. Bull.* 172:1–9.

KLEINHOLZ, L.H., KIMBALL, F., and MCGARVEY, M. 1967. Initial characterization and separation of hyperglycemic (diabetogenic) hormone from the crustacean eyestalk. *Gen. Comp. Endocrin.* 8:75–81.

LEHNINGER, A.L. 1978. Biochemistry, 2nd ed. Worth Publishers, New York.

MASLOW, A.H. 1970. Motivation and Personality, 2nd ed. Harper and Row, New York. 369 pp.

RITTSCHOF, D., and BUSWELL, U.C. 1989. Stimulation of feeding behavior in three species of fiddler crab by hexose sugars. *Chem. Senses* 14:121–130.

ROBERTSON, J.R., BANCROFT, K., VERMEER, G., and PLAISER, K. 1980. Experimental studies on the foraging behavior of the sand fiddler crab *Uca pugilator* (Bosc, 1802). *J. Exp. Mar. Biol. Ecol.* 44:67–83.

ROBERTSON, J.R., FUDGE, J.A., and VERMEER, G.K. 1981. Chemical and live feeding stimulants of the sand fiddler crab, *Uca pugilator* (Bosc). *J. Exp. Mar. Biol. Ecol.* 53:47–64.

SALMON, M., and HYATT, G.W. 1983. Spatial and temporal aspects of reproduction in North Carolina fiddler crabs (*Uca pugilator* Bosc). *J. Exp. Mar. Biol. Ecol.* 70:21–43.

SEARS, M.A., RITTSCHOF, D., and O'BRIEN, T. 1991. Eyestalk factor modulation of chemically stimulated feeding in sand fiddler crabs, *Uca pugilator. J. Exp. Mar. Biol. Ecol.* In press.

WALPOLE, R.E. 1974. *Introduction to Statistics*. Macmillan, New York. 340 pp.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

PEPTIDE ATTRACTION OF HERMIT CRABS *Clibanarius vittatus* BOSC: ROLES OF ENZYMES AND SUBSTRATES

C.M. KRATT and D. RITTSCHOF*

Marine Laboratory and Department of Zoology
Duke University
Beaufort, North Carolina 28516

(Received April 18, 1991; accepted July 29, 1991)

Abstract—Hermit crabs are obligate users of gastropod shells. Shell availability is often the limiting factor for crab population size. Crabs have an extensive behavioral repertoire for obtaining shells. Here we extend our studies of the chemical ability of crabs to locate the shells of dead and dying gastropods from a distance. We show that peptide cues generated by the action of specific proteases on specific substrates attract crabs. The specificity of the crab response is dependent upon the type of substrate as well as the suit of enzymes attacking the substrate. Single specific enzymes are not as effective as mixtures of enzymes in generating cues from pure (and totally foreign) substrates such as ovalbumin. However, the activation of trypsinogen by enterokinase yields only a single hexapeptide and results in potent crab attraction. We conclude that the specific sequence of the peptide determines attraction. Thus, the key to crab attraction is the presence of a particular sequence in a substrate and the ability of enzymes or mixtures of enzymes to release that sequence.

Key Words—Hermit crabs, *Busycon carica*, *Clibanarius vittatus*, peptide attractants, salivary glands, proteases, shell cues.

INTRODUCTION

Marine hermit crabs are obligate users of gastropod shells. The availability and kinds of snail shells control hermit crab population density (Vance, 1972). Proper shell fit is important because it can influence growth (Fotheringham, 1976; Spight, 1985), fecundity (Childress, 1972; Bach et al., 1976; Bertness, 1981),

*To whom correspondence should be addressed.

0098-0331/91/1200-2347$06.50/0

and even copulatory success (Hazlett, 1989; Hazlett and Baron, 1989). Hermit crabs display complex behaviors associated with assessing and trading shells with other hermit crabs (Hazlett, 1966, 1981; Kuris and Brody, 1976; Jackson and Elwood, 1989). New shells enter aquatic crab populations upon death of the gastropods that grew the shells (McLean, 1974; Spight, 1977; Herrnkind et al., 1981; Wilber and Herrnkind, 1982, 1984). Snail mortality may be due to physical occurrences such as large storms or temperature changes (Spight, 1977; Rittschof, 1980a; Wilber and Herrnkind, 1982, 1984) or due to biological occurrences such as snail predation events (McLean, 1974; Wilber and Herrnkind, 1984).

Chemicals released from the flesh of dead and dying gastropods signal the availability of new shells (McLean, 1975; Rittschof, 1980a; Hazlett and Herrnkind, 1980; Gilchrist and Abele, 1984; Wilber and Herrnkind, 1984; Lepore and Gilchrist, 1988). Chemical signals are highly specific (Rittschof, 1980a,b). That is, hermit crab species A is attracted to odors of dead snail species B while hermit crab species C is attracted to the odors of dead snail species D. Most aquatic hermit crabs studied are chemically attracted to obtain shells rather than to feed (McLean, 1975; Rittschof, 1980a). The hermit crabs which are chemically attracted are generally those with small shells (Gilchrist and Abele, 1984), poor fitting shells (Rittschof, 1980a), or heavily fouled shells (Hazlett and Herrnkind, 1980).

Rittschof (1980b) postulated that predatory gastropods had a role in generating chemical crab attractants. That role was the release of proteases upon feeding. The putative proteases were postulated to act upon prey snail muscle proteins to generate peptide crab attractants. Recently, Rittschof et al. (1990b) showed that enzymes from the salivary glands of predatory gastropods (Graham, 1941) could act upon the muscle proteins purified from two gastropod species to attract the striped-legged hermit crab *Clibinarius vittatus*. Attraction was to cues from the flesh of gastropods whose shells were often occupied by *C. vittatus*.

Here we extend the study of Rittschof et al., (1990b) to more clearly understand the substrate–enzyme interactions that generate peptides attractive to hermit crabs. We demonstrate that both the proper substrate(s) and proper enzyme(s) are necessary components for producing cues eliciting hermit crab responses. However, less dramatic responses can be elicited upon changing either substrate or enzyme mixtures. Using a single protease to digest three substrates, we find that whelk flesh produces peptides attractive to *C. vittatus* while digestion of clam flesh or ovalbumin does not. Poor substrates do produce effective attractants when digested with mixtures of proteases. The greater number of cleavage sites results in smaller and more compositionally diverse digestion products. Finally, we show that hermit crab attraction appears to be due to specific peptides. This conclusion is supported by the results of three experi-

ments: (1) mixtures of peptides are not necessarily more attractive than single peptides, (2) putative enzymes of the salivary gland conservatively and specifically cleave 50 kD mollusc muscle proteins to generate highly potent cues, and (3) a specific hexapeptide is a highly potent attractant.

MATERIALS AND METHODS

Study Site and Field Assay

Hermit crab attraction to test substances was determined by field assays (Rittschof et al., 1990b). Assays were performed in the immediate vicinity of the Duke University Marine Laboratory, Beaufort, North Carolina, in low intertidal areas inhabited by the striped-legged hermit crab, *Clibanarius vittatus*. Protein substrates and/or enzymes (see specific experiments below) were mixed in 2 ml of deionized water and 4 ml of sea water. Test substances were prepared and incubated (from 20–60 min depending on the requirements of the reaction) in 2.1-cm diameter 14-kD cutoff dialysis bags (Spectrum Medical Industries, Los Angeles, California). The bags were used directly in field assays.

Each field assay consisted of two or three test substances, one of which was always trypsinized *Busycon carica* flesh. Trypsinized *B. carica* flesh has been documented to be highly attractive to *C. vittatus* (Rittschof et al., 1990b) and was used as a standard attractant to which the attractiveness of other substances was compared. For each type of test substance, eight to ten replicate dialysis bags were prepared. The dialysis bags were placed in individual 100-ml polypropylene cups covered with fiberglass windowscreen, secured with rubber bands.

Cups were buried with the windowscreen flush with the sand along a transect line in the intertidal region during daytime low tides. Water levels varied with the tide between 15 and 45 cm. The cups were placed 2–3 meters apart in a series that alternated treatments.

Cups were monitored for crab attraction for 60 to 90 min by walking up and down the beach parallel to the transect line. A positive response was recorded when a hermit crab touched the windowscreen of a cup. Each responding crab was captured and held until the end of the assay to minimize social interactions (McLean, 1975; Rittschof, 1990b) and to ensure that each crab was counted only once. Response frequencies were analyzed by the G-test, a goodness-of-fit test with expected frequencies based on an extrinsic hypothesis (Sokal and Rohlf, 1981).

Crab responses to treatments could be due to a positive stimulus or to chance. Therefore, the degree of a treatment's potency was determined by comparing the attractiveness of the treatment both to the attractiveness of a known positive stimulus and to a measure of chance encounter. Attractiveness of a

positive stimulus was obtained from trypsinized *Busycon carica* flesh. A measure of chance encounter obtained from crab responses to the test apparatus alone indicated no attractiveness of the latter, i.e., 0 crabs came to the apparatus and 41 crabs came to trypsinized *B. carica* flesh.

Two types of treatments were tested in field assays. Protein digestion assays tested the attraction of crabs to mollusc and commercial protein preparations digested by proteolytic enzymes. Proenzyme activation assays tested the attraction of crabs to proenzymes specifically clipped by activating enzymes.

Whelk Blood and Muscle Proteins

A series of protein digestion experiments utilized specific groups of blood and muscle proteins, extracted from *B. carica*, relaxed overnight in 0.2-M $MgCl_2$ at 4°C. The snails were bled by cutting the foot several times with a scalpel. The blood was collected in a solution of 1 mM phenylmethylsulfonyl fluoride (PMSF) and ethyl alcohol. Tris (0.05 M), ethylenediamine-tetraacetic acid (EDTA, 1 mM), and PMSF (1 mM) pH 7.0 buffer was added to double volume and the solution was centrifuged at 4000 × g for 10 min to remove debris. Half of the solution, hereafter referred to as hemolymph, was stored refrigerated. The remaining hemolymph was ultra-centrifuged at 370,000 × g for 2 hr at 4°C to precipitate hemocyanin from the other blood proteins. The blue hemocyanin pellets were redissolved in fresh buffer solution and stored refrigerated.

Muscle proteins were extracted from whelk flesh by the procedure of Rittschof et al. (1990a,b). In brief, cleaned muscle tissue was coarsely minced and extracted in the cold with 0.3 M KCl buffered with 0.15 M Na_2HPO_4 (pH 7.0). The solution was then clarified by filtration and diluted to 0.05 M KCl to precipitate proteins. Upon centrifugation, pelleted precipitate was redissolved in 2.0 M KCl buffered with 50 mM phosphate to pH 7.0 and clarified supernatant was collected and diluted to ionic strength of 0.25 M KCl. The flocculent white (intermediate ionic strength) precipitate was separated from the supernatant through centrifugation. The ionic strength of the supernatant was then lowered to 0.05 M KCl and a second flocculent white (low ionic strength) precipitate was collected through centrifugation. These procedures generated fractions containing those proteins extracted from muscle in high salt conditions which become insoluble when the ionic strength is lowered.

Whelk Salivary Gland Homogenate

Busycon carica salivary glands have been hypothesized to secrete enzymes which function in the digestion of food (Rittschof et al., 1990a,b); therefore, they were used as a source of predatory snail proteolytic enzymes in some protein digestion assays. Salivary gland homogenate was prepared by the procedure of

Rittschof et al. (1990b). In brief, salivary glands were coarsely dissected from the head region of freshly killed *B. carica*, pooled, homogenized in Tris buffer (0.05 M Tris, 1 mM EDTA, pH 7.5) at 25°C, and stored in aliquots at −20°C.

Commercially Purified Proteins

All commercially purified proteins, including structural proteins, enzymes, and proenzymes, were obtained from Sigma Chemical Company, St. Louis, Missouri. Ovalbumin (chicken egg, grade V, #A5503) was the source of purified protein substrate in several protein digestion assays. Ovalbumin was dissolved in filtered seawater to a concentration of 25 $mgml^{-1}$ immediately prior to experimentation.

Enzymes, used in the protein digestion assays, were trypsin (10,200 BAEE units mg^{-1}, from bovine pancreas, Type III, #T8253), alpha-chymotrypsin (50 BTEE units mg^{-1}, from bovine pancreas, Type VII, #C3142), and trypsin-chymotrypsin (1120 BAEE units mg^{-1} of trypsin activity, 1110 BTEE units mg^{-1} of chymotrypsin activity, from porcine pancreas, Type II, #8128). Enzyme solutions were prepared at a concentration of 1 $mgml^{-1}$ in deionized water and were used immediately. Enzyme and proteins were incubated at RT for 20 min prior to field assays.

Proenzymes used were trypsinogen (from bovine pancreas, #T1143) and α-chymotrypsinogen a (from bovine pancreas, Type II, #C4879). The activating enzymes were enterokinase (from bovine intestine, #E5510), and trypsin (from bovine pancreas, Type III, #T8253), respectively. Incubation procedures were provided by the Sigma Chemical Company, St. Louis, Missouri. Trypsinogen (10 $mgml^{-1}$) and enterokinase (77 $mgml^{-1}$) were incubated in a Tris-HCl buffer (ph 5.8) at RT for 1 hr prior to the field assay. Chymotrypsinogen (50 $mgml^{-1}$) and trypsin (0.5 $mgml^{-1}$) were incubated in 0.05 M phosphate buffer (pH 8.0) at RT for 1 hr prior to the field assay.

Protein Characterization

Proteins of mollusc flesh treatments were characterized by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS PAGE, a 7.5% resolving gel with a 5% stacking gel; Laemmli, 1970). Photographs of gels were subjected to static image analysis (using JAVA software from Jandel Scientific, Corte Madera, California), a densitometry tracing technique. Proteins were characterized both before and after enzymatic digestion.

Experiments

The first series of experiments was designed to test responses of *C. vittatus* to whelk and clam flesh when presented frozen and thawed or when treated with trypsin or chymotrypsin. This series tested the effectiveness of the flesh types

as a source of protein substrate as well as tested the effectiveness of endogenous enzymes, trypsin, and chymotrypsin in generating attractive signals. Five field assays were performed, each comparing hermit crab response to the standard attractant (trypsinized *B. carica* flesh) and a test substance. The test substances were frozen and thawed *B. carica* flesh (1 g per replicate, see *Field assays* above), fresh *B. carica* flesh digested with chymotrypsin, frozen and thawed *Mercenaria mercenaria* flesh (1 g), and fresh *M. mercenaria* flesh (1 g) digested with trypsin (0.5 mg = 5100 BAEE units) or chymotrypsin (2.0 mg = 100 BTEE units).

The second series of experiments tested the effectiveness of an enzyme combination in yielding attractive signals from the potent protein substrate, *B. carica* flesh, and from the less potent protein substrate, *M. mercenaria* (see previous series of experiments). We tested the hypothesis that a less potent protein substrate, if digested with an appropriate enzyme combination, would result in attractant molecules. Four field assays were performed, each comparing trypsinized *B. carica* flesh to a test substance. Test substances were fresh whelk flesh (1 g) or fresh clam flesh (1 g), digested by trypsin-chymotrypsin (0.09 mg #T8128 and 0.5 mg #T8253 = 5,200 BAEE units of trypsin activity and 100 BTEE units of chymotrypsin activity) or *B. carica* salivary gland homogenate (2 mg of protein).

The third series of experiments attempted to pinpoint the source of the attractant protein in *B. carica* flesh. Proteins were extracted from *B. carica* muscle and blood (see above). Muscle proteins included an intermediate ionic strength precipitate and a low ionic strength precipitate. Blood fractions included hemolymph, consisting of all blood proteins, and hemocyanin, the major blood protein. Four field assays were performed to compare the attractiveness of each of the four trypsinized protein fractions with that of trypsinized *B. carica* flesh. Protein amount was 30 mg and trypsin amount was 0.5 mg (5100 BAEE units).

The next series of experiments tested the effectiveness of a single commercially purified protein, rather than a crude protein mixture, as a source of peptide attractants. These experiments tested the hypothesis that if any protein of sufficient complexity, in sufficient quantity is cut with the proper enzymes, its cleavage products can elicit a response. Five field assays were performed to test crab response to 150 mg untreated ovalbumin or ovalbumin digested by trypsin, chymotrypsin, trypsin-chymotrypsin mix, or salivary gland homogenate.

The next two series of experiments tested the responses of hermit crabs to specific peptides rather than to a mixture of many peptides. The experiments tested the hypothesis that hermit crabs are attracted to specific peptides released during activation of predatory snail digestive proenzymes, trypsinogen and chymotrypsinogen, were activated with enterokinase and trypsin, respectively, to yield peptide products. The solution of the proenzymes with their activating

enzymes, as well as different reactant and product combinations, were tested for attractiveness. Mixture amounts per replicate cup were based upon activity units. The incubated mixture of purified bovine trypsinogen (10 mg) and purified bovine enterokinase (77 mg) was tested in a field assay with the following controls: trypsinogen (10 mg), trypsin (10 mg), enterokinase (77 mg), and trypsin (10 mg)-enterokinase (77 mg). The incubated mixture of purified bovine chymotrypsinogen (50 mg) and purified bovine trypsin (0.5 mg) was tested in a field assay with the following controls: chymotrypsinogen (50 mg), trypsin (0.5 mg), and chymotrypsin (50 mg).

RESULTS

Hermit Crab Attraction

C. vittatus has been reported to be readily and specifically attracted to chemicals released from trypsin-treated *B. carica* flesh (Rittschof et al., 1990b). Hermit crabs respond to chemical cues indicating shell availability and not to those indicating food (Rittschof et al., 1990b). Here we compare crab responses to trypsinized *B. carica* flesh to crab responses to *B. carica* and *M. mercenaria* flesh treated with enzyme sources that might release crab attractants. Responses of crabs were used in conjunction with SDS PAGE analysis to test alternative hypotheses about crab attractant molecules. One hypothesis was that potency of attraction was inversely proportional to the amount of protein remaining visible on the gel. The other hypothesis was that potency was due to specific interactions of enzymes and substrates and not necessarily proportional to the amount of protein remaining on the gel.

Attraction to Enzyme Digests of Gastropod Flesh

Overall, whelk flesh is more potent than clam flesh in attracting *C. vittatus* (Figure 1). All whelk flesh treatments attracted crabs as effectively as whelk flesh treated with trypsin ($G < 3.84$, $p > 0.05$, 1 *df*, for all treatments). In contrast, crabs were not attracted to frozen and thawed *M. mercenaria* flesh ($G = 70.30$, $p \ll 0.01$, 1 *df*) or to *M. mercenaria* flesh treated with trypsin ($G = 13.5$, $p < 0.01$, 1 *df*). Clam flesh treated with chymotrypsin attracted 16 crabs, significantly fewer than those attracted to trypsinized whelk flesh ($G = 7.8$, $p < 0.01$, 1 *df*).

Enzymes alone, in amounts equivalent to enzymes in treatments, were compared to responses to whelk flesh and trypsin in field assays. Neither trypsin (four crabs) nor chymotrypsin (no crabs) attracted crabs ($G > 30$, $p < 0.01$, 1 *df* for both enzymes).

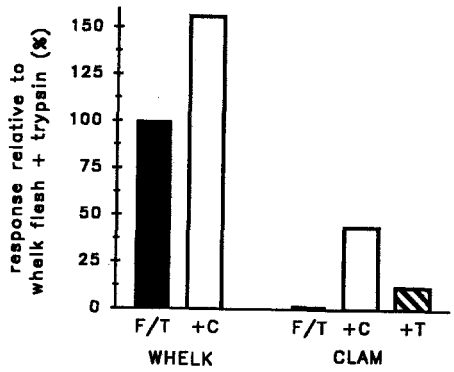


FIG. 1. Responses of *C. vittatus* to *B. carica* or *M. mercenaria* flesh. Frozen and thawed = (F/T); Hydrolyzed by trypsin = +T or chymotrypsin = +C. Assays compared the attractiveness of each treatment with the attractiveness of *B. carica* flesh + trypsin. Actual number of crabs responding (treatment: *B. carica* + trypsin) were: whelk F/T, 15:15 ($G = 0.00$, $p > 0.05$, 1 *df*), whelk + C, 28:18 ($G = 2.19$, $p > 0.05$, 1 *df*), clam F/T, 1:57 ($G = 70.3$, $p \ll 0.01$, 1 *df*), clam +C, 16:36 ($G = 7.89$, $p < 0.01$, 1 *df*), clam + T, 2:17 ($G = 13.55$, $p < 0.01$, 1 *df*).

Attraction to Complex Digests of Mollusc Flesh

Whelk and clam flesh, when treated with a trypsin-chymotrypsin mixture, were equally effective in attracting the crabs ($G < 0.67$, $p > 0.05$, 1 *df*, for both flesh types; Figure 2). Hydrolysis of both flesh types with salivary gland homogenate was also as effective as *B. carica* flesh treated with trypsin ($G < 1.20$, $p > 0.05$, 1 *df*, for both flesh types; Figure 2).

In control tests, comparing the enzymes alone to whelk flesh plus trypsin, both trypsin-chymotrypsin (two crabs) and salivary gland homogenate alone (no crabs; see also Rittschof et al., 1990b) were unattractive ($G > 12$, $p < 0.01$, 1 *df*, for both enzymes).

Thus, all enzymatic treatments of whelk flesh generated responses by crabs that were comparable to responses generated by trypsinized whelk flesh. In contrast, *M. mercenaria* flesh did not attract significant numbers of crabs when frozen and thawed or when treated with trypsin. Clam flesh treated with chymotrypsin was attractive, but significantly less potent than whelk flesh treated with trypsin. Clam flesh treated with either the chymotrypsin–trypsin mixture or the salivary gland extract was as potent as trypsin-treated whelk flesh.

Mollusc Flesh Protein Characterization

Presentation of assay materials in dialysis bags limits crab attraction to molecules < 14 kD. Thus, characterization of proteins by gel electrophoresis detecting molecules > 14 kD is an indirect method of determining the amounts

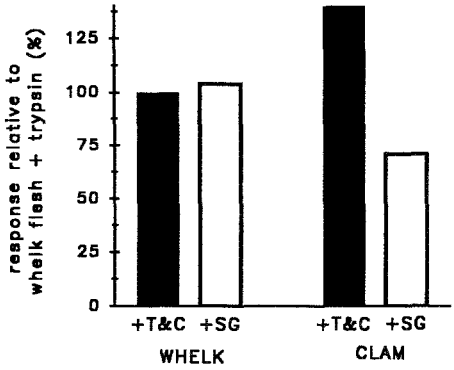


FIG. 2. Responses of *C. vittatus* to *B. carica* or *M. mercenaria* flesh when treated with enzyme mixtures. Field assays were conducted to compare the attractiveness of each treatment with *B. carica* flesh + trypsin. +T&C = +trypsin and chymotrypsin, +SG = +salivary gland homogenate. Actual number of crabs responding (treatment: *B. carica* + trypsin) were: whelk + T&C, 54:54 ($G = 0.00, p > 0.05$, 1 *df*), whelk + SG, 26:25 ($G = 0.02, p > 0.05$, 1 *df*), clam + T&C, 14:10 ($G = 0.67, p > 0.05$, 1 *df*); clam + SG, 17:24 ($G = 1.20, p > 0.05$, 1 *df*).

of material that become available for crab attraction. Densitometry tracings of the gels containing treatments of whelk flesh and clam flesh (Figure 3) were analyzed to determine major protein bands, sensitivity to enzymatic digestion, and potency of total peptides versus specific peptides (Table 1 and see below).

In gel analysis, fresh *B. carica*, unattractive to crabs (Rittschof et al., 1990b), provided a reference protein banding pattern. Fresh *B. carica* flesh contained eight major protein bands of 240, 210, 190, 170, 105, 60, 50, and 40 kD (Figure 3A). Freezing and thawing of flesh released more proteins into solution as indicated by the higher staining intensities. Only the 50 kD protein occurred in lower concentration in the frozen and thawed treatment than in the fresh flesh treatment. The commercially purified enzymes (trypsin, chymotrypsin, and trypsin–chymotrypsin mixture) effectively hydrolyzed all bands and digested over 90% of the total protein. The salivary gland homogenate hydrolyzed approximately 40% of the high molecular weight (>150 kD) proteins and over 90% of 50 kD protein (Table 1A).

Fresh *M. mercenaria* flesh, like fresh *B. carica* flesh, is unattractive to crabs. It provided a standard for protein composition and a reference protein banding pattern. Fresh *M. mercenaria* flesh contained major protein bands of 240, 210, 190, 150, 105, 90, 80, and 40 kD. Freezing and thawing of the flesh promoted minimal hydrolysis. Trypsin, chymotrypsin, and the trypsin–chymotrypsin mixture hydrolyzed all major bands and digested 50% of the proteins. Salivary gland homogenate caused minimal hydrolysis of the high

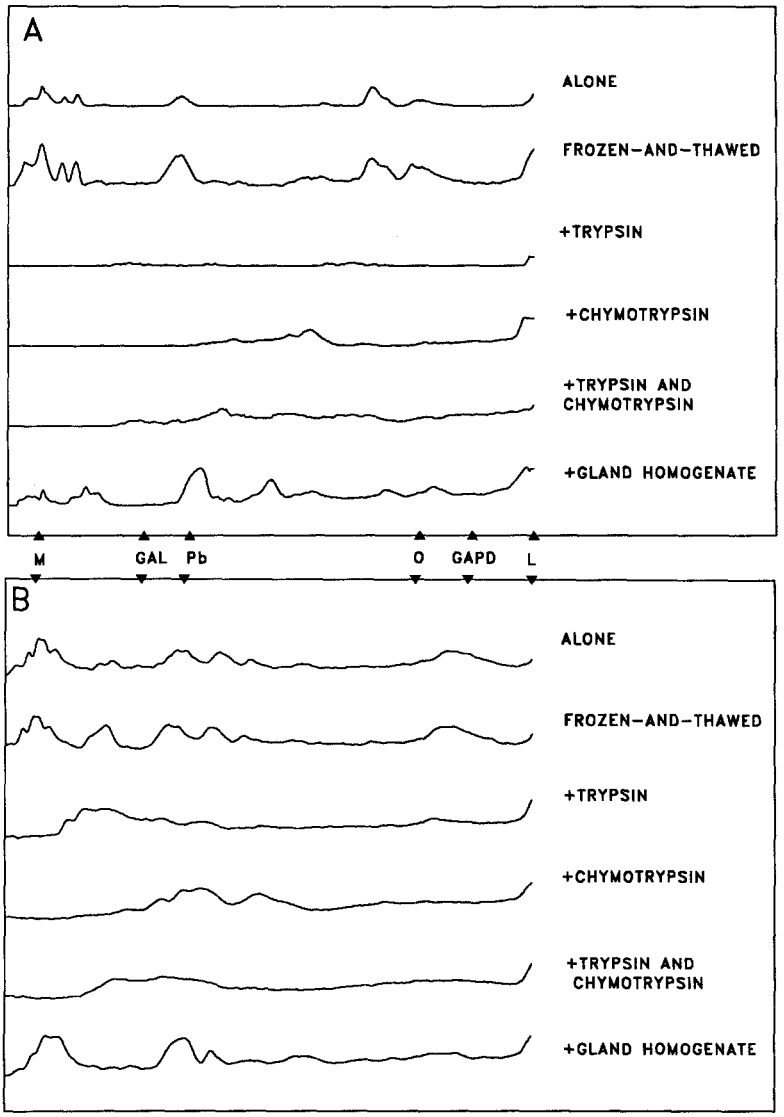


FIG. 3. Densitometry traces from SDS PAGE analysis of protein extracts. (A) *B. carica* proteins extracted from fresh flesh, frozen and thawed flesh, and fresh flesh after incubation for 90 min with catalytic amounts of enzymes. (B) *M. mercenaria* proteins treated as above. The purified enzymes, when presented alone in the same catalyztic amounts as used during incubation, gave no bands. Salivary gland homogenate, when presented similarly, showed bands in the 105–120 kD region. Position of marker proteins are provided between panels. M = rabbit myosin heavy chain, 212 kD, GAL = beta-galactosidase, 116 kD, Pb = phosphorylase b, 97 kD, O = chicken ovalbumin, 45 kD, GAPD = glyceraldehyde 3-phosphate dehydrogenase, 36 kD, L = α-lactalbumin, 14 kD.

TABLE 1. SENSITIVITY OF PROTEINS TO HYDROLYSIS AND EXPECTED AND ACTUAL POTENCY OF TREATMENTS[a]

Treatment	Protein hydrolysis: protein size and (hydrolysis level)	Estimated percent protein exclusion	Expected potency	Actual potency
A. *B. carica* flesh				
Alone	NA	0	Low	Low
Frozen and thawed	50 kD (+)	10	Low	High
+Trypsin	All (+ +)	>95	High	High
+Chymotrypsin	All (+ +)	90	High	High
+Trypsin and chymo.	All (+ +)	90	High	High
+Salivary gland homogenate	170–240 kD (+) 50 kD (+ +)	10	Low	High
B. *M. mercenaria* flesh				
Alone	NA	0	Low	Low
Frozen and thawed	190–240 kD (0)	5	Low	High
+Trypsin	All (+)	50	Moderate	Low
+Chymotrypsin	All (+)	50	Moderate	Moderate
+Trypsin and chymo.	All (+)	70	Moderate	High
+Salivary gland homogenate	190–240 kD (0) 40 kD (+ +)	10	Low	High

[a]Protein size and extent of protein hydrolysis (0 = minimal, + = moderate, + + = extreme) were determined from the desitometry tracings of gels (Figure 1). Percent of protein hydrolyzed to less than 14 kD (dialysis membrane exclusion limit) was estimated from the gels and used to predict expected potency of treatments. Treatments, with < 14 kD proteins comprising 0–20% of total protein, were predicted to be of low potency, 21–40%, moderate potency, and 41–60%, high potency. Predicted potency could be compared to actual biological potency as recorded in field assays (Figures 2 and 4). Treatments which elicited herrmit crab responses of 0–33% were considered to be of low potency, 34–68%, moderate potency, 69–100, high potency.

molecular weight proteins and 10% hydrolysis of the 40 kD protein. Comparison of bioassay data with gel analysis indicates that the level of crab attraction was not inversely proportional to the total amount of protein on the gel (Table 1B). Salivary gland homogenate had minor effects on the baseline protein banding patterns of both whelk and clam flesh but generated potent crab attractants. The frozen and thawed treatment had minor effects on protein banding patterns and effectively generated attractants from whelk flesh, but not from clam flesh. In contrast, trypsin strongly affected the protein banding patterns of both flesh types, but trypsin-treated clam flesh did not attract crabs. Treatment of clam flesh with either chymotrypsin or the trypsin–chymotrypsin mixture altered banding patterns and attracted crabs. Alteration in the 50-kD protein band in whelk flesh and the 40-kD band in clam flesh appeared to correlate with crab attractant potency.

This first series of experiments suggested that both the enzyme and the substrate were important in generating crab attractants. The whelk substrate (the more potent of the mollusc substrates) was composed of two major components, muscle proteins, and blood proteins. In the next series of experiments, each of these components was separated into two fractions and digested with trypsin to determine which fraction produced the most potent attractants when treated with the enzyme.

Attraction to Digests of B. carica *Muscle and Blood Proteins*

The following experiments attempted to further characterize the specific trypsin substrate within *B. carica* flesh. When equal amounts of the fractions were digested by trypsin and presented in field assays, muscle proteins were far more effective substrates than hemolymph or hemocyanin (Figure 4). The intermediate ionic strength muscle precipitate, treated with trypsin was over 2.5 times as potent as flesh treated with trypsin ($G = 9.15$, $p < 0.01$, 1 df). The low ionic strength muscle precipitate treated with trypsin was equally as effective in attracting crabs as flesh treated with trypsin ($G = 0.017$, $p > 0.05$, 1 df). Both precipitates contain proteins of 40–50 kD that are hydrolyzed by both trypsin and salivary gland homogenate (Rittschof et al., 1990a,b). Trypsinized blood proteins, hemolymph and hemocyanin, were significantly less attractive than trypsinized whelk flesh ($G = 12.08$, $p < 0.01$, 1 df; $G = 29.13$, $p << 0.01$, 1 df).

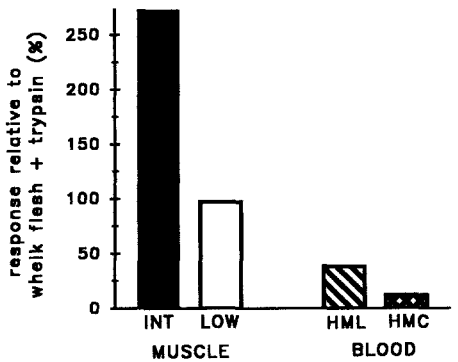


FIG. 4. Responses of *C. vittatus* to *B. carica* muscle and blood proteins after treatment with trypsin. Field assays were conducted to compare the attractiveness of each treatment with the attractiveness *B. carica* flesh + trypsin. Actual number of crabs responding (treatment: *B. carica* + trypsin) were: INT = intermediate ionic strength precipitate, 30:11 ($G = 9.15$, $p < 0.01$, 1 df), LOW = low ionic strength precipitate, 29:30 ($G = 0.017$, $p > 0.05$, 1 df), HML = hemolymph, 16:42 ($G = 12.08$, $p < 0.01$, 1 df), HMC = hemocyanin, 5:42 ($G = 29.13$, $p << 0.01$, 1 df).

Attraction to Digests of Ovalbumin

If the specificity of crab attraction resides in the enzyme, then any substrate of sufficient quantity and complexity, digested by the appropriate enzyme, should attract crabs. Ovalbumin was chosen as a test substrate for this hypothesis. Ovalbumin is approximately the same molecular size as the potential cue substrates—40 kD band from clam and 50 kD band from whelk. Ovalbumin was digested with trypsin, chymotrypsin, a mixture of trypsin and chymotrypsin, and salivary gland homogenate. All treatments were tested in field assays.

All enzymes were effective in cleaving ovalbumin into smaller units which collected in the dye front of the gel (Figure 5). Chymotrypsin was most effective. It eliminated the native ovalbumin band at 45 kD, generating >90% protein < 14 kD. Trypsin and the trypsin–chymotrypsin mixture were moderately effective, yielding 20% and 40% protein < 14 kD.

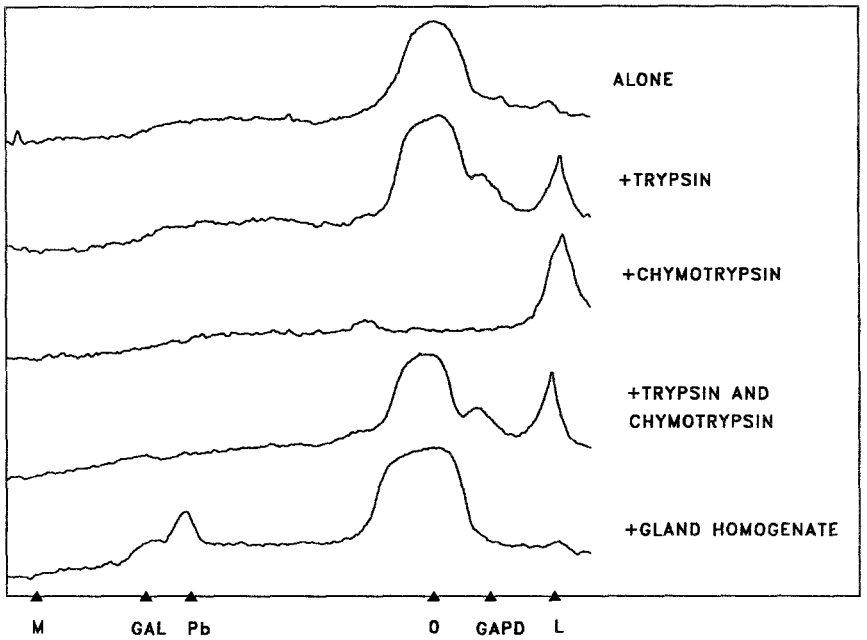


FIG. 5. Densitometry traces from SDS PAGE analysis of ovalbumin. Ovalbumin is presented alone and after incubation for 90 min with catalytic amounts of enzymes. Catalytic amounts of purified enzymes, when presented alone, gave no bands. The salivary gland homogenate, when presented similarly, showed bands in the 105–120 kD region. Position of marker proteins are provided below the panel. M = rabbit myosin heavy chain, 212 kD, GAL = beta-galactosidase, 116 kD, Pb = phosphorylase b, 97 kD, O = chicken ovalbumin, 45 kD, GAPD = glyceraldehyde 3-phosphate dehydrogenase, 36 kD, L = α-lactalbumin, 14 kD.

Overall, in field assays, none of the ovalbumin treatments was as effective as *B. carica* flesh + trypsin in attracting the crabs (Figure 6). Ovalbumin hydrolyzed by chymotrypsin was not attractive ($G = 28.88$, $p << 0.01$, $1df$) even though over 90% of the protein was cleaved to molecules smaller than the dialysis bag exclusion limit. Ovalbumin cleaved with trypsin did not attract crabs even though an amount of the protein was available for detection. In contrast, ovalbumin hydrolyzed by the salivary gland homogenate and ovalbumin treated with the trypsin–chymotrypsin mixture were minimally attractive, attracting 14 crabs ($G = 7.90$, $p < 0.01$, 1 *df*) and 22 crabs ($G = 15.33$, $p < 0.01$, 1 *df*), respectively.

Attraction to Specific Peptides Generated by Proenzyme Activation

Many proteolytic enzymes are activated from proenzymes by proteolytic cleavage that generates small peptides. The next two series of experiments tested the hypothesis that peptides, released upon activation of proenzymes from the predatory snail, could be crab attractants. The proenzymes used in the experiments are well characterized and the cleavage peptides released are known. Two proenzymes, trypsinogen (Figure 7) and chymotrypsinogen, were tested in field assays. *C. vittatus* was attracted to trypsinogen only when activated by enterokinase, generating the hexapeptide, Val-$(Asp)_4$-Lys ($G = 0.224$, $p > 0.05$, 1 *df*). *C. vittatus* was not attracted to the reactants alone nor to other product mixtures ($G > 5.88$, $p < 0.05$, 1 *df*, for all assays). *C. vittatus* was not attracted

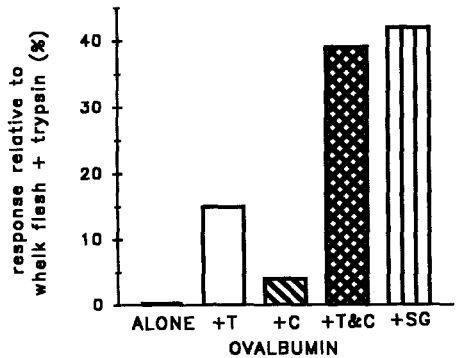


FIG. 6. Responses of *C. vittatus* to ovalbumin when presented alone or when treated with enzymes. Field assays compared the attractiveness of each treatment with the attractiveness of *B. carica* flesh + trypsin. Actual number of crabs responding (treatment: *B. carica* + trypsin) were: ovalbumin alone, 0:16 ($G = >20$, $p < .01$, 1 *df*), +T = +trypsin 3:20 ($G = 14.07$, $p < 0.01$, 1 *df*), +C = +chymotrypsin, 1:26 ($G = 28.88$, $p << 0.01$, 1 *df*), +T&C = +trypsin and chymotrypsin, 22:56 ($G = 15.33$, $p < 0.01$, 1 *df*), +SG = +salivary gland homogenate, 14:33 ($G = 7.90$, $p < 0.01$, 1 *df*).

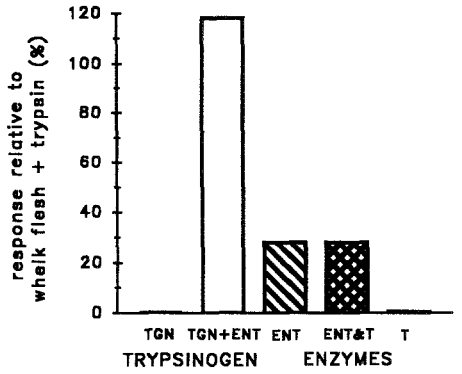


FIG. 7. Responses of *C. vittatus* to trypsinogen–enterokinase incubation, generating Val-(Asp)4-Lys. Five field assays were conducted to compare response to the standard (*B. carica* flesh + trypsin) with response to treatments. Actual number of crabs responding (treatment: *B. carica* + trypsin) were: TGN = trypsinogen alone, 0:24 ($G = 33.27$, $p < 0.01$, 1 *df*), TGN + ENT = trypsinogen activated by enterokinase, 20:17 ($G = 0.244$, $p > 0.05$, 1 *df*), ENT = enterokinase alone, 4:14 ($G = 5.88$, $p < 0.05$, 1 *df*), ENT&T = enterokinase and trypsin, 4:14 ($G = 5.88$, $p < 0.05$, 1 *df*), T = trypsin, 4:64 ($G > 6$, $p < 0.01$, 1 *df*).

in any of the chymotrypsinogen preparations, including the chymotrypsinogen–trypsin incubation (generating serylarginine and threonylasparagissl; Bohinski, 1987), the reactants alone, or the products alone ($G > 13$, $p < 0.01$, 1 *df*, for all assays).

DISCUSSION

The main purpose of this study was to extend our understanding of the substrate/enzyme interactions that generate hermit crab attractant peptides. The experiments show that generation of attractant cues by trypsin and chymotrypsin is substrate dependent. Experiments with endogenous enzymes of frozen and thawed flesh also support this assertion. All three types of enzymes generate attractants from whelk flesh, but not from clam flesh. Analysis of attractive properties of partially purified whelk muscle and blood proteins treated with trypsin (see also Rittschof et al., 1990b) shows that there are at least several protein substrates within whelk flesh that can be cleaved by trypsin to generate crab attractants. Muscle is more effective than blood as a substrate for attractants. Evolutionarily, muscle protein has two obvious advantages over blood protein as substrate: (1) collectively, muscle proteins are far more abundant and diverse than blood proteins, (2) tryptic digestion of muscle proteins generates many small peptides, each capable of escaping the dialysis bag as a chemical

messenger (Rittschof et al., 1990a,b) whereas tryptic digestion of hemocyanin (approximately 90% of total blood protein) generates only two large peptides of 50 kD and 55 kD (Brouwer et al., 1979) molecules, too large to escape from the dialysis bag and, thus, unable to act as chemical messengers.

Single enzyme additions have specific substrate requirements for the effective generation of crab attractants. Trypsin cleaves after arginine and lysine whereas chymotrypsin cleaves after aromatic amino acids (Bohinski, 1987). Specific substrate requirements are relaxed significantly by the use of enzyme mixtures. Mixtures of chymotrypsin and trypsin produce attractants equally well from both clam and whelk flesh. Similar to the effects of single enzymes on clam flesh, digestion of ovalbumin with either trypsin or chymotrypsin alone does not produce crab attractants. Digestion of ovalbumin with the enzyme mixtures generates crab attractants. Enzyme mixtures may generate different peptides (not just more peptides, see gel analysis). This suggests that complex enzyme mixtures can work on a broad spectrum of protein substrates to generate attractants. Enzymatic addition of snail salivary gland homogenate, containing a complex mixture of digestive enzymes (R.L., Ohio Wesleyan and D.R., Duke University, in preparation) produced crab attractants from both clam and whelk flesh as well as from ovalbumin.

Gel analysis shows that there is substantial variability in sensitivity of the major mollusc flesh proteins to enzymatic degradation. The 40 kD proteins and 50 kD proteins of *B. carica* and *M. mercenaria*, respectively, are likely candidates as substrates for cue generation. Crabs were attracted to the treatments in which these proteins were obviously degraded: frozen and thawed whelk flesh, whelk flesh + salivary gland homogenate, and clam flesh + salivary gland homogenate. Purification of the 50 kD protein from *B. carica* and purification and characterization of salivary gland enzymes are planned future studies for determining native crab attractants.

Coupling the above observations with electrophoretic analysis of protein degradation indicates that it is not the absolute amount of protein degradation that attracts crabs, but rather it is the generation of specific peptides (Table 1). This conclusion is supported by the experiments showing that treatments with extensive protein degradation are not always potent and that the single hexapeptide, Val-$(Asp)_4$-Lys, released in low concentration during trypsinogen activation, is as effective as whelk flesh + trypsin at attracting crabs. Although complex mixtures of peptides may function in crab attraction in the native system, a single peptide fragment can serve as a potent crab attractant.

In contrast to trypsinogen activation, which releases a single hexapeptide, chymotrypsinogen activation releases two dipeptides. These dipeptides do not attract *C. vittatus*. They may be too small or have a sequence that does not convey the correct information. Dipeptides are potent cues in other crustacean

systems (for a review, see Rittschof, 1990). Thus, it is more likely that the sequence information is incorrect.

Shell attractant responses have been studied in many species of hermit crabs (see Table 5 in Rittschof, 1980b). In each case, responses of hermit crabs are highly specific. Each species of crab responds only to cues generated from the flesh of certain species of gastropod (McLean, 1974; Rittschof, 1980a). Although it is unclear exactly how such information is stored in the signal molecules, it is clear from the data presented here that detailed discrimination is possible.

There are several molecular mechanisms that could explain how crabs discriminate between shell species by peptide released from flesh. Discrimination could be based upon differences in the relative proportions of peptides. Alternatively, specificity could reside within the peptide sequence, as has been shown for scores of peptide hormones (Litwack and Norman, 1987), for leukocyte attractants (Shiffman, 1982; Unson et al., 1984), and for peptide mimics of crustacean larval release pheromones (Rittschof et al., 1985, 1989, 1990a; Forward et al., 1987; De Vries et al., 1989) and barnacle settlement pheromones (Tegtmeyer and Rittschof, 1989). Studies that use pure peptides and that identify native crab attractant cues will resolve this issue.

Acknowledgments—Thanks to K. Berger, G.W. Cannon, J. Cheu, K.G. Eisenman, M. Erdmann, J.K. Forte, G. Godette, C. Hays, R. Huff, R. Lucas, A. Oliver, D.A. Roberts, and J.P. Sutherland. Supported partially by an NSF Grant No. OCE87-12195 and a special supplement from the NSF Biological Oceanography Program.

REFERENCES

BACH, C., HAZLETT, B., and RITTSCHOF, D. 1976. Effects of interspecific competition on fitness of the hermit crab *Clibanarius tricolor*. *Ecology* 57(3):579–586.

BERTNESS, M.D. 1981. The influence of shell-type on hermit crab growth rate and clutch size (Decapoda, Anomura). *Crustaceana* 40:197–205.

BOHINSKI, R.C. 1987. Modern Concepts in Biochemistry, 5th ed. Allyn and Bacon, Boston. 739 pp.

BROUWER, M., WOLTERS, M., and VAN BRUGGEN, E.F.J. 1979. Proteolytic fragmentation of *Helix pomatia* α-hemocyanin: Isolation of a functionally active chemically pure domain and evidence for subunit heterogeneity. *Arch. Biochem. Biophys.* 193:487–495.

CHILDRESS, J.R. 1972. Behavioral ecology and fitness theory in a tropical hermit crab. *Ecology* 53:960–964.

DE VRIES, M.C., RITTSCHOF, D., and FORWARD, R.B., JR. 1989. Response of rhizocephalan-parasitized crabs to analogues of crab larval-release pheromones. *J. Crus. Biol.* 9:517–524.

FORWARD, R.B., JR., RITTSCHOF, D., and DE VRIES, M. 1987. Peptide pheromones synchronize crustacean egg hatching and larval release. *Chem. Sens.* 12(3):491–498.

FOTHERINGHAM, N. 1976. Effects of shell stress on the growth of hermit crabs. *J. Exp. Mar. Biol. Ecol.* 23:655–671.

GILCHRIST, S., and ABELE, L.G. 1984. Effects of sampling method on the estimation of population parameters in hermit crabs. *J. Crus. Biol.* 4:645–654.

GRAHAM, A. 1941. The oesophagus of the stenoglossan prosobranchs. *Proc. Roy. Soc. Edinb.* 61B:1–22.

HAZLETT, B.A. 1966. Social behavior of the Paguridae and Diogenidae of Curacao. *Stud. Fauna Curacao Other Caribbean Insects* 23:1–43.

HAZLETT, B.A. 1981. The behavioral ecology of hermit crabs. *Annu. Rev. Ecol. Syst.* 12:1–22.

HAZLETT, B.A. 1989. Mating success of male hermit crabs in shell generalist and shell specialist species. *Behav. Ecol. Sociobiol.* 25:119–128.

HAZLETT, B.A., and BARON, L.C. 1989. Influence of shells on mating behavior in the hermit crab *Calcinus tibicen*. *Behav. Ecol. Sociobiol.* 24:369–376.

HAZLETT, B.A., and HERRNKIND, W. 1980. Orientation to shell events by the hermit crab *Clibanarius vittatus* (Bosc) (Decapoda, Saguridra). *Crustaceana* 89:311–314.

HERRNKIND, W., WILBER, P., and LOFTIN, J. 1981. Shells travelling from snails to hermit crabs: A rapid transit system? *Am. Zool.* 21(4):991.

JACKSON, N.W. and ELWOOD, R.W. 1989. Memory of information during shell investigation by the hermit crab *Pagurus bernhardus*. *Anim. Behav.* 37:529–534.

KURIS, A.M., and BRODY, M.S. 1976. Use of principal components analysis to describe the snail shell resource for hermit crabs. *J. Exp. Mar. Biol. Ecol.* 22:69–77.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.

LEPORE, M., and GILCHRIST, S. 1988. Hermit crab attraction to gastropod predation sites. *Am. Zool.* 28(4):93A.

LITWACK, G., and NORMAN, A.W. 1987. Hormones. Academic Press, Orlando, Florida. 806 pp.

MCLEAN, R.B. 1974. Direct shell acquisition by hermit crabs from gastropods. *Experientia* 30(2):206–208.

MCLEAN, R.B. 1975. A Description of a Marine Benthic Faunal Habitat Web. Ph.D. thesis, Florida State University.

RITTSCHOF, D. 1980a. Chemical attraction of hermit crabs and other attendants to gastropod predation sites. *J. Chem. Ecol.* 6(1):103–118.

RITTSCHOF, D. 1980b. Enzymatic production of small molecules attracting hermit crabs to simulated gastropod predation sites. *J. Chem. Ecol.* 6(3):665–675.

RITTSCHOF, D. 1990. Peptide-mediated behaviors in marine organisms: Evidence for a common theme. *J. Chem. Ecol.* 16(1):261–272.

RITTSCHOF, D., FORWARD, R.B., JR., and MOTT, D.D. 1985. Larval release in the crab *Rhithropanopeus harrisii* (Gould): Chemical cues from hatching eggs. *Chem. Sens.* 10(4):567–577.

RITTSCHOF, D., FORWARD, R.B., JR., SIMONS, D.A., REDDY, P.A., and ERICKSON, B.W. 1989. Peptide analogs of the mud crab pumping pheromone: Structure-function studies. *Chem. Sens.* 14(1):137–148.

RITTSCHOF, D., FORWARD, R.B., JR., and ERICKSON, B.W. 1990a. Larval release in brachyuran crustaceans: Functional similarity of the peptide pheromone receptor and the catalytic site of trypsin. *J. Chem. Ecol.* 16:1354–1370.

RITTSCHOF, D., KRATT, C.M., and CLARE, A.S. 1990b. Gastropod predation sites: The roles of predator and prey in chemical attraction of the hermit crab *Clibanarius vittatus* (Bosc). *J. Mar. Biol. Assn. UK* 70:583–596.

SCHIFFMAN, E. 1982. Leukocyte chemotaxis. *Annu. Rev. Physiol.* 44:553–568.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry. W.H. Freeman and Co., San Francisco. 829 pp.

SPIGHT, T.N. 1977. Availability and use of shells by intertidal hermit crabs. *Biol. Bull.* 152:120–133.

SPIGHT, T.N. 1985. Why small hermit crabs have large shells. *Res. Pop. Ecol.* 27(1):39–54.

TEGTMEYER, K., and RITTSCHOF, D. 1989. Synthetic peptide analogs to barnacle settlement pheromone. *Peptides* 9:1403–1406.

UNSON, C.G., ERICKSON, B.W., and HUGLI, T.E. 1984. Active site of C3a anaphylatoxin: Contributions of the lipophilic and orienting residues. *Biochemistry* 23(4):585–589.

VANCE, R.R. 1972. Competition and mechanism of coexistence in three sympatric species of intertidal hermit crabs. *Ecology* 53:1062–1074.

WILBER, T.P., JR., and HERRNKIND, W.F. 1982. Rate of new shell acquisition by hermit crabs in a salt marsh habitat. *J. Crus. Biol.* 2(4):588–592.

WILBER, T.P., JR., and HERRNKIND, W.F. 1984. Predaceous gastropods regulate new-shell supply to salt marsh hermit crabs. *Mar. Biol.* 79:145–150.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

SEQUESTRATION OF INGESTED [^{14}C]SENECIONINE *N*-OXIDE IN THE EXOCRINE DEFENSIVE SECRETIONS OF CHRYSOMELID BEETLES

ADELHEID EHMKE,[1] MARTINE ROWELL-RAHIER,[2] JACQUES M. PASTEELS,[3] and THOMAS HARTMANN[1,*]

[1]*Institut für Pharmazeutische Biologie der Technischen Universität D-3300 Braunschweig, Germany*

[2]*Zoologisches Institut der Universität CH-4051 Basel, Switzerland*

[3]*Laboratoire de Biologie Animale et Cellulaire Université Libre de Bruxelles B-1050 Brussels, Belgium*

(Received March 25, 1991; accepted July 29, 1991)

Abstract—*Oreina cacaliae* (Chrysomelidae) sequesters in its elytral and pronotal defensive secretion the *N*-oxides of pyrrolizidine alkaloids (PA *N*-oxides) from its food plant *Adenostyles alliariae* (Asteraceae). [^{14}C]Senecionine *N*-oxide was applied for detailed studies of PA *N*-oxide sequestration. An average of 11.4% of total radioactivity is taken up by individual beetles which had received [^{14}C]senecionine *N*-oxide with their food leaves 8 days before. An average of 28.9% of the ingested radioactivity could be recovered from the defensive secretions collected twice, i.e., 5 and 8 days after tracer feeding. The tracer transfer into the secretion seems to be a slow but progressive process as indicated by the high percentage of tracer still recovered from the secretion sampled after 8 days. Chromatographic analysis revealed that [^{14}C]senecionine *N*-oxide is the only labeled compound in the defensive secretion. Beetles that fed on tertiary [^{14}C]senecionine sequestered only trace amounts of radioactivity (exclusively present as labeled *N*-oxide) in their secretions. *O. speciosissima*, a species also adapted to PA containing food plants, was shown to sequester [^{14}C]senecionine *N*-oxide with the same efficiency as *O. cacaliae*. *O. bifrons*, a specialist feeding on *Chaerophyllum hirsutum* (Apiaceae), rejected PA treated leaf samples already at very low PA concentrations (10 nmol/leaf piece). In both *O. cacaliae* and *O. speciosissima*, [^{14}C]senecionine *N*-oxide applied by injection into the hemolymph is rapidly transferred into the glands. *O. bifrons*, not adapted to

*To whom correspondence should be addressed.

0098-0331/91/1200-2367$06.50/0

pyrrolizidine alkaloid containing plants was unable to sequester [^{14}C]-senecionine *N*-oxide in the secretion but rapidly eliminated the tracer with the frass. Again, only traces of labeled [^{14}C]senecionine *N*-oxide were found in the defensive secretions of the two PA adapted species if labeled senecionine was injected. It is suggested that the beetles are adapted to the *N*-oxide form of PAs, similarly as their food plants, and that they lack the ability to efficiently *N*- oxidize tertiary PAs. No indication for *de novo* PA synthesis by the beetles was found in tracer feeding experiments with the biogenetic PA precursor putrescine.

Key Words—*Oreina*, Chrysomelidae, leaf beetle, defensive secretion, alkaloid sequestration, pyrrolizidine alkaloid *N*-oxides, tracer feeding, [^{14}C]senecionine *N*-oxide.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are a group of plant secondary compounds which are receiving much attention in the study of insect–plant relationships (see Boppré, 1986, 1990; Schneider, 1987). Although it is likely that they are produced by plants as protective agents against herbivory, some plant feeders have evolved to cope with these compounds or even use them for their own protection or as pheromone precursors. Well-known examples are the arctiid moths and the danaid and ithomiine butterflies.

The leaf beetle *Oreina cacaliae* (Chrysomelidae) was recently shown to sequester plant-derived PAs in the form of their *N*-oxides (PA *N*-oxides) in its defensive glands (Pasteels et al., 1988a). This is the first time that PAs have reported to be involved in exocrine chemical defense. Leaf beetles are specialist herbivores and chemically defended, but sequestration of plant chemicals for defense has been established unambiguously in only a few of them (Pasteels et al., 1988b), e.g., the sequestration in hemolymph and eggs of cucurbitacins and conjugates by cucumber beetles (Galerucinae, Luperini; Ferguson and Metcalf, 1985).

In the subfamily Chrysomelinae, defensive compounds are typically biosynthesized *de novo* and stored in pronotal and elytral glands (Pasteels et al., 1988b). *Oreina cacaliae* was the first example of host plant influence on these secretions. It feeds on *Adenostyles alliariae*, *Senecio fuchsii*, and to a lesser extent on *Petasites paradoxus* (all members of the Asteraceae) but sequesters in its secretions exclusively PA *N*-oxides derived from *A. alliariae* (Rowell-Rahier et al., 1991). *O. cacaliae* does not synthesize the cardenolides found in the secretions of all the other species of the genus and of most members of the related genus *Chrysolina* (van Oycke et al., 1987, 1988; Rowell-Rahier et al., unpublished). Most interestingly, a second species, *O. speciosissima*, feeding on the same host plants as *O. cacaliae*, was also found able to sequester PA

N-oxides from *A. alliariae* but to still possess the capability for *de novo* cardenolide biosynthesis (Rowell-Rahier et al., 1991).

In this study, we investigated by means of ^{14}C-labeled senecionine and its *N*-oxide the ability of *O. cacaliae* to sequester PAs. Labeled PAs were offered either orally or administered by injection into the hemolymph in order to circumvent the gut barrier. In addition, the ability to sequester and *N*-oxidize the tertiary PAs and to synthesize PAs *de novo* was tested. For comparison, *O. speciosissima* and the related *O. bifrons*, a species not adapted to PA-containing plants which feeds on *Chaerophyllum hirsutum* (Apiaceae), were included in the experiments.

MATERIAL AND METHODS

Insects

The beetles were collected in May and June 1988/89 and kept at room temperature on their host plants or at 4°C for 1 to 3 weeks until use.

O. cacaliae specimens originated from Wasserliesen (Vosges, France) and Zastler (Schwarzwald, Germany), *O. speciosissima* from La Lécherette (Vaud, Switzerland) and Zastler (Schwarzwald, Germany), and *O. bifrons* from Hohwald (Vosges, France).

Radioactive Tracers

[^{14}C]Senecionine (1.07 GBq/mmol) and its *N*-oxide were prepared biosynthetically from [1,4-^{14}C]putrescine (4.4 GBq/mmol) using root cultures of *Senecio vulgaris* according to Ehmke et al. (1988). Labeled putrescine was obtained from Amersham Buchler (Braunschweig, Germany).

Tracer Feeding Experiments

The beetles were placed individually in Petri dishes (5 cm diam.), kept at room temperature (20–22°C) and fed during 24 hr with a disk (1 cm diam.) or a piece of host plant leaf treated with 10 μl of a methanolic solution of [^{14}C]senecionine *N*-oxide (8.3 kBq/μl) or [^{14}C]senecionine (8.7 kBq/μl). After 24 hr, the frass and leaf remains were collected, and the beetles were transferred to fresh untreated host plant leaves. This transfer was repeated every day until the end of the experiment. Frass and defensive secretions were collected at the time intervals given in the results section. At the end of the experiment, the beetles were deep frozen prior to chemical extraction.

Injection of Labeled Compounds

An ethanolic solution (0.5 μl) of [^{14}C]senecionine *N*-oxide (1.0 kBq/μl) or 1 μl of [^{14}C]senecionine (0.8 kBq/μl), or 1 μl of [1,4-^{14}C]putrescine (2.1 kBq/μl) was injected into the abdominal cavity of the beetles with a 1 μl-microsyringe (Hamilton) or a individually calibrated micropipette. The beetles were subsequently treated as described above.

Collection of Defensive Secretions

The beetles were manipulated with fine forceps in order to provoke emission of the secretion which was collected on filter papers and immediately placed in MeOH until analysis.

Alkaloid Extraction

Individual deep-frozen beetles were ground with 2.0 ml acidic MeOH (1% HCl) and quartz sand in a mortar for 10 min. The homogenate was suspended in a total volume of 5.0 ml acidic MeOH and after centrifugation the supernatant was used directly for analysis of total radioactivity by scintillation counting or TLC-analysis. Frass samples were extracted in the same way.

Thin-Layer Chromatography (TLC)

MeOH solutions of secretions and crude MeOH extracts of beetles and frass were analyzed by TLC and evaluated quantitatively by means of a TLC multichannel analyzer (Rita-32a, Raytest). Separation was obtained on Silica gel 60 Merck) using the solvent system: CH_2Cl_2:MeOH:NH_4OH (25%) (82:15:3, by vol).

High Pressure Liquid Chromatography (HPLC)

Reverse-phase ion-pair HPLC (Wagner et al., 1986) was used to separate PA *N*-oxides according to Hartmann et al. (1988). ^{14}C-labeled PA *N*-oxides were detected with the HPLC radioactivity monitor LB-506D (Berthold) equipped with a 2 ml flow-cell and the split-mixer LB-5035.

RESULTS

Sequestration of Orally Fed [^{14}C]Senecionine N*-Oxide and [^{14}C]Senecionine by* O. cacaliae

In order to study the efficiency of PA uptake and storage, leaf-disks of *A. alliariae* treated with [^{14}C]senecionine *N*-oxide and [^{14}C]senecionine were offered individually to *O. cacaliae* beetles. The beetles consumed the leaf-disks

usually within 24 hr and were subsequently allowed to feed on untreated food leaves. Specimens with pre-emptied glands were employed. The defensive secretions were collected 5 days after the beginning of the experiment and in the *N*-oxide feeding experiment again 3 days later and were analyzed and compared with the MeOH-soluble radioactivity found in the frass and the bodies of the beetles (Table 1). In spite of great individual variation which is probably mainly due to differences in feeding behavior and activity, the data clearly show that a considerable proportion of the tracer is ingested by the beetles, i.e., an average of 11.4% (senecionine *N*-oxide) and 4.8% (senecionine). Only trace amounts of radioactivity could be recovered from the secretions of beetles that fed an tertiary [^{14}C]senecionine, whereas an average 28.9% of ingested radioactivity was found in the secretions of beetles that fed on [^{14}C]senecionine *N*-oxide. The tracer transfer into the secretion seems to be a slow and progressive process as indicated by the high percentage of tracer still recovered from the second secretion sampled after 8 days. Chromatographic analysis of the secretions revealed absolutely pure [^{14}C]senecionine *N*-oxide. Not even traces of labeled tertiary senecionine or any other metabolite could be detected (Figure 1A). Reversed-phase ion-pair HPLC confirmed the purity; neither labeled seneciphylline *N*-oxide nor any other potential derivative of senecionine *N*-oxide could be detected. On the other hand in crude beetle extracts, [^{14}C]senecionine *N*-oxide was always found to be accompanied by at least trace amounts of labeled senecionine and polar metabolites ($R_f < 0.1$; Figure 1B).

TABLE 1. DISTRIBUTION OF MeOH-EXTRACTABLE RADIOACTIVITY (MEAN % ± *SD*) AFTER INGESTION OF EITHER [^{14}C] SENECIONINE *N*-OXIDE OR [^{14}C] SENECIONINE BY *O. cacaliae*. EACH INDIVIDUAL BEETLE WITH PRE-EMPTIED GLANDS WAS FED WITH 8.3 kBq ([^{14}C] SENECIONINE *N*-OXIDE) OR 8.7 kBq ([^{14}C] SENECIONINE) DEPOSITED ON LEAF-DISCS (*A. alliariae*). AFTER 24 hr, THE BEETLES WERE ALLOWED TO CONTINUE FEEDING ON UNTREATED LEAVES

Sample	Time of sampling (*d*)	Percent of ^{14}C-labeled tracer applied	
		[^{14}C] Senecionine *N*-Oxide ($n = 9$)	[^{14}C] Senecionine ($n = 8$)
Frass I	1	20.0 ± 6.6	18.5 ± 7.2
Frass II	2	3.9 ± 2.0	3.8 ± 2.5
Secretion I	5	1.7 ± 1.1	Traces
Secretion II	8	1.6 ± 1.3	Not collected
Beetle body	8	8.1 ± 4.3	4.8 ± 4.5
Secretions + body		11.4 ± 5.7	4.8 ± 4.5
Percent in secretion[a]		28.9 ± 11.4	

[a] 100% = Total radioactivity in the beetles (secretions + beetle extracts).

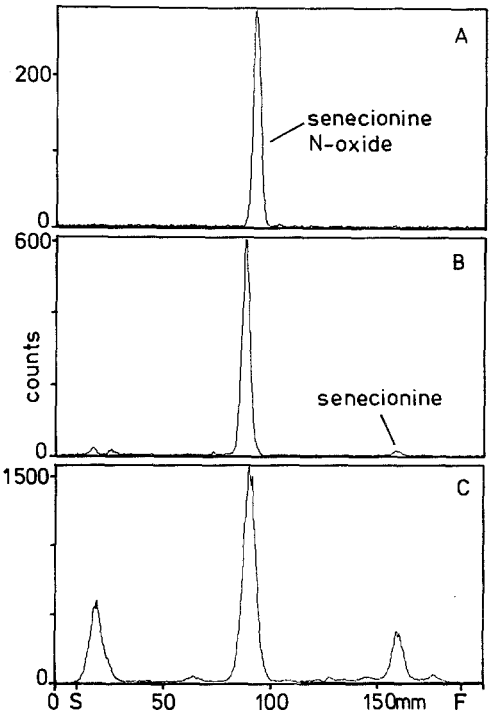


FIG. 1. Sequestration and elimination of [^{14}C]senecionine *N*-oxide fed orally to *O. caliae*. TLC separation of crude MeOH extracts of A: defensive secretion, B: beetle extracts, C = frass (24 hr) extracts. Detection: TLC radioscanning.

The trace amount of radioactivity found in the secretions of [^{14}C]senecionine fed beetles was exclusively in the *N*-oxide form, whereas extracts of these beetles were found to contain mainly tertiary [^{14}C]senecionine and only a small proportion of the *N*-oxide (ca. 20% of total alkaloid in the body).

The total amount of MeOH soluble radioactivity eliminated by the beetles with the frass during 2 days was on average 23.9% (*N*-oxide fed) and 22.3% (senecionine fed), respectively. Most of the radioactivity was eliminated during the first 24 hr (Table 1, Figure 1). Within two days, the elimination of radioactivity decreased to a level of less than 1% per day (Figure 1). Chromatographic analysis of frass extracts showed that during gut passage considerable amounts of [^{14}C]senecionine *N*-oxide were reduced to tertiary senecionine and degraded to polar metabolites (Figure 1C). Only trace amounts of the *N*-oxide form were detected in frass extracts of beetles that fed on [^{14}C]senecionine.

The proportion of total radioactivity recovered as MeOH soluble com-

pounds from secretions, beetles, and frass is on average only about 27% of radioactivity given (Table 1). Apparently, considerable proportions of the tracers fed are transformed into MeOH insoluble material. The fate of insoluble radioactivity has not been followed in the course of the present experiments; however, in comparable feeding studies with *Tyria jacobaeae* (Biller et al., unpublished), the loss of labeled senecionine *N*-oxide into insoluble material occurred most probably during the gut passage, and its endproducts were found to be associated with the frass.

In a second experiment, designed in the same way as that shown in Table 1, ten beetles that were "milked" prior to the tracer feeding were compared to ten individuals that were not "milked." The results show that the incorporation into the secretion is significantly lower (ten times) in the beetles with full glands (1.6% ± 1.1) than in those with empty glands (13.9 ± 8.4) (data not shown).

Sequestration of Orally Fed [^{14}C]senecionine N-*oxide by* O. speciosissima *and* O. bifrons

With *O. speciosissima* beetles fed on labeled senecionine *N*-oxide, results were obtained which are comparable to those described above for *O. cacaliae*. *O. bifrons*, a specialist feeding on *Chaerophyllum hirsutum* (Apiaceae), that during its life history never gains access to pyrrolizidine alkaloids, behaves differently. At alkaloid *N*-oxide concentrations of 10 nmols per leaf disk, the concentration at which the tracer was applied, nine out of ten beetles rejected their alkaloid-treated host plant, whereas control beetles fed well on untreated leaves. No radioactivity was found in the secretions collected from the one individual that was willing to feed on a treated leaf.

Sequestration of Injected [^{14}C]Senecionine N-*Oxide by* O. cacaliae, O. speciosissima, *and* O. bifrons

To test the ability of the three species to sequester and excrete senecionine *N*-oxide, ^{14}C-labeled *N*-oxide was directly injected into the hemolymph of the beetles. The distribution of radioactivity in frass, secretion, and beetle was analyzed (Table 2). The results are given individually for nine specimens in each series, to demonstrate the variability between replicates. To some extent, this variability is due to the difficulty of reproducibly injecting 0.5 μl into the abdominal cavity of the beetle. Nevertheless, the results clearly show that 5 days after injection, considerable amounts of radioactivity are in the defensive secretions and bodies of *O. cacaliae* and *O. speciosissima*. Another part of the radioactivity is rapidly eliminated with the frass. This elimination seems to be more rapid in *O. cacaliae*. Here, only trace amounts of radioactivity were detected in the frass collected on the second day, whereas in *O. speciosissima*, substantial amounts of radioactivity were measured in the frass of the second

TABLE 2. DISTRIBUTION OF MeOH-EXTRACTABLE RADIOACTIVITY AFTER INJECTION OF [^{14}C] SENECIONINE *N*-OXIDE INTO *O. cacaliae*, *O. speciosissima*, AND *O. bifrons*. THE BEETLES (NINE INDIVIDUAL SPECIMENS WITH EMPTY GLANDS) WERE EACH INJECTED WITH 0.6 µl TRACER (ca. 0.5 kBq) AND WERE ALLOWED TO CONTINUE FEEDING ON THEIR RESPECTIVE HOST PLANTS

MeOH extract	Time of sampling (*d*)	Percent of ^{14}C-labeled tracer applied									Mean value (±*SD*)
		1	2	3	4	5	6	7	8	9	
O. cacaliae											
Secretion	5	10.8	22.7	8.3	5.8	6.4	4.2	5.1	19.2[a]	7.4[a]	10.0 ± 6.6
Beetle	5	14.8	15.2	51.1	13.5	15.7	8.5	11.5	39.7[a]	32.2[a]	22.5 ± 14.9
Frass	1	20.8	5.3	5.8	6.4	3.1	25.5	3.6	3.9	7.8	9.1 ± 8.2
Frass	2	<1.0	1.1	1.0	2.1	<1.0	1.2	1.1	nd	nd	1.2 ± 0.4
O. speciosissima											
Secretion	5	14.2	4.7	0.9	2.7	nd	2.2	13.5	11.7	12.3	7.8 ± 5.7
Beetle	5	27.4	3.4	9.9	6.6	2.3	10.4	19.2	28.1	20.3	14.2 ± 9.9
Frass	1	3.0	2.0	2.2	10.4	21.2	6.7	6.5	5.1	4.7	6.9 ± 6.0
Frass	2	5.1	3.0	2.3	2.2	7.1	6.0	1.7	5.1	4.0	4.1 ± 1.9
O. bifrons											
Secretion	5	—	—	—	tr	—	—	—	—	—[a]	traces
Beetle	5	nd	nd	nd	nd	2.1	6.7	nd	1.5	5.4[a]	3.9 ± 2.5
Frass	1	32.1	25.8	78.7	32.5	62.5	40.8	46.8	86.5	51.3	50.1 ± 21.3
Frass	2	3.1	2.1	nd	1.5	1.0	10.5	2.6	2.8	nd	3.4 ± 3.2

[a]sampling after 4 *d*; nd = not determined, tr = traces, — = not detectable.

day. *O. bifrons*, not adapted to pyrrolizidine-containing plants, is, as expected, unable to sequester [^{14}C]senecionine *N*-oxide either in the body or in the secretion. It is, however, as efficient as the other two species in eliminating injected alkaloid from its body. Most of the radioactivity is voided with the frass within the first 24 hr (Figure 2).

Analysis of the different fractions again revealed [^{14}C]senecionine *N*-oxide as the only labeled compound in the defensive secretions, whereas in beetle extracts, a considerable amount of unknown labeled polar metabolites was detected in addition to the *N*-oxide.

Only trace amounts of [^{14}C]senecionine *N*-oxide were found in the secretions of *O. cacaliae* if labeled senecionine was injected instead of its *N*-oxide.

Injection of [^{14}C]*Putrescine into* O. cacaliae

To test the ability of the beetle to synthesize *de novo* pyrrolizidine alkaloids, ten beetles were injected with labeled putrescine, a known alkaloid precursor. In none of the beetles was activity found in the secretions, nor could any traces of alkaloid be detected.

DISCUSSION

[^{14}C]senecionine *N*-oxide is efficiently taken up by *O. cacaliae* and translocated via the exocrine glands into the defensive secretion. On its way from the gut into the secretion, it has to pass at least two sets of membrane barriers,

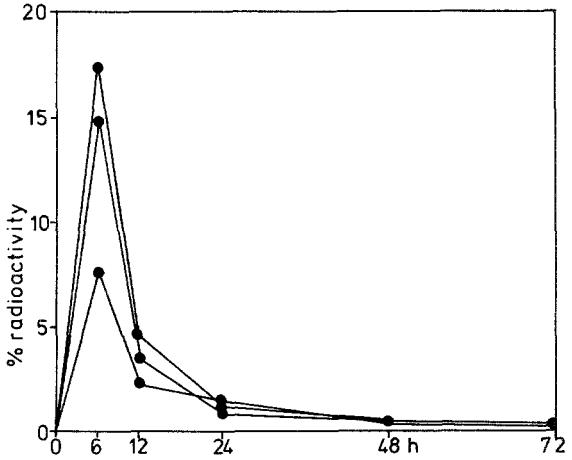


FIG. 2. Time course of occurrence of radioactivity in the frass of *O. cacaliae* following administration of [^{14}C]senecionine *N*-oxide. The tracer was fed orally on *A. alliariae* leaf disks.

the gut epithelium and the gland cell membranes. The rapid uptake of the PA *N*-oxide from the gut and its accumulation in the beetle seems to be followed by a slow and continuous transfer into the defensive glands (see Table 1). This transport is highly specific in two respects: (1) [^{14}C]Senecionine *N*-oxide is recovered from the defensive secretion in a virtually pure state. No labeled transformation or degradation products are detectable. This is in accordance with the observation that PA *N*-oxides from food plants such as *Adenostyles alliariae* and *Senecio fuchsii* are taken up rather unspecifically by the beetle, but only the PA *N*-oxides acquired from *A. alliariae* are transferred into the glands (Rowell-Rahier et al., 1991). (2) Only the *N*-oxide form is translocated into the secretion, not even traces of the tertiary form being detectable (see Figure 1A). This is in good agreement with the identification exclusively of PA *N*-oxides in the secretions of *O. cacaliae* collected in nature (Pasteels et al., 1988a; Rowell-Rahier et al., 1991). In this respect, *O. cacaliae* behaves like other specialized PA sequestering insects, e.g., *Tyria jacobaeae* (Ehmke et al., 1990) and *Creatonotos transiens* (von Nickisch-Rosenegk et al., 1990; Hartmann et al., 1990). In contrast to these insects, however, which easily transform tertiary PAs into the respective *N*-oxides and thus are able to sequester the tertiary form with the same efficiency as the *N*-oxide, *O. cacaliae* does not possess this ability. If labeled senecionine is offered orally or administered by injection into the hemolymph, only traces of radioactivity (exclusively [^{14}C]senecionine *N*-oxide) are detected in the secretions.

This once again emphasizes the physiological importance of the *N*-oxide form of the pyrrolizidine alkaloids in plants as well as in adapted insects. Plants are known to synthesize PAs as *N*-oxides (Hartmann and Toppel, 1987) and to use the polar, salt-like *N*-oxide as the molecular form for specific transport and cellular accumulation (Hartmann et al., 1989). Carrier-mediated membrane transport has been demonstrated for PA-containing plants (*Senecio vulgaris*) (Ehmke et al., 1988) as well as for PA-sequestering insects (*Creatonotos transiens;* Wink and Schneider, 1988). Characterization of the two different carrier systems present in *O. cacaliae* and *O. speciosissima*, i.e., of the gut epithelia cell membranes and the gland cell membranes, will be challenging tasks.

It is surprising that *O. speciosissima* sequesters orally fed or injected [^{14}C]senecionine *N*-oxide with same efficiency as *O. cacaliae*, but it confirms previous findings (Rowell-Rahier et al., 1991). In nature, *O. speciosissima* feeds preferentially on *Petasites paradoxus*, the leaves of which contain no PAs or at least amounts undetectable with our analytical methods (Rowell-Rahier et al., 1991). Specimens collected in nature are reported to sequester either no PA *N*-oxides (Pasteels et al., 1988a) or only very small amounts, which represent the PA pattern found in *A. alliariae* (Rowell-Rahier et al., 1991). On the other hand, in contrast to *O. cacaliae*, *O. speciosissima* still synthesizes cardenolides (Rowell-Rahier et al., 1991). Why does not *O. speciosissima* use in nature its ability to sequester PAs, and why has it evolved if it is not used? These are

intriguing questions which must await a better understanding of the biological significance of this sequestration.

Sequestration in the glands does not seem to have evolved as a detoxification mechanism, since the absolute amount sequestered is negligible compared to the amount ingested and excreted. Furthermore, the mechanisms which eliminate the polar *N*-oxides from the body via the gut seem to be as efficiently developed in unadapted beetles, e.g., *O. bifrons*, as demonstrated by the injection experiment (see Table 2).

The role of PA *N*-oxide sequestration in defense is suggested by the facts that it occurs in secretions which are known to be defensive in related chrysomelids, and that in *O. cacaliae* PAs have completely replaced the production of other toxins, i.e., cardenolides. PA *N*-oxide concentrations as high as 0.3 mol/l reported for the secretion of *O. cacaliae* (Rowell-Rahier et al., 1991) should ensure an efficient protection, judging by the concentrations found in other sequestering insects. In caterpillars of *Tyria jacobaeae* the PA *N*-oxide concentrations may reach concentrations of 10 mmol/kg fresh weight (Ehmke et al., 1990). The tissue concentrations are somewhat higher if we consider the integument as the major site of PA *N*-oxides storage. PAs are said to be strong repellents for a wide range of animals (Boppré, 1986). A convincing example is the giant tropical orb spider *Nephila clavipes*, an important potential predator of butterflies. *Nephila* cuts out field-caught Ithomiinae unharmed from its web, but readily eats freshly emerged adults, which are still free of PAs (Brown, 1984). A *Nephila* bioassay is now used, because the spider rejects sensitively PA-protected butterflies, but eats most other "unpalatable" aposematic insects. *O. bifrons* used in the experiments described here is a second example. Amounts of senecionine *N*-oxides as low as 10 nmol offered with a piece of food leaf prevented the beetles from feeding. For comparison, no feeding deterrency was observed when senecionine or monocrotaline and their *N*-oxides were offered in amounts of 40 μmol per leaf disk to the PA-adapted *O. cacaliae* and *O. speciosissima* (Rowell-Rahier et al., 1991).

The PA *N*-oxides found in the defensive secretions are acquired defensive chemicals which are exclusively derived from the food plant. There is no indication of *de novo* synthesis of PA *N*-oxides by the beetles. Furthermore, there is no evidence for chemical modification of acquired PA *N*-oxides, as documented recently for insect-specific PA *N*-oxides. The callimorphines in *Tyria jacobaeae* (Ehmke et al., 1990) and the creatonotines of *Creatonotos transiens* (Hartmann et al., 1990) are synthesized by the insects from a necine base acquired from the plant, esterified with a necic acid of insect origin.

Acknowledgments—This work was supported by grants of the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie to T.H. We thank Claudine Theuring for skillful technical assistance. J.P. thanks the Belgian Fund for Joint Basic Research for financial support. M.R.R. thanks the Schweizerischer Nationalfonds for financial support. We thank S. Dobler, F. Eggen-

berger, and P. Soetens for help in taking care of the beetles. We are grateful to H. Rowell for linguistic help.

REFERENCES

BOPPRÉ, M. 1986. Insect pharmacophagously utilize defensive plant chemicals (pyrrolizidine alkaloids). *Naturwissenschaften* 73:17–26.

BOPPRÉ, M. 1990. Lepidoptera and pyrrolizidine alkaloids; exemplification of complexity in chemical ecology. *J. Chem. Ecol.* 16:165–185.

BROWN, K.S. 1984. Adult-obtained pyrrolizidine alkaloids defend ithomiine butterflies against a spider predator. *Nature* 309:707–709.

EHMKE, A., VON BORSTEL, K., and HARTMANN, T. 1988. Alkaloid N-oxides as transport and vacuolar storage compounds of pyrrolizidine alkaloids in *Senecio vulgaris* L. *Planta* 176:83–90.

EHMKE, A., WITTE, L., BILLER, A., and HARTMANN, T. 1990. Sequestration, N-oxidation and transformation of plant pyrrolizidine alkaloids by the arctiid moth *Tyria jacobaeae* L. *Z. Naturforsch.* 45c:1185–1192.

FERGUSON, J.E., and METCALF, R.L. 1985. Cucurbitacins. Plant-derived defense compounds for Diabroticites (Coleoptera: Chrysomelidae). *J. Chem. Ecol.* 11:311–317.

HARTMANN, T., and TOPPEL, G. 1987. Senecionine N-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of *Senecio vulgaris*. *Phytochemistry* 26:1639–1643.

HARTMANN, T., SANDER, H., ADOLPH, R., and TOPPEL, G. 1988. Metabolic links between the biosynthesis of pyrrolizidine alkaloids and polyamines in root cultures of *Senecio vulgaris*. *Planta* 175:82–90.

HARTMANN, T., EHMKE, A., EILERT, U., V. BORSTEL, K., and THEURING, C. 1989. Sites of synthesis, translocation and accumulation of pyrrolizidine alkaloid N-oxides in *Senecio vulgaris* L. *Planta* 177:98–107.

HARTMANN, T., BILLER, A., WITTE, L., ERNST, L., and BOPPRÉ, M. 1990. Transformation of plant pyrrolizidine alkaloids into novel insect alkaloids by arctiid moths (Lepidoptera). *Biochem. Syst. Ecol.* 18:549–554.

PASTEELS, J.M., ROWELL-RAHIER, M., RANDOUX, T., BRAEKMAN, J.C., and DALOZE, D. 1988a. Pyrrolizidine alkaloids of propable host-plant origin in the protonal and elytral secretion of the leaf beetle *Oreina cacaliae*. *Entomol. Exp. Appl.* 49:55–58.

PASTEELS, J.M., ROWELL-RAHIER, M., and RAUPP, M.J. 1988b. Plant-derived defense in chrysomelid beetles, pp. 235–272, *in* P. Barbosa and D. Letourneau (eds.). Novel Aspects of Insect–Plant Interactions. J. Wiley & Sons, New York.

ROWELL-RAHIER, M., WITTE, L., EHMKE, A., HARTMANN, T., and PASTEELS, J.M. 1991. Sequestration of plant pyrrolizidine alkaloids by chrysomelid beetles and selective transfer into the defensive secretions. *Chemoecology*. In press.

SCHNEIDER, D. 1987. The strange fate of pyrrolizidine alkaloids, pp. 123–142, *in* R.F. Chapman, E.A. Bernays, and J.G. Stoffolano (eds.). Perspectives in Chemoreception and Behavior. Springer, New York.

VAN OYCKE, S., BRAEKMAN, J.C., DALOZE, J.C., and PASTEELS, J.M. 1987. Cardenolide biosynthesis in chrysomelid beetles. *Experientia* 43:460–462.

VAN OYCKE, S., RENDOUX, T., BRAEKMAN, J.C., DALOZE, D., and PASTEELS, J.M. 1988. New cardenolide glycosides from the defense glands of chrysolinina beetles (Coleoptera: Chrysomelidae). *Bull. Soc. Chim. Belg.* 97:297–311.

VON NICKISCH-ROSENEGK, E., SCHNEIDER, D., and WINK, M. 1990. Time-course of pyrrolizidine alkaloid processing in the alkaloid expoiting arctiid moth, *Creatonotos transiens*. *Z. Naturforsch.* 45c:881–889.

WAGNER, J., DANZIN, C., and MAMONT, P. 1986. Reversed-phase ion-pair liquid chromatographic procedure for the simultaneous analysis of S-adenosylmethionine, its metabolites and the natural polyamines. *J. Chromatogr.* 227:349–368.

WINK, M., and SCHNEIDER, D. 1988. Carrier-mediated uptake of pyrrolizidine alkaloids in larvae of the aposematic and alkaloid-exploiting moth, *Creatonotos. Naturwissenschaften* 75:524–525.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

MORTALITY AND INHIBITION OF *Helicoverpa zea* EGG PARASITISM RATES BY *Trichogramma* IN RELATION TO TRICHOME/METHYL KETONE-MEDIATED INSECT RESISTANCE OF *Lycopersicon hirsutum f. glabratum*, ACCESSION PI 134417

R.K. KASHYAP,[1] G.G. KENNEDY,[2,*] and R.R. FARRAR, JR.[2]

[1]*Department of Entomology*
Haryana Agricultural University
HISAR 125004, India

[2]*Department of Entomology*
North Carolina State University
Raleigh, North Carolina 27695-7630

(Received May 28, 1991; accepted August 5, 1991)

Abstract—The glandular trichome/methyl ketone-mediated insect resistance of the wild tomato, *Lycopersicon hirsutum f. glabratum* C.H. Mull, accession PI 134417, to *Manduca sexta* (L.) and *Leptinotarsa decemlineata* (Say) was shown to affect adversely *Trichogramma pretiosum* Riley. Adult *T. pretiosum* were killed by direct contact with PI 134417 foliage and by exposure to its volatiles. This effect was greatly reduced or eliminated by removing the exudate of the glandular trichomes from the foliage. 2-Tridecanone, a principal consituent of the foliar glandular trichomes of PI 134417, was toxic to adult *T. pretiosum* at concentrations similar to those associated with PI 134417 foliage. Incubation of parasitized *Helicoverpa (=Heliothis) zea* (Boddie) eggs on PI 134417 foliage or 2-tridecanone-treated filter paper significantly reduced the proportion of eggs producing adult parasitoids. Similarly, incubation of parasitized *H. zea* eggs on filter paper treated with 2-undecanone, another constituent of the glandular trichomes of PI 134417, caused an increase in the percentage of host eggs containing dead parasitoid pupae.

Key Words—*Lycopersicon hirsutum f. glabratum*, tomato, glandular trichomes, *Trichogramma pretiosum*, Hymenoptera, Trichogrammatidae, plant-insect interaction, 2-tridecanone, 2-undecanone.

*To whom correspondence should be addressed.

INTRODUCTION

Host plant resistance and biological control have often been cited as compatible tactics in pest management programs (Bergman and Tingey, 1979; Obrycki, 1986). Although host plant resistance is compatible with, and may enhance, biological control of insect pests in many situations, there are many situations in which they are not compatible. Recent research has identified a number of cases in which plant defense characters adversely affect organisms of the third trophic level (Campbell and Duffey, 1981; Duffey and Bloem, 1986; Obrycki, 1986; Price, 1986; van Emden, 1986). Indeed, adverse effects extending to organisms of the fourth trophic level have been demonstrated (Orr and Boethel, 1986). Thus, alteration of plant characters to achieve resistance to insect pests can affect the level of biological control of not only the resisted species, but also other pests not directly affected by the resistance (Kauffman and Kennedy, 1989a,c). Consequently, pest management programs using insect resistant varieties as one component must take into account possible adverse effects on biological control.

Plant resistance characters can affect natural enemies either directly, through contact with the resistant plant, or indirectly, through plant effects on the herbivores that render them more or less suitable as hosts or prey for their natural enemy. Among the parameters that cause direct effects, foliar morphology (Obrycki and Tauber, 1984; Keller, 1987) and plant volatiles (Altieri et al., 1981; Boethel and Eikenbary, 1986) are characteristics that have been shown to adversely affect predators and parasitoids. Perhaps the most consistent pattern to emerge from research on tritrophic level interactions is that high densities of foliar trichomes interfere with the effectiveness of small parasitic or predaceous arthropods (Obrycki, 1986; Kauffman and Kennedy, 1989b).

Foliage of the wild tomato, *Lycopersicon hirsutum f. glabratum* C.H. Mull, accession PI 134417, is characterized by a high density of Type VI (Luckwill, 1943) glandular trichomes, that mediate resistance to the tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae), and the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). Resistance to these insects is due largely to the presence of the 13-carbon methyl ketone, 2-tridecanone, in the tips of these trichomes (Kennedy and Yamamoto, 1979; Williams et al., 1980; Dimock and Kennedy, 1983; Kennedy and Dimock, 1983; Kennedy and Sorenson, 1985, Kennedy et al., 1985; Fery and Kennedy 1987; Kennedy and Farrar, 1987). The trichomes also confer a limited degree of resistance to the tomato fruitworm, *Helicoverpa* (=*Heliothis*) *zea* (Boddie) (Lepidoptera: Noctuidae), although higher levels of resistance to this pest are conferred by lamellar-based factors (Kennedy and Dimock, 1983; Dimock and Kennedy, 1983; Kennedy, 1986; Farrar and Kennedy, 1987a, 1991). The foliar trichomes of PI 134417 do contain quantities of 2-tridecanone that are poten-

tially lethal to *H. zea.* However, because of the means by which exposure to this chemical occurs (primarily through vapors) neonate *H. zea* larvae experience only a narcotizing dose, from which ca. 80% of them soon recover (Kennedy and Dimock, 1983; Kennedy, 1984; Kennedy et al., 1987). The 11-carbon methyl ketone, 2-undecanone, also found in type VI trichomes of PI 134417, is active against *H. zea* and several other pest species (Dimock et al., 1982; Farrar and Kennedy, 1987b, 1988; Lin et al., 1987). 2-Undecanone is both less abundant than 2-tridecanone in PI 134417 (Kauffman and Kennedy, 1989a,b) and less acutely toxic to *H. zea* (LD_{50} = 64.19 $\mu g/cm^2$ on filter paper vs. 17.05 $\mu g/cm^2$ for 2-tridecanone; Dimock et al., 1982). 2-Undecanone however, synergizes the activity of 2-tridecanone against neonate *H. zea*, and also causes high rates of pupal mortality in *H. zea* (Farrar and Kennedy, 1987b, 1988).

Kauffman and Kennedy (1989b) found that parasitism by *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) was a major cause of egg mortality for *H. zea* on cultivated tomato, *L. esculentum* Mill., in the field in North Carolina, but was significantly reduced on resistant PI 134417 plants. They also found reduced levels of parasitism on plant lines with elevated densities of glandular trichomes but no methyl ketones, indicating a likely inhibition of *Trichogramma* by physical effects of trichome densities. However, the lowest rates of parasitism were on PI 134417 and other lines with both methyl ketones and the highest trichome densities. Therefore, they could not determine whether this reduction in parasitism was due entirely to increased trichome density or if the methyl ketones were also involved. Recently, Kashyap et al. (1991) found that rates of movement of *T. pretiosum* Riley on PI 134417 were greatly reduced, and entanglement of wasps in trichome exudate greatly increased, compared to insect-susceptible plant lines. These findings suggest that physical effects of the trichomes could potentially explain all or most of the inhibition of *Trichogramma* on PI 134417 foliage. One implication of these findings is that it may be possible to reduce these effects in insect-resistant tomato cultivars by selecting for decreased trichome density but increased levels of 2-tridecanone per trichome.

Despite the evidence for physical effects of the glandular trichomes of PI 134417 on *Trichogramma*, there remains the potential for toxic or repellent effects of 2-tridecanone and 2-undecanone. Kauffman and Kennedy (unpublished data), using chemically treated filter paper, found an LC_{50} for 2-tridecanone to *T. pretiosum* of approximately one-tenth of the concentration found on PI 134417 foliage. There are, therefore, levels of 2-tridecanone in PI 134417 foliage that are potentially lethal to *Trichogramma*. A reduction in physical effects achieved by breeding for fewer trichomes but more 2-tridecanone per trichome could thus be negated by toxic or repellent effects of 2-tridecanone. This study was therefore undertaken to further evaluate the potential of 2-tridecanone and 2-undecanone to kill or repel *T. pretiosum*.

MATERIALS AND METHODS

H. zea eggs were obtained from a stock culture initiated from field-collected insects and maintained on semi-synthetic diet (Burton, 1970) for less than 1 year. *T. pretiosum* was obtained from a laboratory culture maintained on *H. zea* eggs. In all tests, 0- 24-hr-old *T. pretiosum* adults were used.

Foliage of PI 134417 and *L. esculentum* cv. "Better Boy" was obtained from plants raised in the greenhouse using a commercial soil mix (Metro Mix 220®). Plants were watered daily and fertilized weekly (Peters Professional® fertilizer, 20-20-20 (N-P-K) at 2.8 g/plant). Natural daylengths were supplemented to 16 hr by light from metal halide lamps.

Direct Effects of Foliage on Adult T. pretiosum. The effect of resistant PI 134417 foliage on the mortality of adult *T. pretiosum* was studied by confining parasitoid adults on foliage with the exudate of the glandular trichomes removed or with the trichomes intact. Young, fully expanded foliage was collected from 8- to 10-week-old plants. To remove the trichome exudate, the foliage was first gently wiped on both surfaces with a dry cotton ball, then with a cotton ball soaked in a 0.1% aqueous solution of Triton X-155® (a non-phytotoxic emulsifying agent), then rinsed, first in a 0.1% Triton X-155 solution and finally in clean water.

Leaflets were removed from the petiole and placed on moist filter paper (Fisher brand #8®) in the inverted lid of a 5.5 cm Petri dish. The dish was then placed in a 1.0-l glass jar. Adult *T. pretiosum* were transferred in shell vials from the culture, anesthetized with carbon dioxide, and counted under a stereomicroscope. After they revived, 25 wasps were released into each jar, which was then sealed with filter paper across the top. Jars were held horizontally. Each of the four treatments, i.e. (1) PI 134417 with intact trichomes, (2) PI 134417 with trichome exudate removed, (3) Better Boy, and (4) no foliage (filter paper only), was replicated ten times. Each replicate of each treatment was in a separate jar. Jars were held at 27°C and, after 4 hr, mortality of adult parasitoids was recorded. The data were normalized using an arcsine $\sqrt{\%}$ transformation and subjected to analysis of variance (ANOVA). The Least Significant Difference (LSD) was used to test for differences among treatment means.

Because 2-tridecanone vapors are associated with PI 134417 foliage (Dimock and Kennedy, 1983; Kennedy, 1984), a study was conducted to determine if foliar volatiles from PI 134417 caused mortality of *T. pretiosum*. A moist 5.5 cm filter paper disk was folded and placed in a 30-ml clear plastic cup. One leaflet was placed in the cup and the cup was covered with a piece of nylon organdy cloth (33 × 33 threads/cm). Twenty-five anesthetized *Trichogramma* adults were transferred quickly into another cup (a small amount of a 50% honey solution was wiped on the inside as a food source) which was then inverted over the organdy cloth. The cups were held together with Parafilm® to

avoid the loss of parasitoids, moisture, and leaf volatiles. Wasps were thus separated from the foliage by the organdy. The same four foliage treatments as above were included and were replicated ten times. The test was held at 27°C and the mortality of wasps was recorded at 4 hr and 24 hr. The data were analyzed as above.

Effects of Methyl Ketones on Immature Stages of Trichogramma *in Host Eggs.* Parasitized eggs were obtained by exposing *H. zea* eggs on cheesecloth to several hundred adult *T. pretiosum* for 24 hr. Eggs were then transferred with a small brush onto excised foliage. The foliage was then placed on moist filter paper in a Petri dish and the dish was sealed with Parafilm. The same foliage treatments as above were included. Each of the foliage treatments was replicated ten times with a replicate consisting of 25 parasitized eggs on an excised leaflet. All treatments were held at 27°C. Foliage was replaced with fresh foliage at 2- to 3-day intervals. On 8 and 12 days after the initiation of the test, the number of eggs from which adult *T. pretiosum* emerged was recorded. The experiment was repeated twice.

The effects of 2-tridecanone and 2-undecanone on survival of *T. pretiosum* within *H. zea* eggs were investigated in separate experiments. Parasitized *H. zea* eggs were obtained as above. Groups of 25 parasitized eggs were placed on chemically-treated 5.5-cm filter paper disks in Petri dishes. Treatments included 2-tridecanone (Fluka Chemical Corp., Hauppauge, New York 11788) at concentrations of 22.5, 45.0 or 90.0 $\mu g/cm^2$, or 2-undecanone (Pflatz and Bauer, Inc., Stamford, Connecticut 06902) at concentrations of 4.47, 8.94, or 17.88 $\mu g/cm^2$. The methyl ketones were applied to the filter paper disks as acetone solutions at a volume of 21 $\mu l/cm^2$ and the acetone allowed to evaporate. Controls, consisting of disks similarly treated with acetone only, were included in both experiments. The rates of 45 $\mu g/cm^2$ 2-tridecanone and 8.94 $\mu g/cm^2$ 2-undecanone roughly correspond to the mean levels associated with PI 134417 foliage (Kennedy, 1986).

Treated filter paper disks and parasitized eggs were placed in inverted 5.5-cm Petri dishes and the dishes covered and sealed with Parafilm. Filter papers were replaced with freshly treated papers every third day. After 12 days at 27°C, the number of parasitized *H. zea* eggs producing adult parasitoids, as evidenced by emergence holes, was recorded. No honey was placed in the dishes since it was unnecessary to maintain emerged parasitoids. Treatments were replicated ten times.

A second experiment involving 2-undecanone was conducted and was identical to the above experiment in every respect except that the host eggs were subjected to parasitism by exposing them to *T. pretiosum* adults for only 4 hr. This was done to minimize variation in the time the eggs were parasitized. After 12 days, all eggs were dissected and examined for the presence of dead parasitoid pupae. (Preliminary examinations indicated the presence of dead pupae

in 2-undecanone-treated eggs but not in 2-tridecanone-treated eggs.) Treatments were replicated five times. Data from both of these experiments were normalized by arcsine $\sqrt{\%}$ and analyzed by ANOVA for effects of methyl ketone concentration.

Effects of Methyl Ketone on Rates of Parasitization. The effects of 2-tridecanone on rates of parasitization were studied in both no-choice and choice experiments. In the no-choice experiment, unparasitized *H. zea* eggs were placed on filter paper disks treated with 2-tridecanone (0, 45.0 or 90.0 $\mu g/cm^2$). Groups of 50 eggs were placed with ca. 40 unsexed adults of *T. pretiosum* in 1-l glass jars as above. A small amount of honey was streaked on the side of the jar to provide food for the parasitoids.

After 4 hr, mortality of *T. pretiosum* adults was recorded and each group of *H. zea* eggs was randomly divided into two groups of 25. One group was transferred to filter paper treated with 2-tridecanone at the same concentration as that to which the initial group of 50 eggs was exposed; the other group was transferred to filter paper that had been previously treated only with acetone. The chemical treatments on which the groups of 25 eggs were held are hereafter referred to as post-treatments. This procedure allowed for comparison of long- and short-term exposure periods. Eggs were held at 27°C in covered Petri dishes sealed with Parafilm. Filter papers were replaced with freshly treated papers every 3 days. The proportion of eggs producing adult *T. pretiosum* was recorded after 8 days. This experiment was replicated ten times and the data were transformed to arcsine $\sqrt{\%}$ prior to factorial ANOVA. Because a significant interaction between initial treatment and post-treatment was found, post-treatment effects were also analyzed separately.

The choice experiment was conducted as above except that each glass jar contained both 25 unparasitized *H. zea* eggs on an acetone-treated filter paper disk and 25 unparasitized eggs on a disk treated with 2-tridecanone at one of four concentrations (22.5, 45.0, 67.5, or 90.0 $\mu g/cm^2$). After 4 hr, mortality of parasitoid adults was recorded and the *H. zea* eggs were placed individually in gelatin capsules (#2 Eli Lilly and Co., Indianapolis, Indiana 46285). The eggs were held at 27°C for 14 days, and, beginning at day 7, examined daily for parasitoid emergence. Only eggs from which parasitoids emerged were classified as parasitized.

The effects of 2-undecanone on parasitization were also examined in a choice test. The procedures were identical to those described for 2-tridecanone except that the concentrations of 2-undecanone tested were 0, 4.47, 8.94, 13.41, and 17.88 $\mu g/cm^2$.

In all experiments, percentage parasitization data were transformed to arcsine $\sqrt{\%}$ and subjected to ANOVA. Data on developmental times (days from parasitization to adult wasp emergence) were also subjected to ANOVA.

RESULTS

Direct Effects of Foliage on Adult T. pretiosum. Exposure of *T. pretiosum* adults to contact with PI 134417 foliage or its volatiles caused significant mortality within 4 hr (Table 1). In the experiment in which the wasps contacted foliage, removal of the exudate of the glandular trichomes resulted in a significant reduction in mortality, but did not completely eliminate the effect of PI 134417 foliage. In the experiment in which wasps were exposed only to foliar volatiles, there was no difference in mortality between PI 134417 foliage without trichome exudate and Better Boy foliage. However, in this experiment, mortality on PI 134417 foliage without trichome exudate was greater after 24 hr than that in the experimental control (no foliage). Although visual inspection of PI 134417 foliage indicated that virtually all of the exudate of the glandular trichomes was removed, it is possible that the elevated mortality observed was due to traces of trichome constituents that were not completely removed during the washing process. Type VI glandular trichomes in tomato do not regenerate exudate after they are ruptured.

Effects of Methyl Ketones on Immature Stages of Trichogramma *in Host Eggs*. Incubation of parasitized *H. zea* eggs on PI 134417 foliage with intact glandular trichomes resulted in a significant reduction in the percentage of eggs yielding adult parasitoids in both runs of the experiment. Removal of the trichome exudate eliminated this effect (Table 2).

TABLE 1. MORTALITY OF *T. pretiosum* ADULTS FOLLOWING DIRECT CONTACT WITH FOLIAGE OR EXPOSURE TO FOLIAR VOLATILES OF TOMATO GENOTYPES RESISTANT AND SUSCEPTIBLE TO *H. zea*

	(%) Mortality		
	Direct foliar contact	Foliar volatiles	
Treatment	4 hr[a]	4 hr[a]	24 hr[a]
L. hirsutum f. glabratum PI 134417			
Trichome exudate present	35.3 A	15.7 A	49.3 A
Trichome exudate removed	10.4 B	5.9 B	24.9 B
L. esculentum			
cv. Better Boy	5.1 C	4.6 B	20.0 BC
No foliage (filter paper)	3.4 C	3.6 B	13.0 C

[a] Means separation vertical; means with the same letter are not significantly different at $P \leq 0.05$ (LSD), $df = 3$.

TABLE 2. EMERGENCE OF *T. pretiosum* ADULTS FROM *H. zea* EGGS HELD ON RESISTANT AND SUSCEPTIBLE TOMATO FOLIAGE

	(%) Host eggs yielding parasitoid wasps			
	(Test I)[a] after		(Test II)[a] after	
Treatment	8 days[b]	12 days[b]	8 days[b]	12 days[b]
L. hirsutum f. glabratum PI 134417				
With trichome exudate present	36.6 A	55.8 A	14.5 A	47.4 A
With trichome exudate removed	67.9 B	83.5 B	38.7 B	75.2 B
L. esculentum				
Cv. Better Boy	72.8 B	85.8 B	50.1 B	79.6 B

[a] Test I—January, 1989; Test II—June, 1989. Eggs were on a neutral substrate when exposed for 24 hr to parasitization by adult T. *pretiosum*. Eggs were then transferred to foliage treatments for incubation.

[b] Means separation vertical; means with the same letter are not significantly different at $P \leq 0.05$ (LSD), $df = 2$.

In the experiments in which parasitized eggs were incubated on methyl ketone-treated filter paper, 2-tridecanone significantly reduced the percentage of host eggs yielding adult *T. pretiosum* (Table 3). However, the effect was seen only at the highest rate (90 $\mu g/cm^2$) tested, which represents twice the mean level commonly associated with PI 134417 foliage (Kennedy, 1986).

When eggs were subjected to parasitization by *T. pretiosum* for 24 hr and then incubated on 2-undecanone-treated filter paper, there was no significant effect on the percentage of host eggs yielding parasitoid adults (Table 3). In contrast, when eggs were subjected to parasitism for only 4 hr prior to incubation on a 2-undecanone-treated substrate, significant treatment effects were seen (Table 3). Fewer of these eggs produced one or more adult parasitoids, although the overall effect was not quite statistically significant ($F = 4.15$, $df = 1$, $P = 0.0533$). However, the effect on the percentage of eggs containing dead pupae was highly significant ($F = 15.13$, $df = 1$, $P = 0.0007$).

Effects of Methyl Ketones on Rates of Parasitization. In the no-choice test in which *H. zea* eggs on substrates treated with different concentrations of 2-tridecanone were exposed to parasitism by adult *T. pretiosum*, there was significantly greater mortality of *T. pretiosum* adults in the 2-tridecanone treatments than in the untreated controls (Table 4). There was a significant reduction in the percentage of eggs yielding adult parasitoids by the presence of 2-tridecanone during exposure of the eggs to the wasps. This effect was seen in both the 2-tridecanone and acetone only post-treatments. However, there was a highly significant treatment × post-treatment interaction (Table 4). This interaction

TABLE 3. EMERGENCE OF *T. pretiosum* ADULTS FROM HOST EGGS EXPOSED TO WASPS ON NEUTRAL SUBSTRATES AND INCUBATED ON METHYL KETONE TREATED SUBSTRATES

Treatment	Exposure time of eggs to parasitoids	Methyl ketone rate ($\mu g/cm^2$)	Host eggs yielding wasps (%)	Host eggs with dead parasitoid pupae (%)
2-Tridecanone	24 hr	0	77.8[a]	—
		22.5	65.8	—
		45.0	70.3	—
		90.0	0.0	—
2-Undecanone	24 hr	0	92.3[b]	—
		4.47	91.7	—
		8.94	89.4	—
		17.88	73.7	—
2-Undecanone	4 hr	0	80.8[c]	0.8[d]
		4.47	7.2	82.4
		8.94	37.6	52.8
		17.88	11.2	80.0

[a]For effect of 2-tridecanone rate, $B = -0.0121$, $df = 1$, $F = 138.64$, $P = 0.0001$.
[b]For effect of 2-undecanone rate, $B = -0.0054$, $df = 1$, $F = 0.31$, $P = 0.5800$.
[c]For effect of 2-undecanone rate, $B = 0.0875$, $df = 1$, $F = 4.15$, $P = 0.0533$.
[d]For effect of 2-undecanone rate, $B = -0.1540$, $df = 1$, $F = 15.13$, $P = 0.0007$.

appears attributable to a greater reduction in the percentage of eggs producing adult *T. pretiosum* when eggs were incubated on 2-tridecanone-treated filter paper than when they were incubated on acetone-treated filter paper, indicating that immature parasitoids exposed within the host egg to 2-tridecanone died prior to emergence from the egg. These results are consistent with those of the above experiment in which eggs were parasitized on a neutral substrate and then placed on 2-tridecanone-treated filter paper.

When *T. pretiosum* adults were given a choice between *H. zea* eggs on filter paper disks treated with 2-tridecanone or acetone, they parasitized significantly fewer eggs on 2-tridecanone-treated filter paper ($F = 9.61$, $df = 1$, $P = 0.0027$; Table 5). There was a significant positive relationship between mortality of adult *T. pretiosum* and the amount of 2-tridecanone on the treated disk in the choice arena. This increasing mortality is reflected in the reduced percent parasitism of *H. zea* eggs on both the 2-tridecanone- and the acetone-treated disks (Table 5). In this experiment, increasing concentrations of 2-tridecanone on the disk on which the eggs were exposed were associated with a small but statistically significant increase in the time of emergence of adult *T. pretiosum* ($F = 115.3$, $df = 1$, $P \leq 0.0001$; Table 6).

TABLE 4. EFFECT OF 2-TRIDECANONE ON *T. pretiosum* ADULT MORTALITY AND ON EMERGENCE OF *T. pretiosum* FROM PARASITIZED *H. zea* EGGS HELD IN THE PRESENCE OR ABSENCE OF VARIOUS CONCENTRATIONS OF 2-TRIDECANONE (POST-TREATMENT)

Pre and post-treatment 2-tridecanone conc. ($\mu g/cm^2$)	(%) Adult parasitoid mortality	(%) Eggs producing adult parasitoids on post-treatment[b, c]	
		Acetone	2-Tridecanone
0	26.4[d]	90.3[e]	83.8[f]
45	84.9	17.7	1.8
90	95.9	11.1	0.0

[a] Eggs exposed to parasitization by adults for 4 hr while on filter paper treated at the indicated concentrations of 2-tridecanone (pretreatment).
[b] Eggs from each pretreatment group were divided into two groups for incubation on acetone- or 2-tridecanone-treated filter paper. 2-Tridecanone post-treatment concentration was equal to pre-treatment concentration.
[c] For pre- and post-treatment interaction, $B = 0.0003$, $df = 1$, $F = 19.35$, $P = 0.0001$.
[d] For effect of 2-tridecanone concentration, $B = 0.0073$, $df = 1$, $F = 66.62$, $P = 0.0001$.
[e] For effect of 2-tridecanone pre-treatment rate, $B = -0.0242$, $df = 1$, $F = 22.42$, $P = 0.0001$.
[f] For effect on 2-tridecanone pre- and post-treatment rate, $B = 0.0133$, $df = 1$, $F = 84.26$, $P = 0.0001$.

TABLE 5. PARASITISM OF *H. zea* EGGS BY *T. pretiosum* ADULTS GIVEN A CHOICE BETWEEN EGGS ON 2-TRIDECANONE OR ACETONE-TREATED FILTER PAPER

Filter papers impregnated with 2-tridecanone at ($\mu g/cm^2$)	Wasp mortality (%)	(%) Parasitization of *H. zea* eggs on	
		2-Tridecanone	Acetone[a]
22.5	64	35.3	55.3
45.0[b]	72	46.0	59.5
67.5	75	28.9	45.0
90.0	85	28.1	43.2
F ($df = 1$)	8.13	6.26	11.47
$P > F$	0.0003	0.0168	0.0017

[a] Filter papers treated with acetone were held with 2-tridecanone treated filter papers in a 1-1 glass jar. Eggs were held on treated filter papers only during the 4 hr period of exposure to adult *T. pretiosum* after which they were transferred to gelatin capsules for incubation in the absence of 2-tridecanone.
[b] Plant rate (Dimock and Kennedy, 1983).

In a similar choice test involving eggs on 2-undecanone or acetone treated substrates, there were no significant differences in the percent parasitization of eggs on either substrate over the range of 2-undecanone concentrations tested, although there was a trend toward lower parasitization of eggs on substrates treated with higher concentrations of 2-undecanone (Table 7). There was, however, a significant positive relationship between wasp mortality and 2-undecanone concentration on the treated disk (Table 7). There was no effect of this

TABLE 6. DEVELOPMENTAL TIMES (TO ADULT EMERGENCE) OF *T. pretiosum* FROM *H. zea* EGGS EXPOSED FOR 4 HR TO PARASITIZATION ON FILTER PAPERS TREATED WITH 2-TRIDECANONE[a]

Rate ($\mu g/cm^2$)	Development time (days)
0.0	9.8
22.5	10.1
45.0	10.5
67.5	10.6
90.0	10.8
F ($df = 1$)	115.23
$P > F$	<.0001

[a] Eggs exposed to adult parasitoids for 4 hr after which they were removed from exposure to 2-tridecanone and incubated in gelatin capsules.

TABLE 7. PARASITISM OF *H. zea* EGGS BY *T. pretiosum* ADULTS GIVEN A CHOICE BETWEEN EGGS ON 2-UNDECANONE OR ACETONE-TREATED FILTER PAPER

Filter papers impregnated with 2-undecanone at ($\mu g/cm^2$)	Wasp mortality (%)	(%) Eggs, producing adult *T. pretiosum* on	
		2-Undecanone	Acetone[a]
4.47	38	61.8	66.3
8.94[b]	49	58.6	61.0
13.41	49	46.1	61.7
17.88	66	49.2	60.8
F value ($df = 1$)	13.81	1.55	0.48
$P > F$	0.0006	0.2233	0.4945

[a] Filter papers treated with acetone were held with 2-undecanone treated filter papers in a glass jar (1.0 1). Eggs were held on treated filter papers only during the 4 h period of exposure to adult *T. pretiosum* after which they were transferred to gelatin capsules for incubation.
[b] Plant rate (Dimock and Kennedy, 1983).

brief exposure (up to 4 hr) to 2-undecanone on development time of *T. pretiosum* ($P \geq 0.05$).

DISCUSSION

These results suggest at least a potential role for methyl ketones in the reduction of parasitism of *Heliothis* eggs on PI 134417 by *Trichogramma* spp., including *T. pretiosum*, as previously reported by Kauffman and Kennedy (1989b). Adult *T. pretiosum* contacting PI 134417 foliage or exposed to foliar volatiles of PI 134417 suffered elevated levels of mortality, an effect that was greatly reduced or eliminated by removing the glandular trichomes from the foliage. The effect of foliar volatiles is a strong indication of effects of the methyl ketones beyond physical effects of the trichomes.

In addition to effects on adult *T. pretiosum*, incubation of parasitized eggs on PI 134417 foliage resulted in fewer eggs producing parasitoid adults, apparently because of mortality of immature parasitoids within the eggs. This mortality did not occur when parasitized eggs were incubated on PI 134417 foliage from which the glandular trichomes had been removed or on *L. esculentum* foliage. These effects are also likely attributable to the methyl ketones, although it is possible that physical coating of the eggs with trichome exudate could also be a factor.

Effects similar to those associated with PI 134417 foliage were caused by methyl ketone-treated filter paper. 2-Tridecanone at concentrations commonly found associated with PI 134417 foliage caused mortality of adult *T. pretiosum* and immature *T. pretiosum* within the eggs. 2-Undecanone at rates comparable to those in PI 134417 also caused significant mortality among adult *T. pretiosum* and among developing parasitoids within host eggs. However, significant effects of 2-undecanone on immature parasitoids were seen only when host eggs were exposed to 2-undecanone following a 4-hr exposure, on a neutral substrate, to adult parasitoids, not after 24-hr exposure (Table 3). This difference may indicate that *T. pretiosum* is sensitive to 2-undecanone during its early stages of development. By way of comparison, Farrar and Kennedy (1987b, 1988) found that *H. zea* is sensitive to the toxic effects of low concentrations of 2-undecanone only if it is exposed to the chemical during the fifth larval instar. In the case of *H. zea*, the toxic effects of 2-undecanone are manifest as mortality during the larval/pupal molt and the pupal stage. The presence of dead *T. pretiosum* pupae in host eggs held on 2-undecanone-treated filter paper (Table 3) may indicate a similar pupal toxicity in *T. pretiosum*. The similarity of effects of chemically treated filter paper with those of foliage is further evidence of the potential of the methyl ketones in PI 134417 foliage to adversely affect *T. pretiosum*.

There is thus the potential for 2-tridecanone, and, to a lesser extent, 2-undecanone, in the foliage of PI 134417 to adversely affect *T. pretiosum*. The findings of Kashyap et al. (1991) indicate that, on intact foliage, these effects may be largely masked by physical effects such as inhibition of movement or entanglement of wasps. It may be possible to reduce physical effects of resistant cultivars by selecting for fewer trichomes per unit area but more 2-tridecanone per trichome. However, given the potential effects of 2-tridecanone on *T. pretiosum*, this approach would probably be of limited value.

These findings further substantiate previous findings that the methyl ketone-based resistance of PI 134417 can adversely affect organisms of the third trophic level. In the case of *H. zea*, most of the resistance factors are in the leaf lamella and are not related to methyl ketones (Farrar and Kennedy, 1987a, 1991). In cultivars with methyl ketone-based resistance, biological control of *H. zea* could thus be disrupted, so that in localities where *H. zea* is a major pest, it would thus be necessary to use cultivars with *H. zea* resistance as well. These findings should aid both in understanding the nature of tritrophic level interactions and in the successful integration of host plant resistance and biological control into pest management programs.

Acknowledgments—Supported in part by U.S.D.A. Competitive Grants Program in Biological Stress under Grant No. 88-37252-4017 and the North Carolina Agricultural Research Service. Use of trade names does not imply endorsement of products named nor criticism of similar ones not mentioned. We thank M. Woodall for typing, D. Bumgarner and V.B. Covington for technical assistance, and Drs. J.R. Bradley, Jr., J.W. Van Duyn, and J.F. Walgenbach for comments.

REFERENCES

ALTIERI, M.A., LEWIS, W.J., NORDLUND, D.A., GUELDNER, R.C., and TODD, W.J. 1981. Chemical interactions between plants and *Trichogramma* wasps in Georgia soybean fields. *Prot. Ecol.* 3:259–263.

BERGMAN, J.M., and TINGEY, W.M. 1979. Aspects of interaction between plant genotypes and biological control. *Bull. Entomol. Soc. Am.* 25:275–279.

BOETHEL, D.J., and EIKENBARY, R.D. (eds.). 1986. Interactions of Plant Resistance and Parasitoids and Predators of Insects. Ellis Horwood Ltd., Chichester.

BURTON, R.L. 1970. A low-cost artificial diet for the corn earworm. *J. Econ. Entomol.* 63:1969–1970.

CAMPBELL, B.C., and DUFFEY, S.S. 1981. Alleviation of α-tomatine-induced toxicity to the parasitoid, *Hyposoter exiguae*, by phytosterols in the diet of the host, *Heliothis zea*. *J. Chem. Ecol.* 7(6):927–946.

DIMOCK, M.B., and KENNEDY, G.G. 1983. The role of glandular trichomes in the resistance of *Lycopersicon hirsutum f. glabratum* to *Heliothis zea*. *Entomol. Exp. Appl.* 33:263–268.

DIMOCK, M.B., KENNEDY, G.G., and WILLIAMS, W.G. 1982. Toxicity studies of analogs of 2-tridecanone, a naturally-occurring toxicant from a wild tomato. *J. Chem. Ecol.* 8:837–842.

DUFFEY, S.S., and BLOEM, K.A. 1986. Plant defense-herbivore-parasite interactions and biological control, pp. 135–183, *in* M. Kogan (ed.). Ecological Theory and Integrated Pest Management Practice. Wiley, New York.

FARRAR, R.R., JR., and KENNEDY, G.G. 1987a. Growth, food consumption and mortality of *Heliothis zea* larvae on the foliage of the wild tomato *Lycopersicon hirsutum f. glabratum* and the cultivated tomato, *L. esculentum*. *Entomol. Exp. Appl.* 44:213–219.

FARRAR, R.R., JR., and KENNEDY, G.G. 1987b. 2-Undecanone, a constituent of the glandular trihcomes of *Lycopersicon hirsutum f. glabratum*: Effects on *Heliothis zea* and *Manduca sexta* growth and survival. *Entomol. Exp. Appl.* 43:17–23.

FARRAR, R.R., JR., and KENNEDY, G.G. 1988. 2-Undecanone, a pupal mortality factor in *Heliothis zea*: Sensitive larval stage and in planta activity in *Lycopersicon hirsutum f. glabratum*. *Entomol. Exp. Appl.* 47:205–210.

FARRAR, R.R., JR., and KENNEDY, G.G. 1991. Relationship of leaf lamellar-based resistance to *Leptinotarsa decemlineata* and *Heliothis zea* in a wild tomato, *Lycopersicon hirsutum f. glabratum*, PI 134417. *Entomol. Exp. Appl.* 58:61–67.

FERY, R.L., and KENNEDY, G.G. 1987. Genetic analysis of 2-tridecanone concentration, leaf trichome characteristics, and tobacco hornworm resistance in tomato. *J. Am. Hort. Soc.* 112:886–891.

KASHYAP, R.K., KENNEDY, G.G., and FARRAR, R.R., JR. 1991. Behavioral response of *Trichogramma pretiosum* Riley and *Telenomus sphingis* (Ashmead) to trichome/methyl ketone mediated resistance in tomato. *J. Chem. Ecol.* 17:543–556.

KAUFFMAN, W.C., and KENNEDY, G.G. 1989a. Inhibition of *Campoletis sonorensis* parasitism of *Heliothis zea* and of parasitoid development by 2-tridecanone-mediated insect resistance of wild tomato. *J. Chem. Ecol.* 15:1919–1930.

KAUFFMAN, W.C., and KENNEDY, G.G. 1989b. Relationship between trichome density in tomato and parasitism of *Heliothis* spp. (Lepidoptera: Noctuidae) eggs by *Trichogramma* spp. (Hymenoptera: Trichogrammatidae). *Environ. Entomol.* 18(4):698–704.

KAUFFMAN, W.C., and KENNEDY, G.G. 1989c. Toxicity of allelochemicals from wild insect-resistant tomato *Lycopersicon hirsutum f. glabratum* to *Campoletis sonorensis* a parasitoid of *Heliothis zea*. *J. Chem. Ecol.* 15:2051–2060.

KELLER, M. 1987. Influence of leaf surface on movement by the hymenopterous parasitoid, *Trichogramma exiguum*. *Entomol. Exp. Appl.* 43:55–59.

KENNEDY, G.G. 1984. 2-Tridecanone, tomatoes and *Heliothis zea*: Potential incompatability of plant antibiosis with insecticidal control. *Entomol. Exp. Appl.* 35:305–311.

KENNEDY, G.G. 1986. Consequences of modifying biochemically mediated insect resistance in *Lycopersicon* species, pp. 130–141, *in* M.R. Green and P.A. Hedin (eds.). Natural Resistance of Plants to Pest-Roles of Allelochemicals. ACS Symposium Series 296, Washington, D.C. 243 pp.

KENNEDY, G.G., and DIMOCK, M.B. 1983. 2-Tridecanone: A natural toxicant in a wild tomato responsible for insect resistance, pp. 123–128, *in* J. Miyamoto and P.C. Kearney (eds.). Pesticides Chemistry, Human Welfare and the Environment, Vol. 2. Pergamon Press, Tokyo.

KENNEDY, G.G., and FARRAR, R.R., JR. 1987. Response of insecticide-resistant and susceptible Colorado potato beetles, *Leptinotarsa decemlineata* to 2-tridecanone and resistant foliage: The absence of cross resistance. *Entomol. Exp. Appl.* 45:187–192.

KENNEDY, G.G., and SORENSON, C.E. 1985. Role of glandular trichomes in the resistance of *Lycopersicon hirsutum f. glabratum* to Colorado potato beetle (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 78:547–551.

KENNEDY, G.G., and YAMAMOTO, R.T. 1979. A toxic factor causing resistance in a wild tomato to the tobacco hornworm and some other insects. *Entomol. Exp. Appl.* 26:121–126.

KENNEDY, G.G., SORENSON, C.E., and FERY, R.L. 1985. Mechanisms of resistance to Colorado potato beetle in tomato, pp. 107–116, *in* D.N. Ferro and R.H. Voss (eds.). Proceedings of the Symposium on the Colorado Potato Beetle, XVII International Congress of Entomology. Mass. Agric. Expt. Stn. Res. Bull. No. 704. 144 pp.

KENNEDY, G.G., FARRAR, R.R., JR., and RISKALLAH, M.R. 1987. Induced tolerance in *Heliothis zea* to host plant allelochemicals and carbaryl following incubation of eggs on foliage of *Lycopersicon hirsutum f. glabratum*. *Oecologia* (Berlin) 73:615–620.

LIN, S.Y.H., TRUMBLE, J.T., and KUMAMOTO, J. 1987. Activity of volatile compounds in glandular trichomes of *Lycopersicon* species against two insect herbivores. *J. Chem. Ecol.* 13:837–850.

LUCKWILL, L.C. 1943. The Genus *Lycopersicon*: An Historical, Biological, and Taxonomic Survey of the Wild and Cultivated Tomatoes. Aberdeen Univ. Studies No. 120, Aberdeen Univ. Press, Aberdeen, Scotland.

OBRYCKI, J.J. 1986. The influence of foliar pubescence on entomophagous species, pp. 61–83, *in* D. Boethal and R.D. Eikenbary (eds.). Interactions of Plant Resistance and Parasitoids and Predators of Insects. Wiley and Sons, New York.

OBRYCKI, J.J., and TAUBER, M.J. 1984. Natural enemy activity on glandular pubescent potato plants in the greenhouse: An unreliable predictor of effects in the field. *Environ. Entomol.* 13:679–683.

ORR, D.B., and BOETHEL, D.J. 1986. Influence of plant antibiosis through four trophic levels. *Oecologia* (Berlin) 70:242–249.

PRICE, P.W. 1986. Ecological aspects of host plant resistance and biological control: Interactions among three trophic levels, pp. 11–30, *in* D.J. Boethel and R.D. Eikenbary (eds.). Interactions of Plant Resistance and Parasitoids and Predators of Insects. Ellis Horwood Ltd., Chichester.

VAN EMDEN, H.V. 1986. The interaction of plant resistance and natural enemies: Effects on populations of sucking insects, pp. 138–150, *in* D.J. Boethel and R.D. Eikenbary (eds.). Interactions of Plant Resistance and Parasitoids and Predators of Insects. Ellis Horwood Ltd., Chichester.

WILLIAMS, W.G., KENNEDY, G.G., YAMAMOTO, R.T., THACKER, J.D., and BORDNER, J. 1980. 2-Tridecanone: A naturally-occurring insecticide from the wild tomato *Lycopersicon hirsutum f. glabratum*. *Science* 207:888–889.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

INTERSPECIFIC RECOGNITION AMONG TERMITES OF THE GENUS *Reticulitermes*: EVIDENCE FOR A ROLE FOR THE CUTICULAR HYDROCARBONS

ANNE-GENEVIEVE BAGNERES,[1,*] ARMAND KILLIAN,[1] JEAN-LUC CLEMENT,[1] and CATHERINE LANGE[2]

[1]*CNRS-LNB8*
31, Chemin J. Aiguier
13402 Marseille Cedex 09, France

[2]*U.P.M.C., Lab. Chimie Org. Structurale*
4, Place Jussieu
75005 Paris, France

(Received April 18, 1991; accepted August 5, 1991)

Abstract—Two species of termites, *Reticulitermes* (*lucifugus*) *grassei* and *R.* (*l.*) *banyulensis*, show a high degree of aggressivity toward each other. The epicuticular signature, recognized by contact, can be extracted using organic solvents, and the removal of the signature abolished all types of aggressive behavior. The signature can be transferred to lures, where it triggers interspecies aggression. It was found to be mainly present in the apolar fraction of the cuticular extracts, which contained only hydrocarbons, are determined by GC/MS techniques. Chemical recognition contributes towards isolation of the two species belonging to the *R. lucifugus* complex.

Key Words—Interspecific recognition, Isoptera, termites, *Reticulitermes*, cuticular hydrocarbons, aggression.

INTRODUCTION

In 1903, A.M. Fielde noticed that recognition between insects, such as ants, required antennal contact, and suggested that a system of chemical recognition (Fielde, 1904), and a "specific and progressive" odor exists (Fielde, 1905). Clément (1981a, 1982) described the sequences of contacts among termites

*To whom correspondence should be addressed.

0098-0331/91/1200-2397$06.50/0

Reticulitermes which release aggression, and proposed that substances present on the cuticle were involved.

The cuticle of many insects has been described in detail by Wigglesworth (1933, 1990), and the cuticular lipids have been the subject of numerous reviews (Blomquist and Jackson, 1976; Howard and Blomquist, 1982; Lockey, 1988). They describe the exoskeleton of insects as being covered in a thin layer of lipids, consisting mostly of hydrocarbons, which can be extracted using an organic solvent and separated with chromatographic methods.

These hydrocarbons, as well as serving to protect the insects against dessication, toxins of predators or invasion by microorganisms, play a part in interspecific and intraspecific recognition (Blomquist et al., 1987). They were described in terms of chemical signature among social insects by Howard and his colleagues in 1982 (1982b). Bonavita-Cougourdan et al. (1987) have demonstrated their role in recognition between individuals of *Camponotus vagus* (Scop.). Subsequent studies by Morel et al. (1988) on *Camponotus floridanus* (Buckley), and by Nowbahari et al. (1990) on *Cataglyphis cursor* (Fonsc.), have confirmed this intraspecific function among other ants.

Howard et al. (1980b, 1982a) have shown that some species of termitophiles, such as the staphylinids (*Trichopsenius frosti* Seevers, *T. depressus*, *Philotermes howardi*, etc.) which are associated with termites (*R. flavipes* Kollar and *R. virginicus* Banks) chemically mimic the cuticle of their hosts. Howard et al. (1990a) have also observed a chemical mimicry of the ant *Camponotus modoc* (Wheeler) by a dipteran parasite (*Microdon albicomatus* Novak). Recently, Howard et al. (1990b) have also proved a synthesis *de novo* of hydrocarbons by the same parasite, who mimics another ant, *Myrmica incompleta* (Prov.).

Cuticular signals are used as "camouflage" in order to penetrate termite or ant colonies with impunity. This "exceptional similarity" has also been noted among ant inquilines by Franks et al. (1990).

Espelie and Hermann (1988) have noted another example of chemical convergence between a wasp (*Parachartergus aztecus* Willink), a species of parasitic ant (*Pseudomyrmex ferrugineus* Smith) and their host plant (*Acacia collinsii* Safford). Espelie et al. (1990) have likewise observed a similarity of the cuticular hydrocarbons of a wasp (*Polistes metricus* Say) and the walls and pedicel of its nest.

Obin (1986) conducted laboratory tests on the ability of *Solenopsis invicta* (Buren) ants to distinguish between a familiar and foreign signal, but the behavioral data were not very convincing. Among these Hymenoptera, the glandular signals are very strong and may have masked the cuticular signals or be added to the cuticular hydrocarbons. The studies by Jaffe (1980) suggest that the volatile glandular secretions of *Atta cephalotes* (L.) may play an important part in the recognition mechanism. Today, many authors agree as to the presence on

the cuticle of a signal for specific, colonial, and individual recognition (Blum, 1987; Hölldobler and Carlin, 1987).

Haverty and Thorne (1989), working with different phenotypes of *Zootermopsis* (Isoptera, Termopsidae), noted a correlation between aggressive behavior and cuticular phenotypes, which has led to the separation of two subspecies of *Z. nevadensis* (Hagen), namely *Z. (n.) nevadensis* and *Z. (n.) nuttingi*. With the exception of the work of Howard et al. (1982b), Bonavita-Cougourdan et al. (1987), Morel et al. (1988), and Nowbahari et al. (1990), however, there has been little experimental proof that cuticular extracts are really active in recognition between individuals. On the other hand, these organic extracts can contain polar compounds (ethers, alcohols) and other substances not suitable for gas chromatography (steroids, amino-acids, sugars) which accompany the hydrocarbons when placed on a lure. The volatile secretions of non-cuticular glands may also be spread all over the cuticule (Howse, 1975; Blum, 1987).

The aim of this study was to examine the chemical signals responsible for recognition between the two species of the super-species *Reticulitermes lucifugus* (Rossi). These two species are at the two extremes of a circular cline in Europe (Clément, 1981b), and show aggressive behavior toward each other (Clément, 1978). *Reticulitermes (lucifugus) grassei* (Clément) inhabits southwest France, *R. (l.) banyulensis* (Cl.) southeast France; they are genetically and physiologically isolated (Clément, 1981b).

In order to understand this interspecific aggression, an ethological and chemical study was carried out, using on the one hand, various behavioral tests with living or dead individuals, and lures covered with different organic extracts separated into fractions of different polarities, and, on the other hand, chemical determination of the extracts carrying the specific signature of the termites.

METHODS AND MATERIALS

Biological Material. Colonies of *R. (l.) grassei* were collected near La Rochelle (Forêt de la Coubre, Ile d'Oléron, Charente Maritime, France), in 1986, 1987, and 1988, and colonies of *R. (l.) banyulensis* were collected near Perpignan (Banyuls, Pyrénées Orientales, France), in 1987, 1988, and 1989.

Behavioral Tests. Two series of tests were carried out. The frequencies of eight defined actions (behavioral items) were quantified and the median for each item calculated from ten replicates. At the end of each test the percentage occurrence of each of the eight actions was calculated in relation to the total number of actions. These actions were either antennal (antenna to antenna, antenna to head, antenna to body), palpating (palps to head, palps to body), the number of openings of the mandibles, the number of bites, and the number of trembling

actions. In each case, the confrontations were between a stranger introduced into an arena with 30 resident workers, randomly chosen. For each nest used in the experiment, the level of the aggressive behavior between species was quantified, using the threshold described by Clément in 1986.

Each test was followed by a control test in which a worker of the same species was introduced instead of the stranger. The worker termites were selected from the same advanced larval stage (stage 7 or 8).

In the lure experiment, the chemical extract came from the same colony as the individuals used to control the level of aggression of the resident colony.

The arena consisted of a Petri dish repeatedly washed with acetone in which was placed a filter paper kept humid. The 30 residents were placed in the arena 24 hr before the test, which was the time calculated as being necessary for them to become accustomed to it.

Series 1: Introduction of a Stranger Living, Dead, or Dead and Washed

Living individuals of the stranger species were dyed blue for the first series of tests. This was done by placing part of the colony in a dish containing a filter paper impregnated with a solution of Cresyl-blue in distilled water. The intestines of the termites become stained through their licking and eating some of the paper, and since their cuticle is transparent, the color can be easily seen. This dying technique was described by Goldberg (1971) and used by Guille-Escuret (1981), but with Neutral-red which is too similar to the natural rosy color of the termites. Preliminary tests showed that on being introduced into a group from its own colony, one of these stained individuals is totally accepted and slowly loses its coloring. The various colonies used in the test have the same level of aggressive behavior according to the threshold described previously (Clément, 1986). The stain, therefore, has no effect upon the termites' behavior. The observation period with live individuals was 5 min, because the exchanges are extremely rapid.

Tests were carried out with dead individuals, asphyxiated by nitrogen or carbon dioxide. The observation time for this test was 15 min. The dead individual was placed at the center of the arena.

Tests were also carried out with individuals killed as above. They were then rinsed in three baths of organic solvent (1 ml each) for 5 min.

The observation period was again 15 min. A preliminary study was carried out to determine the type of solvent to be used, the most appropriate extraction time, the number of individuals to be used for extraction purposes, and the amount of solvent required (Bagnères, 1989). The most suitable solvent was found to be pentane, a non-polar solvent which nevertheless extracts other more polar substances, such as fatty acids, in addition to the hydrocarbons. This solvent was then used for the washing experiments and for the following series in which extracts were obtained and deposited on another insect.

Three kinds of tests were carried out each time in both directions: with one *R.* (*l.*) *grassei* and 30 *R.* (*l.*) *banyulensis* (with one *R.* (*l.*) *banyulensis* as control), and with one *R.* (*l.*) *banyulensis* and 30 *R.* (*l.*) *grassei* (using one *R.* (*l.*) *grassei* as control). The 12 replicate tests described above (six for each species: three tests + three controls) were repeated ten times.

Series 2

In a second series of tests, a stranger was asphyxiated, washed, and dehydrated (by critical point drying/CO_2 at 72.9 atm. pressure, 31.2°C). This technique was used to avoid the problem of brittleness of the body after immersion in a solvent and was first used by Howard et al. (1982b) and then readapted by us for this study. Control chromatograms were obtained to check that all the signals from the cuticle had disappeared when a typical extract was produced. The critical-point-dried (CPD) lures (from *R.* (*l.*) *grassei* and *R.* (*l.*) *banyulensis*) were kept in a dry atmosphere in a container with Actigel (silicic acid). A single lure was used only once and received either no treatment (blank) or one kind of treatment, namely either pentane (10 μl) (true control) or a pentane extract (10 μl) of one or the other species, so that each lure was covered with the equivalent of an extract from five workers, since we calculated that in the recovery of this extract about 4/5 of the total was lost (Killian, 1989). These extracts were either total pentane extracts, or the polar fraction, or the non-polar fraction of the total extract of each species (*R.* (*l.*) *grassei* and *R.* (*l.*) *banyulensis*). The nature of these extracts is described in the section on chemical analysis. The deposition of the extract was made with a Hamilton 10 μl syringe. Each test was repeated about ten times.

Statistical Analysis. Various statistical analyses were carried out using a Statgraphics software (version 3.0). The first series of summary statistics was performed to calculate the medians of percentages together with the lower quartiles and the upper quartiles. The fit with the normal distribution was calculated for each set. To compare the various medians, the Mann–Whitney non-parametric test was chosen.

Chemical Analysis. Groups of 100 workers at the same stage were extracted with pentane (2 ml) for 5 min to obtain the extract for the ethological tests. The extracts were adjusted to a volume of 1 ml before fractionation. The extracts were either used as such when deposited on a lure or separated into fractions. Silica Sep-pak (Millipore, Waters Ass.) were used as microcolumns. The non-polar fraction was eluted first with pentane, followed by a further pentane washing, and then the polar fraction was eluted with methanol.

For the identification and quantification of the chemicals in the total extract, in both species, individual workers were counted and weighed, and 35 extracts of 100 workers each were made with each of the two species. Ten portions of

100 μl were made from each extract, to which were added 100 μl of a solution of an internal standard (800 ng of heneicosane, nC21).

The extracts were analyzed by gas chromatography (GC) on a Delsi 300 instrument equipped with a flame ionization detector (FID), a split/splitless injector (maintained splitless for 15 sec after injection), and a capillary column (25 m $\times$ 0.35 mm) coated with a 0.12 μm film of CP Si15. The carrier gas was helium at 1 bar. The temperature was isothermal at 150°C for 5 min and then programmed at 5°C/min to 320°C. The results were collected in an Enica 21 integrator.

The double-bond position in alkenes was determined by the methoxymercuration-demercuration method (Plattner et al., 1976; Blomquist et al., 1980).

Thin layer chromatography was carried out on silica impregnated with 10% silver nitrate, and developed with benzene:hexane (8:92) to separate alkanes and alkenes (Howard et al., 1978).

Compounds were identified by coupled gas chromatography-mass spectrometry (GC-MS). An identical chromatograph to that described above was coupled to a Nermag R1010C quadrupole mass spectrometer, and the data fed into an IBM PC computer using Spectral 30 software. Analysis by electron impact (at 70 ev) was followed by examination under chemical ionization using methane (0.1 Torr, 100 ev). Equivalent chain length (ECL) is calculated for each compound, using a mixture of internal standards from C20 to C38 coinjected with the total extract, and data compared with Howard et al. (1978, 1980a), Lockey (1988, 1991), Lockey and Metcalfe, (1988), Nelson et al. (1988) and Pomonis et al. (1989).

The quantification of each substance (absolute quantity and percentage of the total) was performed using a Lotus software in which the area of each peak was corrected with a response coefficient K for the FID detector (see Bagnères et al., 1990) where $K = 0.042 \times nC + 0.11$ (nC is the number of carbon atoms per molecule of the substance).

RESULTS OF TEST 1

Introduction of a Living Stranger (Table 1)

When two results on the same line have different letters beside them, the difference between them is statistically significant at least at the 95% significance level according to the Mann–Whitney test. If, on the other hand, two results on the same line have one or more letters in common beside them, the difference is not significant at the level chosen. It can be seen from these results that when a stranger-dyed individual was introduced into the group, the last five items described, but particularly the biting, increased greatly, and therefore provided the best index to measure the aggression level. The sequence occurred

TABLE 1. PERCENTAGE OCCURRENCE OF THE EIGHT BEHAVIORAL ITEMS PERFORMED IN AN ARENA CONTAINING EITHER 30 *Reticulitermes (lucifugus) banyulensis* (*R. (l.).b.*), OR 30 *R. (l.) grassei* (*R. (l.).g.*) TOWARDS A LIVING INTRUDER (LIVING), BELONGING TO EITHER THE SAME (CONTROL) OR THE OPPOSITE SPECIES[a]

	30 *R.(l.)b.*		30 *R.(l.)g.*	
	1 *R.(l.)b.* living (control)	1 *R.(l.)g.* living	1 *R.(l.)g.* living (control)	1 *R.(l.)b.* living
Ant./ant.	22 a (14-30)	6 c (4-6)	22 a (14-29)	14 abc (5-28)
Ant./head	15 b (20-33)	15.5 b (23-31)	24 abc (18-20)	11 d (9-17)
Ant./body	54 a (49-62)	38 c (30-43)	52 a (47-59)	52 a (45-63)
Palp./body	0 c (0-0)	4 b (2-7)	0 c (0-0)	2 b (0-9)
Pal./head	0 c (0-0)	1.5 b (0-4)	0 b (0-0)	1 a (0-3)
Open.mand.	0 b (0-0)	6 a (2-7)	0 b (0-0)	2 a (0-3)
Biting	0 c (0-0)	30 a (3-47)	0 c (0-0)	20 b (11-34)
Trembling	0 bc (0-1)	0 c (0-1)	0 a (0-0)	0 a (0-2)

[a] The percentages are the median number of items of each kind. A letter occurring more than once on the same line means that the difference was not significant (Mann–Whitney test, $p < 5\%$). Numbers in parentheses refer to quartiles (lower and upper).

very rapidly, and nine times out of ten, it ended in the death of the introduced stranger, whereas the control-dyed (from the same colony) individual in both cases was incorporated into the group immediately.

When the arena was filled with a group of *R. (l.) banyulensis* workers, they displayed a number of activities and reacted more violently than a group of *R. (l.) grassei* workers (30 biting items giving a highly significant difference between control and stranger of $p < 7 \times 10^{-5}$; 6 openings of mandibles, significantly different at $p < 3 \times 10^{-4}$). When the arena was filled with *R. (l.) grassei* workers, the difference between the scores was equally significant, but this species seems to be a little less aggressive. The experimental conditions did not affect the natural aggressive behavior between the two species.

Introduction of a Dead Stranger (Table 2)

Upon introducing a dead stranger, the same aggressive behavior was observed, but with less intensity than with the living stranger. When the arena was filled with *R.* (*l.*) *banyulensis*, the wounding score was reduced to 18, compared with 30 (above) when alive, but this was still significantly different from the controls at $p < 2 \times 10^{-4}$, and the mandible opening score dropped from 6 (above) to 2. Because the lure was immobile, the duration of the test was prolonged (5 to 15 min), so that more contacts could take place. The chemical signal which triggered aggression was therefore not due to any activities of

TABLE 2. PERCENTAGE OCCURRENCE OF THE EIGHT BEHAVIORAL ITEMS PERFORMED IN AN ARENA CONTAINING EITHER 30 *Reticulitermes* (*lucifugus*) *banyulensis* (*R.* (*l.*).*b.*), OR 30 *R.* (*l.*) *grassei* (*R.* (*l.*).*g.*) TOWARDS A DEAD INDIVIDUAL (DEAD), BELONGING TO EITHER THE SAME (CONTROL) OR THE OPPOSITE SPECIES[a]

	30 *R.*(*l.*)*b.*		30 *R.*(*l.*)*g.*	
	1 *R.*(*l.*)*b.* dead (control)	1 *R.*(*l.*)*g.* dead	1 *R.*(*l.*)*g.* dead (control)	1 *R.*(*l.*)*b.* dead
Ant./ant.	10 b (10-12)	1 d (0-3)	12 bc (9-15)	4 c (2-10)
Ant./head	14 b (9-20)	11 b (8-9)	21 c (16-23)	17.5 bcd (13-28)
Ant./body	49 ab (45-52)	42 c (39-42)	43 ab (41-51)	40.5 b (33-43)
Palp./body	19 a (11-24)	15 a (13-16)	14 a (10-15)	12 a (5-17)
Palp./head	4 a (2-4)	5.5 a (3-8)	2.5 a (0-5)	6 a (0-8)
Open.mand.	1 b (0-1)	2 a (1-4)	0 b (0-0)	2 a (0-3)
Biting	0 c (0-2)	18 b (17-21)	0 c (0-0)	9 a (0-15)
Trembling	2 b (0-2)	3.5 a (3-6)	0 a (0-1)	0 a (0-1)

[a] The percentages are the median number of items of each kind. A letter occurring more than once on the same line means that the difference was not significant (Mann-Whitney test, $p < 5\%$). Numbers in parentheses refer to quartiles (lower and upper).

the stranger, but the signal lost some of its strength because the dead lure was not moving among the group in the arena and did not actively elicit aggression.

Introduction of an Individual Washed with Pentane (Table 3)

An extracted termite produced very little aggressiveness (seven biting items observed in an arena filled with *R. (l.) banyulensis* in 150 min of observation). On the other hand, this lure elicited more contacts or "grooming" with the palps, so that the remaining actions corresponded more to a preliminary feeding than to true aggression.

TABLE 3. PERCENTAGE OCCURRENCE OF THE EIGHT BEHAVIORAL ITEMS PERFORMED IN AN ARENA CONTAINING EITHER 30 *Reticulitermes (lucifugus) banyulensis* (*R. (l.).b.*), OR 30 *R. (l.) grassei* (*R. (l.).g.*) TOWARDS A DEAD INDIVIDUAL WASHED WITH PENTANE (DEAD), BELONGING TO EITHER THE SAME (CONTROL) OR THE OPPOSITE SPECIES[a]

	30 *R.(l.)b.*		30 *R.(l.)g.*	
	1 *R.(l.)b.* washed (control)	1 *R.(l.)g.* washed	1 *R.(l.)g.* washed (control)	1 *R.(l.)b.* washed
Ant./ant.	20 a (17-34)	19.5 a (17-26)	15.5 abc (8-19)	20 ab (14-30)
Ant./head	22.5 a (20-33)	27.5 a (23-31)	29.5 a (25-31)	23.5 ab (21-31)
Ant./body	43 bc (39-48)	47 ab (45-52)	48.5 a (46-57)	50 a (46-55)
Palp./body	3 b (0-8)	1 bc (0-2)	2 b (1-4)	1 b (0-3)
Palp./head	0 a (0-5)	0 b (0-1)	3 a (1-3)	1.5 a (0-3)
Open.mand.	0 b (0-0)	0 b (0-0)	0 b (0-0)	0 b (0-0)
Biting	0 c (0-0)	0 c (0-2)	0 b (0-0)	0 a (0-1)
Trembling	0 c (0-0)	0 c (0-0)	0 c (0-1)	0 c (0-1)

[a]The percentages are the median number of items of each kind. A letter occurring more than once on the same line means that the difference was not significant (Mann–Whitney test, $p < 5\%$). Numbers in parentheses refer to quartiles (lower and upper).

These results confirm the hypothesis that species recognition is based upon a chemical signal of the contact pheromone type, and not upon any movement recognition. When an individual was washed with pentane, it was not perceived differently from a control: the substances responsible for recognition are therefore soluble in pentane. In order to prove this hypothesis, however, it was necessary to transfer these substances.

RESULTS OF TEST 2

A CPD lure covered with different extracts was introduced in an arena filled with *R.* (*l.*) *banyulensis* workers. This species was chosen because it is the more aggressive of the two. First, we checked with the controls (no solvent or just pentane on the lure) compared with total extract from workers of one or the other species, that the extract from foreign *R.* (*l.*) *grassei* elicited significant aggression while that from *R.* (*l.*) *banyulensis* did not. We then proceeded to test the different fractions (either total, non-polar, or polar) on the two types of lures (Table 4a and 4b).

The tests showed that with the total extract, the origin of the lure (same or different species) did not affect the results, so that they were satisfactory tools for our tests. The washing process effectively removed all traces of the signal. Covering with an extract of workers of the same species elicited some biting, but this was nothing like the number of woundings inflicted in the case of true aggression (1% and 1.5%). Finally, covering the lure with an extract from the opposite species produced significantly more cases of biting (3.5% and 4.5%).

The extract consisting of non-polar lipids from an alien species (*R.* (*l.*) *grassei*) provoked much more aggression in every case, not only in comparison with the polar fraction, but also with the total extract (6.5% and 5%). The tests with the alien polar extract produced a significantly weaker response (2.5% and 2%).

In our first experiments, we did not collect an intermediate fraction between the non-polar and polar portions and therefore traces of hydrocarbons were carried over into the polar fraction. Aggression was then proportional to the remaining quantity of hydrocarbons. We therefore made sure that not more than 1% of the hydrocarbons remained in our polar fraction (Killian, 1989).

The decrease in the frequency of aggressive acts can be seen from the experiments to have been due to the fact that it was impossible to reproduce perfectly the natural situation: the immobility of the introduced specimen, or other parameters arising from the difficulty of transferring these substances in a natural manner, or with the accurate concentration of cuticular substances, lowers the aggression scores and raises the scores for palpations. Nevertheless, the total mixture of lipids constituted a true chemical signature for the *Reticu-*

TABLE 4. PERCENTAGE OCCURRENCE OF THE EIGHT BEHAVIORAL ITEMS PERFORMED IN AN ARENA CONTAINING 30 *Reticulitermes (lucifugus) banyulensis* (*R.(l.).b.*), TOWARDS A LURE-CPD (CRITICAL POINT DRIED) BELONGING TO EITHER THE SPECIES *R.(l.).b* (a) OR *R.(l.) grassei* *R.(l.).g.* (b) COATED WITH A MIXTURE EXTRACTED FROM EITHER *R.(l.)b.* OR *R.(l.)g.*, CONSISTING OF EITHER THE TOTAL CUTICULAR EXTRACT (TOT), THE APOLAR FRACTION (APOL), OR THE POLAR FRACTION (POL) OF THE EXTRACT[a]

	(a) LURE *R.(l.)b.* CPD					
	R.(l.)b. (tot)	*R.(l.)b.* (apol)	*R.(l.)b.* (pol)	*R.(l.)g.* (tot)	*R.(l.)g.* (apol)	*R.(l.)g.* (pol)
Ant./ant.	12.5 ab (11-15.5)	4.5 c (3-6)	7.5 bc (3-10)	11.5 abc (6-19)	5.5 bc (2-10)	10 ab (9-11)
Ant./head	29.5 a (26-30.5)	28 a (24-30)	32.5 a (25-33)	28 a (27.5-29.5)	29.5 a (26-31)	23 a (27-29)
Ant./body	34.5 c (31-38)	39.5 abcde (38-40)	41 abc (38-41)	37 bcde (33-40)	39.5 abcd (37-43)	44 ab (36-45)
Palp./body	9 ab (6.5-16.5)	12 a (9-15)	12 a (10-12)	10.5 ab (9-12)	7 ab (4-12)	5 ab (5-12)
Palp./head	9 abc (5-12)	11 ab (8-12)	7.5 bc (5-10)	7 c (4-9)	6.5 c (3-5)	5.5 c (5-9)
Open.mand.	0 bc (0-0.5)	1.5 ab (0-2)	0 abc (0-2)	0.5 abc (0-1)	1 ab (0-1)	0 abc (0-1)
Biting	1 c (0-2)	2 b (2-3)	0.5 c (0-1)	3.5 ab (2-6.5)	6.5 a (5-9)	2.5 bc (1-4)
Trembling	0 bc (0-1)	2 a (1-3)	1 ab (0-1)	0.5 abc (0-1)	0 abc (0-5)	0.5 abc (0-2)
	(b) LURE *R.(l.)g.* CPD					
	R.(l.)b. (tot)	*R.(l.)b.* (apol)	*R.(l.)b.* (pol)	*R.(l.)g.* (tot)	*R.(l.)g.* (apol)	*R.(l.)g.* (pol)
Ant./ant.	14 ab (8.5-15.5)	10.5 abc (2-14)	16.5 a (14-25)	8 bc (4-10.5)	10.5 abc (6-10)	8.5 abc (2-11)
Ant./head	24.5 ab (23-28.5)	30 a (23-32)	29 a (27-31)	22.5 b (21-25)	24.5 ab (23-29)	28 ab (23-30)
Ant./body	36 de (32-39.5)	38.5 abcde (37-39)	39.5 abcde (37-41)	32.5 bcde (29.5-39.5)	37 cde (34-39)	41 a (38-46)
Palp./body	14.5 a (12-18.5)	9 ab (7.5-15)	6 ab (4-16)	16 a (9-21.5)	7 b (4-11)	8 ab (7-15)
Palp./head	9.5 ab (8-10)	8 abc (7.5-5.8)	5 c (4-6)	12 a (9-13.5)	5.5 c (3-9)	1 c (4-7)

TABLE 4. Continued

	(a) LURE *R.(l.)b.* CPD					
	R.(l.)b. (tot)	*R.(l.)b.* (apol)	*R.(l.)b.* (pol)	*R.(l.)g.* (tot)	*R.(l.)g.* (apol)	*R.(l.)g.* (pol)
Open.mand.	0 abc (0-1)	0 abc (0-1)	0 c (0-0)	0 abc (0-0.5)	2 a (0-4)	0.5 ab (0-2)
Biting	1.5 bc (0-2.5)	2 bc (1-2)	0 c (0-1)	4.5 a (3-5.5)	5 a (3-8)	2 b (1-3)
Trembling	0 abc (0-1.5)	1 abc (0-1)	0.5 abc (0-2)	0 bc (0-1)	0 c (0-0)	1 a (0-2)

[a] The percentages are the median number of items of each kind. A letter occurring more than once on the sam line means that the difference was not significant (Mann–Whitney test, $p < 5\%$). Numbers in parenthese refer to quartiles (lower and upper).

litermes workers, and this signature is contained to a large extent in the non-polar fraction extracted with pentane.

Results of the Chemical Investigations on the Cuticular Extracts (Table 5). These results supplement those by Lange et al. (1989). The non-polar chemical signature of *R.* (*l.*) *banyulensis* (Figure 1a) is composed of 48 hydrocarbons containing from 23 to 36 atoms per molecule, and the non-polar chemical signature of *R.* (*l.*) *grassei* (Figure 1b) is composed of 37 hydrocarbons containing from 25 to 33 carbons atoms. These hydrocarbons are principally saturated, and only a very small portion of the total are alkenes (3% in *R.* (*l.*) *b.*, and 2% in *R.* (*l.*) *g.*). The largest group are the monomethylalkanes (63% in *R.* (*l.*) *b.*, and 61% in *R.* (*l.*) *g.*), followed by the *n*-alkanes (29% in *R.* (*l.*) *b.*, and 23% in *R.* (*l.*) *g.*) and then the dimethylalkanes (5% in *R.* (*l.*) *b.* and 14% in *R.* (*l.*) *g.*). It is the latter group which differentiates most the two signatures, followed by the length of the carbon chains. The quantitative results (percentages and absolute quantities) are given in Table 5. It can be seen that 40% of the substances are common to both species, but for the greater part of these common substances, the quantities are quite different, as for example, in the case of pentacosane, 5-methylpentacosane, 5-methylheptacosane, and 5,17-dimethylheptacosane.

DISCUSSION AND CONCLUSION

This study demonstrates the following points:

1. A specific chemical signature exists on the epicuticle, and this can be extracted with pentane and transferred to a lure. This signature provokes aggression between the two species of *Reticulitermes*.

TABLE 5. DETERMINATION OF HYDROCARBONS USING GC-MS EI AND CI ON *Reticulitermes (lucifugus) banyulensis (R.(l.)b.)* AND *R.(l.)grassei (R.(l.)g.)* INCLUDING G.C. PEAK NUMBER, PERCENTAGES, AND QUANTITIES

	Diagnostic EI-MS ions		CI-MS ions	Peaks number			Percentages		Quantities	
Compounds	CN	MW	(a) CH4+, (b)NH4+	ECL	*R.(l.)b.*	*R.(l.)g.*	*R.(l.)b.*	*R.(l.)g.*	*R.(l.)b.*	*R.(l.)g.*
n-Alkanes										
nC21	21	296	295	21.00	T	T				
nC22	22	310	309	22.00	T	T				
nC23	23	324	323	23.00	2	T	0.56(0.25)		0.64(0.33)	
nC24	24	338	337	24.00	4	T	0.96(0.43)		1.07(0.53)	
nC25	25	352	351	25.00	7	2	12.34(4.69)	3.64(8.71)	15.16(10.4)	2.45(4.81)
nC26	26	366	365	26.00	14	6	2.04(0.71)	1.04(0.40)	2.46(1.57)	0.89(0.45)
nC27	27	380	379	27.00	18	11	8.35(5.44)	11.07(4.15)	10.84(10.6)	9.60(5.33)
nC28	28	394	393	28.00	24	18	0.76(0.55)	1.93(0.60)	0.99(0.86)	1.66(0.80)
nC29	29	408	407	29.00	28	25	1.22(0.97)	3.17(1.53)	1.66(1.85)	2.77(1.72)
nC30	30	422	421	30.00	32	31	0.27(0.29)	0.62(0.46)	0.35(0.38)	0.52(0.41)
nC31	31	436	435	31.00	34	34	0.23(0.30)	0.36(0.48)	0.32(0.43)	0.33(0.47)
nC32	32	450	449	32.00	38	T	0.12(0.18)		0.14(0.24)	
nC33	33	464	463	33.00	40	T	0.12(0.21)		0.15(0.25)	
nC34	34	478	479	34.00	44	T	0.14(0.43)		0.09(0.32)	
nC35	35	492	491	35.00	46	—	0.27(0.55)		0.57(0.86)	
Mono-methyl alkanes										
11Me-C23	24	338 168/169, 196/197	337	23.36	3	—	0.46(0.30)		0.50(0.39)	
12Me-C24	25	352 182/183, 196/197	351	24.35	5	—	0.80(0.28)		0.87(0.42)	
11Me-C24	25	352 168/169, 210/211	351	24.35	5	—				
13Me-C25	26	366 196/197	365	25.35	9	—	10.70(3.24)		11.89(6.02)	
11Me-C25	26	366 168/169, 224/225	365	25.35	9	4		0.94(0.72)		0.74(0.59)
9Me-C25	26	366 140/141, 252/253	365	25.37	10	—	0.61(0.17)		0.69(0.34)	
5Me-C25	26	366 84/85, 308/309	365	25.50	11	5	7.46(2.17)	2.11(0.63)	8.45(4.66)	1.71(0.59)
3Me-C25	26	366 56/57, 336/337	365	25.75	12	—	1.30(0.87)		1.39(1.22)	
13Me-C26	27	380 196/197, 210/211	379	26.35	15	7	1.27(0.37)	0.91(0.33)	1.41(0.68)	0.74(0.28)
12Me-C26	27	380 182/183, 224/225	379	26.35	15	7				
11Me-C26	27	380 168/169, 238/239	379	26.35	15	7				

TABLE 5. Continued

Compounds	CN	Diagnostic EI-MS ions MW	CI-MS ions (a) CH4+, (b)NH4+	Peaks number ECL	R.(l.)b.	R.(l.)g.	Percentages R.(l.)b.	R.(l.)g.	Quantities R.(l.)b.	R.(l.)g.
Mono-methyl alkanes										
6Me-C26	27	380 98/99, 308/309	379	26.46	16	8	1.43(0.42)	1.58(0.45)	1.61(0.89)	1.29(0.42)
4/2Me-C26	27	380 70/71, 336/337	379	26.63	17	9	0.64(0.40)	0.88(0.27)	0.85(0.87)	0.72(0.28)
13Me-C27	28	394 196/197, 224/225	393	27.33	19	12	8.22(1.97)	14.49(2.99)	9.33(4.50)	11.79(3.21)
11Me-C27	28	394 168/169, 252/253	393	27.33	19	12				
7Me-C27	28	394 112/113, 308/309	393	27.43	20	13	0.69(0.22)	1.79(0.80)	0.83(0.48)	1.55(0.88)
5Me-C27	28	394 84/85, 336/337	393	27.52	21	14	8.87(2.83)	16.62(3.33)	10.32(6.11)	14.10(5.41)
3Me-C27	28	394 56/57, 365/366	393	27.63	22	16	0.34(0.25)	0.72(0.32)	0.40(0.37)	0.62(0.35)
14Me-C28	29	408 210/211, 224/225	407	28.35	—	20		1.08(0.42)		0.87(0.36)
13Me-C28	29	408 196/197, 238/239	407	28.34	26	20	0.47(0.28)		0.56(0.37)	
11Me-C28	29	408 168/169, 266/267	407	28.33	T	20				
6Me-C28	29	408 98/99, 336/337	407	28.41	27	21	0.56(0.55)	1.84(0.61)	1.82(1.10)	1.49(0.51)
4Me-C28	29	408 70/71, 365/366	407	28.65	—	22		0.80(0.38)		0.67(0.35)
3Me-C28	29	408 56/57, 379/380	407	28.76	—	24		0.46(0.39)		0.38(0.33)
15Me-C29	30	422 224/225	421	29.32	29	27	2.02(0.55)	4.33(1.02)	2.33(1.16)	3.65(1.47)
13Me-C29	30	422 196/197, 252/253	421	29.32	29	27				
11MeC29	30	422 168/169, 280/281	421	29.32	29	27				
7Me-C29	30	422 112/113, 336/337	421	29.52	30	28	3.39(1.07)	7.49(2.37)	4.12(2.37)	6.57(3.48)
5Me-C29	30	422 84/85, 364/365	421	29.52	30	29		0.27(0.34)		0.23(0.30)
6Me-C30	31	436 98/99, 364/365	435	30.61	—	32		0.80(0.56)		0.67(0.50)
5Me-C30	31	436 84/85, 377/378	435	30.57	33	—	0.80(0.73)		0.82(0.80)	
15Me-C31	32	450 224/225, 252/253	449	31.31	—	35		0.99(0.45)		0.84(0.43)
13Me-C31	32	450 196/197, 280/281	449	31.31	35	35	2.35(0.65)		2.69(1.37)	
11Me-C31	32	450 168/169, 308/309	449	31.31	35	—				
5Me-C31	32	450 84/85, 393/394	449	31.56	36	—	1.48(0.54)		1.63(0.83)	
12Me-C32	33	464 182/183, 308/309	463	32.30	39	—	0.41(0.26)		0.50(0.32)	
15Me-C33	34	478 224/225, 280/281	477	33.30	41	—	3.41(1.11)		4.11(2.43)	
13Me-C33	34	478 196/197, 308/309	477	33.30	41	—				
5Me-C33	34	478 84/85, 420/421	477	33.50	42	—	0.96(0.56)		1.08(0.78)	

13Me-C34	35	492	196/197, 322/323	491	34.30	45	—	0.29(0.62)		0.28(0.52)	
11Me-C34	35	492	168/169, 350/351	491	34.30	45	—				
13Me-C35	36	506	196/197, 336/337	505	35.32	47	—	1.59(2.48)		1.37(1.08)	
11Me-C35	36	506	168/169, 364/365	505	35.32	47	—				
Di-methyl alkanes											
9,13diMe-C25	27	380	140, 196, 211, 267	379	25.69	12	—				
5,17diMe-C25	27	380	84, 140, 267, 323	379	25.91	13	—	0.25(0.18)		0.30(0.20)	
5,17diMe-C26	28	394	84, 154, 267, 377	393	26.65	T	—				
11,15diMe-C27	29	408	168, 196, 239, 267	407	27.63	T	15		1.85(0.64)		1.50(0.59)
5,17diMe-C27	29	408	84, 168, 267, 351	407	27.87	23	17	1.53(0.61)	6.39(1.98)	1.72(1.01)	5.17(1.79)
5,17diMe-C29	31	436	84, 196, 267, 379	435	29.82	31	30	0.85(0.32)	2.40(0.90)	0.97(0.60)	1.94(0.81)
7,21diMe-C29	31	436	112, 154, 310, 350	435	29.85	T	—				
5,17diMe-C30	32	450	84, 210, 267, 393	449	30.70	—	33		0.31(0.48)		0.25(0.41)
5,17diMe-C31	33	464	84, 224, 267, 407	463	31.81	37	36	0.21(0.31)	2.17(0.82)	0.23(0.30)	1.80(0.87)
13,17diMe-C33	35	492	196, 252, 267, 322	491	33.50	42	—				
Monoenes											
9-C23:1	23	322	157/241, 171/227	323; 353(a); 340, 372(b)	22.68	1	—	0.88(0.77)		0.91(1.06)	
9-C25:1	25	351	157/269, 171/255	351; 381(a); 368, 400(b)	24.69	6	1	1.72(0.68)	0.44(0.41)	1.84(0.96)	0.36(0.34)
x-C27:1	27	378		379	26.35	—	10		0.90(0.40)		0.75(0.39)
9-C29:1	29	406	157/325, 171/311	407; 437(a); 424, 456(b)	28.72	—	23		0.59(0.44)		0.52(0.46)

[a]The diagnostic ions of monoenes are given after methoxymercuration-demercuration, and chemical ionization (CI) was effected under (a) methane and (b) ammoniac. Percentages and quantities (ng/mg ins.) are given with the standard deviations in parentheses. CN = Carbon number, MW = molecular weight, ECL = equivalent chain length.

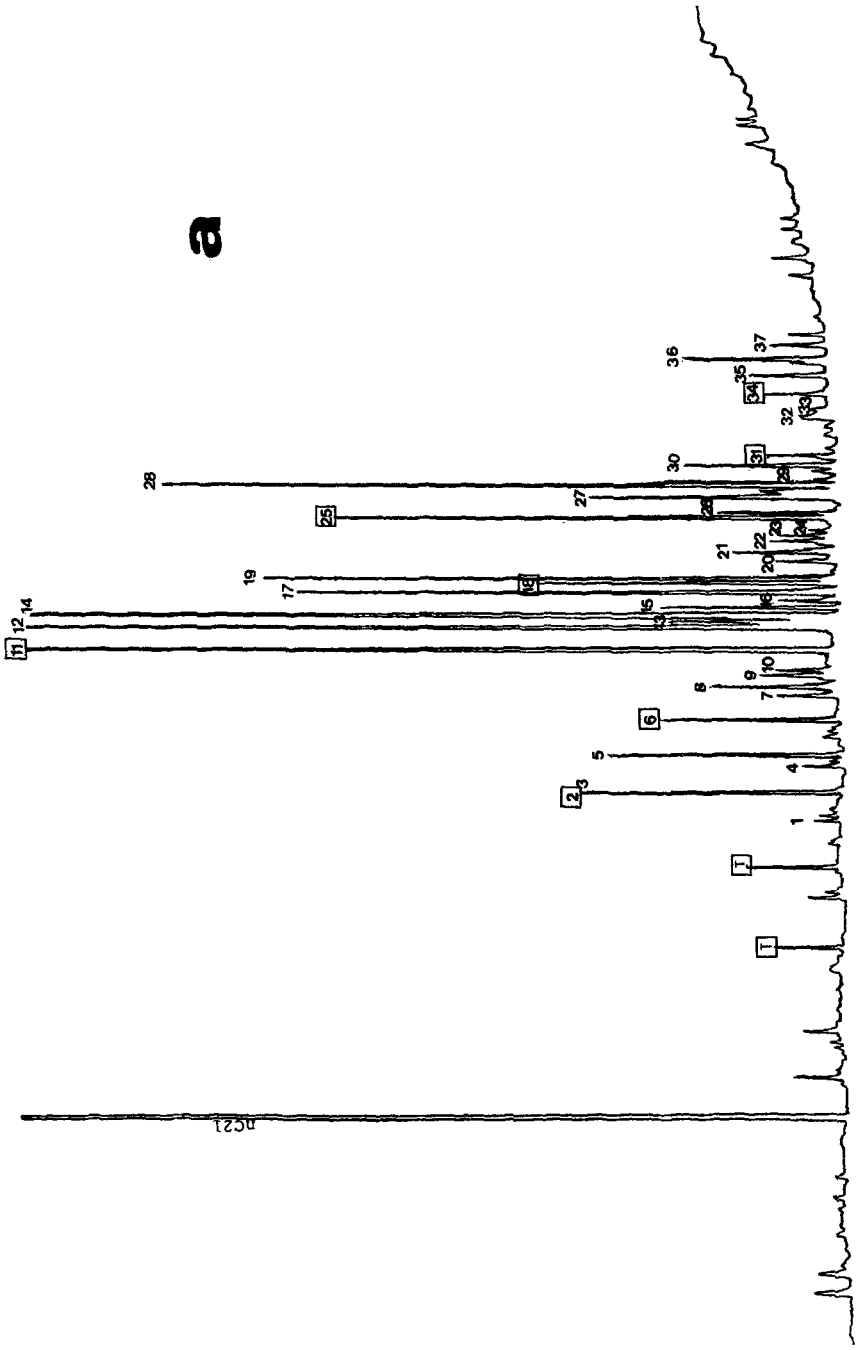


FIG. 1a. Gas chromatogram of cuticular hydrocarbons from *Reticulitermes (lucifugus) grassei*.

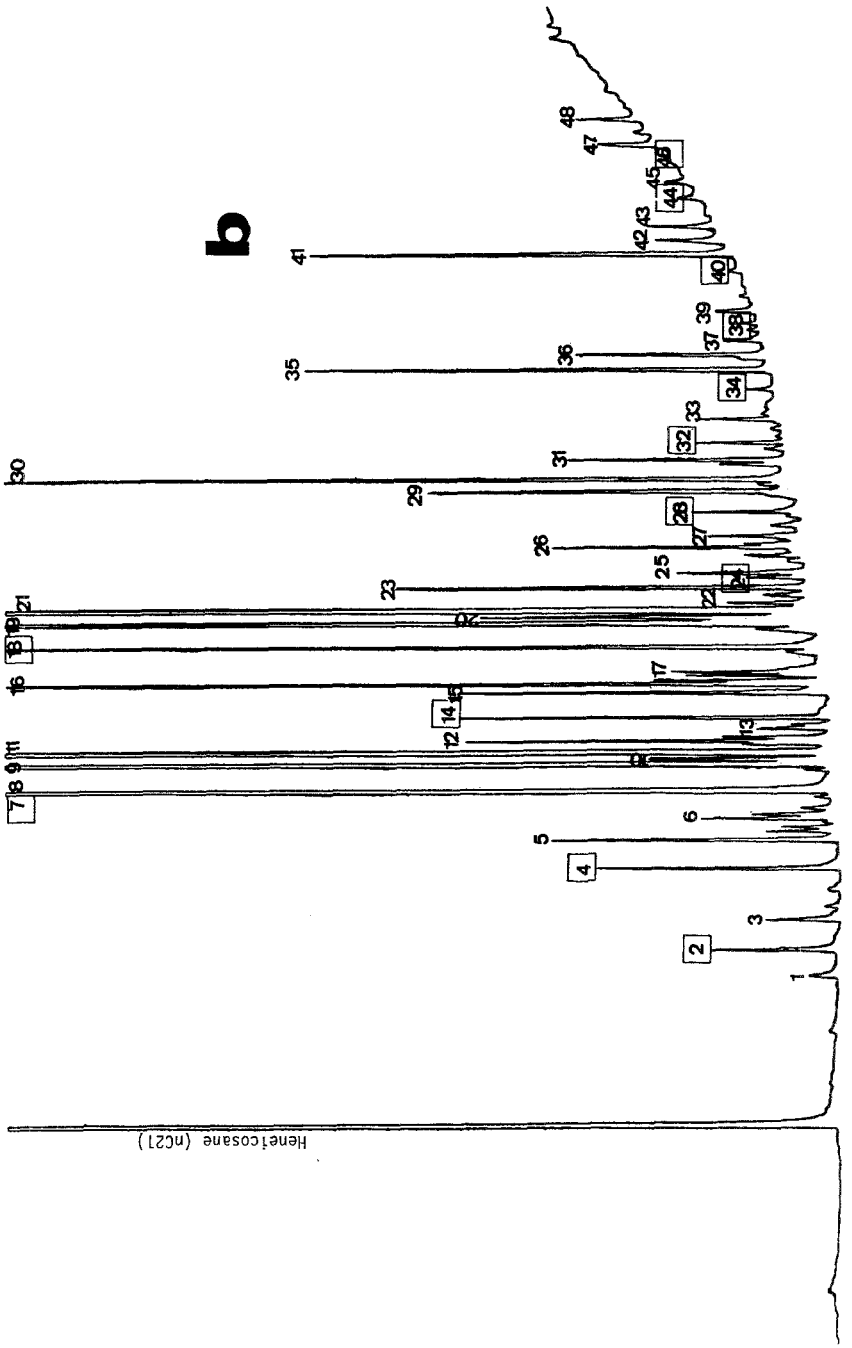


FIG. 1b. Gas chromatogram of cuticular hydrocarbons from *Reticulitermes (lucifugus) banyulensis*.

2. This signature is contained chiefly in the non-polar fraction of the epicuticular extract, which contains only hydrocarbons.

3. Contact is necessary for recognition to occur, but it is preferable for an introduced individual to be mobile, and for a natural encounter to take place.

A few studies have been published previously on aggressive behavior in termites. Those by Clément (1986) on the European *Reticulitermes* provided us here with a basis for the aggression tests, since the author measured the degree of agonism displayed by two intra- or interspecific groups brought into contact. As the result of the investigations by Howard et al. (1982b), the hypothesis was put forward that hydrocarbons may play a role in interspecies recognition between two American *Reticulitermes* species; but the most innovative findings on termite aggression were published by Nel in studies on the closure of *Hodotermes mossambicus* and *Trinervitermes trinervoides* societies (Nel, 1968). The latter author showed that in the former species the aggression displayed by a colony differs significantly depending on whether it is a natural (highly aggressive) colony, or one which has been raised under artificial conditions (non-aggression). Springhetti and Amorelli (1982) have even reported differences in the competitive behavior of *Kalotermes flavicolli* and *Reticulitermes lucifugus*, in the field and in the laboratory with opposite results in the two cases. This conclusion was not confirmed in the present study. It has been observed by us on the contrary, that *Reticulitermes* colonies or species withdraw to their galleries to avoid confrontation with strangers (Bagnères et al., 1990).

As B.L. Thorne (1982) has remarked, "the intensity of agonist encounters depends on which species, colonies and castes (and the respective ratios) are involved."

It does seem likely that hydrocarbons may be a fairly universal factor in insect inter- and intraspecies recognition. Henderson et al. (1990) have reached the same conclusion for the ant *Formica montana*, starting with a hypothesis which seems contradictory to those of most others authors, namely, that in these ants, aggression might be a raising of an inhibition created by the hydrocarbons, in which case these substances would no longer serve as triggering signals as we ourselves have concluded in the present study. The present data certainly showed that in *Reticulitermes*, no aggression is triggered when the recognition signals are missing. Aggression is actively induced by differences in the kind or the proportions of the signals, which are probably compared with a reference mixture which was memorized at emergence.

Some 10 years ago, various authors began to consider, indeed, that cuticular mixtures of hydrocarbons serve as specific markers (Carlson and Service, 1980), and up to the present, many articles have appeared on the chemotaxonomy of numerous species (Flies: Hoppes et al., 1990; Luytens, 1982; Nelson and Carlson, 1986; Nelson et al., 1988; Crickets: Castner and Nation, 1986;

Grunshawn et al., 1990; Beetles: Lockey, 1991; Page et al., 1990). The hydrocarbons have also been dealt with in a number of studies on termites, such as these on *Coptotermes* by Haverty et al. (1990a, 1991, submitted), Su and Haverty (pers. comm.), Brown et al. (1990), on *Zootermopsis* by Blomquist et al. (1979), Haverty et al. (1988), on *Nasutitermes* by Howard et al. (1988) and Haverty et al. (1990b, submitted), and on *Reticulitermes* by Howard et al. (1978, 1982b), Clément et al. (1985, 1986, 1988), Bagnères et al. (1988, 1990), and Haverty et al. (1991).

Very little is known about the mechanisms of regulation involved in chemical signatures and their genetics. Vander Meer et al. (1985), by hybridizing two species of *Solenopsis* (*S. invicta* and *S. richteri*) saw that the cuticular profiles of the hybrids were intermediate between those of the two parents.

The origin of the variations in the spectrum of the cuticular hydrocarbons of *Solenopsis* (and incidentally, the composition of the venom) has been studied by Ross et al. (1987). Bagnères et al. (1991) have observed that the hydrocarbons of the specific signatures of two species of ant were modified when these ants were kept in artificially mixed colonies, in a way that permitted them to live together in the same nest without provoking any aggression (Corbara and Errard, 1991). Likewise, Bonavita-Cougourdan et al. (1989) have transferred larvae of *Camponotus vagus* from one nest to another nest, then these larvae acquired an odor close to that of their new nest and thereby become part of it. There is therefore in these cases a possible plasticity in the specific odor and the colonial odor, or an active transfer of cuticular hydrocarbons from the postpharyngeal gland (Bagnères and Morgan, 1991).

The regulation of these processes no doubt has two origins which are not incompatible. There is a genetic or epigenetic origin and an origin through transfer, because it emerges from our work, both published and unpublished, and from the published work of others (see Howard et al., 1990b), that the secretion of cuticular hydrocarbons is clearly the direct expression of the genome, but that this can be modified through the social environment.

The modifications in this case may be due either to passive transfer (contact and rubbing) or by active transfer (licking and grooming), or to a change in the hydrocarbons synthesis (throught feedback inhibition). Among ants, the products of the postpharyngeal gland may be involved here (Bagnères and Morgan, 1991), but in termites no equivalent gland has ever been described.

It is striking to note that in spite of all the different cuticular profiles, the majority of insect species have somewhat similar profiles, which suggests that evolution of this order has favored a kind of chemical homogeneity, due perhaps to a uniformity at the level of their synthesis and regulation. This may help to explain how the degree of closure or opening of insect societies may depend on the recognition of a complex signal which can sometimes vary very subtly.

The exact sites of synthesis, the mechanisms involved in the transport of precursors and products are not yet fully understood, nor are the receptor sites and the operations taking place at a more central level of control.

To complete the present study, it is necessary to know if mixtures of some compounds are more effective than others, and to carry out tests on the insects. Thus far, a discrimination analysis between hydrocarbons and ethological results within a single superspecies (*R.* (*l.*) *g.*) and (*R.* (*l.*) *b.*) has not led to any conclusive results; it even seems that the whole mixture plays some part (Bagnères, 1989).

Acknowledgments—This study is part of a thesis of the University of Paris VI (U.P.M.C.), France, for the graduation of A.G.B. as "Docteur de l'Université P. et M. Curie," and that of A.K. with a "Diplome d'Etude Approfondie" (D.E.A.). We acknowledge the help of Mrs. C. Joulie, G. Lelait, and A. Poveda. We thank Prof. E.D. Morgan (University of Keele, U.K.) for the English translation, and also J. Blanc.

REFERENCES

BAGNÈRES, A.G. 1989. Les Hydrocarbures Cuticulaires des Insectes Sociaux. Détermination et Rôle dans la Reconnaissance Spécifique Coloniale et Individuelle. Thèse de Doctorat de l'Université P. et M. Curie. 250 pp.

BAGNÈRES, A.G., CLÉMENT, J.L., LANGE, C., and JOULIE, C. 1988. Les hydrocarbures cuticulaires des *Reticulitermes* français: Variations spécifiques et coloniales. *Actes Coll. Ins. Soc.* 4:34–42.

BAGNÈRES, A.G., and MORGAN, E.D. 1991. The postpharyngeal glands and the cuticle of Formicidae contain the same characteristic hydrocarbons. *Experientia* 47:106–111.

BAGNÈRES, A.G., CLÉMENT, J.L., BLUM, M.S., SEVERSON, R.F., JOULIE, C., and LANGE, C. 1990. Cuticular hydrocarbons and defensive compounds of *Reticulitermes flavipes* (Kollar) and *R. santonensis* (Feytaud): Polymorphism and chemotaxonomy. *J. Chem. Ecol.* 16:3213–3244.

BAGNÈRES, A.G., ERRARD, C., MULHEIM, C., JOULIE, C., and LANGE, C. 1991. Induced mimicry of colony odors in ants. *J. Chem. Ecol.* 17:1641–1664.

BLOMQUIST, G.J., and JACKSON, L.L. 1976. Insect waxes, pp. 201–233, *in* P.E. Kolattukudy (ed.). Chemistry and Biochemistry. Elsevier, Amsterdam.

BLOMQUIST, G.J., HOWARD, R.W., and MCDANIEL, C.A. 1979. Structure of the cuticular hydrocarbons of the termite *Zootermopsis angusticollis* (Hagen). *Insect Biochem.* 9:365–370.

BLOMQUIST, G.J., HOWARD, R.W., MCDANIEL, C.A., REMALEY, S., DWYER, L.A., and NELSON, D.R. 1980. Application of methoxymercuration-demercuration followed by mass spectrometry as a convenient microanalytical technique for double-bond location in insect-derived alkenes. *J. Chem. Ecol.* 6:257–269.

BLOMQUIST, G.J., NELSON, D.R., and DE RENOBALES, M. 1987. Chemistry, biochemistry, and physiology of insect cuticular lipids. *Arch. Ins. Biochem. Physiol.* 6:227–265.

BLUM, M.S. 1987. Specificity of pheromonal signals: A search for its recognitive bases in terms of a unified chemisociality, pp. 401–405, *in* J. Peperny (ed.). Chemistry and Biology of Social Insects, Verlag, München.

BONAVITA-COUGOURDAN, A., CLÉMENT, J.L., and LANGE, C. 1987. Nestmate recognition: The role of cuticular hydrocarbons in the ant *Camponotus vagus*. *J. Entomol. Sci.* 22:1–10.

BONAVITA-COUGOURDAN, A., CLÉMENT, J.L., and LANGE, C. 1989. The role of cuticular hydrocarbons in recognition of larvae by workers of the ant *Camponotus vagus*: Changes in the chemical signature in response to social environment (Hymenoptera: Formicidae). *Sociobiology* 16: 49–74.

BROWN, W.V., WATSON, J.A.L., CARTER, F.L., LACEY, M.J., BARETT, R.A., and MCDANIEL, C.A. 1990. Preliminary examination of cuticular hydrocarbons of worker termites as chemotaxonomic characters for some australian species of *Coptotermes* (Isoptera: Rhinotermitidae). *Sociobiology* 16:305–328.

CARLSON, D.A., and SERVICE, M.W. 1980. Identification of mosquitoes of *Anopheles gambiae* species complex A and B by analysis of cuticular components. *Science* 207:1089–1091.

CASTNER, J.L., and NATION, J.L. 1986. Cuticular lipids for species recognition of mole crickets (Orthoptera: Gryllotalpidae): II. *Scapteriscus abbreviatus*, *S. acletus*, *S. vicinus*, *S.* sp., and *Neocurtilla hexadactyla*. *Arch. Ins. Biochem. Physiol.* 3:127–134.

CLÉMENT, J.L. 1978. L'agression interspécifique et intraspécifique des espèces françaises du genre *Reticulitermes* (Isoptère). *C.R. Acad. Sci. Paris* 286:351–354.

CLÉMENT, J.L. 1981a. Comportement de reconnaissance individuelle dans le genre *Reticulitermes* (Isoptera). *C.R. Acad. Sci. Paris* 292:931–933.

CLÉMENT, J.L. 1981b. Autoécologie, distance phénotypiques et génétiques entre population des termites du complexe *Reticulitermes lucifugus*. *Vie Milieu* 31:261–270.

CLÉMENT, J.L. 1982. Signaux de contact responsables de l'agression interspécifique des termites du genre *Reticulitermes* (Isoptères). *C.R. Acad. Sci. Paris* 294:635–638.

CLÉMENT, J.L. 1986. Open and closed societies in *Reticulitermes* termites (Isoptera, Rhinotermitidae): Geographic and seasonal variations. *Sociobiology* 11:311–323.

CLÉMENT, J.L., LANGE, C., BLUM, M.S., HOWARD, R.W., and LLOYD, H. 1985. Chimosystematique du genre *Reticulitermes* (Isoptères) aux U.S.A. et en Europe. *Actes Coll. Ins. Soc.* 2: 123–131.

CLÉMENT, J.L., HOWARD, R.W., BLUM, M.S., and LLOYD, H. 1986. L'isolement spécifique des termites du genre *Reticulitermes* (Isoptera) du sud-est des Etats-Unis. Mise en évidence grâce à la chimie et au comportement d'une espèce jumelle de *R. virginicus*=*R. malletei* sp. nov. et d'une semi-espèce de *R. flavipes*. *C.R. Acad. Sci. Paris* 302:67–70.

CLÉMENT, J.L., LEMAIRE, M., NAGNAN, P., ESCOUBAS, P., BAGNÈRES, A.G., and JOULIE, C. 1988. Chemical ecology of European termites of the genus *Reticulitermes*: Allomones, pheromones and kairomones. *Sociobiology* 14:165–174.

CORBARA, B., and ERRARD, C. 1991. The organisation of artificial heterospecific ant colonies. The case of *Manica rubida/Formica selysi* (Myrmicinae, Formicinae) association: Mixed colony or parallel colonies. *Behav. Proc.* 23:75–87.

ESPELIE, K.E., and HERMANN, H.R. 1988. Congruent cuticular hydrocarbons: Biochemical convergence of a social wasp, an ant and a host plant. *Biochem. Syst. Ecol.* 16:505–508.

ESPELIE, K.E., WENZEL, J.W., and CHANG, G. 1990. Surface lipids of social wasp *Polistes metricus* say and its nest and nest pedicel and their relation to nestmate recognition. *J. Chem. Ecol.* 16:2229–2241.

FIELDE, A.M. 1903. Artificial mixed nests of ants. *Biol. Bull.* V(6):320–325.

FIELDE, A.M. 1904. Power of recognition among ants. *Biol. Bull.* VII(5):227–250.

FIELDE, A.M. 1905. The progressive odor of ants. *Biol. Bull.* X(1):1–15.

FRANKS, N., BLUM, M.S., SMITH, R.K., and ALLIES, A.B. 1990. Behavior and chemical disguise of cuckoo ant *Leptothorax kutteri* in relation to its host. *J. Chem. Ecol.* 16:1431–1444.

GOLDBERG, J. 1971. Mise en évidence du transport d'eau par le termite de Saintonge. *C.R. Acad. Sci. Paris* 272:2820–2822.

GRUNSHAWN, J.P., GUERMOUCHE, H., GUERMOUCHE, S., JAGO, N.D., JULLIEN, R., KNOWLES, E.,

and PEREZ, F. 1990. Chemical taxonomic studies of cuticular hydrocarbons in locusts of the *Schistocerca* americana complex: Chemical relationships between New World and Old World species. *J. Chem. Ecol.* 16:2835–2858.

GUILLE-ESCURET, G. 1981. Contribution à la sociologie du genre *Reticulitermes* (Isoptera: Rhinotermitidae) en charente-maritime. Thèse de Doctorat 3ème cycle, U.P.M.C.

HAVERTY, M.I., and THORNE, B.L. 1989. Agonistic behavior correlated with hydrocarbon phenotypes in dampwood termites, *Zootermopsis* (Isoptera: Termopsidae). *J. Ins. Behav.* 2:523–543.

HAVERTY, M.I., PAGE, M., NELSON, L.J., and BLOMQUIST, G.J. 1988. Cuticular hydrocarbons of dampwood termites, *Zootermopsis*: Intra- and intercolony variation and potential as taxonomic characters. *J. Chem. Ecol.* 14:1035–1058.

HAVERTY, M.I., NELSON, L.J., and PAGE, M. 1990a. Cuticular hydrocarbons of four populations of *Coptotermes formosanus* Shiraki in the United States: Similarities and origins of introductions. *J. Chem. Ecol.* 16:1635–1647.

HAVERTY, M.I., THORNE, B.L., and PAGE, M. 1990b. Surface hydrocarbon components of two species of *Nasutitermes* from Trinidad. *J. Chem. Ecol.* 16:2441–2450.

HAVERTY, M.I., NELSON, L.J., and PAGE, M. 1991. Preliminary investigations of the cuticular hydrocarbons from North American *Reticulitermes* and tropical and subtropical *Coptotermes* (Isoptera: Rhinotermitidae) for chemostaxonomic studies. *Sociobiology.* 19:51–76.

HAVERTY, M.I., NELSON, L.J., THORNE, B.L., COLLINS, M.S., DARLINGTON, J., and PAGE, M. Cuticular hydrocarbons for species determination of tropical termites. Submitted.

HENDERSON, G., ANDERSEN, J.F., PHILLIPS, J.K. and JEANNE, R.L. 1990. Internest aggression and identification of possible nestmate discrimination pheromones in polygynous ant *Formica montana. J. Chem. Ecol.* 16:2217–2228.

HÖLLDOBLER, B., and CARLIN, N.F. 1987. Anonymity and specificity in the chemical communication signals of social insects. *J. Comp. Physiol.* 161A:567–581.

HOPPES, K.L., DILLWITH, J.W., WRIGHT, R.E., and SZUMLAS, D.E. 1990. Identification of horse flies (Diptera: Tabanidae) by analysis of cuticular hydrocarbons. *J. Med. Entomol.* 27:481–486.

HOWARD, R.W., MCDANIEL, C.A., NELSON, D.R., and BLOMQUIST, G.J. 1980a. Chemical ionization mass spectrometry: Application to insect-derived cuticular alkanes. *J. Chem. Ecol.*. 6:609–623.

HOWARD, R.W., and BLOMQUIST, G.J. 1982. Chemical ecology and biochemistry of insect hydrocarbons. *Annu. Rev. Entomol.* 27:149–172.

HOWARD, R.W., MCDANIEL, C.A., and BLOMQUIST, G.J. 1978. Cuticular hydrocarbons of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae). *J. Chem. Ecol.* 4:233–245.

HOWARD, R.W., MCDANIEL, C.A., and BLOMQUIST, G.J. 1980b. Chemical mimicry as an integrating mechanism: Cuticular hydrocarbons of a termitophile and its host. *Science* 210:431–433.

HOWARD, R.W., MCDANIEL, C.A., and BLOMQUIST, G.J. 1982a. Chemical mimicry as an integrating mechanism for three termitophiles associated with *Reticulitermes virginicus. Psyche* 89:157–167.

HOWARD, R.W., MCDANIEL, C.A., NELSON, D.R., BLOMQUIST, G.J., GELBAUM, and ZALKOW, L.H. 1982b. Cuticular hydrocarbons of *Reticulitermes virginicus* (Bank) and their role as potential species- and caste-recognition cues. *J. Chem. Ecol.* 8:1227–1239.

HOWARD, R.W., THORNE, B.L., LEVINGS, S.C., and MCDANIEL, C.A. 1988. Cuticular hydrocarbons as chemotaxonomic characters for *Nasutitermes corniger* (Motschulsky) and *N. ephratae* (Holmgren) (Isoptera: Termitidae). *Ann. Entomol. Soc. Am.* 81:395–399.

HOWARD, R.W., AKRE, R.D., and GARNETT, W.B. 1990a. Chemical mimicry in an obligate predator of carpenter ants (Hymenoptera: Formicidae). *Ann. Entomol. Soc. Am.* 83:607–616.

HOWARD, R.W., STANLEY-SAMUELSON, D.W., and AKRE, R.D. 1990b. Biosynthesis and chemical mimicry of cuticular hydrocarbons from the obligate predator, *Microdon albicomatus* Novak (Diptera: Syrphidae) and its ant prey, *Myrmica incompleta* Provencher (Hymenoptera: Formicidae). *J. Kansas Entomol. Soc.* 63:437–443.

HOWSE, P.E. 1975. Chemical defences of ants, termites and other insects, some outstanding questions, in pheromones and defensive secretions in social insects. Symposium of the I.U.S.S.I., Dijon, pp. 23–40.

JAFFE, K. 1980. Chemical communication systems in the ant *Atta cephalotes, in* Social Insects on the Tropics, Vol. 2, pp. 165–180.

KILLIAN, A. 1989. Etude Comportementale de l'agression interspécifique des *Reticulitermes* (Isoptères). Mémoire de Diplome d'Etude Approfondie. 51 pp.

LANGE, C., BASSELIER, J.J., BAGNÈRES, A.G., ESCOUBAS, P., LEMAIRE, M., LENOIR, A., CLEMENT, J.L., BONAVITA-COUGOURDAN, A., TRABALON, M., and CAMPAN, M. 1989. Strategy for the analysis of cuticular hydrocarbons waxes from insects using gas chromatography/mass spectrometry with electron impact and chemical ionization. *Biomed. Env. Mass Spec.* 18:787–800.

LOCKEY, K.H. 1988. Lipids of the insect cuticule: Origin, composition and function. *Comp. Biochem. Physiol.* 89B:595–645.

LOCKEY, K.H. 1991. Cuticular hydrocarbons of adult *Onymacris bicolor* (Haag) and *Onymacris boschimana* (Péringuey) (Coleoptera: Tenebrionidae). *Comp. Biochem. Physiol.* 98B:151–163.

LOCKEY, K.H. and METCALFE, N.B. 1988. Cuticular hydrocarbons of adult *Himatismus* species and a comparison with 21 other species of adult tenebrionid beetle using multivariate analysis. *Comp. Biochem. Physiol.* 91B:371–382.

LUYTENS, I. 1982. Variations intraspécifiques et interspécifiques des hydrocarbures cuticulaires chez *Drosophila simulans* et des espèces affines. *C.R. Acad. Sci.* 295:733–736.

MOREL, L., VANDER MEER, R.K., and LAVINE, B.K. 1988. Ontogeny of nestmate recognition cues in the red carpenter ant (*Camponotus floridanus*). *Behav. Ecol. Sociobiol.* 22:175–183.

NEL, J.J.C. 1968. Aggressive behaviour of the harvester termites *Hodotermes mossambicus* (Hagen) and *Trinervitermes trinervoides* (Sjöstedt). *Ins. Soc.* 15:145–156.

NELSON, D.R., and CARLSON, D.A. 1986. Cuticular hydrocarbons of the tsetse flies *Glossina morsitans morsitans*, *G. austeni* and *G. pallidipes*. *Insect Biochem.* 16:403–416.

NELSON, D.R., CARLSON, D.A., and FATLAND, C.L. 1988. Cuticular hydrocarbons of tsetse flies II: *Glossina fuscipes fuscipes*, *G. palpalis palpalis*, *G. p. gambiensis*, *G. tachinoides*, and *G. brevipaldis*. *J. Chem. Ecol.* 14:963–987.

NOWBAHARI, E., LENOIR, A., CLÉMENT, J.L., LANGE, C., BAGNÉRES, A.G., and JOULIE, C. 1990. Individual, geographical and experimental variation of cuticular hydrocarbons of the ant *Cataglyphis cursor* (Hymenoptera: Formicidae): Their use in nest and subspecies recognition. *Biochem. Syst. Ecol.* 18:63–73.

OBIN, M.S. 1986. Nestmate recognition cues in laboratory and field colonies of *Solenopsis invicta* Buren (Hymenoptera: Formicidae): Effect of environment and role of cuticular hydrocarbons. *J. Chem. Ecol.* 12:1965–1975.

PAGE, M., NELSON, L.J., HAVERTY, M.I., and BLOMQUIST, G.J. 1990. Cuticular hydrocarbons of eight species of North American cone beetles, *Conophthorus* Hopkins. *J. Chem. Ecol.* 16:1173–1198.

PLATTNER, R.D., SPENCER, G.F., and KLEINAN, R. 1976. Double bond location in polyenoic fatty esters through partial oxymercuration. *Lipids* 11:222–227.

POMONIS, J.G., HAKK, H., and FATLAND, C.L. 1989. Synthetic methyl- and dimethylalkanes: Kovats Indices, [^{13}C]NMR and mass spectra of some methylpentacosanes and 2,X-dimethylheptacosanes. *J. Chem. Ecol.* 15:2319–2333.

ROSS, K.G., VANDER MEER, R.K., FLETCHER, D.J.C., and VARGO, E.J. 1987. Biochemical phenotypic and genetic studies of two introduced fire ants and their hybrid (Hymenoptera: Formicidae). *Evolution* 41:280–293.

SPRINGHETTI, A., and AMORELLI, M. 1982. Competitive behavior between two species of Isoptera: *Kalotermes flavicolli* (Kalotermitidae) and *Reticulitermes lucifugus* (Rhinotermitidae). *Sociobiology* 7:155–164.

THORNE, B.L. 1982. Termite-termite interactions: Workers as an agonistic caste. *Psyche* 89:133–150.

VANDER MEER, R.K., LOFGREN, C.S., and ALVAREZ, F.M. 1985. Biochemical evidence for hybridization in fire ants. *Florida Entomol.* 68:501–506.

WIGGLESWORTH, V.B. 1933. The physiology of the cuticule and of ecdysis in *Rhodnius prolixus* (Triatomodae, Hemiptera); with special reference to the function of the oenocytes and of the dermal glands. *Quart. J. Microsc. Sci.* 76:269–318.

WIGGLESWORTH, V.B. 1990. The Distribution, function and nature of "cuticulin" in the insect cuticule. *J. Ins. Physiol.* 36:307–313.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

WIND TUNNEL STUDIES OF SEX PHEROMONE-MEDIATED BEHAVIOR OF THE HESSIAN FLY (DIPTERA: CECIDOMYIIDAE)

M.O. HARRIS[1,3,*] and S.P. FOSTER[2]

[1]*Department of Entomology*
Kansas State University
Manhattan, Kansas, 66506 USA

[2]*D.S.I.R. Plant Protection*
Private Bag
Palmerston North, New Zealand

(Received May 1, 1991; accepted July 22, 1991)

Abstract—In a wind-tunnel, male Hessian flies flying toward a source of the female-produced sex pheromone exhibited flight maneuvers very similar to those described for male moths. Upwind flight, consisting of zigzagging and straight flight upwind, was initiated within seconds after flies were placed in the odor plume. This upwind flight was sometimes interrupted by casting, which consisted of wide excursions in the horizontal plane ranging 10–35 cm across the central zone of the tunnel. Comparison of the flight maneuvers of males exposed to ten female equivalents of a hexane extract of female ovipositors and males exposed to 20 ng of (2S)-(*E*)-10-tridecen-2-yl acetate (SE10-13:OAc), which has been identified as a component of the Hessian fly sex pheromone, indicated that the sex pheromone probably contains additional components. However, SE10-13:OAc elicited upwind flight and source location by a significant number of males, even at dosages as low as 2 ng on filter paper. At the highest dosage of SE10-13:OAc tested (200 ng on filter paper), there was a significant decrease in net flight velocity and a slight, but not significant, reduction in the number of males contacting the odor source. The addition of increasing amounts of the R enantiomer to the S enantiomer resulted in increased inhibition of upwind flight and source contact by males.

Key Words—Hessian fly, *Mayetiola destructor*, Diptera, Cecidomyiidae, flight, anemotaxis, orientation, olfaction, enantiomer, mating.

*To whom correspondence should be addressed.
[2]Present Address: Department of Plant Health, Massey University, Palmerston North, New Zealand.

0098-0331/91/1200-2421$06.50/0

INTRODUCTION

During the last decade, scientists studying the responses of various species of Lepidoptera to sex pheromones have made significant progress toward understanding how male moths locate odor sources (Baker, 1989). By manipulating concentrations and blends of pheromone components and altering pheromonal plume characteristics, they have shown that behavioral thresholds are lowest for the natural blend of components (Linn et al., 1986, 1987) and that there are two main mechanisms for odor source location (Baker, 1986; Kennedy, 1986), optomotor anemotaxis, i.e., steering with respect to the wind, and self-steered counterturning. Most of these studies have been carried out using a sustained-flight tunnel, an arena that enables the researcher to measure flight responses under controlled conditions.

In contrast to moths, many other insects are either less tractable in the wind tunnel or exhibit a different set of responses to pheromones of conspecifics. Some species of beetles, for example, must fly for a certain time before they become responsive to aggregation and sex pheromones (Choudhury and Kennedy, 1980), whereas others must be starved (Bartelt et al., 1990). However, recent successes with beetles flying in the wind tunnel (Birch and White, 1988; Bartelt et al., 1990; Domek et al., 1990) indicate that behavioral mechanisms used in the location of odor sources by some species of Coleoptera may be similar to those used by moths.

In the Diptera, orientation to sex pheromones over long distances is more common in the so-called primitive flies (suborder Nematocera) than in the more advanced flies (Blomquist et al., 1987). Thus, in the families Sciaridae and Cecidomyiidae, males exposed to sex pheromones move upwind in y-tube olfactometers or individual glass cylinders (Kostelc et al., 1980; Alberts et al., 1981; Williams and Martin, 1986), whereas many species in the suborder Cyclorrhapha produce sex pheromones that do not appear to elicit long-range orientation but rather release short-range courtship (Shorey and Bartell, 1970) and copulatory behaviors (Carlson et al., 1984) or increase mating strike activity (Mackley and Broce, 1981). The exception to this trend within the Cyclorrhapha is the tephritid *Dacus oleae* (Haniotakis et al., 1977); in this species, males respond over distances of > 1 meter to the female-produced sex pheromone.

It has been known for many years that male Hessian flies, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae) respond to virgin females over distances of several meters by flying upwind (Cartwright, 1922). The utilization of a sex pheromone by this species was demonstrated by McKay and Hatchett (1984), who showed that males placed in a y-tube olfactometer responded to hexane extracts of ovipositors of virgin females. Recently, Foster et al. (1991) identified a chemical, (2S)-(*E*)-10-tridecen-2-yl acetate (SE10-13:OAc), from

an extract of Hessian fly ovipositor, that elicited upwind movement by male Hessian flies in a y-tube olfactometer.

Further work characterizing the sex pheromone of the Hessian fly required a more discriminating assay. The y-tube olfactometer, although convenient during isolation and identification of sex pheromone components, only indicates whether the addition of a compound to an airstream elicits more upwind movement than an airstream without the compound. Therefore, for studying the efficacy of sex pheromone blends and observing the actual maneuvers used by insects to locate an odor source, this technique is inadequate. The sustained-flight tunnel is a much more discriminating assay (Baker and Linn, 1984) and is especially useful for measuring subtle effects of pheromone components on flight behavior.

Herein, we describe the pheromone-mediated behavior of male Hessian flies in a sustained flight tunnel. Responses to SE10-13:OAc vs. hexane extracts of female ovipositors were compared to determine whether the sex pheromone of the Hessian fly consisted of a blend of chemicals rather than a single component. Responses of males to the R enantiomer were also of interest, because only SE10-13:OAc, and not (2R)-(*E*)-10-tridecen-2-yl acetate (RE10-13:OAc), is found in the natural pheromone (Foster et al., 1991). Finally, because the sex pheromone of the Hessian fly did appear to be a blend consisting of SE10-13:OAc plus at least one other chemical, we measured the responses of male Hessian flies to binary blends of SE10-13:OAc and three racemic mixtures of three other chemicals, (*Z*)-10-tridecen-2-yl acetate (Z10-13-2:OAc), (*E*)-10-tridecen-2-ol (E10-13-2:OH), and tridecen-2-ol (13-2:OAc), which have also been found in the active fraction of extracts of female Hessian flies (Foster et al., 1991).

METHODS AND MATERIALS

Insects. Puparia were collected in the fall of 1986 from wheat fields in Ellis County, Kansas. Although the Great Plains biotype predominates in Ellis County, other biotypes occur at low frequencies and may have been used in these experiments (biotype A and very low frequencies of B) (J. Hatchett, personal communication). However, allozyme studies on Hessian fly biotypes suggest that gene flow is not restricted among biotypes (Black et al., 1990), and hence the sex pheromone of the different biotypes is likely to be the same. This original culture was increased using the techniques outlined in Harris and Rose (1990). Virgin males and females were obtained from single sex progenies. Males emerged between 15.00 and 21.00 hours and were held overnight in groups of 20–30 in cages (10 cm diam. × 20 cm high) located in an environmental chamber (15°C and ca. 70% RH) before use in experiments. Sex pher-

omone extract was prepared by excising the ovipositors of 0-3-hr-old virgin females and extracting the ovipositors in hexane. Both adult males and females were maintained in a 15:9 (L:D) photoperiod, with lights on at 6.00 hours.

Chemicals. Racemic (*E*)-10-tridecen-2-yl acetate (E10-13-2:OAc), Z10-13-2:OAc, SE10-13:OAc, and RE10-13:OAc were synthesized by Dr. J.G. Millar (Millar et al., 1991). E10-13-2:OH was synthesized by reaction of E10-13-2:OAc with 0.5 M KOH in methanol. Racemic 13-2:OAc was supplied by Dr. R.W. Howard (U.S.D.A.-ARS, Manhattan, Kansas). Hexane solutions of the various mixtures tested in the wind tunnel (Figure 4) were prepared with the dosage of SE10-13:OAc held constant at 20 ng and were checked by capillary gas chromatography prior to use. The ratios used in these mixtures were designed to cover the range observed in extracts of Hessian fly ovipositors (Foster et al., 1991). In contrast, the mixtures of SE10-13:OAc and RE10-13:OAc (also based on a constant dosage of 20 ng SE10-13:OAc) were prepared by weight (and subsequent volumetric dilution) and were not checked by gas chromatography because of the inability of the capillary column (30 m × 0.25 mm Supelcowax, Supelco Inc., Bellefonte, Pennsylvania) to resolve the enantiomers.

Testing Protocol. Pheromone-mediated behaviors of male Hessian flies were quantified in a sustained-flight tunnel based on the design of Miller and Roelofs (1978). The Plexiglass™ tunnel was 182 cm in length, 42 cm in height, and 50 cm wide, with a ca. 1 cm layer of moist soil on the bottom. Soil was used because Hessian fly males are small (ca. 0.5 mg and 4 mm in length) and susceptible to entrapment by electrostatic charges on Plexiglas surfaces. Illumination was provided by a series of six Verilux (Greenwich, Connecticut) full-spectrum fluorescent tubes mounted directly above a light diffusing screen. Light intensity on the floor of the tunnel was measured at 290 microwatts/cm^2 by a United Detector Technology Model 61 Radiometer (Hawthorne, California). The wind speed in the tunnel was measured at 0.3 m/sec (Model AB-27 Teledyne hot-wire anemometer, Teledyne Hastings-Raydist, Hampton, Virginia). The plume, which was visualized with smoke generated from burning incense, was located in the middle of the bottom of the tunnel and contacted the soil surface throughout its length. Widths of the plume, just downwind of the source and upon exiting the tunnel, were ca. 1.5 and 15 cm, respectively. Room temperature was held at 22 ± 1°C.

Males (10–18 hrs old) were allowed to acclimate to the temperature of the wind tunnel room for at least 1 hr before being tested between 07.00 and 10.00 hours. This test period was chosen because it corresponds with the time at which (1) males show higher levels of spontaneous flight activity, and (2) females call, thereby emitting sex pheromone (Bergh et al., 1990). An individual male was removed from a cage using an aspirator and placed in a brass screen cylinder (2.5 cm diam × 3.5 cm length) that had screening at one end and was open at the other end. The cage was then placed (open end upwind) in the center of the

wind tunnel, 85 cm downwind of the odor source. The odor source consisted of a rectangular strip of filter paper (ca. 0.5 × 2 cm) mounted on a partially straightened paper clip, 3 cm above the soil surface. Synthetic pheromone or ovipositor extract was applied to the downwind edge of the paper strip. Males were scored for: taking flight out of the cage (activation), apparent within-plume flight for >10 cm, upwind displacement to within 50, 15, and 2 cm of the source, and source contact. Wing fanning, a pre-flight behavior commonly observed in studies of pheromone-mediated responses of moths, was not evident in male Hessian flies. Observations were terminated when the male (1) remained still in the cage for more than 1 min, (2) contacted the walls of the tunnel, or (3) flew out of the back of the tunnel. Preliminary observations showed that Hessian fly males that lost the pheromone plume typically flew to the side of the tunnel and sat for periods of at least 5 min, and exhibited little, if any, sexual activity. The times from the introduction of the fly into the odor plume to (1) taking flight out of the cage, and (2) to contacting the source were also recorded. Once males contacted the odor source, they were removed from the wind tunnel and were not tested again.

Because of the relatively rapid release rates of volatile chemicals from filter paper sources, only five males were tested for each filter paper source; this procedure was usually completed within ca. 5 min. After each group of five males was tested, a thin layer of fresh soil was added to the tunnel floor and sprayed with a fine mist of water. Flies were tested in a randomized complete block design with five successive males per block per treatment. After testing hundreds of males, we found no evidence to suggest that the soil substrate became contaminated with sex pheromone during the five successive flights to a single source; that is, the last male flying to the source was not more or less likely to contact the source than the first male. Between 45 and 75 males were tested with each of the extracts, chemicals, and chemical blends.

Mean latencies to taking flight and to contacting the source were compared by one-way ANOVA. When the ANOVA indicated significant differences among treatments, 95% confidence intervals were used to separate means. Differences in the distributions of males in response categories were tested using G tests or 2 × 2 or R × C tests of independence using the G statistic, with adjusted G's when there were only two treatments being compared (Sokal and Rohlf, 1969), or by logistic ANOVA (McCullagh, 1980) when variances were sufficiently small to warrant such an analysis.

RESULTS

Description of the Flight Maneuvers of Male Hessian Flies. When placed in the odor plume of ovipositor extract, male Hessian flies took flight within ca. 6 sec and initiated upwind flight. This flight occurred at a height of ca.

2–6 cm above the soil surface and varied, sometimes consisting of narrow zigzags (counterturning with significant resultant progression; see Kennedy, 1986) and at other times appearing to be straight flight upwind (track angles near 0°). Upwind flight was sometimes interrupted by casting (counterturning with no significant net progression upwind; see Kennedy, 1986), which, in the male Hessian fly, consisted of wide sweeps in the horizontal plane, ranging ca. 10–35 cm across the central zone of the tunnel. Frequently, casting was followed by the male again locating the odor plume and flying upwind. Upwind flight was also interrupted when males (1) flew out of the plume without casting, or (2) alighted on the soil beneath the odor plume. Males exhibiting the former behavior generally left the odor plume by flying upward. Once out of the plume, these males typically flew near the upper or side walls for several seconds, and then alighted on the sides of the tunnel, where they remained for at least several minutes. Upwind flight followed by alighting on the soil occurred at a lower frequency (ca. 0.5% of all males). However, when it occurred, the male faced upwind and either resumed upwind flight or flew out of the odor plume. Upon contacting the odor source, all males continued to fan their wings and arch their abdomen, thereby bringing the tip of the abdomen into contact with the filter paper in what appeared to be a copulatory attempt. The few males that flew past the filter paper (without landing) initiated casting after flying upwind 5–10 cm beyond the source, and, then, always regained the odor plume after drifting downwind of the odor source. The shortest and longest flight times from the release cage to the odor source (85 cm) were 6 and 131 seconds, respectively, which are equivalent to average net up-tunnel velocities (Kuenen and Baker, 1982) of 14.2 and 0.65 cm/sec.

Flight Maneuvers in Response to Extract of Sex Pheromone Glands of Females and Synthetic Pheromone. Male Hessian flies exposed to ovipositor extract (ten female equivalents) or to synthetic SE10-13 : OAc (20 ng) exhibited the same maneuvers but with different frequencies. With either odor source, most males took flight (activated) after being placed in the tunnel (Figure 1). Males that took flight did so with a similar latency for both treatments, ranging from 1–51 sec ($x \pm SE = 6.9 \pm 1.8$) for males exposed to extract and 1–17 sec ($x \pm SE = 4.0 \pm 0.6$) for males exposed to SE10-13 : OAc. Only rarely did males that took flight exit the odor plume without exhibiting upwind flight (Figure 1). However, after this stage, a larger number of the males exposed to SE10-13 : OAc lost the odor plume as they progressed up the tunnel to the pheromone source. Having reached the 2-cm point, no male, whether exposed to extract or SE10-13 : OAc, failed to continue upwind and contact the odor source. The overall result of these maneuvers was that 39/45 (87%) of the males exposed to extract contacted the source in contrast to only 25/45 (56%) of the males exposed to SE10-13 : OAc ($G_{adj} = 10.02$, $df = 1$, $P < 0.01$). To compare the overall responses of males to extract vs. SE10-13 : OAc, males were categorized

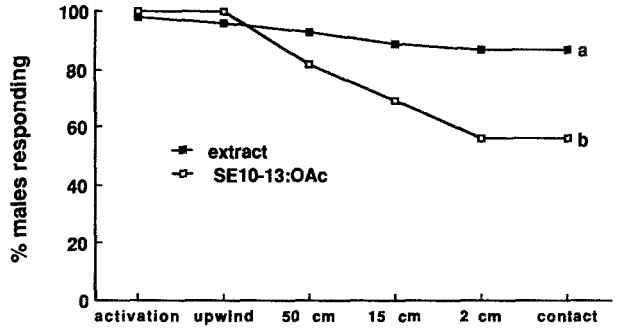


FIG. 1. Flight maneuvers of male Hessian flies exposed to 10 FE sources of extract of female ovipositors (solid squares) and 20 ng sources of (2S)-tridecen-2-yl acetate (SE10-13:OAc-open squares). Activation refers to the percent of males taking flight. Numbers of males contacting the two sources were significantly different at $P < 0.01$ (G test). Number of males tested to each source = 45.

as (1) those that did not exhibit upwind flight, (2) those that exhibited upwind flight but did not contact the source, and (3) those that exhibited upwind flight and contacted the source. Using these response categories, we found that the distribution within these categories differed significantly for males exposed to extract compared to those exposed to SE10-13:OAc (3×2 test of independence, $G = 17.54$, $df = 2$, $P < 0.001$). Net up-tunnel velocities (time from taking flight to contacting the odor source) were similar to extract or to SE10-13:OAc, ranging from 1.0 to 12.1 cm/sec ($x \pm SE = 3.7 \pm 0.4$) and from 1.1 to 8.5 cm/sec ($x \pm SE = 3.5 \pm 0.5$), respectively.

SE10-13:OAc Dose-Response Relationships. Males that flew out of cages after being exposed to 2, 6, 20, 60, and 200 ng of SE10-13:OAc did so with similar latencies, 5.95, 3.33, 5.55, 7.13, and 7.83 sec, respectively ($F_{(4,210)} = 1.50$, $P < 0.20$). Numbers of males contacting the source were also not significantly different for the range of dosages (38%, 49%, 53%, 51%, and 38% for 2, 6, 20, 60, and 200 ng, respectively, $G_{(4)} = 4.56$, $P < 0.50$). However, fitting a quadratic response curve to these data yielded a significant improvement over a linear response curve (chi square = 4.02, $df = 1$, $P < 0.05$), an indication that the number of males contacting the odor source reached a maximum and then decreased with increasing dosage. In addition, in comparison to males flying to the 20 ng source, males exposed to the lowest dosage (2 ng) exited the odor plume during upwind flight more frequently, whereas males exposed to the highest dosage (200 ng) appeared more likely to exit the odor plume before exhibiting upwind flight (Figure 2). Net up-tunnel velocities for males contacting the odor source (Figure 3) were significantly faster with 20 ng than with 200 ng ($F_{(4,98)} = 3.53$, $P < 0.01$, 95% confidence intervals).

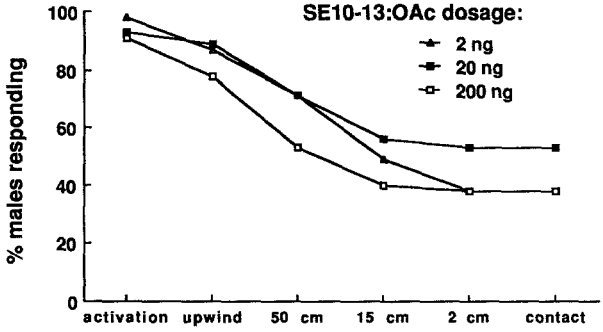


FIG. 2. Behaviors of male Hessian flies exposed to three different dosages of SE10-13:OAc. Males were also exposed to 6 and 60 ng sources; however, these results are not shown. Activation refers to the percent of males taking flight. Numbers of males contacting the source were not significantly different at $P < 0.05$ (G test). Numbers of males tested to each source = 45.

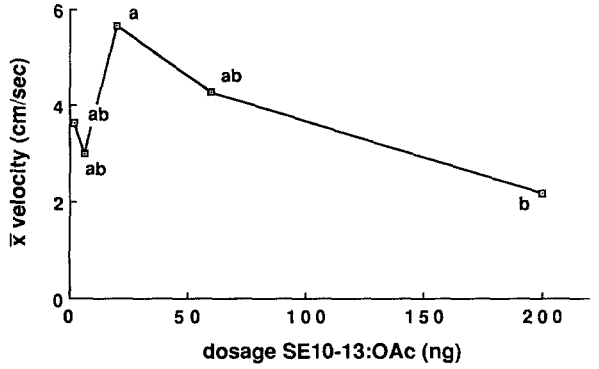


FIG. 3. Net up-tunnel velocities of male Hessian flies flying upwind to and contacting filter paper with different dosages of SE10-13:OAc (2, 6, 20, 60, and 200 ng). Means accompanied by the same letter are not significantly different at $P < 0.05$ (one-way ANOVA, 95% confidence intervals).

Binary Blends of SE10-13:OAc and Other Chemicals Found in an Extract of Female Ovipositors. Similar numbers of males (Figure 4) contacted the odor source when the dosage of SE10-13:OAc was held constant at 20 ng and ratios of Z10-13-2:OAc in SE10-13:OAc ($G_{(3)} = 1.36$, $P < 0.80$), ratios of E10-13-2:OH in SE10-13:OAc ($G_{(2)} = 1.18$, $P < 0.50$), and ratios of 13-2:OAc in SE10-13:OAc ($G_{(3)} = 0.90$, $P < 0.99$) were presented to males. With all binary blends, flight times were not significantly different.

Flight Responses to Mixtures of R and S Enantiomers. The addition of

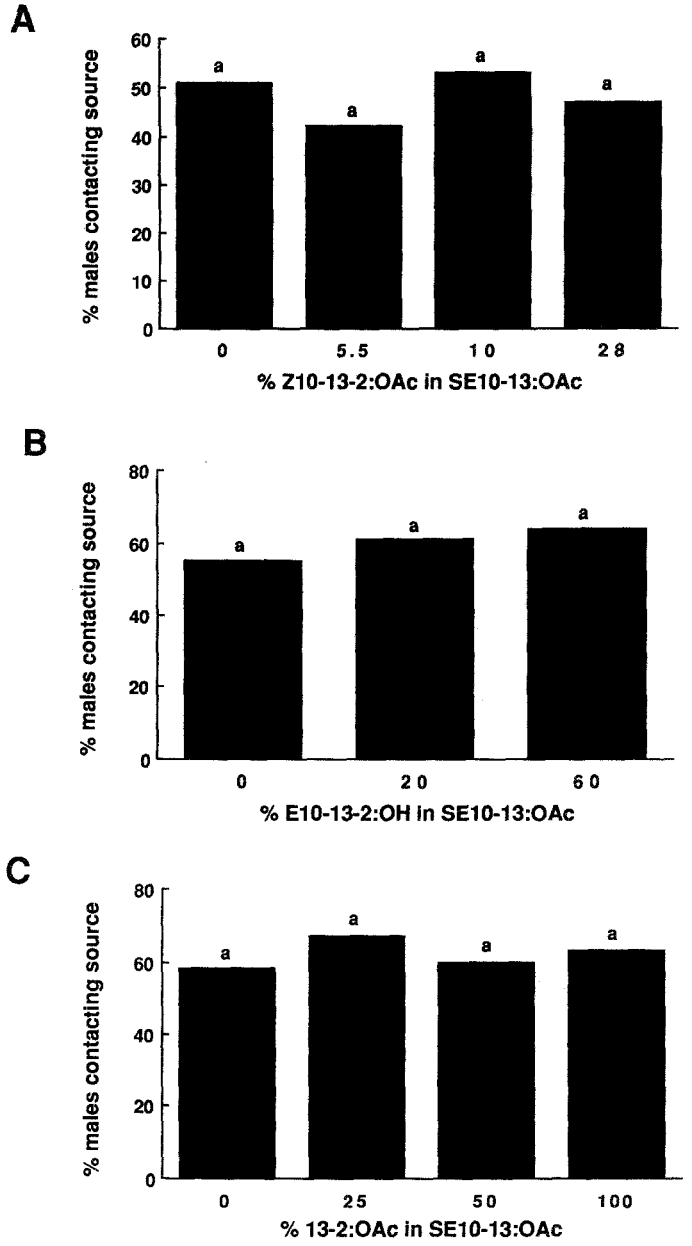


FIG. 4. Percentages of male Hessian flies contacting the odor source given (A) ratios of Z10-13-2:OAc in SE10-13:OAc, (B) ratios of E10-13-2:OH in SE10-13:OAc, and (C) ratios of 13-2:OAc in SE10-13:OAc (acronyms as in text). In all tests, the dosage of SE10-13:OAc was held constant at 20 ng. Within each graph, numbers of males contacting the source were not significantly different at $P < 0.05$ (G test). Numbers of males tested were (A) = 45, (B) = 75, and (C) = 60.

either 20% or 100% RE10-13:OAc to SE10-13:OAc (Figure 5) resulted in more males losing the odor plume during upwind flight and fewer males contacting the odor source (logistic ANOVA, $P < 0.05$). Net up-tunnel velocities towards the 100% S, 100:20 S:R, and 100:100 S:R sources were 3.13, 2.54, and 2.96 cm/sec, respectively, and were not significantly different at $P < 0.05$. A small number of males (4/60) also flew upwind to and contacted the 100% RE10-13:OAc odor source. Males never contacted the source when presented with a filter paper source treated with hexane (Figure 5, blank).

DISCUSSION

The flight maneuvers of male Hessian flies in the presence of the female-produced sex pheromone were very similar to those described for male moths (see reviews, Kennedy, 1986; Baker, 1989). As with moths, flight responses of male Hessian flies varied, sometimes consisting of narrow zigzags and at other times appearing to be straight flight upwind. Casting, a maneuver exhibited by moths when contact with the odor plume is lost, was sometimes exhibited by Hessian fly males flying upwind toward the odor source and also occurred when males flew upwind of the odor source. Upon contacting the source, male Hessian flies arched the abdomen in what appeared to be a copulatory attempt.

To determine whether SE10-13:OAc could account for much or all of the behavioral activity of the sex pheromone, flight maneuvers were contrasted for Hessian fly males flying to SE10-13:OAc vs. a hexane extract of female ovi-

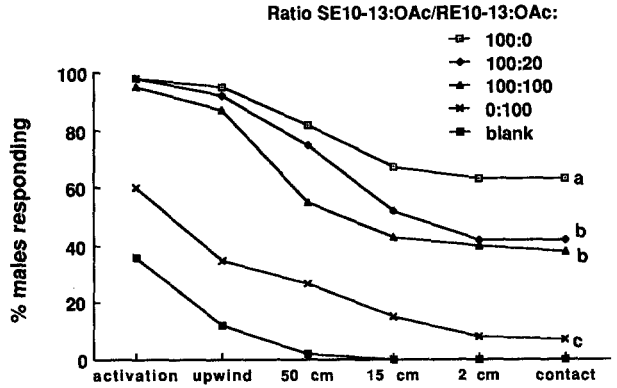


FIG. 5. Behaviors of male Hessian flies exposed to various ratios of the S and R enantiomers of E10-13:OAc. The dosage of SE10-13:OAc was held constant at 20 ng for the first three treatments listed. Activation refers to the percent of males taking flight. Numbers of males contacting the source were not significantly different if accompanied by the same letter ($P < 0.05$, G test). Numbers of males tested with each source = 60.

positors. A ten-female equivalent dosage of extract was contrasted with 20 ng of SE10-13:OAc because females contain, on average, ca. 2.0 ng of SE10-13:OAc (Foster et al., 1992). In contrast to males exposed to female extract, males flying to SE10-13:OAc typically did not fly as far upwind (Figure 1). As a result, only 56% of the males contacted the SE10-13:OAc source, compared to 87% of the males that contacted the female extract source. These data suggest that the sex pheromone of the female Hessian fly consists of a blend of two or more components, rather than just SE10-13:OAc alone. The larger number of male Hessian flies exhibiting upwind flight and source contact to the extract could indicate lower behavioral thresholds for the true pheromone blend and a greater response to the blend at all distances from the odor source as has been shown in several species of moths (Linn et al., 1986, 1987). The alternative explanation, that SE10-13:OAc elicited less upwind flight because of the 1% impurity of the R enantiomer, seems less likely, given the relatively small effect that a larger amount of the R enantiomer had when presented with SE10-13:OAc (Figure 5).

Three other chemicals identified in the extract of female ovipositors, Z10-13-2:OAc, E10-13-2:OH, and 13-2:OAc (Foster et al., 1991), were also tested in binary mixtures with SE10-13:OAc. However, the addition of any of these chemicals to SE10-13:OAc did not affect the percentage of males flying upwind and contacting the odor source. Although it is possible that these components interact, i.e., that tertiary or quaternary blends including SE10-13:OAc would elicit responses in more males (see Linn et al., 1984), we currently have no data to support these chemicals being classified as pheromone components.

That the sex pheromone of the Hessian fly probably consists of a blend of chemicals is not surprising. Blends of chemicals are the rule rather than the exception in the sex pheromones of Lepidoptera and Coleoptera. However, in many insects that use blends of chemicals to locate mates, a single component, when presented by itself, either does not elicit source location and/or upwind flight or only elicits these maneuvers at very high dosages, and even then, only in a small number of males (Linn et al., 1986). This does not appear to be the case with the Hessian fly, at least not within the range of dosages we tested. In the presence of even relatively small dosages of SE10-13:OAc (2 ng, ca. 1 female equivalent), significant numbers of males exhibited activation (98%), upwind flight (62%), and source contact (38%). Thus, male Hessian flies appear to have extremely low thresholds for activation and orientation (Linn and Roelofs, 1989) in response to this component.

At the highest dosage of SE10-13:OAc tested (200 ng/filter paper), there was a slight, but not significant reduction in the number of Hessian fly males flying upwind and contacting the odor source (38% for 200 ng vs. 53% for 20 ng sources). However, net up-tunnel velocities (times in flight from leaving the cage to contacting the source) of the males that did locate the 200 ng source

were two times slower than those of males flying to 20 ng (Figure 3). Kuenen and Baker (1982), studying the effects of pheromone concentration on the flight behavior of the oriental fruit moth, also found a decrease in net up-tunnel velocity with higher concentrations. By analyzing flight tracks, they determined that moths flying to pheromone sources of higher concentration turned more frequently and did so a shorter distance away from the longitudinal axis of the pheromone plume. In-flight arrestment, consisting of long looping turns and movement downwind and out of the plume, was also exhibited more frequently by moths flying towards sources with higher concentration. Although we did not record flight tracks of male Hessian flies (it would be difficult because of the small size of males), upwind flight of males flying to sources loaded with 200 ng of SE10-13:OAc seemed to be interrupted more frequently by bouts of casting (counterturning with no significant net progression), which included looping turns and frequent downwind displacement. Although some of these males subsequently exited the back of the tunnel or alighted on the walls of the tunnel, others relocated the odor plume and eventually contacted the odor source, albeit after significantly longer flight times.

Hessian fly females produce only the S enantiomer and not the R enantiomer of the sex pheromone component E10-13:OAc (Foster et al., 1991). Thus, responses to the other enantiomer were also of interest. Silverstein (1988), in a review of chirality in insect communication, states that there are five possible responses to another enantiomer in insects that produce a single enantiomer: (1) less active or inactive, (2) equally active, (3) more active, (4) synergistic, and (5) inhibitory. The first of these responses is the most common, with several cases of inhibition also reported. When inhibition does occur, it often takes only small amounts of one enantiomer to strongly inhibit or virtually block responses to the other enantiomer (Tumlinson et al., 1977; Birch et al., 1980).

Inhibition of behavioral responses with the addition of the R enantiomer to the S enantiomer also occurred in the Hessian fly (Figure 5). However, relatively large amounts of the R enantiomer were necessary to inhibit flight responses of males; even with 100:100 ratios of S:R, 38% of the males contacted the source, in contrast to the 63% of all males flying toward the 100:0 S:R sources. These data suggest that male Hessian flies have olfactory receptors that detect the R enantiomer. However, whether the R enantiomer interacts with the S-receptor cell or with its own specific receptor cell is not known.

The apparently contradictory result of a small percent of male Hessian flies (6.7%) flying to the source loaded with nominal 100% R enantiomer is probably explained as the combination of the relatively weak inhibitory effect of the R enantiomer and the small impurity of the S enantiomer present in our sample. It is not unreasonable that the impurity of the S enantiomer (1% = 0.2 ng) could have triggered orientation responses in 6.7% of the males, given the rel-

atively high percentage of males (38%) flying upwind to and contacting the source with 2 ng of the S enantiomer.

It has been suggested that studies on insects in orders other than the Lepidoptera may reveal that optomotor anemotaxis and self-steered counterturning are not the only mechanisms available to insects flying towards pheromone sources (Cardé, 1986). Our study of the Hessian fly and recent studies of Coleoptera (Bartelt et al., 1990; Domek et al., 1990) suggest that the converse may also be true, i.e., that many insects in distantly related orders may have evolved behavioral mechanisms similar to those that have been so rigorously studied in the Lepidoptera. Further studies on insects such as the Hessian fly will help to determine if the orientation mechanisms they use differ in any way from those used by moths, and whether, given the constraints of sensory and central processing systems in insects and the vagaries of odor plumes in natural environments, flying insects have additional behavioral options for odor source location.

Acknowledgments—Our thanks to Dr. Chris Bergh for building the wind tunnel, conducting initial studies on male flight responses to female sex pheromone, and for rearing Hessian flies, to Susan Rose for technical help, to Mr. John Maindonald (D.S.I.R. Physical Sciences, Auckland) for advice on statistical analyses, to Dr. Jocelyn Millar (U.C. Riverside) for synthesizing several of the chemicals used in this study, and to Eileen Schofield (Kansas Agricultural Experiment Station) for editorial comments. This work was supported by KAES Hatch funds and is contribution No. 91-238-J from the Kansas Agricultural Experiment Station, Manhattan.

REFERENCES

ALBERTS, S.A., KENNEDY, M.K., and CARDÉ, R.T. 1981. Pheromone-mediated anemotactic flight and mating behavior of the sciarid fly *Bradysia impatiens*. *Environ. Entomol.* 10:10–15.

BAKER, T.C. 1986. Pheromone-modulated movements of flying moths, pp. 39–48, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

BAKER, T.C. 1989. Sex pheromone communication in the Lepidoptera: New research progress. *Experientia* 45:248–262.

BAKER, T.C., and LINN, C.E. 1984. Wind tunnels in pheromone research, pp. 45–73, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research. Springer, New York.

BARTELT, R.J., DOWD, P.F., PLATTNER, R.D., and WEISLEDER, D. 1990. Aggregation pheromone of driedfruit beetle, *Carpophilus hemipterus*: Wind-tunnel bioassay and identification of two novel tetraene hydrocarbons. *J. Chem. Ecol.* 16:1015–1039.

BERGH, J.C., HARRIS, M.O., and ROSE, S. 1990. Temporal patterns of emergence and reproductive behavior of the Hessian fly, *Mayetiola destructor* (Diptera: Cecidomyiidae). *Ann. Entomol. Soc. Am.* 83:998–1004.

BIRCH, M.C., and WHITE, P.R. 1988. Responses of flying male *Anobium punctatum* (Coleoptera: Anobiidae) to female sex pheromone in a wind tunnel. *J. Insect Behav.* 1:111–115.

BIRCH, M.C., LIGHT, D.M., WOOD, D.L., BROWNE, L.E., SILVERSTEIN, R.M., BERGOT, B.V., OHLOFF, G., WEST, J.R., and YOUNG, J.C. 1980. Pheromonal attraction and allomonal interruption of *Ips pini* in California by the enantiomers of ipsdienol. *J. Chem. Ecol.* 6:703–717.

BLACK, W.C., HATCHETT, J.H., and KRCHMA, J.L. 1990. Allozyme variation among populations of the Hessian fly (*Mayetiola destructor*) in the United States. *J. Hered.* 81:331–335.

BLOMQUIST, G.L., DILLWITH, J.W., and ADAMS, T.S. 1987. Biosynthesis and endocrine regulation of sex pheromone production in Diptera, pp. 217–250, *in* G.D. Prestwich and G.J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, Orlando.

CARDÉ, R.T. 1986. Epilogue: Behavioral mechanisms, pp. 175–186, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

CARLSON, D.A., NELSON, D.R., LANGLEY, P.A., COATES, T.W., DAVIS, T.L., and LEEGWATER-VAN DER LINDEN, M.E. 1984. Contact sex pheromone in the tsetse fly *Glossina pallidipes* (Austen): Identification and synthesis. *J. Chem. Ecol.* 10:429–450.

CARTWRIGHT, W.B. 1922. Sexual attraction of the female Hessian fly. *Can. Entomol.* 54:154.

CHOUDHURY, J.H., and KENNEDY, J.S. 1980. Light versus pheromone-bearing wind in the control of flight direction by bark beetle, *Scolytus multistriatus*. *Physiol. Entomol.* 5:207–214.

DOMEK, J.M., TUMLINSON, J.H., and JOHNSON, D.T. 1990. Responses of male green June beetles *Cotinus nitida*(L.)(Coleoptera: Scarabaeidae) to female volatiles in a flight tunnel. *J. Insect Behav.* 3: 271–276.

FOSTER, S.P., HARRIS, M.O., and MILLAR, J.G. 1991. Identification of the sex pheromone of the Hessian fly, *Mayetiola destructor* (Say). *Naturwiss.* 78:130–131.

FOSTER, S.P., BERGH, J.C., ROSE, S., and HARRIS, M.O. 1992. Aspects of pheromone biosynthesis in the Hessian fly, *Mayetiola destructor* (Say). *J. Insect Physiol.* In press.

HANIOTAKIS, G.E., MAZOMENOS, B.E., and TUMLINSON, J.H. 1977. A sex attractant of the olive fruit fly, *Dacus oleae* and its biological activity under laboratory and field conditions. *Ent. Exp. Appl.* 21:81–87.

HARRIS, M.O., and ROSE, S. 1990. Chemical, color, and tactile cues influencing oviposition behavior of the Hessian fly (Diptera: Cecidomyiidae). *Environ. Entomol.* 19:303–308.

KENNEDY, J.S. 1986. Some current issues in orientation to odour sources, pp. 11–26, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

KOSTELC, J.G., GIRARD, J.E., and HENDRY, L.B. 1980. Isolation and identification of a sex attractant of a mushroom-infesting sciarid fly. *J. Chem. Ecol.* 6:1–11.

KUENEN, L.P.S., and BAKER, T.C. 1982. The effects of pheromone concentration on the flight behavior of the oriental fruit moth, *Grapholita molesta*. *Physiol. Entomol.* 7:423–434.

LINN, C.E., and ROELOFS, W.L. 1989. Response specificity of male moths to multicomponent pheromones. *Chemical Senses* 14:421–437.

LINN, C.E., BJOSTAD, L.B., DU, J.W., and ROELOFS, W.L. 1984. Redundancy in a chemical signal: Behavioral responses of male *Trichoplusia ni* to a 6-component sex pheromone blend. *J. Chem. Ecol.* 10:1635–1658.

LINN, C.E., CAMPBELL, M.G., and ROELOFS, W.L. 1986. Male moth sensitivity to multicomponent pheromone: Critical role of female-released blend in determining the functional role of components and active space of the pheromone. *J. Chem. Ecol.* 12:659–668.

LINN, C.E., CAMPBELL, M.G., and ROELOFS, W.L. 1987. Pheromone components and active spaces: What do moths smell and where do they smell it? *Science* 237:650–652.

MACKLEY, J.W., and BROCE, A.B. 1981. Evidence of a female sex recognition pheromone in the screwworm fly. *Environ. Entomol.* 10:405–408.

MCCULLAGH, P. 1980. Regression models for ordinal data. *J. Roy. Stat. Soc. B* 42:109–142.

MCKAY, P.A., and HATCHETT, J.H. 1984. Mating behavior and evidence of a female sex pheromone in the Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae). *Ann. Entomol. Soc. Am.* 77:616–620.

MILLAR, J.G., FOSTER, S.P., and HARRIS, M.O. 1991. Synthesis of the stereoisomers of the sex pheromone of the Hessian fly. *J. Chem. Ecol.* 17:2437–2448.

MILLER, J.R., and ROELOFS, W.L. 1978. Sustained-flight tunnel for measuring insect responses to wind-borne sex pheromones. *J. Chem. Ecol.* 4:685–699.

SHOREY, H.H., and BARTELL, R.J. 1970. Role of a volatile female sex pheromone in stimulating male courtship behavior in *Drosophila melanogaster. Anim. Behav.* 18:159–164.

SILVERSTEIN, R.M. 1988. Chirality in insect communication. *J. Chem. Ecol.* 14:1981–2004.

SOKAL, R.R., and ROHLF, F.J. 1969. Biometry. W.H. Freeman and Company, San Francisco. 776 pp.

TUMLINSON, J.H., KLEIN, M.G., DOOLITTLE, R.E., LADD, T.L., and PROVEAUX, A.T. 1977. Identification of the female Japanese beetle sex pheromone: Inhibition of male response by an enantiomer. *Science* 197:789–792.

WILLIAMS, I.H., and MARTIN, A.P. 1986. Evidence for a female sex pheromone in the brassica pod midge *Dasineura brassicae. Physiol. Entomol.* 11:353–356.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

SYNTHESIS OF THE STEREOISOMERS OF THE FEMALE SEX PHEROMONE OF THE HESSIAN FLY, *Mayetiola destructor*

JOCELYN G. MILLAR,[1,*] STEPHEN P. FOSTER,[2] and MARION O. HARRIS[3,4]

[1]*Department of Entomology*
University of California
Riverside, California 92521

[2]*D.S.I.R. Plant Protection*
Private Bag
Palmerston North, New Zealand

[3]*Department of Entomology*
Kansas State University
Manhattan, Kansas 66506

(Received June 10, 1991; accepted August 5, 1991)

Abstract—The female-produced sex pheromone of the Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), has been identified as (10*E*)-tridecen-2-yl acetate. A flexible synthetic route was developed which allowed access to the chiral and racemic forms of the pheromone, and to the 10*Z* stereoisomer of the pheromone. The natural compound was determined to have the 2*S* configuration by hydrolysis of the acetate function, derivatization of the resulting alcohol with (2*S*)-2-acetoxypropionyl chloride, and capillary gas chromatographic comparison of the derivative with the corresponding derivatives prepared from the synthetic enantiomers. Trace amounts of the 10*Z* isomer of the pheromone have also been detected in extracts of female Hessian fly ovipositors, along with (10*E*)-tridecen-2-ol and 2-tridecanyl acetate. Due to the small quantities of these compounds available from ovipositor extracts, the chirality of the trace components has not yet been determined.

Key Words—(10*E*)-tridecen-2-yl acetate, (10*Z*)-tridecen-2-yl acetate, sex pheromone, Diptera, Cecodomyiidae, Hessian fly, *Mayetiola destructor*.

*To whom correspondence should be addressed.
[4]Present address: Department of Plant Health, Massey University, Private Bag, Palmerston North, New Zealand.

0098-0331/91/1200-2437$06.50/0

INTRODUCTION

Wheat is an important cereal crop in many countries of the world, providing some 20% of the food calories consumed by mankind (Feldman, 1976). One of the most destructive insect pests of wheat is the Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae). Current methods of controlling Hessian fly damage include breeding of resistant wheat varieties, using cultural practices such as rotation of crops and delaying planting of wheat until late fall, and occasionally use of systemic insecticides (Foster et al., 1986). In addition, natural enemies of the Hessian fly often build up late in the season, but by this time most of the crop damage has already occurred (Foster et al., 1986).

It has been known for many years that adult female Hessian flies produce a sex pheromone which is attractive to male flies over distances of at least several meters (Cartwright, 1922; Mackay and Hatchett, 1984). The identity of an attractive sex pheromone component from female Hessian fly was very recently reported as (2*S*,10*E*)-tridecen-2-yl acetate in a preliminary communication (Foster et al., 1991). We report here the details of the synthesis of the racemic and chiral forms of the pheromone, and the synthesis of a geometric isomer of the pheromone. We also report the details of the determination of the chirality of the pheromone.

EXPERIMENTAL

Proton NMR spectra ($CDCl_3$) were recorded on a GE-300 NMR spectrometer, at 300 MHz. IR spectra were recorded as films of neat compounds on NaCl plates, with a Perkin–Elmer 137 spectrometer. Mass spectra (electron impact, 70 eV) were recorded with a Hewlett–Packard (H–P; Avondale PA) 5970 mass selective detector, interfaced to a H–P 5890 capillary GC. An HP-1 capillary column (12.5 m × .2 mm ID, 0.33 micron film; H–P) was used for all gas chromatography-mass spectrometry (GC-MS) work. Routine gas chromatography (GC) runs were carried out with an H–P 5890 instrument with flame ionization detection (FID), fitted with a DB-5 column (20 m × .32 mm ID, 0.25 micron film; J&W Scientific, Folsom CA) in split mode, using He carrier gas. GC signals were integrated with an H–P 3396A integrator. Optical rotations were measured with a Perkin–Elmer Model 241 polarimeter, using a 1 decimeter cell with a volume of approx. 1 ml.

All reagents were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin) or Fisher Scientific (Pittsburgh, Pennsylvania) unless otherwise stated.

Unless otherwise stated, reaction mixtures were extracted three times with the stated solvent, and the combined extracts were washed with brine, dried over anhyd. Na_2SO_4, and concentrated on a rotary evaporator under water aspi-

rator vacuum. Flash chromatography was carried out with 230–400 mesh silica gel (Aldrich Chemical Co.).

Enantiomeric excesses (e.e.) of alcohols 3–5 and 8 were determined by derivatization of the alcohols with (2*S*)-2-acetoxypropionyl chloride. Thus, two equivalents of the acid chloride were added to dilute solutions (~0.1 M) of the alcohols (1 equivalent) in a mixture of ether and triethylamine (approx. five equivalents) at room temperature. The mixtures were mixed thoroughly, and allowed to stand overnight. The mixtures were then washed thoroughly with excess saturated aqueous $NaHCO_3$, the organic portions were dried over anhyd. Na_2SO_4, and the resulting solutions of diastereomeric esters were analyzed by capillary GC on the DB-5 column as described above. Enantiomeric excesses were determined by comparison of the integrated peak areas of the diastereomers. In all cases, baseline separation of the two diastereomers was achieved. In addition, there was no trace of the alcohol starting material, ensuring that the chirality determination had not been distorted by diastereoselective kinetic effects during the derivatization.

Insects and Extracts. Hessian flies were from a laboratory culture originally established from collections from wheat fields in Ellis County, Kansas in fall of 1986. These insects were probably of the Great Plains biotype, although there may also have been low numbers of biotypes A and B. The insects were reared on Newton wheat in the 2–3 leaf stage (Harris and Foster, 1991). As the majority of Hessian fly females lay all male or all female progenies (Gallun et al., 1961), virgin male or female insects were harvested off the individual wheat plants shortly after they emerged, at approx. 15–21:00 hr (for males) and 4:30–7:30 hr (for females). Males were stored in cages in an environmental chamber at 15°C and 70% relative humidity, and 15:9 light:dark cycle until used the following morning.

For analyses of natural pheromone, and for use in olfactometer tests, the terminal abdominal segments of females, 0–3 hr old, were dissected under a binocular scope, placed in hexane, and allowed to extract for at least an hour before use.

The double bond geometry of the pheromone component in the extract was determined by comparison of its retention times with those of synthetic standards on the DB-5 column (70–200°C @ 5°C/min) and on a 30 m × 0.25 mm ID SP2340 column (Supelco, Bellefonte, Pennsylvania; same temperature program as for DB-5 column). (4*E*)-Tridecenyl acetate was used as an internal standard.

The chirality of the Hessian fly-produced compound was determined by hydrolysis of an extract of 200 female abdominal tips (0.5 M KOH in MeOH), and reaction of the resulting free alcohol with excess (2*S*)-2-acetoxypropionyl chloride in 10% pyridine in ether for approx. 24 hr at ambient temp. (Slessor et al., 1985). The retention time on the DB-5 column and the mass spectrum

of the reaction product were compared with those of the compounds obtained from reaction of the synthetic enantiomers of (10*E*)-tridecen-2-ol with (2*S*)-2-acetoxypropionyl chloride.

Bioassays. The Y-tube olfactometer was similar to that described by Chaudhury et al. (1972). The two equivalent arms of a glass Y-tube were attached to two straight glass tubes (2.9-cm diam.) which functioned as traps for the responding male flies. These glass tubes were attached to two larger tubes (4-cm diam. × 15-cm long) into which the sample being tested and a solvent blank were placed (one into each of the respective arms). The sample consisted of the appropriate amount of chemical in *n*-hexane absorbed onto a quadrant of filter paper (2.5-cm radius); the blank consisted of an equal amount of *n*-hexane absorbed onto a similar piece of filter paper. The third arm of the Y-tube was attached to a large glass chamber (19-cm long × 6.3-cm diam.) into which male flies were introduced at the commencement of the test. Air, purified by passage through activated carbon filters and subsequently humidified by being passed through water, was pumped through the olfactometer at a rate of 250 ml/min/arm. Each test was run for 10 min. At the end of each test, the number of males in the sample trap, the blank trap, the Y-tube, and the introduction chamber were recorded. Twenty-five insects were used in each test, and each assay was replicated six times, with the exception of the bioassay of the ovipositor extract, which was run with ten insects/replicate, and four replicates. All tests were run between 6–10 a.m., with freshly emerged insects, under fluorescent light, and at ambient air temperatures (~20–25°C). Data on the distribution of flies between the control and test stimulus arms of the olfactometer were compared against equal distributions of flies between the two arms using Chi-square tests.

Synthesis

4-Undecyn-2-ol 3. n-BuLi (60 ml of a 2.5 M solution in hexanes, 150 mmol) was added dropwise to a cooled (< −10°C) solution of 1-octyne 1 (16.5 g, 150 mmol) in THF (250 ml). The mixture was stirred for 30 min, then cooled to −20°C. HMPA (50 ml) was added, followed by dropwise addition of racemic propylene oxide 2 (10.15 g, 175 mmol). The solution was warmed to 20°C overnight, then poured into water and extracted with hexane, and worked up as described above. The crude product was distilled under vacuum, yielding alcohol 3 (24.18 g, 96%), bp 68–70°C (0.2 mm Hg). Lit bp, 65–72°C (0.2 mm Hg) (Oehlschlager et al., 1985). ^{1}H NMR δ: 3.90 (m, 1H; H-2), 2.37 (ddt, 1H, J = 16.4, 4.9, 2.4 Hz; H-3), 2.27 (ddt, 1H, J = 16.4, 6.8, 2.3 Hz; H-3′) 2.15 (tt, 2H, J = 6.9, 2.4 Hz; H-6), 1.98 (d, 1H, J = 4.7 Hz; -OH), 1.55–1.44 (m, 2H; H-7), 1.43–1.25 (m, 6H; H-8,9,10), 1.23 (d, 3H, J = 6.2 Hz; H-1), 0.89 (t, 3H, J = 6.8 Hz; H-11). IR λ max cm^{-1}: 3350 (s, broad),

2960 (s), 2860 (m), 1465 (m), 1375 (w), 1075 (m), 945 (m). The NMR and IR spectral data were a good match with literature data (Utimoto et al., 1984). MS *m/z* (rel. abundance): 124 (2, M^+-CH_3COH), 109 (3), 96 (15), 95 (23), 82 (17), 81 (21), 68 (31), 67 (39), 54 (100), 45 (68).

The reaction was repeated, substituting *S*-(−)-propylene oxide (Fluka Chemical Corp., Ronkonka, New York) for racemic propylene oxide, yielding (2*S*)-4-undecyn-2-ol (2*S*-3) in 68% isolated yield, $[\alpha]_D^{28} = +20.4 \pm 0.3°$ (c 1.22, $CHCl_3$), and an enantiomeric excess (e.e.) of 98.3%, as determined by capillary GC (190°C, isothermal) of the diastereomeric ester formed by derivatization of 2*S*-3 with (2*S*)-2-acetoxypropionyl chloride. Spectra and chromatographic retention times of 2*S*-3 matched those of racemic 3.

10-Undecyn-2-ol 4. Lithium wire (5.21 g, 750 mmol) was dissolved in approx. 250 ml of 1,3-diaminopropane. The resulting blue solution was heated at 65°C until the blue color was discharged (approx. 2 hr). The resulting white slurry was cooled to 20°C, and potassium *t*-butoxide (66 g, 590 mmol) was added in one portion. The orange slurry was stirred 30 min, followed by dropwise addition of racemic alcohol 3 (21.0 g, 125 mmol). The mixture was stirred 45 min (reaction complete by GC), poured onto 1 kg of ice, and extracted with hexane. The hexane extracts were backwashed with 1 M HCl, then worked up as usual. The crude product was distilled under vacuum, giving alcohol 4 (19.63 g, 93%), bp ~65–70°C (0.18 mm Hg). ^{1}H NMR δ: 3.79 (m, 1H; H-10), 2.17 (td, 2H, J = 7.0, 2.7 Hz; H-3), 1.93 (t, 1H, J = 2.7 Hz; H-1), 1.50 (m, 2H; H-9), 1.45–1.22 (m, 11H; H-4 to H-8, −OH), 1.19 (t, 3H, J = 7.8 Hz; H-11). IR λ max cm^{-1}: 3350 (s, broad), 3300 (m), 2940 (s), 2860 (s), 2135 (w), 1470 (m), 1375 (w), 1135 (m), 1105 (m), 1065 (m). MS *m/z* (rel. abundance): 135 (1, M^+-CH_3-H_2O), 125 (1), 107 (4), 95 (8), 93 (12), 81 (23), 79 (18), 67 (36), 55 (34), 45 (100), 41 (55).

The reaction was repeated on smaller scale with 3.07 g of 2*S*-3 (18.3 mmol), yielding 2*S*-4 in 78% yield, $[\alpha]_D^{30} = +7.2 \pm 0.3°$ (c 0.54, $CHCl_3$), with e.e. > = 98.3% by capillary GC (190°C isothermal) of the diastereomeric derivatives formed as for 3 above. Spectra and chromatographic retention times of 2*S*-4 matched those of racemic 4.

10-Tridecyn-2-ol 5. *n*-BuLi (9 ml of a 2.5 M solution in hexanes, 22.5 mmol) was added to a cooled (<0°C) of alkynol 4 (1.68 g, 10 mmol) in 25 ml of THF. The resulting slurry was cooled to −40°C, HMPA (5 ml) was added, followed by dropwise addition of ethyl bromide (1.42 g, 15 mmol) in THF (5 ml). The mixture was warmed to 20°C overnight, poured into water and extracted three times with hexane. After workup as usual, the residue was purified by flash chromatography (15% EtOAc in hexane), giving alkynol 5 (1.73 g, 88%). NMR δ: 3.79 (m, 1H; H-2), 2.14 (m, 4H; H-9,12), 1.55–1.23 (m, 13H; H-3 to H-8, -OH), 1.19 (d, 3H, J = 6.2 Hz; H-1), 1.12 (t, 3H, J = 7.4 Hz; H-13). IR λ max cm^{-1}: 3400 (s, broad), 2950 (s), 2865 (s), 1465 (m),

1375 (m), 1330 (m), 1135 (m), 1065 (m). MS *m/z* (rel. abundance): 163 (1, M^+-H_2O-CH_3), 149 (5), 135 (5), 121 (10), 107 (22), 93 (37), 82 (31), 81 (41), 79 (49), 67 (100), 55 (56), 45 (99), 41 (98).

Compound 2*S*-5 was prepared in identical fashion in 91% yield, $[\alpha]_D^{31} = +5.7 \pm 0.2°$ (c 0.76, $CHCl_3$), with an e.e. of 98.1%, as determined by capillary GC (210°C isothermal) of the diastereomeric derivatives, formed as for 3 above. Spectra and chromatographic retention times of 2*S*-5 matched those of racemic 5.

(10Z)-tridecen-2-yl acetate 7. Nickel acetate tetrahydrate (63 mg, 0.25 mmol) was dissolved in 10 ml of 95% EtOH. Under a strong flush of argon, 250 μl of a 1 M solution of $NaBH_4$ in EtOH was added in one portion. The flask was flushed with H_2 and ethylene diamine (33 μl, 0.5 mmol) was added, followed by alkynol 5 (0.5 g, 2.55 mmol). The mixture was stirred under a slight positive pressure of H_2 until the reduction was complete (~2 hr). The mixture was then filtered with suction through a plug of activated charcoal, rinsing the charcoal well with ethanol. The filtrate was concentrated, taken up in hexane, washed with 1 M HCl, and worked up as usual. The crude product 6 (0.37 g, 74%) was 98% pure by capillary GC, and so was used without further purification.

Alcohol 6 was taken up in ether (25 ml), cooled in an ice bath, and triethylamine (1 ml) and acetyl chloride (0.29 ml, 4 mmol) were added sequentially. The mixture was warmed to room temperature overnight, poured into hexane, washed with 1M HCl, and worked up as usual. The crude product was purified by flash chromatography on 10% $AgNO_3$ on silica gel, eluted with 5% ether in hexane. The purified acetate was Kugelrohr-distilled (oven temp. 110°C, 0.2 mm Hg), yielding 0.27 g (44% from 5) of acetate 7, >99.5% chemically pure by capillary GC. NMR δ: 5.33 (m, 2H; H-10,11), 4.88 (m, 1H; H-2), 2.02 (s, 3H; acetate CH_3), ~2.02 (m, 4H; H-9,12), 1.6–1.4 (m, 2H; H-3), 1.4–1.25 (m, 10H; H-4 to H-8), 1.20 (d, 3H, J = 6.3 Hz; H-1), 0.96 (t, 3H, J = 7.5 Hz; H-13). IR λ max cm^{-1}: 2950 (s), 2865 (s), 1750 (s), 1465 (m), 1370 (m), 1250 (s, broad), 1070 (m, broad). MS *m/z* (rel. abundance): 225 (trace, M^+-15), 180 (3, M^+-CH_3COOH), 138 (5), 124 (5), 110 (8), 96 (17), 82 (41), 68 (47), 55 (36), 43 (100), 41 (56).

(10E)-tridecen-2-yl acetate 9. Sodium pellets (0.52 g, 23 mmol) were dissolved in 40 ml of a 1:1 solution of THF:liquid NH_3. Alcohol 5 (0.41 g, 21 mmol) was added, and the mixture was stirred under reflux for 5.5 hr. The reaction was quenched by addition of solid NH_4Cl (5 g) and the NH_3 was allowed to evaporate overnight. The residue was taken up in water and extracted with hexane, followed by the usual workup, giving crude alcohol 8 (0.41 g). The alcohol was acetylated and purified as described for 6 above, giving acetate 9 (0.39 g, 78% from 5). NMR δ: 5.40 (m, 2H; H-10,11), 4.88 (m, 1H; H-2), 2.02 (s, 3H; acetate CH_3), 1.97 (m, 4H; H-9,12), 1.6–1.4 (m, 2H; H-3), 1.4–1.25 (m, 10H; H-4 to H-8), 1.20 (d, 3H, J = 6.3 Hz; H-1), 0.96 (t, 3H, J =

7.4 Hz; H-13). IR λ max cm^{-1}: 2950 (s), 2860 (s), 1755 (s), 1465 (m), 1370 (m), 1250 (s, broad), 1030 (broad), 972 (m). MS *m/z* (rel. abundance): 180 (5, M^+-CH_3COOH), 138 (5), 124 (6), 110 (9), 96 (17), 95 (17), 82 (45), 68 (55), 55 (38), 43 (100), 41 (57).

2*S*,10*E*-tridecen-2-ol [2*S*-8], $[\alpha]_D^{31} = +5.8 \pm 0.1°$ (c 1.32, $CHCl_3$), and 2*S*,10*E*-tridecen-2-yl acetate [2*S*-9], $[\alpha]_D^{31} = +0.8 \pm 0.2°$ (c 0.80, $CHCl_3$) were prepared in identical fashion from 2*S*-5, in 87% yield overall. The e.e. of 2*S*-8 was determined as before to be ≥96.7% (GC temp., 210°C isothermal). Spectra and chromatographic retention times of 2*S*-8 and 2*S*-9 matched those of racemic 8 and 9 exactly.

(2R,10E)-tridecen-2-yl acetate (2R-9). Methanesulfonyl chloride (0.52 g, 4.5 mmol) was added to an ice-cold solution of (2*S*,10*E*)-tridecen-2-ol (0.594 g, 3.0 mmol) in 20 ml of 20:1 CH_2Cl_2:triethylamine. The solution was stirred at 0° for 1 hr, then washed twice with cold saturated aqueous $NaHCO_3$, once with brine, then dried and concentrated *in vacuo* (0.2 mm Hg) for 2 hr, yielding 2*S*-10 as a yellow oil (0.82 g, 98%), chromatographically homogeneous by silica gel TLC (20% EtOAc in hexane). The oil was used without further purification.

Cesium propionate:propionic acid complex (~1:1 complex; 500 mg, ~1.5 mmol; Aldrich) was added to a solution of mesylate 2*S*-10 (0.44 g, 1.5 mmol) in dry DMF (15 ml, dried over activated 3 Angstrom molec. sieve). The mixture was stirred for 33 hr at 40°C under Ar, then poured into water (100 ml). The mixture was extracted three times with hexane, and the combined hexane extracts were worked up as usual. The residue was flash chromatographed (7.5% EtOAc in hexane), yielding propionate 2*R*-11 (0.33 g, 87%).

A mixture of the propionate 2*R*-11 (0.21 g, 1.22 mmol) and NaOH (25 mg) were stirred in MeOH (10 ml) for 36 hr. Water (0.5 ml) was added, and the mixture was stirred a further 16 hr. The mixture was concentrated, taken up in hexane, washed with water, and worked up as usual. The residue was flash chromatographed (20% EtOAc in hexane), yielding (2*R*)-tridecen-2-ol (2*R*-8, 200 mg, 83%), $[\alpha]_D^{31} = -5.3 \pm 0.3°$ (c 0.34, $CHCl_3$), with an e.e. of ≥96.4%, determined as described for 2*S*-8 above.

The purified 2*R*-8 was acetylated as described above for the 2*S* enantiomer, yielding 2*R*-9 (92%), $[\alpha]_D^{32} = -0.5 \pm 0.2°$ (c 0.80, $CHCl_3$). Spectra and chromatographic retention times of 2*R*-8 and 2*R*-9 matched those of racemic 8 and 9.

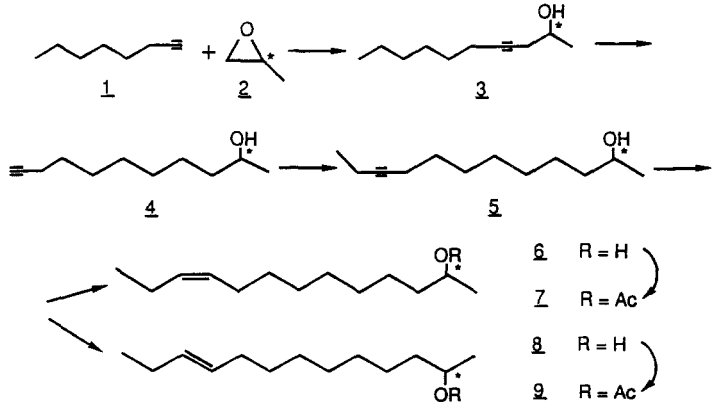
RESULTS AND DISCUSSION

The female-produced sex pheromone of the Hessian fly consists of 2*S*,10*E*-tridecen-2-yl acetate, which contains one disubstituted double bond and one chiral center. Natural pheromone was only available in very small quantities

from extracts of female ovipositors, and it was not possible to ascertain either the double bond geometry or the configuration of the chiral center by analytical chemistry methods alone. Consequently, a synthetic route was devised that would give access to all possible stereoisomers of the basic structure of the Hessian fly pheromone in high stereoisomeric purity, and which could be readily scaled up to obtain multigram yields if required. The synthesis shown in Scheme 1 had the required flexibility. The synthesis was carried through with racemic material initially, and the double bond geometry of the natural pheromone was determined by comparison of the retention times of the synthetic *Z* and *E* isomers with that of the insect-produced material. The synthesis was then repeated with (*S*)-(−)-propylene oxide, to produce the enantiomers of the pheromone.

Thus, the lithium salt of 1-octyne 1 was reacted with racemic propylene oxide 2 in THF-HMPA, to give alkynol 3 (Oehlschlager et al., 1985). The triple bond was then isomerized to the terminal position using the modified acetylene zipper reaction (Abrams and Shaw, 1988), yielding alkynol 4 (Oehlschlager et al., 1985). The lithium salt of 4 was alkylated with ethyl bromide in THF-HMPA, completing the carbon skeleton. Reduction of the product 5 with sodium in liquid ammonia (Henrick et al., 1982), or P-2 nickel and hydrogen (Brown and Ahuja, 1973), gave (10*E*)- and (10*Z*)-tridecen-2-ols 8 and 6, respectively. Acetylation of the alcohols with acetyl chloride and triethylamine gave the racemic forms of the two possible pheromone structures 7 and 9. The isomers were readily distinguishable by capillary GC (DB-5 and SP2340 columns). The retention time of the major component of the natural pheromone extract exactly matched that of the 10*E* isomer 9, confirming the double bond geometry as *E*.

With the question of the alkene geometry settled, the enantiomers of 9



SCHEME 1

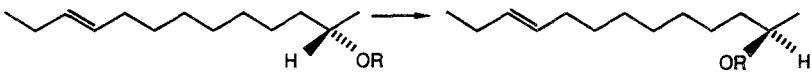
were prepared by the same route, substituting (*S*)-(−)-propylene oxide [*S*-(−)-2] for racemic 2 in the first step. Ring opening of the epoxide was accomplished with little or no loss of chirality (Brandsma, 1971), yielding 2*S*-3 with an enantiomeric excess (e.e.) of >98% (vide infra). 2*S*-3 was then carried through the same synthetic steps as racemic 3, with similar yields, and minimal loss of chirality, yielding (2*S*,10*E*)-tridecen-2-yl acetate, 2*S*-9.

2*R*-9 was synthesized via the intermediate alcohol, 2*R*-8, which in turn was prepared by inversion of the chiral center in alcohol 2*S*-8 by Kruizinga's method (Kruizinga et al., 1981; Johnston and Oehlschlager, 1984; Scheme 2). Thus, 2*S*-8 was converted to mesylate 2*S*-10 by the usual conditions (MesCl/triethylamine in methylene chloride), followed by stereoselective nucleophilic displacement of the mesylate with cesium propionate in DMF. Base hydrolysis of the propionate 2*R*-11 gave alcohol 2*R*-8 (e.e. >96%), which was acetylated as before, giving 2*R*-9, with minimal loss of chirality.

Enantiomeric excesses of alcohols 3–5 and 8 were determined by derivatization with (2*S*)-2-acetoxypropionyl chloride (Slessor et al., 1985). The resulting diastereomeric esters were readily resolved to baseline by capillary GC (DB-5 column). This method was also used to determine the chirality of the insect-produced compound. Thus, a preparative GC fraction of ovipositor extract containing the active compound was hydrolysed with 0.5M KOH in methanol, and the resulting alcohol was derivatized as described above. Only the 2*S* enantiomer was detectable from the insect extract (detection limit of 2*R* enantiomer estimated at <1% of the 2*S* enantiomer).

In Y-tube olfactometer bioassays, the 2*S* enantiomer was shown to be the attractive enantiomer (Table 1). The 2*R* enantiomer was much less attractive, with the residual activity possibly being due to the small amount of 2*S* contaminant present in the synthetic enantiomer. In addition, the racemic mixture was as attractive as the 2*S* enantiomer alone, indicating that the 2*R* enantiomer was not significantly inhibitory.

The Hessian fly ovipositor extract also contained lesser amounts of several related compounds (Foster et al., 1991), including the double bond isomer (10*Z*)-tridecen-2-yl acetate, which was present in amounts equal to approximately 10% of the biologically active *E* isomer, and the saturated analog tri-



2 S-8: R = H
2 S-10: R = Mes

2 R-11: R = C_2H_5CO
2 R-8: R = H
2 R-9: R = Ac

SCHEME 2

TABLE 1. RESPONSES OF VIRGIN MALE HESSIAN FLIES TO FEMALE ABDOMEN TIP EXTRACTS OR TO CHIRAL AND RACEMIC FORMS OF THE MAJOR PHEROMONE COMPONENT, (10*E*)-TRIDECEN-2-YL ACETATE

Stimulus	Mean number of males[a]		Ratio, source/control[c]
	In stimulus arm	In control arm	
Extract (10 FE)[b]	7.8	0.25	31 : 1[c]
Racemate (150 ng)	20.5	2.3	8.9 : 1[c]
S-enantiomer (75 ng)	11.8	1.2	9.8 : 1[c]
R-enantiomer (75 ng)	5.2	1.8	2.9 : 1[c]
Blank	3.4	4.0	0.85 : 1 NS[d]

[a] Trial using extract, mean of four reps, ten insects/rep; other trials, six reps, 25 insects/rep.
[b] 10 FE = ten female equiv.; amounts of synthetic chemicals ~ 10 FE.
[c] Chi-square test probabilities: $P = 0.01$.
[d] NS = not significant.

decan-2-yl acetate. (10*E*)-Tridecen-2-ol and tridecan-2-ol were also detected in extracts. However, in bioassays, the racemic forms of these compounds were not significantly attractive, nor did they increase the response of male flies when added to lures containing the attractive pheromone (Harris and Foster, 1991). The absolute configuration of the minor components has not yet been determined because of the trace levels at which they were present in the natural extract.

Acknowledgments—This work was supported in part by funds from the Hatch foundation.

REFERENCES

ABRAMS, S.R., and SHAW, A.C. 1988. Triple bond isomerizations: 2- to 9-decyn-1-ol. *Org. Syn.* 66:127–131.

BRANDSMA, L. 1971. Preparative Acetylenic Chemistry. Elsevier Publishing Co., New York.

BROWN, C.A., and AHUJA, V.K. 1973. "P-2 nickel" catalyst with ethylenediamine, a novel system for highly stereospecific reduction of alkynes to *cis* olefins. *J.C.S. Chem. Commun.* 553–554.

CARTWRIGHT, W.B. 1922. Sexual attraction of the female Hessian fly. *Can. Entomol.* 54:154.

CHAUDHURY, M.F.B., BALL, H.J., and JONES, C.M. 1972. A sex pheromone of the female face fly, *Musca autumnalis* (Diptera: Muscidae) and its role in sexual behavior. *Ann. Entomol. Soc. Am.* 65:607–612.

FELDMAN, M. 1976. *in* N.W. Simmons (ed.). Evolution of Crop Plants. Longman, London.

FOSTER, J.E., TAYLOR, P.L., and ARAYA, J.E. 1986. The Hessian fly. *Purdue Univ. Ag. Exp. Stn. Bull. No. 502*, West Lafayette, Indiana, 21 pp.

FOSTER, S.P., HARRIS, M.O., and MILLAR, J.G. 1991. Identification of the sex pheromone of the Hessian fly, *Mayetiola destructor* (Say). *Naturwissenschaften* 78:130–131.

GALLUN, R.L., DEAY, H.O., and CARTWRIGHT, W.B. 1961. Four races of Hessian fly selected and developed from an Indiana population. *Purdue Univ. Agric. Expt. Stn. Res. Bull. 732*, Lafayette, Indiana.

HARRIS, M.O., and FOSTER, S.P. 1991. Wind tunnel studies of sex pheromone-mediated behavior of the Hessian fly (Diptera: Cecodomyiidae). *J. Chem. Ecol.* Submitted.

HENRICK, C.A., CARNEY, R.L., and ANDERSON, R.J. 1982. Some Aspects of the Synthesis of Insect Sex Pheromones. *in* B.A. Leonhardt and M. Beroza (eds.). Insect Pheromone Technology: Chemistry and Applications. American Chemical Society, Washington D.C. pp. 27–60.

JOHNSTON, B.D., and OEHLSCHLAGER, A.C. 1984. Synthesis of the aggregation pheromone of the square-necked grain beetle *Cathartus quadricollis*. *J. Org. Chem.* 51:760–763.

KRUIZINGA, W.H., STRIJTVEEN, B., and KELLOGG, R.M. 1981. Cesium carboxylates in dimethylformamide. Reagents for introduction of hydroxyl groups by nucleophilic substitution and for inversion of configuration of secondary alcohols. *J. Org. Chem.* 46:4321–4323.

MACKAY, P.A., and HATCHETT, J.H. 1984. Mating behavior and evidence of a female sex pheromone in the Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae). *Ann. Entomol. Soc. Am.* 77:616–620.

OEHLSCHLAGER, A.C., CZYZEWSKA, E., AKSELA, A., and PIERCE, H.D., Jr. 1985. Improved syntheses of hydroxy acid precursors of macrolide pheromones of cucujid grain beetles. *Can. J. Chem.* 64:1407–1413.

SLESSOR, K.N., KING, G.G.S., MILLER, D.R., WINSTON, M.L., and CUTFORTH, T.L. 1985. Determination of chirality of alcohol or latent alcohol semiochemicals in individual insects. *J. Chem. Ecol.* 11:1659–1667.

UTIMOTO, K., LAMBERT, C., FUKUDA, Y., SHIRAGAMI, H., and NOZAKI, H. 1984. The catalytic action of trimethylgallium on the reaction of alkynyllithium with epoxides. *Tetrahedron Lett.* 25:5423–5426.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

SIGNIFICANCE OF MEDIUM CHAIN *n*-ALKANES AS ACCOMPANYING COMPOUNDS IN HEMIPTERAN DEFENSIVE SECRETIONS: AN INVESTIGATION BASED ON THE DEFENSIVE SECRETION OF *Coridius janus*

N.E. GUNAWARDENA* and H.M.W.K.B. HERATH

Department of Chemistry
University of Kelaniya
Kelaniya, Sri Lanka

(Received January 3, 1991; accepted August 12, 1991)

Abstract—A mixture of *trans*-2-hexenal; *n*-tridecane (60:40, w/w), the natural combination present in the defensive secretion of *Coridius janus* (Hemiptera; Pentatomidae) was shown by comparison with similar aldehyde mixtures having longer and shorter chain *n*-alkanes, to be the optimal combination as a fumigant and a repellent against three test insect species, *Anoplolepis longipes*, *Sitotroga cerealella*, and *Culex quinquefasciatus*. Toxic values obtained for the three insects, respectively: $1/LC_{50}$, 1.72, 4.54, and 6.66 ppm while repellencies were 63%, 50%, and 69%. This study revealed that among *t*-2-hexenal: *n*-alkane combinations those with medium carbon chains, *viz* C-11 and C-12, also possessed high toxicities and repellencies comparable to that of the natural combination while those with shorter and longer *n*-alkanes possessed lower activity. Toxicities and repellencies of *n*-alkane series were only moderate to low showing highest toxic values for *n*-tridecane at $1/LC_{50}$, 0.39, 2.32, and 2.32 ppm and repellencies at 31%, 30%, and 32% for the three test insects, respectively. This series, nevertheless, showed similar variation, medium length chains, C-11, C-12, and C-13 showing comparatively higher activity than other alkanes of shorter and longer chains. This study also revealed that the fumigant property of both alkane and aldehyde are of equal importance while repellency is heavily dependent on the aldehyde.

Key Words—*Coridius janus*, Pentatomidae, Hemiptera, Defensive secretion, *n*-alkanes, acetates, toxicants.

*To whom correspondence should be addressed.

0098-0331/91/1200-2449$06.50/0

INTRODUCTION

A self-protective role against their predators as the primary function of insect defensive secretions has been established (Blum, 1964; Peschke and Eisner, 1987; Roth et al., 1956). Often these secretions are multicomponent, presumably relating to their specific function/s described as repellents, deterrents, toxicants, alarm, or aggregation pheromones (Staddon, 1979; Aldrich, 1988). Hydrocarbons are a frequently found in insect defensive secretions with structural diversity ranging from simple *n*-alkanes to cyclic, mono-, di-, tri-unsaturated, and branched molecules (Weatherston and Percy, 1978). One of the characteristic features of hemipteran defensive secretions is that they often consist of one or more deterrents accompanied by alkanes or acetates (Aldrich, 1988; Eisner et al., 1961; Dettner, 1984). Pentatomidae and Coreidae, the two most thoroughly studied families of Hemiptera conform to the above, possessing simple alkanes and acetates, respectively, as accompanying compounds (Weatherston and Percy, 1978; Weatherston, 1967). Besides serving as solvents that modulate evaporation of volatile irritants, these accompanying compounds act synergistically as spreading and penetrating agents as shown by Staddon (1979) and Remold (1962).

In our previous study, the toxicity of the defensive secretion of *Coridius janus* Fabricius (Hemiptera; Pentatomidae) was shown toward three household pests: *Anoplolepis longipes* (Formicidae), *Culex quinquefasciatus* (Culicidae), and *Sitotroga cerealella* (Gelechiidae) (Gunawardena and Herath, 1991). A mixture of *t*-2-hexenal and *n*-tridecane (60/40, w/w), the two major components of the above secretion was shown to be responsible for the toxic action. Our interest was to examine the effectiveness of the above natural formula against other similar mixtures having longer and shorter chain *n*-alkanes as accompanying compounds. Is there an optimum chain length for effectiveness? What is the contribution of *n*-alkanes toward overall toxicity and repellency of the mixture? These aspects of accompanying compounds have not been studied before.

MATERIALS AND METHODS

Chemical Samples and Chemical Analyses

n-Alkanes were purchased from Fluka Chemicals, Switzerland, and *t*-2-hexenal from Aldrich Chemicals Co. Ltd., U.K. Purity of these chemicals was found to be 99.9% by GLC analysis. Gas Liquid Chromatography was carried out on a Shimadzu GC-6A instrument with FID, a He flow of 30 ml/min., temperature program of 40–225°C at 8°C/min, 240°C detector and injector temperature, glass columns (0.25mm id × 30M; SPB 1 stationary phase fused silica).

Bioassay of n-*Alkanes or* t-2-*Hexenal*/n-*Alkane Mixtures for Toxicity by Fumigant Action*

Test insects, *Anoplolepis longipes* (Formicidae), a natural enemy of *C. janus*, females of *Culex quinquefasciatus* (Culicidae) and *Sitotroga cerealella* (Gelechiidae), a storage product pest, were collected from their respective natural habitats a few hours prior to the experiments and were fed with sugar solutions, avian blood, and paddy grains, respectively, before being subjected to bioassay. Batches consisting of 20–30 insects were confined to test tubes (20 ml) just before the experiment. Test mixtures were prepared by first mixing neat liquids of *t*-2-hexenal and *n*-alkanes in the ratio of 60:40 (w/w) until a single phase was obtained and weighing out from these solutions appropriate amounts (Table 1) in small glass vials (2 ml). A large glass jar (5L) with a small opening at the bottom was used as the fumigating chamber. Glass vials filled with test mixtures (Table 1) were inserted partially into the fumigating jar

TABLE 1. THE AMOUNTS OF *t*-2-HEXENAL: *n*-ALKANE MIXTURES OR *n*-ALKANES EVAPORATED INTO THE FUMIGATION CHAMBER

t-2-Hex.: *n*-alk. mixtures[a] or *n*-Alk.	Amounts used (mg)[b]								
	A. longipes			*C. quinque.*			*S. cerealla*		
	I	II	III	I	II	III	I	II	III
t-2-Hexenal:									
Octane	3.0	3.8	8.8	1.5	3.0	4.0	1.5	3.0	4.0
Decane	3.0	3.8	8.8	0.5	2.5	3.5	1.0	2.5	3.5
Undecane	2.0	3.0	4.5	0.4	0.5	3.0	0.4	0.5	3.0
Dodecane	2.3	3.0	3.8	0.4	0.5	3.0	0.4	0.5	3.0
Tridecane	1.5	3.0	4.0	0.4	0.5	2.5	0.4	1.0	2.5
Tetradecane	3.0	3.8	8.8	1.0	2.5	3.5	1.0	3.0	4.0
Hexadecane	3.0	3.8	8.8	1.5	3.0	4.0	1.5	3.0	4.0
Octane	35	75	150	2.5	3.5	6.0	2.5	3.5	6.0
Decane	13	14	15	2.5	3.0	4.5	1.5	2.5	4.5
Undecane	10	13	15	1.5	2.5	4.0	1.5	2.5	4.5
Dodecane	10	13	15	1.5	2.5	4.0	2.0	3.0	4.5
Tridecane	10	13	15	1.0	2.5	4.0	1.0	2.5	4.0
Tetradecane	15	35	75	2.5	3.0	4.5	2.5	3.0	4.5
Hexadecane	75	125	150	2.5	3.5	5.0	2.5	4.0	6.0

[a] All mixtures were in the ratio 60:40 (w/w) and in single phase.
[b] All experiments were carried out in 5L fumigating chamber and for the calculation of LC_{50} values complete evaporation of compounds were assumed by weighing empty vials before introducing compounds into them and after evaporation of compounds from them. Each dose was tested in three replicates.

through the opening at the bottom in such a manner that the volatiles were allowed to escape into the chamber. The bottom of each vial was heated slowly until all the test compounds/mixtures evaporated into the jar. Complete evaporation was assumed by weighing empty vials before the introduction of chemicals and after their evaporation into the fumigating jar. Subsequently, the test insects were introduced into the jar through the same opening and the jars were tightly closed. During all bioassays, temperature was maintained between 30–31°C. Blank experiments were conducted under the same conditions using only pure water and without any other liquid. Dead insects were separated and kept under observation for a further 24 hr, before mortalities were recorded. In the calculation of mean LC_{50} values, three doses (Table 1) of each test compound/mixture were subjected to three assays.

Repellency Assay

Preliminary tests indicated neat liquid mixtures of *t*-2-hexenal : *n*-tridecane (w/w) repel test insects with 100% efficiency. A 15% solution of *n*-alkane or *t*-2-hexenal/*n*-alkane mixture in paraffin oil was therefore used in all experiments. For each assay, 20–30 test insects were used, at a constant temperature of 30–31°C and 5μl of each *n*-alkane or *t*-2-hexenal/*n*-alkane mixture tested in three replicates. Blank experiments were conducted using pure water, paraffin oil and without any other liquid.

Anoplolepis longipes

Insects were confined to a glass tube (30 cm long × 5 cm ID) with both ends open by spreading a sugar solution inside the tube. This tube was kept in parallel direction to a slow stream of air. After 10 min, the outer surface of a glass funnel was treated with 5 μl of test compounds/mixtures, as 15% solutions in paraffin oil and inserted into one end of the tube. The air flow could thus carry the repellent throughout the tube. This arrangement was deemed most satisfactory since the loss of repellent against the direction of the air flow was minimized. The number of insects in the original tube was counted 5 min after the introduction of test compounds/mixtures.

Sitotroga cerealella

A long glass tube (50 cm long × 3 cm ID) was divided in the middle and the two parts marked A and B. Test insects were introduced into this tube in a dark room and were phototropically lured into part A. The test compound/mixture (5 μl), spread in a glass funnel as above, was introduced into tube A. The number of insects in the tube A was counted 10 min after the introduction of test compounds.

Culex quinquefasciatus

A long glass tube (50 cm long × 3 cm ID) was divided similarly into two parts A and B and the former was covered with black paper. Test insects were introduced into this end and were allowed to settle down. Afterward, the compounds/mixtures (5 μl) were introduced as above from the same end A. The number of mosquitoes in part A was counted 10 min after the introduction of test compounds.

RESULTS

Toxicity Assay

Plot of toxicity (given in 1/LC50 ppm) vs. *t*-2-hexenal: *n*-alkane (60:40, w/w) combinations showed high toxicities for those of *t*-2-hexenal: C-11, *t*-2-hexenal: C-12 and, *t*-2-hexenal: C-13 (Figure 1). The highest toxicity, however, was exhibited by the natural combination, *t*-2-hexenal: *n*-tridecane which gave toxic values of (1/LC 50 ppm) 1.72, 4.54, and 6.66 ppm for *A. longipes*, *S. cerealella*, and *C. quinquefasciatus*, respectively.

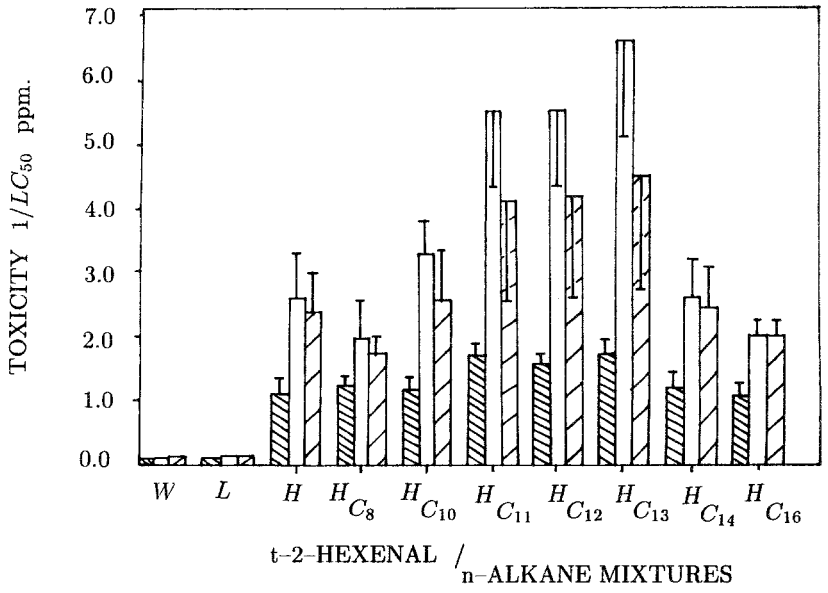


FIG. 1. Variation of toxicities with 60:40 (w/w) *t*-2-hexenal/*n*-alkane combinations. In Fig. 1–4, each column is a mean of three replicates. Vertical bars indicate ∓ SEM. *A. longipes* ≡ ▨, *C. quinquefasciatus* ≡ □, *S. cerealella* = ▨, *t*-2-hexenal = H, paraffin oil = P, distilled water = W, without liquid = L.

n-Alkane series also showed a similar profile with maxima around C-11, C-12, and C-13 but with comparatively lower toxicities than those of their respective combinations (Figure 2). Highest toxicity was shown by tridecane having 1/LC50 values of 0.395, 2.32, and 2.32 ppm for *A. longipes*, *S. cerealella*, and *C. quinquefasciatus*, respectively. Toxicity of *t*-2-hexenal was somewhat higher than those of *n*-alkanes, the 1/LC50 value being 1.14, 2.43, and 2.56 ppm for the three test insects in the above order.

Repellency Assay

Our data showed that repellencies of both series, *t*-2-hexenal: *n*-alkane (60:40, w/w) combinations, and *n*-alkanes varied in a similar manner, only the latter showing somewhat lower values. These variations were quite parallel to those obtained in toxicity assay. High repellencies of combinations from 2-hexenal: C-11 alkane up to 2-hexenal: C-13 alkane were observed with a maxima at the mixture of 2-hexenal: C-13 giving % repellencies of 63, 55, and 65 for *A. longipes*, *S. cerealella*, and *C. quinquefasciatus*, respectively. Along the *n*-alkane series, highest activity was obtained for tridecane, repelling 30%, 31%, and 32% of test insects in the above order. *t*-2-Hexenal, however, was shown to be far superior in its repellent properties compared with hydrocarbons

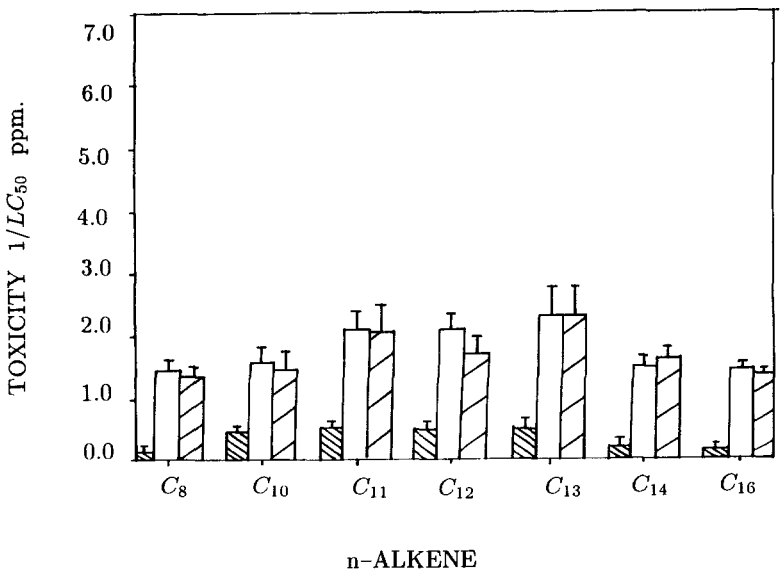


FIG. 2. Variation of toxicities with *n*-alkanes (see Figure 1 legend for explanation of symbols).

as it repelled 44%, 44%, and 55% of *A. longipes*, *S. cerealella*, and *C. quinquefasciatus*, respectively (Figures 3 and 4).

DISCUSSION

Synergistic action of all combinations of *t*-2-hexenal: *n*-alkane (60:40, w/w) is shown by this study. The natural combination, *t*-2-hexenal: C-13 (60:40, w/w) is shown to be the optimal fumigant compared to other possible aldehyde combinations with longer and shorter *n*-alkanes in the ratio 60:40 (w/w). It is also apparent from this study that among other combinations, those with medium carbon chains C-11 and C-12 are much more effective than those with shorter and longer chains.

n-Alkanes, although moderate to low in toxicity, showed similar variation along the series suggesting that *n*-alkanes also contribute directly toward toxicity of the defensive secretions. It is also interesting to note that 2-hexenal by itself is only a little more toxic ($1/LC_{50}$ 1.14, 2.56, and 2.43 ppm for *A. longipes*, *C. quinquefasciatus*, and *S. cerealella*, respectively) than *n*-tridecane, the most toxic alkane in the series ($1/LC_{50}$ 0.395, 2.32, and 2.32 ppm for the above insects, respectively). It is clearly shown from our results that for overall

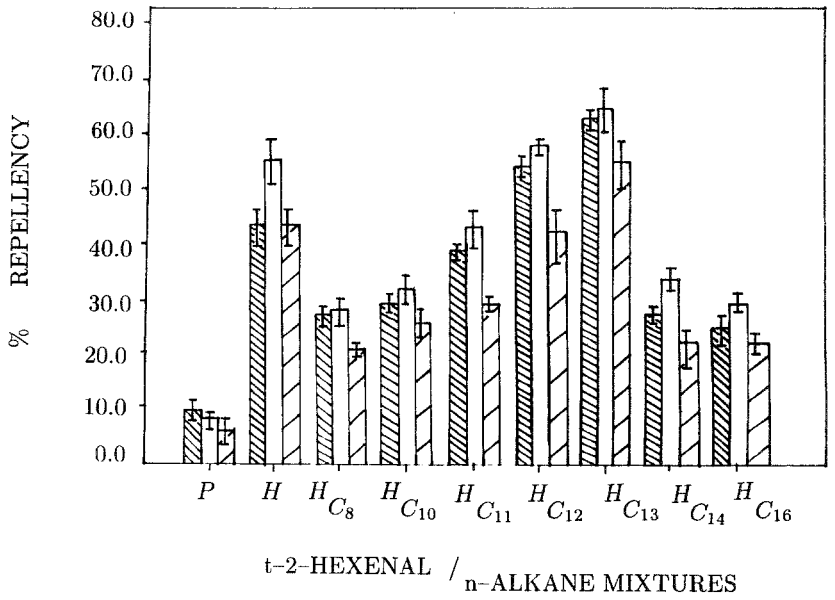


FIG. 3. Variation of repellencies with *t*-2-hexenal/*n*-alkane combnations (see Figure 1 legend for explanation of symbols).

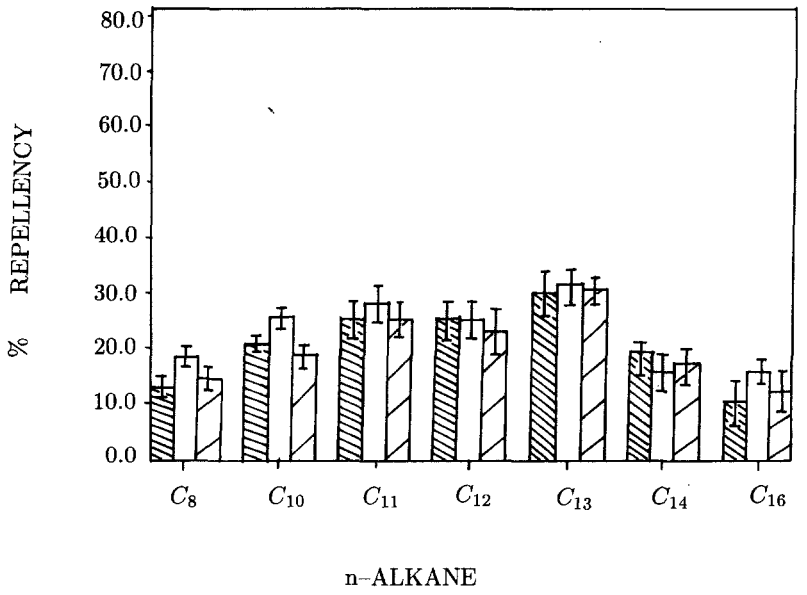


FIG. 4. Variation of repellencies with *n*-alkanes (see Figure 1 legend for explanation of symbols).

high toxicity of the combinations both *n*-alkane and the aldehyde seem to be equally important. Accordingly, the role of hydrocarbon in the defensive secretion of *C. Janus* could be twofold: while acting as a toxicant by itself, it could also enhance the toxicity of the major toxicant, probably by acting as the carrier, as was shown by Remold (1962). For both purposes, medium chain *n*-alkanes, undecane, dodecane, and tridecane seem to be ideally suited.

Repellencies of both series again followed more or less the similar pattern to those of toxicities, the natural combination having the highest repellency (*A. longipes:* 63%, *C. quinquefasciatus:* 65%, and *S. cerealella:* 55%) while those combinations with C-11 and C-12 also showed high activity. Unlike toxicities, however, overall repellency of the combination seems to be entirely derived from *t*-hexenal having repellencies 44%, 55%, and 44% for insects in the above order. This could presumably be the reason for the natural occurrence of *t*-2-hexenal without any accompanying compounds in the defensive secretions of some hemipterans as well as other arthropods (Weatherston and Percy, 1978). Alkenal probably becomes the only constituent when repellency is the only desired mode of defense of an insect. Presence of alkanes in the defensive secretion is known to delay the evaporation of volatile alkenal and this could obviously be unfavorable in some situations (Aldrich, 1988).

These findings on the importance of medium chain *n*-alkanes were com-

pared with their frequency of natural occurrence in defensive secretions. Our search of 25 hemipteran species which use alkanes as accompanying compounds in their defensive secretions revealed that *n*-tridecane occurs exclusively (100%) in all cases. More interestingly, it was found to be the major or the only alkane in 95% of the sample. Other *n*-alkanes found in combination with tridecane were indeed C-11 and or C-12 (50%) while some of the species use, in addition to C-13, C-12, and C-11, some C-15 (14%). No other *n*-alkanes were found as accompanying compounds in the above sample (Weatherston and Percy, 1978; Aldrich et al., 1984, 1986; Staddon, 1979; Kathuria et al., 1975; Remold, 1962; Taketoshi, 1974; Prestwich, 1976).

In conclusion, this study reveals two aspects clearly, *viz*, the natural combination in the defensive secretion of *C. janus* which employs *t*-2-hexenal: tridecane is the most effective toxicant and repellent compared to other combinations of the alkenal having shorter and longer *n*-alkanes. Second, in addition to functions already attributed to alkanes, they contribute to defensive function in their own right. This effect is most significant in the medium chain *n*-alkanes undecane, dodecane, and tridecane which are often found naturally in the defensive secretions of hemipterans.

Acknowledgments—The authors thank the International Foundation for Science (Sweden) and the Natural Resources, Energy and Science Authority of Sri Lanka for funding this project.

REFERENCES

ALDRICH, J.R. 1988. Chemical ecology of the Heteroptera *Annu. Rev. Entomol.* 33:211–238.

ALDRICH, J.R., KOCHANSKY, J.P., LUSBY, W.R., and SERETON, J.D. 1984. Semiochemicals from predaceous stink bug, *Podisus maculiventris, J. Wash. Acad. Sci.* 74(2):39–46.

ALDRICH, J.R., LUSBY, W.R., and KOCHANSKY, J.P. 1986. Identification of a new predaceous stink bug pheromone and its attractiveness to the eastern yellow jacket. *Experientia* 42(5):583–585.

BLUM, M.S. 1964. Insect defensive secretions; hex-2-enal-1 in *Pelmatosilpha coriacea* and its repellent values under natural conditions. *Ann. Entomol. Soc. Am.* 57:600–602.

DETTNER, K. 1984. Isopropylesters as wetting agents from the defensive secretion of the rove beetle, *Coprophilus striatulus* F. (Coleoptera; Staphylinidae). *Insect Biochem.* 14:383–390.

EISNER, T., MEINWALD, J., MONRO, A., and GHENT, R. 1961. Defense mechanisms of Arthropods I, the composition and function of the spray of the whipscorpion, *Mastigoproctus giganteus* Lucas (Arachnida, Pedipalpia), *J. Insect Physiol.* 6:272–298.

GUNAWARDENA, N.E., and HERATH, H.M.W.K.B. 1991. *t*-2-Hexenal/*n*-tridecane combination; A natural fumigant of the defensive secretion of *Coridius janus* (Hemiptera; Pentatomidae) *J. Nat. Sci. Council, Sri Lanka.* Accepted for publication.

KATHURIA, O.P., BROWN, W.V., and GILBY, A.R. 1975. The defense secretion of *Apongopus janus* (Fabricius), Hemiptera; Pentatomidae, *Indian J. Entomol.* 36:31–33.

PESCHKE, K. and EISNER, T. 1987. Defensive secretion of the tenebrionid beetle, *Blaps mucronata;* physical and chemical determinants of effectiveness, *J. Comp. Physiol., A* 161:377–388.

PRESTWICH, G.D. 1976. Composition of the scent of eight East African hemipterans, nymph-adult chemical polymorphism in coreids. *Ann. Entomol. Soc. Am.* 69:812–814.

REMOLD, H. 1962. Scent glands of land bugs, their physiology and biological function. *Nature* 198:764–768.

ROTH, L.M., NIEGISCH, W.D., and STAHL, W.H. 1956. Occurrence of 2-hexenal in the cockroach, *Eurycotis floridana*, *Science* 123:670–671.

STADDON, B.W. 1979. The scent glands of Heteroptera. *Adv. Insect Physiol.* 14:351–418.

TAKETOSHI, I. 1974. The scent of stink bugs (Hemiptera: Pentatomidae). *Appl. Entomol. Zool.* 9:153–158.

WEATHERSTON, J. 1967. The chemistry of arthropod defense substances. *Quarterly Rev.* 21:287–300.

WEATHERSTON, J., and PERCY, J.E. 1978. Venoms of Rhyncota, *in* S. Bettini (ed.). Arthropod Venoms. Springer, Verlag Berlin/Heidelberg.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

IN SEARCH OF A MODEL SYSTEM FOR EXPLORING THE CHEMICAL LEGACY HYPOTHESIS: *Drosophila melanogaster* AND GERANIOL

CLARE J. VELTMAN[1,*] and SARAH A. CORBET[2]

[1]*Department of Botany and Zoology*
Massey University
Palmerston North, New Zealand
[2]*Department of Zoology*
University of Cambridge
Cambridge CB2 3EJ, United Kingdom

(Received February 14, 1991; accepted August 13, 1991)

Abstract—We required an experimental system in which to explore elements of the chemical legacy hypothesis, according to which molecules of environmental chemicals may persist on or in the body or immediate surroundings of larval or pupal insects and influence their habitat preferences in adulthood by affecting the maturation of the chemosensory pathways. Using a purpose-built apparatus which exploited the geotactic and phototactic behavior of *Drosophila melanogaster*, we examined the pattern of responses to a range of dilutions of geraniol, a terpene of plant origin. Fruit flies avoided the geraniol at high concentrations. Although attraction at low doses and reduced aversion after larval, pupal, or early adult exposure to geraniol have been reported, we did not find either. Thus, we failed to generate the phenomenon described as "pre-imaginal conditioning" required for the model system. We discuss the results in relation to designing suitable assays for testing the Chemical Legacy Hypothesis.

Key Words—Chemical legacy hypothesis, pre-imaginal conditioning, Hopkins host-selection principle, *Drosophila melanogaster*, Diptera, Drosophilidae, geraniol.

INTRODUCTION

According to the chemical legacy hypothesis, correlations between adult habitat preferences and larval rearing experiences in insects may arise from direct

*To whom correspondence should be addressed.

0098-0331/91/1200-2459$06.50/0

chemical intervention in development of the chemosensory machinery (Corbet, 1985). Minute traces of chemicals from the larval environment persisting on the surface of the animal's body or cocoon might influence chemosensory development, perhaps acting at or soon after adult emergence (Herard et al., 1988). Corbet (1985) and Vet et al. (1990) reviewed evidence for such plasticity of behavioral responses in insects, but the mechanism underlying this process has not yet been elucidated. We wanted to see whether the effect of early exposure on adult responsiveness could be interpreted in terms of a change in chemosensory threshold, reflected in a shift of a humped dose-response curve along the dose axis. We therefore sought a system showing dose-dependent reversal of response, with attraction at low doses and aversion at high ones, and modification of adult chemosensory responses to a compound by exposure to it in the pre-imaginal or immediately post-emergence environment.

Systems reported to show these features include *Drosophila* with peppermint oil (Hershberger and Smith, 1967; Jaenike, 1982) and geraniol (Manning, 1967). We chose geraniol, a terpene of plant origin, because it is a pure substance whereas peppermint oil is a mixture. In adults of an unnamed species of *Drosophila*, Manning (1967) reported attraction to geraniol at low concentrations and aversion at high concentrations with reduced aversion in flies reared as larvae in agar to which the chemical had been added.

Here, we describe our assay and conditioning methods, and report our failure to find attraction at low concentrations or to modify the adult response to geraniol. We discuss this result in relation to designing suitable model systems for exploring the chemical legacy hypothesis.

METHODS

Drosophila melanogaster were reared on a maizemeal/sugar/agar medium under a 12L:12D lighting regime at 25°C. Since the testing procedure exploited the negative geotaxis and positive phototaxis of adult fruitflies, we waited until flies were 36 hours post-emergence and had reached physical maturity (Ford, et al. 1989) before using them.

Odorant was presented in chambers constructed from the barrels of 7-ml nonsterile polyethylene Pasteur pipettes (Elkay Eireann). We cut off and discarded the tubular portion of each pipette approximately 2 mm below the barrel, and inserted the mouth of the barrel into a hole of 7.5-mm diameter drilled through 3-mm thick perspex. Six barrels were fitted like this in a 35-mm diameter circle on a 100 × 75-mm piece of perspex which had been painted black on the upper side.

To form the testing arena, this barrel assembly was placed face down on a flared plastic dish (volume 85 ml, upper diameter 65 mm, lower diameter

55 mm) coated on the outside with black insulating tape (Figure 1). Each arena was used once, after which the perspex and dish were washed and the barrels were discarded and replaced with new ones for the next test.

Testing was conducted at 25°C under twin fluorescent lights. The barrel assembly was inverted while 5 μl diethyl ether was pipetted into alternate barrels (controls) and 5 μl of a solution of geraniol in ether was pipetted into the remaining three barrels (tests). The barrel entrances were marked "control" or "test" using a water-soluble felt-tip pen. The droplets of both ether and the geraniol solution visibly evaporated while the assembly was in this position.

Approximately 50 flies were drawn from a culture bottle and held in the pooter while the barrel assembly was inverted over the plastic dish. With the barrel assembly in place we pooted the flies into the dish and left the apparatus undisturbed for 30 sec. After that time, an acetate sheet was slipped under the perspex to trap the flies in the barrels, and the entire apparatus was secured with a rubber band. Flies were killed by freezing and then counted by removing each barrel. During the testing process, the experimenter was unable to identify the test or the control tubes.

The geraniol solution was prepared by dissolving geraniol (Sigma Chemical Company) in diethyl ether. We used dilutions from 0.1 to 0.0000001, and express them as log concentration on figures and tables to follow. Since the assay procedure depended on the solvent, ether, evaporating from the barrels and leaving geraniol behind, we checked whether geraniol influenced the evaporation of ether by weighing a sample of barrels to 0.00001 g continuously for

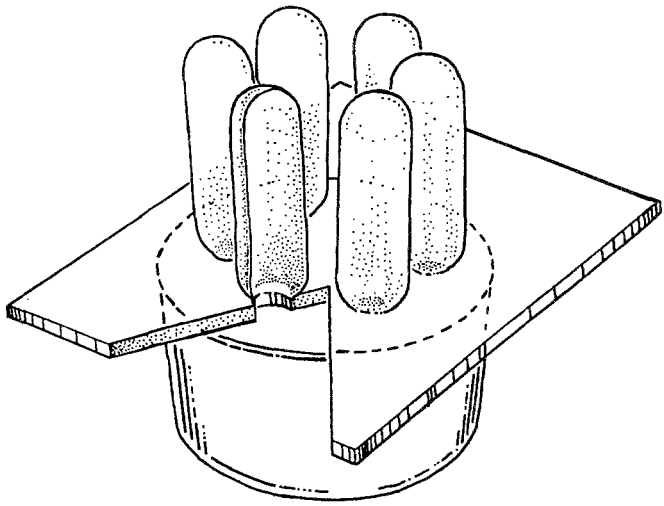


FIG. 1. The testing apparatus.

300 sec while 5 μl of 0.1 geraniol solution or diethyl ether evaporated. We found that evaporation indicated by weight loss was significantly slower in the presence of geraniol at intervals up to 150 sec (Table 1).

We checked whether these differences in evaporation kinetics influenced the proportion of flies entering geraniol-scented barrels by presenting 0.43–0.56 μl droplets of neat geraniol in alternate barrels and leaving control barrels blank. The results for ten such replicates were compared with ten replicates using 5 μl of 0.1 geraniol solution in the test barrels and 5 μl ether in the control barrels, and were significantly different (Mann–Whitney, $U = 55.0$, $P < 0.05$). On average, 41% of respondents entered geraniol tubes in the absence of ether, and 27% entered scented tubes when ether was used as the solvent.

We inferred that geraniol acted as a "keeper" delaying evaporation of ether and that the flies' apparent aversion to the geraniol–ether solution may have resulted, at least in part, from a differential response to persisting traces of the solvent, perhaps aversion to excess ether in the test barrels or attraction to excess ether in the controls.

To guard against this effect during testing, we delayed introducing flies into the arena after pipetting solvent and solution into the barrels, and used a weaker geraniol solution when testing whether flies had reduced aversion to geraniol after pre-imaginal exposure. We assumed that the "ether-holding" property of geraniol would reduce as the concentration of the solute reduced.

We gave flies prior exposure to geraniol in two ways. Flies collected within 30 min of emergence were placed in a small vial stoppered with cotton wool with 5 μl neat geraniol on a small disk of filter paper. After 30 min exposure to geraniol, the flies were transferred to normal culture jars. They were tested at 48 hr post-emergence. We also prepared geraniol-agar by mixing 0.25% geraniol by volume into the agar during the cooling stage, and flies reared in this

TABLE 1. EVAPORATION AS INDICATED BY WEIGHT LOSS OF 5 μl DIETHYL ETHER AND 5 μl 0.1 GERANIOL-ETHER

Time (sec)	n	Mean (SE) weight loss in previous 30 sec (g × 10^{-5})		df	T	
		Ether	Geraniol-ether			
60	9	59.5 (11.1)	37.0 (9.8)	15	4.557	$P < 0.001$
90	9	26.8 (3.2)	17.5 (2.8)	15	6.530	$P < 0.001$
120	9	13.5 (1.8)	10.0 (1.2)	13	4.757	$P < 0.001$
150	9	7.8 (1.3)	6.5 (0.7)	12	2.735	$P = 0.018$
180	10	5.6 (0.9)	4.7 (0.8)	16	1.991	$P > 0.05$

medium were collected either at the moment of emergence or 12 hr later and held in clean vials with damp cottonwool for 5 hr before testing.

RESULTS

We tested about 5000 flies in 93 replicates over seven concentrations of geraniol ranging down to a dilution of 10^{-7} in ether. Like Dudai et al. (1976) and Manning (1967), we found that *Drosophila melanogaster* avoided geraniol at relatively high concentrations. At lower concentrations, the flies did not discriminate between test and control barrels (Figure 2), and unlike Manning (1967), we did not observe any behavior consistent with attraction to geraniol. A polynomial regression model incorporating a cubic term adequately described the dose–response function (Table 2). Response values predicted by the model are shown in Figure 2.

When newly emerged flies were exposed for 30 min to 5 μl neat geraniol, their response to 0.01 geraniol when tested 48 hr later was not significantly different from that of flies without prior exposure ($t = 1.157$, $df = 32$, $P > 0.05$). We conclude that exposure of up to 30 min immediately after emergence had no detectable effect on subsequent adult responsiveness.

Flies raised on agar tainted with geraniol and tested 5 or 17 hr after emergence did not exhibit reduced aversion to 0.01 geraniol ($F_{2,35} = 0.19$, $P > 0.05$), and like the controls, were equally likely to approach scented and unscented barrels when tested with 10^{-4} geraniol ($F_{2,32} = 2.52$, $P > 0.05$) and 10^{-6} geraniol ($t = 0.704$, $df = 19$, $P > 0.05$). We therefore found no

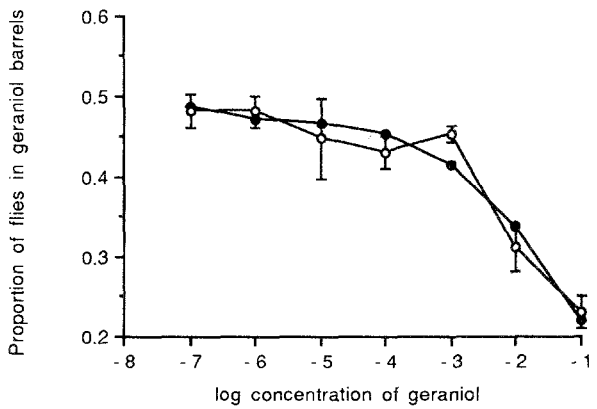


FIG. 2. Proportion of flies attracted to a range of geraniol dilutions. Open circles give replicates at each concentration, with the standard error. Closed circles give the predicted value from the regression model.

TABLE 2. RESULTS OF FITTING A POLYNOMIAL REGRESSION MODEL TO THE DOSE–RESPONSE DATA

Model	RSS	Residual *df*	Source	χ^2	*df*	*P*
$y = \beta^a$	213.85	92				
$y = \beta + \beta_1 x$	143.29	91	Concentration	70.76	1	[b]
$y = \beta + \beta_1 x + \beta_2 x^2$	123.14	90	Squared term	20.15	1	[b]
$y = \beta + \beta_1 x + \beta_2 x^2 + \beta_3 x^3$	120.35	89	Cubed term	2.79	1	[c]

[a] Where β refers to a constant.
[b] Indicates $P < 0.001$.
[c] Indicates $P < 0.10$.

evidence for an effect of pre-imaginal exposure on the adult response to geraniol.

DISCUSSION

In our study, fruit flies appeared unable to detect geraniol diluted below 10^{-3}. We therefore used 10^{-2} geraniol as a test stimulus after prior exposure to geraniol to see whether a change in responsiveness had been induced by a chemical legacy.

Manning (1967) showed that flies reared as larvae and maintained as adults in the presence of geraniol found this compound less aversive than flies reared as larvae and maintained as adults in its absence. The finding that this effect was almost abolished if all flies were removed to geraniol-free vials at emergence indicates that at least part of the effect of early exposure on adult behavior depends on post-emergence exposure to geraniol. Further evidence came from the finding that when flies were reared as larvae on uncontaminated medium, those exposed to geraniol on emergence for 18 hr found geraniol less aversive in subsequent tests than did those kept in unscented vials after emergence.

These results are compatible with the postulate of the chemical legacy hypothesis that effects of chemicals from the larval medium on adult behavior may involve persistent chemical cues acting on the development of the chemosensory machinery in the adult during a "sensitive period" at or soon after emergence (Corbet, 1985). To elucidate the mechanism by which experience of a compound from the larval medium might modify the subsequent responsiveness of adult flies to it, we wanted to see how the adult dose–response curve would be affected by exposure to a compound in the larval medium. We chose the geraniol/*Drosophila* system because it involves a pure substance rather than

a mixture, because exposure to geraniol in the larval medium had been reported to reduce adult aversion to that compound (Manning, 1967), and because Manning (1967) reported a dose-dependent reversal of response, with aversion at high doses and attraction at low ones (see Knight and Corbet, 1991).

We did not find any evidence for attraction to geraniol at low doses, or for effects of pre-exposure before or up to 12 hr after emergence on the proportion entering scented barrels in subsequent tests. The failure of our fly/geraniol bioassay system to reveal phenomena reported by Manning prompts us to consider the features that are desirable in a model system designed to explore the mechanism by which chemicals from the larval environment might influence adult chemosensory responses. First, we consider properties of suitable chemical stimuli.

Natural materials, such as fruits, are mixtures of compounds. Even if natural materials are more appropriate when the ecological implications are being explored (Jaenike, 1983; Papaj and Prokopy, 1988), pure substances are preferred for studies of the mechanism of this effect (Manning, 1967; Jaenike, 1982). In nature, the effect may often depend on persistence of chemical cues on or near the insect's body until adult emergence. With some possible exceptions (ethanol; Jaenike, 1982; perhaps sinigrin), candidate compounds will have features associated with environmental persistence: low solubility in water, and relatively low volatility. In general, the effect on adult responsiveness has been clearest when the stimulus chemical is initially aversive or of low attraction; the response to highly attractive chemicals is less susceptible to changes (Jaenike, 1983, 1988; Vet et al., 1990). Further, the dose–response curve is expected to be more informative if there is initial attraction at very low doses. This has been demonstrated for some compounds (e.g., Rodrigues, 1980), but we have not been able to corroborate the claim that it applies to geraniol.

The manner in which the stimulus chemical is presented to insects may also be critical. The range of doses required for the construction of a dose–response curve is often achieved by diluting the candidate compound in a more volatile solvent such as diethyl ether (e.g., Borst, 1983; Quinn et al., 1974), and it is usual to wait until the solvent can no longer be smelled by the experimenter before conducting the bioassay. It is then assumed that any difference in the insect's response to the test chemical and the solvent control is due to the test chemical itself, and not to persistent traces of the solvent. However, this assumption might not be valid if the test chemical were to affect the evaporation kinetics of the solvent. That this may sometimes be so can be inferred from the perfumers' practice of using fixatives in the formulation of scents to delay evaporation (Moncrieff, 1967), and from the finding of Kaib and Dittebrand (1990) that less volatile components of an ant pheromone delayed evaporation of limonene. We found an effect of geraniol on the rate of evaporation of ether, and modified our procedure in the hope of minimizing it. If traces of solvent remain

in the test chamber, the flies' apparent response to the test chemical might be modified by, or dominated by a response to the solvent. Such solvent–solute interactions are not usually taken into account.

The allelochemicals acting as cues to specialized herbivorous insects in nature are often toxic to non-adapted insects (Spencer, 1988), but if there is substantial mortality during pre-exposure in these experiments, any treatment effects on adult responsiveness may be due to differential survival, rather than to modification of a chemosensory pathway (Jaenike, 1982, 1983). Manning (1967) reported reduced adult aversion to geraniol in a sample of 56 fruitflies reared in agar medium containing 0.5% geraniol by volume. We found that adult flies died in culture jars with medium containing geraniol at that strength, so we halved the geraniol concentration to 0.25%. Even then, flies in geraniol-agar delayed oviposition by about 3 days compared with flies in unadulterated medium.

We also suspect that the timing of exposure and testing may be critical. According to the chemical legacy hypothesis, some effects on adult responsiveness are attributable to exposure of the new adult to chemicals derived from the pre-imaginal environment during a "sensitive period," at or near the time of emergence from the pupa or cocoon, as illustrated by the elegant experiments of Jaenike (1982) and subsequently Herard et al. (1988). In these cases, the behavioral induction is not strictly pre-imaginal, although caused by chemical cues associated with the pre-imaginal environment. To eliminate these cues by washing pupae until no statistically significant effect remains is difficult (Thorpe, 1939; Hershberger and Smith, 1967), but not impossible (Jaenike, 1982). In experiments on modification of adult chemosensory responsiveness, the exact age at exposure of "newly emerged" adults may prove to be important. In our experiments, adults were collected as they left the puparium, but other workers may have timed emergence less precisely, using adults within perhaps a few hours of emergence. It is not yet clear how critical the timing of exposure in relation to emergence may be. The timing of testing is also important; for example, the effect of early exposure to a chemical stimulus wanes over succeeding days of adult life in *Drosophila* (Hershberger and Smith, 1967) and in the ant *Camponotus floridanus* (Carlin and Schwartz, 1989).

The extent to which the design of the bioassay apparatus influences the results remains unknown. Some experiments on olfactory conditioning in *Drosophila* have involved oviposition, being scored in terms of the numbers of eggs laid on two or more different substrate types available at the same time. Others depended on displacement of flies, by walking or in flight, sometimes taking advantage of geotactic and/or phototactic responses. They have used moving air in a Y-tube (Manning, 1967; Fuyama, 1976; Hershberger and Smith, 1967; Kikuchi, 1973) or two-way (Tully and Quinn, 1985; Tully, 1987) or four-way olfactometers (Vet et al., 1983), or still air offering flies a choice between two

chambers (Borst, 1983; Dudai et al., 1976). In some bioassays, some flies are allowed not to respond (e.g., Fuyama, 1976). In our experiments, the proportion of nonrespondents varied with the conditions.

Compounds attractive at low concentration may repel at high concentration (Knight and Corbet, 1991). A fly approaching a scent source in still air will encounter a concentration gradient. It will encounter a progressive increase in concentration of the test chemical, and may experience an aversive dose before reaching the source. At the same time, its chemosensory threshold may be elevated as a result of continuing exposure. The net effect on the fly's displacement is expected to depend on the form of the dose–response curve, the concentration gradient and the rates of approach and sensory adaptation, and on whether or not a non-return valve prevents departure from the source. These complications may not affect the result in cases where the entire gradient within the flies' arena is aversive, but may do so if it spans both concentrations high enough to repel and concentrations low enough to be neutral or attractive. A moving air olfactometer can be arranged so that concentration gradients are less steep, reducing this source of error.

Few experimenters have drawn conclusions on the basis of a single run (Manning, 1967). More often, runs are replicated for each treatment. These often reveal high variability, perhaps due to the numbers of flies per run, or the presence of one or both sexes of flies. Given an estimate of the mean and the variance in the response, it would be possible to calculate the number of runs required to establish a difference of a given magnitude at a given probability level (Elliott, 1977, p. 129).

Finally, chemosensory responsiveness varies throughout the 24-hr period such as in moths (Linn and Roelofs, 1986). Tests to be compared should therefore be run at about the same subjective biological time each day. We ran ours 3 hr after the onset of subjective dawn. Because of the need for thorough cleaning or replacement of the apparatus between tests to avoid scent contamination, this means that multiple copies of the equipment must be available if several tests are to be performed each day. Ideally, then, the apparatus on which the experimental model is based should be inexpensive and/or disposable.

Acknowledgments—We thank Stuart Green for technical assistance in setting up the project, and Bryan Grenfell for help with statistical computing. We also thank Zak Erzincliogl, Koos de Kramer, Peter Kearns, Aubrey Manning, Tim Tully, and Robert Brooks for their suggestions and advice. Figure 1 was drawn by Elizabeth A. Grant.

REFERENCES

BORST, W. 1983. Computation of olfactory signals in *Drosophila melanogaster*. *J. Comp. Physiol. A* 152:373–383.

CARLIN, N.F., and SCHWARTZ, P.H. 1989. Pre-imaginal experience and nestmate brood recognition in the carpenter ant, *Camponotus floridanus*. *Anim. Behav.* 38:89–95.

CORBET, S.A. 1985. Insect chemosensory responses: A chemical legacy hypothesis. *Ecol. Entomol.* 10:143–153.

DUDAI, Y., JAN, Y-H., BYERS, D., QUINN, W.G., and BENZER, S. 1976. *Dunce*, a mutant of *Drosophila* deficient in learning. *Proc. Natl. Acad. Sci. USA* 73:1684–1688.

ELLIOTT, M.M. 1977. Some methods for the statistical analysis of samples of benthic invertebrates, 2nd Ed. Ambleside, Freshwater Biological Association Scientific Publication No. 25.

FORD, S.C., NAPOLITANO, L.M., MCROBERTS, S.P., and TOMPKINS, L. 1989. Development of behavioral competence in young *Drosophila melanogaster* adults. *J. Insect Behav.* 2:575–588.

FUYAMA, Y. 1976. Behavior genetics of olfactory responses in *Drosophila*. I. Olfactory and strain differences in *Drosophila melanogaster*. *Behav. Genet.* 6:407–420.

HERARD, F., KELLER, M.A., LEWIS, W.J., and TUMLINSON, J.H. 1988. Beneficial arthropod behavior mediated by airborne semiochemicals. IV. Influence of host diet on host-oriented flight chamber responses of *Microplitis demolitor* Wilkinson. *J. Chem. Ecol.* 14:1597–1606.

HERSHBERGER, W.A., and SMITH, M.P. 1967. Conditioning in *Drosophila melanogaster*. *Anim. Behav.* 15:259–262.

JAENIKE, J. 1982. Environmental modification of oviposition behavior in *Drosophila*. *Am. Natur.* 119:784–802.

JAENIKE, J. 1983. Induction of host preference in *Drosophila melanogaster*. *Oecologia* 58:320–325.

JAENIKE, J. 1988. Effects of early adult experience on host selection in insects: Some experimental and theoretical results. *J. Insect Behav.* 1:3–15.

KAIB, M., and DITTEBRAND, H. 1990. The poison gland of the ant *Myrmicaria eumenioides* and its role in recruitment communication. *Chemoecology* 1:3–11.

KIKUCHI, T. 1973. Specificity and molecular features of an insect attractant in a *Drosophila* mutant. *Nature, Lond.* 243:36–38.

KNIGHT, J.C., and CORBET, S.A. 1991. Compound affecting mosquito oviposition: Structure-activity relationships and concentration effects. *J. Am. Mosquito Control Assoc.* 7:37–41.

LINN, C.E., and ROELOFS, W.L. 1986. Modulatory effects of octopamine and serotonin on male sensitivity and periodicity of response to sex pheromone in the cabbage looper moth, *Trichoplusia ni*. *Arch. Insect Biochem. Physiol.* 3:161–171.

MANNING, A. 1967. "Pre-imaginal conditioning" in *Drosophila*. *Nature* 216:338–340.

MONCRIEFF, R.W. 1967. The Chemical Senses. Leonard Hill, London.

PAPAJ, D.R., and PROKOPY, R.J. 1988. The effect of prior adult experience on components of habitat preference in the apple maggot fly (*Rhagoletis pomonella*). *Oecologia* 76:538–543.

QUINN, W.G., HARRIS, W.A., and BENZER, S. 1974. Conditioned behavior in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.* 71:708–712.

RODRIGUES, V. 1980. Olfactory behavior of *Drosophila melanogaster*, pp. 361–371, *in* O. Sidiqi, P. Babu, L.M. Hall, and J.C. Hall (eds.). Development and Neurobiology of *Drosophila*. Plenum Press, New York.

SPENCER, K.C. 1988. Chemical Mediation of Coevolution. Academic Press, San Diego. 609 pp.

THORPE, W.H. 1939. Further studies on pre-imaginal olfactory conditioning in insects. *Proc. Roy. Soc. Lond. B* 127:424–433.

TULLY, T. 1987. *Drosophila* learning and memory revisited. *TINS* 10:330–335.

TULLY, T., and QUINN, W.G. 1985. Chemical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J. Comp. Physiol. A.* 157:263–277.

VET, L.E.M., VAN LENTEREN, J.C., HEYMANS, M., and MEELIS, E. 1983. An airflow olfactometer for measuring olfactory responses of hymenopterous parasitoids and other small insects. *Phys. Entomol.* 8:97–106.

VET, L.E.M., LEWIS, W.J., PAPAJ, D.R., and VAN LENTEREN, J.C. 1990. A variable-response model for parasitoid foraging behavior. *J. Insect Behav.* 3:471–490.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

IDENTIFICATION OF FOOD VOLATILES ATTRACTIVE TO *Glischrochilus quadrisignatus* AND *Glischrochilus fasciatus* (COLEOPTERA: NITIDULIDAE)

HENGCHEN LIN* and P. LARRY PHELAN

Department of Entomology
Ohio Agricultural Research and Development Center
The Ohio State University
1680 Madison Avenue
Wooster, Ohio 44691

(Received June 3, 1991; accepted August 13, 1991)

Abstract—Seven volatile compounds identified from the headspace of whole wheat bread dough were investigated for their role in attracting *Glischrochilus quadrisignatus* and *G. fasciatus* in the field. Traps baited with either whole wheat bread dough or a synthetic seven-component bread dough odor caught similar numbers of these beetles, suggesting that the seven-compound combination could simulate the behavioral effect of bread dough. A series of trials using traps baited with various combinations of these chemicals showed that five compounds were significantly active in attracting *G. quadrisignatus* and *G. fasciatus*, but not all were essential for maximum response. The simplest blend eliciting a level of response comparable to the seven-component combination included ethyl acetate, acetaldehyde, ethanol, and racemic 2-methylbutanol, of which ethyl acetate, ethanol, and acetaldehyde were essential and 2-methylbutanol was replaceable with 2-methylpropanol for *G. quadrisignatus* attraction. Ethyl acetate and ethanol were essential for comparable attraction of *G. fasciatus*. The chemical mediation of food finding in *G. quadrisignatus* and *G. fasciatus* is discussed in the context of volatile blends characterized for other nitidulid species.

Key Words—Coleoptera, Nitidulidae, *Glischrochilus quadrisignatus*, *Glischrochilus fasciatus*, field traps, volatile attractants, host volatiles.

INTRODUCTION

Glischrochilus quadrisignatus (Say) and *G. fasciatus* (Olivier) are insect pests of many small fruits and vegetables. Severe damage to corn, strawberry, cane

*To whom correspondence should be addressed.

0098-0331/91/1200-2469$06.50/0

berry, and tomato caused by *Glischrochilus* species throughout the continental United States and Southern Canada frequently have been reported (Pree, 1969; Foott and Hybsky, 1976; Miller and Williams, 1981). In addition, nitidulid beetles are considered the primary vectors of oak wilt disease (Appel et al., 1986), and *G. quadrisignatus* and *G. fasciatus* are among the principal insect species associated with oak wilt-infected trees (Dorsey and Leach, 1956; Juzwik and French, 1983). *G. quadrisignatus* is also associated with *Fusarium* pathogens of corn roots, stalks, and ears (Windels et al., 1976), and is capable of transmitting conidia and ascospores of *Gibberella zea* (the perithelial state of *Fusarium graminearum*) causing rot to corn ears (Attwater and Busch 1983). Furthermore, *Glischrochilus* species can be a considerable nuisance by disrupting picnics and other outdoor activities (Luckmann, 1963; Miller and Williams, 1981).

Glischrochilus species are attracted to a variety of host odors and traps baited with foods have been used in monitoring these and other nitidulid beetles (Foott and Hybsky, 1976; Alm et al., 1985, 1986, 1989; Peng and Williams, 1991). Given that these beetles attack fruits and vegetables late in the maturation process, conventional pesticide control may be unacceptable. Mass trapping using odor baits has been conducted for control and has been suggested as an alternative to broadcast application of pesticides to control these pests (Warner, 1960; Pree, 1969; Foott and Hybsky, 1976); however, the chemical basis of nitidulid food finding is incompletely understood. Our laboratory has recently characterized volatiles mediating food-finding in two other nitidulids, *Carpophilus hemipterus* and *C. lugubris* (Phelan and Lin, 1991; Lin and Phelan, 1991). In this study, we sought to elucidate chemicals mediating food finding in *Glischrochilus quadrisignatus* and *G. fasciatus* to allow a broader interspecific comparison of this process among members of this group of insect herbivores, and more specifically to aid in the development of monitoring tools and alternative control strategies for these insects.

MATERIALS AND METHODS

Odor Baits. Natural odor baits used in this study were either 30 g of whole wheat bread dough inoculated with baker's yeast (Lin and Phelan, 1991) or 30 g of banana inoculated with the yeast *Saccharomyces cerevisiae* (Phelan and Lin, 1991). Although a variety of fruits and vegetables were attractive to *Glischrochilus quadrisignatus* and *G. fasciatus*, whole wheat bread dough was one of the most attractive substrates (Blackmer, 1991). Thus, bread dough odor was used as model for synthetic odor. Yeast-inoculated banana, another attractive substrate, was tested in the first trial to confirm that bread dough and yeast-inoculated banana were equally effective in attracting these beetles.

Headspace volatile profiles of whole wheat bread dough and synthetic blends were analyzed using a Tenax-based volatile-collection device integrated

into a Hewlett-Packard 5890A capillary gas chromatograph (GC) and a Hewlett-Packard 5970C Mass Selective Detector (MS). Methods for collection and analysis of bread dough odor have been previously described (Lin and Phelan, 1991). GC separations were conducted on a DB-1 capillary column (30 m × 0.32-mm ID, 5.0-μm film thickness; J & W Scientific, Folsum, California) with a temperature program of 30–200°C, increased at 10°C/min.

Based on our previous chemical characterization of bread dough odor (Lin and Phelan, 1991), a synthetic odor bait was generated based on seven compounds: acetaldehyde, ethyl acetate, ethanol, propanol, 2-methylpropanol, 2-methylbutanol and 3-methylbutanol. A racemic mixture of 2-methylbutanol was used in this study. Chemicals were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin) and were diluted individually with mineral oil, except ethanol, which was diluted by double-deionized water due to its insolubility in mineral oil. Synthetic odor blends were created by adjoining 1.2-mL glass vials (9 mm ID × 30 mm H), each of which contained 1 mL of an individual component solution. This method effectively prolonged the release of the chemicals and enabled us to change the resultant odor by removing or manipulating the release rate of individual components without altering the release rate of other odor components. Through repetitively adjusting the concentration of each component solution, we generated a synthetic odor profile that closely mimicked that of whole wheat bread dough using the synthetic-blend composition listed in Table 1. The release rate of components in the synthetic blend were

TABLE 1. COMPOSITION OF A SYNTHETIC BREAD DOUGH ODOR, RELEASE RATES OF INDIVIDUAL COMPONENTS, AND COMPARISON OF THEIR HEADSPACE CONCENTRATIONS WITH THOSE OF NATURAL BREAD DOUGH ODOR[a]

Components		Synthetic odor composition[b]	Release rate[c] (mg/24 hr)	SY:BD[d] (absolute quantity)			
Abbr.	Identity			12 hr	24 hr	36 hr	Mean
EtAc	Ethyl acetate	1 ml, 0.25%	0.58	1.67	1.19	1.25	1.37
AcAl	Acetaldehyde	3 ml, 1%	14.65	1.60	0.54	0.44	0.86
EtOH	Ethanol	3 ml, 25%	236.6	0.99	1.02	1.35	1.12
PrOH	1-Propanol	1 ml, 0.5%	2.11	2.40	1.76	2.49	2.22
MPrOH	2-Methylpropanol	1 ml, 0.5%	2.04	0.80	1.11	1.30	1.07
3MBOH	3-Methylbutanol	1 ml, 0.5%	2.39	1.22[e]	1.10	1.17	1.16
2MBOH	2-Methylbutanol	1 ml, 0.5%	2.38				

[a] Measurements of release rates were conducted in the laboratory under conditions of 25–28°C and 65–75% RH.
[b] Solution (v/v) in mineral oil except ethanol, which was diluted with distilled water.
[c] Based on the total release rate in 48 hr ($N = 3$).
[d] Ratio between absolute concentration of each component in the headspace of the synthetic blend (SY) relative to bread dough (BD) at 12, 24, and 36 hr, respectively ($N = 3$).
[e] 3-Methylbutanol and 2-methylbutanol were combined due to unresolved GC peaks.

compared to those in the natural odor after 12, 24, and 36 hr by comparison of GC peak areas. The release rates of 2-methylbutanol and 3-methylbutanol were measured in combination due to their similar retention time in our GC/MS analysis (Lin and Phelan, 1991). The cumulative release over time of each of these seven compounds was measured individually using an electronic balance (Mettler H54AR, $d = 0.01$ mg). Traps for volatile collection and measurement were set in the laboratory at 25–28°C and 65–75% RH. Vials filled with 1 mL mineral oil and with 1 mL double-deionized water were set under the same conditions and their weight losses were also measured as control.

Field Trap and Experimental Procedure. Skalbeck traps (Skalbeck, 1976) were used in this study, because of their trapping efficiency and economy compared with other trap designs (Peng and Williams, 1991). Briefly, the trap consisted of a pint-size canning jar, a screen cap, a hardboard rain shield over the screen cap, and a plastic funnel-shaped cup with the bottom removed placed in the jar underneath the screen cap. The odor bait was held in a small plastic cup covered with fine-mesh organdy to keep beetles out, and the cup was placed at the bottom of the jar. Bread dough was incubated in a plastic bag at room temperature for about 12 hr and the synthetic combinations were prepared immediately prior to trap deployment. Traps were set up in early afternoon and removed after 48 hr.

The trials were conducted in an abandoned apple orchard at Wooster, Ohio, in July and August of 1990. Traps were set at ground level, which was shown to be the height most effective in trapping *Glischrochilus* species (Alm et al., 1989; Peng and Williams, 1991). A randomized complete-block design was used in all trials. Original data were subjected to the transformation of $\log(x + 1)$ before analysis of variance, and Duncan's multiple range test was used for comparing means where the ANOVA indicated a significant F value (SAS Institute, 1985).

RESULTS

Odor Samples. Using the synthetic composition listed in Table 1, the average release rate of individual components over 48 hr ranged from 0.58 mg/24 hr (ethyl acetate) to 236.6 mg/24 hr (ethanol; Table 1). Concentrations of the seven components above the headspace of the synthetic blend and bread dough were similar at 12, 24, and 36 hr after trap setup, and mean ratios of individual compounds between the synthetic blend and bread dough ranged from 0.86–2.22, which we regarded as very good considering the dynamic nature of fermenting bread dough (Table 1).

Beetle Attraction. The mean numbers of *G. quadrisignatus* and *G. fasciatus* caught in traps baited with the synthetic odor of seven compounds was not significantly different from those trapped with bread dough (Figure 1), suggesting that the synthetic blend was equally as effective in attracting both these

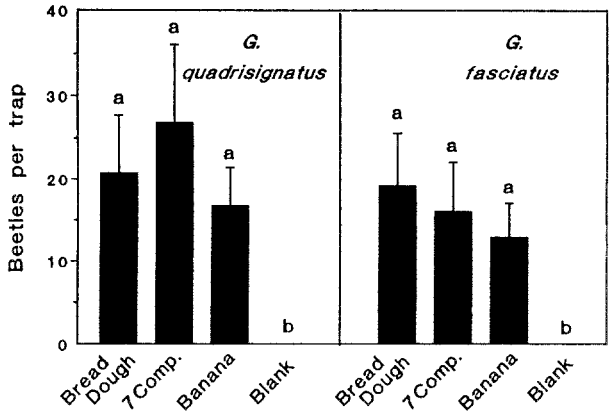


FIG. 1. Catch of *G. quadrisignatus* and *G. fasciatus* ($\bar{x} \pm$ SEM) from traps baited with bread dough (Dough), the synthetic bread dough odor (7 Comp.), or yeast-inoculated banana (Banana), and from blank traps. Bars marked by the same letter are not significantly different (Duncan's multiple-range test, N = eight traps, $P < 0.05$).

species as natural bread dough odor. Beetle responses to bread dough or to the blend of seven compounds were not significantly different from that to yeast-inoculated banana, confirming that bread dough and yeast-inoculated banana were equally attractive to these two species. Blank traps used throughout this study never caught any beetles.

When components of the synthetic odor blend were removed selectively, based on chemical functionality, the absence of acetaldehyde (AcAl), ethyl acetate (EtAc), or all five alcohols (5OH) reduced the beetle response, although the reduction in *G. fasciatus* response caused by the removal of acetaldehyde was not statistically significant (Figure 2). These results suggested that acetaldehyde, ethyl acetate, and one or more alcohols were required for a full-level attraction of *G. quadrisignatus* and *G. fasciatus*.

Next, two sets of trials focused on the relative activity of bread dough odor alcohols. For both species, the addition of individual alcohols to the basic combination of ethyl acetate and acetaldehyde did not significantly enhance beetle response (Figure 3). The addition of ethanol as the only alcohol significantly reduced the catch of *G. quadrisignatus*, with a similar nonsignificant trend for *G. fasciatus* (Figure 3). However, in the subsequent experiment, in which individual alcohols were removed from the seven-component blend, only the combination lacking ethanol caught significantly fewer individuals of either species than the full blend (Figure 4). In both cases, this treatment was not significantly different from that of the two-component blend lacking all alcohols. These results suggested that ethanol was an essential component and that some of the other alcohols were required for a full attraction of both *G. quadrisignatus* and *G. fasciatus*.

In the final trial, the addition of 2-methylbutanol or 2-methylpropanol to

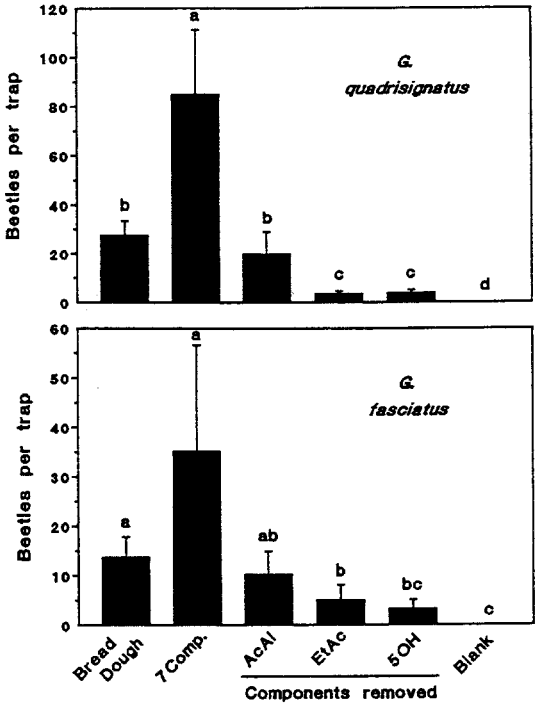


FIG. 2. Catch of *G. quadrisignatus* and *G. fasciatus* ($\bar{x} \pm$ SEM) from traps baited with bread dough, the synthetic bread dough odor (7 Comp.), or synthetic blends with components selectively removed, and from blank traps. Bars marked by the same letter are not significantly different (Duncan's multiple-range test, N = eight traps, $P < 0.05$; see Table 1 for explanation of compound abbreviations).

the three-compound blend of ethyl acetate, acetaldehyde, and ethanol significantly improved the attraction of *G. quadrisignatus* to the level of the seven-component blend (Figure 5). The addition of either propanol or 3-methylbutanol did not significantly enhance trap catch above that of the three-component blend, and the addition of 2-methylpropanol plus 3-methylbutanol to the three-component blend was no more effective than the addition of 2-methylpropanol alone. The response of *G. fasciatus* to these synthetic blends was similar in trend, although some of the differences in trap catch were not significant, probably due to the very low catch overall for this species. This trial demonstrated that at least one bread dough odor alcohol in addition to ethanol was required for full attraction of *G. quadrisignatus*, and probably for *G. fasciatus* as well.

DISCUSSION

In this study, we demonstrated that traps baited with whole wheat bread dough and the seven-component synthetic blend mimicking bread dough odor

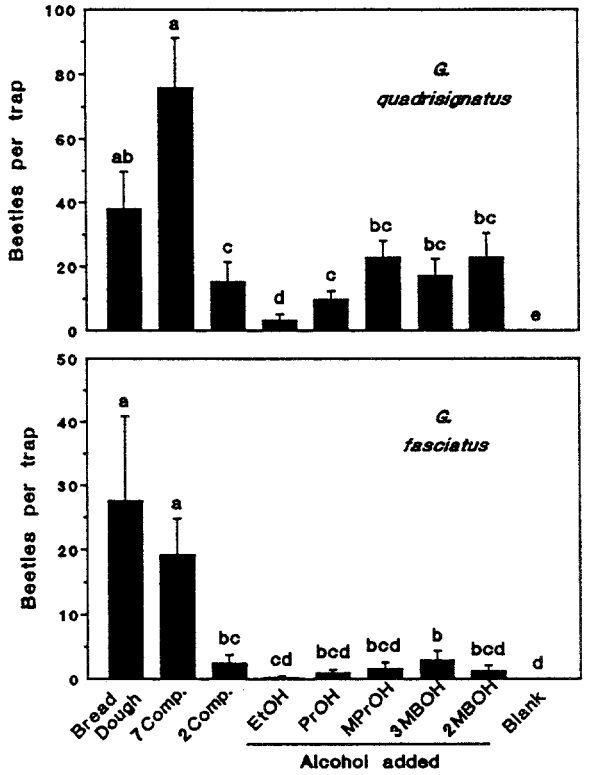


FIG. 3. Catch of *G. quadrisignatus* and *G. fasciatus* ($\bar{x} \pm$ SEM) from traps baited with bread dough, the synthetic bread dough odor (7 Comp.), a base blend of acetaldehyde and ethyl acetate (2 Comp.), or synthetic blends with each bread dough odor alcohol added to the base blend, and from blank traps. Bars marked by the same letter are not significantly different (Duncan's multiple-range test, N = eight traps, $P < 0.05$; see Table 1 for explanation of compound abbreviations).

caught comparable numbers of *G. quadrisignatus* and *G. fasciatus* in the field (Figure 1). In a series of trials that employed the removal and addition of individual components from the seven-component synthetic blend, both species demonstrated a similar trend in response. The effect of ethyl acetate was clear for both species, as its absence significantly reduced the beetle catch (Figure 2). Beetle catch was also reduced by the removal of acetaldehyde, although this reduction was not statistically significant for *G. fasciatus* (Figure 2). Among the bread dough odor alcohols, ethanol represented a third essential component as its removal significantly reduced capture of both species (Figure 4); however, a minimum of one remaining alcohols was also needed for full beetle attraction and it was at least partially interchangeable in this role (Figures 3 and 5).

Alm et al. (1985, 1986, 1989) tested a number of short chain esters, alcohols, and acids individually using traps in the field and found that butyl acetate,

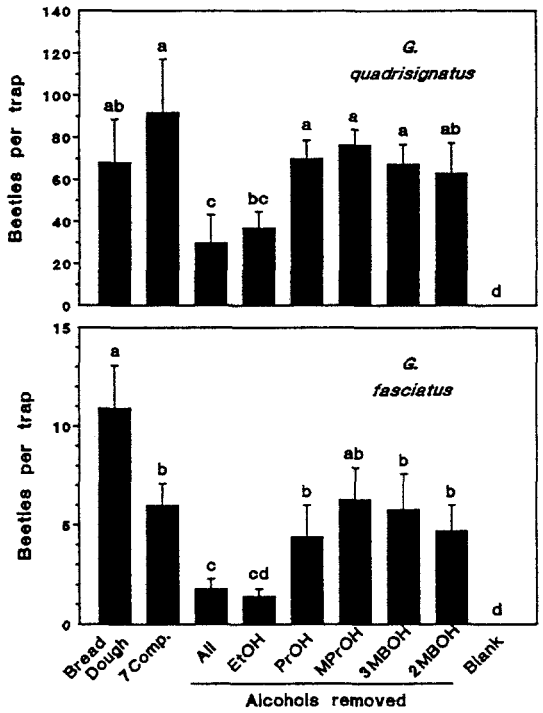


FIG. 4. Catch of *G. quadrisignatus* and *G. fasciatus* ($\bar{x} \pm$ SEM) from traps baited with bread dough, the synthetic bread dough odor (7 Comp.), or synthetic blends with each bread dough odor alcohol removed, and from blank traps. Bars marked by the same letter are not significantly different (Duncan's multiple-range test, N = eight traps, $P < 0.05$; see Table 1 for explanation of compound abbreviations) .

propyl propionate, and allyl propionate were attractive to *G. quadrisignatus*. However, the role of these three components for host finding of *G. quadrisignatus* in nature is not clear, because the release rates used in these studies were 0.061–2 ml/24 hr, which were considerably higher than from natural odor sources. Butyl acetate also was identified in the headspace above yeast-inoculated banana (Phelan and Lin, 1991), but it was not found in bread dough odor (Lin and Phelan, 1991). The beetle catch with yeast-inoculated banana was not significantly different from that with bread dough or the synthetic bread dough odor (Figure 1), suggesting that butyl acetate does not play a major role in the attraction of *G. quadrisignatus* at the rate of release from yeast-inoculated banana.

Bread dough was used as a positive control throughout this study. The response of *G. quadrisignatus* seemed to be lower to bread dough than to the seven-component blend, and the difference was statistically significant in one trial (Figure 2); however, the response of *G. fasciatus* was significantly higher to bread dough than to the seven-component blend in two trials (Figures 4 and

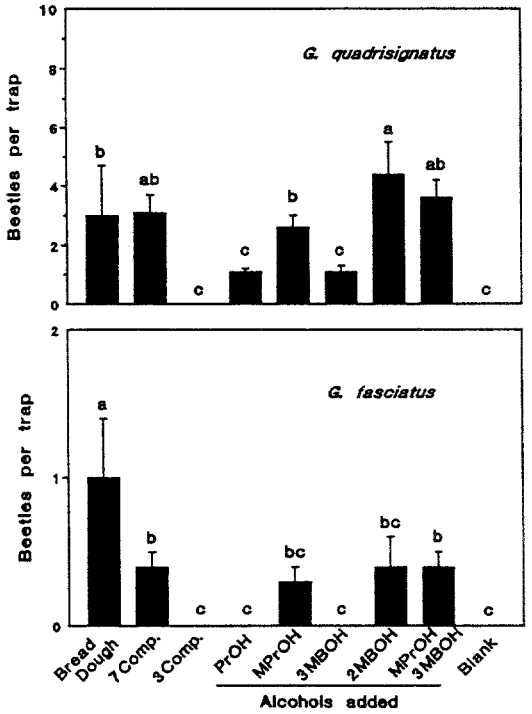


FIG. 5. Catch of *G. quadrisignatus* and *G. fasciatus* ($\bar{x} \pm$ SEM) from traps baited with bread dough, the synthetic bread dough odor (7 Comp.), a base blend of acetaldehyde, ethyl acetate, and ethanol (3 Comp.), or synthetic blends with bread dough odor alcohols added to the base blend, and from blank traps. Bars marked by the same letter are not significantly different (Duncan's multiple-range test, $N = 20$ traps, $P < 0.05$; see Table 1 for explanation of compound abbreviations).

5). This suggests that volatile components mediating the host-finding behavior of these two closely-related species are not identical and that some additional unidentified minor constituents in bread dough odor may be important for *G. fasciatus*, although both species demonstrated a similar trend in response to the seven bread dough odor constituents tested. Alm et al. (1985) also found the difference of these two species in response to volatile chemicals in the field: *G. quadrisignatus* was attracted to butyl acetate, and *G. fasciatus* was not, although both species were attracted to natural substrates.

Agriculturally-related sap beetles including *Glischrochilus* species are attracted to a variety of fruits, vegetables, and foods in the field and in the laboratory, and bread dough was one of the most attractive and commonly used substrates (Pree, 1969; Foott and Hybsky, 1976; Alm et al., 1985; Blackmer, 1991; Peng and Williams, 1991). Volatile components produced by bread dough are also identified from fruits and vegetables, although bread dough odor consists of fewer components than the others (Phelan and Lin, 1991; Lin and

Phelan, 1991). By closely simulating the release rate for each of the volatile constituents identified from bread dough or from a fruit substrate, we were better able to identify the active components attracting the beetles, and to deduce the relative complexity of interactions between components in the mediation of the beetles' host-finding behavior. Food odor attractants for four nitidulid species now have been identified as summarized in Table 2. In general, the active components are short-chain compounds with various functionalities, and they can be clustered into groups based on behavioral activity. Eliciting maximum beetle attraction requires a blend including one or more components from each group. Components within a group may be partially or fully interchangeable in eliciting beetle orientation, while those in different groups are not. Relative effectiveness of components within a group may be the result of qualitative differences or of quantitative differences, since compounds were tested at the concentrations measured in the natural odors.

Acetaldehyde plays an important role in attracting all four species of sap beetles (Table 2). The role of acetaldehyde was replaceable in part by a combination of the banana odor ketones for *C. hemipterus*. This relationship has not been investigated for the other three species, since ketones were not present in bread dough odor. The role of ethyl acetate is clear: it is essential in attracting *C. hemipterus* and two *Glischrochilus* species, but its activity in attracting *C.*

TABLE 2. ACTIVITY GROUPS OF FOOD VOLATILE CONSTITUENTS FOR FOUR NITIDULID SPECIES[a]

Volatile constituents	*C. hemipterus*[b]	*C. lugubris*[c]	*G. quadrisignatus*	*G. fasciatus*
Aldehyde ketones				
Acetaldehyde	1	1	1	1
2-pentanone	1	nt	nt	nt
3-hydroxy-2-butanone	1	nt	nt	nt
Esters				
Ethyl acetate	2	2	2	2
Alcohols				
Ethanol	ne	2,3	3	3
Propanol	3	2,3	4	4
2-methylpropanol	3	3	4	4
3-methylbutanol	3	3	4	4
2-methylbutanol	nt	3	4	4
Butanol	3	nt	nt	nt
2-pentanol	4	nt	nt	nt

[a] For each species, constituents with the same number are at least partially interchangeable and those with numbers not shared with other components are essential in eliciting attraction. An "ne" indicated that the component did not significantly enhance attraction and an "nt" indicated that the component was not tested for the species.
[b] From Phelan and Lin (1991).
[c] From Lin and Phelan (1991).

lugubris is partially replaceable with ethanol and also probably propanol (Table 2). Although seven more ester compounds were identified in the banana odor, they showed no effect on the attraction of *C. hemipterus* and had not been tested for the other three species.

Seven alcohols were identified from banana odor and five were identified from bread dough odor, their roles in attracting these beetles are very diverse (Table 2). We did not find ethanol to be attractive for *C. hemipterus* when presented at the concentration in inoculated banana odor (Phelan and Lin, 1991), although Smilanick et al. (1978) did suggest that it was active when presented in combination with ethyl acetate and acetaldehyde in equal ratio. In attracting *Glischrochilus* species, ethanol is an essential component and its activity is not replaceable with any other bread dough odor components. For *C. lugubris*, ethanol demonstrated dual roles: it is partially interchangeable with ethyl acetate, and it also is active in the role played by the other bread dough odor alcohols. All other bread dough odor alcohols play the same role in attracting these four species at various levels. The fourth role in the attraction of *C. hemipterus* is played by 2-pentanol. It was not detected in bread dough odor and this was not tested for other three species, but it is unlikely to play an essential role in attracting the other three species, because bread dough elicits a response from *C. lugubris* (Blackmer, 1991) and from the two *Glischrochilus* species (this study) comparable to that of yeast-inoculated banana.

Our present and previous studies on food odor attractants of four nitidulid species suggest that the host-finding behavior of each species is mediated by broad overlapping blends of common volatile constituents from fruits and vegetables. This chemically-mediated response mechanism is consistent with the broad host ranges exhibited by all four species, and with their demonstrated response to a variety of food substrates. Further work is required to determine if the subtle difference in optimal blends for each species serves any adaptive function, such as in determining levels of substrate decomposition.

Acknowledgments—The authors thank Dr. R.W. Williams for providing traps and D. Fickle for assistance in data collection in some of the trials. Critical reviews of the manuscript were made by Drs. R.K. Lindquist, T.L. Ladd, Jr., and R.W. Williams. Salaries and research support were provided by state and federal funds, as well as by a grant from the USDA Competitive Research Grants Office (87-CRCR-1-294), appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University, Journal Article No. 106-91.

REFERENCES

ALM, S.R., HALL, F.R., LADD, T.L., and WILLIAMS, R.N. 1985. A chemical attractant for *Glischrochilus quadrisignatus* (Coleoptera: Nitidulidae). *J. Econ. Entomol.* 78:839–843.

ALM, S.R., HALL, F.R., MCGOVERN, T.P., and WILLIAMS, R.N. 1986. Attraction of *Glischrochilus quadrisignatus* (Coleoptera: Nitidulidae) to semiochemicals: Butyl acetate and propyl propionate. *J. Econ. Entomol.* 79:654–658.

ALM, S.R., WILLIAMS, R.N., MCGOVERN, T.P., and HALL, F.R. 1989. Effective chemical struc-

tures, release methods, and trap heights for attracting *Glischrochilus quadrisignatus* (Coleoptera: Nitidulidae). *J. Econ. Entomol.* 82:477–481.

APPEL, D.N., ADERSEN, K., and LEWIS, R. 1986. Occurrence of nitidulid beetles (Coleoptera: Nitidulidae) in Texas oak wilt centers. *J. Econ. Entomol.* 79:1276–1279.

ATTWATER, W.A., and BUSCH, L.V. 1983. Role of the sap beetle *Glischrochilus quadrisignatus* in the epidemiology of gibberella corn ear rot. *Can. J. Plant Pathol.* 5:158–163.

BLACKMER, J. 1991. Factors Affecting Host Orientation and Selection in Nitidulidae (Coleoptera). Ph.D. thesis, The Ohio State University. 141 pp.

DORSEY, C.K., and LEACH, J.G. 1956. The bionomics of certain insects associated with oak wilt with particular reference to the Nitidulidae. *J. Econ. Entomol.* 49:219–230.

FOOTT, W.H., and HYBSKY, J.E. 1976. Capture of *Glischrochilus quadrisignatus* (Coleoptera: Nitidulidae) in bait traps, 1970–1974. *Can. Entomol.* 108:837–839.

JUZWIK, J., and FRENCH, D.W. 1983. *Ceratocystis fagacearum* and *C. piceae* on the surfaces of free-flying and fungus-mat-inhibiting nitidulids. *Phytopathology* 73:1164–1168.

LIN, H., and PHELAN, P.L. 1991. Identification of food volatiles attractive to *Carpophilus lugubris* (Coleoptera: Nitidulidae). *J. Chem. Ecol.* 17:1273–1286.

LUCKMANN, W.H. 1963. Observations on the biology and control of *Glischrochilus quadrisignatus*. *J. Econ. Entomol.* 56:681–686.

MILLER, K.V., and WILLIAMS, R.N. 1981. An Annotated Bibliography of the Genus *Glischrochilus* Reitter (Coleoptera: Nitidulidae, Cryptarchinae). Research Circular 266, Ohio Agricultural Research and Development Center, Wooster, Ohio.

PENG, C., and WILLIAMS, R.N. 1991. Effect of trap design, trap height and habitat on the capture of sap beetles (Coleoptera: Nitidulidae) using whole wheat bread dough. *J. Econ. Entomol.* In press.

PHELAN, P.L., and LIN, H. 1991. Chemical characterization of fruit and fungal volatiles attractive to the driedfruit beetle, *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae). *J. Chem. Ecol.* 17:1253–1272.

PREE, D.J. 1969. Control of *Glischrochilus quadrisignatus* (Say) (Coleoptera: Nitidulidae), a pest of fruit and vegetables in southern Ontario. *Proc. Entomol. Soc. Ont.* 99:60–64.

SAS INSTITUTE. 1985. User's Guide: Statistics. SAS Institute, Cary, North Carolina.

SKALBECK, T.C. 1976. The Distribution of Nitidulidae in Deciduous Forests of Minnesota. Ph.D. thesis, University of Minnesota. 204 pp.

SMILANICK, J.M., EHLER, L.E., and BIRCH, M.C. 1978. Attraction of *Carpophilus* spp. (Coleoptera: Nitidulidae) to volatile compounds present in figs. *J. Chem. Ecol.* 4:701–707.

WARNER, R.W. 1960. Area baiting to control drosophila and nitidulid beetles. *Proc. 14th Ann. Res. Conf.*, Calif. Fig Inst., pp. 35–38.

WINDELS, C.E., WINDELS, M.B., and KOMMEDAHL, T. 1976. Association of Fusarium species with picnic beetles on corn ears. *Phytopathology* 66:328–331.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

AGE-RELATED RESPONSE OF MALE MELON FLIES *Dacus cucurbitae* (DIPTERA: TEPHRITIDAE) TO CUE-LURE

TIM T.Y. WONG,* D.O. McINNIS, MOHSEN M. RAMADAN, and JON I. NISHIMOTO

Tropical Fruit and Vegetable Research Laboratory
USDA/ARS, P.O. Box 2280
Honolulu, Hawaii 96804

(Received April 17, 1991; accepted August 19, 1991)

Abstract—Laboratory-reared and wild adults of the melon fly, *Dacus cucurbitae* Coquillett, were tested for response to cue-lure at various ages. Virgin laboratory (4, 6, 8, 10, 12, and 14 days old) and wild (10, 12, 14, 16, 18, 20, and 22 days old) flies were released into outdoor field cages and trapped from 0800 until 1600 hr. Response of males to cue-lure increased with age and corresponded with sexual maturity for each strain. Females of both strains were relatively nonresponsive to cue-lure. Failure to eradicate in past male annihilation programs against *D. cucurbitae* may be explained in part by the fact that only older males, which may have already mated with gravid females, respond to cue-lure.

Key Words—*Dacus cucurbitae*, Diptera, Tephritidae, melon fly, cue-lure, age effects, pheromone.

INTRODUCTION

Cue-lure, a synthetic lure for male melon flies, *Dacus cucurbitae* Coquillett, has been in use since 1960 (Beroza et al., 1960). It is still the most effective attractant available for survey and detection of this insect (Keiser et al., 1973, Harris et al., 1986, Wong et al., 1989b). Cue-lure also attracts other tephritids. Drew (1974) and Drew and Hooper (1981) reported over 80 species of Dacini responded to cue-lure. The possible use of cue-lure in male annihilation programs against *D. cucurbitae* has been demonstrated in several large area field

*To whom correspondence should be addressed.

tests. Cunningham and Steiner (1972) and Cunningham et al. (1970, 1975) reported reductions of up to 99% of male melon flies in plots up to 15 km^2. However, these test populations were only semi-isolated; therefore, as a result, the tests could not demonstrate eradication. Furthermore, in the 10-km^2 plot test, a meaningful reduction in the infestation rate of *D. cucurbitae* in its wild host fruit, *Momordica charantia* L., failed to occur (Cunningham and Steiner, 1972). Bateman et al. (1966a,b) failed to reduce fruit infestation rates by the Queensland fruit fly, *Dacus tryoni* (Froggatt), in semi-isolated populations in small towns in Australia. Bateman (1973) did, however, achieve eradication of *D. tryoni* from Easter Island by using a combination of protein bait sprays and cue-lure male-annihilation treatments.

Here we report studies on the response to cue-lure of various ages of *D. cucurbitae* reared in the laboratory on artificial diet (Tanaka et al., 1969, 1982) compared with wild flies from naturally infested fruits. Also, we compare the present data with sexual maturation data of *D. cucurbitae* from Wong et al. (1986).

METHODS AND MATERIALS

Laboratory-reared melon flies used in the study had been in culture for over 400 generations (at least 33 years, with about 12 generations per year). Throughout the testing period from 1984 to 1985, wild flies were collected as mature larvae and pupae from naturally infested squashes, *Cucurbita pepo* L., and melons, *Cucumis melo* L., from Waimanalo, Oahu, Hawaii. Two days before eclosion, pupae were dyed with powdered pigments (Day-Glo Neon Red, Day-Glo Signal Green, Day-Glo Saturn Yellow, Day-Glo Blaze Orange, Day-Glo Invisible Blue, Calco Blue, and Tinopal SFG) using different colors for different ages of flies. Dyed pupae were placed in (61 × 38 × 37 cm) screened cages separated by age group and provided with ICN hydrolyzed protein (Nutritional Biochemical, Cleveland, Ohio), sugar, honey, and water, as needed for survival of newly emerged adults (Keiser and Schneider, 1969). One day after eclosion, virgin flies were separated by sex while immobilized in a refrigerated room (5–7°C) and then transferred to screened cages and held at ambient room condition (average temperature 26°C, range 24.5–28°C, 66–76% relative humidity under natural daylight supplemented with fluorescent light) until ready for testing. Previous laboratory tests using the same laboratory strain and the same wild population (Wong et al., 1986) had shown differences (both sexes) in physiological ages of the two strains of flies, i.e., sexual maturation of 90% or more was reached under these conditions in 12 days with laboratory-reared flies and in 20 days with wild flies. Therefore, in this study, tests were made when laboratory flies were 4, 6, 8, 10, 12, and 14 days old and when wild flies

were 10, 12, 14, 16, 18, 20, and 22 days old. The tests were made in two outdoor screened cages (2.5 × 2.5 × 2.5 m). In each of two cages, we released 100 unmated flies of each sex and age, laboratory-reared flies in one and wild flies in the other. Water and sugar were provided in each cage. All releases were made at 0745 hr. A plastic trap (Steiner, 1957) containing a 10-ml mixture of 99% cue-lure and 1% naled by volume in a cotton wick (4 cm long × 2 cm diam.); (Keiser et al., 1973) was placed in each outdoor cage at 0800 hr, then replaced with another identical baited trap containing cue-lure and naled hourly until 1600 hr. The flies become inactive at night and during this time are not attracted to cue-lure (Nakagawa, unpublished data). Therefore, we did not continue the experiment beyond 1600 hr. The test was replicated ten times. Total flies trapped for each testing day were recorded. Temperature and relative humidity ranged from 17° to 32°C and from 56 to 88%, respectively, during testing days. Mean percentages of flies captured were transformed using the arcsine procedure before analysis (Snedecor and Cochran, 1967). Mean captures, by age class, within laboratory or wild classes were compared with Duncan's multiple-range test at $P = 0.05$ level (SAS Institute, 1985).

Sexually mature virgin females of some tephritids respond strongly to certain synthetic male lures when males are scarce or absent. Steiner et al. (1965) reported this behavior for females of the oriental fruit fly, *Dacus dorsalis* Hendel, and Nakagawa et al. (1970, 1981) for females of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). We therefore conducted two cage tests to determine the response of mature virgin female *D. cucurbitae* to cue-lure in the absence of males. In one test, 500 6- to 18-day-old virgin laboratory-reared females were released in an outdoor cage. This was repeated six times, each on different dates. In another test, 300 20- to 26-day-old virgin wild females were released into a cage. This test was repeated four times, each on different dates. Testing procedures for these two tests were similar to previous tests with males and females.

RESULTS AND DISCUSSION

In the first experiment involving the releasing of both sexes, captures of males of both fly strains by traps baited with cue-lure increased gradually as adults increased in age (Table 1). A few (5.3%) laboratory males were caught in traps at age 4 days., more than 60% at age 8 days, and over 96% at age 14 days. However, males of the wild strain required a much longer period before they responded to cue-lure: only 27% at age 10 days, 57% at age 14 days, and full response (>93%) was not reached until age 22 days. Females of both strains were seldom attracted to the cue-lure (Table 1). Maximum average female capture was 1.0 (10 days old) and 0.6 (18 days old) for laboratory and

TABLE 1. RESPONSE OF LABORATORY-REARED AND WILD *Dacus cucurbitae* OF DIFFERENT AGES TO TRAPS BAITED WITH CUE-LURE AND 1% NALED IN OUTDOOR CAGES, HONOLULU, HAWAII

Strain	Age (days)	No. flies captured (mean ± SEM)[a]	
		Males	Females
Laboratory	4	5.30 ± 1.26 e	0.5 · 0.3 a
	6	43.20 ± 5.32 d	0.2 · 0.2 a
	8	63.10 ± 4.44 c	0.4 · 0.2 a
	10	79.70 ± 3.04 b	1.0 · 0.3 a
	12	88.40 ± 2.25 ab	0.4 · 0.3 a
	14	96.50 ± 1.07 a	0.4 · 0.3 a
Wild	10	27.20 ± 4.28 d	0 b
	12	32.60 ± 3.32 d	0 b
	14	57.20 ± 4.49 c	0.1 · 0.1 b
	16	63.80 ± 4.22 c	0.3 · 0.2 ab
	18	75.40 ± 5.46 b	0.6 · 0.3 a
	20	81.60 ± 3.94 b	0.3 · 0.2 ab
	22	93.90 ± 1.51 a	0.1 · 0.1 b

[a] Numbers of laboratory-reared or wild flies trapped between the hours of 0800–1600 in a single day. Means based on ten replicates of 100 flies per sex per strain released into cages. Means followed by the same letter within the same sex and strain are not significantly different ($P = 0.05$; Duncan's (1955) multiple range test). Data were arcsine transformed before analysis of variance; untransformed means are presented.

wild flies, respectively. Female and male flies caught in traps were observed nearly touching the wick treated with cue-lure but never observed to be feeding on it.

Response of males to cue-lure increased with age and corresponded with sexual maturity within each fly strain (Figure 1). Best fitting linear regressions for percentage response (Y) of laboratory-reared and wild males of different ages (X) to traps baited with cue-lure are: $Y = 8.69\ (X) - 15.5\ (r = 0.91, df = 59, P \leq 0.05)$ and $Y = 5.65\ (X) - 28.7\ (r = 0.87, df = 69, P \leq 0.05)$, respectively, and for laboratory-reared and wild males mated at different ages are: $Y = 12.19\ (X) - 34.1\ (r = 0.89, df = 79, P \leq 0.05)$ and $Y = 9.1\ (X) - 73.0\ (r = 0.91, df = 109, P \leq 0.05)$, respectively. When males were older (7-day-old laboratory and 12-day-old wild males), response to the lure matched sexual maturation rates for each strain. Of the 3762 laboratory-reared and 4317 wild *D. cucurbitae* males recovered from traps baited with cue-lure, 97.4 and 98.6%, respectively, were captured during the first 3 hr of the daylight period. Responses of *D. cucurbitae* to cue-lure were highest during the first hour of testing ending at 0900 hr (80.9% for laboratory-reared and 64.2% for wild

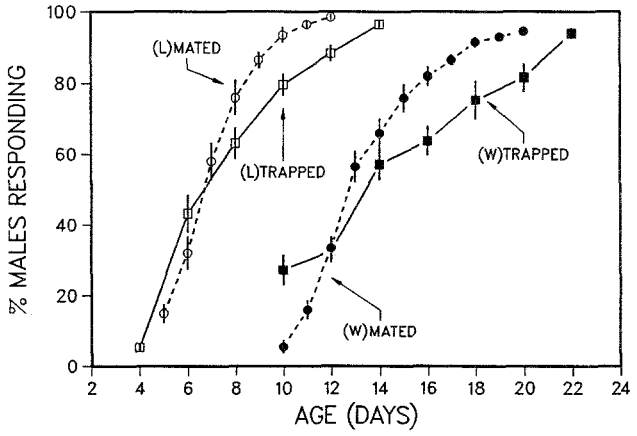


FIG. 1. Response of laboratory-reared (L) and wild (W) *Dacus cucurbitae* males of different ages to traps baited with cue-lure and 1% naled in outdoor cages, Honolulu, Hawaii. Values are arcsine transformed percentage means for five replicates (trapped) and ten replicates (mated). Line at each point represent ±2 standard errors. Data on mating of laboratory-reared and wild flies obtained from Wong et al. (1986).

males), followed by the second hour ending at 1000 hr (12.4% laboratory-reared and 28.2% for wild males). Trap catches dropped sharply after 1000 hr with no response after 1500 hr. These data indicate that the pattern of response for the laboratory-reared flies was very similar to that of the wild flies. The pattern of fly response during the morning hours could be attributed to the release of flies during those hours. However, similar diurnal patterns of lure response are known for field populations of melon fly (Nakagawa and Urago of this laboratory; unpublished data). They found that the peak response period of wild *D. cucurbitae* males occurred in the morning from 0830 to 1030 hr with no response occurring after 1530 hr.

The finding that newly-emerged, sexually immature males of *D. cucurbitae* did not respond to cue-lure differs from that of an earlier study. Beroza et al. (1960) observed responses by immature *D. cucurbitae* to cue-lure. The relation between response to male lure and sexual maturity has been studied previously in other *Dacus* species, but reports on the nature of this relationship are also conflicting. Fitt (1981) found that in male *Dacus opiliae*, the development of the behavioral response to methyl eugenol was related directly to reproductive development. Similarly, *D. tryoni* males do not respond to cue-lure (Monro and Richardson 1969; Fletcher, 1974) until some days after emergence. Steiner (1952) and later Wong et al. (1989a) reported that sexually immature *D. dorsalis* males responded to methyl eugenol. However, Umeya et al. (1973) and

Ito and Iwahashi (1974) found that attraction of *D. dorsalis* males commenced some days after emergence and was correlated with sexual maturation.

Of the six releases of laboratory-reared virgin melon fly females consisting of 2800 individuals (6–18 days old) and four releases of 900 wild virgin females (24–27 days old) released into cages without males, only 14 laboratory females (0.5%) and no wild females were caught in the cue-lure traps. Thus, we conclude virgin, gravid melon fly females are not responsive to cue-lure at any age.

The use of cue-lure against melon fly as a male annihilation technique would probably be much more difficult than is the highly effective use of methyl eugenol against the oriental fruit fly. The problem is that with the melon fly, males respond to cue-lure at a later stage in their sexual maturation than oriental fruit fly males do to methyl eugenol. Wong et al. (1989a) reported that 40–50% of male oriental fruit flies are attracted to methyl eugenol-toxicant mixtures and killed before any became sexually mature. Also, whereas methyl eugenol attracts virgin female oriental fruit flies in the absence of males, cue-lure does not attract virgin female melon flies. With methyl eugenol in male annihilation applications, female oriental fruit flies respond and are killed in ever greater numbers as the male population is depleted, but cue-lure can not do the same with the melon fly.

REFERENCES

BATEMAN, M.A. 1973. The eradication of Queensland fruit fly from Easter Island. *FAO Plant protection Bull.* 21:114.

BATEMAN, M.A., FRIEND, A.H., and HAMPSHIRE, F. 1966a. Population suppression in the Queensland fruit fly, *Dacus (Strumeta) tryoni*. I. The effects of male depletion in a semi-isolated population. *Austr. J. Agric. Res.* 17:687–697.

BATEMAN, M.A., FRIEND, A.H., and HAMPSHIRE, F. 1966b. Population suppression in the Queensland fruit fly, *Dacus (Strumeta) tryoni*. II. Experiments on isolated populations in Western New South Wales. *Austr. J. Agric. Res.* 17:699–718.

BEROZA, M., ALEXANDER, B.H., STEINER, L.F., MITCHELL, W.C., and MIYASHITA, D.H. 1960. New synthetic lures for the melon fly. *Science* 131:1044–1045.

CUNNINGHAM, R.T., and STEINER, L.F. 1972. Field trial of cue-lure + naled on saturated fiberboard blocks for control of the melon fly by the male annihilation technique. *J. Econ. Entomol.* 65:505–507.

CUNNINGHAM, R.T., STEINER, L.F., OHINATA, K., and FARIAS, G.B. 1970. Mortality of male melon flies and male Mediterranean fruit flies treated with aerial sprays of lure and naled formulated with a monoglyceride or siliceous extender. *J. Econ. Entomol.* 63:106–110.

CUNNINGHAM, R.T., CHAMBERS, D.L., STEINER, L.F., and OHINATA, K. 1975. Thixcin-thickened sprays of cue-lure + naled: Investigation of rates of application for use in male annihilation of melon fly. *J. Econ. Entomol.* 68:857–860.

DREW, R.A.I. 1974. The responses of fruit fly species (Diptera: Tephritidae) in the South Pacific area to male attractants. *J. Austr. Entomol. Soc.* 13:267–270.

DREW, R.A.I., and HOOPER, G.H.S. 1981. The responses of fruit fly species (Diptera: Tephritidae) in Australia to various attractants. *J. Austr. Entomol. Soc.* 20:201–205.

DUNCAN, D.G. 1955. Multiple range and Multiple F tests. *Biometrics* 11:1–41.

FITT, G.P. 1981. The influence of age, nutrition, and time of day on the responsiveness of male *Dacus opiliae* to the synthetic lure, methyl eugenol. *Entomol. Exp. Appl.* 30:83–90.

FLETCHER, B.S. 1974. The ecology of a natural population of the Queensland fruit fly, *Dacus tryoni*. VI. Seasonal changes in fruit fly numbers in the areas surrounding the orchard. *Austr. J. Zool.* 22:333–363.

HARRIS, E.J., TAKARA, J.M., and NISHIDA, T. 1986. Distribution of the melon fly, *Dacus cucurbitae* (Diptera: Tephritidae) and host plants on Kauai, Hawaiian Islands. *Environ. Entomol.* 15:488–493.

ITO, Y., and IWAHASHI, O. 1974. Ecological problems associated with an attempt to eradicate *Dacus dorsalis* (Tephritidae: Diptera) from the southern islands of Japan with a recommendation on the use of sterile male technique, pp. 43–53, *in* The Sterile Insect Technique and Its Field Applications (Proceedings of a Panel, Vienna, 1972). IAEA, Vienna.

KEISER, I., and SCHNEIDER, E.L. 1969. Need for immediate sugar and ability to withstand thirst of newly emerged oriental fruit flies, melon flies, and Mediterranean fruit flies, untreated and sexually sterilized with gamma radiation. *J. Econ. Entomol.* 62:539–540.

KEISER, I., NAKAGAWA, S., KOBAYASHI, R.M., CHAMBERS, D.L., URAGO, T., and DOOLITTLE, R.E. 1973. Attractiveness of cue-lure and the degradation product 4(p-hydroxyphenyl)-2-butanone to male melon flies in the field in Hawaii. *J. Econ. Entomol.* 66:112–114.

MONRO, J., and RICHARDSON, N.L. 1969. Traps, male lures, and a warning system for Queensland fruit fly, *Dacus tryoni* (Frogg) (Diptera: Tephritidae). *Austr. J. Agric. Res.* 20:325–328.

NAKAGAWA, S., FARIAS, G.J., and STEINER, L.F. 1970. The response of female Mediterranean fruit flies to male lures in the relative absence of males. *J. Econ. Entomol.* 63:227–229.

NAKAGAWA, S., STEINER, L.F., and FARIAS, G.J. 1981. Responses of female Mediterranean fruit flies to mature normal males, sterile males, and trimedlure in plastic traps. *J. Econ. Entomol.* 74:566–567.

SAS Institute. 1985. SAS user's guide: Statistics. SAS Institute, Cary, North Carolina.

SNEDECOR, G.W., and COCHRAN, W.G. 1967. Statistical Methods. Iowa State University, Ames, Iowa.

STEINER, L.F. 1952. Methyl eugenol as an attractant for Oriental fruit fly. *J. Econ. Entomol.* 45:241–248.

STEINER, L.F. 1957. Low-cost plastic fruit fly trap. *J. Econ. Entomol.* 50:508–509.

STEINER, L.F., MITCHELL, W.C., HARRIS, E.J., KOZUMA, T.T., and FUJIMOTO, M.S. 1965. Oriental fruit fly eradication by male annihilation. *J. Econ. Entomol.* 58:961–964.

TANAKA, N., STEINER, L.F., OHINATA, K., and OKAMOTO, R. 1969. Low-cost larval rearing medium for mass production of oriental and Mediterranean fruit flies. *J. Econ. Entomol.* 62:967–968.

TANAKA, N., MYERS, A.L., KOMURA, M., CHANG, H., and HARRIS, E.J. 1982. Automated larval rearing system for tephritids. *J. Econ. Entomol.* 75:517–519.

UMEYA, K., SEKIGUCHI, Y., and USHIO, S.I. 1973. The reproductive ability of the Oriental fruit fly, *Dacus dorsalis* Hendel and the response of adults to methyl eugenol. *Jpn. J. Appl. Entomol. Zool.* 17:63–70.

WONG, T.T.Y., MCINNIS, D.O., and NISHIMOTO, J.I. 1986. Melon fly (Diptera: Tephritidae): Sexual maturation rates and mating responses of laboratory-reared and wild flies. *Ann. Entomol. Soc. Am.* 79:605–609.

WONG, T.T.Y., MCINNIS, D.O., and NISHIMOTO, J.I. 1989a. Relationship of sexual maturation rate to response of oriental fruit fly strains (Diptera: Tephritidae) to methyl eugenol. *J. Chem. Ecol.* 15:1399–1405.

WONG, T.T.Y., CUNNINGHAM, R.T., MCINNIS, D.O., and GILMORE, J.E. 1989b. Seasonal distribution and abundance of *Dacus cucurbitae* (Diptera: Tephritidae) in Rota, Commonwealth of the Northern Mariana Islands. *Environ. Entomol.* 18:1079–1082.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

OVIPOSITIONAL RESPONSE OF TOBACCO BUDWORM MOTHS (LEPIDOPTERA: NOCTUIDAE) TO CUTICULAR LABDANES AND SUCROSE ESTERS FROM THE GREEN LEAVES OF *Nicotiana glutinosa* L. (SOLANACEAE)[1]

D. MICHAEL JACKSON,[2,*] R.F. SEVERSON,[3] V.A. SISSON,[2] and M.G. STEPHENSON[4]

[2]*ARS, USDA*
Crops Research Laboratory
Oxford, North Carolina 27565

[3]*ARS, USDA*
Phytochemical Research Unit
Athens, Georgia 30613

[4]*ARS, USDA*
Georgia Coastal Plain Experiment Station
Tifton, Georgia 31793

(Received June 13, 1991; accepted August 19, 1991)

Abstract—Field plots of three accessions of *Nicotiana glutinosa* L. (*Nicotiana* species accessions 24, 24A, and 24B) at Oxford, North Carolina and Tifton, Georgia were heavily damaged by natural populations of tobacco budworms, *Heliothis virescens* (F.), during 1985–1989. Experiments in outdoor screen cages demonstrated that all accessions of *N. glutinosa* were as prone to oviposition by *H. virescens* moths as was NC 2326, a commercial cultivar of flue-cured tobacco, *N. tabacum* L. However, in greenhouse experiments, tobacco budworm larvae did not survive or grow as well when placed on plants of *N. glutinosa* as they did when placed on plants of NC 2326. Four labdane diterpenes (manool, 2-hydroxymanool, a mixture of sclareols, and labda-13-ene-8α,15-diol [labdenediol]) and two sucrose ester fractions (2,3,4-tri-*O*-acyl-3′-*O*-acetyl-sucrose [G-SE-I] and 2,3,4,-tri-*O*-acyl-sucrose [G-SE-

*To whom correspondence should be addressed.

[1] This article reports the results of research only. Mention of a proprietary product does not constitute endorsement or recommendation for its use by USDA, ARS, or by the North Carolina Agricultural Research Service.

II]) were isolated from green leaves of the three accessions of *N. glutinosa*. These components were bioassayed for their effects on the ovipositional behavior of tobacco budworm moths using small screen cages in a greenhouse at Oxford, North Carolina. Labdenediol, manool, and both sucrose ester fractions stimulated tobacco budworm moths to oviposit on a tobacco budworm-resistant Tobacco Introduction, TI 1112 (PI 124166), when these materials were sprayed onto a leaf.

Key Words—Insecta, Lepidoptera, Noctuidae, *Heliothis virescens*, tobacco budworm, *Nicotiana*, ovipositional stimulants.

INTRODUCTION

Nicotiana glutinosa L. is a coarse-viscid pubescent annual in the family Solanaceae (Subgenus Tabacum, Section Tomentosae). It is native to Peru and southern Ecuador, where it has adapted to semi-arid areas such as rocky slopes and ditch banks (Goodspeed, 1954; Tatemichi, 1990). There are three accessions of *N. glutinosa* (accessions 24, 24A, and 24B) in the USDA-National Plant Germplasm System Collection at the Crops Research Laboratory in Oxford. The Section Tomentosae ($2n = 24$) is the group of *Nicotiana* species most closely related to cultivated tobacco, *N. tabacum* L. (Subgenus Tabacum, Section Genuinae; $2n = 48$). *N. glutinosa* is currently used as a source of desirable traits in tobacco-breeding programs, and chromosomes from this species have been incorporated into commercial tobacco cultivars by conventional breeding techniques (Gerstel, 1945).

N. glutinosa is resistant to tobacco mosaic virus (TMV), powdery mildew, *Erysiphe cichoracearum* DC (Cohen et al., 1983), and cyst nematodes, *Globodera solanacearum* (Miller & Gray) Behrens. It is the source of resistance to TMV in all commercial tobacco cultivars (Stavely, 1979). However, *N. glutinosa* is susceptible to several other diseases (Stavely, 1979), and it is also attacked by most common insect pests of tobacco in the United States, including the tobacco hornworm, *Manduca sexta* (L.), the tobacco aphid, *Myzus nicotianae* Blackman, and the tobacco budworm, *Heliothis virescens* (F.) (Thurston, 1961; Thurston et al., 1966; Parr, 1967; Parr and Thurston, 1968; Burk and Stewart, 1969, 1971; Greene and Thurston, 1971). *N. glutinosa* is preferred for oviposition by moths of tobacco hornworms and tobacco budworms over tobacco, most *Nicotiana* species, and tomato, *Lycopersicon esculentum* Mill. (Thurston et al., 1966; Parr, 1967; Greene and Thurston, 1971; Jackson and Severson, 1989; Jackson et al., 1988, 1989; Severson et al., 1990).

N. glutinosa does not produce the cuticular duvane diterpenes found on *N. tabacum* and *N. sylvestris* Spegazzini & Comes, but it does produce labdane diterpenes (Colledge et al., 1974; Reid, 1979). Severson et al. (1990) listed ten

cuticular labdanes produced by the *Nicotiana* species. The primary labdanes of *N. tabacum* are (12*Z*)-labda-12,14-dien-8α-ol (*cis*-abienol) and (13*E*)-labda-13-ene-8α,15-diol (labdenediol) (Reid, 1974; Severson et al., 1985b). One accession of *N. glutinosa* (acc. 24) produces (13*R*)-labda-8,14-dien-13-ol (manool), (13*R*)-labda-8,14-dien-2-oxy-13-ol (2-oxymanool), (13*R*)-labda-8,14-diene-2α,13-diol (2-hydroxymanool), (13*R*)-labda-14-ene-8α,13-diol (sclareol), (13*S*)-labda-14-ene-8α,13-diol (13-episclareol), and labdenediol. The second accession of *N. glutinosa* (acc. 24A) produces only the sclareols and labdenediol, while the third accession of *N. glutinosa* (acc. 24B) does not produce labdanes (Severson et al., 1988a; Figure 1).

An epimeric mixture of sclareol and 13-episclareol inhibits the growth of a wide range of fungal species (Bailey et al., 1974, 1975). Cohen et al. (1983) reported that oxymanool suppresses the emergence of conidial germ tubes of *E. cichoracearum*, and thus inhibits development of powdery mildew. *Cis*-abienol from *N. tabacum*, and 2-hydroxymanool, manool, oxymanool, sclareol, and the eutectic mixture of sclareol and 13-episclareol from *N. glutinosa* inhibit the growth of wheat coleoptiles (Cutler et al., 1977). We previously reported that *cis*-abienol does not stimulate oviposition by tobacco budworm moths (Jackson et al., 1986).

All three accessions of *N. glutinosa* produce sucrose esters (SE), which were isolated and characterized by Arrendale et al. (1990). They classified the SE from *Nicotiana* species based upon the position of the acetyl moieties on the sucrose molecule. The SE from *N. glutinosa* in this paper are designated G-SE-I (2,3,4-tri-*O*-acyl-3′-*O*-acetyl-sucrose) and G-SE-II (2,3,4-tri-*O*-acyl-sucrose), while the SE from *N. tabacum* (6-*O*-acetyl-2,3,4-tri-*O*-acyl-sucrose; Severson et al., 1985b, 1990) is designated T-SE-I. Severson et al. (1990) listed a total of eight SE types and two types of glucose esters (GE) in a survey of all of the *Nicotiana* species. The SE from *N. tabacum* (TI 165, PI 405002) (Cutler et al., 1986) and *N. glutinosa* (Severson et al., 1990) inhibit growth of wheat coleoptiles and the growth of some gram positive bacteria. We previously reported that the sugar esters from *N. tabacum* (TI 165) (SE), *N. kawakamii* Ohashi (SE & GE), *N. alata* Link & Otto (SE), and *N. trigonophylla* Dunal (SE & GE) stimulate oviposition by tobacco budworm moths (Jackson et al., 1989; Severson et al., 1990). However, oviposition by tobacco budworm moths is not affected by simple sugars such as sucrose, glucose, or fructose (Jackson et al., 1989).

Because the cuticular components from *N. glutinosa* may be transferred to *N. tabacum* during the development of tobacco breeding lines, it is of interest to know how these components affect the behavior of associated insect pests. In this paper, we describe the effects that cuticular components from *N. glutinosa* have on the ovipositional behavior of tobacco budworm moths.

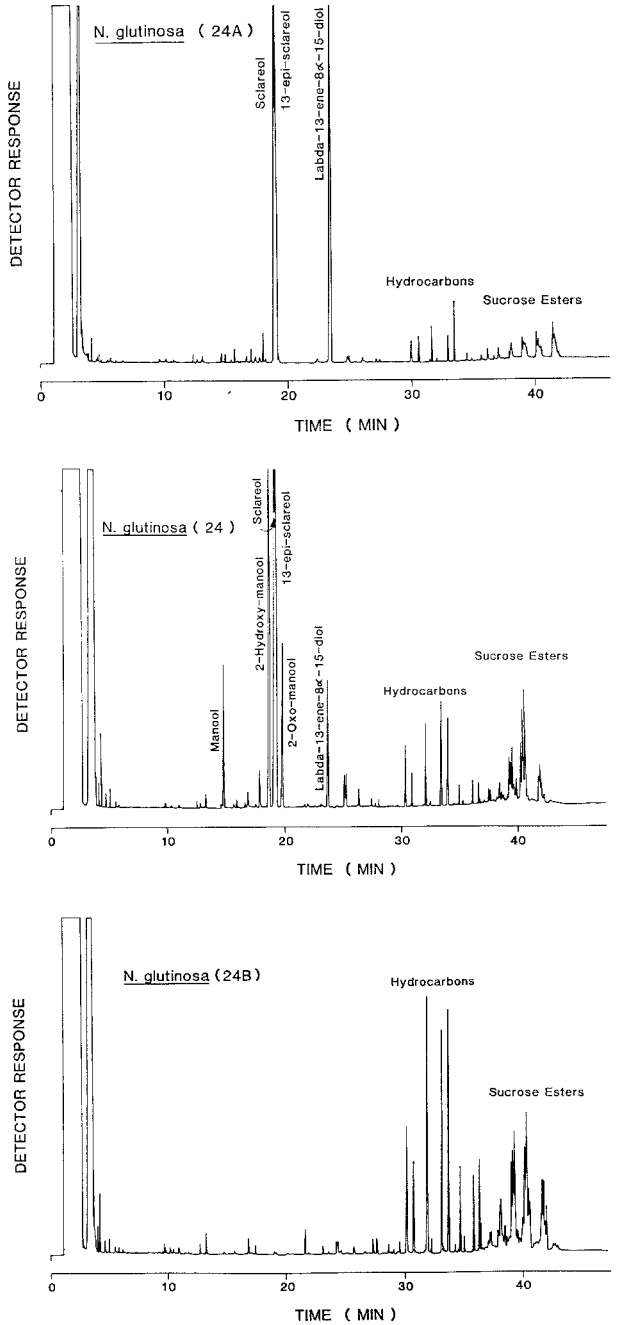


FIG. 1. Capillary gas chromatograms of the cuticular components from the green leaves of three accessions of *Nicotiana glutinosa* grown in field plots at Oxford, North Carolina, 1985.

MATERIALS AND METHODS

Field Plots

Seventy accessions of 66 *Nicotiana* species (including the three accessions of *N. glutinosa*) were grown in 20-plant plots at Tifton, Georgia and Oxford, North Carolina during 1985 and 1986. Plots were arranged in a randomized complete block design with three replications. Fields were fumigated with methyl bromide before planting to control soil-borne diseases. Napropamide (Devrinol 50WP®, ICI Americas Inc., Wilmington, Delaware) and metalaxyl (Ridomil 2E®, Ciba-Geigy Corp., Greensboro, North Carolina) were incorporated into the soil 2 weeks before transplantation for control of weeds and blue mold, *Peronospora tabacina* D. B. Adam. Plots were cultivated, fertilized, and irrigated as needed. The numbers of tobacco budworm larvae on each plant were counted at peak infestations 1–2 times each year at each location.

Field plants were sampled for cuticular components once each season at each location. One leaf disk (2-cm diam) was removed from each of five plants per treatment, with two replications per location per year. Disks were taken from the center (including midrib) of a fully expanded but not senescent leaf near the top of each plant. Leaf disks were briefly dipped ten times in succession into 10 ml of methylene chloride (distilled-in-glass grade, American Burdick & Jackson, Muskegon, Michigan) in scintillation vials. Samples were chilled on dry ice and kept at −17°C until analyzed by gas chromatography (GC).

Ovipositional Choice Experiments with Whole Plants

Whole plants of *N. glutinosa* were bioassayed for tobacco budworm ovipositional preference in choice tests vs. NC 2326, a commercial flue-cured tobacco cultivar, using 16 outdoor screen cages (2.4 × 2.4 × 2.0 meters high; Jackson et al., 1983, 1984, 1986). Each cage had a wooden floor and waterproof gabled roof. Three 7.5 watt light bulbs were positioned over the center of each cage and aimed upward under the roof. Lights were shaded from below by 30.5-cm diam. metal pans, and they were controlled by a central rheostat so that the light intensity was equal within all cages. The cages were three meters apart and they were located in an isolated area of the Tobacco Research Station in Oxford, where there were no extraneous light sources within view of moths inside the cages. These experiments were conducted July 3–31, 1984 and May 31–August 15, 1985. Maximum daytime temperatures ranged from 22 to 37°C and minimum nighttime temperatures ranged from 8 to 23°C during these periods.

Plants used in these experiments were germinated in the greenhouse (ambient photoperiod, temperature maintained below 35°C) and later transplanted to 12.7-cm diam. plastic pots containing sterilized soil and a time-release

fertilizer (14-14-14, N-P-K) (Osmocote®, Sierra Chem. Co., Milpitas, California). At transplantation, each seedling was treated with 0.04 ml of metalaxyl (Ridomil 2E) in 100 ml water to prevent fungal diseases. Plants were grown in the greenhouse for an additional 2 weeks then moved to a shady area of mowed grass until they were large enough for testing at 5–7 weeks after transplantation. When aphids were a problem, plants were sprayed with pirimicarb (Pirimor 50 W®, ICI Americas Inc., Wilmington, Delaware) at 0.01 g per plant.

Tobacco budworms were from a laboratory colony started each fall from larvae collected in tobacco fields near Oxford. This colony was cultured for 7–10 generations on an artificial diet (Baumhover, 1985) before the start of these experiments. Female moths were 3–4 days old after eclosion, and they were caged together as groups of ten female and ten male moths in 3.8-liter paper cartons for two nights before testing to insure mating. The mating containers and colony moths were kept in rooms at ca. 25°C, ca. 70% RH, and a photoperiod of 14 : 10 (L : D).

For choice experiments, four *N. glutinosa* plants were placed in one corner of a cage, and four NC 2326 plants of a similar size were placed in the opposite corner of the cage. Plants ranged in height from ca. 15–30 cm. Ten female tobacco budworm moths were placed into each cage a few hours before sunset. Moths were provided water, but no food. The following morning, the total number of tobacco budworm eggs on the four plants of each entry was counted. Each cage-night was treated as one replication. Data were analyzed by paired *t*-tests (Steel and Torrie, 1960). For no-choice experiments, eight plants of a treatment and eight plants of the control (NC 2326) were placed in separate cages set up as pairs, and the data were analyzed by paired *t*-tests.

Ovipositional Bioassays of Cuticular Components

Manool, hydroxymanool, labdenediol, a sclareol mixture (sclareol plus episclareol), two SE fractions (G-SE-I and G-SE-II), and a mixture of α-+ β-4,8,13-duvatriene-1,3-diols (duvatrienediols) were bioassayed for ovipositional response by tobacco budworm moths in "hoop cages" (0.46 × 1.31 × 0.46 meters high). Twelve hoop cages were located inside a glass greenhouse (7.3 × 9.1 × 4.9 meters high). The walls of this greenhouse were covered with black plastic to block extraneous lights, but the gabled roof was left uncovered. No artificial light sources were used for these experiments. The greenhouse was maintained at 25–30°C during the test period, and the humidity was kept high by hosing down the concrete floor several hours before each experiment. Each hoop cage had a rectangular plywood floor (0.46 × 1.31 meters) and two semicircular end pieces (0.46 meters high). It was covered with 0.32-cm hardware cloth. In the center of the floor of each cage was a trap door large enough to

introduce potted plants and moths. Cages were painted flat black. In the center of each plywood end piece was a 10.2-cm diam. hole through which was pushed the end of a frustum (11.7-cm diam. bottom, 9.5-cm diam. top, and 8.9-cm high) formed from a 12.7-cm black plastic pot with the end removed. A detached leaf was placed over the end of an intact 12.7-cm plastic pot, and the frustum was pushed over the end of the pot to secure the leaf in place so that its abaxial surface was exposed. After the exposed surface of the leaf was treated with a cuticular component, the frustum was pushed through the hole in the end of the cage and held fast with Velcro® fasteners so that the leaf protruded into the cage ca. 2 cm. For a choice test, a frustum with attached leaf was pushed into each end of the cage. Thus, the two excised leaves at opposite ends of the cage each exposed a circular area of 71 cm^2 to ovipositing moths.

Leaves from Tobacco Introduction (TI) 1112 (PI 124166) were used in these experiments. TI 1112 has glandless trichomes on its leaves and, in contrast to most tobacco cultivars and *Nicotiana* species, it has negligible levels of the common leaf surface constituents (Severson et al., 1984). Plants were grown in 12.7-cm diam. plastic pots under high pressure sodium lights in a greenhouse. Plants grown under these lights are similar in physical and chemical characteristics to field-grown plants (Severson et al., 1985b).

Tobacco budworm moths were reared and prepared for bioassay in the same manner as those for the outdoor cage experiments described above. Five female moths were introduced into each cage in the late afternoon after temperatures in the greenhouse had fallen below 30°C. Eggs on each exposed leaf surface were counted the following morning.

Each cuticular component was bioassayed in a choice test vs. a solvent blank (SB) (0.5 mL methylene chloride in 1.5 mL of 3:1 acetone:water) and vs. duvatrienediols. We previously reported that duvatrienediols are strong ovipositional stimulants for tobacco budworm moths (Jackson et al., 1986, 1989). Spray applications were made in the late afternoon with a small air brush (Model 250, Badger Air-Brush Co., Franklin Park, Illinois). This apparatus had a 20-mL reservoir, a Teflon® delivery tube, and it was attached to an air compressor that would deliver up to 200 kPa. About 35 kPa were optimal for spraying the plants, because that pressure produced a fine mist that did not run off the leaves. One milliliter of a cuticular component was mixed with 1.0 mL of 1:1 acetone:distilled water. Control plants were sprayed with 2.0 mL of SB or with 1.0 mL duvatrienediols plus 1.0 mL of 1:1 acetone:water. Thus, 2.0 mL of spray was applied to the surface of each leaf. Leaf disks were allowed to air-dry (ca. 30 min) before they were inserted into the oviposition cage. The following morning after tobacco budworm eggs had been removed, four 2-cm diam leaf disks were taken from each excised leaf. These disks were dipped ten times each into scintillation vials containing 10 mL of methylene chloride (dis-

tilled-in-glass grade, American Burdick & Jackson). Samples were frozen on dry ice and kept frozen at −17°C until they were analyzed for chemical components by capillary GC.

Before analysis of variance (ANOVA) for each experiment, data were transformed first to a percentage of the total eggs counted per replication (x), and then transformed to arcsine (square root x). Preference for egg laying on a particular treatment vs. the control was determined using a paired t-test for each experiment (SAS Institute, 1985).

Feeding Experiment

In order to determine how well tobacco budworm larvae survive and develop on the three *N. glutinosa* accessions vs. NC 2326, greenhouse-grown plants were artificially infested with second instars. Plants were grown in the greenhouse under supplemental high pressure sodium lights for 6–8 weeks after transplantation from seed pots in a manner similar to that used to produce plants for ovipositional cage bioassays described above. Five-second instars of *H. virescens* from the laboratory colony described above were placed on the terminal leaf bud of each plant. Larvae were allowed to feed for 1 week, then they were weighed.

Extraction of Cuticular Components for Bioassays

Cuticular components were extracted from all three accessions of *N. glutinosa* from field plots at Oxford and Tifton. Plant tops (ca. 6–10 weeks old) were cut off and dipped ten times into 4-liter beakers of methylene chloride (Resi-analyzed reagent grade, J.T. Baker Chemical Co., Phillipsburg, New Jersey; Jackson et al., 1984, 1986). Approximately 100 plants were extracted with ca. 3 liters of solvent. These crude extracts were cooled with dry ice and stored at −17°C until needed. After warming to room temperature, the extracts were filtered through folded filter paper containing anhydrous Na_2SO_4, and the solvent was removed on a roto-evaporator under vacuum at 40°C.

Isolation of Labdanes and Sucrose Esters

The cuticular extract from *N. glutinosa* (acc. 24A) (10.9 g) was dissolved in 1:1 hexane:methylene chloride, deposited onto 50 g of silicic acid, and loaded onto a 50-g silicic acid column. The column was eluted with hexane (500 mL), 1:3 hexane:methylene chloride (750 mL), methylene chloride (400 mL), 1:9 acetone:methylene chloride (400 mL), and 1:4 acetone:methylene chloride (1000 mL). Fractions rich in the sclareol mixture (2.1 g, 99+%) eluted in the third 100 mL portion of the 1:9 acetone:methylene chloride fraction. Re-crystallization from hexane yielded a constant melting mixture of sclareols

(1.8 g, melting point [*mp*] 96–97°C, 99+%) (reported literature *mp* = 95°C; Reid, 1974). Combination of the last 100 mL of the 1:9 acetone:methylene chloride and the first 400 mL of the 1:4 acetone:methylene chloride elutants produced 5.8 g of the sclareols plus labdenediol mixture. A portion of this mixture (2.8 g) was chromatographed on a Sephadex LH-20 column (2.54 cm id × 100 cm) using chloroform (0.75% EtOH as preservative) as the eluting solvent, and 5 mL fractions were collected. After solvent removal and re-crystallization, fractions 77–84 produced 0.9 g of the sclareol-episclareol mixture (*mp* 96–97°C, 99+%). Solvent removal and re-crystallization of fractions 95–107 produced 99+% labdenediol (*mp* 131–132°C) (reported literature *mp* = 131–131.5°C; Reid, 1974).

Cuticular extract from *N. glutinosa* (acc. 24) (6.2 g) was deposited onto 50-g silicic acid, loaded into a 50-g silicic acid column, and eluted with hexane (150 mL), 3:1 hexane:methylene chloride (200 mL), hexane:methylene chloride (1000 mL), and 1:19 acetone:methylene chloride (1000 mL). The first 200-mL elutant of the 1:19 acetone:methylene chloride fraction yielded 1.7 g of a mixture of 2-oxymanool (9%), sclareols (53%), and 2-hydroxymanool (28%). This mixture was re-chromatographed on the Sephadex LH-20 column as described above. After solvent removal and re-crystallization, combinations of fractions 95–100 yielded 99+% 2-hydroxymanool (0.22 g, *mp* = 131–133°C).

The G-SE-I and G-SE-II fractions were isolated as described by Arrendale et al. (1990). Manool was obtained from Aldrich Chemical Company (Milwaukee, Wisconsin). The duvatrienediols were isolated as described by Severson et al. (1988b).

Gas Chromatography

Chemical samples were analyzed by capillary GC and GC/MS as described by Severson et al. (1984, 1985a,b). Cuticular extracts, fractions, and isolates were taken to dryness under N_2 in a micro autosampler vial (100 μL), then 50 μL of 1:1 N-*O-bis*(trimethylsilyl)trifluoroacetamide:dimethylformamide (Pierce Chemical Company, Rockford, Illinois) was added. The vial was capped and heated at 76°C for 45 min to convert hydroxylated components to trimethylsilyl ethers. After cooling, the samples were analyzed on a Hewlett Packard 5880 gas chromatograph equipped with a splitless injector (injector temp. 250°C, purge activation time of 1.5 min) and flame ionization detector (detector temp. 350°C) using a 0.3-mm id × 30-m bonded SE54 fused silica capillary column (Arrendale and Martin, 1988). A temperature program was run at 100°C for 5 min, followed by a temperature increase of 10°C/min to 170°C, 3°C/min from 170–220°C, and 5°C/min from 220–290°C. The final temperature was held at 290°C until all components had eluted.

RESULTS AND DISCUSSION

Results from field plots showed that *N. glutinosa* was one of the *Nicotiana* species most frequently infested with tobacco budworm larvae (Table 1). Although numerically lower, the percentage infestations on the three accessions of *N. glutinosa* were not significantly different from infestation levels seen on *N. tabacum* cultivars such as NC 2326 and Samsun, an oriental tobacco (Severson et al., 1985b). *Nicotiana kawakamii* was the only species that had a significantly higher average infestation rate in the field than *N. glutinosa* (Table

TABLE 1. PERCENTAGE INFESTATION BY NATURAL POPULATIONS OF LARVAE OF THE TOBACCO BUDWORM ON SINGLE ROW PLOTS OF 70 ACCESSIONS OF 66 *Nicotiana* SPECIES AT OXFORD, NORTH CAROLINA AND TIFTON, GEORGIA, 1985–1986

Nicotiana species	Percent of plants[a] with tobacco budworm larvae	Cuticular components ($\mu g/cm^2$)[b]			
		Total duvanes	Total labdanes	SE[c]	GE[d]
N. kawakamii (72)[e]	46.3 a[f]	—[g]	38	24	88
N. tabacum cv. Samsun	33.9 ab	93	24	57	—
N. tabacum cv. NC 2326	23.9 abc	105	—	5	—
N. alata (3)	21.3 cdef	—	—	56	—
N. debneyi (17)	18.0 bcde	—	—	15	2
N. glutinosa (24)	17.6 bcd	—	79	85	—
N. glutinosa (24A)	13.7 cdefg	—	144	20	—
N. bigelovii (10)	13.6 cdef	—	—	75	15
N. clevelandii (14)	9.6 cdefg	—	—	66	7
N. otophora (38)	9.1 cdefg	1	—	32	—
N. acuminata (2)	8.5 cdefg	—	—	179	—
N. nesophila (34A)	8.1 cdefg	Trace	—	—	—
N. rustica v. *pavonii* (44)	8.1 defg	—	—	—	—
N. glutinosa (24B)	7.4 cdefg	—	—	38	—
N. setchellii (51)	6.5 cdefg	—	56	67	10
24 *Nicotiana* species	0.1–6.4 cdefg				
31 *Nicotiana* species	0.0g				

[a] Average for three data sets: Oxford—1985, Tifton—1985, and Tifton—1986; Three replications per location with 12-plant plots.
[b] Data from Severson et al. (1990).
[c] Sucrose esters.
[d] Glucose esters.
[e] *Nicotiana* species seed accession number: for species authority see Goodspeed (1954) or Tatemichi (1990).
[f] Means followed by the same letter are not significantly different, Waller–Duncan K-ratio t-test ($K = 100$, $\alpha = 0.05$); data transformed to square root ($X + 0.5$) before ANOVA (SAS Institute, 1985).
[g] Below detection limits.

1). However, data from field infestation rates must be interpreted cautiously since they are only a relative measure of host suitability (Jackson and Severson, 1989). This is because several mortality factors (predation, abiotic factors, plant antibiosis) may kill eggs or larvae of *H. virescens* once they are on a plant. Oviposition by moths of *H. virescens* may be heavy on certain tobacco types, but they may show little insect damage in the field because of high larval mortality due to their antibiotic defenses (Jackson et al., 1988, 1989; Jackson and Severson, 1989). However, because larvae of *H. virescens* seldom leave the plants upon which they hatched (Jackson et al., 1989), we may assume that a plant type heavily damaged in the field is highly susceptible to oviposition. The three accessions of *N. glutinosa* fall in this latter group, making them quite susceptible to oviposition by tobacco budworm moths.

The propensity of female tobacco budworm moths to oviposit on *N. glutinosa* was confirmed by both choice and no-choice oviposition cage experiments when compared with the highly susceptible *N. tabacum* cv. NC 2326 (Table 2). In no-choice tests, *N. glutinosa* (acc. 24) was equally acceptable as an ovipositional site as NC 2326 with no significant difference in the number of eggs that were laid on either host. Similarly, when given a choice between one of the accessions of *N. glutinosa* and NC 2326, tobacco budworm moths deposited equal numbers of eggs on each plant type despite their differences in cuticular chemical profiles (Figure 1). This probably indicates that more than one cuticular component is utilized by moths of *H. virescens* as ovipositional stimulants.

TABLE 2. OVIPOSITIONAL RESPONSE OF FEMALE TOBACCO BUDWORM MOTHS TO THREE ACCESSIONS OF *Nicotiana glutinosa* IN SCREEN FIELD CAGES, OXFORD, NORTH CAROLINA, 1984–1985

	Choice experiments[a]			No-choice experiments[b]		
Species accession	Percent of eggs on *N. glutinosa*	Total no. of eggs	No. of reps	Percent of eggs on *N. glutinosa*	Total no. of eggs	No. of reps
N. glutinosa (24)	50.6 NS[c]	5489	20	46.3 NS[c]	4452	15
N. glutinosa (24A)	45.7 NS	2624	10	—[d]	—	—
N. glutinosa (24B)	51.6 NS	2749	10	—	—	—

[a] Paired choice tests with four plants of *N. glutinosa* vs. four plants of *N. tabacum* cv. NC 2326 flue-cured tobacco in the same cage.
[b] Paired no-choice tests with eight plants of *N. glutinosa* in one cage vs. eight plants of NC 2326 in a separate cage.
[c] Paired *t*-test; NS = not significant. For no-choice experiments, data from separate cages containing either NC 2326 or an *N. glutinosa* accession were paired.
[d] Not tested.

While there did not appear to be any difference between *N. glutinosa* and NC 2326 for ovipositional preference, feeding tests using greenhouse-grown plants showed that tobacco budworm larvae survived and grew better on NC 2326 than on any of the accessions of *N. glutinosa* (Table 3). This result differs from Burk and Stewart (1971) who listed only one of the accessions of *N. glutinosa*, 24A, as inducing significantly higher mortality on tobacco budworm larvae than those feeding on a flue-cured control. Differences in survival rates of larvae may help explain the lower percentages of *N. glutinosa* plants infested with tobacco budworm larvae in the field compared to *N. tabacum* cultivars (Table 1). Reasons for lower survival and developmental rates for tobacco budworm larvae on *N. glutinosa* have not been studied, but differences in cuticular components or internal leaf components such as flavanols (Jurzysta, 1983; Snook et al., 1986) or pyridine alkaloids (Sisson and Severson, 1990) may be important. These classes of compounds are known to be toxic to larvae of *H. virescens* (Lukefahr and Martin, 1966; Shaver and Lukefahr, 1969; Granzow et al., 1985; Jackson et al., 1989; Gunasena et al., 1990). Interestingly, *N. glutinosa* is not resistant to feeding by tobacco hornworm larvae (Parr and Thurston, 1968).

Chromatograms of the cuticular components of the three accessions of *N. glutinosa* are given in Figure 1, and levels of duvanes, labdanes, and SE found on *N. glutinosa* and *N. tabacum* in the field are shown in Table 4. Both SE fractions (G-SE-I and G-SE-II), manool, and labdenediol stimulated tobacco budworm oviposition when these compounds were sprayed onto TI 1112 leaves (Table 5). These components stimulated oviposition to a level not significantly

TABLE 3. AVERAGE SURVIVAL AND WEIGHT GAIN AFTER 1 WEEK FOR SECOND INSTAR *Heliothis virescens* LARVAE PLACED ON GREENHOUSE-GROWN *Nicotiana glutinosa* PLANTS COMPARED TO THOSE PLACED ON FLUE-CURED TOBACCO, NC 2326 AT OXFORD, NORTH CAROLINA, 1985–1986

Species[a] accession	Number of[b] replications	Average[c] percent survival	Average[d] weight gain per larva (mg)
N. glutinosa (24)	8	17.0 a	18.1 a
N. glutinosa (24A)	9	27.6 a	53.2 b
N. glutinosa (24B)	8	19.6 a	22.3 a
N. tabacum (NC 2326)	17	43.3 b	91.0 c

[a] Six- to 8-week-old plants grown in 12.7-cm diam. pots.
[b] Five plants with five larvae per plant made up one replication.
[c] Means in the same column followed by the same letter are not significantly different, Waller-Duncan *K*-ratio *t*-test ($K = 100$, $\alpha = 0.05$); SAS Institute, 1985).

TABLE 4. LEVELS OF MAJOR CUTICULAR COMPONENTS FOUND ON GREEN LEAVES ON FIELD-GROWN PLANTS OF THREE ACCESSIONS OF *Nicotiana glutinosa* (24, 24A, AND 24B) AND TWO *N. tabacum* CULTIVARS AT OXFORD, NORTH CAROLINA, 1985

Cuticular component	$\mu g/cm^2$ on green leaves[a]				
	Nicotiana tabacum		*Nicotiana glutinosa*		
	NC 2326	Samsun	24	24A	24B
Duvatrienediols[b]	105.0	93.0	—[c]	—	—
Labdenediol[d]	—	4.0	6.0	50.0	—
Cis-Abienol[e]	—	20.0	—	—	—
Manool[f]	—	—	7.0	—	—
Hydroxymanool[g]	—	—	23.0	—	—
Oxymanool[h]	—	—	5.0	—	—
Sclareols[i]	—	—	43.0	94.0	—
Sucrose Esters[j]	5.0	57.0	85.0	18.0	38.0

[a] Average from two replications of composite samples from five plants, Oxford, North Carolina, 1985.
[b] α-+β-4,8,13-duvatriene-1,3-diols.
[c] Below detection levels.
[d] (13*E*)-labda-13-ene-8α,15-diol.
[e] (12*Z*)-labda-12,14-dien-8α-ol.
[f] (13*R*)-labda-8,14-dien-13-ol.
[g] (13*R*)-labda-8,14-diene-2α,13-diol.
[h] (13*R*)-labda-8,14-dien-2-oxy-13-ol.
[i] Mixture of (13*R*)-labda-14-ene-8α,13-diol (sclareol) and (13-S)-labda-14-ene-8α,13-diol (13-epi-sclareol).
[j] *N. glutinosa*: G-SE-I (2,3,4-tri-*O*-acyl-3′-*O*-acetyl-sucrose) and G-SE-II (2,3,4-tri-*O*-acyl-sucrose); *N. tabacum*: T-SE-I (6-*O*-acetyl-2,3,4-tri-*O*-acyl-sucrose).

different from the effects of the duvatrienediols (Table 5), known ovipositional stimulants for *H. virescens* (Jackson et al., 1986). The mixture of sclareols gave conflicting results in the two types of paired tests. Although comparison of sclareol-treated leaves vs. solvent blank-treated leaves indicated no ovipositional activity (statistically nonsignificant), comparison of sclareol-treated leaves vs. duvatrienediol-treated leaves indicate activity similar to that of the duvatrienediols (Table 5). We have no suitable explanation for this apparent discrepancy. Hydroxymanool had no effect on oviposition by tobacco budworm moths, as indicated by the nonsignificant difference from the solvent blank and by the significantly lower egg deposition when compared with the duvatrienediol-treated leaves.

In this paper, we have demonstrated that three accessions of *N. glutinosa* are attacked by larvae of *H. virescens* in the field, but that infestation levels of tobacco budworm larvae on these accessions are slightly less than are found on

TABLE 5. OVIPOSITIONAL RESPONSE OF TOBACCO BUDWORM MOTHS TO CUTICULAR COMPONENTS ISOLATED FROM *Nicotiana glutinosa* AND APPLIED TO TI 1112 LEAVES IN CHOICE TESTS IN SMALL SCREEN CAGES IN A GREENHOUSE SECTION, OXFORD, NORTH CAROLINA, 1988

Entry A (TI 1112 with:)	Entry B[a] (TI 1112 with:)	Percent of eggs[b] on entry A	No. of replications
Labdenediol[c]	SB	67.8 [l]	15
Labdenediol	DVT	50.5 NS	14
Manool[d]	SB	71.9 [l]	12
Manool	DVT	46.3 NS	13
2-Hydroxymanool[e]	SB	40.8 NS	13
2-Hydroxymanool	DVT	24.8 [l]	12
Sclareols[f]	SB	53.7 NS	13
Sclareols	DVT	46.5 NS	10
G-SE-I[g]	SB	72.6 [l]	11
G-SE-I	DVT	44.7 NS	12
G-SE-II[h]	SB	63.7 [l]	11
G-SE-II	DVT	52.5 NS	17
T-SE-I[i]	SB	63.4 [m]	41
Duvatriendiols[j]	SB	74.4 [m]	15
NC 2326[k]	TI 1112[k]	77.8 [m]	42

[a] TI 1112 leaves sprayed either with a solvent blank (SB) or with $\alpha + \beta$-4,8,13-duvatriene-1,3-diols (DVT).
[b] Paired *t*-test; NS = not significant.
[c] Applied at 50.0 $\mu g/cm^2$; avg. recovery of 62.3% from 36 samples.
[d] Applied at 50.0 $\mu g/cm^2$; avg. recovery of 78.7% from 36 samples.
[e] Applied at 50.0 $\mu m/cm^2$; avg. recovery of 68.4% from 35 samples.
[f] Mixture of sclareol and 13-episclareol applied at 50.0 $\mu g/cm^2$; avg. recovery of 73.5% from 36 samples.
[g] G-SE-I = 2,3,4,tri-*O*-acyl-3′-*O*-acetyl-sucrose applied at 50.0 $\mu g/cm^2$; avg. recovery of 52.5% from 38 samples.
[h] G-SE-II = 2,3,4,tri-*O*-acyl-sucrose applied at 50.0 $\mu g/cm^2$; avg. recovery of 57.2% from 39 samples.
[i] T-SE-I = 6-*O*-acetyl-2,3,4-tri-*O*-acyl-sucrose, tested 1983–1985 in field cages (see Jackson et al., 1989).
[j] α-+β-4,8,13-duvatriene-1,3-diols applied at 50.0 $\mu g/cm^2$: avg. recovery of 66.9% from four samples.
[k] Unsprayed NC 2326 and TI 1112 leaves.
[l] Significant difference ($P = 0.05$).
[m] Significant difference ($P = 0.01$).

TABLE 6. ACID COMPOSITION (C_3-C_8) OF THE SUCROSE ESTER FRACTIONS FROM *Nicotiana tabacum* AND *N. glutinosa*

Acid	Mole percent[a]		
	N. tabacum[b] (T-SE-I)[c]	*N. glutinosa* G-SE-I[c]	*N. glutinosa* G-SE-II[c]
Propanoic	0.3	0.7	0.2
Isobutanoic	2.7	9.9	2.8
Butanoic	1.0	0.3	0.5
2-Methylbutanoic	8.1	20.1	8.5
3-Methylbutanoic	13.9	3.1	1.3
Pentanoic	0.7	1.0	0.7
3-Methylpentanoic	68.8	3.7	1.1
4-Methylpentanoic	1.4	4.7	2.4
Hexanoic	0.7	1.3	0.7
4-Methylhexanoic	1.2	27.5	42.3
5-Methylhexanoic	0.8	32.0	30.6
Heptanoic	—[d]	0.2	1.4
Methylheptanoic	0.3	1.1	1.7
Octanoic	—	0.3	0.6

[a] Based on total C_3-C_8 acids as determined by GC analysis of butylesters after hydrolysis of sucrose ester (Severson et al., 1990).
[b] Isolated from TI 165 (Severson et al., 1985a).
[c] T-SE-I = 6-*O*-acetyl-2,3,4-tri-*O*-acyl-sucrose; G-SE-I = 2,3,4-tri-*O*-acyl-3′-*O*-acetyl-sucrose, and G-SE-II = 2,3,4-tri-*O*-acyl-sucrose.
[d] Below detection limits.

oriental or flue-cured tobacco cultivars. We have also shown that each of the accessions of *N. glutinosa* is equally susceptible to oviposition by tobacco budworm moths as is a standard flue-cured cultivar, NC 2326. Differences in the infestation levels of tobacco budworm larvae on tobacco and *N. glutinosa* appear to be due to larvae not surviving or developing as well on the accessions of *N. glutinosa* as they do on tobacco cultivars.

We have also shown that two labdane diterpenes and two SE types from the cuticulae of *N. glutinosa* accessions stimulate oviposition by tobacco budworm moths in cage bioassays. We previously reported (Jackson et al., 1989) that GE and SE type compounds from other *Nicotiana* species also stimulate oviposition by these insects. There are additional labdanes and GE and SE compounds from the *Nicotiana* species (Severson et al., 1990) that we have not bioassayed for ovipositional activity. Several of the *Nicotiana* species possessing these untested components are also susceptible to tobacco budworm damage in the field (Table 1) and to oviposition by tobacco budworm moths (Jackson

et al., 1989; Severson et al., 1990). Sucrose esters from the *Nicotiana* species differ not only in esterification positions on the sucrose molecule, but also in the acid moiety compositions which vary considerably within the genus (Severson et al., 1990). For example, the major acid moieties of the SE from *N. tabacum* (T-SE-I from TI 165) are methylpentanoics, whereas the major acid moieties of the SEs from *N. glutinosa* (G-SE-I and G-SE-II) are methylhexanoics (Table 6). Thus, it appears that several different SE fractions from the genus *Nicotiana* elicit the same behavioral response, oviposition, by tobacco budworm moths. We are investigating how structural differences and variations in the acid compositions of the SE types from the *Nicotiana* species affect the ovipositional response of tobacco budworm moths. The components tested in this study and those listed by Severson et al. (1990) might also affect pest or beneficial insects other than tobacco budworms which are associated with tobacco culture, and we are investigating these interactions.

Acknowledgments—We thank James S. Cheatham, James M. Hobgood, Jr., Linda Smith, Brenda King, and Sara J. Mueller for their excellent technical assistance.

REFERENCES

ARRENDALE, R.F. and MARTIN, R.M. 1988. The preparation of immobilized stationary phase fused silica capillary columns with OV-1701-vinyl deactivation. *J. High Resolut. Chromatogr. Chromatogr. Commun.* 11:157–161.

ARRENDALE, R.F., SEVERSON, R.F., SISSON, V.A., COSTELLO, C.E., LEARY, J.A., HIMMELSBACH, D.S., and VAN HALBEEK, H. 1990. Characterization of the sucrose ester fraction from *Nicotiana glutinosa*. *J. Agric. Food Chem.* 38:75–85.

BAILEY, J.A., VINCENT, G.G., and BURDEN, R.S. 1974. Diterpenes from *Nicotiana glutinosa* and their effects on fungal growth. *J. Gen. Microbiol.* 85:57–64.

BAILEY, J.A., CARTER, G.A., BURDEN, R.S., and WAIN, R.S. 1975. Control of rust diseases by diterpenes from *Nicotiana glutinosa*. *Nature* 255:328–329.

BAUMHOVER, A.H. 1985. *Manduca sexta*, pp. 387–400, *in* P. Singh and R.F. Moore (eds.). Handbook of Insect Rearing, Vol. 2. Elsevier, Amsterdam.

BURK, L.G., and STEWART, P.A. 1969. Resistance of *Nicotiana* species to the green peach aphid. *J. Econ. Entomol.* 62:1115–1117.

BURK, L.G., and STEWART, P.A. 1971. Survey of resistance among *Nicotiana* species to tobacco hornworm and budworm larvae. *Tobacco Sci.* 15:32–34.

COHEN, Y., EYAL, H., GOLDSCHMIDT, Z., and SKLARZ, B. 1983. A preformed chemical inhibitor of tobacco powdery mildew on leaves of *Nicotiana glutinosa*. *Physiol. Plant Pathol.* 22:143–150.

COLLEDGE, A., REID, W.W., and RUSSELL, R.A. 1974. A survey of surface diterpenoids of green leaves. *Ann. du Tabak, Sec. 2*, 11:159–164.

CUTLER, H.G., REID, W.W., and DELETANG, J. 1977. Plant growth inhibiting properties of diterpenes from tobacco. *Plant Cell Physiol.* 18:711–714.

CUTLER, H.G., SEVERSON, R.F., COLE, P.D., JACKSON, D.M., and JOHNSON, A.W. 1986. Secondary metabolites from higher plants: Their possible role as biological control agents, pp. 178–196, *in* M.B. Green and P.A. Hedin (eds.), Natural Resistance of Plants to Pests: Roles of Allelochemicals. Am. Chem. Soc. Symp. Ser. No. 296.

GERSTEL, D.U. 1945. Inheritance in *Nicotiana tabacum*. XX: The addition of *N. glutinosa* chromosomes to tobacco. *J. Hered.* 36:197–206.

GOODSPEED, T.H. 1954. The genus *Nicotiana*. Chronica Botanica, Waltham, Massachusetts.

GRANZOW, P., SHALOSKY, T., and JOHNSON, A. 1985. Effect of nicotine concentration in an artificial diet on the development and survival of newly hatched tobacco budworm larvae. *Tobacco Sci.* 29:111–112.

GREENE, G.L., and THURSTON, R. 1971. Ovipositional preference of *Heliothis virescens* for *Nicotiana* species. *J. Econ. Entomol.* 64:641–643.

GUNASENA, G.H., VINSON, S.B., and WILLIAMS, H.J. 1990. Effect of nicotine on growth, development, and survival of the tobacco budworm (Lepidoptera: Noctuidae) and the parasitoid *Campoletis sonorensis* (Hymenoptera: Ichneumonidae). *J. Econ. Entomol.* 83:1777–1782.

JACKSON, D.M., and SEVERSON, R.F. 1989. Evaluating tobacco for resistance to insect pests, pp. 101–124, *in* T. Stalker and C. Chapman (eds.), IBPGR Training Courses: Lecture Series 2. Scientific Management of Germplasm: Characterization, Evaluation and Enhancement. Internat. Board Plant Genetics Resources, Rome. 194 pp.

JACKSON, D.M., CHEATHAM, J.S., PITTS, J.M., and BAUMHOVER, A.H. 1983. Ovipositional response of tobacco budworm moths (Lepidoptera: Noctuidae) to Tobacco Introduction 1112 and NC 2326 in cage tests. *J. Econ. Entomol.* 76:1303–1308.

JACKSON, D.M., SEVERSON, R.F., JOHNSON, A.W., CHAPLIN, J.F., and STEPHENSON, M.G. 1984. Ovipositional response of tobacco budworm moths (Lepidoptera: Noctuidae) to cuticular chemical isolates from green tobacco leaves. *Environ. Entomol.* 13:1023–1030.

JACKSON, D.M., SEVERSON, R.F., JOHNSON, A.W., and HERZOG, G.A. 1986. Effects of cuticular duvane diterpenes from green tobacco leaves on tobacco budworm (Lepidoptera: Noctuidae) oviposition. *J. Chem. Ecol.* 12:1349–1359.

JACKSON, D.M., SEVERSON, R.F., JOHNSON, A.W., GWYNN, G.R., CHAPLIN, J.F., SISSON, V.A., and HERZOG, G.A. 1988. Host plant resistance in tobacco to *Heliothis* species, pp. 31–49, *in* G.A. Herzog, S. Ramaswamy, G. Lentz, J.L. Goodenough, and J.J. Hamm (eds.), Theory and Tactics of *Heliothis* Population Management: III. Emerging Control Tactics and Techniques. South. Coop. Ser. Bull. No. 337.

JACKSON, D.M., SEVERSON, R.F., and JOHNSON, A.W. 1989. Effects of natural tobacco constituents on insect survival, development, and behavior. *Recent Adv. Tobacco Sci.* 15:26–116.

JURZYSTA, A. 1983. Flavanoids from leaves from some *Nicotiana* species. *Pamiet. Pulawski* 79:231–237.

LUKEFAHR, M.J., and MARTIN, D.F. 1966. Cotton-plant pigments as a source of resistance to the bollworm and the tobacco budworm. *J. Econ. Entomol.* 59:176–179.

PARR, J.C. 1967. Resistance in *Nicotiana* and Some Other Plants of the Family, Solanaceae, to the Tobacco Hornworm, *Manduca sexta* (Johan.): Larval Survival and Oviposition Responses. Master's thesis, University of Kentucky. 69 pp.

PARR, J.C., and THURSTON, R. 1968. Toxicity of *Nicotiana* and *Petunia* species to larvae of the tobacco hornworm. *J. Econ. Entomol.* 61:1525–1531.

REID, W.W. 1974. The cuticular and cytoplasmic lipids of *Nicotiana tabacum*. *Ann. du Tabak, SEITA* 11:151–159.

REID, W.W. 1979. The diterpenes of *Nicotiana* species and *N. tabacum* cultivars, pp. 273–278, *in* J.G. Hawkes, R.N. Lester, and A.D. Skelding (eds.), The Biology and Taxonomy of the Solanaceae. Linnean Soc. Symp. Ser. No. 7, Academic Press, New York.

SAS Institute. 1985. SAS User's Guide: Statistics, Version 5 Ed. SAS Institute, Cary, North Carolina.

SEVERSON, R.F., ARRENDALE, R.F., CHORTYK, O.T., JOHNSON, A.W., JACKSON, D.M., GWYNN, G.R., CHAPLIN, J.F., and STEPHENSON, M.G. 1984. Quantitation of cuticular components of green tobacco leaf. *J. Agric. Food Chem.* 32:566–570.

SEVERSON, R.F., ARRENDALE, R.F., CHORTYK, O.T., GREEN, C.R., THOME, F.A., STEWART, J.L., and JOHNSON, A.W. 1985a. Isolation and characterization of the sucrose esters of the cuticular waxes of green tobacco leaf. *J. Agric. Food Chem.* 33:870–875.

SEVERSON, R.F., JOHNSON, A.W., and JACKSON, D.M. 1985b. Cuticular constituents of tobacco: Factors affecting their production and their role in insect and disease resistance and smoke quality. *Recent Adv. Tobacco Sci.* 11:105–174.

SEVERSON, R.F., MUELLER, S.J., SISSON, V.A., JACKSON, D.M., and STEPHENSON, M.G. 1988a. Isolation of the major cuticular diterpenes from *Nicotiana glutinosa*. 42nd Tobacco Chem. Res. Conf., 42:29.

SEVERSON, R.F., STEPHENSON, M.G., JOHNSON, A.W., JACKSON, D.M., and CHORTYK, O.T. 1988b. Isolation and preparative chromatography of the major cuticular diterpenes of green tobacco. *Tobacco Sci.* 32:99–103.

SEVERSON, R.F., JACKSON, D.M., JOHNSON, A.W., SISSON, V.A., and STEPHENSON, M.G. 1990. Ovipositional behavior of tobacco budworm and tobacco hornworm: Effects of cuticular components from *Nicotiana* species, pp. 264–277, *in* P.A. Hedin (ed.), Naturally Occurring Pest Bioregulators. Am. Chem. Soc. Symp. Ser. No. 449, Am. Chem. Soc., Washington, D.C.

SHAVER, T.N., and LUKEFAHR, M.J. 1969. Effect of flavanoid pigments and gossypol on growth and development of the bollworm, tobacco budworm, and pink bollworm. *J. Econ. Entomol.* 62:643–646.

SISSON, V.A., and SEVERSON, R.F. 1990. Alkaloid composition of the *Nicotiana* species. *Beitrage Tabakforsch. Internat.* 14:327–339.

SNOOK, M.E., MASON, P.F., and SISSON, V.A. 1986. Polyphenols in the *Nicotiana* species. *Tobacco Sci.* 30:43–49.

STAVELY, J.R. 1979. Disease resistance, pp. 87–110, *in* R.D. Durbin (ed.), *Nicotiana*: Procedures for Experimental Use. U.S. Dept. Agric. Tech. Bull. No. 1586.

STEEL, R.G.D., and TORRIE, J.H. 1960. Principles and procedures of statistics with special reference to the biological sciences. McGraw-Hill Book Co. 481 pp.

TATEMICHI, Y. 1990. Illustrated Book of the Genus *Nicotiana*. Japan Tobacco Inc., Plant Breed. Genet. Res. Lab., Shizuokaken, Japan. 234 pp.

THURSTON, R. 1961. Resistance in *Nicotiana* to the green peach aphid and some other tobacco insect pests. *J. Econ. Entomol.* 54:946–949.

THURSTON, R., PARR, J.C., and SMITH, W.T. 1966. The phylogeny of *Nicotiana* and resistance to insects, pp. 424–430, *in* Proc. 4th Internat. Tobacco Sci. Congr., Athens, Greece.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

CHEMISTRY OF VENOM ALKALOIDS IN THE ANT *Megalomyrmex foreli* (MYRMICINAE) FROM COSTA RICA

T.H. JONES,[1,*] P.J. DEVRIES,[2] and P. ESCOUBAS[3]

[1]*Laboratory of Biophysical Chemistry*
National Heart, Lung, and Blood Institute
Bethesda, Maryland 20892

[2]*Department of Zoology*
University of Texas
Austin, Texas 78712

[3]*Plant Ecochemicals Project*
Research Development Corporation of Japan
Megumino Kita 3-1-1
Eniwa-shi 061-13, Japan

(Received June 26, 1991; accepted August 19, 1991)

Abstract—Chemical analysis of the venom of the myrmicine ant *Megalomyrmex foreli* from Costa Rica revealed the presence of four major alkaloidal components. Two of these, 2-butyl-5-(*E*, 1-heptenyl)-5-pyrroline **(3)** and 2-butyl-5-(*E*, *E*, 1,3-heptadienyl)-5-pyrroline **(4)**, constitute a new functional class of ant venom alkaloids, whose structures were assigned from their spectral and chemical behavior and unambiguous syntheses. The function of these compounds is suggested by field observations of the behavior of *M. foreli*, its sting morphology, and the relative toxicity of **3** and **4** against termite workers.

Key Words—*Megalomyrmex*, Hymenoptera, Myrmicinae, venom, alkaloids, ants, pyrrolines, alarm behavior, stridulation, toxicity.

INTRODUCTION

The venoms of many species of the two related myrmicine genera *Solenopsis* and *Monomorium* have been shown to contain 2,5-dialkylpyrrolidines. These compounds have been found in some *Solenopsis* (*Diplorhoptrum*) species and in nearly every species of *Monomorium* where alkaloids have been detected

*To whom correspondence should be addressed.

0098-0331/91/1200-2507$06.50/0

(Jones et al., 1982). Recently, we have also found 2,5-dialkylpyrrolidines in two *Megalomyrmex* species (Jones et al., 1991). In a number of cases, smaller amounts of 1-pyrrolines may also be also present (Fales et al., 1980; Pedder et al., 1976; Jones et al., 1982, 1988a,b) although they may be instrumental artifacts. The only other unsaturation detected in the naturally occurring 2,5-dialkylpyrrolidines has been the presence of one or more terminal double bonds on the alkyl groups (Jones et al., 1982).

Other species in the genus *Megalomyrmex* have been found to produce saturated 2,5-dialkylpyrrolidines, and in one case, a 3,5-dialkylpyrrolizidine (Jones et al., 1991). In the present paper, we report the elucidation of two novel 1-pyrrolines with conjugated unsaturation, produced by workers of *M. foreli*. Some insight into the chemical ecology of this species has been gained from field observations and consideration of its sting morphology and the topical toxicities of its venom alkaloids.

METHODS AND MATERIALS

Chemical Analyses. Gas chromatographic analyses were carried out using a Shimadzu GC-9A equipped with a 30 m × 0.5 mm i.d. open DB-17 column (1 μm film thickness). The temperature for the analyses was programed from 60–215°C at 10°/min, and the carrier gas flow rate was 15 mL/min. On a given day, retention temperatures were reproducable to 1°C. Preparative gas chromatography was conducted with a Varian model 1400 gas chromatograph equipped with a 2 m × 5 mm i.d. column packed with 10% OV-17 on 100–120 mesh Supelcoport. IR spectra were obtained using a Hewlett–Packard model 5965A FTIR. ^{1}H and ^{13}C NMR spectra were obtained from $CDCl_3$ solutions using a Varian XL-200 spectrometer. ^{13}C assignments were made on the basis of standard 2D COSY and HETCOR experiments. Electron impact mass spectra were obtained using a LKB-9000 GC/MS equipped with a 30 m × 0.52 mm i.d. open DB-17 column (1 μm film thickness), a LKB-2091 equipped with a 25 m × 0.31 mm i.d. HP-5 column, or a Finnigan ion trap model 800 equipped with a 25 m × 0.31 mm i.d. HP-5 column. High resolution mass spectra were obtained using a VG 7070F instrument in the EI mode at an ionizing voltage of 70 eV.

Ants. Collections and observations on *Megalomyrmex foreli* were made at Jardin Botanico Wilson, San Vito de Java, Puntarenas Province, 1100 m in southwestern Costa Rica. Intermittent observations on *M. foreli* behavior were made from February 1990 through March 1991 on nine separate colonies. These were: five colonies with ca. 100 individuals/nest found in the sheathing stems of *Calathea* spp. (Marantaceae) from 0.5–1 m above the ground in secondary forest, and four subterranean nests in open second growth: three at the base of

shrubs and one in a rock wall. None of the subterranean nests had any tumulus at the entrance, and overall colony sizes were estimated to range from 100–300 individuals. Ants were immediately placed in glass vials containing 1–2 ml of CH_2Cl_2. Specimens of *M. foreli* were deposited in the collection of the Los Angeles County Museum of Natural History, Los Angeles, California, and have been accessed by Roy R. Snelling. For analysis, these mixtures were reduced to ca. 0.2 ml with a slow stream of nitrogen. GC/MS and GC/FTIR analyses revealed the presence of four **(1–4)** nitrogen containing components in a 2 : 1 : 7 : 5 ratio with the following spectral data: **1:** IR 1644 cm^{-1}; MS *m/z* (relative intensity) 223 (M^+, 2), 222 (2), 208 (2), 194 (4), 180 (7), 166 (14), 152 (23), 139 (44), 124 (4), 110 (7), 96 (14), 83 (13), 82 (100), 69 (6), 68 (6), 67 (8), 55 (23), 41 (34). **2:** MS *m/z* (relative intensity) 225 (M^+, 2), 169 (3), 168 (65), 127 (10), 126 (100), 109 (10), 95 (10), 82 (15), 67 (30), 55 (40). **3:** IR 1648, 1594, 1459, 1331, and 973 cm^{-1}; MS *m/z* (relative intensity) 221 (25, M^+), 179 (24), 178 (72), 166 (14), 165 (87), 164 (100), 150 (29), 122 (37), 109 (15), 108 (77), 106 (18), 94 (58), 80 (33), 79 (21), 67 (32), 55 (52), 54 (34), 53 (23), 41 (97). **4:** IR 1622, 1578, 1459, 1330, and 987 cm^{-1}; MS *m/z* (relative intensity) 219 (25, M^+), 190 (29), 177 (26), 176 (99), 163 (45), 162 (45), 148 (22), 134 (44), 132 (18), 121 (22), 120 (100), 119 (29), 118 (29), 107 (18), 106 (27), 105 (12), 94 (13), 93 (18), 91 (26), 80 (24), 79 (31), 77 (25), 67 (19), 65 (18), 55 (25), 53 (17), 41 (60).

In addition, a small quantity (<5% of **4**) of an earlier eluting isomer of **4** was also detected, with a mass spectrum identical to that of **4,** and the following infrared spectrum: IR 1642, 1458, 1320, 989, and 887 cm^{-1}.

Hydrogenation. A slow stream of hydrogen was passed through a small portion of the extract containing ca. 5 mg of PtO_2 for 5 min. Reexamination by GC/MS revealed only two major nitrogen containing components whose gas chromatographic retention times, and infrared and mass spectra were identical to those of a sample of synthetic *cis/trans* **2.**

2-Butyl-5-methyl-5-pyrroline (5). A solution containing 5.26 g (40 mmol) of 1-nitropentane and 3.7 ml of methyl vinyl ketone in 70 ml of benzene was cooled to 0°C and treated with 0.2 g of tributylphosphine. The mixture was stirred overnight, treated with 0.5 ml of iodomethane, and filtered through a short overnight, treated with 0.5 ml of iodomethane, and filtered through a short Florisil column. Gas chromatographic analysis revealed the presence of one product. The solution was heated to reflux under a Dean–Stark trap with 5 ml of ethylene glycol and 0.3 g of *p*-toluenesulfonic acid for 3 hr until the formation of water ceased. The mixture was cooled, washed with saturated $NaHCO_3$, dried over anhydrous $MgSO_4$, and filtered. GC/MS analysis showed the presence of one major long retention time component, **2,** MS *m/z* 206 (8, $M-CH_3$), 99 (11), 88 (5), 87 (100), 55 (10), and 43 (58). After the solvent was removed, the residue was taken up in EtOH and hydrogenated over 5 g of 10%

Pd/C at 3 atm pressure for 3 days. After filtration, the solvent was removed, and the residue was stirred with 70 ml of 10% HCl containing 1 ml of 60% $HClO_4$ for 2 hr. The aqueous solution was washed with ether, and carefully made alkaline with 10% NaOH. The aqueous solution was extracted with ether (4 × 50 ml), and the combined ether layers were dried over anhydrous K_2CO_3. Distillation provided 3.2 g (58% from 1-nitropentane) of **5**, bp 65–68°/13 mm Hg: IR 1652 cm^{-1}; [^{1}H] NMR δ = 3.9 (1H, m), 2.48 (2H, m), 2.05 (1H, m), 2.02 (3H, s), 1.7 (1H, m), 1.5–1.3 (4H, m), 0.9 (3H, br t), ^{13}C NMR δ = 173.57, 72.93, 38.84, 36.52, 29.17, 29.02, 22.89, 19.81, 14.08; MS *m/z* (relative intensity) 139 (6, M^+), 124 (3), 97 (20), 96 (37), 83 (78), 82 (100), 81 (8), 69 (10), 68 (46), 55 (38), 54 (13), 53 (11), 43 (6), 42 (82), 41 (59), 39 (39); HRMS *m/z* 139.1355 ($C_9H_{17}N$ M^+, calcd. 139.1361).

2-(Diethylphosphonomethyl)-5-butyl-2-pyrroline ***(6).*** A solution containing 0.14 g of **5** in 2 ml of THF was added over 15 min to a solution containing 2 mmol of diisopropylamide at −78°C in 5 ml of THF (from 0.28 ml of diisopropylamine and 1.3 ml of 1.6 M *n*-butyllithium) under a nitrogen atmosphere. After 1 hr, a solution containing 0.14 ml of diethyl chlorophosphate in THF was added slowly and the mixture was stirred for 3 hr. The reaction was quenched with 1 ml of saturated $NaHCO_3$ and warmed to room temperature. After the addition of 10 ml of ether, the aqueous layer was separated, and the organic mixture was dried over anhydrous $MgSO_4$. Kugelrohr distillation (210–220°/0.3 mm Hg) of the filtered mixture provided 0.25 g (90%) of **6** as a yellow oil: IR 3316, 1619, 1279, 1040, 950, and 787 cm^{-1}; ^{1}H NMR δ = 7.13 (br s), 4.12 (m), 3.95 (m), 3.6 (m), 3.5 (d, J = 14.3 Hz), 3.0 (d, J = 22.3 Hz), 2.55 (m), 2.0 (m), 1.5–1.2 (m), 0.8 (m); ^{13}C NMR δ = 168.3, 167.4, 72.90, 64.30, 62.12, 60.69, 60.35, 59.36, 38.18, 36.04, 33.42, 33.06, 31.46, 29.08, 28.92, 28.68, 28.47, 22.71, 16.35, 16.16, 13.91; MS *m/z* (relative intensity) 275 (7, M^+), 219 (26), 218 (76), 190 (23), 162 (22), 144 (28), 138 (30), 137 (32), 136 (16), 122 (27), 82 (86), 81 (26), 80 (100), 55 (19); HRMS *m/z* 275.1657 ($C_{13}H_{26}NO_3P$ M^+, calcd. 275.1650).

2-Butyl-5-(E,1-heptenyl)-5-pyrroline ***(3).*** A solution containing 0.4 g (1.45 mmol) of **6** in 10 ml of THF was cooled to −78°C under a nitrogen atmosphere and treated with 0.9 ml of 1.6 M *n*-butyllithium in hexane. After 30 min, a solution containing 0.145 g of hexanal in 2 ml of THF was added and the mixture was allowed to warm to room temperature over 12 hr. The mixture was acidified with 10% HCl, and after separation, the aqueous phase was washed once with ether. The aqueous solution was then made alkaline with 10% NaOH and extracted 2 × 10 ml with ether. The combined ether extracts were dried, filtered, and the solvent removed *in vacuo*, to provide 0.14 g (45% yield) of **3** as a single component by GC analysis. The IR and mass spectra, and gas chromatographic retention time of **3** were identical to those observed for natural **3** from *M. foreli*. ^{1}H NMR δ = 6.34 (1H, d, J = 16 Hz), 6.12 (1H, d of t, J =

16, 6.6 Hz), 3.92 (1H, br m), 2.6 (2H, complex m), 2.1 (2H, m), 1.95 (1H, m), 1.7 (1H, m), 1.5–1.2 (13H, m), 0.85 (6H, m); ^{13}C[NMR δ = 172.33 (C=N), 141.99 ($\underline{C}H_2$–CH=), 127.55 (=$\underline{C}$H–C=N), 72.65 (C=N–$\underline{C}$H), 36.47 (N–CH–$\underline{C}H_2$–*n*-propyl), 33.59 ($\underline{C}H_2$–C=N), 32.76 ($\underline{C}H_2$–CH=), 31.34 ($\underline{C}H_2$–CH_2–CH=), 29.02, 28.46 ($\underline{C}H_2$–CH_2–C=N), 28.32, 22.83 ($\underline{C}H_2$–CH_3), 22.44 ($\underline{C}H_2$–CH_3), 13.99 (CH_3), 13.90 (CH_3); HRMS *m/z* 221.2155 ($C_{15}H_{27}N$ M^+, calcd. 221.2144).

2-Butyl-5-(E,E,1,3-heptadienyl)-5-pyrroline ***(4).*** In the same manner as described for **3,** a solution containing 0.4 g (1.47 mmol) of **6** in 10 ml of THF was treated sequentially with *n*-butyllithium and E-2-hexenal, and the reaction was worked up as described above to give 0.12 g (37% yield) of **4** as a single component by GC analysis. The IR and mass spectra, and gas chromatographic retention time of **4** were identical to those of natural **4** from *M. foreli*. ^{1}H NMR δ = 6.56 (1H, d of d, J = 15.8, 9.8 Hz), 6.38 (1H, d, J = 15.8 Hz), 6.17 (1H, d of d of t, J = 15.0, 9.8, 1.2 Hz), 5.90 (1H, d of t, J = 15.0, 7.1 Hz), 4.0 (1H, m), 2.6 (2H, m), 2.1 (2H, m), 1.7 (1H, m), 1.5–1.3 (9H, m), 0.9 (6H, br t); ^{13}C NMR 172.4 (C=N), 139.49 (CH_2–$\underline{C}$H=), 138.92 (CH_2–CH=CH–$\underline{C}$H=), 130.13 (CH_2–CH=$\underline{C}$H), 126.68 (=$\underline{C}$H–C=N), 72.91 (C=N–$\underline{C}$H), 36.47 (N–CH–$\underline{C}H_2$–*n*-propyl), 34.92 ($\underline{C}H_2$–CH=), 33.58 ($\underline{C}H_2$–C=N), 29.00, 28.53, 22.86 ($\underline{C}H_2$–CH_3), 22.25 ($\underline{C}H_2$–CH_3), 14.02 ($\underline{C}H_3$), 13.62 ($\underline{C}H_3$); HRMS *m/z* 219.1989 ($C_{15}H_{25}N$ M^+, calcd. 219.1987).

Toxicity Determinations. The toxicities of **3** and **4** were determined from topical application bioassays against *Reticulitermes speratus* collected near Yokkaichi Mie prefecture, Japan, as previously described (Jones et al., 1988a). The LD_{50} of **3** was 1.06 $\pm$ 0.93 μg/mg termite, and the LD_{50} of **4** was 2.39 $\pm$ 0.69 μg/mg termite.

RESULTS

Field Observations. The diet of *M. foreli* consisted mainly of secretions produced by plants and insects. Workers were observed consistently to harvest secretions from at least three species of Membracidae (Homoptera), extrafloral nectar of *Cassia* sp., *Inga* spp. (Leguminoseae), and secretions of the (both Riodinidae). Just after dawn, some workers also occasionally harvested dead arthropods that had collected under a porch light. Although workers foraged throughout a 24-hr cycle, typically the greatest abundance of individual workers per colony was at night, and there was no indication that any foraged more than 3–5 m from the nest entrance. When nests located in *Calthea* stems were broken into, there was a rapid scatter response; individuals ran excitedly over the stems, dropped to the ground, or retreated further into the stem. While in this state, individuals produced stridulations ranging from 500 to 2100 Hz at 20–25 pulses/sec (DeVries, 1991a).

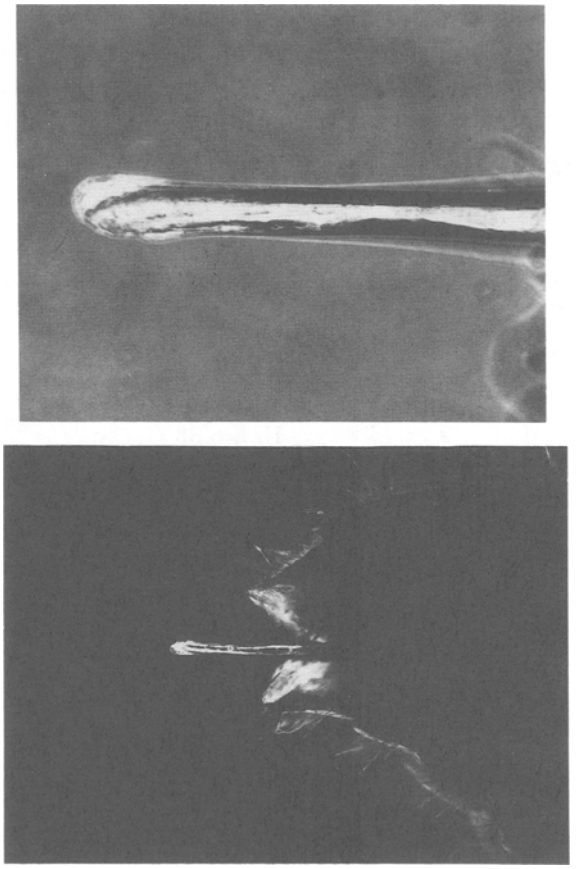


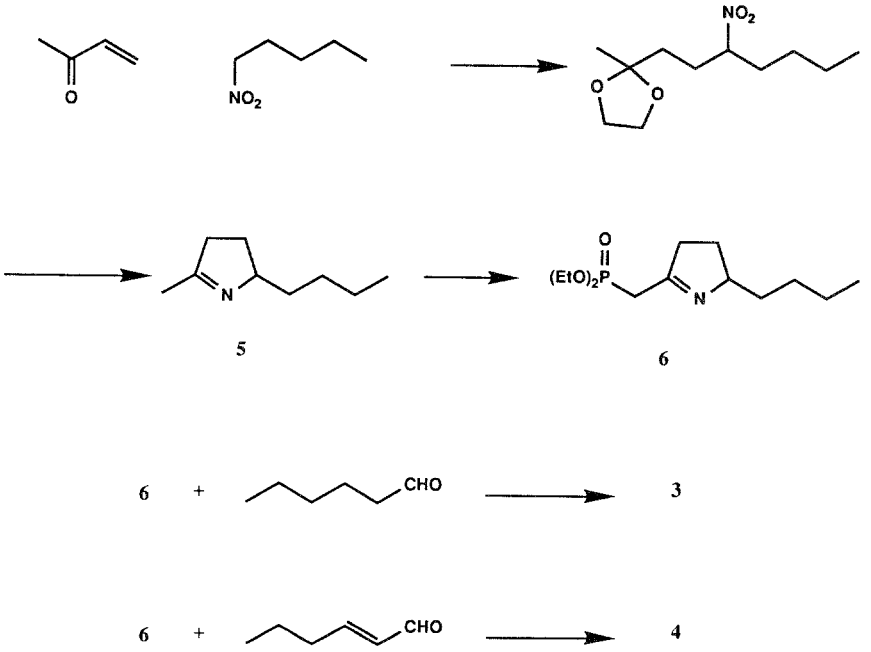
FIG. 1. The spatulate sting of *Megalomyrmex foreli* (Bottom: × 50; top × 200).

The easily visible sting was angled slightly dorsally and was extruded when an individual was held between two fingers, although restrained individuals of *M. foreli* did not sting readily. Apparently, the sting could not pierce the callous tissue at the fingertips. A microscopic examination of the sting revealed the spatulate nature of its lancets (Fig. 1).

Alkaloid Analysis. Analysis of the methylene chloride extracts from all of the collections of *Megalomyrmex foreli* revealed the presence of four major alkaloidal components in a 2:1:7:5 ratio with parent ions at m/z = 223, 225, 221, and 219, respectively. Comparison of their mass and infrared spectra and retention times to those of authentic samples showed that the first two components were 2-butyl-5-heptyl-5-pyrroline **(1)** and *trans*-2-butyl-5-heptylpyrrolidine **(2)** (Jones et al., 1982). Hydrogenation of a portion of the extract over

PtO_2 gave an isomeric mixture of *cis/trans* **2,** demonstrating that the carbon–nitrogen skeleton of **2** was common to the remaining alkaloids. The longer retention times and the pair of strong bands between 1650 and 1550 cm^1 in the infrared spectra of the third and fourth eluting alkaloids indicated the presence of one or two units of conjugated unsaturation in these compounds. In addition, strong out-of-plane bending bands at 973 and 987 cm^{-1}, respectively, were evidence for the *E* geometry of the added double bonds in these compounds. The mass spectra of the third and fourth eluting compounds both showed ions attributable to loss of propene $(M\text{-}42)^+$ and the loss of C_4H_9 that would be expected if they were unsaturated analogues of **1** such as 2-butyl-5-(*E*,1-heptenyl)-5-pyrroline **(3)** and 2-butyl-5-(*E*,*E*, 1,3-heptadienyl)-5-pyrroline **(4)** (Fig. 2).

In order to confirm these assignments, pyrroline **3** and **4** were synthesized as shown in Scheme I. The ethylene ketal of the nitro ketone formed by the addition of 1-nitropentane to methylvinylketone (Miyakoshi, 1986) was hydrogenated, and following acidification, this sequence provided 2-butyl-5-methyl-5-pyrroline **(5)** in nearly 60% yield. Reaction of the kinetic enolate of **5** with diethyl chlorophosphate gave a good yield of the pyrroline phosphonate **6.** The condensation of the anion of **6** (*n*-butyllithium, −78°) with hexanal or *E*,2-



SCHEME 1.

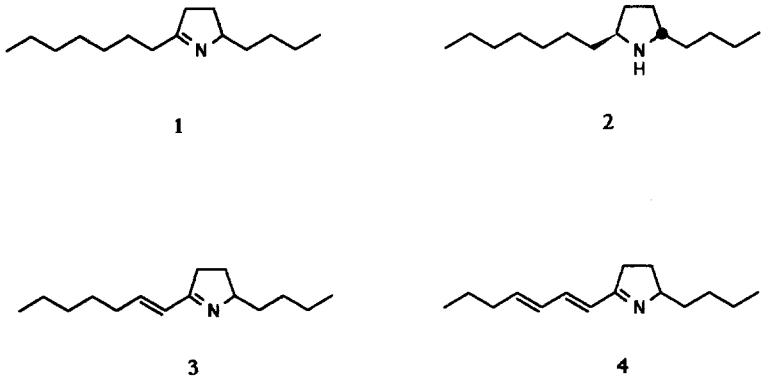


FIG. 2. The major alkaloids from the venom of *Megalomyrmex foreli*.

hexenal in a Wadsworth–Emmons-type reaction formed **3** and **4**, respectively, in moderate yield. In their ^{1}H NMR spectra, the olefinic coupling constants of 16 Hz in **3** and 15.8 Hz and 15.0 Hz in **4** confirm the *E* geometry of the side chain double bonds in these products. The retention times as well as the infrared and mass spectra of **3** and **4** were identical to those of the third and fourth eluting alkaloids from *M. foreli*.

Finally, a minor component with a retention time between that of **3** and **4** and a mass spectrum identical to that of **4** was detected. The band at 887 cm^{-1} in the infrared spectrum of this compound along with its retention time suggested that it was very likely a geometrical isomer of one of the double bonds of **4**.

Since **3** and **4** are new ant venom alkaloids, their toxicity against termites (*Reticulitermes* spp.) in topical application bioassays was determined. At 1.06 μg/mg and 2.39 μg/mg of termite, respectively, the LD_{50}'s of **3** and **4** were within the range of those found for other ant-venom-derived 1-pyrrolines (Escoubas et al., 1988; Jones et al., 1988a). For comparison, the LD_{50} of nicotine is 0.5 μg/mg termite. No "knock-down" effect was observed, even when the mortality became 100%.

DISCUSSION

The venom alkaloids **1–4** (Fig. 2) present in *M. foreli* share a common carbon–nitrogen skeleton, and the presence of pyrrolidine **2** suggests employment of the venom as a repellent, since this compound has been shown to have a strong repellent effect on other ants (Blum et al., 1980). *M. leoninus* produces only **2** in its venom (Jones et al., 1991), which may emphasize its taxonomic

relationship to *M. foreli*, as *M. foreli* is a member of the *M. leoninus* species group (Brandao, 1990). Saturated 2,5-dialkylpyrrolidines, including **2**, are frequently encountered venom components in other myrmicine species (Jones et al., 1982), and it may be that the pyrrolines **1**, **3**, and **4** are the result of an incomplete or modified biosynthetic pathway to **2**.

The unsaturated pyrrolines **3** and **4** constitute a new functional class of ant venom alkaloids. Their conjugated imine groups are reminiscent of the vinyl ketones produced in the defensive secretions of some termites, e.g., *Schedorhinotermes* spp., whose toxicity has been attributed to their strongly, electrophilic nature (Quennedey et al., 1973; Prestwich and Collins, 1982; Prestwich et al., 1975). The toxicities of **3** and **4** in topical application against termites (*Reticulitermes* spp.) are similar to those found for other ant-derived 1-pyrrolines, and are somewhat less than those reported for the corresponding pyrrolidines (Escoubas et al., 1988; Jones et al., 1988a, 1989). It is not surprising that the more polar **3** and **4** are not exceptionally toxic to termites compared to other more lipophilic ant venom alkaloids, since topical activity requires cuticular penetration. Our observations suggest that termites and *M. foreli* seldom, if ever, have strong interactions. Thus, it is unclear if these results can be taken as an indication that *M. foreli* evolved as a defense against termites. On the other hand, the functionally analogous vinyl ketones, produced by termites (*Schedorhinotermes*) for defense, have demonstrable *in vivo* toxicity (Spanton and Prestwich, 1981; Prestwich and Collins, 1982), and are inhibitors of *in vitro* pheromone degradation (Prestwich et al., 1989; Tasayco and Prestwich, 1990a,b). As defensive chemicals, the unsaturated pyrrolines **3** and **4**, functioning as irreversible electrophiles, may be used by *M. foreli* as a defense by disrupting the enzymatic degradation of chemical signals in the antennal sensilla of would be predators in a similar fashion.

The reluctance of *M. foreli* to sting led to an examination of the sting apparatus. The terminal lobate portion of the sting (Fig. 1) indicates some function other than piercing, and fortifies the suggestion that *Megalomyrmex* stings may be used as a chemical applicator (Kugler, 1979). Indeed, as has been observed in the genus *Tetraponera*, a characteristic of ants with well-developed chemical defenses may be replacement of stinging by some other mode of defense (Kugler, 1979; Braekman et al., 1987). The alarm-defense behaviors in *M. foreli* parallel those of other ant taxa. The rapid scatter response and stridulations produced by these ants when they are disturbed are similar to such responses commonly observed in the genera *Azteca*, *Dolichoderus*, *Tapinoma* (Dolichoderinae), *Oecophylla*, *Camponotus* (Formicinae), and in the myrmicine genus *Crematogaster* (DeVries, personal observation), all taxa with vestigal stings and highly evolved chemical defenses (Hölldobler and Wilson, 1990). Additionally, it is well established that alkaloid producing ants can utilize these compounds in the form of an aerosol (Obin and Vander Meer, 1985).

Taken together, the rapid scatter response, apparently non-penetrating sting, and relatively low topical toxicity of **3** and **4**, along with their electrophilic functionality, suggest that the venom of *M. foreli* may be deployed in much the same way.

The most recent review (Hölldobler and Wilson, 1990) indicates that little is published on the natural history of *Megalomyrmex*. It has been observed that some species of *Megalomyrmex* are apparently xenobiotic parasites of its nearest relatives, *Cyphomyrmex* and *Sericomyrmex*, and that in some species in the *M. leoninus* group, reproduction, or the queen caste, has been taken over by the workers (Brandao, 1987; Hölldobler and Wilson, 1990). The present observations of *M. foreli* suggest that the unique venom chemistry and modified sting play a role in the foraging and defense behaviors of these ants. Furthermore, these behaviors converge upon other, distantly related ant taxa that posses highly developed chemical defenses. Since plants and insects that form associations with ants receive survival benefit from the symbiotic interaction (Way, 1963; Beattie, 1985; Pierce, 1987; DeVries and Baker, 1989; DeVries, 1991b), it is likely that the *E. lycisca* and *N. cachrus* caterpillars reported here benefit from forming symbioses with *M. foreli*. Thus, although the defensive chemistry in *M. foreli* undoubtedly evolved in response to specific selective pressures, totally unrelated organisms like plants and other insects may benefit from the unique venom alkaloids of this ant.

Acknowledgments—We wish to thank Dr. H.M. Garraffo and Mr. Noel F. Whittaker of the laboratory of Bioorganic Chemistry, NIDDK for FTIR and high resolution mass spectra, respectively, and Dr. R.J. Highet of the laboratory of biophysical chemistry, NHLBI for the NMR spectra. We also thank Mr. R. Dreyfuss of the medical arts and photography branch, NIH, for the photomicroscopy. PJD would like to thank J. Longino, B. Hawkins, J. Clark, and the MacArthur Foundation for field assistance, and dedicate this paper to the work of P. Martino.

REFERENCES

BEATTIE, A.J., 1985. The Evolutionary Ecology of Ant–Plant Mutualisms. Cambridge University Press, Cambridge.

BLUM, M.S., JONES, T.H., HÖLLDOBLER, B., FALES, H.M., and JAOUNI, T. 1980. Alkaloidal venom mace: Offensive use by a thief ant. *Naturwissenschaften*. 67:144–145.

BRAEKMAN, J.C., DALOZE, D., PASTEELS, J.M., VAN HECKE, P., DECLERCQ, J.P., SINNWELL, V., and FRANCKE, W. 1987. Tetraponerine-8, an alkaloidal contact poison in a Neoguinean pseudomyrmecine ant, *Tetraponera* sp. *Z. Naturforsch.* 42c:627–630.

BRANDAO, C.R.F. 1987. Queenlessness in *Megalomyrmex* (Formicidae: Myrmicinae) with a discussion of the effects of the loss of true queens in ants, pp. 111–1112, *in* J. Eder and H. Rembold (eds.). *Chemistry and Biology of Social Insects*. Proc. 10th IUSSI, Munich 1986, Verlag J. Peperny, Munich.

BRANDAO, C.R.F. 1990. Systematic revision of the neotropical ant genus *Megalomyrmex* Forel (Hymenoptera: Formicidae: Myrmicinae). *Arquivos de Zoologica* 31: 411–481.

DEVRIES, P.J. 1991a. Detecting and recording the calls produced by butterfly caterpillars and ants. *J. Lep. Res.* In press.

DEVRIES, P.J. 1991b. Mutualism between *Thisbe irenea* larvae and ants, and the role of ant ecology in the evolution of myrmecophilous butterflies. *Biol. J. Linn. Soc.* 43:178–195.

DEVRIES, P.J., and BAKER, I., 1989. Butterfly exploitation of a plant–ant mutualism: Adding insult to herbivory. *J. N.Y. Entomol. Soc.* 97:332–340.

ESCOUBAS, P., CLEMENT, J.L., BLUM, M.S., JONES, T.H., LHOMMET, G., and CELERIER, J.P. 1988. Toxicité des alcaloides de fourmis. *Actes Coll. Insectes Sociaux.* 4:51–58.

FALES, H.M., COMSTOCK, W., and JONES, T.H. 1980. Test for dehydrogenation in gas chromatography–mass spectrometry systems. *Anal. Chem.* 52:980–982.

HÖLLDOBLER, B., and WILSON, E.O. 1990. The Ants. Harvard University Press, Cambridge.

JONES, T.H., BLUM, M.S., and FALES, H.M. 1982. Ant venom alkaloids from *Solenopsis* and *Monomorium* species. *Tetrahedron* 38:1949–1958.

JONES, T.H., BLUM, M.S., ANDERSEN, A.N., FALES, H.M., and ESCOUBAS, P. 1988a. Novel 2-ethyl-5-alkylpyrrolidines in the venom of an Australian ant of the genus *Monomorium. J. Chem. Ecol.* 14:35–45.

JONES, T.H., STAHLY, S.M., DON, A.W., and BLUM, M.S. 1988b. Chemotaxonomic implications of the venom chemistry of some *Monomorium "antarcticum"* populations. *J. Chem. Ecol.* 14:2197–2212.

JONES, T.H., BLUM, M.S., ESCOUBAS, P., and MUSTHAK ALI, T.M. 1989. Novel pyrrolidines in the venom of the ant *Monomorium indicum. J. Natl. Prod.* 52:779–784.

JONES, T.H., BLUM, M.S., FALES, H.M., BRANDAO, C.R.F., and LATTKE, J. 1991. Chemistry of venom alkaloids in the ant genus *Megalomyrmex. J. Chem. Ecol.* 17:1897–1908.

KUGLER, C. 1979. Evolution of the sting apparatus in the myrmicine ants. *Evolution* 33:117–130.

MIYAKOSHI, T. 1986. A convenient synthesis of 4-oxoalkanals. *Synthesis* 1986:766–768.

OBIN, M.S., and VANDER MEER, R.K., 1985. Gaster flagging by fire ants (*Solenopsis* spp.): Functional significance of venom dispersal behavior. *J. Chem. Ecol.* 11:1757–1768.

PEDDER, D.J., FALES, H.M., JAOUNI, T., BLUM, M.S., MACCONNELL, J., and CREWE, R.M. 1976. Constituents of the venom of a South African fire ant (*Solenopsis punctaticeps*). *Tetrahedron.* 32:2275–2279.

PIERCE, N.E. 1987. The evolution and biogeography of associations between lycaenid butterflies and ants. *Oxford Surveys Evolutionary Biol.* 4:89–116.

PRESTWICH, G.D., and COLLINS, M.S. 1982. Chemical defense secretions of the termite soldiers of *Acorhinotermes* and *Rhinotermes* (Isoptera: Rhinoterminae). *J. Chem. Ecol.* 8:147–161.

PRESTWICH, G.D., KAIB, M., WOOD, W.F., and MEINWALD, J. 1975. 1,13-Tetradecadien-3-one and homologs: New natural products isolated from *Schedorhinotermes* soldiers. *Tetrahedron Lett.* 4701–4704.

PRESTWICH, G.D., GRAHAM, S. MCG., HANDLEY, M., LATLI, B., STREINZ, L., and TASAYCO, J., M.L. 1989. Enzymatic processing of pheromones and pheromone analogs. *Experientia* 45:263–270.

QUENNEDEY, A., BRULE, G., RIGAUD, J., DUBOIS, P., and BROSSUT, R. 1973. La glande frontale des soldats de *Shedorhinotermes putorius* (Isoptera): Analyse chimique et fonctionnement. *Insect Biochem.* 3:67–74.

SPANTON, S.G., and PRESTWICH, G.D. 1981. Chemical self-defense by termite workers: Prevention of autotoxication in two rhinotermitids. *Science* 214:1363–1365.

TASAYCO, J., M.L. and PRESTWICH, G.D. 1990a. Aldehyde-oxidizing enzymes in an adult moth:

In vitro study of aldehyde metabolism in *Heliothis virescens*. *Arch. Biochem. Biophys.* 278:444–451.

TASAYCO, J., M.L., and PRESTWICH, G.D. 1990b. A specific affinity reagent to distinguish aldehyde dehydrogenases and oxidases. *J. Biol. Chem.* 265:3094–3101.

WAY, M.J. 1963. Mutualism between ants and honeydew producing Homoptera. *Annu. Rev. Entomol.* 8:307–344.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

RELATIONSHIP BETWEEN DETERRENCE AND TOXICITY OF PLANT SECONDARY COMPOUNDS FOR THE GRASSHOPPER *Schistocerca americana*

E.A. BERNAYS

Department of Entomology and Center for Insect Science
University of Arizona
Tucson, Arizona 85721

(Received July 29, 1991; accepted August 19, 1991)

Abstract—A variety of plant secondary compounds, several of which are quite widespread in nature were tested for their deterrence to the generalist grasshopper *Schistocerca americana* in short-term behavioral assays. The compounds were coumarin, salicin, tannic acid, gramine, nicotine, quinine, carvone, geraniol, abietic acid, umbelliferone, and ursolic acid. These were then tested for their post-ingestional effects over the whole of the last larval instar. Different methods were employed to mask the taste of compounds that were deterrent in order to ensure that any effects were not due to reduced feeding. In no case was there any indication of a detrimental effect or any trend suggesting one. In two cases, there was a significant increase in growth rate with the addition of the secondary compound to the diet. The evolutionary implications of these findings are discussed.

Key Words—*Schistocerca americana*, Orthoptera, Acrididae, deterrence, toxicity, plant defense, diet breadth.

INTRODUCTION

The possible evolutionary reasons for deterrent effects of plant secondary compounds on herbivores are numerous, but usually it is assumed that avoidance of chemicals or plants is an evolutionary response to some noxious quality of these materials. Yet many deterrent responses appear to be unrelated to post-ingestional toxicity (Cottee et al., 1988; Bernays and Chapman, 1987; Bernays and Graham, 1988). The question needs answering on a series of different insect species and plant compounds because, if behavioral deterrence is not associated with post-ingestional toxicity, the evolutionary reasons for the behavioral sen-

0098-0331/91/1200-2519$06.50/0

sitivity need reevaluation with respect to whether they are primarily ecological or physiological. Among grasshoppers, deterrence of chemicals in non-host plants appears to be the primary basis for rejection of plants as food (Bernays and Chapman, 1977, 1978; Chapman and Bernays, 1977; Chapman et al., 1988), and there is interest in whether rejection responses are indicators of unsuitability. The aim of this paper is to examine the behavioral effects of a range of plant secondary compounds and then to examine whether measurable noxious effects follow ingestion of them, using the acridid *Schistocerca americana* Drury. This species is polyphagous but many native American plants tested are quite unacceptable, or become so after the initial meal (Chapman and Sward, unpublished).

METHODS AND MATERIALS

Stock colonies of *Schistocerca americana* Drury (Orthoptera: Acrididae) were reared in rectangular metal cages of 64 L capacity according to the procedures used at the Centre for Overseas Pest Research (Hunter-Jones, 1961). Each day, individuals that had newly molted to the sixth instar were removed and kept in 8 L cylindrical plexiglass cages in an environment chamber kept at L:D, 12:12 and temperature, 32:28°C. They were fed on seedling wheat.

Behavioral Choice Tests. Chemicals were tested for their deterrence on 3-day-old sixth instar nymphs by presentation in choice tests on sucrose-impregnated glass fiber disks, with sucrose-only control disks for 3–4 hr at 30°C (Navon and Bernays, 1978). Experiments were also carried out in which the chemicals were presented on wheat blades, but the measurements on acceptability were done by relative amounts of leaf area removed in a 5-hr period.

Chemicals chosen for experiments were expected to be deterrent at some concentration. The ten compounds and their commercial sources are listed in Table 1. For application to wheat blades, concentrations were made up either in water with detergent, in acetone, or in alcohol, such that an appropriate dry weight concentration of the desired level was achieved. This was found to vary by about 20% around the mean value, which was considered a realistic level of variation that could occur naturally. Concentrations tested were at about the maximum recorded levels found naturally, and at one fifth of that concentration.

Performance Tests. To test the potential effects of ingestion of the plant secondary compounds, various techniques were employed. For those that had no deterrent effect on feeding behavior (tannic acid, coumarin, abeitic acid, and carvone), the material was simply added to leaves as described above (Table 1) while the controls had solvent-only treated leaves. In each experiment, each individual was fed daily on either wheat leaves with an added chemical, or control wheat leaves without the chemical. Insects were weighed and selected

TABLE 1. COMPOUNDS USED FOR THE TESTS ON BEHAVIOR AND PHYSIOLOGY OF *Schistocerca americana*, AND THEIR SOURCES

Compound	Source	Application method	
		To leaves	To insects
Phenolics			
Coumarin	Eastman	Water	On leaves
Umbelliferone	Sigma	Alcohol	s.m. capsule
Salicin	Sigma	Water	Wax coat
Tannic acid	Sigma	Water	On leaves
Alkaloids			
Gramine	Sigma	Alcohol	s.m. capsules
Nicotine (hydrogen tartrate)	Sigma	Water	s.m. capsules
Terpenoids			
Carvone	Kodak	Alcohol	On leaves
Geraniol	Kodak	Alcohol	Cyclodextrin
Abeitic acid	Sigma	Alcohol	On leaves
Ursolic acid	Sigma	Acetone	Gelatin microcaps

for each treatment in such a way that there was considerable overlap in the weight range and no significant difference between the test and control groups. They were kept individually in 5 L tubs with gauze lids. Conditions were the same as for the sorted and aged insects described above. The amounts of leaf material given to each insect each day were approximately of the same weight as the insect. In this way, most or all of the food was eaten each 24 hr except toward the end of the instar (Bernays, 1990). There were usually at least ten test and ten control insects in an experiment. The insects were weighed daily and the dry weight measured after the final ecdysis. The growth rate and efficiency of conversion of ingested food to body mass (ECI) were calculated in the usual way (e.g. Scriber, 1978).

In some cases (umbelliferone, gramine, and nicotine) insects were dosed daily using semimicro gelatin capsules prepared as described by Szentesi and Bernays (1984). The feeding regime was as described above, but twice daily a capsule containing the chemical under test, or an empty capsule, was inserted under the labrum and between the bases of the mandibles, from which it was swallowed. The amounts of the chemical in the capsules were calculated to give the approximate percentage dry weight of food assuming all food was eaten (Table 2).

With geraniol, we attempted to mask the taste by inclusion in betacyclodextrin, as described by Usher et al. (1989). This appeared to be successful

TABLE 2. EFFECTS OF COMPOUNDS ON FEEDING BEHAVIOR OF *Schistocerca americana*, NEAR OR ABOVE THEIR LIKELY MAXIMUM NATURAL CONCENTRATIONS, AND AT ONE-FIFTH OF THAT CONCENTRATION[a]

Compound	Higher concentration used (%dw)	Effect on feeding		
		Disk high	Disk low	Wheat high
Phenolics				
Coumarin	1	− (0.06)	0	0
Umbelliferone	1	− (0.03)[b]	0	− (0.05)[b]
Salicin	1	− (0.05)[b]	0	− (0.08)
Tannic acid	5	+ (0.06)	+ (0.01)[b]	0
Alkaloids				
Gramine	1	− (0.07)	+ (0.05)[b]	0
Nicotine	1	− (0.05)[b]	0	− (0.05)[b]
Terpenoids				
Carvone	0.1	+ (0.05)[b]	+ (0.025)[b]	0
Geraniol	0.01	− (0.07)	+ (0.08)	0
Abeitic acid	0.1	− (0.08)	0	0
Ursolic acid	0.1	− (0.07)	0	0

[a] + = stimulatory, 0 = no effects, − = deterrent. *P* values (less than the value in parenthesis) based on Wilcoxon's Signed Ranks test.
[b] Significant effect.

since no more food remained in the tubs at the end of each day relative to controls.

Crystals of salicin and methylene blue dye (mixed together) were wax-coated in a simplified version of the fluid-bed Wurster chamber process (see Usher et al., 1989 for details of this chamber). Crystals were placed in a hand-pulled funnel made from glass tubing 2 cm in diameter with a plug of glass wool at the bottom. Warm air was fed into the upright funnel from below, and this airstream suspended the particles at a level somewhat above the region where the diameter expanded to that of the tubing which extended about 10 cm above. From the top of the tubing, a solution of beeswax in chloroform was sprayed onto the moving particles using a chromatographic spray. The air stream was continued for about 5 min and then turned off allowing the newly-coated particles to fall onto the plug. Blue color was not observed at this stage. When the resulting wax-coated particles were extracted with chloroform, it was found that the salicin made up about 10% of the total weight. A behavioral assay was used to test deterrency by giving individuals a choice test between wax-coated cellulose and methylene blue crystals, and wax-coated salicin and methylene blue crystals, spread on sucrose-impregnated filter paper disks. The concentration was determined to be approximately 2% dw. No deterrency was detected

and subsequent dissection showed that the dye had been released in the gut. Wax-coated particles were tested for post-ingestional effects over the instar by spreading them onto leaves using a thin layer of office glue stick material. Coated cellulose (a carbohydrate that is not digested) was used as the control. Concentrations of salicin were approximately 1–3% dw of the leaf.

Finally, with ursolic acid, gelatin walled microcapsules were prepared by complex coacervation (Thies, 1986). Afterward, these were spread as an aqueous slurry onto the wheat leaves and allowed to dry. The difficulty of controlling the concentration obtained, and of variable levels of agglomeration, meant that the amounts ingested probably varied between about 0.1 and 0.5% of the dry weight of the leaf.

RESULTS AND DISCUSSION

Eight of the ten compounds deterred feeding at some concentration, although in only four cases were there significant effects at concentrations in the likely natural range, while three were significantly phagostimulatory at lower concentrations (Table 2). In no case was there any significant depression of growth rate or efficiency of conversion of food to body mass when insects ingested the chemicals over a whole instar (Tables 3 and 4). On the contrary, tannic acid and salicin appeared to cause slight increases in growth rates (Table 3). Similarly, Cottee et al. (1988) found a singular lack of oral toxicity in eight

TABLE 3. EFFECTS OF INGESTED PLANT SECONDARY COMPOUNDS ON GROWTH RATE OF SIXTH INSTAR *Schistocerca americana* NYMPHS[a]

	Test	Control	*F*	*P*
Coumarin	13.5(0.2)	13.3(0.2)	1.13	0.3618
Umbelliferone	13.8(0.2)	14.0(0.3)	1.42	0.2588
Salicin	14.7(0.2)	13.6(0.3)	4.54	0.0481[b]
Tannic acid	14.5(0.2)	13.6(0.3)	4.43	0.0400[b]
Gramine	13.4(0.3)	13.4(0.2)	0.66	0.5462
Nicotine	13.7(0.2)	13.4(0.2)	0.08	0.5611
Carvone	13.6(0.2)	13.2(0.3)	0.09	0.6302
Geraniol	13.5(0.3)	13.6(0.2)	0.93	0.3259
Abietic acid	14.7(0.3)	14.0(0.3)	1.11	0.4333
Ursolic acid	14.7(0.3)	14.8(0.3)	0.12	0.5639

[a] Values are given in mg dw increase per day, with standard error in parenthesis. *F* and *p* values are from analyses of covariance where weight gain is examined in relation to initial weight as well as treatment.

[b] Significant effects.

TABLE 4. EFFECTS OF INGESTED PLANT SECONDARY COMPOUNDS ON ECI OF SIXTH INSTAR *Schistocerca americana* NYMPHS[a]

	Test	Control	*p*
Coumarin	12.5 (0.4)	12.0 (0.5)	NS
Umbelliferone	13.1 (0.5)	12.6 (0.6)	NS
Salicin	13.3 (0.5)	12.8 (0.4)	NS
Tannic acid	14.1 (0.6)	13.0 (0.4)	<0.05
Gramine	11.9 (0.6)	12.7 (0.4)	NS
Nicotine	13.3 (0.5)	13.3 (0.4)	NS
Carvone	11.8 (0.4)	12.2 (0.5)	NS
Geraniol	12.0 (0.3)	12.4 (0.4)	NS
Abietic acid	14.5 (0.6)	13.7 (0.5)	NS
Ursolic acid	13.0 (0.5)	14.1 (0.6)	NS

[a] Number in each test and control between 10 and 15. Values are given in percentages with standard error in parenthesis. *P* values are from *t*-tests.

plant secondary compounds when tested with the related species, *Schistocerca gregaria* (the desert locust), and Bernays (1990) found no detrimental post-ingestive effects of several deterrents for *Locusta migratoria*.

The lack of postingestive effects of the secondary compounds tested precluded the establishment of a meaningful ranking so that correlation analyses between deterrence and toxicity were not really possible. Cottee et al. (1988) demonstrated a weak correlation between behavioral deterrence of compounds and their toxicity following injection into the hemolymph in both *Locusta migratoria* and *Schistocerca gregaria* (when this is re-analyzed and Spearmans rank correlation done, $p < 0.05$). On the other hand, when compounds were administered by cannulation into the midgut, no correlation was found when deterrence was compared with oral toxicity.

In a comprehensive study by Harley and Thorsteinson (1967) on another polyphagous grasshopper, *Melanoplus bivittatus*, results with steroids and alkaloids also indicate a total lack of correlation between deterrence and toxicity. Interestingly, in that study, care was taken to use plant chemicals occurring in the habitat in which the insect occurs.

Although these experiments were undertaken to examine components of fitness, the tests are obviously not definitive. Feeding on such chemicals over a lifetime may be detrimental while feeding over one instar is possibly not long enough for effects to be seen. However, it might be expected that with below optimal levels of food, additional stresses involved would render insects vulnerable to any deleterious effects of plant secondary compounds. For example, protein shortage may lead to less effective detoxification processes and water

shortage to less effective excretion via the Malpighian tubules. Experiments by Boys (1981) in which *Locusta migratoria* was dosed with gramine at several concentrations covering the natural range, showed no effects on growth rate, fecundity, or longevity. Usher et al. (1989) also found that grindelic acid was deterrent to *Schistocerca americana*, but that when the taste was masked and the food eaten, no detrimental effects could be detected. Tannic acid has been tested with the related *Schistocerca gregaria* and also found to have no detrimental effects even when it is ingested at relatively high levels for the whole of nymphal life (Bernays et al., 1980).

Perhaps, if levels are high enough, detrimental effects would ensue (Cottee et al., 1988), but there is an interesting possibility that grasshoppers are much more highly sensitive behaviorally to plant secondary compounds, than is required for reasonable protection from ingestion of them.

From the evolutionary point of view, a poor correlation between deterrency and oral toxicity would indicate that avoidance responses may often have evolved under selective pressures unrelated to post-ingestional toxicity. Similarly, the tendency toward restriction of host range observed in insect herbivores may be unrelated to their inability to tolerate plant secondary compounds. Host range may be restricted for quite diverse and ecological reasons rather than for mainly physiological reasons. In such a case, non-host plants may become deterrent and unacceptable even when they are chemically suitable for growth and development (Bernays and Chapman, 1987; Bernays and Graham, 1988).

Acknowledgments—This work was funded by NSF Grant BSR-8705014. Various students and other colleagues helped in different ways, but special thanks go to Barbara Usher, Roger Wrubel, Ray Barbehenn, Michelle Murgia, and Junji Hamai.

REFERENCES

BERNAYS, E.A. 1990. Plant secondary compounds deterrent but not toxic to the grass specialist acridid *Locusta migratoria*: Implications for the evolution of graminivory. *Entomologia Exp. Appl.* 54:53–56.

BERNAYS, E.A., and CHAPMAN, R.F. 1977. Deterrent chemicals as a basis of oligophagy in *Locusta migratoria* (L.). *Ecol. Entomol.* 2:1–18.

BERNAYS, E.A., and CHAPMAN, R.F. 1978. Plant chemistry and acridoid feeding behaviour, pp. 99–141, *in* J.B. Harborne (ed.). Biochemical Aspects of Plant and Animal Co-evolution. Phytochem. Soc. Europe Symp. No. 15.

BERNAYS, E.A., and CHAPMAN, R.F. 1987. Evolution of plant deterrence to insects, pp. 159, *in* R.F. Chapman, E.A. Bernays, J.G. Stoffolano (eds.). Perspectives in Chemoreception and Behavior. Springer-Verlag, New York.

BERNAYS, E.A., and GRAHAM, M. 1988. On the evolution of host specificity in phytophagous arthropods. *Ecology* 69:886–892.

BERNAYS, E.A., CHAMBERLAIN, D.J., and MCCARTHY, P. 1980. The differential effects of ingested tannic acid on different species of Acridoidea. *Entomologia Exp. Appl.* 28:158–166.

BOYS, H. 1981. Food selection by some graminivorous Acrididae. D. Phil. thesis, University of Oxford, England.

CHAPMAN, R.F. and BERNAYS, E.A. 1977. The chemical resistance of plants to insect attack. *Pont. Acad. Sci. Scripta varia* 41:603–643.

CHAPMAN, R.F., BERNAYS, E.A., and WYATT, T. 1988. Chemical aspects of host-plant specificity in three *Larrea*-feeding grasshoppers. *J. Chem. Ecol.* 14:561–579.

COTTEE, P.K., BERNAYS, E.A., and MORDUE, A.J. 1988. Comparisons of deterrency and toxicity of selected secondary plant compounds to an oligophagous and a polyphagous acridid. *Entomologia. Exp. Appl.* 46:241–247.

HARLEY, K.L.S., and THORSTEINSON, A.J. 1967. The influence of plant chemicals on the feeding behavior, development, and survival of the two-striped grasshopper, *Melanoplus bivittatus* (Say), Acrididae, Orthoptera. *Can. J. Zool.* 45:305–319.

HUNTER-JONES, P. 1961. Rearing and Breeding Locusts in the Laboratory. Anti-Locust Research Centre, London. 12 pp.

NAVON, A., and BERNAYS, E.A. 1978. Inhibition of feeding in acridids by non-protein amino acids. *Comp. Biochem. Physiol.* 59A:161–164.

SCRIBER, J.M. 1978. The effects of larval feeding specialization and plant growth form on the consumption and utilization of plant biomass and nitrogen: An ecological consideration. *Entomologia Exp. Appl.* 24:695–710.

SZENTESI, A., and BERNAYS, E.A. 1984. A study of behavioural habituation to a feeding deterrent in nymphs of *Schistocerca gregaria. Physiol. Entomol.* 9:329–340.

THIES, C. 1986. How to Make Microcapsules. Course manual, Washington University, St. Louis, Missouri.

USHER, B.F., BERNAYS, E.A., BARBEHENN, R.V., and WRUBEL, R.P. 1989. Oral dosing of insects with feeding deterrent compounds. *Entomologia Exp. Appl.* 52:119–133.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

ELECTROANTENNOGRAM RESPONSES OF THE SOUTHERN PINE BEETLE PARASITOID *Dinotiscus dendroctoni* (ASHMEAD) (HYMENOPTERA: PTEROMALIDAE) TO POTENTIAL SEMIOCHEMICALS

S.M. SALOM,[1,*] G. BIRGERSSON,[2,3] T.L. PAYNE,[1] and C.W. BERISFORD[2]

[1]*Department of Entomology*
Virginia Polytechnic Institute and State University
Blacksburg, Virginia 24061-0319

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

(Received June 17, 1991; accepted August 26, 1991)

Abstract—In two studies using the electroantennogram (EAG) technique, bark beetle- and tree-produced semiochemicals were presented to *Dinotiscus dendroctoni* (Ashmead), a larval parasitoid of *Dendroctonus frontalis* Zimm. In the first study, 20 test compounds and a standard mixture of oxygenated monoterpenes were presented individually at one concentration to the parasitoids. In the second study, the nine compounds that elicited the greatest EAGs in study 1 were then tested as serial dilutions of 10 to 0.0001 $\mu g/\mu l$. The individual compounds did not elicit responses greater than the standard mixture of oxygenated monoterpenes. Males and females exhibited similar dose responses, although females showed lower thresholds of response than males to frontalin, terpinen-4-ol, *E,Z*-chalcogran, and *exo*-brevicomin. In both studies, pino-/isopinocamphone elicited the greatest responses at high concentrations. Tests of different ratios of the camphone mixture indicated that pinocamphone elicited the greatest response. Most of the test compounds elicited similar responses which suggests that several of the compounds may be used together by *D. dendroctoni* in habitat and/or host community location.

Key Words—*Dendroctonus frontalis*, Coleoptera, Scolytidae, *Dinotiscus*

*To whom correspondence should be addressed.
[3]Present address: Department of Chemical Ecology, Göteberg University, Reuteragaten 2C, S-413 20, Göteberg, Sweden.

0098-0331/91/1200-2527$06.50/0

dendroctoni, Hymenoptera, Pteromalidae, parasitoid, kairomone, electroantennogram.

INTRODUCTION

Numerous parasitoid species are associated with bark beetles in the family Scolytidae (Dahlsten, 1982). The ability of these natural enemies to locate their hosts has, in many cases, been determined to be mediated by semiochemicals (Borden, 1982; Payne, 1989; Mills et al., 1991). For the southern pine beetle, *Dendroctonus frontalis* Zimm., parasitoids generally begin arriving on beetle-attacked trees about 3 weeks after initial attack by their host (Camors and Payne, 1973). This coincides with the presence of *D. frontalis* brood in the late larval stadia (Thatcher and Pickard, 1967; Mizell and Nebeker, 1978), the host stage attacked by most of its parasitoids (Berisford, 1980).

Parasitoids arrive after peak pheromone production. Thus, it has been suggested that they may respond to pheromones remaining in the frass after initial attack by the bark beetles (Berisford and Franklin, 1971; Dixon and Payne, 1980) and/or olfactory stimuli from larvae feeding under the bark (Camors and Payne, 1973; Kudon and Berisford, 1981). Other possibilities include pheromones of *D. frontalis* produced later in the attack phase, i.e., verbenone and *exo*-brevicomin, and compounds produced by yeasts (Leufvén and Birgersson, 1987) and fungi (Birgersson and Leufvén, 1988) that are carried into the trees by the beetles.

Dinotiscus (=*Cecidostiba*) *dendroctoni* (Ashmead) (Hymenoptera: Pteromalidae), one of the more abundant species found parasitizing *D. frontalis* (Hetrick, 1940; Dixon and Payne, 1980; Goyer and Finger, 1980), is attracted to *D. frontalis*-infested loblolly pine *Pinus taeda* L. traps in the field (Camors and Payne, 1973). The specific olfactory cues used by this and other bark beetle parasitoid species are unknown. The following studies were conducted to determine the receptivity and sensitivity of the antennal olfactory receptor system of *D. dendroctoni* to potential semiochemicals.

METHODS AND MATERIALS

Insects. Pitch pine, *Pinus rigida* Mill., logs from Georgia, infested with heavily parasitized *D. frontalis* brood, served as our source of *D. dendroctoni* adults. Logs were stored in a cooler at 4°C. As adults were needed, logs were placed in rearing/emergence chambers (Browne, 1972). The chambers were checked daily and emerged adults were placed individually in 1 dram screw-top vials with a 30 × 7 mm section of filter paper moistened with a 1 : 1 honey–water solution. Adults were stored at 4°C in a domestic refrigerator without

photoperiodic control, for no longer than 21 days. To allow the adults to feed on the honey solution, they were removed from the refrigerator daily and exposed to room temperature (ca. 23°C) for 30 min. The procedure prolonged the lives of the parasitoids.

Compounds. Twenty bark beetle- and tree-produced compounds were tested in the study. The source and purity of the compounds are listed in Table 1. All compounds were diluted in redistilled pentane. Pino-/isopinocamphone was oxidized from precursor isopinocampheol with CrO_3/H_2SO_4 (Jones reagent). Due to changes in oxidizing procedures, the ratio of pino-/isopinocamphone varied from 3:2 (used in study 1), to 1:2 and 1:19 (used in study 2). The relative heights of the peaks obtained from GC-MS analysis were used to determine the ratio of pinocamphone to isopinocamphone.

Electrophysiology. Electroantennogram (EAG) techniques (Dickens and

TABLE 1. SOURCE AND PURITY OF BARK BEETLE PHEROMONES AND HOST TREE VOLATILES USED IN THE ELECTROANTENNOGRAM STUDIES

Compound	Type of compound[a]	Source of supply	Chemical purity (%)[b]
alpha-Pinene	OM	Aldrich Chemical Co.	97
alpha-Terpineol	OM	Aldrich Chemical Co.	98
Borneol	OM	Aldrich Chemical Co.	97
Bornyl Acetate	OM	Aldrich Chemical Co.	97
Camphor	OM	Aldrich Chemical Co.	95
cis-Verbenol	P	Phero Tech	97
MAD[c]	P	W. Francke, Germany	95
E,Z-chalcogran	P	H. Hogberg, Sweden	95
endo-Brevicomin	P	Chem. Samp. Co.	99
exo-Brevicomin	P	Semiochemicals Internat.	94
Fenchone	OM	Aldrich Chemical Co.	98
Frontalin	P	BASF	99
4-Allylanisol	OM	Aldrich Chemical Co.	99
Ipsenol	P	Borregard	96
Ipsdienol	P	Borregard	96
Myrtenol	P	Aldrich Chemical Co.	88
Pino-/Isopinocamphone[d]	OM	Aldrich Chemical Co.	95
Terpinen-4-ol	OM	Aldrich Chemical Co.	98
trans-Verbenol	P or OM	Aldrich Chemical Co.	91
Verbenone	P or OM	Bedoukian	98

[a] OM = oxygenated monoterpene; *P* = pheromone of a scolytid beetle.
[b] Determined from GLC analysis.
[c] MAD = E-7 methyl-1,6-dioxaspiro [4.5] decane.
[d] Used in three ratios (3:2, 1:2, and 1:19) and oxidized (Jones reagent) from isopinocampheol (Aldrich).

Payne, 1977) were modified from earlier techniques (Schneider, 1957; Payne, 1970, 1975). Glass capillary, Ag-AgCl electrodes filled with insect Ringer's solution (Barbosa, 1974) were used. A scalpel was used to sever the tip of the distal flagellum segment from the insect's antenna. The recording electrode was then inserted into the open end of the segment; the indifferent electrode was inserted into the insect's oral cavity. EAGs were displayed on a Tektronix 5223 digitizing oscilloscope and recorded on a Soltec x–y plotter. Stimuli were delivered at 5 min intervals as 5 μl aliquots on filter paper (20 $\times$ 7 mm) placed into glass cartridges (75 mm long, 5 mm ID), and oriented toward the antennal preparation from ca. 1 cm. Stimulus duration was 1 sec in a 1 liter/min airflow filtered through activated charcoal. The initial depolarization upon stimulation was recorded as response to a given stimulus.

Study 1. A screening study was undertaken, in which all compounds were presented to *D. dendroctoni* at one of two concentrations (10 or 100 μg/μl), except for pino-/isopinocamphone (3:2) which was presented at 1 μg/μl. The pentane solvent (5 μl on filter paper) was used as a control. A standard stimulus was used as a basis against which to normalize parasitoid response to the compounds. This helped control for variation between and within preparations over time (Payne, 1975). The standard consisted of an oxygenated mixture of several volatiles emanating from trees containing heavily parasitized *D. frontalis* brood. The mixture contained *E,Z*-chalcogran (16 μg/μl), fenchone (93 μg/μl), camphor (96 μg/μl), bornyl acetate (122 μg/μl), pino-/isopinocamphone (1:2) (90 μg/μl), terpinen-4-ol (91 μg/μl), 4-allylanisol (98 μg/μl), *trans*-verbenol (91μg/μl), borneol (98 μg/μl), verbenone (99 μg/μl), and myrtenol (88 μg/μl).

Each insect was exposed to all test compounds randomly. Both the control and the standard were presented to the insect after every four stimulations by candidate compounds. Response to each compound was calculated as a percent of the response to the standard closest in sequence. Not all insects survived to be stimulated by all compounds. Therefore, on several occasions more than one insect was needed to complete a replicate. Essentially, equal numbers of males and females were tested for each compound.

Responses to compounds were ranked into four "tiers." Compounds eliciting EAGs greater than or equal to those elicited by the standard were ranked in the first and second tier, respectively. The third tier consisted of compounds which elicited EAGs not significantly different from the lowest ranking tier 2 compound. Tier 4 compounds elicited EAGs significantly lower than the lowest ranking tier 2 compound.

Study 2. Serial dilutions of compounds ranked in tiers 1–3 were tested in concentrations ranging from 10 to 0.0001 μg/μl. The dilution series for each randomly-assigned compound were presented to the insects randomly. Pentane solvent was used as the control. The same standard mixture used in study 1 was used here, except all compounds in the mixture were diluted to ca. 10 μg/μl.

The control and standard stimuli were presented to the insect after each dilution series. Response to each dilution in the series was calculated as a percent of the mean of the two closest responses to the standard. Ten females and nine males were tested for each dilution series.

Statistical Analysis. Study 1 was designed as a randomized complete block, with each insect representing a block. Blocks were nested within sex and analyzed as an incomplete block design using the General Linear Models (GLM) procedure in SAS (SAS, 1985). Mean comparisons, using least square means ($P \leq 0.05$), were determined *a priori* to compare all compounds in tiers 1–3 to the standard. Mean comparisons were not run on tier 4 compounds.

Dose-response to serial dilutions in study 2 were first analyzed by determining the low threshold of response. This was calculated, relative to the standard, as the minimum stimulus concentration at which the percent EAG to the stimulus was significantly greater ($P \leq 0.05$) than the percent EAG to the corresponding pentane control, as determined by *t*-test comparisons. The GLM procedure was used to compare percent response values between compounds for each sex at and above the low threshold (SAS, 1985). Means were separated using the Student–Newman–Kuels' test. The *t*-test was used to compare percent response values between sexes for each compound at each dose.

RESULTS AND DISCUSSION

Study 1. EAGs were not significantly different between male and female *D. dendroctoni* in response to the candidate compounds ($F_{1,493} = 1.05$; $P > 0.05$), relative to the EAGs elicited by the standard for each sex. In addition, male and female EAGs elicited by the standard were also not significantly different ($t_{(106)} = 0.45$; $P > 0.05$). Therefore, the data were pooled. This is surprising, since male parasitoids normally mate on the tree from which they emerge, aggregating to sites where females emerge (Mills, personal communication). After mating, only females are attracted to scolytid infested trees (Camors and Payne, 1973).

The only compound to elicit significantly greater response than the standard was pino-/isopinocamphone (3:2) (tier 1 compound), which elicited EAGs of 126% relative to the standard (Table 2). Tier 2 compounds consisted of frontalin, *E,Z*-chalcogran, *E*-7 methyl-1,6-dioxaspiro[4.5]decane (MAD), with mean EAG responses ranging from 92 to 107%. Compounds ranked in the third tier were *exo*-brevicomin, *alpha*-pinene, *alpha*-terpineol, and terpinen-4-ol, with mean EAGs ranging from 77 to 82%.

Study 2. Electroantennograms between sex for each compound were similar except for two compounds, *alpha*-terpineol and frontalin at 1 $\mu g/\mu l$ (Figure 1). However, females had lower response thresholds than males for several

TABLE 2. MEAN PERCENT EAGs, RELATIVE TO A STANDARD, BY *Dinotiscus dendroctoni*, TO POTENTIAL BARK BEETLE- AND HOST TREE-PRODUCED SEMIOCHEMICALS IN STUDY 1

Compounds	Concentration ($\mu g/\mu l$)	N	$\bar{X}$ + SE[a] (%)
Pino-/Isopinocamphone (3:2)	10^0	13	126.1 ± 9.5 A
Frontalin	10^2	17	106.6 ± 9.7 B
Standard[b]	10^2	81	100.0 ± 0.0 B
E,Z-chalcogran	10^1	20	93.7 ± 5.9 B
E-7 methyl-1,6-diox-aspiro [4.5] decane	10^1	20	91.6 ± 6.3 BC
exo-brevicomin	10^2	19	81.7 ± 5.2 CD
alpha-pinene	10^2	19	81.5 ± 5.8 CD
alpha-terpineol	10^2	19	77.8 ± 7.1 CD
Terpinen-4-ol	10^2	20	76.7 ± 4.6 CD
Ipsenol	10^2	18	71.1 ± 5.0 D
Verbenone	10^2	18	67.3 ± 4.3
Myrtenol	10^2	20	67.0 ± 4.6
endo-Brevicomin	10^2	19	66.4 ± 5.6
Fenchone	10^2	14	65.7 ± 4.2
trans-Verbenol	10^2	20	64.6 ± 5.0
Bornyl Acetate	10^2	19	61.6 ± 5.3
cis-Verbenol	10^2	20	61.5 ± 3.1
4-Allylanisol	10^1	13	60.2 ± 5.0
Ipsdienol	10^2	17	60.0 ± 5.5
Borneol	10^2	19	58.2 ± 4.2
Camphor	10^2	14	57.2 ± 5.0
Pentane	neat	90	34.7 ± 2.3

[a] Means followed by different letters are significantly different from each other, based on *a priori* comparisons (LS means; $p < .05$). Tier 4 compounds were not subject to mean comparisons; therefore, those means are not followed by letters.

[b] Standard is a mixture of *E,Z*-chalcogran, fenchone, camphor, bornyl acetate, pino-/isopinocampone (1:2), terpinen-4-ol, allylanisol, *trans*-verbenol, borneol, verbenone, and myrtenol.

compounds; *exo*-brevicomin, *E,Z*-chalcogran, frontalin, and terpinen-4-ol (Table 3). Lowest response thresholds for females were elicited by terpinen-4-ol and frontalin, followed by *E,Z*-chalcogran. Dickens (1981) suggested that compounds which elicited responses to low concentrations and wider concentration ranges may function in long-range orientation of the insects to the host or host habitat.

The standard elicited significantly higher EAGs from both sexes than any individual compound tested at 1 $\mu g/\mu l$ (Table 4). The ranking of EAGs to compounds at 10 $\mu g/\mu l$ was quite similar to the ranking at 1 $\mu g/\mu l$, except for MAD which elicited relatively low EAGs at 1 $\mu g/\mu l$ and relatively high EAGs at 10 $\mu g/\mu l$, and terpinen-4-ol which elicited similar responses at both concen-

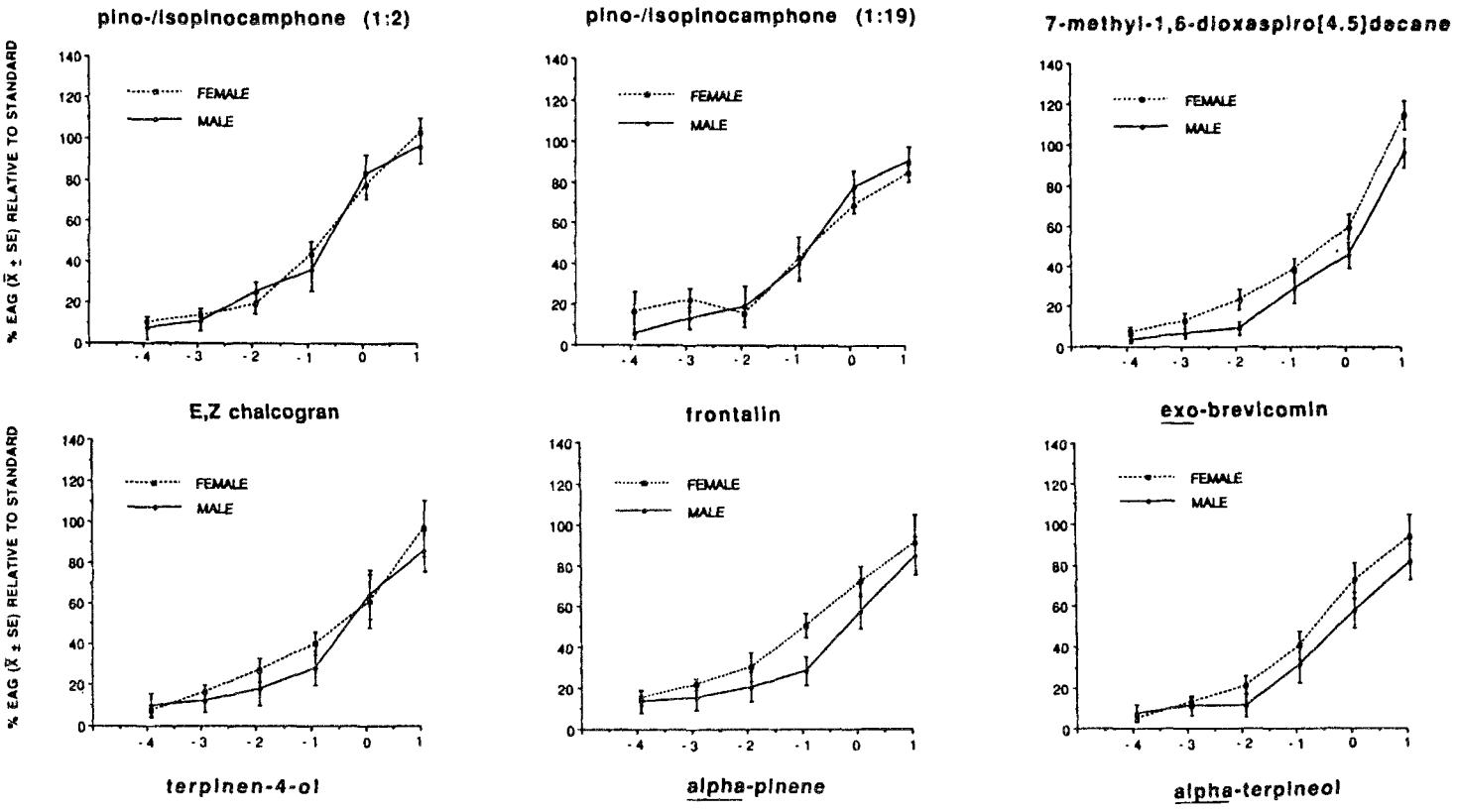


FIG. 1. Mean percent EAGs ± SE from *Dinotiscus dendroctoni* to bark beetle pheromones and tree-produced volatiles, relative to EAG responses by each sex to the standard mixture of oxygenated monoterpenes. Each concentration was presented as 5 μl aliquots.

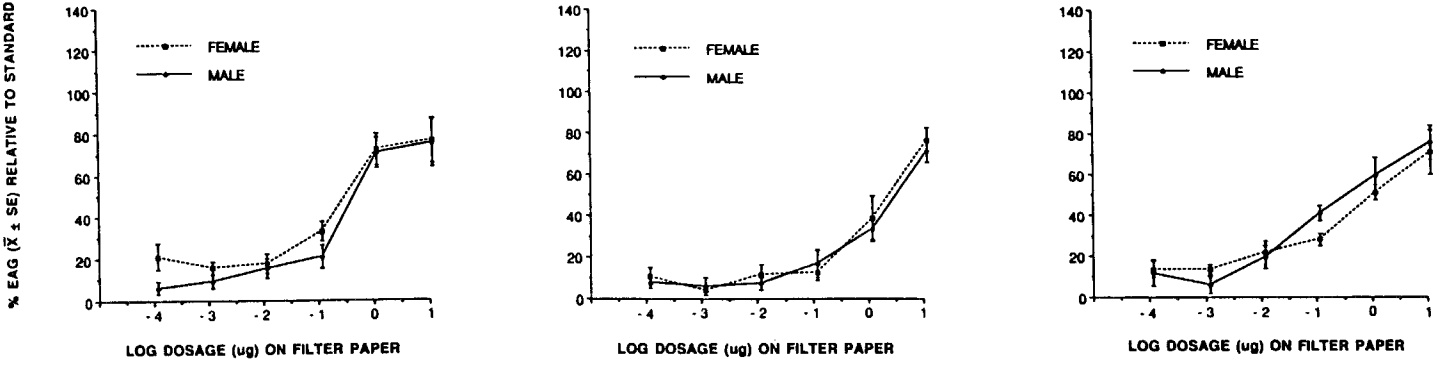


FIG. 1. Continued

TABLE 3. EAG THRESHOLDS OF *Dinotiscus dendroctoni* TO VOLATILES EXTRACTED FROM TREES WITH HEAVY PARASITIZATION OF *Dendroctonus frontalis*[a]

Compound	Concentration of female threshold	Concentration of male threshold
alpha-Pinene	10^{0}	10^{0}
alpha-Terpineol	10^{-2}	10^{-2}
exo-Brevicomin	10^{-2}	10^{-1}
E,Z-chalcogran	10^{-3}	10^{-1}
Frontalin	10^{-4}	10^{-2}
Fenchone	10^{-1}	10^{-1}
MAD[b]	10^{-1}	10^{-1}
Pino-/Isopinocamphone (1:2)	10^{-2}	10^{-2}
Pino-/Isopinocamphone (1:19)	10^{-1}	20^{-1}
Terpinen-4-ol	10^{-4}	10^{-2}

[a] Based on *t*-test comparison ($P \leq 0.05$) of EAG responses between compounds and the pentane control.
[b] MAD = *E*-7 methyl-1,6-dioxaspiro [4.5] decane.

trations (Table 4). Pino-/isopinocamphone (1:2) consistently ranked first or second for both males and females at both concentrations, yet responses to the compound were only significantly different from a few of the other compounds. Pino-/isopinocamphone (1:19) consistently ranked lower than the (1:2) mixture. In study 1, pino-/isopinocamphone (3:2) elicited higher responses than did the standard, even though its concentration was 1/100th the standard. The data suggest that *D. dendroctoni* is more sensitive to pinocamphone than isopinocamphone.

An exponential increase in EAGs, at the highest concentrations tested, was recorded for males and females exposed to MAD and *alpha*-pinene, and for females exposed to *E,Z*-chalcogran (Figure 1). This pattern suggests that *D. dendroctoni* may use the increasing concentration gradients of one or all of these compounds as directional cues for close range orientation of the insect to its host.

The results show that *D. dendroctoni* has receptors for many of the oxygenated monoterpenes collected from host trees containing *D. frontalis* brood. Based on our electrophysiological data, where most of the compounds elicited similar EAGs, it can be suggested that several of the compounds may be used by *D. dendroctoni* as cues in habitat and host community location. In field tests, the standard mixture attracted *D. dendroctoni* and other parasitoid species at a level comparable to crude extracts taken from *D. frontalis*-infested trees exhibiting high levels of parasitism (Birgersson et al., unpublished data). This is consistent with studies of parasitoids of other plant-inhabiting insect host spe-

TABLE 4. MEAN PERCENT EAGs, RELATIVE TO THE STANDARD AT 1 $\mu g/\mu l$, OF *Dinotiscus dendroctoni* TO VOLATILES ASSOCIATED WITH *Dendroctonus frontalis* BROOD

Concentration ($\mu g/\mu l$ of solvent)	Chemical	$\bar{X}$ Female response ± SE	Chemical	$\bar{X}$ Male response ± SE
1	Standard[a]	100.0 ± 0.0 A[b]	Standard	100.0 ± 0.0 A
	P/I (1:2)[c]	75.7 ± 7.7 B	P/I (1:2)	80.7 ± 9.4 B
	Terpinen-4-ol	70.7 ± 7.7 BC	P/I (1:19)	75.7 ± 7.8 BC
	exo-Brevicomin	70.3 ± 8.5 BC	Terpinen-4-ol	69.0 ± 7.4 BC
	Frontalin	70.1 ± 7.4 BC	*E,Z*-chalcogran	62.2 ± 12.1 BCD
	P/I (1:19)	67.0 ± 3.9 BC	*alpha*-Terpineol	57.8 ± 8.8 CD
	E,Z-chalcogran	59.1 ± 13.2 BC	*exo*-Brev.	55.7 ± 8.7 CD
	MAD[d]	57.8 ± 6.2 BC	Frontalin	55.4 ± 8.6 CD
	alpha-Terpineol	49.3 ± 3.6 CD	MAD	43.8 + 6.5 DE
	alpha-Pinene	36.9 ± 10.4 D	*alpha*-Pinene	31.7 ± 5.9 E
10	MAD	112.9 ± 7.2 A	P/I (1:2)	94.6 ± 8.6 A
	P/I (1:2)	100.7 ± 7.0 AB	MAD	94.4 ± 8.6 A
	E,Z-chalcogran	94.8 ± 13.8 AB	P/I (1:19)	88.6 ± 7.2 A
	exo-Brevicomin	91.3 ± 11.1 AB	*E,Z*-chalcogran	83.6 ± 9.9 A
	Frontalin	89.1 ± 13.8 AB	Frontalin	82.7 ± 9.4 A
	P/I (1:19)	82.5 ± 4.1 AB	*exo*-Brevicomin	79.3 ± 8.6 A
	Terpinen-4-ol	74.9 ± 10.9 AB	*alpha*-Terpineol	73.8 ± 8.3 A
	alpha-Pinene	74.5 ± 5.8 AB	Terpinen-4-ol	73.6 ± 11.5 A
	alpha-Terpineol	69.1 ± 10.9 AB	*alpha*-Pinene	69.5 ± 5.6 A

[a] The standard is a mixture of oxygenated monoterpenes.
[b] Means in columns for each concentration, followed by different letters are significantly different ($P \leq 0.05$; SNK test).
[c] P/I = Pino-/Isopinocamphone.
[d] MAD = *E*-7 methyl-1,6-dioxaspiro [4.5] decane.

cies that have been found to use a complex of insect and plant-produced compounds as habitat and host community location cues (Vinson, 1981, 1984; Lewis and Martin, 1990).

Subtractive assays of the standard mixture, used to evaluate parasitoid attraction, revealed that parasitoids were most attracted to traps baited with the complete standard mixture blend and less so to the blends missing one of the compounds (Birgersson et al., unpublished data). No one compound tested was predominantly attractive to parasitoids. Additionally, most parasitoids attracted to these blends were female. This suggests that although males respond similarly to these compounds as females at the sensory level, they may respond differently or not at all at the behavioral level.

One compound that has consistently been extracted from *D. dendroctoni* oviposition sites may serve as a short range oviposition cue for the parasitoid

(Birgersson et al., unpublished data). Unfortunately, it has not yet been identified. Sensory and behavioral experiments with the compound, as well as behavioral studies with the test compounds from this study, need to be carried out to further identify the semiochemicals used by *D. dendroctoni* during the host-searching process.

Acknowledgments—The authors wish to thank M.J. Dalusky, D.M. Grosman, and T.L. Coffey for technical assistance; and N.J. Mills and E.H. Baehrecke for critical review of the manuscript. The research was supported by USDA Competitive Grants Office Agreement No. 90-37250-5267, and NATO Grant 0710/86 to T.L. Payne.

REFERENCES

BARBOSA, P. 1974. Manual of Basic Techniques in Insect Histology. Autumn Publishers, Amherst, Massachusetts. 240 pp.

BERISFORD, C.W. 1980. Natural enemies and associated organisms, pp. 31–54, *in* R.C. Thatcher, J.L. Searcy, J.E. Coster, and G.D. Hertel (eds.). The Southern Pine Beetle. USDA For. Serv. Tech. Bull. 1631.

BERISFORD, C.W., and FRANKLIN, R.T. 1971. Attack patterns of *Ips grandicollis* (Coleoptera: Scolytidae) on four species of southern pines. *Ann. Entomol. Soc. Am.* 64:894–897.

BIRGERSSON, G., and LEUFVÉN, A. 1988. The influence of host tree response to *Ips typographus* and fungal attack on production of semiochemicals. *Insect Biochem.* 18:761–770.

BORDEN, J.H. 1982. Aggregation pheromones, pp. 74–139, *in* J.B. Mitton and K.B. Sturgeon (eds.). Bark Beetles in North American Conifers. University of Texas Press, Austin.

BROWNE, L.E. 1972. An emergence cage and refrigerated collector for wood-boring insects and their associates. *J. Econ. Entomol.* 65:1499–1501.

CAMORS, F.B., JR., and PAYNE, T.L. 1973. Sequence of arrival of entomophagous insects to trees infested with the southern pine beetle. *Environ. Entomol.* 2:267–270.

DAHLSTEN, D. 1982. Relationships between bark beetles and their natural enemies, pp. 140–182, *in* J.B. Mitton and K.B. Sturgeon (eds.). Bark Beetles in North American Conifers. University of Texas, Austin.

DICKENS, J.C. 1981. Behavioral and electrophysiological responses of the bark beetle, *Ips typographus*, to potential pheromone components. *Physiol. Entomol.* 6:251–261.

DICKENS, J.C., and PAYNE, T.L. 1977. Bark beetle olfaction: Pheromone receptor system in *Dendroctonus frontalis* Zimm. (Coleoptera: Scolytidae). *J. Insect Physiol.* 23:481–489.

DIXON, W.N., and PAYNE, T.L. 1980. Attraction of entomophagous and associate insects of the southern pine beetle to beetle- and host tree-produced volatiles. *J. Georgia Entomol. Soc.* 15:378–389.

GOYER, R.A., and FINGER, C.K. 1980. Relative abundance and seasonal distribution of the major hymenopterous parasites of the southern pine beetle, *Dendroctonus frontalis* Zimmerman, on loblolly pine. *Environ. Entomol.* 9:97–100.

HETRICK, L.A. 1940. Some factors in natural control of the southern pine beetle, *Dendroctonus frontalis* Zimm. *J. Econ. Entomol.* 33:554–556.

KUDON, L.H., and BERISFORD, C.W. 1980. Influence of brood hosts on host preferences of bark beetle parasites. *Nature* 283:288–290.

KUDON, L.H., and BERISFORD, C.W. 1981. An olfactometer for bark beetle parasites. *J. Chem. Ecol.* 7:359–366.

LEUFVÉN, A., and BIRGERSSON, G. 1987. Quantitative variation of different monoterpenes around

galleries of *Ips typographus* (Coleoptera: Scolytidae) attacking Norway spruce. *Can. J. Bot.* 65:1038–1044.

LEWIS, W.J., and W.R. MARTIN, JR. 1990. Semiochemicals for use with parasitoids: Status and future. *J. Chem. Ecol.* 16:3067–3089.

MILLS, N.J., KRUGER, K., and SCHLUP, J. 1991. Short-range host location mechanisms of bark beetle parasitoids. *J. Appl. Entomol.* 111:33–43.

MIZELL, R.F., III, and NEBEKER, T.E. 1978. Estimating the developmental time of the southern pine beetle *Dendroctonus frontalis* as a function of field temperatures. *Environ. Entomol.* 7:592–595.

PAYNE, T.L. 1970. Electrophysiological investigations on response to pheromones in bark beetles. *Contrib. Boyce Thomp. Inst.* 24:275–282.

PAYNE, T.L. 1975. Bark beetle olfaction responsiveness of *Dendroctonus frontalis* Zimmerman and *D. brevicomis* LeConte (Coleopt.: Scolyt.) to aggregation pheromones and host tree terpene hydrocarbons. *J. Chem. Ecol.* 1:233–242.

PAYNE, T.L. 1989. Olfactory basis for insect enemies, pp. 55–69, *in* D.L. Kulhavey and M.C. Miller (eds.). Potential for Biological Control of *Dendroctonus* and *Ips* Bark Beetles. Stephen F. Austin University, Texas.

SAS Institute. 1985. SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina.

SCHNEIDER, D. 1957. Elektrophsiologische Untersuchungen von Chemo- und Mechanorezeptoren der Antenne des Seidenspinners *Bombyx mori* L. *Z. Vergl. Physiol.* 40:8–41.

THATCHER, R.C., and PICKARD, L.S. 1967. Seasonal development of the southern pine beetle in East Texas. *J. Econ. Entomol.* 60:656–658.

VINSON, S.B. 1981. Habitat location, pp. 51–78, *in* D.A. Nordland, R.L. Jones, and W.J. Lewis (eds.). Semiochemicals: Their role in Pest Control. John Wiley & Sons, New York.

VINSON, S.B. 1984. Parasitoid-host relationship, pp. 205–236. *in* W.J. Bell and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

TAXON-SPECIFIC DIFFERENCES IN RESPONSIVENESS TO CAPSAICIN AND SEVERAL ANALOGUES: CORRELATES BETWEEN CHEMICAL STRUCTURE AND BEHAVIORAL AVERSIVENESS

J. RUSSELL MASON, [1,*] N. JAY BEAN,[2] PANKAJ S. SHAH,[3] and LARRY CLARK[1]

[1]*U.S. Department of Agriculture*
Animal and Plant Health Inspection Service, Science and Technology
Denver Wildlife Research Center
c/o Monell Chemical Senses Center
3500 Market Street
Philadelphia, Pennsylvania 19104-3308

[2]*Psychology Department*
Vassar College
Poughkeepsie, New York 12601

[3]*Monell Chemical Senses Center*
3500 Market Street
Philadelphia, Pennsylvania 19104-3308

(Received July 8, 1991; accepted August 26, 1991)

Abstract—The present set of experiments was designed to explore avian insensitivity to capsaicin. Based upon a molecular model of avian chemosensory repellency, we hypothesized that structural modifications of the basic capsaicin molecule, which is itself not aversive to birds, might produce aversive analogues. To this end, European starlings (*Sturnus vulgaris*) and Norway rats (*Rattus norvegicus*) were given varied concentrations of synthetic capsaicin and four analogues (methyl capsaicin, veratryl amine, veratryl acetamide, vanillyl acetamide) in feeding and drinking tests. The results agreed with a model that we are developing to describe the chemical nature of avian repellents. Synthetic capsaicin and vanillyl acetamide were not repellent to birds, owing to the presence of an acidic phenolic OH group. Conversely, veratryl acetamide was aversive, due to the basic nature of this compound. For rats, repellent effectiveness among compounds was reversed: synthetic capsaicin was the best repellent while veratryl acetamide was the worst. We speculate that this taxonomic reversal may reflect basic differences in trige-

*To whom correspondence should be addressed.

minal chemoreception. In any case, it is clear that chemical correlates of mammalian repellents are opposite to those that predict avian repellency.

Key Words—Capsaicin, chemosensory, irritation, rat, *Rattus norvegicus*, starling, *Sturnus vulgaris*, trigeminal.

INTRODUCTION

Avoidance of a chemical can be learned by associating sensory features of the chemical with post-ingestional effects, e.g., sickness (Riley and Clarke, 1977). Alternatively, avoidance of a chemical can occur in the absence of learning, provided that the chemical stimulates trigeminal free nerve endings (Parker, 1912) in the nose, eyes, or mouth. While the morphological organization of the trigeminal system in birds is not very different from that found in mammals (Dubbeldam and Veenman, 1978), there appear to be dramatic behavioral discrepancies (Kare and Mason, 1986). Although the avian trigeminal system responds to odorants (e.g., Walker et al., 1979, Mason and Silver, 1983) and mediates avoidance of avian irritants, e.g., methyl anthranilate (Mason et al., 1989), it does not respond to strong mammalian irritants. Thus, pigeons (*Columba livia*) and grey partridges (*Perdix perdix*) are indifferent to ammonia (Soudek, 1929), and parrots (*Amazona* spp., Mason and Reidinger, 1983), pigeons (Szolcsanyi et al., 1986), red-winged blackbirds (*Agelaius phoeniceus*; Mason and Maruniak, 1983), European starlings (Mason and Clark, 1990), cedar waxwings (*Bombycilla cedrorum*), and house finches (*Carpodacus mexicanus*; D. Norman, unpublished observation) , are insensitive to capsaicin, the pungent principle in *Capsicum* peppers.

The finding that birds are unresponsive to capsicum is especially interesting, because mammals uniformly avoid this substance. The present set of experiments was designed to explore this instance of avian insensitivity. On the basis of a model relating avian repellency with chemical structure (Mason et al., 1991; Clark and Shah, 1991; Clark et al., 1991; Shah et al., 1991), we hypothesized that structural modifications of the basic capsaicin molecule might produce analogues that were aversive to birds. To this end, starlings and Norway rats (*Rattus norvegicus*, laboratory strain) were given varied concentrations of capsaicin and four analogues (methyl capsaicin, veratryl amine, veratryl acetamide, vanillyl acetamide) in feeding and drinking tests.

Our choice of analogue structures was guided by the following considerations. First, methyl capsaicin was selected because its additional OCH_3 group (Figure 1) made it more basic than capsaicin, and molecular basicity is positively associated with avian repellency (Clark and Shah, 1991). Second, vanillyl acetamide was chosen because the removal of the alkyl group (relative to capsaicin) should enhance repellency by contributing to the electron richness of

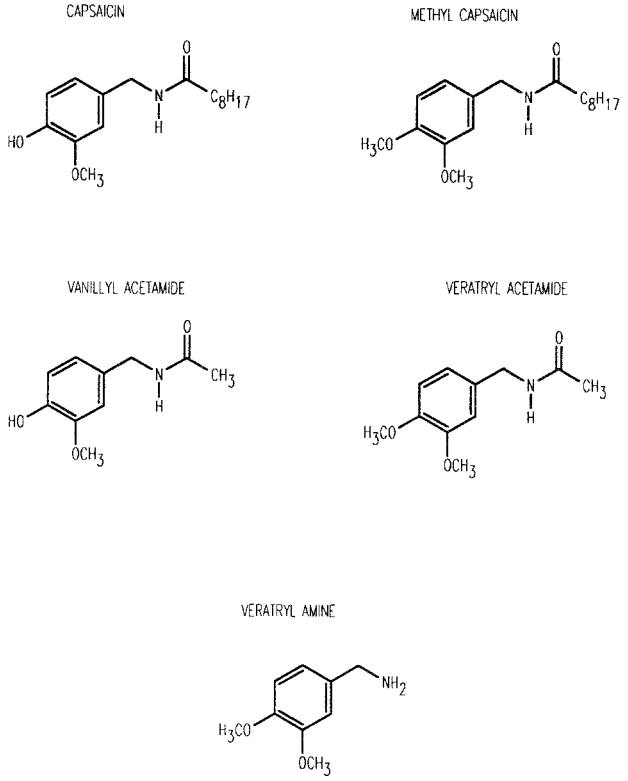


FIG. 1. Molecular structure of synthetic capsaicin, methyl capsaicin, veratryl amine, vanillyl acetamide, and veratryl acetamide.

the phenyl ring (Shah et al., 1991). Third, veratryl amine was evaluated because it is more basic than capsaicin and because there is no possibility of side chain interference in effectiveness, e.g., no possibility of electronic interference (Clark et al., 1991). Finally, we selected veratryl acetamide both because it is basic relative to capsaicin or vanillyl acetamide, and because it is more lipophilic than veratryl amine. Because lipophilicity is positively correlated with repellency (Mason et al., 1989), we predicted that veratryl acetamide should be the most effective bird repellent of the derivatives tested.

MATERIALS AND METHODS

Subjects. Adult European starlings (*Sturnus vulgaris*) were decoy-trapped in Pilesgrove township, Salem County, New Jersey, and transported to the laboratory. Birds were held for 3 weeks prior to testing. We chose to use this

species because (1) they show good chemical sensing ability (e.g., Clark and Mason, 1987), and (2) comparable data exist concerning the responses of starlings to other repellents, e.g., anthranilate derivatives (Mason et al., 1989) and mammalian irritants, e.g., piperine, zingerone, allyl isothiocyanate, and gingerol (Mason and Otis, 1990).

Upon arrival, the birds were individually caged (61 × 36 × 41 cm) under a 12:12 light-dark cycle with light onset at 0700 hr. Food and grit [Purina Flight Bird Conditioner (PFBC) and medicated oyster shells] were available *ad libitum*; apples were provided twice a week. Before experiments began, birds were permitted free access to tap water.

Sixty-day-old Sprague–Dawley Norway rats were obtained from the Vassar College colony. The rats were individually caged (17.7 × 24.2 × 17.7 cm) under a 12:12 light/dark cycle (light onset 0700 hours). During a 2-week adaptation period prior to testing, all rats were permitted free access to crumbled Purina rat chow and tap water.

Chemicals. Veratryl amine was purchased from Fluka Chemical Co. (Buchs, Switzerland), while synthetic capsaicin was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Vanillyl acetamide was synthesized according to the method described by Helson (1919). Methyl capsaicin (corresponding to synthetic capsaicin, i.e., veratryl nonamide) and veratryl acetamide were synthesized by a simple acetylation procedure, as follows. Veratryl amine (10 g) was dissolved in triethylamine (20 ml) and the solution was cooled to 0°C. The nonanoyl chloride or acetic anhydride (two equivalents) was added dropwise and the solution was allowed to stand at room temperature for 1 hr. The reaction mixture was poured into ice water (50 ml) and extracted with dichloromethane (2 × 25 ml). The organic layer was successively washed with 5% HCl (25 ml), 11% sodium hydrogen carbonate (25 ml), and water (25 ml), dried and evaporated under reduced pressure. The residue was purified by column chromatography (on silica gel) to obtain pure samples of veratryl nonamide and veratryl acetamide.

For the bird experiments, each chemical constituted an independent test for both the feeding and drinking experiments. For each chemical in drinking trials, stimuli were dissolved in water to prepare the following concentrations (w/v): 0.001, 0.005, 0.01, 0.05, 0.1, and 1.0%. For each chemical in feeding trials, stimuli were first dissolved in ether, and the solutions applied to bird feed. The ether was then evaporated (Jakubas et al., 1991). Chemical concentrations used in the bird feeding trials were: 0.001, 0.01, 0.1, and 1.0% (w/w).

Procedures. In *drinking trials*, one-bottle tests similar to those described by Clark and Shah (1991) were used. Briefly, water consumption for starlings was monitored for a total of 6 hr for each of 3 days. At the end of this period, individuals whose variance was $> \pm 1$ standard deviation of the population vari-

ance were excluded from the trials. Those birds with stable daily water consumption were ranked according to mean water consumption and assigned to one of the six treatment concentration groups (hereafter called concentration group). That bird with the highest water consumption was assigned to the 0.5% concentration group, that bird with the second highest consumption, to the 0.1% concentration group, and so forth, until all birds were assigned to a group. This assured that all concentration groups were balanced with respect to drinking when trials began. A total of 36 birds was used for each experiment, with six birds per concentration group. During any given experiment, only a single chemical was tested.

After assignment to a concentration group, a 1-day pre-treatment drinking trial began. At 0930, tap water consumption for birds within each concentration group was measured every 2 hr for a total of 6 hr. These measures served as the control level of water consumption. After the pre-treatment trial, drinking tubes were switched (to maintain consistent protocol) and the birds were provided free access to fresh tap water. The following day, at 0930, tap water was replaced with a preassigned concentration of chemical in water, and consumption was recorded every 2 hr for the next 6 hr. After the test, birds were again provided free access to tap water. Consumption of tap water was monitored overnight. Overnight consumption was monitored to evaluate whether birds made up for any water deficits resulting from experiments. During the post-treatment trial, tap water consumption was monitored every 2 hr for a total of 6 hr. Mean posttreatment water consumption within concentration groups was compared with mean pre-treatment water consumption to determine whether consumption had returned to pre-treatment levels; i.e., were there carryover effects due to consumption of treated water? If pre-treatment consumption was equal to posttreatment consumption, then the next compound was tested following the above procedures. During subsequent experiments with other chemicals, each group was assigned a different concentration, as determined by a counterbalanced predetermined schedule. This was done to minimize the possibility that strength of stimulus, rather than quality *per se* affected drinking behavior. Previous experience suggested that these precautions were adequate, though we did not specifically test for this effect.

Protocol specified that an individual's intertrial water consumption be maintained within ± 1 standard error of its pre-treatment value. If this were the case, the bird was used in the next experiment; otherwise, it was replaced with a new bird whose baseline water consumption was matched for the group mean. Individual birds showed remarkable consistency for water consumption. Only one bird was replaced throughout the drinking trials.

Birds were checked for health condition after each daily trial, e.g., unusual inactivity, vent fouling, and piloerection. There were no mortalities, and birds were released to the wild at the end of the experiment. Naive birds were not

used for each test because it was impractical to capture the 180 starlings required for all trials.

In *feeding trials*, 24 starlings were used with each chemical. In every test, the birds were given a 4-day pre-treatment period, during which each was presented with a cup containing 50 g of feed during the first 2 hr following light onset. Consumption was then assessed and birds were left with undisturbed access to the maintenance diet until lights were out. Feed was removed from the cages overnight so that the birds were moderately food deprived. On the fourth day, birds were assigned to four concentration groups ($n = 6$/group) on the basis of consumption. That bird with the greatest consumption was assigned to the first group, that with the second highest was assigned to the second group, and so forth.

A 4-day treatment period immediately followed pre-treatment. During the first 2 hr following light onset, each bird was given 1 cup containing 50 g of feed. The feed was adulterated with one of the stimulus chemicals, and different groups were presented with different concentrations. As in pre-treatment, consumption was measured after 2 hr, and birds were food-deprived overnight.

Rat feeding trials were similar to starling feeding trials, with two differences. First, rats were given only one concentration of each chemical (1.0%, w/w). We had planned to use the same range of concentrations presented to birds, but only capsaicin was aversive in pilot testing at a concentration of 0.1%. Second, a control group that received untreated chow during the treatment period was included. Drinking trials were not conducted because 1.0% concentrations of the chemicals were near the limit of solubility in the drinking trials with birds.

Analyses. For starling drinking trials, data were transformed using a difference score to control for individuals' pre-treatment water consumption, i.e., treatment–pre-treatment, and posttreatment–pre-treatment. Difference scores were evaluated in a two-factor analysis of variance (ANOVA), with repeated measures between difference scores (two levels). The independent factor was treatment concentration group. Missing data was only a problem for the drinking experiments. At times, individuals would rest atop the drinking tubes and spill water. If this occurred during any of the test periods (pre-treatment, treatment, or posttreatment), that individual's consumption was not included in the analysis.

For starling feeding trials, mean consumption was evaluated using three-factor ANOVAs with repeated measures between periods (two levels: pre-treatment, treatment) and among days (four levels). The independent factor was concentration group (four levels).

For rat feeding trials, mean consumption during the treatment period was assessed in a two-factor ANOVA with repeated measures among days. The independent factor was chemicals (six levels, the five chemicals, and a control).

In all cases, Tukey *b post-hoc* tests were used to isolate significant differences among means ($P < 0.05$). Unless otherwise indicated, all data were tested and found to be homogeneous using Bartlett's-Box method.

RESULTS

Avian Drinking Trials

Synthetic Capsaicin. There were no significant concentration, treatment period (block), or interaction effects (Figure 2A).

Methyl Capsaicin. The interaction between concentration groups and period was significant ($F = 5.3$; 5,29 df; $P < 0.001$). *Post-hoc* tests showed that, relative to posttreatment, there was a significant, albeit slight reduction in treatment difference scores for the highest methyl capsaicin concentration group (Figure 2B).

Vanillyl Acetamide. The interaction between concentration groups and period was significant ($F = 6.3$; 5,29 df; $P < 0.001$). *Post-hoc* tests showed that the mean treatment difference score for the highest concentration group was significantly less than the mean posttreatment score for that concentration group (Figure 2C).

Veratryl Amine. The interaction between concentration groups and period was significant ($F = 5.5$; 5,29 df; $P < 0.001$). *Post-hoc* tests showed that the treatment difference scores for the two highest concentrations of veratryl amine were smaller than the posttreatment difference scores for these concentrations. The highest concentration produced the greater effect (Figure 2D).

Veratryl Acetamide. The interaction between concentration groups and period was significant ($F = 14.4$; 5,29 df; $P < 0.001$). *Post-hoc* tests showed that while posttreatment difference scores remained constant or became larger, treatment scores decreased with increasing concentration. The two highest concentrations produced a drop from pre-treatment consumption that was significantly greater than the drop produced by any other concentration (Figure 2E).

Avian Feeding Trials

Synthetic Capsaicin. There were no significant differences for concentration, treatment period, day, or the interaction effects (Figure 3A).

Methyl Capsaicin. The day by period by concentration interaction was significant ($F = 3.0$, 9,60 df, $P < 0.005$). *Post-hoc* tests showed that the profile for consumption for the first day of pre-treatment differed from all other profiles. Indeed, all interaction terms and main effects involving days pointed to decreased consumption on the first day. However, more importantly to the hypothesis of interest, there was no period effect ($P = 0.789$), even though the

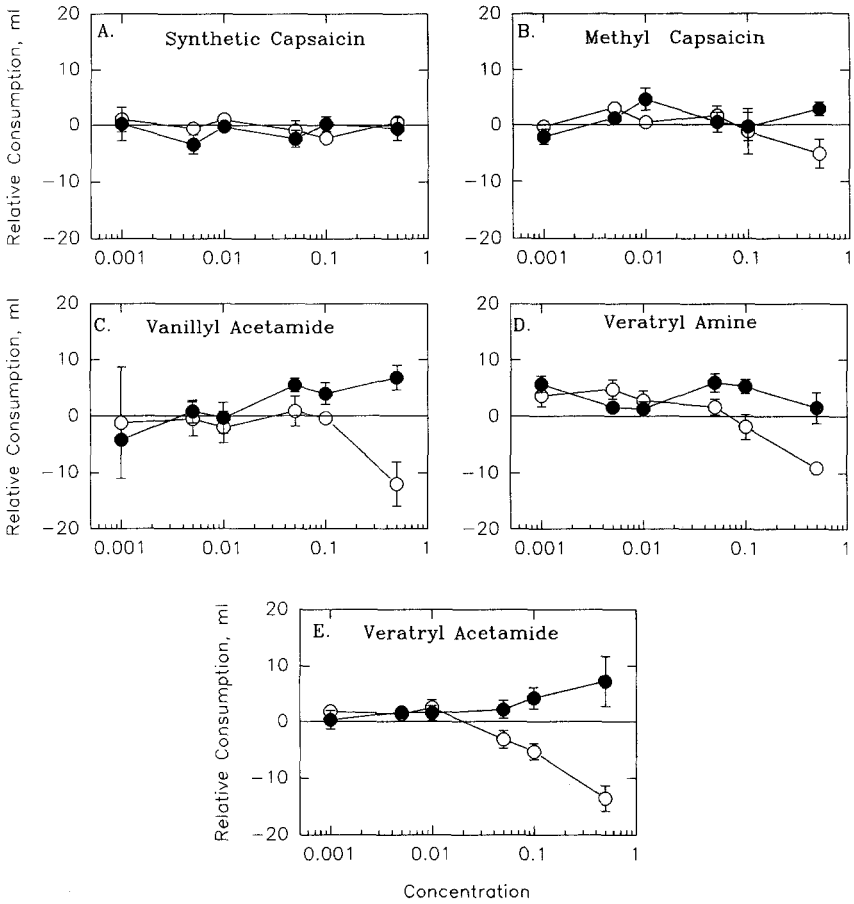


FIG. 2. Mean consumption of synthetic capsaicin and the four capsaicin analogues in avian drinking trials. Open circles represent treatment–pre-treatment values, while filled circles represent mean posttreatment–pre-treatment values. Capped vertical bars represent standard errors of the means.

period by concentration profiles differed ($F = 5.4$, 3,20 df, $P < 0.007$; Figure 3B).

Vanillyl Acetamide. Again, there were no significant effects for treatment ($P = 0.51$), concentration ($P = 0.23$), or their interaction ($P = 0.69$; Figure 3C).

Veratryl Amine. Overall, the consumption of food was less during the treatment period relative to the pre-treatment period ($F = 635.4$; 1,20 df; $P < 0.001$). Inspection of the concentration by period interaction (Figure 3D) showed

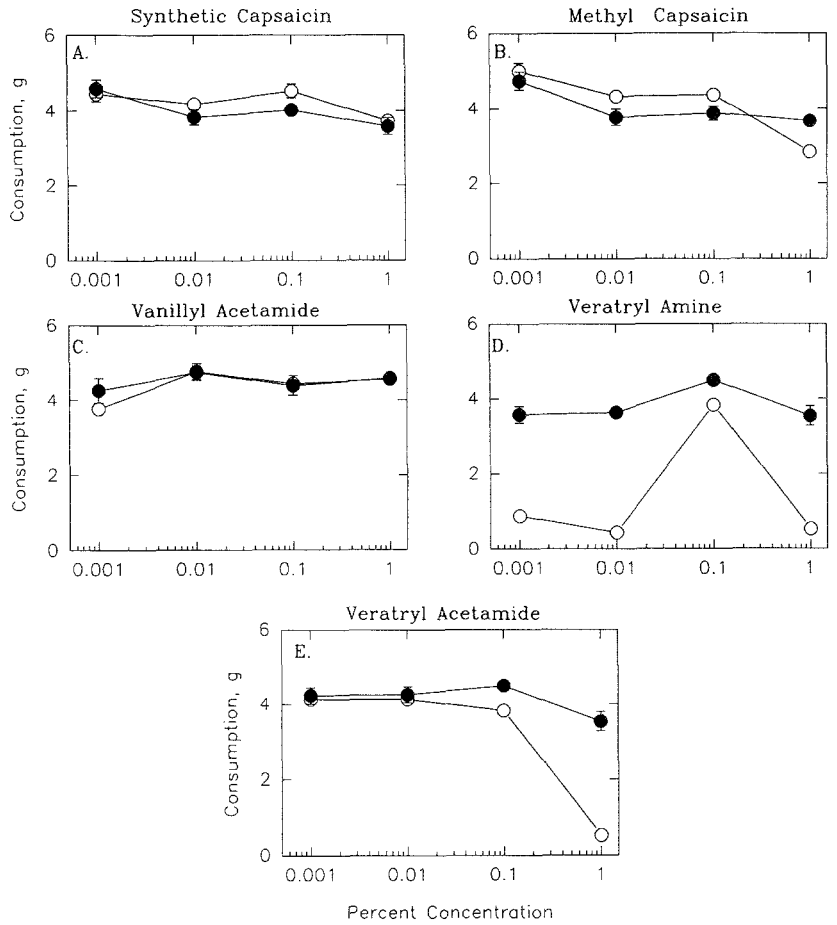


FIG. 3. Mean consumption of synthetic capsaicin and the four capsaicin analogues in avian feeding trials. Open circles represent treatment consumption while filled circles represent pre-treatment consumption. Capped vertical bars represent standard errors of the means.

that this relationship held down to the limits of concentrations tested, i.e., 0.001% ($F = 44.62$; 1,20 *df*, $P < 0.001$). *Post-hoc* tests showed that consumptions for birds assigned to the 0.1% concentration group did not differ between treatment periods.

Veratryl Acetamide. This compound did not appear to be as effective as veratryl amine, but was more effective than capsaicin, methyl capsaicin, and vanillyl acetamide. Overall, the treatment period consumption was less than the pre-treatment control ($F = 67.15$; 1,20 *df*; $P < 0.001$). The interaction of

period by concentration profiles indicated that the reduction in consumption of treated food was accentuated at higher concentrations (Figure 3E; $F = 33.63$; 3,220 df; $P < 0.001$). *Post-hoc* tests indicated that only the 1% concentration differed from pre-treatment levels and the lower levels of treated food.

Rodent Feeding Trials

Synthetic Capsaicin, Methyl Capsaicin, Veratryl Amine, Veratryl Acetamide, and Vanillyl Acetamide. There were significant differences among compounds ($F = 2.8$; 5,18 df; $P < 0.05$). *Post-hoc* examination of this effect indicated that synthetic capsaicin significantly reduced consumption relative to consumption by the group that received untreated feed. The least effective repellent was vanillyl acetamide; consumption by rats given this substance was significantly higher than the consumption exhibited by control group rats (Figure 4).

DISCUSSION

Although the peripheral trigeminal system in birds and mammals is morphologically similar, there are broad functional differences between the taxa. Birds are uniformly unresponsive to most (if not all) mammalian sensory irri-

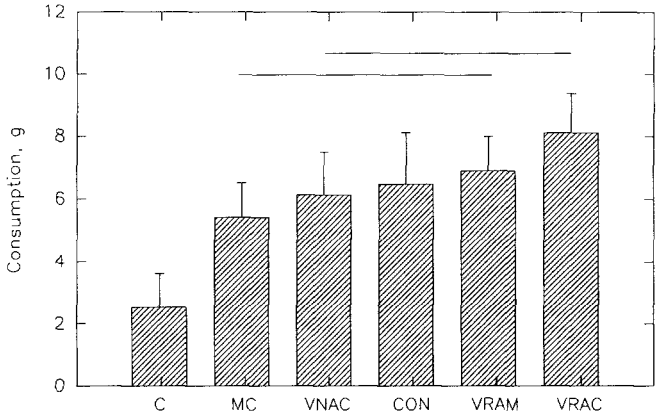


FIG. 4. Mean consumption in rat feeding trials with synthetic capsaicin and the four capsaicin analogues. All chemicals were presented at a concentration of 1.0% (w/w). Capped vertical bars represent standard errors of the means. The horizontal lines depict homogeneous group means as determined by a *post-hoc* Tukey's *b* test. Abbreviations: C = synthetic capsaicin, MC = methyl capsaicin, VRAM = veratryl amine, VRAC = veratryl acetamide, VNAC = vanillyl acetamide, CON = control.

tants at biologically relevant concentrations. The present set of experiments was designed to explore avian insensitivity.

Overall, the present results are consistent with our model describing the chemical nature of avian repellents (Clark et al., 1991; Clark and Shah, 1991; Mason et al., 1991; Shah et al., 1991). According to that model, the electron richness of the phenyl ring and basicity are positively related to repellency, whereas acidic functionalities are negatively associated with effectiveness. Hence, vanillyl amides, i.e., synthetic capsaicin and vanillyl acetamide, should be weakly repellent (if at all), owing to the presence of the acidic phenolic OH group. Conversely, the veratryl amides should be aversive.

Veratryl amine and veratryl acetamide were aversive to birds in both drinking and feeding trials. The former substance was the most basic of the compounds tested. These data agree with our earlier work on vanillyl and cinnamyl alcohol derivatives. In those experiments (Shah et al., 1991; Jakubas et al., 1991, respectively), veratryl alcohol was more active than vanillyl alcohol and dimethoxy cinnamyl alcohol and its benzoate were more active than the corresponding coniferyl derivatives.

Methyl capsaicin was not significantly repellent to birds in the present experiments, although the data hint that it may be aversive, relative to capsaicin, but only at very high concentrations. The higher activity of veratryl acetamide compared to veratryl nonamide (methyl capsaicin) may be due to stereoelectronic factors. For example, the nine-carbon chain on the amino group may sterically hinder the molecule's activity, e.g., structural and conformational effects. Alternatively, this long chain could effectively block electronic effects of the electron-rich phenyl ring. However, steric effects alone are not sufficient to explain the inhibition of repellency. Indeed, the feeding experiments indicate that vanillyl acetamide was ineffective as a repellent. The fact that vanillyl acetamide was aversive at the highest concentration in drinking trials may reflect the tendency for drinking trials to be more sensitive (Clark et al., 1991), possibly because the active ingredients in solution have greater access to receptors than those attached to particles of food.

The interaction of steric and basic effects may be important for repellency. It is worthwhile to note that, structurally, both veratryl amine and veratryl acetamide have short (two-carbon) or no side chains. At present, the nature of the assay (feeding vs. drinking) precludes solution of which of these points is more important. Molecular modeling may clarify the importance of steric and electronic effects of the side chain on the basic molecule, as well as the importance of hydrophobic and hydrophilic interactions.

In mammals, repellent effectiveness among compounds was essentially reversed. Capsaicin was the best repellent while veratryl acetamide was the worst. These results are consistent with the available evidence. We speculate that this taxonomic reversal may reflect several factors, e.g., differences in tri-

geminal chemoreception. In any case, it is clear that chemical correlates of mammalian repellents are opposite to those that predict avian repellency. Thus, acidity, a long side chain (lipophilicity), and an electron-poor phenyl ring may correlate positively with mammalian irritancy. According to the results obtained in this study and also the available data on the mammalian repellency of capsaicin derivatives (e.g., Green et al., 1990), the former two factors can be substantiated. More experiments are needed to test the third attribute.

LITERATURE

CLARK, L., and MASON, J.R. 1987. Olfactory discrimination of plant volatiles by the European starling. *Anim. Behav.* 35:227–235.

CLARK, L., and SHAH, P.S. 1991. Resonance and basicity as predictors of avian repellency and irritation. *J. Wildl. Manage.* 55:539–546.

CLARK, L., SHAH, P.S., and MASON, J.R. 1991. Chemical repellency in birds. Relationship between structure of anthranilate and benzoic acid derivatives and avoidance response. *J. Expl. Zool.* In press.

DUBBELDAM, J.L., and VEENMAN, C.L. 1978. Studies on the somatotopy of the trigeminal system in the mallard, *Anas platyrhyncous* L.: The Ganglion Trigeminale. *Netherlands J. Zool.* 28:150–160.

GREEN, B.G., MASON, J.R., and KARE, M.R. 1990. Chemical Senses, Vol. II: Irritation. Marcel-Dekker, New York. 361 pp.

HELSON, E.K. 1919. Vanillyl acyl-amides. *J. Amr. Chem. Soc.* 41:2121–2123.

JAKUBAS, W.J., MASON, J.R., CLARK, L., SHAH, P.S., and NORMAN, D. 1991. Avian feeding deterrence as mediated by coniferyl benzoate: A structure-activity approach. *Ecol. Appl.* In press.

KARE, M.R., and MASON, J.R. 1986. The chemical senses in birds, pp. 59–74, *in* P.D. Sturkie (ed.). Avian Physiology. Springer-Verlag, New York. 516 pp.

MASON, J.R., and CLARK, L. 1990. Evaluation of Blockout (Northwest Chemicals, Inc.) and Its Chemical Components as Avian Contact Repellents. Denver Wildlife Research Center Bird Section Research Report 457. 10 pp.

MASON, J.R., and MARUNIAK, J.A. 1983. Behavioral and physiological effects of capsaicin in red-winged blackbirds. *Pharmacol. Biochem. Behav.* 19:857–862.

MASON, J.R., and OTIS, D.L. 1990. Chemical nociception in birds, pp. 309–322, *in* B. Green, J.R. Mason, and M.R. Kare (eds.). Chemical Senses, Vol. II: Irritation. Marcel Dekker, New York. 361 pp.

MASON, J.R., and REIDINGER, R.F. 1983. Exploitable characteristics of neophobia and food aversions for improvements in rodent and bird control, pp. 20–42, *in* D.E. Kaukienen (ed.). Vertebrate Pest Control and Management Materials: Fourth Symposium. American Society for Testing and Materials , Philadelphia, Pennsylvania. 315 pp.

MASON, J.R., and SILVER, W.L. 1983. Trigeminally mediated odor aversions in starlings. *Brain Res.* 269:196–199.

MASON, J.R., ADAMS, M.A., and CLARK, L. 1989. Anthranilate repellent to starlings: Chemical correlates and sensory perception. *J. Wildl. Manage.* 53:55–64.

MASON, J.R., CLARK, L., and SHAH, P.S. 1991. Ortho-aminoacetophenone repellency to birds: Similarities to methyl anthranilate. *J. Wildl. Manage.* 55:334–340.

PARKER, G.H. 1912. The reactions of smell, taste, and the common chemical sense in vertebrates. *J. Acad. Natl. Sci. Phila.* 15:221–234.

RILEY, A.L., and CLARKE, C.M. 1977. Conditioned taste aversions: A bibliography, pp. 593–632, *in* L.M. Barker, M.R. Best, and M. Domjan (eds.). Learning Mechanisms in Food Selection. Baylor University Press, Waco, Texas 632 pp.

SHAH, P.S., CLARK, L., and MASON, J.R. 1991. Prediction of avian repellency from chemical structure: The aversiveness of vanillin, vanillyl alcohol and veratryl alcohol. *Pest. Biochem. Physiol.* 40:1–7.

SOUDEK, J. 1929. The sense of smell in birds. *Proc. Intl. Congr. Zool.* 1929:755.

SZOLCSANYI, J., SANN, H., and PIERAU, 1986. Nociception in pigeons is not impaired by capsaicin. *Pain* 27:247–260.

WALKER, J.C., TUCKER, D., and SMITH, J.C. 1979. Odor sensitivity mediated by the trigeminal nerve in the pigeon. *Chem. Senses Flav.* 4:107–116.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

POSTEMBRYONIC DEVELOPMENT AND REPRODUCTION IN *Corcyra cephalonica* (STAINTON) (LEPIDOPTERA: PYRALIDAE) ON EXPOSURE TO EUCALYPTUS AND NEEM OIL VOLATILES

P.H. PATHAK and S.S. KRISHNA*

Entomology Laboratory
Department of Zoology
University of Gorakhpur
Gorakhpur-273009 (U.P.), India

(Received June 27, 1991; accepted September 4, 1991)

Abstract—Postembryonic development and adult emergence of *Corcyra cephalonica* (Stainton) were adversely affected in varying degrees when individuals were reared for the first 2 weeks of larval life or for a similar duration from the sixteenth day of their lives in an environment of eucalyptus oil volatiles. This was, however, not so in the progeny of parents exposed to these volatiles for only 5 min. Exposure of this pest to neem oil volatiles during larval stages for similar periods failed to produce such adverse effects. A marked decline in the reproductive potential, in terms of egg output and egg hatchability, of the moth was observed when the larvae were reared for the first 15 days in the presence of eucalyptus oil volatiles or when the parents were exposed for 5 min to such an environment during adult life. Thus, a "carry over" of the detrimental effect of the volatile(s) of this oil on the reproductive potential of the pyralid was indicated.

Key Words—Plant oils, *Eucalyptus*, neem, postembryonic development, reproductive potential, *Corcyra cephalonica*, Lepidoptera, Pyralidae.

INTRODUCTION

The rice moth *Corcyra cephalonica* (Stainton) is a major pest of stored commodities in the tropics (Piltz, 1977). Information on the effects of volatiles of selected plant-derived stored food products (Krishna and Mishra, 1985; Kumar

*To whom correspondence should be addressed.

0098-0331/91/1200-2553$06.50/0

and Krishna, 1991) or of specified plant components (Pathak and Krishna, 1985, 1992; Ansari and Krishna, 1987) on the insect's reproductive biology, particularly egg output and egg hatchability, have been reported. However, nothing is known about the changes that are likely to occur in the postembryonic development and reproduction of the insect following programmed exposure of individuals to the volatiles from such sources during rearing or breeding. We present our findings on these aspects with respect to eucalyptus and neem oils.

METHODS AND MATERIALS

Insects

A culture of *C. cephalonica* was maintained in the laboratory on a diet composed of "Jowar" (*Sorghum bicolor* (L.) Moench) and 5% yeast (Mishra and Krishna, 1979). Newly emerged larvae of males and females (<24 hr old) were drawn from the culture for this investigation. The oil of eucalyptus tested was a commercial brand (M/S Vivek Pharma, Mazagon, Bombay, India) and that of neem was a product extracted from the decorticated seeds of locally grown Indian neem trees (*Azadirachta indica* A. Juss).

Larval Exposure to the Oils

For assessing the impact of the volatiles of these oils on the postembryonic development, adult emergence and subsequent reproductive potential of the pest, two series of tests were conducted, each comprising five sets of 20 larvae (a set constituting a replicate). These larvae were housed in glass containers (70 mm diameter; 90 mm height) covered on the top by black muslin fastened by elastic bands and provided with an adequate quantity of the same diet mixture as that used in the culture. In one series, the larvae were maintained for the first 15 days of their lives, in a chamber permeated by the volatiles emanating from 6 ml of eucalyptus or neem oil contained in glass vials (23 mm bottom diameter and 13 mm top open end diameter; 35 mm height) placed on the floor of the chamber. In the second series, the larvae were similarly exposed to the volatiles but commencing from the sixteenth day after hatching. The number of adult males and females that finally emerged and their total postembryonic developmental period were recorded in all the sets of both series of experiments.

Reproductive performance of these moths was then evaluated by holding a freshly emerged male and a female (<24 hr old and both belonging to the same series) in single pairs for mating and egg laying inside clean and empty black muslin-covered glass containers described above. The number of eggs deposited by the females was recorded daily for the first 4 days, the period when the females were generally prolific in their egg laying, and the hatchability of the

eggs were also noted. The data, pooled from five (four in one case only) independently run tests, were subjected to *t*-tests (Paterson, 1939; Snedecor, 1956) to ascertain statistical significance between treatments.

Adult Exposure to the Oils

To examine the effects of exposure of adults to eucalyptus oil volatiles on the postembryonic development and breeding efficiency of the progeny, five newly emerged males and females were arranged in single pairs. They were exposed inside parafilm-covered glass containers to the vapors of the oil for 5 min. Longer exposure led to their death. The oil was held in wire-mesh capped glass vials kept at the base of the containers. The vapor-exposed pairs were transferred to another set of clean, similar glass containers with their top ends closed by black muslin for copulation and subsequent oviposition. Eggs laid on the first day were removed from all the containers and arranged in rows on a glass petri dish for hatching. One hundred larvae that hatched from this pooled egg mass were divided into five lots of 20 each, and individuals of every such lot were then kept on the culture diet in glass containers. Numbers of adult males and females that emerged, the total postembryonic developmental period and reproductive potential, in terms of egg output and egg hatchability, of the mated females were recorded as described before.

All the tests, performed at 27°C ± 2°C under 85% ± 5% r.h., were accompanied by appropriately designed controls wherein the insects were not exposed to the oil vapors.

RESULTS AND DISCUSSION

Considerable lengthening of total postembryonic developmental period and substantial reduction in the numbers of adults that finally emerged were characteristically noticed in both sexes of *C. cephalonica* that passed their first 2 weeks of larval life in an environment laden with eucalyptus oil vapor (Table 1). Presumably the volatile(s) released from eucalyptus oil detrimentally affected the basic insect development regulating neuroendocrinal mechanisms (Wigglesworth, 1972; Chapman, 1982) in these treated individuals. Deferment of the oil vapor treatment to the growing caterpillars to a later period commencing from the 16th day of their life for 15 days failed to produce any adult (data relating to this treatment not shown in Table 1). Possibly, these mature larvae, whose orientation behavior changed from one of remaining deep within the diet to that of emerging to the surface of the food in search of favorable sites for pupation, became easily subjected to the adverse effect of the oil vapor leading to their death. The same did not occur when this pest, during larval rearing, likewise interacted with neem oil vapor or in the progeny of parents exposed to eucalyptus

TABLE 1. POSTEMBRYONIC DEVELOPMENT PERIOD AND EMERGENCE OF ADULT MALES AND FEMALES OF *Corcyra cephalonica* EXPOSED OR UNEXPOSED TO EUCALYPTUS/NEEM OIL VAPORS (DATA POOLED FROM FIVE REPLICATES OF 20 LARVAE EACH PER TEST)[a]

Treatment	*N*	Adult score: mean nos. emerging (±SE)		Mean no. of days to adult stage ± SE	
		M	F	M	F
I Eucalyptus oil:					
A. Larval exposure:					
(i) First 15 days after hatching	100	2.2 ± 0.7[b]	2.0 ± 0.7[b]	55.7 ± 1.1[b]	62.4 ± 2.4
(ii) Unexposed (control)	100	9.2 ± 1.0	6.0 ± 0.7	29.6 ± 0.5	32.6 ± 0.6
B. Adult exposure:					
(i) Freshly emerged (<24 hr old) males and females for 5 min	100[e]	8.2 ± 1.4[d]	9.0 ± 1.6[d]	32.1 ± 0.3[d]	34.4 ± 0.3[c]
(ii) Unexposed (control)	100[e]	7.8 ± 1.0	8.6 ± 1.2	32.4 ± 0.4	35.5 ± 0.3
II Neem oil:					
A. Larval exposure:					
(i) First 15 days after hatching	100	6.8 ± 1.0[d]	7.0 ± 0.5[d]	29.6 ± 0.7[d]	32.7 ± 1.6[d]
(ii) Unexposed (control)	100	9.2 ± 1.0	6.0 ± 0.7	29.7 ± 0.5	32.6 ± 0.6
(iii) For 15 days from the 16th day after hatching	100	10.2 ± 1.8[d]	6.8 ± 0.7[d]	26.9 ± 1.6[d]	32.8 ± 0.5[d]
(iv) Unexposed (control)		9.2 ± 1.0	6.0 ±0.7	29.7 ± 0.5	32.6 ± 0.6

[a] F = female; M = male; *N* = total number of larvae from all the five replicates employed at the beginning of each treatment, SE = standard error.
[b] $P < 0.01$.
[c] $P < 0.05$.
[d] Not significant ($P > 0.05$; *t*-test; Paterson, 1939).
[e] Larvae of the progeny.

oil vapor, as adults, for only 5 min; however, curiously enough, in the latter situation, a statistically significant reduction ($P < 0.05$) was noticed in the postembryonic developmental period of females.

There was a marked decline both in egg output and egg hatchability in reproductive pairs whose larval development for the first 15 days took place in an eucalyptus oil environment ($P < 0.01$; Table 2). A similar reduction in the moth's reproductive potential was again noticed in the breeding pairs of progeny whose parents were exposed during their adult life to this oil vapor for only 5 min. These observations reflect a "carry over" of the deleterious effect of the volatile(s) from this oil on the reproductive fitness of the insect. Data comparisons of larval treatments involving neem oil did not manifest this outcome although Pathak and Krishna (1985) reported a significant fall in egg yield and egg hatchability in this species following a 30-min exposure of adults to vapors arising from 160 μl of this oil.

TABLE 2. EGG YIELD AND EGG HATCHABILITY IN *Corcyra cephalonica* ADULTS EXPOSED OR UNEXPOSED TO EUCALYPTUS/NEEM OIL VAPORS[a]

Treatment	*N*	Mean egg yield (±SE)	Mean egg hatchability (±S.E.)
I Eucalyptus oil:			
(a) Adults (both sexes) exposed during larval life for first 15 days after hatching	5[b]	175.2 ± 30.8[d]	141.4 ± 28.6[d]
(b) Adults (both sexes) not exposed during larval life (control)	5[b]	367.2 ± 15.2	336.4 ± 12.1
(c) Moths whose both parents, as freshly emerged adults (<24 hr old), were exposed for 5 min	5[b]	68.8 ± 28.1[d]	1.8 ± 00.8[d]
(d) Moths whose both parents, as freshly emerged adults (<24 hr old), were unexposed (control)	5[b]	343.2 ± 8.5	329.4 ± 09.4
II Neem oil:			
(a) Adults (both sexes) exposed during larval life for first 15 days after hatching	4[c]	316.3 ± 32.6[c]	289.8 ± 32.4[c]
(b) Adults (both sexes) not exposed during larval life (control)	5[c]	335.4 ± 17.9	311.0 ± 11.7
(c) Adults (both sexes) exposed during larval life for 15 days from the 16th day after hatching	5[b]	266.8 ± 27.3[e]	247.8 ± 36.2[e]
(d) Adults (both sexes) not exposed during larval life (control)	5[b]	335.4 ± 17.9	311.0 ± 11.7

[a] *N* = Number of ovipositing females; SE = standard error.
[b] *t*-test for samples of equal size (Paterson, 1939).
[c] *t*-test for samples of unequal size (Snedecor, 1956).
[d] $P < 0.01$.
[e] Not significant ($P > 0.05$).

Acknowledgments—This work was supported by research Grant No. 9/57 (73) 87/EMR-1 provided to the senior author by the Council of Scientific and Industrial Research, New Delhi.

REFERENCES

ANSARI, A.A., and KRISHNA, S.S. 1987. Clove oil vapour lowers reproductive efficiency in *Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae). *Biol. Bull. India*, 9:7–10.

CHAPMAN, R.F. 1982. The Insects: Structure and Function. ELBS and Hodder and Stoughton, Hong Kong.

KRISHNA, S.S., and MISHRA, S.N. 1985. A behavioural assessment of the impact of some environmental and physiological factors on the reproductive potential of *Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae). *Proc. Indian Acad. Sci. (Anim. Sci.)* 94:249–264.

KUMAR, A., and KRISHNA, S.S. 1991. Rice moth reproduction in relation to quality of stored products in the insect's breeding environment. *Uttar Pradesh J. Zool.* In press.

MISHRA, S.N., and KRISHNA, S.S. 1979. Influence of some specific time- and age-related mating schedules on oviposition and egg fertility in *Corcyra cephalonica* (Stainton) (Lepidoptera: Galleridae). *Entomon.* 4:197–199.

PATERSON, D.D. 1939. Statistical Technique in Agricultural Research. McGraw-Hill, New York.

PATHAK, P.H., and KRISHNA, S.S. 1985. Neem seed oil, a capable ingredient to check rice moth reproduction. *Z. Ang. Ent.* 100:33–35.

PATHAK, P.H., and KRISHNA, S.S. 1992. Effects of odours of certain plant oils or leaves on the egg hatchability in *Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae). *Mitt. Zool. Mus. Berl.* 68. In press.

PILTZ, H. 1977. *Corcyra cephalonica* (Stainton), pp. 439–440, *in* J. Kranz, H. Schmutterer, and W. Koch (eds.). Diseases, Pests and Weeds in Tropical Crops. Verlag Paul Parey, Berlin.

SNEDECOR, G.W. 1956. Statistical Methods. Iowa State College Press, Ames.

WIGGLESWORTH, V.B. 1972. The Principles of Insect Physiology. ELBS and Chapman & Hall, London.

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

ERRATUM

The following figure originally appeared on page 2038 of Volume 17, Number 10 (October 1991) of Journal of Chemical Ecology in the article, "Evidence for Presence and Nature of Female Sex Pheromone of *Brithys crini* Fabricius (Lepidoptera: Noctuidae)" by R. Kou, Y. S. Chow, S. Takahashi, and R. Yamaoka. It appears on the following page in a better quality reproduction.

0098-0331/91/1200-2559$06.50/0

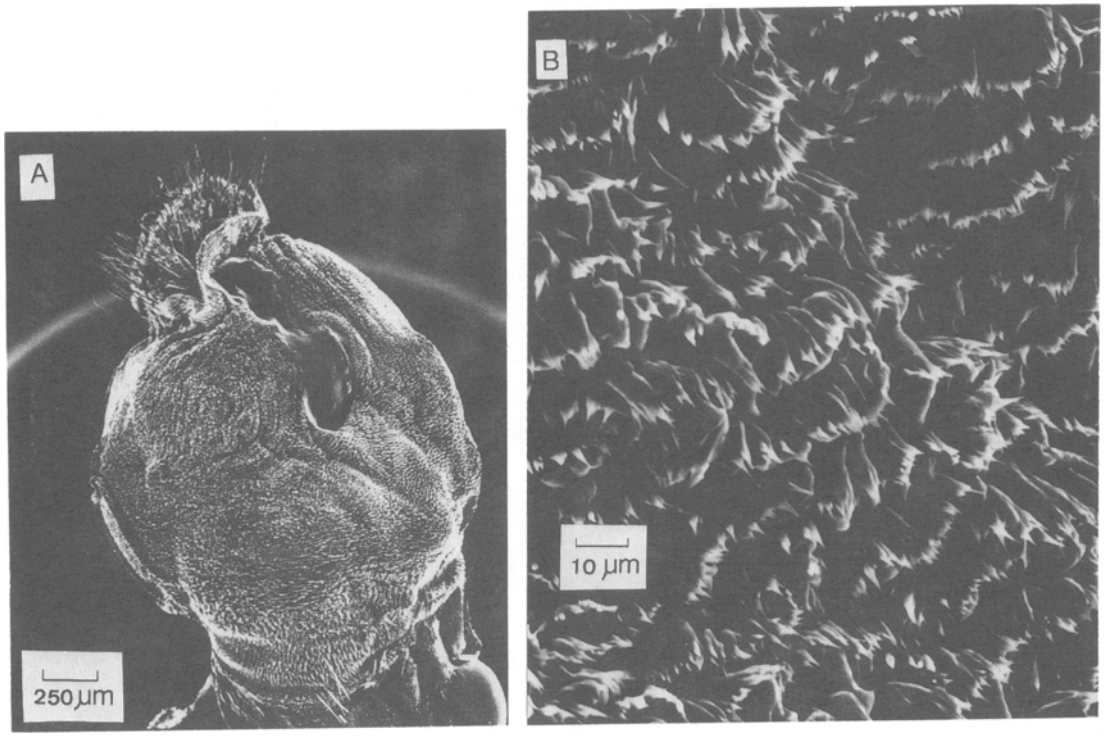


FIG. 1. (A) Scanning electron microscope view of the female *Brithys crini* sex pheromone gland (37X, 15 kV). (B) Scanning electron microscope view of the surface folding of female *Brithys crini* sex pheronome gland (720X, 15 kV).

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

ANNOUNCEMENT

International Society of Chemical Ecology

The 9th annual meeting of the International Society of Chemical Ecology will take place in Kyoto, Japan, July 6–9, 1992. Sessions will consist of 30 min presentations by invited speakers and 12 min presentations by contributing speakers, with five min and three min discussion periods, respectively. Thus far, organizers have planned sessions on the Chemical Ecology of Medicinal Plants (K. Fuji), Allelopathy (C.H. Chou), Aqueous Biosphere (D. Daloze), Natural Products (K. Mori, K. Nakanishi, A.B. Smith III), and Fruit Flies (J.L. Nation). Poster presentations on session themes, as well as general chemical ecology, are invited.

An abstract is required for oral and poster presentations, and should include author's name, title, organization, and address, paper title, and subject matter. A form for this purpose should be requested. Program details, abstract forms, registration forms, and accommodation applications are available from

Dr. Shozo Takahashi
Pesticide Research Institute
Faculty of Agriculture
Kyoto University
Kyoto 606, Japan

0098-0331/91/1200-2561$06.50/0

Journal of Chemical Ecology, Vol. 17, No. 12, 1991

ANNOUNCEMENT

The newly established Indian Society of Allelopathy (ISAL) is sponsoring a first National Symposium, "Allelopathy in Agroecosystems (Agriculture and Forestry)" at Haryana Agricultural University, Hisar 125 004, Haryana, India on February 12–14, 1992. Additional information and registration forms can be obtained from

Shamsher S. Narwal
Organizing Secretary
Department of Agronomy
Haryana Agricultural University
Hisar 125 004
Haryana
India

0098-0331/91/1200-2563$06.50/0