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PROPHEROMONES DERIVED FROM CODLEMONE

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Abstract—Tricarbonyl [(8,9,10,11-η)-8,10-dodecadien-1-ol] iron and the corresponding acetate prepared from 8,10-dodecadien-1-ol or its acetate, comprise the protected double-bond system of the molecule. After coming in contact with ambient oxygen, the iron complexes in question slowly release the corresponding pheromones of, for example, the codling moth, *Cydia pomonella*, and the pea moth, *Cydia nigricana* in high *E,E* purity and amounts that are sufficient for pest monitoring. A simple dispenser for propheromone application is proposed. Results of release rates in laboratory conditions and field trials are given.

Key Words—Insect pheromones, *C. pomonella*, *C. nigricana*, Lepidoptera, Tortricidae, propheromone, codlemone, conjugated dienes, pheromone protection, pheromone release.

INTRODUCTION

The efficiency of synthetic insect sex pheromones used in the field is usually influenced by: (1) the presence of the reactive functional groups in the pheromone molecule, (2) the compound's volatility, (3) the type of substrates used for dispensers, and (4) environmental conditions.

For example, pheromones that contain aldehydic groups undergo oxidation and trimerization, with subsequent loss of biological activity (Dunkelblum et al., 1984). The pheromones with conjugated double bonds easily undergo rapid isomerization (Brown and McDonough, 1986; Vrkoč et al., 1988), oxidation (Shani and Klug, 1980; Ideses et al., 1982), and polymerization, especially

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when exposed to higher temperatures and/or sunlight, air oxygen, humidity, etc. In general, conjugated double bonds are both photoisomerized to the equilibrium mixture of four possible geometric isomers and/or oxidized to give endoperoxides that are then converted to furans (Ideses and Shani, 1987). The degradation can take place not only during field application but also during manufacture, packaging, and storage of the pheromone dispensers.

Individual insect species differ in their sensitivity to the degradation products present in lures. The pea moth, *Cydia nigricana*, probably uses the same major pheromone component (i.e., *E*8,*E*10–12:Ac; Greenway, 1984) as the lucerne moth, *Cydia medicaginis*. According to Horák et al. (1989), the difference among various pheromone formulations affects the degree of pea moth sex pheromone isomerization, consequently resulting in discrepancies that have been reported previously (Wall et al., 1976; Horák et al., 1980; Greenway and Wall, 1981; Greenway et al., 1983; Horák and Hrdý, 1988). Unlike *C. nigricana*, the repulsion of *C. medicaginis* from *E*8,*E*10–12:Ac lures was not complete, and such lures can be used to detect and monitor this pest (Bournoville, 1979; Wall et al., 1987).

The introduction of propheromones (Liu, et al., 1984) seems to be one of the possibilities to improve the release of pure pheromone components in different field applications. The improvement arises through the derivatization of reactive functional groups in the molecule of biologically active compounds, to form a relatively stable molecule with substantial changes in volatility. Furthermore, such a propheromone system (and an application form based upon it) should enable suitable release of the volatile active compounds (the protective groups must be removable under ambient factors, and therefore the dispensers have to be designed to ensure good conditions for decomposition).

The conjugated double bonds can be protected as the iron diene carbonyls (Greé, 1989; Knox and Thom, 1981). These complexes are stable in the presence of many organic reagents as long as these reagents are not oxidative. This feature stimulated us to find out whether such protection of the conjugated double bond system in codlemone-like pheromones meets the propheromone demands. The codling moth, *Cydia pomonella*, and the pea moth, *Cydia nigricana*, were chosen as the model insects for field trials because of known variations in sensitivity between the two species to the products of the double bond isomerization (Hrdý et al., 1986; Vrkoč et al., 1988; Horák et al., 1989; Vrkoč and Streinz, 1990).

METHODS AND MATERIALS

Chemicals. Iron pentacarbonyl was obtained from Fluka and was used without additional purification. Diethyleneglycol dimethylether (DGDE, Fluka) was distilled from sodium and then kept over molecular sieves. (8*E*,10*E*)-8,10-

dodecadien-1-ol (Ia, *E*8,*E*10–12:OH) was purchased from Slušovice Coop., Czechoslovakia, which according to GC contained 93% of the *E*,*E* isomer and 7% of other isomers. The acetate Ib (*E*8,*E*10–12:Ac) was obtained from Ia by simple acetylation using acetyl chloride and pyridine in ether.

Gas chromatography. A Hewlett-Packard 5880 chromatograph, equipped with FID and 25 m × 0.3 mm ID capillary silica fused column coated with HP-5 phase (splitless injection), was used. The programming was as follows: 50°C, 30 sec, 30°C/min, 190°C, 20 min, 10 min purge at 25°C.

Nuclear Magnetic Resonance (NMR). Spectra were taken on a Varian Unity-200 (200 MHz) spectrometer in deuterochloroform and TMS as an internal standard. The data are in δ units.

Synthesis. To the 1.0 g (5.5 mmol) of 8,10–12:OH in 5 ml of DGDE was added 1.2 ml (9.2 mmol) of iron pentacarbonyl. The reaction mixture was then stirred under argon at 140°C for 5 hr (TLC) and worked up. The reaction mixture was diluted by 10 ml of light petroleum, filtered through 5 g of Celite 545, and, after evaporation of the solvent, chromatographed on 100 g of silica. The mixture of light petroleum and diethyl ether in a 3:1 ratio was used as a mobile phase. The yield was 1.1 g (62%) of propheromone IIa. For $C_{15}H_{22}FeO_4$ (322.18) calculated: 55.92% C, 6.88% H, 17.64% Fe. Found: 56.35% C, 7.17% H, ^{1}H NMR: 0.89–1.75 (m, 17H, CH_2, CH_3), 3.63 (t, 2H, CH_2OH), 4.94, 5.02 [2 × br s, 4H, CH=CH−CH=CH·Fe(CO)$_3$]. IR (CCl_4): 3640, 2043, 1977, 1968, 1073, 1059, 1035 cm^{-1}. The propheromone IIb was prepared by the same method except that the 8,10–12:Ac was used as a starting material. During the final work-up, the light petroleum ether–diethylether 95:5 was used as a mobile phase. For $C_{17}H_{24}FeO_5$ (364.22) calculated: 56.06% C, 6.64% H. Found: 56.21% C, 6.67% H. ^{1}H NMR: 1.10–1.73 (m, 15H, CH_2), 2.04 (s, 3H, CH_3CO), 4.07 (t, 2H, CH_2OH), 4.92, 5.03 [2 × br s, 4H, CH=CH−CH=CH·Fe(CO)$_3$]. IR (CCl_4): 3075, 3040, 3005, 2045, 1970, 1240, 1046 cm^{-1}.

Determination of Evaporation Rates. The samples were tested in the laboratory where the temperature was kept at 24–26°C and relative humidity at 70%. The samples were kept out of direct sunlight. Filter papers (Schleicher–Schuel A.G. No. 350γ) of different areas were used as dispensers. The propheromone was applied to the dispenser in benzene (20% solution) using a Hamilton syringe. For every test, five samples were prepared, and the results were expressed as an average value. The determination of evaporation rate was carried out according to Baker et al., (1980).

Field Trials. For *Cydia pomonella*, the experiment was performed in an apple orchard in Prague–Zbraslav from July 1 to August 22, 1991. Two cardboard sticky Delta traps baited with *E*8,*E*10–12:Ac (0.5 mg) on the rubber septum and IIa on the filter paper were used and catches of males were counted in two-day intervals.

For *Cydia nigricana*, the field experiment was done in peas (*Pisum sativum* L.) in the Breeding Station Horní Moštěnice (North Moravia) from June 10 to July 12, 1988. Catches were evaluated in cylindrical sticky traps, CN ETO-KAP, baited with *E*8,*E*10–12:Ac (0.1 mg) on the rubber septum and IIb on the cellulose (5 mg/3 cm^2) at two-day intervals.

RESULTS AND DISCUSSION

Tricarbonyl [(8,9,10,11-η)-8,10-dodecadien-1-ol] iron (IIa), and its acetate (IIb) were prepared using a modification of the procedure described for butadiene derivatives (Mahler et al., 1963). The compounds with dienic systems other than the *E*,*E* configuration can also undergo this reaction, but under these reaction conditions the rearrangement to the more stable *E*,*E* configuration takes place (DePuy et al., 1974; Morey et al., 1987). This is advantageous for us because the synthetic mixture of geometrical isomers of the alcohole (Ia) can be used as a starting material. The *E*,*E* isomer is then the only product obtained after oxidative cleavage. It was found that the propheromones IIa and IIb are also slowly decomposed by exposure to ambient air. For the controlled release of pheromones, the dispenser technique is used (Zeoli et al., 1982). Based on the development of new substrates and on the specific demands of insect behavior, known types of formulations have varied widely. Of these, rubber septa are most commonly used as a substrate for monitoring dispensers. This type of dispenser tends to follow first-order kinetics of evaporation loss. In contrast, the controlled release formulations, where emission depends on the parameters of the porous barrier membrane, follow zero-order kinetics if the concentration gradient remains constant (Nightingale, 1979). However, neither type of dispenser is utilizable for propheromone applications, where direct contact of propheromone with ambient factors plays an important role. Porous substrates with a large surface area appeared to be optimal as dispensers for propheromones. The best substrate for this purpose was found to be filter paper, where nearly all propheromone applied is located on the surface, affording efficient decomposition. In both cases, the pure *E*,*E* isomer (IIIa, IIIb) was the only one detected by the GLC analysis, using either the oxidant or the ambient air for decomposition. The release kinetics from propheromone IIa applied to the filter paper (Figure 1) differs from first-order evaporation kinetics of *E*8,*E*10–12:OH from the rubber dispenser (Figure 2). Although it varies depending on the amount of propheromone applied and on the surface area of the dispenser, the evaporation rate of pheromone is rather constant over 20–100 days. Simple calculation reveals that the amount of pheromone freed during this time is roughly in direct proportion to the amount of propheromone applied at the start of the experiment and to the dispenser area in contact with ambient air. The length of the part of

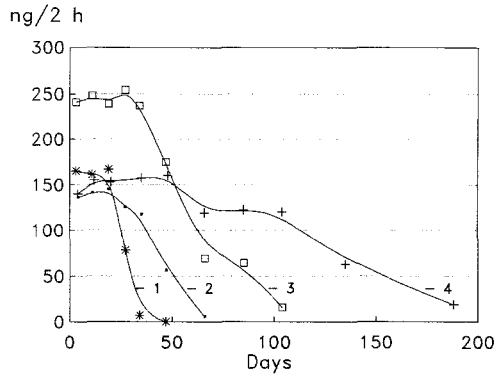


FIG. 1. The effect of dose and surface area of dispenser on the evaporation rate of (8*E*,10*E*)-8,10-dodecadien-1-ol from propheromone IIa ($N = 5$; 1, 5 mg of IIa on 9 cm^2 of dispenser; 2, 5 mg/3 cm^2; 3, 15 mg/9 cm^2; 4, 15 mg/3 cm^2).

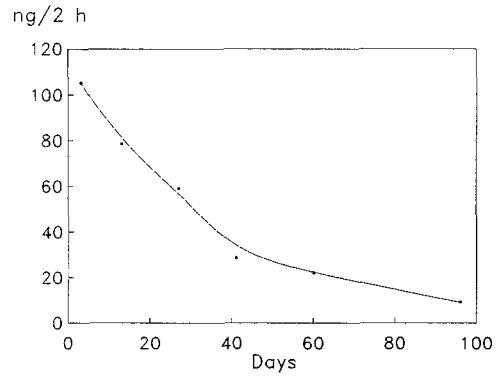


FIG. 2. The evaporation of (8*E*,10*E*)-8,10-dodecadien-1-ol (codlemone) from rubber septum (0.5 mg of active compound).

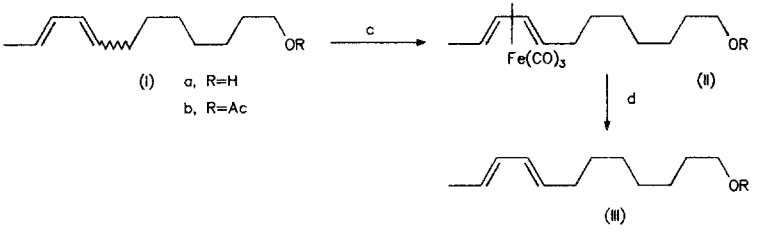
the curves parallel with the time axis is, on the other hand, proportional to the amount of propheromone applied to the square unit of the dispenser (Table 1). This is rather advantageous because one can simply achieve the optimum evaporation rate by changing the application parameters (under the full suppression of the isomerization and other pheromone activity degradation). The drop in the curves is probably due to ferric salts, which cover the surface of the dispenser, thus preventing further decomposition of propheromone.

In the open environment, the procedure outlined in Scheme 2 for decomposition of propheromone can be adopted (McDonough and Butler, 1983) [P_{pr}, P, P_v, and P_i are, respectively, concentrations of propheromone, active com-

TABLE 1. EFFECT OF PROPHEROMONE AMOUNT AND SURFACE AREA OF DISPENSER ON EVAPORATION RATE OF PHEROMONE IIIa

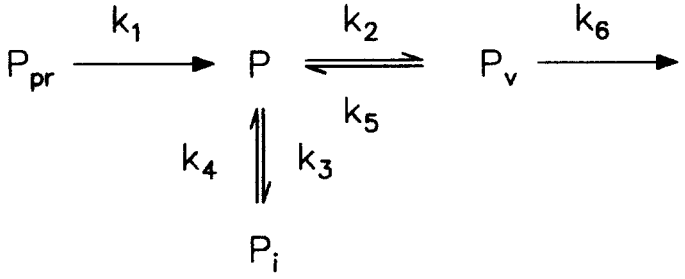
Amount (mg)[a]	Surface area (cm^2)[b]	Amount (mg/cm^2)[c]	Released (ng 2 hr)[d]	Duration[e]
5	3	1.7	150	25
5	9	0.65	160	20
15	3	5.0	150	100
15	9	1.7	250	30

[a] Propheromone applied to the dispenser.
[b] Area of filter paper used as a dispenser.
[c] Amount of propheromone applied to 1 cm^2 of dispenser.
[d] Amount of released pheromone.
[e] Approximate duration of straight part of the curve in days (Fig. 1).



c, $Fe(CO)_5$, 140°C, 6 h, d, air oxygen

SCHEME 1. Synthesis and principle of propheromone utilization.



SCHEME 2. Description of propheromone decomposition.

pound, vapor near the dispenser, and concentration of other than *E,E* isomers; k_1-k_6 are, respectively, the rate constants for decomposition of propheromones (k_1), loss of free active compound from the dispenser (k_2), two constants of isomerisation (k_3, k_4), readsorption of free compound to the dispenser (k_5) and evaporation into the environment (k_6)]. As was already mentioned, only the *E,E* isomer was detected in our experiment. Taking this fact into consideration, we believe that the relative magnitude of the above rate constants is as follows: $k_6 \geq k_1, k_2 >> k_3, k_5$ and k_1 P process is therefore the rate-controlling step. The pheromone is evaporated from the dispenser surface as fast as the propheromone is decomposed. Both the readsorption process and isomerization to the equilibrium are suppressed.

During the 1988 and 1991 seasons, the propheromones were tested in the field for male attraction in both the pea and the codling moths and the results are summarized in Table 2. The data confirmed that pure *E*8,*E*10–12:OH from the propheromone, is less attractive (about 20% attractivity) compared with that of the *E*8,*E*10–12:OH applied to the rubber septum. This indicates that the presence of isomers is, to some extent, necessary to obtain good attractivity.

Results of the field trial for male attractivity in the pea moth, *Cydia nigri-*

TABLE 2. CATCHES OF MALE CODLING MOTHS WITH PROPHEROMONE IIa (1991)

	Mean catch/trap/2 days		
Treatment	July 4–17	July 18–August 22	Total (48 days)
IIa[a]	8.8	4.2	6.5
*E*8, *E*10–12:OH[b]	50.2	13.4	31.8

[a] 5 mg of propheromone IIa on 5 cm^2 filter paper.
[b] 0.5 mg of (8*E*, 10*E*)-8,10-dodecadien-1-ol on rubber septum.

TABLE 3. CATCHES OF MALE PEA MOTHS WITH PROPHEROMONE IIb (1988)

	Mean catch/trap/2 days		
Treatment	June 10–21	June 22–July 3	Total (24 days)
*E*8,*E*10–12:0Ac[a]	6.2	2.5	4.3
IIb	19.2	55.0	37.1

[a] 0.1 mg of (8*E*,10*E*)-8,10-dodecadien-1-ol acetate on the rubber septum; IIb—propheromone on cellulose (5 mg/3 cm^2)

cana, are summarized in Table 3. While the codling moth is rather tolerant of geometrical isomers of *E*8,*E* 10–12:OH, the pea moth males responded to pure *E*8,*E*10–12:Ac (IIIb) released from propheromone IIb (Table 3). This corresponds to the results reported by Horák et al. (1989): the main factors that affect the efficacy of *E*8,*E*10–12:Ac as an attractant for the males of *C. nigricana* are the purity of the substance in the lure (*E*,*E* isomer) and the rate of release. Generally, if an unsuitable formulation is used, isomerization proceeds to an equilibrium mixture of all four geometrical isomers (Brown and McDonough, 1986; Vrkoč et al., 1988) followed by strong inhibition. The data confirmed that isomerization in lures with propheromones was suppressed and the lures were effective for more than 20 days under field conditions.

The propheromones of this type proved to be very useful as controlled slow-release formulations when there is: (1) easy accessibility, (2) simple control of the amount of released pheromone, and (3) prevention of pheromone decomposition. This type of compound application can also be used in pheromones containing conjugated dienic systems other than *E*,*E* (which is under preparation).

REFERENCES

BAKER, T.C., CARDÉ, R.T., and MILLER, J.R. 1980. Oriental fruit moth pheromone component. Emission rates measured after collection by glass surface absorption. *J. Chem. Ecol.* 6:749–758.

BOURNOVILLE, R. 1979. Determination, au champ, d'attractifs sexuels de synthèse des mâles de deux Tortricidae (Lep.): *Laspeyresia medicaginis* Kuzn. et *Laspeyresia nigricana* F., nuisibles aux legumineuses cultivées. *Rev. Zool. Agric. Pathol. Veg.* 78: 41–48.

BROWN, D.F., and MCDONOUGH, L.M. 1986. Insect sex pheromones. Formulation to increase the stability of conjugated dienes. *J. Ecol. Entomol.* 79:922–927.

DEPUY, C.H., JONES, T., and PARTON, R.L. 1974. Chemistry of substituted hydroxybutadienetricarbonyliron complexes. *J. Am. Chem. Soc.* 96:5602–5604.

DUNKELBLUM, E., KEHAT, M., KLUG, J.T., and SHANI, A. 1984. Trimerization of *Erias insultana* sex pheromone, (*E*,*E*)-10,12-hexadecadienal. A phenomenon affecting trapping efficiency. *J. Chem. Ecol.* 10(3):421–428.

GREÉ, R. 1989. Acyclic butadiene-iron tricarbonyl complexes in organic synthesis. *Synthesis* 341–355.

GREENWAY, A.R., 1984. Sex pheromone of the pea moth, *Cydia nigricana* (F.) (Lepidoptera: Olethreutidae). *J. Chem. Ecol.* 10:973–982.

GREENWAY, A.R., and WALL, C. 1981. Attractant lures for males of the pea moth, *Cydia nigricana* (F.) containing (*E*)-10-dodecen-1-yl acetate and (*E*,*E*)-8,10-dodecadien-1-yl acetate. *J. Chem. Ecol.* 7:563–573.

GREENWAY, A.R.C., WALL, C., BOURNOVILLE, R., and EINHORN, J., 1983. Sex attractants for pea moth. Rep. Rothamstead Exp. Sta. 1982, Pt. 1, p. 128.

HORÁK, A., and HRDÝ, I. 1988. Attractiveness of (*E*)-10-dodecen-1-yl acetate for the pea moth, *Cydia nigricana*: modification by codlemone, codlemone acetate and dodecyl acetate. *Acta Entomol. Bohemoslov.* 85:174–181.

HORÁK, A., HRDÝ, I., KRAMPL, F., and KALVODA, L. 1980. Polní zkoušky feromonových lapáku

pro monitorová-ní obaleče hrachového, *Cydia nigricana. Sbor. UVTIZ-Ochr. Rostl.* 16:213–225.

HORÁK, A., HRDÝ, I., KONEČNÝ, K., and VRKOČ, J. 1989. Effect of substrate formulation on the efficacy of the pea moth, *Cydia nigricana*, sex pheromone lures. *Entomol. Exp. Appl.* 53:125–131.

HRDÝ, I., KONEČNÝ, K., and VRKOČ, J. 1986. The effect of formulation on the effectiveness of codlemone for monitoring codling moth, *Cydia pomonella. Sbor. UVTIZ-Ochr. Rostl.* 22:33–52 (in Czech).

IDESES, R., and SHANI, A. 1987. Chemical protection of pheromones containing internal conjugated diene system, from chemical degradation in mating disruption: Behaviour of moth on molecules. Proc. Conf. 10CB-WPRS. H. Arn (ed.). pp. 66–67.

IDESES, R., SHANI, A., and KLUG, J.T. 1982. Sex pheromone of European grapevine moth (*Lobesia botrana*): Its chemical transformation in sunlight and heat. *J. Chem. Ecol.* 8:973–980.

KNOX, G.R., and THOM, I.G. 1981. Simple stereospecific syntheses of some insect pheromones using the $Fe(CO)_3$ protecting group. *J. Chem. Soc. Chem. Commun.* pp. 373–374.

LIU, X., MACAULAY, E.D.M., and PICKETT, J.A. 1984. Propheromones that release pheromonal carbonyl compounds in light. *J. Chem. Ecol.* 10:809–822.

MAHLER, J.E., GIBSON, D.H., and PETIT, R. 1963. Organoiron complexes. III. Reaction of the syn-1-methylpentadienyl iron tricarbonyl cation. *J. Am. Chem. Soc.* 85:3955–3963.

MCDONOUGH, L.M., and BUTLER, L.I. 1983. Insect sex pheromones: Determination of half-lives from formulations by collection of emitted vapor. *J. Chem. Ecol.* 9:1491–1502.

MOREY, J., GREÉ, D., MOSSET, P., TOUPET, L., GREÉ, R. 1987. Synthesis and reactivity of *E,Z*-butadiene–iron tricarbonyl complexes. *Tetrahedron Lett.* 28(26):2959–2961.

NIGHTINGALE, W.M. 1979. Improved means of obtaining sustained uniform emission of bioactive substances p. 401, *in* Proceedings, 1979 British Crop Protection Conference—Pests and Diseases. British Crop Protection Council, London.

SHANI, A., and KLUG, J.T. 1980. Sex pheromone of Egyptian cotton leafworm (*Spodoptera litoralis*): Its chemical transformation under field conditions. *J. Chem. Ecol.* 6:875–881.

VRKOČ, J., and STREINZ, L. 1990. Conference on pheromones in Mediterranean pest management. Pheromones with conjugated double bonds: Problems in system for pest monitoring and mating disruption. Granada, 10-15 September.

VRKOČ, J., KONEČNÝ, K., VALTEROVÁ, I., and HRDÝ, I. 1988. Rubber substrates and their influence on isomerization of conjugated dienes in pheromone dispensers. *J. Chem. Ecol.* 14:1347–1358.

WALL, C., GREENWAY, A.R., and BURT, P.E. 1976. Electroantennographic and field responses of the pea moth, *Cydia nigricana*, to sex attractants and related compounds. *Physiol. Entomol.* 1:151–157.

WALL, C., BOURNOVILLE, R., GREENWAY, A.R., and EINHORN, J. 1987. Efficacy and stability of sex attractant lures for *Cydia nigricana* (F.) and *Cydia medicaginis* (Kuzn.). *Crop protect.* 6:7–12.

ZEOLI, L.T., KYDONIEUS, A.F., and QUISUMBING, A.R. 1982. Controlled release technologies, pp. 131–144, *in* A.F. Kydonieus and M. Beroza (eds.). Insect Suppression with Controlled Release Pheromone System, Vol. I. CRC Press, Boca Raton, Florida.

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STRUCTURE–ACTIVITY RELATIONSHIPS FOR ANALOGS OF (+)-(*E*)-*endo*-β-BERGAMOTEN-12-OIC ACID, AN OVIPOSITION STIMULANT OF *Helicoverpa zea* (BODDIE)

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Abstract—β-Bergamotenoic acid, a compound previously shown to stimulate oviposition in *H. zea*, was converted into a set of bicyclic analogs and tested with a set of acyclic side chain analogs to ascertain the molecular structure that maximizes insect behavioral response. While changes in the bicyclic ring elicited no variation in response, alteration in the side chain structure of β-bergamotenoic acid resulted in significant changes in moth preference. Free rotation about the C—C bond proximal to the carboxylic acid group appears to be an important structural factor, since saturation of the side chain double bond significantly increased activity. The carboxylic acid group seems to be required for strong oviposition stimulation, since analogs lacking the carboxylic acid group exhibited no significant oviposition activity. Oviposition preference of *H. zea* was also influenced by the length of the hydrocarbon chain to which the carboxylic acid is attached. While hexanoic acid was found inactive, the ovipositional preference for the heptanoic and octanoic acids was greatest for the one 8-carbon tested. This and other work suggest that carboxylic acids of specific chain lengths influence the oviposition behavior of both *Helicoverpa* and *Heliothis* species and may be associated with host-plant selection. The potential use of this information in designing integrated pest management strategies for control of *H. zea* is discussed.

Key Words—Oviposition, insect chemoreception, corn earworm, *Heli-*

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coverpa zea, Lepidoptera, Noctuidae, *Lycopersicon hirsutum*, host-plant selection, sesquiterpenes, carboxylic acids.

INTRODUCTION

The larvae of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) are a major agricultural pest in the United States. The costs associated with damage and control of *H. zea* and the related pest, *Heliothis virescens*, are estimated to reach one billion dollars annually on a range of crops, including cotton, corn, tobacco, and tomato (Johnson et al., 1986). Because the primary method used for controlling *H. zea* is through the repeated application of insecticides, there is a need for other pest management strategies that reduce chemical inputs into the crop ecosystem.

Accurate pest population monitoring is an important component in any integrated pest management system for *Helicoverpa* and *Heliothis* control because it indicates to the grower when the application of insecticides is warranted. Currently, most *H. zea* populations are monitored by field scouting, which involves field egg and larval counts and/or damage evaluation (University of California, 1985). Because scouting is costly and labor intensive, other monitoring methods are being investigated. Current research involving semiochemicals, such as pheromones, which influence insect behavior, suggests that monitoring and/or control involving the use of attractant or repellent compounds could play a significant role in insect control practices.

While pheromone traps have been used effectively for monitoring levels of some insect pests, there are contradictory reports about their reliability for monitoring *H. zea* population levels. Leonard et al. (1989) observed a significant correlation ($r = 0.69$) between pheromone trap captures of male *H. zea* and *H. virescens* moths and levels of larval infestation in cotton fields. This report states that pheromone traps should only be used to complement field scouting. In contrast, Raulston et al. (1990) did not find a correlation between trap captures and the levels of infestation in corn fields.

Most research into utilizing semiochemicals for insect control has focused on insect pheromones and feeding incitants, with very little work on oviposition stimulants. Monitoring procedures that utilize oviposition stimulants, in contrast to pheromones, would estimate female moth populations, providing a more accurate estimate of resultant larval epidemiology. If particularly effective at modifying insect behavior, these attractants may also confuse moths and interfere with the process of host-plant selection. Information on compounds that attract and stimulate oviposition of *H. zea* may also be used in breeding programs to develop cultivars that lack these chemical cues, improving host-plant resistance to insects and reducing damage from larval feeding. Identification of the phytochemicals involved in oviposition stimulation will aid in understanding the

basis of insect chemoreception and determination of the most effective compounds for use in field traps.

Previous research in these laboratories has found that, (+)-(*E*)-α-santalen-12-oic acid, (+)-(*E*)-*endo*-β-bergamoten-12-oic acid **(1)**, and (−)-(*E*)-*endo*-α-bergamoten-12-oic acid **(7)**, three structurally similar sesquiterpene carboxylic acids exuded from trichomes on the leaf surfaces of the wild tomato accession LA 1777 (*Lycoperscion hirsutum*), are powerful oviposition stimulants of *H. zea* (Coates et al., 1988; Juvik et al., 1988). These compounds have key structural features that can be altered to determine what molecular properties are important for *H. zea* oviposition stimulation and to aid in basic studies of plant volatiles, chemoreception, and insect behavioral response. In this study, a set of cyclic and acyclic analogs (see Table 1) of (+)-(*E*)-*endo*-β-bergamoten-12-oic acid was assayed for kairomonal activity to determine the effect of molecular structure on female *H. zea* oviposition preference.

METHODS AND MATERIALS

Synthesis of Bicyclic and Acyclic Analogs. Purity of chemical samples was determined by gas chromatography (GC) conducted using a Hewlett Packard model 5760 gas chromatograph with a HP 7671A autosampler, an HP Ultra Performance 12.5-m cross-linked methylsilicone capillary column (0.2 mm ID), a flame ionization detector, and an integrator (HP 3390A). The column temperature at the time of injection was maintained at 90°C for 1 min and then increased to 170°C at a rate of 10°/min followed by an increase to 320°C at a rate of 15°/min. A split–splitless ratio of 100:1 was employed with a column flow rate of 1 ml helium/min.

All chemical structures were confirmed by infrared (IR) and proton nuclear magnetic resonance (^{1}H NMR) spectra. NMR chemical shifts were referenced to tetramethylsilane. Flash chromatography was performed as described by Still (1978) using Merck 0.040–0.063 mm silica gel or Merck silica gel impregnated with 10% silver nitrate. Thin-layer chromatographic (TLC) analysis was performed using plastic-backed plates precoated with 0.2 mm of silica gel F-254 with fluorescent indicator manufactured by EM Science. Thin-layer plates for argentic chromatography were obtained from Alltech Associates, Inc. Plates were 20 × 20 cm, 250 μm thick, and coated with 10% silver nitrate–silica gel. Plates were visualized by ultraviolet exposure or spraying with 5% phosphomolybdic acid in 95% ethanol and heating.

All reactions were carried out under a nitrogen atmosphere unless specified otherwise. All reagents and solvents used were reagent grade and used without further purification unless specified otherwise. Pentane used in recrystallizations was distilled prior to use. Diethyl ether and dichloromethane were freshly dis-

tilled from sodium benzophenone ketyl and calcium hydride, respectively, before their use as reaction solvents. Ethereal diazomethane was produced from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald) in ether with potassium hydroxide in ethanol following the procedure described by De Bore and Backer (1963). The ethereal diazomethane was stored in a −80°C freezer between uses.

(+)-(E)-endo-β-Bergamoten-12-oic Acid ***(1).*** The sesquiterpene acid was isolated by extraction of whole, undamaged leaves of *Lycopersicon hirsutum* accession LA 1777, esterification, chromatographic fractionation, saponification, and recrystallization as described by Coates et al. (1988). IR and ^{1}H NMR spectra are in agreement with those obtained previously.

(+)-Methyl-(E)-endo-β-Bergamotenoate ***(2),*** *(+)-(E)-endo-β-Bergamotenol* ***(3),*** *and (−)-(E)-endo-β-Bergamotene* ***(5).*** These known derivatives of **1** were prepared as described previously (Coates et al., 1988). IR and ^{1}H NMR spectra correspond with those obtained previously. The purities of **2, 3,** and **5** were 97%, 91%, and 100%, respectively, according to GC analysis. The 9% component present in **3** may be the 10,11-dihydro alcohol arising from conjugate reduction.

(+)-(E)-endo-β-Bergamotenyl Acetate ***(4).*** A solution of **3** (102 mg, 0.466 mmol), in 1 ml of pyridine was stirred as 0.3 ml of acetic anhydride (3.18 mmol) was added. After 4 hr at room temperature, the reaction mixture was diluted with 10 ml of ether and water. The organic layer was washed twice with 3 N HCl, once with sat. $NaHCO_3$, and once with saturated NaCl. The organic layer was dried over $MgSO_4$ and evaporated leaving 122 mg of 92% pure acetate. Purification by flash chromatography on 25 g silica gel with 5% ether–hexane as eluent afforded 87.9 mg (72%) of the acetate **4.** GC analysis revealed two peaks: R_t 8.95 (4%) and R_t 9.47 min (96%): IR (CCl_4), 2926, 1740 (C=O), 1375, 1234, 1024 (C−O), 871 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.14 (m, 2H, CH_2CH_2CH=), 1.25 (s, 3H, CH_3), 1.42 (d, J = 9.4 Hz, 1H, C-7 endo-H), 1.63 (s, 3H, =CCH_3), 1.81 (m, 2H, $CHCH_2CH_2$), 1.88 (m, 1H, $CHCH_2CH_2$), 2.01 (m, 2H, CH_2CH=), 2.07 (s, 3H, $COCH_3$) 2.25 (m, 1H, CHC=CH_2), 2.31 (dt, J = 5.7, 8.5 Hz, 1H, C-7 exo-H), 2.52 (t, J = 5.5 Hz, 2H, CH_2C=CH_2), 4.43 (s, 2H, CH_2O), 4.56 and, 4.65 (2s, 2H, =CH_2), 5.41 (t, J = 7.5 Hz, 1H, vinyl H).

10,11-Dihydro-β-Bergamotenoic Acid ***(6).*** Conjugate reduction of methyl β-bergamotenoate **2** was carried out by the procedure of Fortunato and Ganem (1976) for α,β-unsaturated esters. Purification of the organic product by flash chromatography on 15 g silica gel with 20% ether–hexane as eluent, afforded 97.6 mg (80%) of **6,** which was presumably a mixture of two side-chain diastereomers: IR (CCl_4), 2932, 1740, 1462, 1433, 1192, 1167, 874 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.07 [m, 2H, $(CH_2)_3CH$], 1.12 [d, 3H, $CH(CH_3)$], 1.1 (s, 3H, CH_3), 1.40 (d, J = 9.4 Hz, 1H, C-7 endo-H), 1.58 [m, 1H, $CH_2CH(CH_3)CO_2$], 1.80 (m, 2H, $CHCH_2CH_2$), 2.00 (m, 1H, $CHCH_2CH_2$),

2.27 (m, 1H, C*H*C=CH_2), 2.31 (dt, J = 5.7, 8.5 Hz, 1H, C-7 exo-H), 2.45 (m, 2H, CH_2C=CH_2), 3.69 (s, 3H, OCH_3), 4.54, and 4.64 (2s, 2H, =CH_2). Saponification of the ester to the acid was done following the same procedure described previously in the purification of β-bergamotenoic acid (Coates et al., 1988). This yielded 88 mg (96%) of **6.** GC analysis showed two peaks: R_t 13.67 min (3%) and 8.22 min (97%).

(−)-(E)-endo-α-Bergamotenoic Acid ***(7).*** A solution of HCl was generated by reaction of 76.1μl (0.6 mmol) of chlorotrimethylsilane with 75 μl (1.85 mmol) of methanol in 1.5 ml of CH_2Cl_2 for 10 min at 0°C. β-Bergamotenoic acid **1** (150 mg, 0.64 mmol) in 3.5 ml of CH_2Cl_2 was added to this solution. After 25 min, the solution was diluted with ether and washed with saturated NaCl. The aqueous layer was extracted three times with ether and the combined organic layers were washed twice with sat. NaCl. The solution was dried over $MgSO_4$ and evaporated, leaving 150.0 mg of crude **7** (85% purity). Purification by flash chromatography on 30 g of silica gel with 50% ether–hexane as eluent, and recrystallization in pentane at −78°C afforded 87.9 mg (59%) **7** as a white solid. GC analysis showed one peak with a retention time of 8.69 min (100%): mp = 51–52°C, $[\alpha]_D^{28}$ = −15.6° (C = 1, $CHCl_3$), IR (CCl_4), 2959, 2924, 2662, 2558, 1688 (CO_2H), 1644, 1422, 1287 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz), δ 1.18 (d, J = 8.3 Hz, 1H, C-7 endo-H), 1.28 (s, 3H, CH_3), 1.51 (m, 2H, C$H_2$$CH_2$CH=), 1.70 [d, J = 1.3 Hz, 3H, CH=C(CH_3)CH_2], 1.83 [s, 3H, =C(CH_3)CO_2], 1.95 (m, 1H, C*H*$CH_2$$CH_2$), 2.04 (m, 2H, CH*C*$H_2$$CH_2$), 2.15 (m, 2H, C$H_2$CH=), 2.20 (m, 1H, C*H*C=CH_2), 2.36 (dt, J = 5.7, 8.4 Hz, 1H, C-7 exo-H), 5.22 (br s, 1H, C=C*H*CH_2), 6.87 (t, J = 7.4 Hz, 1H, vinyl H). All spectral data are in agreement with the partial values given by Coates et al. (1988).

(−)-(E)-endo-α-Bergamotene ***(8).*** Isomerization of the ring double bond occurred during preparation of the chloride utilizing a procedure similar to that described previously for the β-isomer **(5)** (Coates et al., 1988) with the exception that the hindered amine buffer (2,2,6,6-tetramethylpiperidine) was not used. The yield was 59 mg (54%) of **8** as a colorless oil. GC analysis revealed one peak at 4.36 min. (100%). All spectral data are in agreement with values given by Coates et al. (1988).

Acyclic Analogs. Hexanoic acid **(9),** 1-hexanol **(10),** heptanoic acid **(11),** 1-heptanol **(12),** and *n*-octanol **(20)** were commercial chemicals from the Aldrich Chemical Company. Octanoic acid **(19)** was purchased from Matheson, Coleman, and Bell. Compounds **13–18** were prepared according to standard synthetic procedures that are given briefly below. All were purified by Kugelrohr distillation, sometimes after initial purification by flash chromatography on silica gel with ether–pentane or ether–hexane mixtures as eluant. High purity (>97%) was verified by capillary GC analysis. The ^1H NMR and IR spectra of the compounds are consistent with the structures and with spectral data in the lit-

erature. References are given to document the synthetic procedures and/or the physical properties of the compounds.

(±)-2-Methylheptanoic Acid ***(13)*** (Kawashima et al., 1989). The acid (135 mg, 93%) was obtained by saponification of **14** according to the conditions given below for **16.**

(±)-Methyl 2-Methylheptanoate ***(14)*** (Dobner et al., 1989). The ester was prepared by Li/NH_3 reduction (li, NH_3-ether; reflux 0.5 hr; NH_4Cl quench) (Caine, 1976) of **16.** Purification by flash chromatography and Kugelrohr distillation (65°C, 3.0 mm) afforded 471 mg (66%) of **14.**

(±)-2-Methyl heptan-1-ol ***(15)*** (Dorough et al., 1941). This alcohol was prepared by reduction ($LiAlH_4$, ether; 25°C, 2 hr; H_2O, 15% aq. NaOH; H_2O quench) (Micovic and Mihailoviz, 1953). The yield was 110 mg (89%) after flash chromatography and Kugelrohr distillation.

(E)-2-Methyl-2-heptenoic Acid ***(16).*** This acid was prepared by Wittig reaction of valeraldehyde and ethyl 2-(triphenylphosphoranylidene)propionate (CH_2Cl_2, reflux, 6 hr) (White et al., 1985) to give (*E*)-ethyl-2-methyl-2-heptanoate (1.35, 79%) followed by saponification (1:4 2 N KOH/ethanol, 25°C, 18 hr). Kugelrohr distillation (130°C, 1.0 mm) afforded 652 mg (92%) of **16.**

(E)-Methyl 2-methyl-2-heptenoate ***(17).*** This ester was obtained by diazomethane esterification of **16** in ether. Purification by flash chromatography and Kugelrohr distillation (70°C, 4.5 mm) gave 145 mg (79%) of **17.**

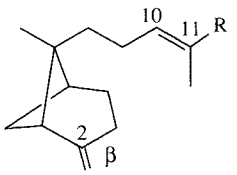
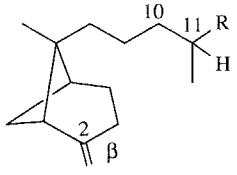
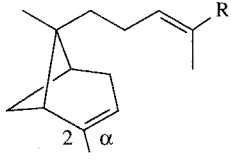
(E)-2-Methyl-2-hepten-1-ol ***(18)*** (White et al., 1985). This alcohol was prepared by aluminum hydride reduction of (*E*) -ethyl-2-methyl-2-heptenoate (−10°C, 10 min; H_2O quench) according to a published procedure (Coates et al., 1978). Kugelrohr distillation (70°C, 3.0 mm) gave 130 mg (85%) of **18.**

The structures and structure numbers of all 20 compounds are listed in Table 1.

Determination of β-Bergamotenoic Acid in Leaf Tissue of LA 1777. Three separate hexane extractions of 10 individual leaflets from five mature leaves of different LA 1777 plants were conducted to determine the average concentration of β-bergamotenoic acid **(1).** GC analysis indicated that the sesquiterpene accounted for 45.2% of the extract's dry weight or 0.116 mmol β-bergamotenoic acid/g fresh leaf tissue. With surface areas of LA 1777 leaflets averaging 56.3 cm^2/g, concentrations of **1** would be 0.48 mg/cm^2. When applied to filter paper disks in the oviposition assays whose surface areas were 14.2 cm^2, this would equate to 6.82 mg/disk. To provide some similarity between compound concentrations per unit surface area on leaves and in the oviposition assays, 7.0 mg of **1** or 0.0299 mmol of the various compounds were used as a standard rate of application per filter disk.

Oviposition Assays. All *Helicoverpa zea* moths used in these experiments were obtained from colonies maintained by Dr. J.A. Juvik at the University of Illinois. The compounds tested in arena assays are shown in Table 1. Arena

TABLE 1. BICYCLIC AND ACYCLIC ANALOGS OF (+)-(*E*)-*endo*-β-BERGAMOTEN-12-OIC ACID (**1**) TESTED FOR *Helicoverpa zea* OVIPOSITION PREFERENCE

Bicyclic analogs[a]			Acyclic analogs		
No.	Structure	End group (R)	No.	Structure	End group (R)
1		CO_2H	**9**	$CH_3(CH_2)_4R$	CO_2H
2		CO_2CH_3	**10**		CH_2OH
3		CH_2OH	**11**	$CH_3(CH_2)_5R$	CO_2H
4		CH_2OCOCH_3	**12**		CH_2OH
5		CH_3	**13**[c]	$CH_3(CH_2)_4-CHR$ (CH$_3$ branch)	CO_2H
6[b]		CO_2H	**14**[c]		CO_2CH_3
			15[c]		CH_2OH
			16	$CH_3(CH_2)_3$, CH_3, H, R	CO_2H
7		CO_2H	**17**		CO_2CH_3
8		CH_3	**18**		CH_2OH
			19	$CH_3(CH_2)_6R$	CO_2H
			20		CH_2OH

[a] α Symbol designates endocyclic double bond at C−2 (**7** and **8**); β symbol designates exocyclic double bond at C−2 (**1–6**).
[b] Mixture (presumed) of diastereomers at C−11.
[c] 1:1 mixture of enantiomers.

oviposition studies were conducted as described by Juvik et al. (1988). Moth oviposition preference values were calculated by recording the number of eggs oviposited on Whatman No. 1 filter paper disks (4.25 cm) attached to the sides of cylindrical arenas. Dividing the number of eggs laid on sample disks by the mean number of eggs found on the two adjacent untreated disks provided a measure of the sample's oviposition preference.

Arena Assay-Experiments. In experiment 1, 7.0-mg amounts of each bicyclic compound **(1–8)** in 0.5 ml of hexane were applied to the disks with one compound per disk. Disks were placed under a hood for at least 15 min to allow for complete evaporation of the solvent. Experimental disks and a control disk, to which 0.5 ml hexane had been applied, were randomly arranged in each arena, and each disk was separated by an untreated paper disk. This experiment contained six replicates of each arena. Twenty mated female moths were then released into each arena and egg counts recorded at 24, 48, and 72 hr after moth release.

In experiment 2, the amount of compound applied to each disk differed to allow for their relative volatilities. To the disks were applied 7 mg of **1,** 6.11 mg of **2,** 5.62 mg of **3,** 6.11 mg of **4,** 5.74 mg of **6,** and 6.04 mg of **7,** with one compound per disk. These values were determined by quantifying the concentrations of equimolar amounts of the various compounds in the vapor phase in sealed vials (see Bengtsson et al., 1990). As before, these disks and a control were randomly arranged in the arena and separated by untreated disks. This experiment contained three replicates of each arena.

In experiment 3, equimolar amounts of the acyclic compounds **9–20** were applied to the disks. The equimolar amounts were based on 7.0 mg or 0.0299 mmol of β-bergamotenoic acid. The disks were randomly arranged in each arena, but unlike the other arena experiments, two experimental disks were placed together and were flanked by untreated disks. This experiment contained four replicates of each arena.

In experiment 4, compounds from the above experiments that displayed significant oviposition preference were tested together. This experiment compared the bicyclic compounds from experiments 1 and 2 with the acyclic analogs of experiment 3. In this experiment 1.71×10^{-5} mol of **6, 11, 13, 14,** and **19** were applied to individual disks. These disks and a hexane control were randomly arranged in each arena and were separated by blank disks. This experiment contained four replicates of each arena.

Data obtained for oviposition preference were analyzed for significant differences by analysis of variance (ANOVA) and by Fisher's protected least significant difference test (LSD) as described by J.H. Goodnight (1982).

Oviposition Studies of Caged Plants Sprayed with Selected Structural Analogs. Leaves were removed from 1-month-old Chico III plants and grown from seed in the greenhouse, so that only the top two leaf nodes and the growing

point remained. Solutions containing 8 mg each of **1, 3, 4, 6,** and **8** in 3 ml of acetone were evenly layered over the surface of each leaf of individual plants using a syringe and allowed to dry. This was estimated to be 33–50% of typical sesquiterpene concentrations found on the leaf surface of LA 1777. All the plants, including a control plant to which acetone alone was applied, were evenly spaced in a random order in a 1.2 × 1.2 × 1-m cage while providing maximal distances between each plant. The cage was set up as described by Juvik et al. (1988). This experiment was repeated two more times with new plants, and data for oviposition preference were analyzed for significant differences by both ANOVA and protected LSD test (Goodnight, 1982).

RESULTS

Oviposition Assays of Bicyclic Analogs (Table 2). Assays were conducted both in arenas and on actual plants. The experiment utilizing real plants was conducted to determine if the compounds observed to stimulate *H. zea* ovipo-

TABLE 2. ARENA AND PLANT CAGE OVIPOSITION PREFERENCE DATA FOR BICYCLIC ANALOGS

Bicyclic analog			Oviposition preference[a]		
Number	C=C position	End group (R)	Experiment 1[b]	Experiment 2[c]	Plants[d]
6	β	CO_2H[e]	9.60 a[f]	20.46 a[f]	104.8 a[f]
7	α	CO_2H	5.71 b	12.48 b	NT[g]
1	β	CO_2H	4.85 bc	9.31 bc	84.7 ab
3	β	CH_2OH	3.80 c	5.10 bcd	57.7 bc
4	β	CH_2OCOCH_3	1.66 d	1.72 d	61.8 bc
2	β	CO_2CH_3	1.58 d	1.99 cd	NT
8	α	CH_3	1.52 d	NT	NT
5	β	CH_3	1.45 d	NT	74.2 bc
Hexane control			1.11 d	1.24 d	43.4 c
LSD (P = 0.05)			1.45	7.54	35.7

[a] For experiments 1 and 2 oviposition preference equals the mean number of eggs on a sample disk/average number of eggs on adjacent untreated disks. In the experiment using plants, oviposition preference equates to the mean number of eggs laid on the treated leaves.
[b] In experiment 1, equivalent amounts of the bicyclic analogs were applied to individual disks.
[c] In experiment 2, varying amounts of the compounds were applied to individual disks on the basis of the relative volatility of the specific analog.
[d] Experiment where *H. zea* moths were released in cages containing plants treated with the compounds.
[e] 10,11-dihydro.
[f] Means with dissimilar letters are significantly different at P = 0.05, using Fisher's protected LSD test.
[g] NT = not tested.

sition in the arena studies would also modify insect host selection on plants. These experiments were designed to indicate whether leaf trichome synthesis of phytochemicals can significantly influence female *H. zea* host-plant selection for oviposition. They also address the potential for the artificial modification of female moth host selection behavior in agricultural ecosystems.

Experiments that tested the bicyclic analogs (arena experiments 1 and 2, and the plant cage study) demonstrate that significant differences in moth oviposition preference occur when changes are made to the side chain of β-bergamotenoic acid (Table 2). In contrast, a change in the double bond position from exocyclic (β) to endocyclic (α) did not result in significant differences. In previous work by Coates et al. (1988, α-bergamotenoic acid **(7)** had not been tested for ovipositional activity because it was present in smaller amounts and could not be separated cleanly from α-santalenoic acid. In this study **7** was synthesized through isomerization of the exocyclic double bond in β-bergamotenoic acid to the more stable endocyclic position. While the α-isomer appears to be ovipositionally preferred over the β-isomer, the preference values are not significantly different (Table 2). These results suggest that receptor stimulation is not particularly sensitive to the three-dimensional shape of the bicyclic nucleus.

It is interesting that the α- and β-bergamotenoic acids that occur naturally in LA 1777 leaves are less active oviposition stimulants than the ostensibly unnatural dihydro analog **6.** Even with standardization for its higher relative volatility, **6** induced significantly more biological activity than either α- or β-bergamotenoic acid (experiment 2, Table 2). Furthermore, in the plant cage study, leaves treated with this compound were the most preferred sites for oviposition. In this experiment, application of **6** was the only treatment that resulted in observable leaf damage. The phytotoxic effects associated with the application of **6** may have influenced the ovipositional preference of these plants and may explain why a significant difference between plants treated with **6,** β-bergamotenoic acid **(1),** and β-bergamotene **(5)** (oviposition preference values of 104.8, 84.7, and 74.2, respectively) was not observed.

The functional group on the side-chain of β-bergamotenoic acid **(1)** appears to play the greatest role in oviposition stimulation. Analogs in which the functional group was modified showed decreases in oviposition preference. Reduction to the corresponding alcohol, β-bergamotenol **(3),** showed a lower oviposition preference in all experiments. This decrease is significantly different from α-bergamotenoic acid (Table 2, experiments 1 and 2) but not significantly different from β-bergamotenoic acid. While β-bergamotenol retains biological activity, the ester analogs, methyl β-bergamotenoate **(2)** and β-bergamotenyl acetate **(4),** and the bicyclic hydrocarbons, β-bergamotene **(5)** and α-bergamotene **(8)** are significantly less preferred then β-bergamotenoic acid and not statistically different from the hexane control in the arena experiments. In the plant study, variations in oviposition stimulation between the treatments were not as

great. It is likely that differences in treatment means were partially masked by variation due to the differences in volatilities of each compound, interaction of other physical plant properties with oviposition, and the fact that actual amounts of the compounds per unit surface area were considerably less (33–50%) than levels used in the arena studies. However, as in the arena studies, plants treated with the 10,11-dihydro analog **(6)** and β-bergamotenoic acid were the most preferred.

Oviposition Assays of Acyclic Analogs. In experiment 3, acyclic alcohols, acids, and esters with a chain length of six to eight carbons and structural similarity to the side chain of β-bergamotenoic acid were tested (see Table 1). However, only the acids with a chain length of seven or eight carbons were found to be active (Table 3). Furthermore, as seen in the results of the bicyclic compounds, the saturated side-chain analogs are more active. Thus, the saturated 2-methylheptanoic acid **(13)** exhibited significantly greater oviposition stimulatory activity than 2-methyl-2-heptenoic acid **(16)** (2.57 vs. 1.98). A further increase in oviposition was observed for heptanoic acid (oviposition preference value of 3.75), which lacks the branching methyl group at C-2. The compound with the greatest oviposition preference was octanoic acid (oviposition preference value of 5.00).

In experiment 4, analogs that displayed significant oviposition activity in

TABLE 3. ARENA OVIPOSITION PREFERENCE FOR ACYCLIC ANALOGS

Number	Chain length	Other group(s)	End group	Oviposition preference[a]
19	8		CO_2H	5.00 a[b]
11	7		CO_2H	3.75 b
13	7	2-CH_3	CO_2H	2.57 c
16	7	2-CH_3-2-ene	CO_2H	1.98 d
15	7	2-CH_3	CH_2OH	1.30 e
14	7	2-CH_3	CO_2CH_3	1.18 e
18	7	2-CH_3-2-ene	CH_2OH	1.15 e
12	7		CH_2OH	1.10 e
17	7	2-CH_3-2-ene	CO_2CH_3	1.05 e
10	6		CH_2OH	1.01 e
9	6		CO_2H	1.01 e
20	8		CH_2OH	0.95 e
Control				0.94 e

[a] Oviposition preference equals the number of eggs on a sample disk divided by the average number of eggs on adjacent untreated disks.

[b] Means with dissimilar letters are significantly different at $P = 0.05$ using Fisher's protected LSD test.

TABLE 4. ARENA OVIPOSITION PREFERENCE COMPARISON OF BICYCLIC AND ACYCLIC ACID ANALOGS

Number	Compound name	Structure	Oviposition preference[a]
16	2-Methyl-heptanoic acid	acyclic	3.20 a[b]
19	Octanoic acid	acyclic	3.09 a
14	2-Methyl-2-heptanoic acid	acyclic	2.97 a
11	Heptanoic acid	acyclic	2.64 a
6	10,11-Dihydro	bicyclic	2.39 a
	Control		1.07 b

[a] Oviposition preference equals the number of eggs on sample disks divided by the average number of eggs on adjacent untreated disks.
[b] Means with dissimilar letters are significantly different at $P = 0.05$ using Fisher's protected LSD test.

experiment 3 (octanoic acid, heptanoic acid, 2-methylheptanoic acid, and 2-methyl-2-heptenoic acid) were compared on a equimolar basis to the most active bicyclic compound, 10,11-dihydro-β-bergamotenoic acid. This experiment was conducted to determine if differences exist between the biological activity of bicyclic and acyclic side-chain analogs. In this assay, all of the compounds tested were statistically greater in oviposition preference than the control. However, while all chain analogs displayed higher levels of oviposition than the bicyclic compound, the numerical preference were not significantly different (Table 4).

DISCUSSION

A body of evidence suggests that phytochemicals influence insect behavior through interaction with specific insect chemoreceptors (Moncrieff, 1967; Evershed, 1988). Previous research has demonstrated that insects detect pheromones through molecular interactions with specific chemoreceptors. For this interaction to occur, the chemicals must have specific molecular structures that are complementary to the receptor protein. Several studies have used structural analogs of a naturally occurring compound eliciting a behavioral response to determine receptor configuration (Tai et al., 1971; Metcalf et al., 1983, 1986; Bengtsson et al., 1990). These studies have found that changing the molecular structure can either increase or decrease the capability of the molecule to activate the receptor. Metcalf (1983, 1986) found that in order for optimal olfactory response to occur in the melon fly, the molecule must have a parahydroxy group attached to an aromatic ring, with a carbonyl group two atoms away from that

ring. While there may be other explanations of how insects perceive and respond to their chemical environment, current theory suggests that insect chemoreception is a result of the binding of a molecule to the insect receptor protein.

The effects of changes in molecular structure we have observed on *H. zea* oviposition behavior suggest specific chemoreceptors are involved in oviposition stimulation. In this study, β-bergamotenoic acid, a compound previously identified to stimulate oviposition in *H. zea* (Coates et al., 1988), was converted into a set of analogs to ascertain the molecular structure that maximizes insect behavioral response. The side chain of 10,11-dihydro-β-bergamotenoic acid **(6)** and 2-methyl-heptanoic acid share structural homology, and they induce similar oviposition stimulation (2.97 and 2.39, respectively), suggesting that the bicyclic ring of β-bergamotenoic acid is not essential for activity. However, as shown in experiment 4, the bicyclic ring does not appear to hinder receptor binding, since **6** and its acyclic carboxylic acid analogs with seven or eight carbons display similar activity.

It appears that free rotation about the C—C bond proximal to the carboxylic acid group is an important structural factor. Reduction of the double bond is also important since the 10,11-dihydro and saturated acyclic analogs showed significantly enhanced oviposition activities compared to those of the corresponding α,β-unsaturated acids. It is plausible that the structural rigidity imposed by the α,β double bond impedes optimal interaction with the receptor. This increase in activity due to saturation of the double bond could result from either a better physical fit of the molecule to the receptor or could provide for the movement of a previously confined functional group to a site to interact more effectively with the receptor.

While saturation of the side-chain double bond significantly increases activity, the carboxylic acid group appears to be required for strong oviposition stimulation. With only one exception, analogs lacking the carboxylic acid group exhibited no statistically significant oviposition activity. The low but detectable activity of β-bergamotenol **(3)** seems to indicate that the CO_2H end group can be replaced by the H-bond donor CH_2OH without complete loss of oviposition response. However, the same CO_2H to CH_2OH replacement abolished the strong activity of the acyclic analog octanoic acid **(19).** Nevertheless, it seems reasonable to postulate as a tentative hypothesis that one component of the kairomone–receptor interaction is a hydrogen bond from the carboxylic acid end groups to a hydrogen acceptor on the receptor. Oviposition preference of *H. zea* is also influenced by the length of the hydrocarbon chain to which the carboxylic acid is attached. While hexanoic acid is inactive, the ovipositional preference for the heptanoic and octanoic acids was greatest for the one eight-carbon acid tested. Further studies will be needed to determine at what chain length *H. zea* response is maximized.

Published work on phytochemicals that influence *H. zea* oviposition behav-

ior is scarce. Phenylacetaldehyde, a volatile compound isolated from corn silks, was found to stimulate oviposition (Cantelo and Jacobson, 1979), while Jones et al. (1973) discovered that triacetin, a volatile compound present in the ink of marking pens, acted as an attractant to female *H. zea* moths. Neither of these compounds share any structural similarity to the carboxylic acids tested in this study. This lack of similarity may indicate that multiple moth chemoreceptors are involved in oviposition behavior, particularly since the sesquiterpenes in *L. hirsutum* are of comparatively low volatility and are presumably acting as contact ovipositional stimulants.

In a recent report by Severson et al. (1991), cuticular compounds from plant species within the genus *Nicotiana* were identified that influenced the ovipositional response of *Heliothis virescens*, a species closely related to *H. zea*. Among the compounds eliciting ovipositional stimulation were a set of sugar esters containing complex mixtures of C_3–C_8 fatty acids attached to the 2, 3, and 4 positions of the glucose moiety. This information, and the observation of oviposition preference of *Helicoverpa armigera* for leaves of LA 1777 (Juvik et al., 1982) and the significant *H. virescens* oviposition stimulation in arenas of hexane extracts of LA 1777 (85–90% sesquiterpene content by weight) (Juvik, unpublished), suggest that the females of these species share a similarity in the chemical reception systems that influence host-plant selection for oviposition.

Walters and Steffens (1990) have recently described the chemical diversity of a mixture of 2,3,4-tri-*O*-acylated glucose esters synthesized by glandular trichomes on leaves of the wild tomato species, *Lycopersicon pennellii*, which act as aphid feeding deterrents (Goffreda et al., 1989). A major fraction of the acyl moieties are branched and straight chain C_{10}, C_{11}, and C_{12} fatty acids. The observation that purified extracts of sugar esters from *L. pennellii* elicit significant ovipositional stimulation in female *H. zea* (Juvik et al., in preparation) provides additional evidence that acid esters of specific chain lengths influence the oviposition behavior of *Helicoverpa* and *Heliothis* species and may be associated with host-plant selection.

Straight-chain aliphatic acids are relatively ubiquitous among flowering plants, where they occur as free fatty acids or bound to carbohydrates, lipids, amino acids, terpenes, waxes, etc. *H. zea* is a highly polyphagous insect having a known host range of 238 species in 36 different plant families (Kogan et al., 1978). It is interesting to speculate whether the broad host range of *Helicoverpa zea* may in any way be associated with the presence of aliphatic acids in host-plant tissues. The answer to this question awaits a more comprehensive evaluation of the surface chemistry of *H. zea* host plants.

The evidence suggesting that the side-chain aliphatic acid in β-bergamotenoic acid is the putative chemical structure associated with oviposition stimulation and host-plant selection also helps to clarify questions regarding the

dramatic preference shown by *H. zea* for LA 1777 plants. In an evolutionary sense, it is difficult to explain why a sesquiterpene found in only a few accessions of *L. hirsutum* (Juvik et al., 1988) should act as a ovipositional kairomone for *H. zea* and its sibling species. This ephemeral wild tomato species of low population density inhabits a limited zone on the western slopes of the Andes from 1000 to 3300 m elevation from central Peru to northern Ecuador (Rick, 1973). In addition, previous work has shown that foliage of LA 1777 provides the poorest diet for survival and growth of larvae of *H. armigera* (Juvik et al., 1982) and *H. zea* (Sinha and Juvik, 1987). *H. zea* larvae after six days on diets supplemented with sesquiterpene extracts from LA 1777 (at concentrations below those observed on leaf tissue) displayed 62% greater mortality and growth rates 1/30th of those seen in larvae reared on control diets (Juvik, unpublished). The sesquiterpenes from LA 1777 are apparently toxic to *H. zea*.

The most likely explanation for the observed ovipositional stimulation of β-bergamotenoic acid to *H. zea* with its complementary toxicity to the larvae, lies in the hypothesis that moth chemoreception evolved in response to the presence of acyl acids of specific chain length in other plant species that became important habitat resources for completion of *H. zea*'s life cycle. In contrast, evolution may have favored the synthesis of sesquiterpene carboxylic acids in accessions of *L. hirsutum* as allomonal protectants from colonization of insects and diseases. In this evolutionary scenario, aliphatic acid side chains may provide an adaptive advantage to the plants because they result in greater sesquiterpene stability, volatility, or more likely modify the melting point so that the leaf glandular exudates are fluids of the appropriate viscosity for defense.

Understanding what compounds are important for host-plant identification and oviposition stimulation could potentially be used for developing methods of controlling *H. zea*. For example, compounds conferring maximal oviposition stimulation could be synthesized and used to spray on indicator plants. The presence and number of eggs could indicate to growers when pest populations have reached a density where spraying of pesticides is necessary. Measurement of egg densities on indicator plants would provide a more direct and accurate estimate of potential larval pest epidemics than pheromone traps that only measure relative numbers of male moths. *H. zea* larvae, after their first instar, tunnel into tomato fruit, ears of corn, cotton bolls, or fruit tissue of other host plants, rendering them partially protected from pesticide sprays. If growers begin to see an abundance of eggs on indicator plants, they can estimate when to apply the pesticides when early instar larvae are feeding on the plant surface and are more susceptible to insecticidal sprays.

Knowledge of the chemical structure of ovipositional kairomones could also be used by plant breeders to develop crop plants with improved host-plant resistance. If plants could be created that do not contain key chemical cues, they may not be recognized by *H. zea* as appropriate hosts for oviposition. This

could potentially lead to a decrease in damage because of the lower population numbers of *H. zea* larvae. Furthermore, since evidence suggests that the compounds that influence oviposition behavior in *H. zea* also display kairomonal activity to *H. virescens* and *H. armigera*, methods of pest management developed for *H. zea* using oviposition stimulants may also have implications in the control of the other two species.

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REFERENCES

BENGTSSON, M., LILJEFORS, T., HANSSON, B.S., LOFSTEDT, 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.

CANTELO, W.W., and JACOBSON, M. 1979. Corn silk volatiles attract many species of moths. *J. Environ. Sci. Health* 14:695–707.

CAINE, D. 1976. Reduction and related reactions of α, β-unsaturated carbonyl compounds with metals in liquid ammonia. *Org. React.* 23:1–258.

COATES, R.M., LEY, D.A., and CAVENDER, P.L. 1978. Synthesis and carbon-13 nuclear magnetic resonance spectra of all-*trans*-geranylgeraniol and its Nor analogues. *J. Org. Chem.* 43:4915.

COATES, R.M., DENNISEN, J.F., JUVIK, J.A., and BABKA, B.A. 1988. Identification of α-santalenoic and *endo*-β-bergamotenoic acids as moth oviposition stimulants from wild tomato leaves. *J. Org. Chem.* 53:2186.

DEBORE, T.J., and BACKER, H.J. 1963. Diazomethane. *Org. Synth. Coll.* Vol. 4:250–253.

DOBNER, B., ELSNER, B., and NUHN, P. 1989. Synthese mittelständig verzweigter Fettsäuren. *Pharmazie* 44:758.

DOROUGH, G.L., GLASS, H.B., GRESHAM, T.L., MALONE, G.B., and REID, E.E. 1941. Structure–property relationships in some isomeric octanols. *J. Am. Chem. Soc.* 63:3100.

EVERSHED, R.P. 1988. Insect olfaction and molecular structure, pp. 1–33, *in* D.E. Morgan, and N.B. Mandava (eds.). CRC Handbook of Natural Pesticides. Vol IV, Part A. Academic Press, New York.

FORTUNATO, J.M., and GANEM, B. 1976. Lithium and potassium trialkylborohydrides. Reagents for direct reduction of α,β-unsaturated carbonyl compounds to synthetically versatile enolate anions. *J. Org. Chem.* 41:2194.

GOFFREDA, J.C., MUTSCHLER, M.A., AVE, D.A., TINGEY, W.M., and STEFFENS, J.C. 1989. Aphid deterrence by glucose esters in glandular trichome exudate of the wild tomato *Lycopersicon pennellii*. *J. Chem. Ecol.* 15:2135–2147.

GOODNIGHT, J.H. 1982. ANOVA, pp. 113–137, *in* SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina.

JOHNSON, S.J., KING, E.G., and BRADLEY, J.R. 1986. Theory and tactics of *Heliothis* control. *S. Coop. Ser. Bull.* 316:161 pp.

JONES, R.L., BURTON, R.L., MCGOVERN, T.P., and BEROSA, M. 1973. Potential oviposition inducers for corn earworms. *Environ. Entomol.* 66:921–925.

JUVIK, J.A., BERLINGER, M.J., BEN-DAVID, T., and RUDICH, J. 1982. Resistance among accessions

of the genus *Lycopersicon* and *Solanum* to four of the major insect pests of tomatoes in Israel. *Phytoparasitica* 10:145–156.

JUVIK, J.A., BABKA, B.A., and TIMMERMANN, E.A. 1988. Influence of trichome exudates from species of Lycopersicon on oviposition behavior of *Heliothis zea* (Boddie). *J. Chem. Ecol.* 14:1261.

KAWASHIMA, M., SATO, T., and FUJISAWA, T. 1989. A facile method for synthesis of three carbon-homologated carboxylic acid by regioselective ring-opening of β-propiolactones with organocopper reagents. *Tetrahedron* 45:403.

KOGAN, J., SELL, D.K., STINNER, R.E., BRADLEY, J.R., JR., and KOGAN, M. 1978. A bibliography of *Heliothis zea* and *Heliothis virescens. Intsoy* 17:1–8.

LEONARD, B.R., GRAVES, J.B., BURRIS, E., PAVLOFF, A.M., and CHURCH, G. 1989. *Heliothis* spp. (Lepidoptera: Noctuidae) captures in pheromone traps: Species composition and relationship to oviposition in cotton. *J. Econ. Entomol.* 82:574–579.

METCALF, R.L., MITCHELL, W.C., and METCALF, E.R. 1983. Olfactory receptors in the melon fly, *Dacus cucurbitae* and the oriental fruit fly *Dacus dorsalis. Proc. Natl. Acad. Sci. U.S.A.* 80:3143–3147.

METCALF, R.L., METCALF, E.R., and MITCHELL, W.C. 1986. Benzyl acetates as attractants for the male oriental fruit fly *Dacus dorsalis*, and the male melon fly, *Dacus cucurbitae. Proc. Natl. Acad. Sci. U.S.A.* 78:4007–4010.

MICOVIC, V.C., and MIHAILOVIC, M.L.J. 1953. The reduction of acid amides with lithium aluminum hydride. *J. Org. Chem.* 18:1190.

MONCRIEFF, R.W. 1967. The Chemical Senses, 3rd ed. Leonard Hill, London.

RAULSTON, J.R., SUMMY, K.R., LOERA, J., PUIR, S.D., and SPARKS, A.N. 1990. Population dynamics of corn earworm larvae (Lepidoptera: Noctuidae) on corn in the lower Rio Grande Valley. *Envirn. Entomol.* 19:274–280.

RICK, C.M. 1973. Potential genetic resources in tomato species; clues from observations in native habitats, pp. 255–269, *in* A. Hollaender et al. (eds.). Genes, Enzymes, and Populations. Plenum Press, New York.

SEVERSON, R.F., JACKSON, D.M., JOHNSON, A.W., SISSON, V.A., and STEPHENSON, M.G. 1991. Ovipositional behavior of tobacco budworm and tobacco hornworm, pp. 265–278, *in* P.A. Hedin (ed.). Naturally Occurring Pest Bioregulators. American Chemical Society, Washington, D.C.

SINHA, N., and JUVIK, J.A. 1987. Chemical factors associated with resistance to *Heliothis zea* in wild accessions of tomatoes. *HortSci.* 22:1105.

STILL, W.C., KAHN, M., and MITRA, A. 1978. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 43(14):2923–2925.

TAI, A., MATSUMURA, F., and COPPEL, H.C. 1971. Synthetic analogues of the termite trail following pheromone, structure and biological activity. *J. Insect Physiol.* 17:181.

UNIVERSITY OF CALIFORNIA. 1985. Integrated pest management for tomatoes. Division of Agriculture and Natural Resources. Pub. 3274. 104 pp.

WALTERS, D.S., and STEFFENS, J.C. 1990. Branched chain amino acid metabolism in the biosynthesis of *Lycopersicon pennellii* glucose esters. *Plant Physiol.* 93:1544–1551.

WHITE, J.D., TAKABE, K., and PRISBYLLA, M.P. 1985. Steroselective synthesis of trisporic acids A and B, their methyl esters, and trisporols A and B, hormones and prohormones of mucoraceous fungi. *J. Org. Chem.* 50:5233–5244.

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CUCURBITACINS FROM *Cucurbita texana:* EVIDENCE FOR THE ROLE OF ISOCUCURBITACINS

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Abstract—In addition to cucurbitacins E and I, cucurbitacins D, **1**, 3-*epi*-isocucurbitacin D, **2**, and B, **3**, were isolated from the fruits of *Cucurbita texana* and structurally identified by UV, IR, ^{1}H NMR, ^{13}C NMR, and MS, and 2-*O*-β-glucopyranosylcucurbitacin I was identified. These compounds have not been reported previously as constituents of this species. The isolation of 3-*epi*-isocucurbitacin D **2** together with normal cucurbitacins suggests that isocucurbitacins occur naturally. Evidence is also discussed that isocucurbitacins are biosynthesized one step ahead of normal cucurbitacin.

Key Words—*Cucurbita texana*, corn rootworm, phagostimulant, cucurbitacin D, isocucurbitacin, glycosides.

INTRODUCTION

The cucurbitacins are a group of highly oxygenated tetracyclic triterpenes having a unique 19(10→9β)*abeo*-10-α-lanostane (cucurbitane) skeleton. Biological activity of this group has been recognized for centuries and has been used as a laxative, an emetic, and in the treatment of malaria, dysentery, and dysmenorrhea (Lavie and Glotter, 1971; Halaweish, 1987). In addition, the cucurbitacins have received a great deal of attention because of their cytotoxic and anticancer activities (Witkowski et al., 1984; Whithouse and Doskotch, 1969).

Cucurbitacins occur widely in plants of the family Cucurbitaceae and, because of their extreme bitterness, are thought to be involved in plant protection against herbivores (Metcalf, 1985; Tallamy and Krischik, 1989). Nevertheless, cucurbitacins are phagostimulants for both adult (Metcalf, 1985) and larval

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diabroticite chrysomelid beetles (DeHeer and Tallamy, 1991) and can have important ecological consequences for plants that possess them. Adult diabroticites can detect cucurbitacins in nanogram quantities and readily devour bitter plant material. These compounds influence the behavior of a number of important crop pests including the banded cucumber beetle (*Diabrotica balteata* Le Conte), the northern corn rootworm (*D. barberi* Smith & Lawrence), the southern corn rootworm (*D. undecimpunctata howardii* Barber and its western relative *D.u. undecimpunctata* Mannerheim) and the western corn rootworm (*D. virgifera virgifera* Le Conte).

The pharmacopetic response of diabroticite beetles to cucurbitacins is critical to the integrated pest management of cucumber beetles and corn rootworms throughout the world and has led to the development of promising control products containing cucurbitacin baits (Ferguson and Metcalf, 1984; Metcalf and Rhodes, 1985; Metcalf et al., 1987).

In view of the importance of these compounds to pharmacology and agriculture, there is a need to elucidate the chemistry of cucurbitacin-bearing plants. This is particularly true for species containing high concentrations of cucurbitacins such as the gourd, *Cucurbita texana* Gray, in which fruits have been shown to contain concentrations of cucurbitacins E and I of about 0.44 mg/g fresh weight and 0.75 mg/g cucurbitacin E glycoside (Metcalf et al., 1982). Since past identification of *C. texana* cucurbitacins was based mainly on comparison with standard materials (Metcalf et al., 1982), we have reexamined the cucurbitacin chemistry of this gourd to obtain a more complete cucurbitacin profile.

METHODS AND MATERIALS

Plant Material. C. texana (Cucurbitaceae) was grown to maturity in 25-cm pots in a greenhouse (Agriculture Experiment Station, University of Delaware, Newark, Delaware) and plants were hand pollinated. Fruits were collected when ripe and held in a freezer until extraction.

Extraction. The fruits (1.3 kg) were cut into small pieces, homogenized in chloroform (4 × 300 ml) for 10 min and filtered. The filtrate was concentrated in a rotary evaporator to about 100 ml (aqueous), then partitioned with hexane (4 × 150 ml) and $CHCl_3$ (4 × 150 ml). The $CHCl_3$ extract was concentrated and evaporated to dryness (1.6 g). The residue was adsorbed onto the top of a silica gel column 60 A (80 g, 70–230 mesh ASTM) and chromatographed using hexane–EtOAc [100, 9:1, 4:2, 3:2, 2:3, 1:8, 400 ml each, EtOAc–MeOH (1:1) 600 ml]. Fractions of 100 ml were collected, screened with TLC, and grouped by similarity. Silica gel GF_{254} plates (20 × 10 0.2 mm thickness) were used and developed with toluene–EtOAc (25:75) and examined under UV.

Fractions 11–18 showed cucurbitacin E (R_f 0.8), but in very low concentration and highly contaminated with chlorophyll; fractions 19–22 provided a yellowish residue, which was further purified with preparative HPLC (described below) to give compound **3** (R_f 0.7). Fractions 23–25, 26–30, and 31–36 all provided compounds with R_f values of 0.56 (cucurbitacin I), 0.47, **2**, and 0.37, **1**, respectively. All compounds were purified by preparative HPLC and crystallized from MeOH. HPLC separation was performed with a Perkin–Elmer S-100 with UV detector at 229 nm, preparative column Spherisorb-ODS-2 5 μm (20 cm × 10 mm ID) with gradient separation using solvent A (ACN–H_2O, 30:70) and solvent B (ACN–H_2O, 45:50) with B 0% to 100% in 30 min at a flow rate of 2 ml/min. When developed further with EtOAc–MeOH (1:1), fractions 37–44 showed the presence of two glycosides, which, after preparative TLC (solvent, $CHCL_3$–MeOH 9:1), were shown to be the glycosides of cucurbitacin E and I (2-*O*-β-D-glucopyranosyl-cucurbitacin E, and 2-*O*-β- D-glucopyranosyl-cucurbitacin I, respectively) by comparison with standard samples (R_f 0.34 and 0.24, respectively).

Visualization. Vanillin–phosphoric acid reagent was used (Duncan et al., 1968). TLC plates were heated at 120°C for 10 min and examined under UV light at 365 nm. Cucurbitacins appeared as red or yellow spots. α-Ketol cucurbitacins were visualized by spraying with triphenyltetrazolium chloride reagent (TTC) (Duncan et al., 1968) and appeared as red spots after heating to 100°C for 5 min, which intensified over steam. Diosphenol cucurbitacins were visualized with $FeCL_3$ reagent (Polman, 1975) and appeared as blue spots.

UV analysis was performed with a Hewlett Packard 8452A Diode Array Spectrophotometer. IR spectra were analyzed by Perkin-Elmer FT-IR Spectrometer 1720X. NMR spectra were examined on a Brucker AM 250 MHz, and MS spectra on a VG 7070F, EI 70 eV. Melting points were measured using a Kofler hot-stage microscope. Optical rotation was measured on Perkin-Elmer model 241 polarimeter.

Compound 1 (cucurbitacin D) (Fig. 1). White crystals (84 mg from MeOH), mp 150–152°C, $[\alpha]_D^{25}$ + 55 (c = 1, MeOH), positive reaction with TTC reagent (i.e., α-ketol system in ring A), λ_{max} (MeOH) 230 nm; IR (KBr) 3450, 1725, 1705, 1685, 1450, 1375, 1225 and 1085 cm_1; MS *m/z* (rel. int.) 516.31 (0.5), 499.30 ($M^+ - H_2O$ + H) (3.9), 481.29 ($M^+ - 2H_2O$ + H) (1.8), 464.26 (1.6), 403.23 (3.9), 369.20 (6.1), 203.12 (7.2), 189.10 (10.4), 166.07 (11.3), 113.05 (87.7), 111.05 (75), and 96.04 (100%).

Compound 2 (3-epi-*isocucurbitacin D) (Fig. 1).* White crystals (15 mg, from MeOH), mp 178–180°C, $[\alpha]_D^{25}$ − 78° (c = 1, $CHCl_3$), positive reaction with TTC reagent (i.e., α-ketol system in ring A), λ_{max} (MeOH) 230 nm, IR (KBr), 3450, 1725, 1705, 1685, 1450, 1375, 1235, 1085 cm^{-1}. MS *m/z* (rel. int.): 516.31 (0.4), 499.30 ($M^+ - H_2O$ + H) (4.8), 480.29 (2.1), 464.26 (1.6), 403.23 (2.7), 369.20 (8), 203.12 (8.8), 189.10 (13.6), 166.07 (8.3), 115 (22.6), 113.05 (95.7), and 111.05 (100%).

TABLE 1. ^{1}H NMR SHIFTS OF RELEVANT PROTONS OF COMPOUNDS **1**, **2** AND **3**[a]

	Compound		
Proton No.	1	2	3
2	4.45 dd(14, 7)		4.44 dd(14, 7)
3		4.10	
6	5.75 m	5.75 m	5.75 m
12β	2.74 br d(15)	2.72 br d(15)	2.74 br d(15)
12α	3.28 br d(15)	3.20 br d(15)	3.20 br d(15)
16	4.34 br t(7)	4.33 br t(7)	4.34 br t(7)
17	2.52 d(7)	2.40 d(7)	2.60 d(7)
23	6.62 br d(15)	6.62 br d(15)	6.62 br d(15)
24	7.12 br d(15)	7.12 br d(15)	7.12 br d(15)
Methyls:			
18	0.95	0.95	0.98
19	1.09	1.10	1.08
21	1.34	1.34	1.35
26	1.34	1.36	1.40
27	1.38	1.38	1.40
28	1.32	1.36	1.25
29	1.34	1.34	1.35
30	1.30	1.34	1.35
25-OAc			2.02

[a]Coupling constant (J) in Hz is quoted in parentheses. Signals are singlets unless otherwise indicated.

Compound 3 (cucurbitacin B) (Fig. 1). White crystals (30 mg, from MeOH), mp 186–188°C [lit. (Jacobs et al., 1990) mp 184°C], $[\alpha]_D^{25}$ + 83 (c ; eq 1, MeOH), positive reaction with TTC reagent (i.e., α-ketol in ring A); λ_{max} (MeOH) 230 nm; IR (KBr) 3450, 1725, 1707, 1690, 1450, 1375, 1225, and 1085 cm^{-1}; MS m/z (rel. int.), 499.30 (M^+ − acetic acid + H) (0.7), 498.30 (4), 480.30 (2.7), 465.25 (4.6), 403.23 (1.5), 369.20 (18.5), 203.12 (10.9), 189.10 (23.9), 166.07 (16.3), 113.05 (21.2), 111.05 (89.5), 96.04 (99.4), 60.03 (32.5), and 43.02 (CH_3CO) (100%).

The ^{1}H NMR and ^{13}C NMR data of compounds **1**, **2**, and **3** are presented in Tables 1 and 2.

RESULTS AND DISCUSSION

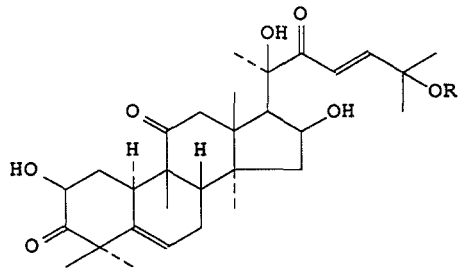
The mass spectrum of compound **1** showed the (M^+) peak at m/z 516.3098 ($C_{30}H_{44}O_7$) with the two fragments at m/z 499.2988 and 480.2879, which is in accordance with the molecular formulas of $C_{30}H_{42}O_6$ + H and $C_{30}H_{40}O_5$,

TABLE 2. ^{13}C NMR SHIFTS OF COMPOUNDS **1**, **2** AND **3**[a]

Carbon No.	Compound 1	2	3
1	36.02	36.45	35.98
2	71.16	210.98	71.63
3	212.38	79.45	212.51
4	50.67	47.86	50.66
5	140.35	140.13	140.41
6	120.10	121.3	120.45
7	24.28	24.36	23.85
8	41.32	42.4	42.37
9	48.30	48.73	48.42
10	34.63	32.36	33.72
11	213.12	212.39	213.07
12	48.27	48.83	48.09
13	50.24	50.63	50.21
14	48.27	48.35	48.63
15	45.42	45.63	45.21
16	70.40	71.14	71.25
17	58.10	57.43	58.19
18	20.17	19.98	19.81
19	20.20	20.40	20.08
20	77.98	78.09	77.20
21	23.76	23.94	23.94
22	202.44	202.68	202.51
23	119.01	119.01	120.33
24	154.80	155.73	152.00
25	72.68	72.41	79.30
26	30.31	29.51	26.38
27	28.30	29.01	26.00
28	28.26	28.69	29.33
29	21.01	19.98	21.22
30	19.04	18.68	18.98
25-OAc			170.25

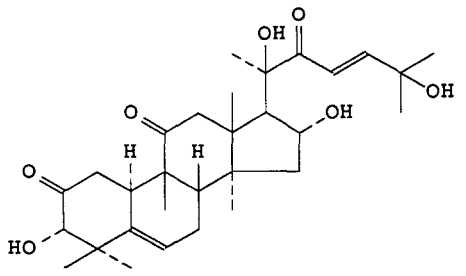
[a]Shifts in ppm, solvent $CDCl_3$ and TMS as standard.

respectively, which arise due to the consecutive loss of two molecules of water. A cucurbitane nucleus was shown by ^1H NMR data (Table 1) such as δ 5.75 (m), δ 3.28 (d, J = 15), δ 2.74 (d, J = 15) and δ 4.34 (t, J = 7) assigned for H-6, 12α-H, 12β-H and H-16, respectively (Velde and Lavie, 1983). In addition, the olefinic center in the side chain at C-23, 24 was identified at δ 6.61 (d, J = 15) and δ 7.12 (d, J = 15) assigned for H-23 and H-24, respectively, and the double doublet at δ 4.45 [dd, J = 14, 7] was assigned for H-2 in the



1 R= H

3 R= COCH3



2

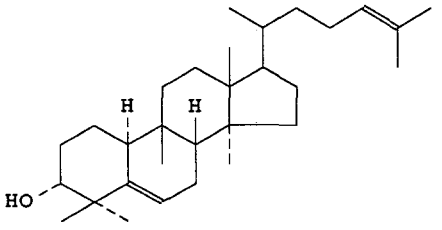


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FIG. 1. Structures of cucurbitacins D, **1**, 3-epi-isocucurbitacin D, **2**, and B, **3**, and the cucurbitacin precursor 10α-cucurbitan-5,24-dien-3β-ol, **4**.

α-ketol system in ring A (Velde and Lavie, 1983; Jacobs et al., 1990). The latter statement indicated that compound **1** was a normal cucurbitacin (i.e., 2-hydroxy-3-oxo) in ring A. The rest of the proton shifts were assigned by comparison, which all support the structure of compound **1** as cucurbitacin D (Velde and Lavie, 1983; Jacobs et al., 1990). ^{13}C NMR data (Table 2) showed

complete similarity with published data (Velde and Lavie, 1983; Jacobs et al., 1990) and also confirmed that compound **1** is cucurbitacin D.

The mass spectrum of compound **2** showed the (M^+) peak at *m/z* 516.3083 ($C_{30}H_{44}O_7$) with the mass fragments at 499.2872 [$M^+ - H_2O$ + H] and *m/z* 480.2981 ($M^+ - 2H_2O$), which is in accordance with the molecular formulas of { $C_{30}H_{42}O_6$ and $C_{30}H_{40}O_5$ and confirms the (M^+) peak. The main features of its ^1H NMR (Table 1) spectrum were of a cucurbitane nucleus, such as δ 5.75 for H-6, δ 3.28 (d, J = 15) for 12α-H, δ 2.70 (D, J = 15) for 12β-H, δ 4.3 (t, J = 7) for H-16, and $\Delta^{23,24}$ protons at δ 6.60 (d, J = 15) and δ 7.12 (d, J = 15) for H-23 and 24, respectively (Velde and Lavie, 1983; Jacobs et al., 1990). The latter unsaturated bond was confirmed by the presence of the major peak in the MS spectra at *m/z* 111.043 (100%) (Kupchan et al., 1970). A sharp singlet at δ 4.1 was assigned for the C-3 methine hydrogen in the α-ketol and indicated that compound **2** is a member of the isocucurbitacin series (Kupchan et al., 1978; Hylands and Magd, 1986). The 2-hydroxyl-3-oxo-cucurbitacins, such as **1,** show the signal for H-2 at δ 4.45 as a double doublet (Velde and Lavie, 1983). The fact that H-3 in compound **2** resonated at δ 4.1 is evidence for an axial hydrogen (i.e., equatorial hydroxy group) and indicative of an epimeric isocucurbitacin known as 3-*epi*-isocucurbitacin D **2,** which was reported previously from *Phormium tenax* Forest (Arisawa et al., 1984). The structure of the rest of the molecule was established primarily by comparison of its ^1H NMR data with those known for cucurbitacins and further confirmed with ^{13}C NMR (Table 2), which showed complete similarity to cucurbitacin data (Velde and Lavie, 1983; Jacobs et al., 1990). Furthermore, the α-ketol system in ring A was positively confirmed by reaction with TTC reagent (Duncan et al., 1968). All of these data proved compound **2** to be 3-*epi*-isocucurbitacin D.

Compound **3** showed chemical shifts (Tables 1 and 2) identical with those of cucurbitacin D (compound 1), except for a sharp singlet at δ 2.02 assigned to an acetate group and accommodated at C-25 hydroxyl, which was also confirmed by ^{13}C NMR shifts at 170.25 and 20.25 ppm. (Velde and Lavie, 1983; Jacobs et al., 1990). This indicated that compound **3** is cucurbitacin B, which was also proven through comparison of the physical and MS published data (Arisawa et al., 1984).

This represents the first isolation and identification of cucurbitacins B, D, and 3-*epi*-isocucurbitacin D, and the identification of cucurbitacin I glucosides from *C. texana.* The latter glucoside was identified by comparison with known standard. It is also the first isolation of 3-*epi*-isocucurbitacin D **3** from any member of the genus *Cucurbita.* Of further significance is the presence and isolation of 3-*epi*-isocucurbitacin D **3** under the mildest conditions of extraction and preparation, confirming previous suggestions (Halaweish, 1987; Hylands and Magd, 1986) that isocucurbitacins are naturally occurring compounds rather than artifacts (Hylands and Mansour, 1983; Kupchan et al., 1978; Arisawa et

al., 1984). The presence of 3-*epi*-isocucurbitacin D **3** in *C. texana* tissues was also confirmed in a fresh fruit sample extract (screened with HPTLC and HPLC), which excludes the possibility of formation during the purification processes.

The results of this study suggest an important step in the biosynthetic pathway of cucurbitacins which, to date, has not been considered in as much detail as have the pathways of other related groups of triterpenes and steroids. The order of oxidation in ring A primarily determines the configuration of cucurbitacins as normal or iso. From our results and some earlier work (Halaweish, 1987), we offer the following support for the hypothesis that isocucurbitacins are formed one step prior to normal cucurbitacins. Cucurbitacin formation begins with squalene-2, 3-epoxide and proceeds through other intermediates to the main cucurbitacin precursor, 10α-cucurbita-5,24-dien-3β-ol **4** (Fig. 1) (Balliano et al., 1983). The latter compound **4** and isocucurbitacins are hydroxylated at C-3 rather than C-2. Other related aglycones and glycosides such as bryogenin and bryosides (isolated from *Bryonia dioica*) are also hydroxylated only at C-3 in ring A (Hylands and Kosugi, 1982). Further evidence is provided by the transformation of the intermediate **4** to cucurbitacin C after incubation with seedlings of *Cucumis sativus* L; cucurbitacin C has only C-3 OH in ring A (Balliano et al., 1983). Moreover, cucurbitacins such as F, O, P and Q (2,3 diol in ring A) (Lavie and Glotter, 1971) provide a possible intermediate in the process of transformation of iso to normal cucurbitacin. Perhaps the best evidence comes from the fact that labeled isocucurbitacin B has been retrieved from seedlings and tissue cultures of *B. dioica* previously fed with labeled precursors (Halaweish, 1987). All of these data suggest that hydroxylation takes place at C-3 first rather than at C-2 and indicate that isocucurbitacin formation occurs one step ahead of normal cucurbitacin biosynthesis.

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REFERENCES

ARISAWA, M., PEZZUTO, J.M., KINGHORN, A.D., CORDELL, G.A., and FARNSWORTH, N.R. 1984. Plant anticancer agents XXX: Cucurbitacins from *Ipomopsis aggregata* (Polemoniaceae). *J. Pharm. Sci.* 73:411-413.

BALLIANO, G., CAPUTO, O., VIOLA, F., DELPRINO, L., and CATTEL, L. 1983. The transformation of 10α-cucurbita-5-24-dien-3β-ol into cucurbitacin C by seedlings of *Cucumis sativus*. *Phytochemistry* 22:909-913.

DEHEER, C.J., and TALLAMY, D.W. 1991. Cucumber beetle larval affinity to cucurbitacins. *Environ. Entomol.* 20:775-781.

DUNCAN, G.R., LEVI, D.D., and PYTTEL, R. 1968. Bitter principles of Cucurbitaceae—*Bryonia dioica*. *Planta Med.* 16:224-229.

FERGUSON, J.E., and METCALF, R.L. 1984. Cucurbitacins: Plant-derived defense compounds for controlling Diabroticite (Coleoptera Chrysomelidae). *J. Chem. Ecol.* 11:311.

HALAWEISH, F. 1987. Cucurbitacins in tissue cultures of *Bryonia dioica* Jacq. PhD thesis. University of Wales, Cardiff, U.K.

HYLANDS, P.J., and KOSUGI, J. 1982. Bryonoside and bryoside, new triterpene glycosides from *Bryonia dioica*. Jacq. *Phytochemistry* 21:1379–1385.

HYLANDS, P.J., and MAGD, M. 1986. Cucurbitacins from *Acanthosicyos horridus*. *Phytochemistry* 25:1681–1684.

HYLANDS, P.J., and MANSOUR, El-S.S. 1983. New cucurbitacin derivatives from *Bryonia dioica* Jacq. *J. Chem. Soc. Perkin Trans.* 1:2821–2825.

JACOBS, H., SINGH, T., REYNOLDS, W.F., and MCLEAN, S. 1990. Isolation and ^{13}C-NMR assignments of cucurbitacins from *Cyanoponia angustloba*, *C. racemosa* and *Gurania submrellata*. *J. Nat. Prod.* 53:1600–1605.

KUPCHAN, S.M., SMITH, R.M., AVNEHCHI, Y., and MARUYAMA, M. 1970. Tumor inhibitors LVI. cucurbitacins O, P, and Q, the cytotoxic principles of *Brandegea bigelovii*. *J. Org. Chem.* 35:2891–2994.

KUPCHAN, S.M., MESHULAM, H., and SNEDEN, A.T. 1978. New cucurbitacins from *Phorium tenax* and *Marah oreganus*. *Phytochemistry* 17:767–769.

LAVIE, D., and GLOTTER, D. 1971. The cucurbitacins, a group of tetracyclic tritepenes. *Fortschr. Chem. Org. Naturst.* 29:306–364.

METCALF, R.L. 1985. Plant kairmones and insect pest control. Bull. III Natur. Hist. Surv. 33:175.

METCALF, R.L., and RHODES, A.M. 1985. Ca. Pat. 1,195,992.

METCALF, R.L., RHODES, A.M., METCALF, R.A., FERGUSON, J.E., METCALF, E.R., and PO-YUNG, L. 1982. Cucurbitacin contents and diabroticite (Coleoptera: Chrysomelidae) feeding upon *Cucurbita* spp. *Environ. Entomol.* 11:931–937.

METCALF, R.L., FERGUSON, J.E., LAMPMAN, R., and ANDERSON, J.F. 1987. Dry cucurbitacin-containing baits for controlling diabroticite beetles (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 80:870–875.

POLMAN, J. 1975. Die cucurbitacine in *Bryonia alba* and *Bryonia dioica*. *Phytochemistry* 14:1587–1589.

TALLAMY, D.W., and KRISCHIK, V.A. 1989. Variation and function of cucurbitacins in *Cucurbita*: An examination of current hypotheses. *Am. Nat.* 133:766–786.

VELDE, V.V., and LAVIE, D. 1983. ^{13}C NMR spectroscopy of cucurbitacins. *Tetrahedron* 39:317–321.

WITKOWSKI, A., and KONOPA, J. 1981. Selective inhibition of thymidine incorporation into lymphocytes by cucurbitacin B and D. *Biochem. Biophys. Acta* 674:246–250.

WITKOWSKI, A., WOYNAROWSKA, B., and KONOPA, J. 1984. Inhibition of the biosynthesis of deoxyribonucleic acid, ribonucleic acid and protein in HeLa S3 cell by cucurbitacins, glucocorticoid-like cytotoxic triterpenes. *Biochem. Pharmacol.* 33:995–998.

WHITHOUSE, M., and DOSKOTCH, W. 1969. Binding of the cytotoxic and antitumor tritepenes, cucurbitacins, to glucocorticoid receptor of HeLa cells. *Biochem. Pharmacol.* 18:1790–1793.

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CHEMICAL IDENTIFICATION OF DEFENSIVE SECRETION OF STICK INSECT, *Megacrania tsudai* SHIRAKI

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Abstract—Volatile constituents of the defensive secretion of the stick insect, *Megacrania tsudai* Shiraki, in Taiwan have been analyzed by gas chromatography–mass spectrometry (GC-MS). In addition to the major component, actinidine, three more minor components of the secretion are identified as boschniakine and two stereoisomers of 1-acetyl-3-methylcyclopentane by comparing GC retention times with the authentic samples and synthetic compounds. Other components are also tentatively assigned as derivatives of actinidine.

Key Words—Stick insect, *Megacrania tsudai*, Orthoptera, Phasmatidae, actinidine, 1-acetyl-3-methylcyclopentane, boschniakine, screw pine, *Pandanus tectorius*.

INTRODUCTION

Iridodial was first described as a defensive compound from the species of Phasmida, viz. *Anisomorpha buprestoides* by Meinwald et al. (1962). Later on, in addition to iridodial, nepetalactone was also detected in the defensive secretion of the coconut stick insect, *Graeffea crouani* (Smith et al., 1979). In our previous report (Chow and Lin, 1986), a third compound, actinidine, was found to be the major component of the defensive secretion of the local stick insect, *Megacrania alpheus* Westwood. These compounds could be used as insecticidal fumigants for other insects (Dettner et al., 1992). The scientific name of our stick insect has recently been corrected to *M. tsudai* Shiraki according to Hsiung

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(1991). In the current investigation new minor components were identified by GC-MS: 1-acetyl-3-methylcyclopentane and derivatives of actinidine. Actinidine was also found in the insect's dietary plant, the screw pine, *Pandanus tectorius*, but at a lower concentration. Apparently the stick insect fed on the plant and therefore concentrated the actinidine in its gland for defensive purposes as the ant does. (Tomalski et al., 1987).

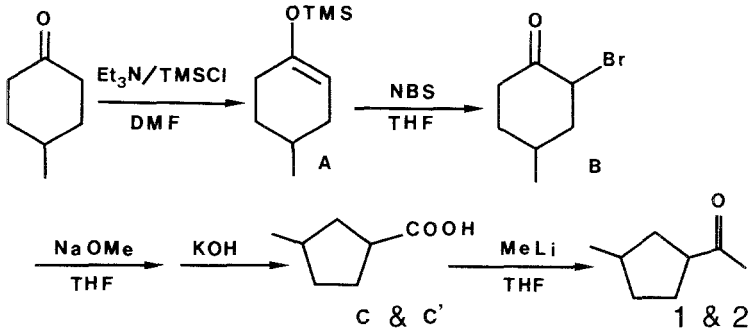
METHODS AND MATERIALS

Biological Material. Adults of *M. tsudai* were collected at the O-luan-bi, southern coast area of Taiwan. The adults were then reared on the leaves of screw pine, *Pandanus tectorius*, collected on the coast of Taiwan. The stick insect reared in the laboratory reproduced by parthenogenesis, and the eggs hatched to give nymphs of different stages. After the stick insect hatched, the eggshell was collected, ground, and extracted with methanol. The leaves of the screw pine were also ground and extracted with MeOH overnight. Extraction was repeated three times, and the extract was concentrated for GC-MS analysis. The defensive secretion of the stick insect after irritation by handling was collected with a microsyringe and directly injected into GC-MS for analysis.

Analytical Techniques. The GC-MS analysis was carried out on a Varian 3400 gas chromatograph coupled to a Finnigan MAT Incos 50 mass spectrometer. GC-MS data acquisition was made using electron impact ionization (EI) as well as chemical ionization (CI) (with methane as reactant gas). GC-MS analysis was performed on a DB-5 fused silica capillary column (0.25 mm × 30 m). Elution was programmed from 40°C (hold for 2 min), then from 40°C to 200°C with a heating rate of 2°C/min, and from 200°C to 250°C with a heating rate of 10°C/min (carrier gas: helium).

Benzophenone was used as internal standard to determine the peak areas of the GC-MS chromatogram. 1-Acetyl-3-methylcyclopentane was prepared from 4-methylcyclohexanone according to literature procedures as shown in Scheme 1. 4-Methylcyclohexanone was obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin).

Silyl Enol Ether (A). To freshly distilled trimethylsilyl chloride (15.2 ml) and triethylamine (33.4 ml) in dimethylformamide was added 4-methylcyclohexanone (11.2 g). The mixture was refluxed with stirring for 24 hr, then cooled, diluted with 50 ml pentane, and washed with 50 ml of cold aqueous $NaHCO_3$. The organic layer was washed rapidly in succession with portions of cold aqueous 1.5 M HCl and cold aqueous $NaHCO_3$. The pentane solution was dried and concentrated (House et al., 1969). The product, silyl enol ether (compound A), was distilled under vacuum (20 torr, 70°C). Mass spectral data are as follows: *m/z* 184(30), 169(60), 142(62), 127(100), 75(80), 73(50).



SCHEME 1. Preparation of 1-acetyl-3-methylcyclopentane.

2-Bromo-4-methyl-cyclohexanone (B). To 3.03 g of silyl enol ether (A) in 50 ml dry tetrahydrofuran (THF) at 0°C was added 3 g of *N*-bromosuccinimide (NBS). After stirring for 15 min, the reaction mixture was poured into aqueous $NaHCO_3$ and NaCl solution. The mixture was extracted with petroleum ether. The organic extracts were dried over $NaSO_4$ and concentrated under vacuum to afford the crude product (Reuss and Hassner, 1974). The crude was distilled under reduced pressure (0.1 torr, 62–64°C) to afford the pure product (B), with mass spectral data as follows: *m/z* 192(15), 190(15), 148(9), 146(9), 135(3), 133(3), 111(45), 55(100).

3-Methyl-cyclopentanecarboxylic Acid (C and C'). To dry THF solution of 0.09 g NaOMe in an ice bath was added 0.3 g of bromoketone (compound B) in 10 ml dry THF. The reaction mixture was then refluxed for 2 hr (Goheen and Vaughan, 1963). After the reaction mixture cooled, an alcoholic solution of KOH was added, and the mixture was stirred under room temperature overnight. Then the mixture was poured into ice-water and extracted with ether. The ether layer was discarded. The aqueous layer was acidified with dilute HCl solution and then extracted with ether. The ether extract was dried over $MgSO_4$, concentrated under vacuum to afford the product compound C and C'. According to GC-MS analysis there were two isomers with mass spectral data as follows: compound C *m/z* 128(5), 113(13), 100(10), 86(58), 73(85), 67(40), 56(100); compound C' *m/z* 128(5), 113(13), 100(10), 86(42), 73(93), 67(40), 56(100). Compound C and C' are stereoisomers with retention times of 15 min 3 sec and 14 min 48 sec on column DB-WAX with heating rate of 10°/min from 50°C to 240°C.

1-Acetyl-3-methylcyclopentane (1 and 2). To a solution of compound C and C' in dry THF maintained at 0°C under N_2 atmosphere, was added methyllithium in ether (2 ml of 1.4 M) over a period of 3 min. After about 20 min, the reaction mixture was poured into water. The organic phase was separated,

washed with water, dried over Na_2SO_4 and the solvent evaporated to give the product mixture, compound **1** and compound **2** (Reddy et al., 1986).

RESULTS AND DISCUSSION

The gas chromatogram of the defensive secretion of the stick insect is shown in Figure 1. Table 1 lists the molecular weights, mass spectral data of the nine components, amount (milligrams) of each component contained per milliliter of the secretion and the identities of the components.

From the mass spectral data of peaks 1 and 2, both unknowns give the molecular ion of *m/z* 126 and major fragmentation ion of *m/z* 111 ($M^+ - 15$),

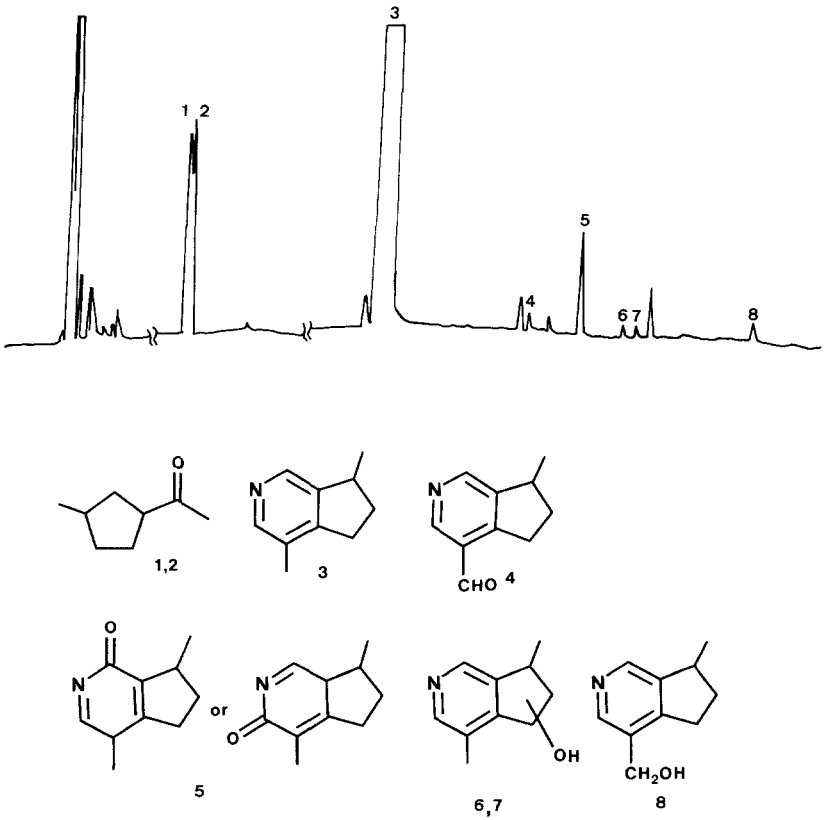


FIG. 1. Gas chromatogram of the defensive secretion of the stick insect, *Megacrania tsudai*.

TABLE 1. VOLATILE CHEMICAL CONSTITUENTS OF DEFENSIVE SECRETION OF STICK INSECT, *Megacrania tsudai*

Peak	R_t	Mol wt	Confirmed	Amount (mg)	Compound assigned	Mass spectral data *m/z* (%)
1	18 min 11 sec	126	yes	0.25	1-acetyl-3-methylcyclopentane	126(19), 111(14), 108(9), 83(35), 71(100), 55(68), 43(70)
2	18 min 17 sec	126	yes	0.33	1-acetyl-3-methylcyclopentane	126(25), 111(17), 108(3), 83(61), 71(80), 55(100), 46(95)
3	44 min 33 sec	147	yes	77	actinidine	147(52), 132(100), 117(50), 103(8), 91(10), 77(18), 63(15), 51(30)
4	54 min 14 sec	161	yes	0.03	boschniakine	161(100), 146(90), 132(50), 118(55), 117(90), 91(55), 77(35), 63(40)
5	59 min 20 sec	163	no	0.17	dehydro lactam of actinidine	163(5), 148(100), 133(5), 120(18), 91(10), 77(80), 65(10)
6	61 min 33 sec	163	no	0.02	hydroxyactinidine	163(50), 148(70), 145(100), 144(98), 130(50), 120(60), 91(42), 77(48)
7	62 min 01 sec	163	no	0.03	hydroxyactinidine	163(75), 148(100), 145(90), 130(70), 120(80), 91(55), 77(60)
8	67 min 08 sec	163	no	0.08	hydroxyactinidine	163(38), 148(28), 145(50), 130(100), 118(40), 117(40), 103(20), 91(30), 77(30)

108 ($M^+ - 18$), 83 ($M^+ - 43$, corresponding to $M^+ - CH_3CO$), 71 ($C_5H_{11}^+$), indicating that there was an acetyl group and a cyclopentyl group in the structure. By comparison of the fragmentation patterns and retention times of possible defensive pheromones with a molecular weight of 126 and the cyclopentyl group (Wheeler et al., 1974), 1-acetyl-3-methylcyclopentane was suggested to be a possible candidate. When 1-acetyl-3-methylcyclopentane was synthesized (a mixture of *cis* and *trans* stereoisomers), it was found to coelute with the unknowns 1 and 2. Thus, it was concluded that peaks 1 and 2 were stereoisomers of 1-acetyl-3-methylcyclopentane (compounds **1** and **2**).

Peak 3 constitutes the major component of the secretion, i.e., actinidine (compound **3**), which was identified in our previous report (Chow and Lin, 1986) and the mass spectrum is the same as reported by Tomalski et al. (1987). The component of peak 4 has a retention time of 54 min 14 sec and a similar mass spectral pattern as that of actinidine, even though each fragment ion differs by 14 mass units. The major fragment ions are ions with *m/z* 161 (M^+), 146 ($M^+ - 15$) and 132 ($M^+ - 29$). When picric acid was added to precipitate actinidine, peak 4 also disappeared, suggesting that this component may relate to actinidine. As the M^+ is 14 mass units higher than that of actinidine, it was assumed that this compound is a derivative of actinidine with one of the methyl groups changed to CHO, based on the peak *m/z* 132, which corresponds to $M^+ - CHO$. A literature search led to boschniakine as a possible candidate. Coinjection with authentic boschniakine (compound **4**) (a gift from Dr. Stermitz of Colorado State University; McCoy and Stermitz, 1983) presented unambiguous evidence supporting the original assignments.

The compound of peak 5 at retention time of 59 min 20 sec showed mass fragmentation of *m/z* 163 (M^+), 148 ($M^+ - 15$), 133 ($M^+ - 30$), 120 ($M^+ - 43$). This compound did not give precipitates with picric acid since this peak still remained in the GC-MS analysis of the supernatant after the picric acid treatment. This compound was tentatively assigned as the dehydrolactam of actinidine (compound **5**).

The amount of actinidine in the leaves of the screw pine was about 0.02 mg/g of wet leaves, and the secretion of the stick insect contained about 77 mg/ml of the secretion; the actinidine was concentrated more than 3500 times in the gland of the stick insect. The stick insects fed on the plants containing actinidine and presumably concentrated it in gland tissue for defensive purposes (Tomalski et al., 1987). No actinidine was found in the eggshell of the stick insect.

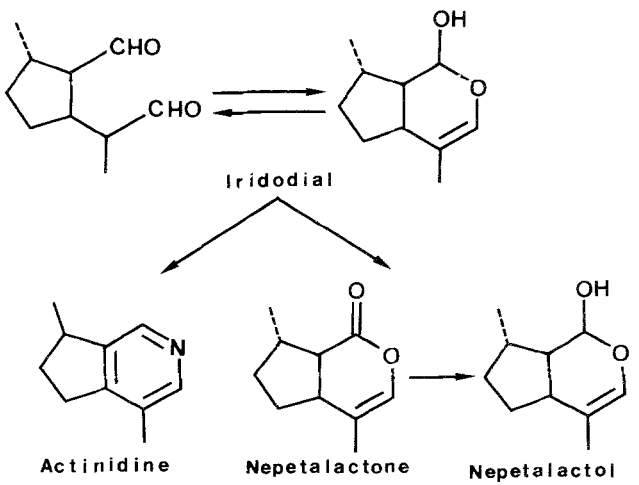
The amounts of other components in the secretion were about 0.03 mg boschniakine and 0.6 mg of 1-acetyl-3-methylcyclopentane per milliliter of the secretion.

There was another compound (peak 8) with retention time of 67 min 08 sec and mass fragmentation of *m/z* 163 (M^+), 148 ($M^+ - 15$), 145 ($M^+ -$

18). Since the fragmentation pattern was similar to both actinidine and boschniakine, the peak with *m/z* 145 corresponding to $M^+ - H_2O$, indicated an alcohol group in the structure. It is proposed that this compound might be the reduced form of boschniakine (compound **8**). Peaks 6 and 7, with a molecular weight of 163 (16 mass units higher than actinidine) show a peak at *m/z* 145, corresponding to $M^+ - 18$ ($M^+ - H_2O$). Because their mass spectrum is similar to actinidine, they were tentatively assigned as isomers of hydroxyactinidine (compounds **6** and **7**).

In conclusion, the major component of the defensive secretion of the *Megacrania* stick insect was actinidine, comprising 99% of the volatile components of the secretion. Other minor compounds were found to be 1-acetyl-3-methylcyclopentane and compounds related to actinidine. Actinidine was also found in its dietary plant, the screw pine, *Pandanus tectorius*, but at a very low concentration. Although iridodials and nepetalactone described in other stick insects have not been found in this species, the biosynthetic pathway of actinidine and nepetalactone from the iridodial had been proposed by Cavill and Hinterberger (1960, 1962, cited in Blum, 1981). Their simplified biogenetic pathway is shown in Scheme 2.

According to Scheme 2, both actinidine and nepetalactone could be derived from iridodial. Actinidine from the local stick insect had a strong defensive action against its parasites and against predatory spiders and birds (Chow and Lin, 1986; Carlberg, 1986; Nentwig, 1990). On the other hand, nepetalactone and nepetalactol were recently identified as the sex pheromones of aphids, *Megoura vicial* (Dawson et al., 1987, 1990). It is interesting to note that the



SCHEME 2. Biosynthetic pathway of actinidine and nepetalactone.

biological functions of the above chemicals are quite different. Actinidine acts as a repellent between different species in tropical areas, whereas nepetalactone or nepetalactol acts as a sex attractant within aphid species in temperate zones; yet both compounds might derive from the same precursor.

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REFERENCES

BLUM, M.S. 1981. Chemical Defenses of Arthropods. Academic Press, New York, p. 390.

CARLBERG, U. 1986. Chemical defence in *Sipyloides sipylu* (Westwood) (Insecta: Phasmida). *Zool. Anz.* 217:31–38.

CHOW, Y.S., and LIN, Y.M. 1986. Actinidine, a defensive secretion of stick insect, *Megacrania alpheus* Westwood (Orthoptera: Phasmatidae). *J. Entomol. Sci.* 21(2):97–101.

DAWSON, G.W., GRIFFITHS, D.C., JANES, N.F., MUDD, A., PICKETT, J.A., WADHAMS, L.J., and WOODCOCK, C.M. 1987. Identification of an aphid sex pheromone. *Nature* 325: 614–616.

DAWSON, G.W., GRIFFITHS, D.C., MERRITT, L.A., MUDD, A., PICKETT, J.A., WADHAMS, L.J., and WOODCOCK, C.M. 1990. Aphid semiochemicals—a review and recent advances on the sex pheromone. *J. Chem. Ecol.* 16:3019–3030.

DETTNER, K., FETTKOTHER, R., ANSTEEG, O., DEML, R., LIEPERT, C., PETERSEN, B., HASLINGER, E., and FRANCKE, W. 1992. Insecticidal fumigants from defensive glands of insects—a fumigant test with adults of *Drosophila melanogaster*. *Z. Angew. Entomol.* 113:128–137.

GOHEEN, D.W., and VAUGHAN, W.R. 1963. Organic Syntheses Collective Vol IV. John Wiley & Sons, London, pp. 594–596.

HOUSE, H.O., CZUBA, L.J., GALL, M., and OLMSTEAD, H.D., 1969. The chemistry of carbanions. XVIII Preparation of trimethylsilyl enol ethers. *J. Org. Chem.* 34:2324–2336.

HSIUNG, C.C. 1991. The identity of *Megacrania species* of Taiwan (Cheleutoptera: phasmatidae). *Oriental Insects* 25:171–177.

MCCOY, J.W., and STERMITZ, F.R. 1983. Alkaloids from *Castilleja miniata* and *Penstemon whippleanus*, two host species for the plume moth, *Amblyptilia (platyptilia) pica*. *J. Nat. Prod.* 46:902–907.

MEINWALD, J., CHADHA, M.S., HURST, J.J., and EISNER, T. 1962. Defense mechanism of arthropods—IX Anisomorphal, the secretion of a phasmid insect. *Tetrahedron Lett.* 1:29–33.

NENTWIG, W. 1990. Stick insect (Phasmida) as prey of spiders: Size, palatablity and defence in feeding test. *Oecologia* 82:446–449.

REDDY, S.M., DURAISAMY, M., and WALBORSKY, H.M. 1986. Low valent titanium induced cross-coupling of chiral α,β-unsaturated ketones with acetone. *J. Org. Chem.* 51:2361–2366.

REUSS, R.H., and HASSNER, A. 1974. Halogenation of carbonyl compounds via silyl enol ether. *J. Org. Chem.* 39:1785–1787.

SMITH, R.M., BROPHY, J.J., CAVILL, G.W.K., and DAVIK, N.W. 1979. Iridodials and nepetalactone in the defensive secretion of the coconut stick insects, *Graeffea crouani*. *J. Chem. Ecol.* 5:727–735.

TOMALSKI, M.D., BLUM, M.S., JONES, T.H., FALES, H.M., HOWARD, D.F., and PASSERA, L. 1987. Chemistry and functions of exocrine secretions of the ants *Tapinoma melanocephalum* and *T. erraticum*. *J. Chem. Ecol.* 13:253–263.

WHEELER, J.W., EVANS, S.L., BLUM, M.S., and TORGERSON, R.L. 1974. Cyclopentyl ketones: Identification and function in *Azteca* ants. *Science* 187:254–255.

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RELATIVE IMPORTANCE OF INFOCHEMICALS FROM FIRST AND SECOND TROPHIC LEVEL IN LONG-RANGE HOST LOCATION BY THE LARVAL PARASITOID *Cotesia glomerata*

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Abstract—Recently parasitoids were hypothesized to encounter a reliability–detectability problem relating to chemical stimuli from the first and second trophic level, when searching for hosts. The relative role of infochemicals originating from the host, *Pieris brassicae* (second trophic level), and its food plant, cabbage (first trophic level), have been investigated with respect to long-range host location by the larval parasitoid *Cotesia glomerata*. Flight-chamber dual choice tests showed that uninfested cabbage plants are least attractive to female wasps. Host larvae and their feces were more attractive than clean plants but far less attractive than artificially damaged and herbivore-damaged plants. The plant–host complex, with host larvae actively feeding on the plant, was the most attractive odor source for the parasitoids. The data indicate that one of the solutions *C. glomerata* uses to solve the reliability–detectability problem is to respond to infochemicals that are emitted from herbivore-damaged plants. Whether these infochemicals are herbivore-induced synomones that are produced by the plant remains to be demonstrated. Infochemicals emitted by the herbivore or its by-products are of little importance in the foraging behavior of *C. glomerata*.

Key Words—Hymenoptera, Braconidae, Lepidoptera, Pieridae, Cruciferae, tritrophic interactions, foraging behavior, host-habitat location, herbivore-induced synomones, flight chamber, infochemicals.

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INTRODUCTION

Natural enemies of insect herbivores are faced with a variety of stimuli that may be used to locate their victims, even when only one sensory modality is considered, i.e., chemoreception. They may use, for example, chemical stimuli from the herbivore, from the food of the herbivore, or from interactions between the herbivore and the food plant (Nordlund et al., 1981; Vinson, 1976, 1981; Weseloh, 1981). The utility of this chemical information depends on: (1) its reliability in indicating herbivore presence, identity, accessibility and suitability; and (2) its detectability. In this respect the information value of stimuli from the herbivore and stimuli from its host plant differ: herbivore-produced chemicals are obviously the most reliable source of information about herbivore presence and identity, but this information is presumably not very detectable at a distance. Herbivores are but a small component in a complex environment, and their odor production thus constitutes a minor constituent of environmental odors. Moreover, natural selection on reduction of chemical emissions that may attract the herbivore's enemies will be strong. In fact the literature documents only a few examples of natural enemies that are attracted from a distance to volatiles from the herbivore other than pheromones (Monteith, 1964; Nettles, 1980; Ullyett, 1953). In the case of attraction to herbivore pheromones, natural enemies exploit the moments that herbivores are conspicuous because of intraspecific communication (e.g., Vité and Williamson, 1970; Mizell et al., 1984; Lewis et al., 1982; Noldus et al., 1991). On the other hand, information from the plant is expected to be relatively well detectable, but it is much less reliable in indicating herbivore presence and identity. Thus, natural enemies of herbivores are hypothesized to be faced with a reliability–detectability problem (Vet and Dicke, 1992; Wäckers and Lewis, 1992). One of the solutions to this problem is to use herbivore-induced synomones, which are chemicals that are produced by plants in response to (specific) herbivore damage and which attract natural enemies of the herbivore (Vet and Dicke, 1992): these stimuli are both highly detectable (Dicke and Sabelis, 1988; Dicke et al., 1990a, Turlings et al., 1990b, 1991a,b) and very reliable because of their high degree of specificity (Dicke, 1988; Turlings et al., 1990a; Dicke et al., 1990b; Takabayashi et al., 1991a; Dicke and Takabayashi, 1991).

Many natural enemies of herbivores are known to discriminate between the volatile chemicals emitted by uninfested plants and plants infested by a particular herbivore species (e.g., Sabelis et al., 1984; Vet, 1985; Dicke et al., 1989; Eller et al., 1988b; Roth et al., 1982; Turlings et al., 1991a; Steinberg et al., 1992). Some studies have demonstrated that the infochemicals involved are not emitted by the herbivore, but by the plant upon infestation by the herbivore (Sabelis et al., 1984; Turlings et al., 1991a,b; Roth et al., 1982). Other investigations show that the chemicals are not only emitted but also produced by the

plant in response to herbivory, which indicates that the stimuli are herbivore-induced synomones (Dicke et al., 1990a,b, 1993; Turlings et al., 1990b; Takabayashi et al., 1991b; Turlings and Tumlinson, 1992). The production of these chemicals by the plant may be induced by application of oral secretion of the herbivore onto mechanical damage while the oral secretion itself is not attractive to the parasitoids (Turlings et al., 1990b). In some cases, arrestment after contact with oral secretions has been reported (Weseloh, 1981). In this paper, we investigate the reliability–detectability problem for the parasitoid *Cotesia glomerata* and elucidate the relative role of volatile infochemicals from the first and second trophic level during long-range host location by the larval parasitoid *C. glomerata*.

Cotesia (= *Apanteles*) *glomerata* (L.) (Hymenoptera: Braconidae) is a gregarious larval endoparasitoid of several pierid species such as the cabbage caterpillars *Pieris brassicae* (L.) and *Pieris rapae* (L.). Initial studies by Kitano (1978) and Sato (1979) suggested that odors emanating from the plant mediate host-habitat or long-range host location by this parasitoid. Recently, while establishing a basic bioassay to investigate the response of *C. glomerata* to volatile infochemicals, it was found that females are by far more attracted to odors emitted by the complex of host larvae feeding on cabbage plants than by uninfested cabbage plants (Steinberg et al., 1992). Yet the exact origin(s) of those attractive volatiles remains to be elucidated.

We compare the role of three groups of volatile stimuli in the long-range host location (i.e., host location through olfaction, *sensu* Weseloh, 1981) by *C. glomerata*: (1) chemicals emitted from cabbage plants upon mechanical damage, (2) chemicals emitted by larvae of *P. brassicae* or their feces, and (3) chemicals emitted from plants upon infestation by *P. brassicae* larvae.

METHODS AND MATERIALS

Bioassay. In a previous study we have shown that the strongest and most consistent response of *C. glomerata* towards the plant–host complex (PHC) was obtained in a glasshouse flight-chamber set-up by 4- to 5-day-old females that had either experienced feeding damage of first-instar hosts and their products or had oviposited in a first-instar *P. brassicae* larva on a host-damaged leaf 24 hr before the experiment. In this bioassay, parasitoids could fly towards either of two plants that were placed on a table in a white cloth tent in a greenhouse compartment. Two fans positioned behind the plants generated an airstream, and a screen was hung downwind of the plants between these and the point of parasitoid release to avoid visible differences between the two plants tested (see Steinberg et al., 1992, for details). Based on that study, the following conditions were adopted in the following experiments where the response of *C. glomerata*

to various odor sources was examined in three series of dual-choice tests. The parasitoids used were 4- to 5-day-old females that had contacted a leaf that had been fed upon by first-instar *P. brassicae*, but from which the herbivores were removed. This experience was given 24 hr prior to the experiment and lasted for 20 sec. The standard odor source consisted of ca. 2-month-old individually potted Brussels sprouts plant (*Brassica oleracea* cv. Titurel) bearing 10–12 leaves. Damage was inflicted by 60 ± 10 newly hatched *P. brassicae* larvae, distributed in three clusters on three separate leaves. The larvae were allowed to feed for a maximum of 24 hr under climate room conditions of 20 ± 2°C, 50–70% relative humidity, and light regime of 16 : 8 hr light–dark (see Steinberg et al., 1992, for more details on plants, herbivores, parasitoids and procedures).

Series 1: Artificially Damaged Plants. An artificially damaged plant (AD) was obtained by punching three holes, each on a separate leaf, using a 0.8-cm-diameter cork borer. AD had two categories: (1) damage caused only once, just prior to the initiation of the experiment (AD_I); and (2) damage maintained continuously by punching three holes every 15 min during the experiment (AD_{II}); this was done in order to mimic the feeding bouts of first-instar *P. brassicae* larvae (J.J.A. van Loon, personal communication). The artificially damaged plant was tested against two alternating control odors: a clean cabbage plant (CC) and a PHC.

Series 2: Herbivore-Damaged Plants. A herbivore-damaged plant (HD) was obtained by taking a PHC plant and removing the feeding larvae of *P. brassicae* just prior to the experiment. Host by-products such as feces and silk were also removed from the damage spots by means of a fine brush. The herbivore-damaged plant was tested against two alternating control odors: an artificially damaged plant (AD_{II}) and a PHC. In an additional experiment, a choice was given between HD and CC.

Series 3: Herbivore Larvae and Their Feces. The larval odor source (L) was prepared by punching three holes of 0.8 cm diameter in three separate leaves of a plant, 24 hr before the experiment. The hole mimicked the feeding hole through which air could pass over the larvae (see below). The hole was made 24 hr prior to the experiment to avoid the release of plant volatiles that might attract the parasitoid (based on results from series 1; see Figure 1 below). A 3 × 3-cm fine mesh gauze was attached by two entomological pins over each hole on the underside of the leaf. Just prior to the experiment, 50–60 24-hr-old feeding larvae were removed from the PHC and placed in groups of 17–20 larvae at the center of each gauze. A ring of Tanglefoot glue smeared around the larvae prevented them from escaping from the gauze. Thus, the herbivore larvae were isolated, not being able to feed, while their natural spatial distribution on the plant remained closely simulated. The nonfeeding larvae were still producing feces throughout the experiment, but most of the feces fell on the

table, which was well below the height at which the parasitoids were released. Moreover, because we switched the position of the two odor sources after every five or ten parasitoids (see experimental procedure below), the feces were soon on the table at both locations where the two plants were placed. Thus, the feces can not have played an important role in this particular set of experiments. The plant with nonfeeding larvae was tested against two alternating control odors: a clean plant and a herbivore-damaged plant. To control for any volatiles emanating from the holes, gauze, glue, and/or the means of attaching the gauze to the leaf, the same procedure (excluding the addition of the larvae) was carried out on the CC plant and HD plant.

The feces odor source (F) was prepared by placing a clean plant adjacent to a PHC carrying 50–60 newly hatched *P. brassicae* larvae. Three leaves of the clean plant were located under three leaves of the PHC, and for 24 hr before the experiment, feces of the feeding caterpillars dropped on the upper surface of the clean leaves. The plant with feces produced by 50–60 feeding larvae during 24 hr was tested against two alternating control odors: a clean plant and a herbivore-damaged plant. In an additional experiment, a plant with feces was compared with a plant with nonfeeding larvae.

It should be emphasized that the L, F, and HD odor sources that were offered as alternatives to the parasitoids in this series were always derived from a breakdown of the same PHC.

Experimental Procedure, Test Factors, and Data Analysis. In the experiments in which the test odor source was compared with two control odor sources, the latter were alternated after every five replicates (=tested wasps). In addition, the position of the test and control plants was switched left-to-right and vice versa every 10 replicates (five replicates of each choice test) to control for any asymmetry in the setup. The treatments in series 1 were conducted in 34–61 replicates while treatments of the other series consisted of 51–63 replicates. Each treatment was performed over several consecutive days, 10–15 wasps a day (0900–1300 hr), controlling for the day effect (Steinberg et al., 1992).

An oriented flight that ended in first landing of *C. glomerata* on the screen in front of one of the odor source plants, which were separated by an imaginary vertical line (see description of bioassay by Steinberg et al., 1992), was recorded as "choice." In contrast, a nonoriented flight, which resulted in first landing elsewhere in the bioassay arena, was recorded as "no response."

Both the choice and no-response records were analyzed as binary (two-category) data by G test for goodness-of-fit with Williams' correction and correction for continuity or G test of independence with Williams' correction, respectively (Sokal and Rohlf, 1981). The choice analysis indicated the attractiveness of the odor sources within every choice test, whereas the no-response analysis evaluated the responsiveness of the wasps among the choice tests.

RESULTS

Artificially Damaged Plants. Figure 1 shows that few females of *C. glomerata* made a choice when CC and AD_I plants were offered, and those that did so did not distinguish between CC and AD_I odor sources. On the other hand, the PHC was significantly more attractive than AD_I. When AD_{II} was offered vs. CC, all wasps that made a choice did so for AD_{II}, whereas a significantly higher proportion of parasitoids chose for PHC when offered against AD_{II}.

The analysis of responsiveness among the choice tests revealed that the odor sources CC and AD_I were poor, yielding a high proportion of nonresponding individuals (more than 80%) while in the presence of AD_{II}, PHC, or both, the responsiveness of the wasps was higher, i.e., the rate of no response was less than 20% (Figure 1).

Herbivore-Damaged Plants. A herbivore-damaged plant (HD) attracted the majority of parasitoids (more than 95%) when offered versus a clean plant

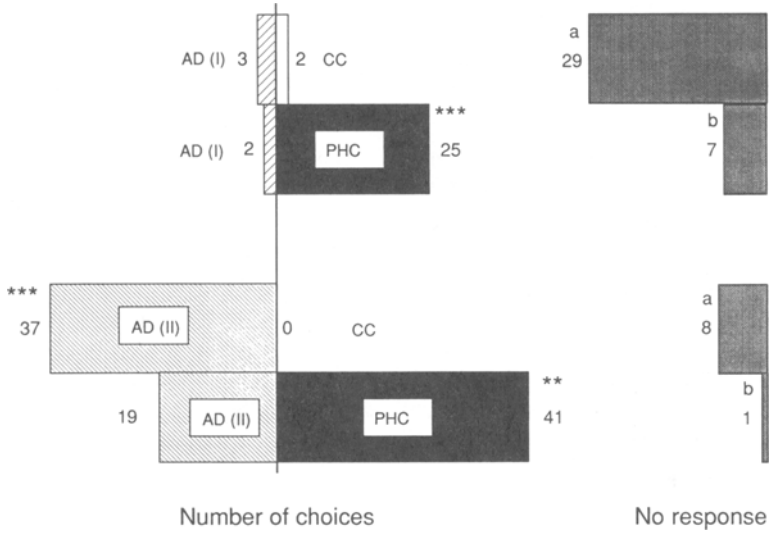

FIG. 1. The effect of infochemicals from artificially damaged plants on foraging behavior of *C. glomerata* in a flight chamber. Results of dual-choice tests with the following odor sources: AD_I—plant with artificial damage inflicted once, just prior to the experiment; AD_{II}—plant with continuously maintained artificial damage; CC—clean cabbage plant; PHC—plant–host complex. Asterisks indicate significant difference within a choice test: $^{**}P < 0.01$; $^{***}P < 0.005$; G test for goodness of fit (Sokal and Rohlf, 1981). Lowercase letters left of no-response bars indicate significant difference within a pair, G test of independence ($P < 0.05$) (Sokal and Rohlf, 1981). Numbers next to bars correspond to numbers of parasitoids.

(Figure 2). Foraging females of *C. glomerata* did not distinguish between HD and AD_{II} plants. However, once a PHC odor source was offered next to HD, the wasps chose the PHC significantly more often (Figure 2).

In all three choice tests involving herbivore-damaged plants, *C. glomerata* showed high responsiveness, with the no-response rate being less than 4% (Figure 2).

Herbivore Larvae and Their Feces. Plants with nonfeeding larvae of *P. brassicae* attracted significantly more wasps than clean plants, while herbivore-damaged plants were significantly more attractive than the nonfeeding larvae (Figure 3). An identical trend was found when larval feces were offered next to CC or HD odor sources. On the other hand, females of *C. glomerata* did not distinguish between L and F plants. It should be noted that most of the feces produced by the nonfeeding larvae during the experiment fell on the table below the plant. Moreover, the amount of feces produced during the experiment by the nonfeeding larvae was much smaller than the amount of feces on the F plant, which was produced by 50–60 feeding larvae during 24 hr.

The analysis of responsiveness showed that L and F were relatively poor

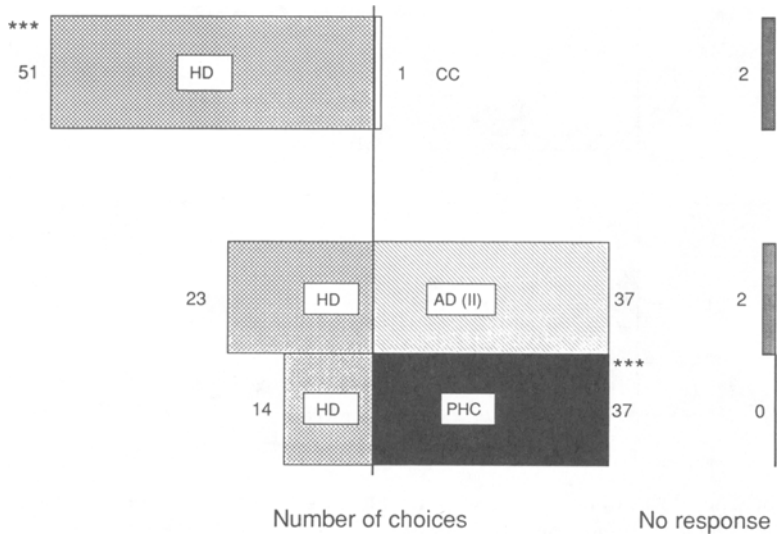

FIG. 2. The effect of infochemicals from herbivore-damaged plants on foraging behavior of *C. glomerata* in a flight chamber. Results of dual-choice tests with the following odor sources: HD—herbivore-damaged plant from which herbivores and their by-products have been removed; AD_{II}—plant with continuously maintained artificial damage; CC—clean cabbage plant; PHC—plant–host complex. Asterisks indicate significant difference within a choice test: $***P < 0.005$; G test for goodness of fit (Sokal and Rohlf, 1981). Numbers next to bars correspond to numbers of parasitoids.

odor sources with no-response rates higher than 50%. Conversely, the presence of HD plants in the choice tests significantly increased the responsiveness of the wasps, i.e., reduced the rate of no response to less than 10% (Figure 3).

DISCUSSION

In all experiments, clean cabbage plants were the least attractive infochemical source for foraging *C. glomerata* females. Infochemicals emitted by caterpillars or their by-products were somewhat attractive to *C. glomerata*, but the percentage of no response was very high. Even though fecal odors may be partially responsible for the observed response of the parasitoids toward larvae, the attractiveness of the larvae is very low (>50% no response). Herbivore-damaged plants (HD) were far more attractive than these odor sources and the percentage of no response was low (<10%) when HD was offered. The most attractive odor source was the PHC. A similar order of attractiveness of PHC components was reported by Elst et al. (1991), although the overall percentage

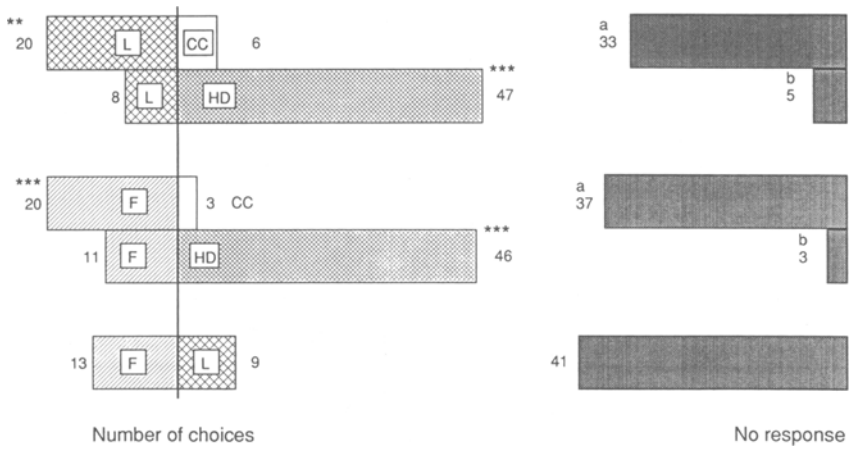

FIG. 3. The effect of infochemicals from nonfeeding first-instar *P. brassicae* larvae and *P. brassicae* feces on foraging behavior of *C. glomerata* in a flight chamber. Results of dual-choice tests with the following odor sources: L—nonfeeding larvae on a cabbage plant; F—feces on a cabbage plant; CC—clean cabbage plant; HD—herbivore-damaged cabbage plant from which herbivores and their by-products have been removed. Asterisks indicate significant difference within a choice test: $^{**}P < 0.01$; $^{***}P < 0.005$; G test for goodness of fit (Sokal and Rohlf, 1981). Lowercase letters left of no-response bars indicate significant difference within a pair, G test of independence ($P < 0.05$) (Sokal and Rohlf, 1981). Numbers next to bars correspond to numbers of parasitoids.

of response by *C. glomerata* females did not exceed 20% in their wind-tunnel experiments.

The experiments with mechanical damage showed that the response to the volatiles emitted from such damage wanes very quickly unless the damage is inflicted at regular intervals (Figure 1). This may explain the observations that herbivore-infested plants from which the herbivores have been removed prior to the experiment (HD) are less attractive than herbivore-infested plants on which the herbivores are still feeding during the experiment (PHC). This situation is comparable to the observations on *Cotesia marginiventris* (Cresson): this parasitoid preferred volatile infochemicals from host-infested corn plants to those from host-infested corn plants from which the hosts were removed or on which the hosts were prevented from feeding during the experiment (Turlings et al., 1991a).

The observation that mechanical damage attracts parasitoids has been reported for other parasitoid species as well (Elzen et al., 1983; Eller et al., 1988a,b; Ding et al., 1989; Turlings et al., 1990b; McAuslane et al., 1991).

Although host odors may be perceived at a distance by *C. glomerata* to some extent, and may be a very reliable indication of actual presence of the host, infochemicals from herbivore-damaged plants evoke the strongest response. This parallels other studies in which herbivore-infested plants were more attractive to natural enemies than the herbivore, its products, or mechanically damaged plants. Some of the natural enemies involved in those studies are the predatory mite *Phytoseiulus persimilis* Athias-Henriot, which feeds on the spider mite *Tetranychus urticae* Koch (Sabelis et al., 1984); the larval parasitoids *Microplitis croceipes* (Cresson) (Eller et al., 1988b) and *Campoletis sonorensis* (Cameron) (Elzen et al., 1984), which parasitize *Heliothis* species; and the larval parasitoid *C. marginiventris*, which parasitizes *Spodoptera* species (Turlings et al., 1991a). For the tritrophic systems bean plants–*T. urticae*–*P. persimilis* and corn plants–*Spodoptera*–*C. marginiventris*, it was demonstrated that the attractive volatiles that were released in large amounts by herbivore-damaged plants were not produced by the herbivore but by the plant (Dicke and Sabelis, 1988, Dicke et al., 1990a,b, 1993; Turlings et al., 1990b; Turlings and Tumlinson, 1992). In addition, hardly any volatiles were detected from *Spodoptera exigua* larvae or their feces (Turlings et al., 1991b) or from *T. urticae* (M. Dicke and M.A. Posthumus, unpublished data) and certainly not the volatiles that are known to attract the natural enemies of these herbivores. These findings suggest that low detectability of herbivore-produced cues is a constraint on the effective use of these infochemicals. Chemical analysis for the current tritrophic system is needed to elucidate the relative amounts of infochemicals released by the plant and herbivore.

Our data show that mechanical damage causes the release of plant chemicals that evoke a strong response in the parasitoid for only a short period of time

after infliction of damage. Moreover, our findings suggest that cabbage plants are involved in the production of the volatile chemicals that are emitted by *Pieris*-damaged plants, since herbivore-infested plants from which the herbivores are removed prior to the experiment are still very attractive to *C. glomerata* (Figure 2). The plants are attractive during at least several hours after removal of the larvae. This indicates that effects of larval damage (which includes mechanical damage) are different from the effects of mechanical damage alone. In order to demonstrate that the chemicals emitted from *Pieris*-damaged plants are actively produced by the plant, more evidence is needed. Evidence for active involvement of the plant is available for two other tritrophic systems consisting of: (1) Lima bean plants, the spider mite *T. urticae*, and the predatory mite *P. persimilis* (Dicke and Sabelis, 1988; Dicke et al., 1990a,b, 1993; Takabayashi et al., 1991b) and (2) corn plants, the army worm *Spodoptera exigua* (Hübner), and the parasitoid *C. marginiventris* (Turlings et al., 1990b, 1991a,b; Turlings and Tumlinson, 1992).

Our data show that the volatiles of the PHC evoke the strongest response. In addition to the involvement of the cabbage plant in the production of these cues, it remains to be investigated how specific the infochemicals in this cabbage–*Pieris*–*Cotesia* system are; i.e., how reliable their information is to the foraging parasitoid. Herbivore-induced synomones may be specific for the herbivore species (Dicke, 1988; Turlings et al., 1990a; Takabayashi et al., 1991a), for the plant species (Dicke et al., 1990b), and for the plant cultivar (Dicke et al., 1990b; Takabayashi et al., 1991a). The infochemicals emitted from *Pieris*-infested cabbage plants seem to be herbivore-species specific: in their flight towards herbivore-infested plants, the parasitoid *Cotesia rubecula* (Marshall) discriminates between plants infested by *P. brassicae* and *P. rapae*, whereas *C. glomerata* does not (Wiskerke and Vet, 1991). Of these two parasitoid species, *C. glomerata* is a generalist parasitoid that attacks several herbivores on cruciferous plants (Sato, 1979; Laing and Levin, 1982), whereas *C. rubecula* preferentially attacks *P. rapae* on cruciferous plants. The response to general cues from cruciferous plants, such as cues resulting from mechanical damage, may be adaptive to a generalist such as *C. glomerata*, whereas a more specialized parasitoid such as *C. rubecula* may need more specific information on the presence of its host species (cf. Vet and Dicke, 1992). Our data on the polyphagous parasitoid *C. glomerata* compare well with the data for the polyphagous species *C. marginiventris* (Turlings et al., 1991a). Although it is known that the specialist parasitoid *C. rubecula* responds to infochemicals from the PHC or from clean cabbage plants (Nealis, 1986; Keller, 1990; Wiskerke and Vet, 1991; Kaiser and Cardé, 1992), no information on the relative importance of infochemicals from the first and second trophic level is available yet for this parasitoid. To obtain information on the adaptiveness of the observed use of infochemicals from the first and second trophic level, comparative studies of

phylogenetically closely related parasitoid species that differ in host and host plant range are essential (Wiskerke and Vet, 1991; Vet and Dicke, 1992). In addition to the comparative study on *C. glomerata* and *C. rubecula* by Wiskerke and Vet (1991), a more extensive comparison related to the way these parasitoid species use infochemicals is presently being undertaken in our laboratory.

In conclusion, infochemicals from the second trophic level seem to be less important in long-range host location by *C. glomerata* than infochemicals from the first trophic level. However, the active production by the plant of the infochemical that is released from the plant upon feeding by *P. brassicae* remains to be demonstrated.

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REFERENCES

DICKE, M. 1988. Prey preference of the phytoseiid mite *Typhlodromus pyri:* 1. Response to volatile kairomones. *Exp. Appl. Acarol.* 4:1–13.

DICKE, M., and SABELIS, M.W. 1988. How plants obtain predatory mites as bodyguards. *Neth. J. Zool.* 38:148–165.

DICKE, M., and TAKABAYASHI, J. 1991. Specificity of induced indirect defence of plants against herbivores. *Redia* 74(3)Appendix:105–113.

DICKE, M., JONG, M. DE, ALERS, M.P.T., STELDER, F.C.T., WUNDERINK, R., and POST, J. 1989. Quality control of mass-reared arthropods: Nutritional effects on performance of predatory mites. *J. Appl. Entomol.* 108:462–475.

DICKE, M., BAARLEN, P. VAN, WESSELS, R., and DÖKMAN, H. 1993. Herbivory induces systemic production of plant volatiles that attract the herbivore's predators: extraction of endogenous elicitor. *J. Chem. Ecol.* 19 (in press).

DICKE, M., BEEK, T.A. VAN, POSTHUMUS, M.A., BEN DOM, N., BOKHOVEN, H. VAN, and GROOT, AE. DE 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.

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.* 62:164–176.

ELLER, F.J., TUMLINSON, J.H., and LEWIS, W.J. 1988a. Beneficial arthropod behavior mediated by airborne semiochemicals. VII 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.

ELLER, F.J., TUMLINSON, J.H., and LEWIS, W.J. 1988b. Beneficial arthropod behavior mediated by airborne semiochemicals. II. Olfactometric studies of host location by the parasitoid *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae). *J. Chem. Ecol.* 14:425–434.

ELST, I.F.A.M., VERNEDE, R., and PAK, G.A. 1991. Response of the parasitoid wasp *Cotesia*

glomerata to odour cues from the host-habitat complex. *Proc. Exp. Appl. Entomol.* 2:109–114.

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.* 12:1873–1877.

ELZEN, G.W., WILLIAMS, H.J., and VINSON, S.B. 1984. Role of diet in host selection of *Heliothis virescens* by parasitoid *Campoletis sonorensis* (Hymenoptera: Ichneumonidae). *J. Chem. Ecol.* 10:1535–1541.

KAISER, L., and CARDÉ, R.T. 1992. In-flight orientation to volatiles from the plant–host complex in *Cotesia rubecula* (Hym.: Braconidae): increased sensitivity through olfactory experience. *Physiol. Entomol.* 17:62–67.

KELLER, M.A. 1990. Responses of the parasitoid *Cotesia rubecula* to its host *Pieris rapae* in a flight tunnel. *Entomol. Exp. Appl.* 57:243–249.

KITANO, H. 1978. Studies on the ovipositing activity and host-searching behavior of *Apanteles glomeratus* L. (Hymenoptera, Braconidae), a parasitoid on the cabbage white butterfly. *Kontyu Tokyo* 46:152–161.

LAING, J.E., and LEVIN, D.B. 1982. A review of the biology and bibliography of *Apanteles glomeratus* (L.) (Hymenoptera: Braconidae). *Biocontrol News Information* 3:7–23.

LEWIS, W.J., NORDLUND, D.A., GUELDNER, R.C., TEAL, P.E.A., and TUMLINSON, J.H. 1982. Kairomones and their use for management of entomophagous insects. XIII. Kairomonal activity for *Trichogramma* spp. of abdominal tips, excretion, and a synthetic sex pheromone blend of *Heliothis zea* (Boddie) moths. *J. Chem. Ecol.* 8:1323–1331.

MCAUSLANE, H.J., VINSON, S.B., and WILLIAMS, H.J. 1991. Stimuli influencing host microhabitat location in the parasitoid *Campoletis sonorensis*. *Entomol. Exp. Appl.* 58:267–277.

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.

MONTEITH, L.G. 1964. Influence of the health of food plant of the host on host-finding by tachinid parasites. *Can. Entomol.* 96:1477–1482.

NEALIS, V.G. 1986. Responses to host kairomones and foraging behaviour of the insect parasite *Cotesia rubecula* (Hymenoptera: Braconidae). *Can. J. Zool.* 64:2393–2398.

NETTLES, W.C. 1980. Adult *Eucelatoria* sp.: Response to volatiles from cotton and okra plants and from larvae of *Heliothis virescens*, *Spodoptera eridania*, and *Estigmene acrea*. *Environ. Entomol.* 9:759–763.

NOLDUS, L.P.J.J., LENTEREN, J.C. VAN, and LEWIS, W.J. 1991. How *Trichogramma* parasitoids use moth sex pheromones as kairomone: orientation behaviour in a wind tunnel. *Physiol. Entomol.* 16:313–327.

NORDLUND, D.A., JONES, R.L., and LEWIS, W.J. (eds.). 1981. Semiochemicals, Their Role in Pest Control. Wiley & Sons, New York, 306 pp.

ROTH, J.P., KING, E.G., and HENSLEY, S.D. 1982. Plant, host, and parasite interactions in the host selection sequence of the tachinid *Lixophaga diatraeae*. *Environ. Entomol.* 11:273–277.

SABELIS, M.W., AFMAN, B.P., and SLIM, P.J. 1984. Location of distant spider mite colonies by *Phytoseiulus persimilis*: localization and extraction of a kairomone. *Acarology* 6 (1): 431–440.

SATO, Y. 1979. Experimental studies on parasitization by *Apanteles glomeratus*. IV. Factors leading a female to the host. *Physiol. Entomol.* 4:63–70.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry. W.H. Freeman & Company, New York.

STEINBERG, S., DICKE, M., VET, L.E.M., and WANNINGEN, R. 1992. Response of the braconid parasitoid *Cotesia* (=*Apanteles*) *glomerata* to volatile infochemicals: Effect of bioassay set-up, parasitoid age and experience and barometric flux. *Entomol. Exp. Appl.* 63:163–175.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M. A. 1991a. Variation in composition of predator-

attracting allelochemicals emitted by herbivore-infested plants: relative influence of plant and herbivore. *Chemoecology* 2:1–6.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M.A. 1991b. Induction of indirect defence against spider-mites in uninfested lima bean leaves. *Phytochemistry* 30:1459–1462.

TURLINGS, T.C.J., and TUMLINSON, J.H. 1992. Systemic release of chemical signals by herbivore-injured corn *Proc. Natl. Acad. Sci. U.S.A.* 89:8399–8402.

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 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. 1991a. 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.

TURLINGS, T.C.J., TUMLINSON, J.H., HEATH, R.R., PROVEAUS, A.T., and DOOLITTLE, R.E. 1991b. Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. *J. Chem. Ecol.* 17:2235–2251.

ULLYET, G.C. 1953. Biomathematics and insect population problems. *Entomol. Soc. South Afr. Mem.* 2:1–89.

VET, L.E.M. 1985. Olfactory microhabitat location in some eucoilid and alysiine species (Hymenoptera), larval parasitoids of Diptera. *Neth. J. Zool.* 35:720–730.

VET, L.E.M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies of herbivores in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

VITÉ, J., and WILLIAMSON, D.L. 1970. *Thanasimus dubius*: prey perception. *J. Insect Physiol.* 16:233–239.

VINSON, S.B. 1976. Host selection by insect parasitoids. *Annu. Rev. Entomol.* 21:109–134.

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. Wiley, New York.

WÄCKERS, F.L., and LEWIS, W.J. 1992. Olfactory and visual learning and their interaction in host site location by *Microplitis croceipes*. *Biol. Control* In press.

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. Wiley, New York.

WISKERKE, J.S.C., and VET, L.E.M. 1991. Comparison of two *Cotesia* species foraging for solitarily and gregariously feeding *Pieris* host species. *Proc. Exp. Appl. Entomol.* 2:190–195.

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EFFECT OF RELATIVE HUMIDITY CONDITIONS ON RESPONSIVENESS OF EUROPEAN CORN BORER (*Ostrinia nubilalis*) MALES TO FEMALE SEX PHEROMONE IN A WIND TUNNEL

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Abstract—The responsiveness of 3-day-old European corn borer males to three concentrations of 97:3 Z:E 11-tetradecenyl acetate, the female sex pheromone, was studied over a range of relative humidities (43–100%) in a wind tunnel. The proportion of males taking flight and reaching the source decreased, while the proportion exhibiting in-flight arrestment of upwind progress increased under high humidity conditions at all three concentrations of pheromone tested. The relationships between relative humidity and these behaviors were best described by polynomial equations.

Key Words—European corn borer, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, male responsiveness, relative humidity, wind tunnel.

INTRODUCTION

Finding a mate is a key event in sexual reproduction, being the first step in assuring a genetic representation in future populations. In some insect species, mate seeking is facilitated by the aggregation of females at specific zones within the habitat, termed conventional encounter sites (Parker, 1978a; Thornhill and Alcock, 1983), and/or by the emission of a volatile sex pheromone by females (Thornhill and Alcock, 1983). European corn borer (ECB), *Ostrinia nubilalis* (Hbn), (Lepidoptera, Pyralidae) adults aggregate in tall dense vegetation outside fields of host plants (Caffrey and Worthley, 1927; Showers et al., 1976) where

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females call (overt behavior associated with the emission of the sex pheromone) and matings occur (Showers et al., 1976; Sappington and Showers, 1983). However, in arid southeastern Alberta, where weeds are unavailable outside cultivated areas, ECB moths are found at the center of irrigated cornfields, the most humid zone of the habitat (Lee, 1988). These observations, together with the adverse effect of low humidity on the calling behavior (Webster and Cardé, 1982; Royer and McNeil, 1991) and on the length of the premating period of ECB females (Royer and McNeil, 1991), suggest that high humidity is an important ecological factor responsible for the selection of the observed conventional encounter site of this species. As no detailed information is available on the effect of relative humidity on the receptivity of males to the female sex pheromone in this or any other lepidopteran, we examined the responsiveness of 3-day-old ECB males to pheromone lures under wind tunnel conditions.

METHODS AND MATERIALS

The responsiveness of 3-day-old univoltine ECB males to sex pheromone sources loaded with 30 (W. Roelofs, Geneva, New York), 100, or 200 μg of 97:3 Z:E 11-tetradecenyl acetate (Raylo Chemicals Ltd), was independently tested in a 0.25 m/sec laminar air current (determined using a TA3000T thermal anemometer, Airflow Developments Ltd) from the fifth through the seventh hour of the scotophase. One hour before the beginning of the test, naive males, maintained at 25 $\pm$ 0.5°C and 45 $\pm$ 5% relative humidity under a 16:8 hr light–dark photoperiod where they had access to a source of water to drink, were transferred to the environment chamber housing the wind tunnel (Fitzpatrick et al., 1988) at 20 $\pm$ 1°C. When different concentrations were tested on the same day, clean air was allowed to circulate in the tunnel during a period of at least 3 min between trials. We recorded the following behaviors: taking flight, in-flight arrestment of upwind progress (Willis and Baker, 1988) following the initiation of oriented flight toward the source, and reaching the lure. Males not responding within 60 sec but that took flight when physically disturbed were classified as unresponsive. Relative humidity conditions (measured using an Abbeon certified hygrometer, model AB 167B) varied from 43% to 100% over the experimental period of several months, but remained constant during trials on any given day. A total of 99, 182, and 316 ECB males were tested to pheromone sources loaded with 30, 100, and 200 μg, respectively.

Best curvilinear regressions, weighted for the sample size of each point (Snedecor and Cochran, 1978), were fitted to describe the relationship between humidity conditions and different parameters of the flight response of males to the sex pheromone. Each point corresponds to the proportion of males exhibiting a given behavior in any given replicate, when at least five individuals were tested.

RESULTS

Male flight capacity remained unchanged over the range of humidity conditions tested, for all males not responding to the female sex pheromone promptly took flight when physically disturbed. However, humidity conditions explained at least 41% of the variation observed in different parameters of the ECB males' responsiveness to the female sex pheromone (Figures 1–3). At all pheromone

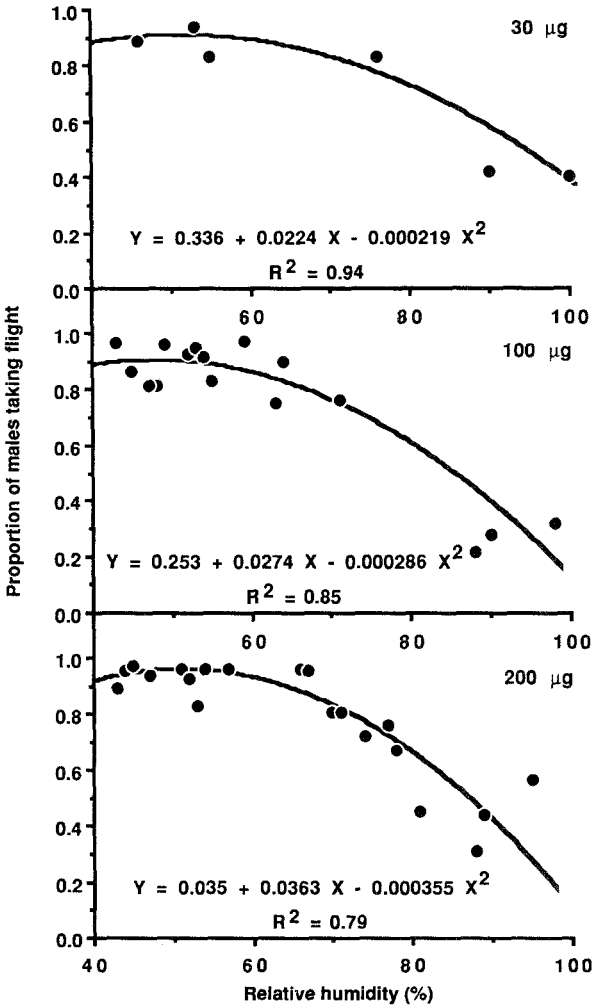

FIG. 1. Relation between humidity conditions and the proportion of ECB males taking flight to 30, 100, and 200 µg of the sex pheromone at 20°C.

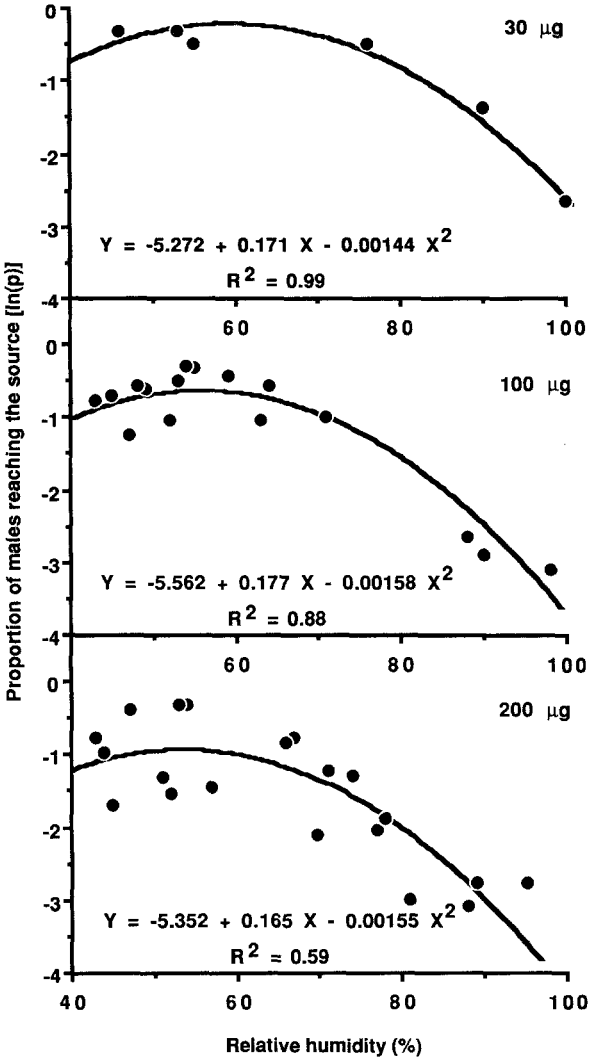

FIG. 2. Relation between humidity conditions and the proportion of males reaching sex pheromone sources loaded with 30, 100, and 200 µg, at 20°C.

concentrations tested, the percent of males taking flight (Figure 1) and the proportion of those taking flight that reach the source (Figure 2) decreased as relative humidity increased. The reduction in the number of receptive males reaching the lure was due to the increased proportion of males exhibiting in-

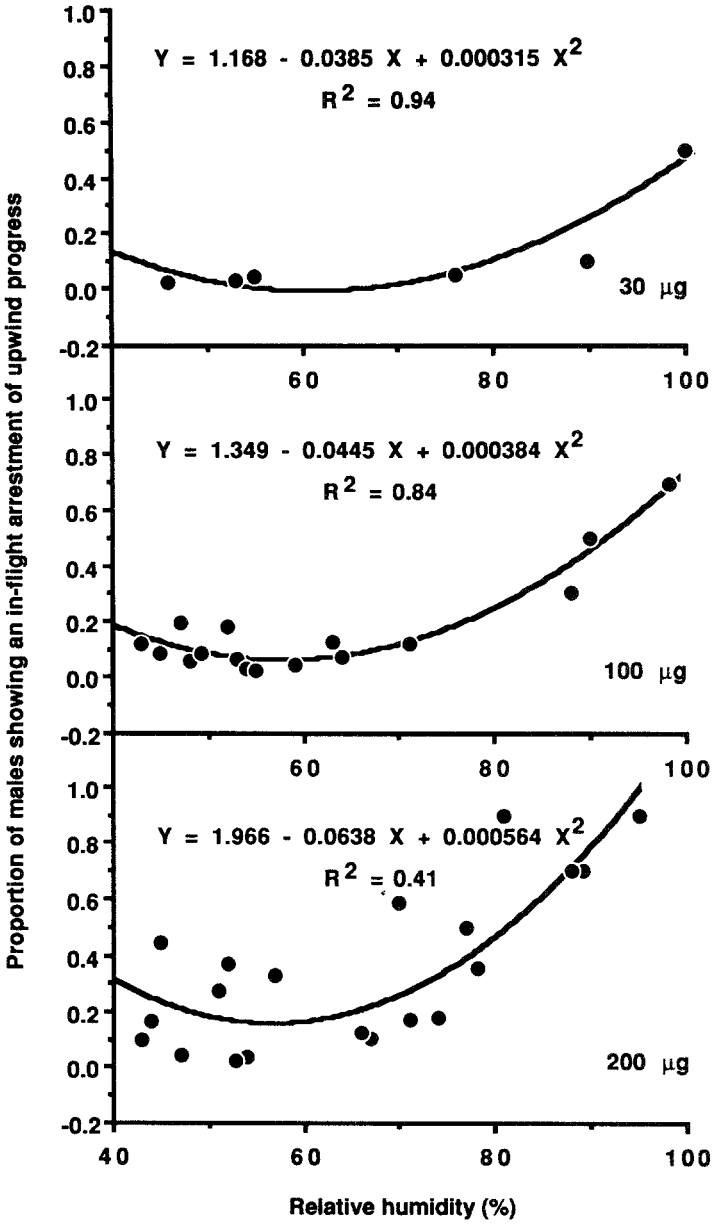

FIG. 3. Relation between humidity conditions and the proportion of ECB males exhibiting in-flight arrestment of upwind progress to 30, 100, and 200 μg of the sex pheromone at 20°C.

flight arrestment of upwind progress with increasing relative humidity (Figure 3).

DISCUSSION

Direct comparisons of the results obtained with the three different pheromone concentrations are not possible for two reasons. We were not always able to test a sufficient number of individuals at each concentration during the same trial as the availability of 3-day-old virgin males fluctuated. Furthermore, as the lures came from different sources, they could vary slightly with respect to the isomeric ratio or chemical purity. However, the relationships between relative humidity and the three parameters measured were similar for all concentrations of the female sex pheromone, clearly indicating that relative humidity affects the male receptivity in the ECB.

At all pheromone concentrations tested, there was a decrease in male receptivity with an increase in humidity (Figure 1). Furthermore, at high humidities fewer receptive males reached the source (Figure 2), due to an increase of in-flight arrestment of upwind progress (Figure 3). In-flight arrestment is correlated with either complete adaptation of antennal neurons or attenuation of their firing rate (Baker et al., 1988; Baker and Haynes, 1989) to high pheromone concentration (Willis and Baker, 1988). Thus, the increase in arrestment with increasing humidity at a given concentration of pheromone suggests that high relative humidity enhances stimulus perception. The same modification of stimulus perception by humidity could also explain why males, capable of flight, did not always respond when exposed to pheromone. The antennae of several Lepidoptera respond to humid air (Grant, 1975), and cleloconica sensilla, known to perceive humidity in other species (Altner et al., 1977), are found adjacent to trichodea sensilla on the antennae of *O. nubilalis* (Cornford et al., 1973). Thus, the observed responses could result from an interaction of pheromonal and humidity stimuli at the level of either peripheral receptors or the central nervous system, whereby the quantity of pheromone required to stimulate the male is less at higher than at lower relative humidities. An effect at the peripheral level would be analogous to the finding in *Trichoplusia ni*, where receptor response to the major component of the sex pheromone increased as the concentration of a minor component, not directly perceived by the receptor tested, increased (O'Connell, 1986).

Loughner and Brindley (1971) reported that decreasing temperature conditions in the scotophase were important for onset of sexual activities by the ECB. However, in subsequent analyses under a range of field conditions DeRozari et al. (1977) found that the availability of free water, in the form of dew, was the most important cue. Obviously, as DeRozari et al. pointed out,

the drop in temperature would contribute directly to the formation of dew. The decline in temperature and dew formation would also be accompanied by an increase in relative humidity, which can affect the mating success in certain lepidopteran species (Kanno and Sato, 1980; Mbata, 1986). Humidity conditions are important for reproduction in the ECB (Loughner and Brindley, 1971; Royer and McNeil, 1991), and the decline in mating success at low relative humidity could be due to reduced adult flight activity (Broersma et al., 1976) or female calling behavior (Webster and Cardé, 1982; Royer and McNeil, 1991). It could also result from a change in male receptivity, as the number of individuals reaching the 30-μg source declined from 70.4 (N = 27) to 42.1% (N = 19) when tested at 40 (35–44) and 20 (15–24) % relative humidity (unpublished data). Males not reaching the source appeared to loose the pheromone plume rather than exhibit the arrestment behavior observed at high humidities. Thus, it is not surprising that receptive *Ostrinia nubilalis* females aggregate in humid microhabitats (Showers et al., 1976; Sappington and Showers, 1983; Lee, 1988).

However, the decline in male receptivity at the higher humidity conditions seems counterintuitive, given that the lifetime reproductive success of a male depends on the number of mates acquired (Royer and McNeil, 1992), which, in part, is determined by his ability to find receptive females (Parker, 1978b; Bell, 1990) that, as mentioned above, aggregate at high humidity sites. A plausible explanation for this apparent contradiction is possible if the emission rate from a calling female is significantly less than the lures used in these experiments. Thus, even if there is an increased sensitivity to the pheromone at high humidity, males would be able to respond to and locate calling females due to the low concentrations of pheromone present. The possibility that the emission rate of females is low does not seem unreasonable, as ECB female pheromone glands only contain approximately 2.5 ng (Glover et al., 1987). However, additional data on the actual release rates from calling females and the response of males to such concentrations under different environmental conditions are required to validate this explanation.

Roelofs (1978), when examining the effect of concentration on the male response to different pheromone blends, suggested that environmental factors could also modify male responsiveness. This has since been demonstrated experimentally for temperature (Linn et al.,1988) and, if our hypothesis concerning the interaction between pheromone and relative humidity stimuli is correct, blend perception may change with relative humidity conditions. The modification of blend perception by environmental conditions could explain why, in field trials, some males of one ECB pheromonal strain went to the wrong blend (Klun and Huettel, 1988), even though male responsiveness is genetically determined (Roelofs et al., 1987). It is clear that if we are to use pheromone traps effectively in management programs we must understand the effects of different environmental factors on the pheromone-mediated mating behavior of both sexes

(McNeil, 1991) and, for species that use specific encounter sites, particular attention should be given to the ecological factors in these microhabitats. This knowledge could help explain fluctuations in pheromone trap efficiency, both within and between years.

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REFERENCES

ALTNER, H., SASS, H., and ALTNER, I. 1977. Relationship between structure and function of antennal chemo-, hygro-, and thermo-receptive sensilla in *Periplaneta americana*. *Cell Tissue Res.* 176:389–405.

BAKER, T.C., and HAYNES, K.F. 1989. Field and laboratory electroantennographic measurements of pheromone plume structure correlated with Oriental fruit moth behaviour. *Physiol. Entomol.* 14:1–12.

BAKER, T.C., HANSSON, B.S., LÖFSTEDT, C., and LÖFQVIST, J. 1988. Adaptation of antennal neurons in moths is associated with cessation of pheromone-mediated upwind flight. *Proc. Natl. Acad. Sci. U.S.A.* 85:9826–9830.

BELL, W.J. 1990. Searching behavior patterns in insects. *Annu. Rev. Entomol.* 35:447–467.

BROERSMA, D.B., BARRETT, J.R., and SILLINGS, J.O. 1976. Activity and blacklight induced flight of black cutworm and European corn borer as related to temperature and relative humidity. *Environ. Entomol.* 5:1191–1194.

CAFFREY, D.J., and WORTHLEY, L.H. 1927. A progress report on the investigations of the European corn borer. Report #1476, U.S. Department of Agriculture.

CORNFORD, M.E., ROWLEY, W.A., and KLUN, J.A. 1973. Scanning electron microscopy of antennal sensilla of the European corn borer, *Ostrinia nubilalis*. *Ann. Entomol. Soc. Am.* 66:1079–1088.

DEROZARI, M.B., SHOWERS, W.B., and SHAW, R.H. 1977. Environment and the sexual activity of the European corn borer. *Environ. Entomol.* 6:657–665.

FITZPATRICK, S.M., MCNEIL, J.N., and DUMONT, S. 1988. Does male pheromone effectively inhibit competition among courting true armyworm males (Lepidoptera: Noctuidae)? *Anim. Behav.* 36:1831–1835.

GLOVER, T.J., TANG, X.H., and ROELOFS, W.L. 1987. Sex pheromone blend discrimination by the male moths from *E* and *Z* strains of European corn borer. *J. Chem. Ecol.* 13:143–151.

GRANT, G.G. 1975. Preuve électrophysiologique d'un récepteur d'humidité sur les antennes de plusieurs Lépidoptères. *Rev. Bimestrielle Rech. Environ. Canada* 31:28.

KANNO, H., and SATO, A. 1980. Mating behaviour of the rice stem borer moth, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) III. Joint action of temperature and relative humidity on mating activity. *Appl. Entomol. Zool.* 15:111–112.

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.

LEE, D.A. 1988. Moth density and oviposition patterns of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae), in Alberta. *Environ. Entomol.* 17:220–224.

LINN, C.E., CAMPBELL, M.G., and ROELOFS, W.L. 1988. Temperature modulation of behavioural thresholds controlling male moth sex pheromone response specificity. *Physiol. Entomol.* 13:59–67.

LOUGHNER, G.E., and BRINDLEY, T.A. 1971. Mating success of the European corn borer, *Ostrinia nubilalis*, as influenced by environmental factors. *Ann. Entomol. Soc. Am.* 64:1091–1094.

MBATA, G.N. 1986. Combined effect of temperature and relative humidity on mating activities and commencement of oviposition in *Plodia interpunctella* (Hübner) (Lepidoptera: Phycitidae). *Insect Sci. Appl.* 7:623–628.

MCNEIL, J.N. 1991. Behavioral ecology of pheromone-mediated communication in moths and its importance in the use of pheromone traps. *Annu. Rev. Entomol.* 36:407–430.

O'CONNELL, R.J. 1986. Electrophysiological responses to pheromone blends in single olfactory receptor neurones, pp. 217–224, *in* T. L. Payne, M. C. Birch and C. E. J. Kennedy (eds.). Mechanisms of Insect Olfaction. Clarendon Press, Oxford.

PARKER, G.A. 1978a. Evolution of competitive mate searching. *Annu. Rev. Entomol.* 23:173–196.

PARKER, G.A. 1978b. Searching for mates, pp. 214–244, *in* J.R. Krebs and N.B. Davies (eds.). Behavioural Ecology: An Evolutionary Approach. Sinauer Associates, Sunderland, Massachusetts.

ROELOFS, W.L. 1978. Threshold hypothesis for pheromone perception. *J. Chem. Ecol.* 4:685–699.

ROELOFS, W.L., GLOVER, T., TANG, X.H., SRENG, I., ROBBINS, P., ECKENRODE, C., LÖFSTEDT, C., HANSSON, B.S., and BENGTSSON, B.O. 1987. Sex pheromone production and perception in European corn borer moths is determined by both autosomal and sex-linked genes. *Proc. Natl. Acad. Sci. U.S.A.* 84:7585–7589.

ROYER, L., and MCNEIL, J.N. 1991. Changes in calling behaviour and mating success in the European corn borer (*Ostrinia nubilalis*), caused by relative humidity. *Entomol. Exp. Appl.* 61:131–138.

ROYER, L., and MCNEIL, J.N. 1993. Male investment in the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae): Impact on female longevity and reproductive performance. *Funct. Ecol.* In press.

SAPPINGTON, T.W., and SHOWERS, W.B. 1983. Adult European corn borer (Lepidoptera: Pyralidae) flight activity in and away from aggregation sites. *Environ. Entomol.* 12:1154–1158.

SHOWERS, W.B., REED, G.L., ROBINSON, J.F., and DEROZARI, M.B. 1976. Flight and sexual activity of the European corn borer. *Environ. Entomol.* 5:1099–1104.

SNEDECOR, G.W., and COCHRAN, W.G. 1978. Statistical Methods. The Iowa State University Press, Ames.

THORNHILL, R., and ALCOCK, J. 1983. The Evolution of Insect Mating Systems. Harvard University Press, Cambridge, Massachusetts.

WEBSTER, R.P., and CARDÉ, R.T. 1982. Influence of relative humidity on calling behaviour of the female European corn borer moth (*O. nubilalis*). *Entomol. Exp. Appl.* 28:925–933.

WILLIS, M.A., and BAKER, T.C. 1988. Effects of varying sex pheromone component ratios on the zigzagging flight movements of the Oriental fruit moth, *Grapholita molesta*. *J. Insect Behavior* 1:357–371.

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EFFECT OF REARING TEMPERATURE ON PHEROMONE COMPONENT RATIO IN POTATO TUBERWORM MOTH, *Phthorimaea operculella*, (LEPIDOPTERA: GELECHIIDAE)

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Abstract—The ratio of two components in the sex pheromone of the potato tuberworm moth, *Phthorimaea operculella*, was influenced by the rearing temperature. The percentage of the (*E*,*Z*,*Z*)-4,7,10-tridecatrienyl acetate dropped as the rearing temperature was raised. The total amount of the pheromone did not change parallel with the change of ratio. The critical period sensitive to temperature seemed to be the pupal stage. Temperature in the larval stage may also influence the ratio slightly. The biological significance of the phenomenon was also discussed.

Key Words—Sex pheromone, pheromone components ratio, (*E*,*Z*,*Z*)-4,7,10-tridecatrienyl acetate, (*E*,*Z*)-4,7-tridecadienyl acetate, rearing temperature, Lepidoptera, Gelechiidae, potato tuberworm moth, *Phthorimaea operculella*.

INTRODUCTION

The individual variation in the ratio of components of moth pheromone has been documented in a number of species. Typically, there is little variation among individuals (Roelofs and Brown, 1982). This fact presumably is related to the precise ratio required for male response. On the other hand, considerable individual variation in the pheromone component ratio has been shown in a species such as the potato tuberworm moth, *Phthorimaea operculella* (Ono et al., 1990). In this species, a precise component ratio is not required for male attraction (Ono and Orita, 1986).

There are some studies on the control mechanisms of the pheromone com-

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ponent ratio. Collins and Cardé (1989) showed genetic control of component ratio and pheromone content in the pink bollworm moth, *Pectinophora gossypiella*. In the cabbage looper moth, *Trichoplusia ni*, the presence of a mutant in the pheromone component ratio, which was controlled by a single autosomal gene, was shown (Haynes and Hunt, 1990). However, the effect of environmental factors on the component ratio has never been demonstrated.

The sex pheromone of *P. operculella* is composed of (*E*,*Z*,*Z*)-4,7,10-tridecatrienyl acetate (triene) and (*E*,*Z*)-4,7,-tridecadienyl acetate (diene) (Persoons et al., 1976). Since there are no studies on factors controlling the pheromone component ratio in the species, this work was conducted to clarify the effect of environmental factors, especially the rearing temperature, on the pheromone component ratio of *P. operculella*, in addition to some preliminary genetic studies.

METHODS AND MATERIALS

The insects used originated from a field population of the Shiratsuchi area in Nagoya, Japan. The collected insects were reared on potatoes in a rearing room (25°C and 14:10 hr light–dark regime) over 80 generations. The experiments using field-collected females were conducted on samples collected from the same locality in November 1989. Full-grown larvae collected from the potato field were kept at room temperature (10–20°C) and allowed to pupate and emerge.

A preliminary genetic experiment was conducted by using pairs of a female and male moth collected from the stock culture at random. The progeny from each female–male pair were reared in a plastic container (10 cm diameter and 8 cm high) separately. These containers were put on a plastic plate, on which a sticky material prevented mixing of larvae that escaped from the container. Each of these pairs was inbred successively between full siblings from the same parents (one pair of a female and male per generation for a family). During these procedures, 10 females in each generation were used for individual quantification of sex pheromone components by GLC.

The rearings at different temperature conditions were conducted in a rearing chamber (temperature gradient chamber TG-100-AD, Nippon Medical & Chemical Instruments Co., Ltd.). There were five chambers set at different temperatures (every 5°C between 15°C and 35°C with ±0.5°C fluctuation). The light and humidity conditions were not controlled. The light–dark cycle was the same as in the laboratory (about 13–15 hr light). The eggs were collected at 25°C in all experiments and kept at the same conditions for two days. The eggs were then put into each temperature chamber with potatoes (var. Danshaku). The pupae collected from each condition were kept in the same chamber until gland

extraction two days after adult eclosion. All the extractions were done in the afternoon (about 2–5 hr before natural sunset).

Experiments replicating different groupings of temperature conditions were conducted. A 25°C condition was included in all groupings. In each grouping of temperature conditions, both the eggs and the food were drawn from the same moths and potatoes. All the data were combined because there were no significant differences among data at the 25°C condition in each grouping (Mann-Whitney U test).

Glandular pheromone content of individual females was analyzed by GLC. The procedure of sample preparation and analyses was basically similar to that of Ono et al. (1990). The glands were extracted by 50 μl hexane, and 5 ng (*E*)-11-tetradecenyl acetate was added as an internal standard. Analyses were performed on a Shimadzu GC-15A GLC with an SP-2340 fused silica capillary column (30 m × 0.25 mm ID; 0.20 μm film thickness; Supelco Inc.). The initial column temperature was 80°C, then increased 15°C/min to a final temperature of 160°C with a 10-min hold. Peaks were characterized by retention times, and peak areas were quantified by a Shimadzu C-R3A chromatopack.

RESULTS

Figure 1 shows the change in mean percentages of triene for total pheromone amount and its standard deviation (SD) through generations in a typical case. The mean percentages of triene fluctuated through the generations, and the decrease of SD was not clear. This seems to show that there were some environmental factors controlling the pheromone component ratio in *P. operculella*.

In November 1989, I collected some final-instar larvae of *P. operculella* from a potato field in Shiratsuchi (same locality as the data in Ono et al., 1990) and kept them in the laboratory at about 13–15 hr light and 10–20°C temperature range. The pheromone component ratio of the females from these larvae was then analyzed. The frequency distribution of percentage of triene in the total pheromone amount is shown in Figure 2a. The percentage of triene was extremely high in the population and was very different from the former study (Ono et al., 1990). It seemed that there were two possible explanations for this biased distribution: genetic change in the field population or some environmental factor, especially the low temperature condition in the end of November (about 10–15°C range in the field at Nagoya). The second generation from the Shiratsuchi population was reared in a 25°C room. The pheromone component ratio of these females was changed and was similar with the data of Ono et al. (1990) (Figure 2b). These results suggested that an environmental factor, possibly rearing temperature, modified the pheromone component ratio in this species. Rearing

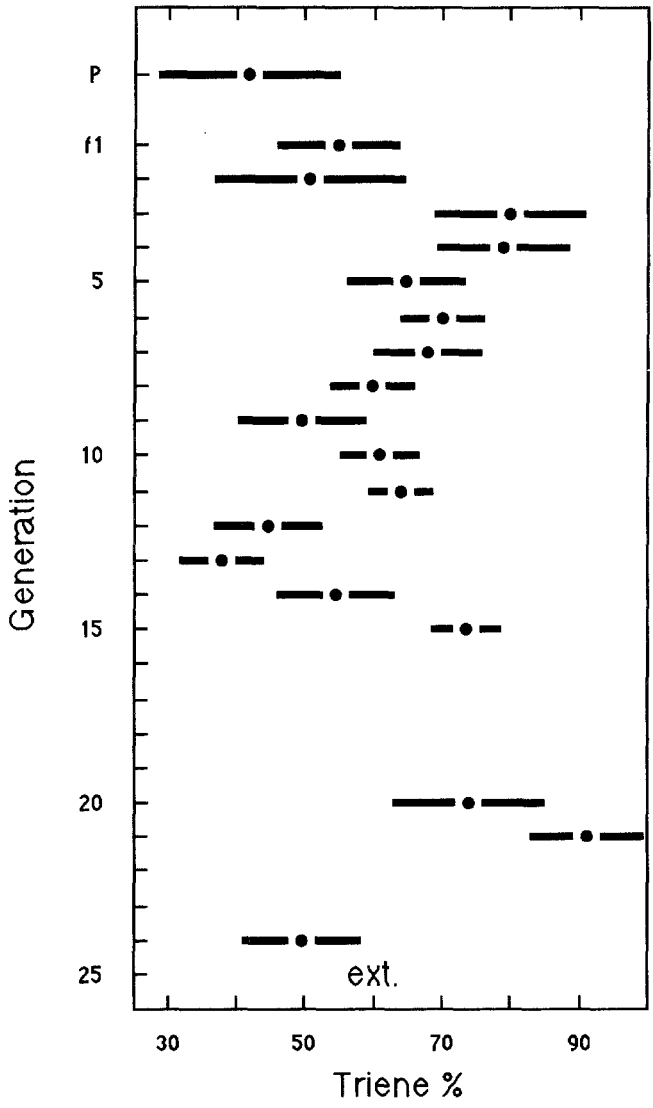

FIG. 1. A typical change of mean percentages of triene in total pheromone amount and its SD through generations. "P" and "f" mean parents and filial, respectively, and "ext." means extinction.

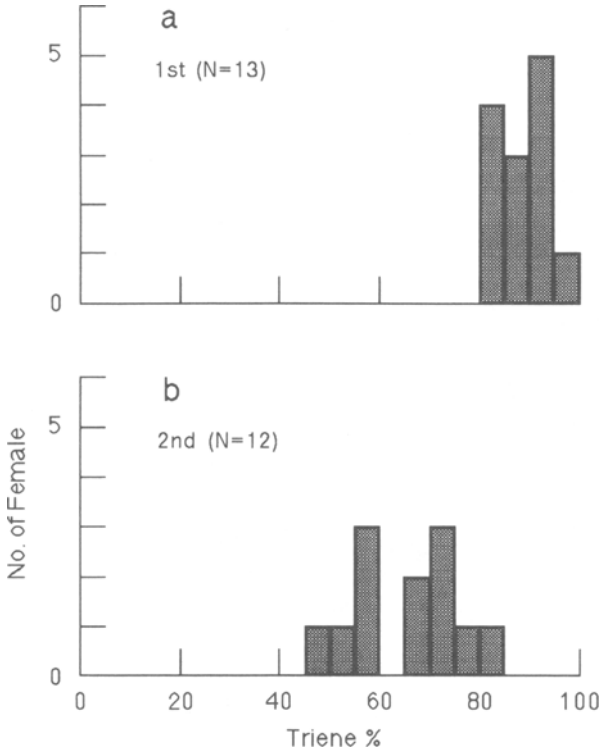

FIG. 2. Frequency distributions of pheromone component ratio in the females collected from a potato field November 1989 (a) and in the females of their progeny reared at 25°C (b).

experiments at different temperatures were, therefore, conducted to clarify this point.

The results are summarized in Figure 3. The percentage of triene in the total pheromone amount dropped as the rearing temperature increased. All points were significantly different ($P < 0.05$, Mann-Whitney U test) from each other. The results show that rearing temperature influences the pheromone component ratio in *P. operculella*. The diene component increased at high temperature and the triene component increased at low temperature.

On the other hand, the total amount of pheromone did not change in parallel with the change of ratio, although there are significant differences among these rearing conditions (Figure 4). This means that both components of pheromone were influenced by rearing temperature. Actually, the amounts of each of triene and diene are correlated significantly with rearing temperature ($r = -0.991$, P

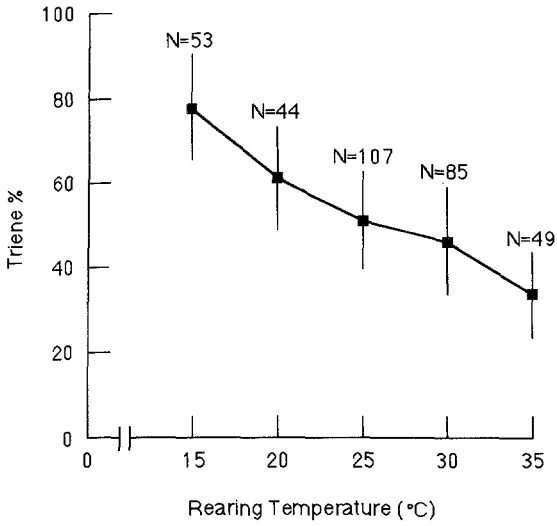

FIG. 3. Change of pheromone component ratio with different rearing temperature conditions. Solid squares show mean values and bars show SD. All the points were significantly different ($P < 0.05$, Mann-Whitney U test).

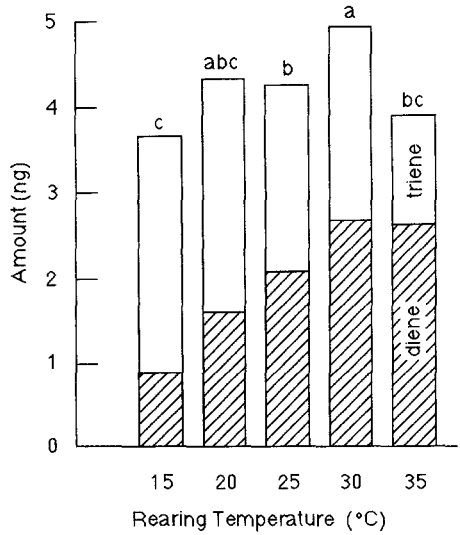

FIG. 4. Change of total amount of pheromone at different rearing temperatures. The SE values of total amount at 15, 20, 25, 30, and 35°C were 0.19, 0.22, 0.17, 0.19, and 0.24, respectively. Histograms with the same letters are not significantly different ($P < 0.05$, Duncan's multiple-range test).

= 0.03 and $r = 0.956$, $P = 0.01$, respectively), while the total amount is not ($r = 0.377$, $P = 0.53$).

Temperature may influence the component ratio in the latter half of the period from egg to adult, because the females kept at 25°C during the early half (about 10 days, average period from egg to adult was about 20 days at 25°C) and moved to 15°C during the latter half (about 35 days, average period from egg to adult was about 70 days at 15°C) contained a higher percentage of the triene pheromone component, while those kept in the reversed temperature combination (about 35 days at 15°C and about 10 days at 25°C) contained a higher diene percentage (Figure 5). The critical sensitive period seems to be in the pupal stage. Figure 6 shows the pheromone component ratio of females kept at 15, 25, or 35°C after pupation. All these pupae were reared at 25°C from egg to final-instar larvae before moving to each temperature condition. Females in each temperature condition contained a similar, but not the same, pheromone component ratio as those reared at corresponding temperature conditions for the whole egg-to-adult growth period of Figure 3. In Figure 6, only the data of 25→15°C were significantly different ($P < 0.05$ in Mann-Whitney U test) from others. The data in Figures 5 and 6 suggest that the rearing temperature before pupation may also influence the pheromone component ratio.

This suggestion was also supported by comparison of the frequency distribution in the component ratio among several rearing conditions (Figure 7). Three groups of different rearing conditions were compared; 15°C rearing from egg to adult (data of 15°C in Figure 3), 15°C rearing in the latter half of the growing period (data of 25–15°C in Figure 5), and 15°C rearing in pupal stage (data of 25–15°C in Figure 6). Only in the last case (Figure 7c), was the variance very

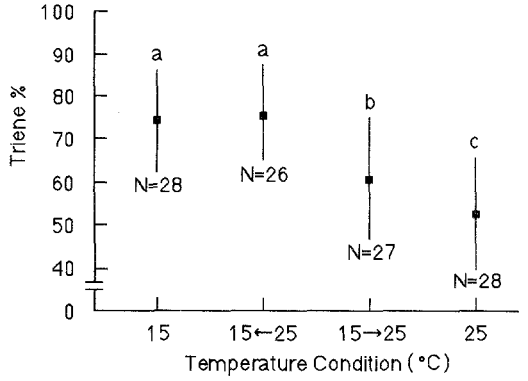

FIG. 5. Pheromone component ratio of females, when the rearing temperature was changed at half of the period from egg to adult. See text for details of the rearing conditions. The solid squares show mean values and bars show SD. Bars with the same letters are not significantly different ($P < 0.05$, Duncan's multiple-range test).

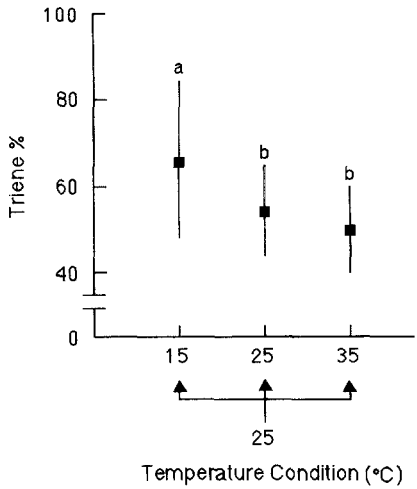

FIG. 6. Pheromone component ratio of females, when the rearing temperature was changed at pupal period. See text for details on rearing conditions. The solid squares show mean values and bars show SD ($N = 30$ in each condition). Bars with the same letters are not significantly different ($P < 0.05$, Duncan's multiple-range test).

wide and the distribution pattern seemed to differ from others (significantly different at $P < 0.05$ in F test).

DISCUSSION

The results show that the temperature during the growing stage influenced the pheromone component ratio of females. There are some reports on lepidopterous species in which the pheromone component ratio is controlled genetically (Roelofs et al., 1986; Hunt et al., 1990). This case is the first to show that temperature during the growing period modifies the pheromone component ratio in females.

The temperature condition did not control synthesis of only one pheromone component, but modulated synthesis of two components, because total amounts of pheromone were similar in all conditions tested (Figure 4).

The critical period sensitive to ambient temperature conditions seems to be in the pupal stage (Figures 5 and 6). However, there may be other factors influencing the component ratio during earlier stages, because a slightly different distribution pattern was found in the experiment shown in Figure 7c. Some other factors effective in earlier stages may induce the lower-ratio individuals in this population. The pathway inducing the change of the component

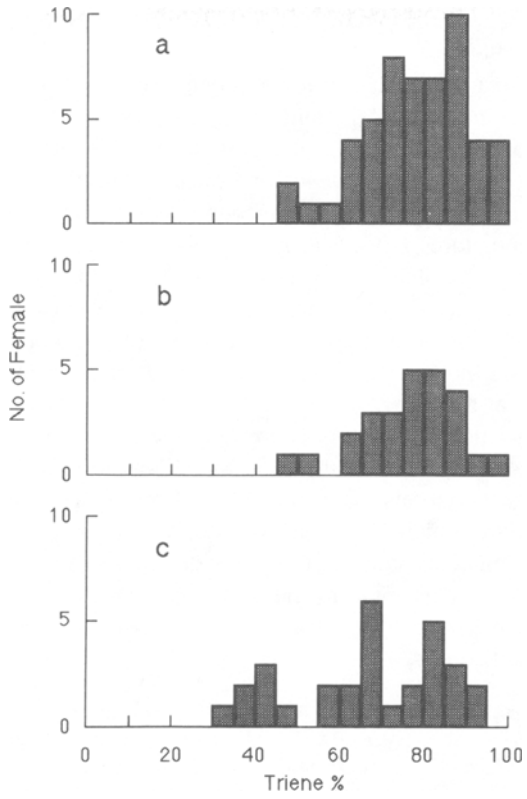

FIG. 7. Frequency distribution of pheromone component ratio in the females reared at 15°C from egg to adult (a), 15°C in the latter half of the growing period (b), and 15°C in the stage after pupation.

ratio by temperature is not known. It will be difficult to answer until the biosynthetic pathway is clarified in this species.

Ono et al. (1990) pointed out that there was a difference in mean triene percentage between Nagoya and California strains and suggested that this might be an interpopulational difference. We should, however, add another possibility caused by rearing temperature condition as shown here.

Maximal male responsiveness to the pheromone blend seems to be tuned to the female-releasing ratio in some species (for example in the European corn borer, *Ostrinia nubilalis*, reviewed by Cardé, 1986). In *P. operculella*, the effect of rearing temperature on males is unknown and, even if present, may be difficult to detect because males have a much wider latitude of responsiveness to the

pheromone component ratio than female variability of pheromone production (Ono and Orita, 1986).

The high triene percentage in low temperature conditions is thought to be adaptive from the viewpoint of the female–male communication system, because the triene component is the essential factor in male orientation (Ono and Ito, 1989) and because dispersal efficiency and male sensitivity must be low at low temperature. In other words, the phenomenon may be a female strategy to adapt to changing temperature. However, it may not be true if the females cannot regulate their own pheromone. As shown above, the males of *P. operculella* have a broad response ratio. This may mean that females cannot regulate their pheromone component ratio. If so, it should be concluded that the change in ratio only reflects a biosynthetic limitation. The latter seems to be a more plausible explanation at this time.

Finally, other possible factors controlling the pheromone component ratio should be pointed out. It is difficult to explain all variations of ratio in past data (for example, the variations of intergeneration in Figure 1) as being due to ambient temperature alone. Nearly all females were reared at "about" 25°C, yet pheromone component ratios fluctuated widely in these results. Other factors must, therefore, also contribute to the variation. Some candidate factors controlling the ratio might include food quality, temperature differences between night and day, and so on.

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REFERENCES

CARDÉ, R.T. 1986. The role of pheromones in reproductive isolation and speciation of insects, pp. 303–317, *in* M.D. Huettel (ed.). Evolutionary Genetics of Invertebrate Behavior, Progress and Prospects. Plenum, New York.

COLLINS, R.D., and CARDÉ, R.T. 1989. Selection for altered pheromone-component ratios in the pink bollworm moth, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). *J. Insect Behav.* 2:609–621.

HAYNES, K.F., and HUNT, R.E. 1990. A mutation in pheromonal communication system of cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 16:1249–1257.

HUNT, R.E., ZHAO, B.-G., and HAYNES, K.F. 1990. Genetic aspects of interpopulational differences in pheromone blend of cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 16:2935–2946.

ONO, T., and ITO, M. 1989. Pattern of pheromone-oriented flight in male potato tuberworm moths. *J. Chem. Ecol.* 15:2357–2368.

ONO, T., and ORITA, S. 1986. Field trapping of the potato tuber moth, *Phthorimaea operculella* (Lepidoptera: Gelechiidae), with the sex pheromone. *Appl. Entomol. Zool.* 21:632–634.

ONO, T., CHARLTON, R.E., and CARDÉ, R.T. 1990. Variability in pheromone composition and periodicity of pheromone titer in potato tuberworm moth, *Phthorimaea operculella* (Lepidoptera: Gelechiidae). *J. Chem. Ecol.* 16:531–542.

PERSOONS, 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., and BROWN, R.L. 1982. Pheromones and evolutionary relationships of Tortricidae. *Annu. Rev. Ecol. Syst.* 13:395–422.

ROELOFS, W.L., DU, J.-W., LINN, C., GLOVER, T.J., and BJOSTAD, L.B. 1986. The potential for genetic manipulation in the redbanded leafroller moth sex pheromone blend, pp. 263–272, *in* M.D. Huettel (ed.). Evolutionary Genetics of Invertebrate Behavior, Progress and Prospects. Plenum, New York.

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OLFACTORY DISCRIMINATION BY *Heteropsylla cubana* (HOMOPTERA: PSYLLIDAE) BETWEEN SUSCEPTIBLE AND RESISTANT SPECIES OF *Leucaena* (LEGUMINOSAE)

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Abstract—In the field, adult psyllids, *Heteropsylla cubana* Crawford, oriented significantly more towards the caged seedlings of susceptible *Leucaena leucocephala* (Lam.) de Wit than to those of the resistant tree species, *Leucaena collinsii* Britton & Rose, or the nonhost *Amaranthus spinosus* L. In a dual-choice bioassay using a still-air olfactometer, the females demonstrated a strong positive response to the hexane extract of *L. leucocephala* leaves at 1×10^{-3} g equivalents (g eq) of leaf material. The females did not orient to leaf extracts of *L. collinsii* at high concentrations but responded positively at a lower concentration of 1×10^{-4} g eq. Olfactory discrimination by *H. cubana* between resistant and susceptible host species should be considered in selection and breeding programs.

Key Words—*Leucaena*, *Heteropsylla cubana*, Homoptera, Psyllidae, plant–insect interaction, plant resistance, kairomones.

INTRODUCTION

The psyllid, *Heteropsylla cubana* Crawford, has recently become a serious pest of the ipil-ipil tree, *Leucaena leucocephala* (Lam.) de Wit (Leguminosae) in the South Pacific Islands and Southeast Asia (McFadden, 1986). *H. cubana* is oligophagous and feeds strictly within a narrow host range among

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members of the genus *Leucaena*. However, even within its host range, differential preference among *Leucaena* spp. was exhibited by the alighting adult insect (Lapis and Borden, 1993). Significantly more adults alighted and more eggs were laid on *L. leucocephala* than on *Leucaena collinsii* Britton & Rose.

Although adults were highly attracted to yellow-colored card traps, Lapis (1991) concluded that the slight difference in the peak yellow reflectance (550 nm) between the leaves of the susceptible *L. leucocephala* and the resistant *L. collinsii* did not account for the preference of *H. cubana* adults for the susceptible species. Prokopy and Owens (1983) cautioned that plant spectral quality is unlikely to constitute a host-plant-specific character for phytophagous insects because of its similarity among most plants.

In contrast, chemical characteristics are often typical to a plant species and play a major role in host selection by ovipositing or feeding adult insects (Dethier, 1980, 1982; Tingle et al., 1990). To date, practically no research has addressed the role of olfaction in host selection by *H. cubana*. Our objective was to determine if *H. cubana* adults would demonstrate olfactory discrimination between *L. leucocephala* and the resistant species *L. collinsii*.

METHODS AND MATERIALS

Field Experiment. Three 6-month-old seedlings, about 40 cm tall, each of *L. leucocephala*, *L. collinsii* and a nonhost, *Amaranthus spinosus* L. (age not known), were placed inside separate Saran-screen cages, that completely obscured visual perception of plant color or form to the human eye. All the plants used had well-developed shoots. The cages were pentagonal and measured 30 cm on each side and 100 cm in height. The cages were placed along the periphery of an infested *L. leucocephala* stand in Los Baños, Philippines.

To measure olfactory attraction of the plants, freely flying, wild psyllids were captured on white cardboard traps (15 × 15 cm) coated with Stickem Special (Seabright Enterprises, Emeryville, California), and clipped on the outside middle portion of all sides of the screen cage. After 24 hr, the traps were collected and replaced with clean traps, and the total number of *H. cubana* adults for all the traps for each species was recorded. The cages together with plants were rerandomized every collection time, usually in the morning. The experiment was replicated daily for 15 days. Plants that did not produce well-developed shoots were replaced with new plants.

The data were analyzed using multisample hypothesis analysis of variance after $\log_{10}(X + 1)$ transformation, and the means were ranked by Tukey's test, $\alpha = 0.05$ (Zar, 1984).

Laboratory Bioassays. Extracts were prepared by placing excised unexpanded leaves of both *Leucaena* spp. in screw-cap vials with hexane (HPLC

grade) for 24 hr, after which the solute was transferred into another vial and stored in the freezer for future use. For every gram of plant tissue a corresponding 5 ml of hexane was used as solvent.

H. cubana used in laboratory bioassays were from cultures maintained on *L. leucocephala* plants in the laboratory for about two years. The test insects were adults of undetermined age but were all mature, as evidenced by their dark green to orange–green color. They were held individually in gelatin capsules for at least 30 min before each test; each insect was used only once.

The attractiveness of extracts of *L. leucocephala* and *L. collinsii* to adult *H. cubana* was tested using a still-air olfactometer similar to that developed by Smith (1989) for the pear psylla. It was made from a rectangular piece of opaque Plexiglas, 5 × 5 × 12 cm in dimension, and divided crosswise into three equal parts, each 4 cm long. A circular chamber, 25 mm in diameter and 10 mm deep, was made in each of the two lateral parts while a 6-mm-deep chamber was made in the middle portion. A straight canal, about 6 mm deep and 12 mm wide, connected the three chambers to one another. The two lateral chambers were both 2.5 cm distant on either side of the central chamber. A transparent sheet of Plexiglas, about 4 mm thick, covered the top of the device. The cover was pegged to one corner of each lateral part to fit and secure all the parts of the olfactometer together when a bioassay was in progress, but still allowing full view of the insect inside the olfactometer. This set-up allowed easy access to all three chambers by sliding out the bottom parts, either singly or simultaneously.

A bioassay was done by putting the extract and solvent each on a circular white filter paper, about 15 mm in diameter, placed on an 18-mm-diameter microscope cover glass in either of the lateral (test) chambers. Each test insect was introduced individually into the central (release) chamber, and its response observed. A response was defined as movement of a test insect into either of the test chambers and making contact with the filter paper wick before 10 min had elapsed. During the bioassay, an upright, white cardboard cylinder, measuring 25 cm in diameter and 30 cm tall, and open on both ends enclosed the olfactometer. A lamp with white light was placed 10 cm above the top open end of the cylinder to provide uniform lighting. The cylinder housed two olfactometers that were observed simultaneously. Bioassays were conducted between 1300 and 1800 hr.

Several experiments were conducted to test the response of adult *H. cubana* to host volatiles. The first experiment compared the responses of males and females to extracts of the susceptible *L. leucocephala*. Succeeding experiments compared the responses of females to solvent or extracts of *L. collinsii*, to a choice of extracts of *L. leucocephala* or *L. collinsii*, and to the hexane solvent or an *L. leucocephala–L. collinsii* extract mixture. In all experiments 5 μl of the prepared extract [1×10^{-3} g equivalents (g eq) of leaf material] and control

solvent was used as stimulus. A final experiment tested *L. collinsii* extract at four concentrations ranging from 1×10^{-3} to 1×10^{-5} g eq. Data were analyzed separately for each set of bioassays using the fixed-ratio hypothesis χ^2 test (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

When plants were caged outdoors in such a manner as to obstruct color perception, adult *H. cubana* preferentially oriented to the cage containing the more susceptible *L. leucocephala* than to the cages holding either the less susceptible *L. collinsii* or the nonhost plant (Figure 1). This result suggested that *H. cubana* had responded to host-plant volatiles. This hypothesis of attractive, host-plant kairomones was further supported by laboratory experiments using the two-choice olfactometer, in which females responded more strongly to volatiles from *L. leucocephala* leaf extracts than to the solvent control (Table 1).

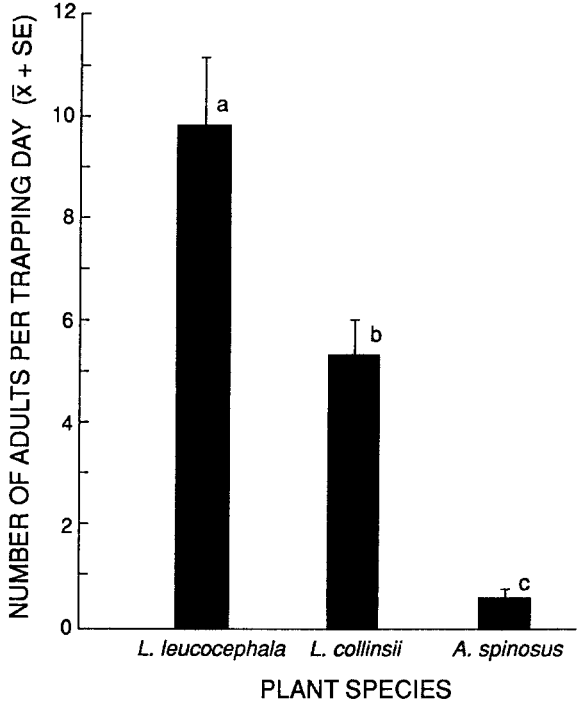

FIG. 1. Comparative numbers of adult *H. cubana* caught on white sticky card traps in response to caged host and nonhost plants. Means topped by the same letter are not significantly different, Tukey's test, $\alpha = 0.05$.

TABLE 1. RESPONSE OF MALE AND FEMALE *H. cubana* TO EXTRACTS OF *L. leucocephala* LEAVES IN A TWO-CHOICE OLFACTOMETER[a]

Sex	*N*	Number of responders	Percent response (responders only)		Chi-square value[b]
			Extract	Solvent	
Male	41	32	68.8	31.2	3.78 NS
Female	33	24	76.9	23.1	6.50*

[a]Experimental stimuli presented as 1×10^{-3} g eq of plant material in 5 µl of solvent.
[b]NS = not significant; * significant, $P < 0.05$.

Although there was no significant difference between the numbers of males responding to the two stimuli, 68.8% of the responsive males contacted the filter paper impregnated with leaf extract. Such differential response between sexes to plant volatiles has been demonstrated in several phytophagous insects, e.g., the cotton boll weevil, *Anthonomus grandis* Boh. (Dickens, 1984), the flea beetle, *Phyllotreta cruciferae* (Goeze) to allyl isothiocyanate (Vincent and Stewart, 1984), and the grasshopper, *Hypochlora alba* (Dodge) (Blust and Hopkins, 1987), and also in the parasitoids *Microplitis croceipes* (Cresson) and *Netelia heroica* Townes (Whitman and Eller, 1987).

Significantly more females were attracted to *L. leucocephala* extract than to hexane (Table 2). On the contrary, the females were not attracted to *L. collinsii* extract when tested against the solvent control. When *L. leucocephala* was tested against *L. collinsii*, there was no significant difference between the numbers of females contacting the two extracts. However, when equal amounts of extracts of *L. leucocephala* and *L. collinsii* were mixed, significantly more females went to the mixture than to the solvent control. When tested against a series of *L. collinsii* extracts at graded, low concentrations, *H. cubana* females responded positively to a stimulus of 1×10^{-4} g eq, but not to stimuli at higher or lower concentrations (Table 3).

The stronger response by *H. cubana* females to leaf extracts of *L. leucocephala* than to extracts of *L. collinsii*, and the positive response to *L. collinsii* extract only at one low concentration, suggest that *L. collinsii* has only a weak olfactory attraction. The possibility of a strong repellent is negated by the lack of a preference when *L. leucocephala* was tested against *L. collinssi* and by the strong response to the mixture of *L. leucocephala* and *L. collinsii* extracts. If the active chemicals are the same in both species, it is possible that in nature *L. collinsii* produces so much of these chemicals that *H. cubana* do not normally respond positively. In the two-spotted strawberry mite, *Tetranychus urticae* Koch, the "standard essential oil mixture" containing low concentrations of the

TABLE 2. RESPONSES OF FEMALE *H. cubana* TO EXTRACTS OF *L. leucocephala* AND *L. collinsii* LEAVES TESTED AT CONCENTRATIONS OF 10^{-3} g eq IN TWO-CHOICE OLFACTOMETER

Experiments	*N*	Number of responders	Percent response (responders only)				Chi-square value[a]
			Leucaena leucocephala	*Leucaena collinsii*	Both species	Hexane	
L. leucocephala vs. hexane	33	28	75.0			25.0	6.04*
L. collinsii vs. hexane	30	26		50.0		50.0	0.04 NS
L. leucocephala vs. *L. collinsii*	32	28	42.9	57.1			0.42 NS
Both species vs. hexane	39	35			74.3	25.7	7.31*

[a]NS = not significant; * significant, $P < 0.05$.

TABLE 3. RESPONSES OF FEMALE *H. cubana* TO DIFFERENT CONCENTRATIONS OF EXTRACTS OF *L. collinsii* LEAVES IN TWO-CHOICE OLFACTOMETER

Stimulus strength (g eq)	*N*	Number of responders	Percent response (responders only)		Chi-square value[a]
			Extract	Solvent	
1×10^{-3}	32	28	50.0	50.0	0.04 NS
5×10^{-4}	34	31	54.8	45.2	0.13 NS
1×10^{-4}	32	27	74.1	25.9	5.33*
1×10^{-5}	33	26	65.4	34.6	1.88 NS

[a]NS = not significant; * significant, $P < 0.05$.

volatile methyl salicylate attracted females but repelled them at high concentrations (Rodriguez et al., 1976). It is also possible that at high concentrations of *L. collinsii* extract, some volatiles are present at sufficient concentrations to mask the activity of an attractant. Visser and Avé (1978) explained this phenomenon as a "disturbance of the attractive complex by artificially changing the relative proportions of the components." However, in interpreting the results one should be wary of the possibility that Hopkins' host selection principle (Beck and Schoonhoven, 1980) may be operational in *H. cubana*. All the insects used in the bioassays were reared on *L. leucocephala*, which could influence the adults' predilection to prefer the plant species on which they were reared during their nymphal stages (Yamamoto et al., 1969; Eijackers and van Lenteren, 1970).

Nevertheless, the difference in the attractiveness of the caged plants and extracts of the two *Leucaena* spp. to female *H. cubana* at the same concentration strongly indicates that such differences contribute substantially to the preference of *H. cubana* adults for *L. leucocephala*. The demonstration that *H. cubana* uses host-plant odor in its host-plant preference behavior could be exploited and included in selection and breeding programs for resistant hybrids of the agronomically desirable *L. leucocephala*.

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REFERENCES

BECK, S.D., and SCHOONHOVEN, L.M. 1980. Insect behavior and insect resistance, pp. 115–135, *in* F.G. Maxwell and P.R. Jennings (eds.). Breeding Plants Resistant to Insects. Wiley, New York.

BLUST, M.H., and HOPKINS, T.L. 1987. Olfactory responses of a specialist and a generalist grasshopper to volatiles of *Artemisia ludoviciana* Nutt. (Asteraceae). *J. Chem. Ecol.* 13:1893–1902.

DETHIER, V.G. 1980. Evolution of receptor sensitivity to secondary plant substances with special reference to deterrents. *Am. Nat.* 115:45–66.

DETHIER, V.G. 1982. Mechanisms of host plant recognition. *Entomol. Exp. Appl.* 31:49–56.

DICKENS, J.C. 1984. Olfaction in the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae): Electroantennogram studies. *J. Chem. Ecol.* 10:1759–1785.

EIJACKERS, H.J.P., and VAN LENTEREN, J.C. 1970. Host choice and host discrimination in *Pseudeucoila bochei*. . *Neth. J. Zool.* 20:414.

GOMEZ, K.A., and GOMEZ, A.A. 1984. Statistical Procedures for Agricultural Research. Wiley, New York.

LAPIS, E.B. 1991. A comparative study on the susceptibility of two species of *Leucaena* to *Heteropsylla cubana* Crawford (Homoptera; Psyllidae). PhD thesis. Simon Fraser University, Burnaby, British Columbia, Canada.

LAPIS, E.B., and BORDEN, J.H. 1993. Components of resistance in *Leucaena collinsii* (Leguminosae) to the psyllid, *Heteropsylla cubana* (Homoptera: Psyllidae). *Environ. Entomol.* (in press).

MCFADDEN, M.W. 1986. Evaluation of *Leucaena/Heteropsylla* problem in Hawaii, the Philippines and New Caledonia. Forestry Support Program, USDA Forest Service, Washington, D.C.

PROKOPY, R.J., and OWENS, E.D. 1983. Visual detection of plants by herbivorous insects. *Annu. Rev. Entomol.* 28:337–364.

RODRIGUEZ, J.G., KEMP, T.R., and DABROWSKI, Z.T. 1976. Behavior of *Tetranychus urticae* toward essential oil mixtures from strawberry foliage. *J. Chem. Ecol.* 2:221–230.

SMITH, R.F. 1989. Exploitation of seasonal development and semiochemicals for refinement of pest manageement programs involving the Müllein bug, *Campylomma verbasci* (Meyer) and pear psylla, *Psylla pyricola* Foerster. PhD thesis. Simon Fraser University, Burnaby, British Columbia, Canada.

TINGLE, F.C., MITCHELL, E.R., and HEATH, R.R. 1990. Preference of mated *Heliothis viriscens* and *H. subflexa* females for host and nonhost volatiles in a flight tunnel. *J. Chem. Ecol.* 16:2889–2898.

VINCENT, C., and STEWART, R.K. 1984. Effect of allyl isothiocyanate on field behavior of crucifer feeding flea beetles (Coleoptera: Chrysomelidae). *J. Chem. Ecol.* 10:33–39.

VISSER, J.H., and AVÉ, D.A. 1978. General green leaf volatiles in the olfactory orientation of the Colorado potato beetle, *Leptinotarsa decemlineata*. *Entomol. Exp. Appl.* 24:538–549.

WHITMAN, D.W., and ELLER, F.J. 1987. Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1:69–75.

YAMAMOTO, R.T., JENKINS, R.Y., and MCCLUSKY, R.K. 1969. Factors determining the selection of plants for oviposition by the tobacco hornworm *Manduca sexta*. *Entomol. Exp. Appl.* 12:504–508.

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

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RESPONSE OF BROWN TREE SNAKES (*Boiga irregularis*) TO HUMAN BLOOD

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Abstract—Ten specimens of *Boiga irregularis* were presented with clean or bloody tampons. The latter were used by women during menses. Trial duration was 60 sec, intertrial interval was 24 hr, and the dependent variable was rate of tongue flicking (a measure of chemosensory investigation). Bloody tampons elicited significantly more tongue flicking than did control tampons. An additional snake is shown attacking and ingesting a soiled tampon, confirming that chemosensory interest was associated with predatory behavior.

Key Words—Brown tree snake, *Boiga irregularis*, Reptilia, Colubridae, human blood, attraction.

INTRODUCTION

The brown tree snake (*Boiga irregularis*) was accidentally introduced on Guam during or shortly after World War II and has become a serious pest because of its predation upon native mammals, birds, and lizards, and because of its interactions with the electrical transmission system on the island (Engbring and Fritts, 1988; Fritts et al., 1987; Fritts, 1988; Rodda et al., 1992; Savidge, 1987, 1988). The snake attains considerable length (>3 m), and is nocturnal and arboreal. Since *B. irregularis* is a rear-fanged colubrid with large Duvernoy's glands, there is also a medical dimension in the suite of problems arising from its presence on Guam. Analysis of venom toxicity has started (Zalisko and Kardong, 1992), and the general view based on clinical experience is that the venom has only mild effects on adult humans, but it can have more serious, sometimes life-threatening consequences in neonates (Fritts et al., 1990, 1992).

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For three reasons, we infer that snake attacks on neonates have been predatory attempts rather than defensive behaviors. First, it is unlikely that sleeping babies annoyed the snakes, provoking defensive strikes. Second, defensive strikes frequently involve only the snake's front teeth, with very brief contact between the snake's mouth and the threatening target. Hence, there is little opportunity for venom to flow along the grooved rear fangs into the target during such strikes. Third, envenomated babies had multiple pairs of fang punctures along fingers and hands, strongly suggesting that the snakes were attempting to swallow these appendages.

This raises the question of what attracted the snakes to the babies. Hungry snakes may have been hunting for rodents or geckos, encountering the babies more or less by chance. Perhaps chemical cues arising from neonates are attractive to snakes (Chiszar et al., 1988). Both of these considerations are reasonable and cannot be eliminated. Another possibility is that the snakes were attracted by maternal blood (e.g., blood that might be on sanitary napkins present in indoor waste containers). Five experiments showed that *B. irregularis* investigated and struck cotton-tipped applicators soaked with rat blood (Chiszar et al., 1992). Accordingly, there is little doubt about the ability of fresh mammalian blood to elicit predatory behavior in *B. irregularis* (see also Weldon et al., 1992). These data led us to wonder whether the menstrual or postpartum (lochia) blood of human females would be attractive to brown tree snakes. Positive results from an experimental test of this idea are presented below.

METHODS AND MATERIALS

Ten specimens of *B. irregularis* were randomly selected from our laboratory population of 23 animals from Guam. The 10 subjects ranged in weight from 56 g to 940 g. All had been in captivity for at least two years and had been feeding on rodents (*Mus musculus* and *Rattus norvegicus*; one prey/14 days). Snakes were maintained individually in glass cages (62 × 32 × 32 cm), containing paper floor coverings and ad libitum water. Laboratory photoperiod was controlled by automatic switching devices (12 : 12 hr light–dark, photophase 0700–1900 hr); relative humidity was elevated to 40% by vaporizers; and temperature was kept at 26°C by electric heaters. Snakes had last eaten 10–12 days prior to this study.

Each snake received two tests with an intertrial interval of 24 hr. In one test (control), a clean tampon was suspended into the snake's cage and held 2–4 cm from the snake's snout for 60 sec. During this time we recorded the number of tongue flicks emitted by the snake (see Chiszar et al., 1981, for data on the reliability of this measure; see Burghardt, 1970, and Halpern, 1984, for reviews of chemosensation in reptiles; and see Cooper and Burghardt, 1990, for

an analysis of the sensitivity of this dependent variable in chemical discrimination studies). Control tampons were moistened with 10 cc of tap water to induce slight deformation from their packaged shape, making them comparable in conformation to the experimental tampons. The latter were used by women during menses and were presented to snakes as soon as possible thereafter (within 6 hr; tampons were refrigerated at 13°C and then warmed to 26°C just prior to tests). Accordingly, the experimental tampons in this study were representative of ordinary soiled tampons of the sort that would be present in disposal containers in the homes of menstruating women. We suspect that blood in the experimental tampons was also representative of postpartum blood.

Five snakes received experimental trials first and control trials 24 hr later. The remaining five snakes received the reverse order of presentation. Snakes were not permitted to strike tampons in this study because it is extremely difficult to remove these fibrous objects from the snakes' teeth to prevent swallowing. Accordingly, if snakes advanced toward the tampons, they were moved away to keep the distance between snake snouts and tampons at least 2–4 cm. This procedure raises the question of whether or not chemosensory investigation of experimental tampons was associated with predatory interest. To resolve this matter, we observed an additional snake on two control and two experimental trials, but no precaution was taken to prevent strikes.

RESULTS

The additional snake ignored the control tampons, but both experimental ones were struck, grasped, and swallowed (Figure 1). The tampons were excreted nine days later, with most of the red color gone, probably because the blood had been digested and presumably absorbed by the snake.

Mean rates of tongue flicking in the two conditions are shown in Table 1, where it is clear that experimental tampons elicited significantly more chemosensory investigation than did the controls ($F_{1,9} = 48.68$, $P < 0.01$). Order effects (Table 1) were examined in a mixed analysis of variance treating order of presentation as a between-subjects factor and control versus experimental trials as a repeated-measures factor. The main effect of order and the order × trial interaction were both insignificant ($F_{1,8} = 1.57, 3.47$, respectively, $Ps > 0.05$). Because the interaction had an F that neared significance ($0.10 > P > 0.05$), it is worth noting that this factor accounted for only 4.9% of the variance observed in this study, whereas the main effect of control versus experimental trials accounted for 68.4% of the variance. Accordingly, a robust effect of trials occurred in both orders of presentation (Table 1). Post hoc ANOVAs verified this fact, revealing significant differences between the means within rows two and three of Table 1 ($F_{1,4} = 72.93, 21.49$, respectively, $Ps < 0.01$).

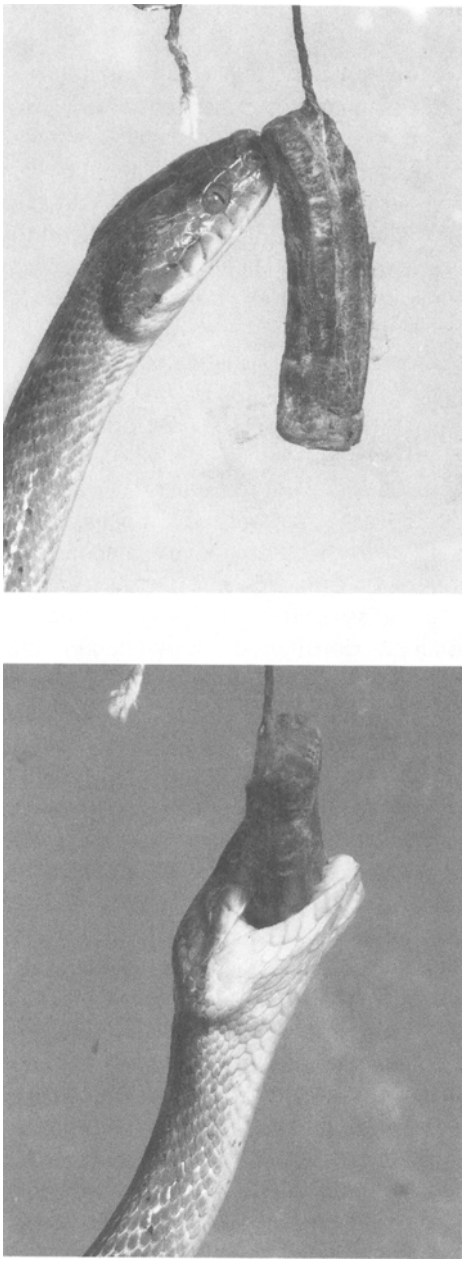

FIG. 1. (A) A brown tree snake investigates a soiled tampon suspended into its cage. (B) Seconds later the snake bites the tampon. (C) About 2 min following the bite, the snake is shown with only the string remaining unswallowed. This snake then struck and swallowed a second soiled tampon.

FIG. 1. Continued

TABLE 1. MEAN NUMBER OF TONGUE FLICKS (SE) BY BROWN TREE SNAKES (*Boiga irregularis*), DURING CONTROL AND EXPERIMENTAL TRIALS

	Control	Experimental
All snakes (N = 10)	26.5 (3.1)	61.7 (4.6)
Snakes that received control followed by experimental trials (N = 5)	27.8 (4.0)	53.6 (3.3)
Snakes that received experimental followed by control trials (N = 5)	25.2 (5.3)	69.8 (7.3)

DISCUSSION

In agreement with the results of Chiszar et al. (1992), the present study verified that human menstrual blood inspired lingual investigation and predatory behavior in brown tree snakes. Two implications arise from these data. First, it is desirable to identify the functional chemicals in mammalian blood that are responsible for this effect. Perhaps these cues might serve as attractants (i.e., baits), capable of luring brown tree snakes into traps on Guam. Second, we take these data as justification for the hypothesis that human postpartum and menstrual blood can play a role in attracting *B. irregularis* into homes on Guam and, hence, into the vicinity of neonates. Field experiments will be necessary to test this hypothesis.

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REFERENCES

BURGHARDT, G.M. 1970. Chemical perception in reptiles, pp. 241–308, *in* J.W. Johnston, D.G. Mouton, and A. Turk (eds.). Communication by Chemical Signals. New York, Appleton-Century-Crofts.

CHISZAR, D., RADCLIFFE, C.W., O'CONNELL, B., and SMITH, H.M. 1981. Strike-induced chemosensory searching in rattlesnakes (*Crotalus viridis*) as a function of disturbance prior to presentation of rodent prey. *Psychol. Rec.* 32:57–62.

CHISZAR, D., KANDLER, K., LEE, R., and SMITH, H.M. 1988. Stimulus control of predatory attack in the brown tree snake (*Boiga irregularis*). 2. Use of chemical cues during foraging. *Amphibia-Reptilia* 9:77–88.

CHISZAR, D., FOX, K., and SMITH, H.M. 1992. Stimulus control of predatory behavior in the brown tree snake (*Boiga irregularis*). IV. Effect of mammalian blood. *Behav. Neural Biol.* 57:167–169.

COOPER, W.E., JR., and BURGHARDT, C.M. 1990. A comparative analysis of scoring methods for chemical discrimination of prey by squamate reptiles. *J. Chem. Ecol.* 16:45–65.

ENGBRING, J., and FRITTS, T.H. 1988. Demise of an insular avifauna: The brown tree snake on Guam. *Trans. West. Sect. Wildl. Soc.* 24:31–37.

FRITTS, T.H. 1988. The brown tree snake, *Boiga irregularis*, a threat to Pacific islands. *U.S. Fish Wildl. Serv. Biol. Rep.* 88, p. 31.

FRITTS, T.H., SCOTT, N.J., JR., and SAVIDGE, J.A. 1987. Activity of the arboreal brown tree snake (*Boiga irregularis*) on Guam as determined by electrical outages. *The Snake* 19:51–58.

FRITTS, T.H., MCCOID, M.J., and HADDOCK, R.L. 1990. Risks to infants on Guam from bites of the brown tree snake (*Boiga irregularis*). *Am. J. Trop. Med. Hyg.* 42:607–611.

FRITTS, T.H., MCCOID, M.J., and HADDOCK, R.L. 1992. Symptoms and circumstances associated with bites by the brown tree snake (Colubridae: *Boiga irregularis*) on Guam. *Am. J. Trop. Med. Hyg.* In press.

HALPERN, M. 1984. The role of the ophidian vomeronasal system in species-typical behavior. *Trends Neurosci.* 7:472–477.

RODDA, G.H., FRITTS, T.H., and CONRY, P.J. 1992. Origin and population growth of the brown tree snake, *Boiga irregularis*, on Guam. *Pacific Science* 46:46–57.

SAVIDGE, J.A. 1987. Extinction of an island forest avifauna by an introduced snake. *Ecology* 68:660–668.

SAVIDGE, J.A. 1988. Food habits of *Boiga irregularis*, an introduced predator on Guam. *J. Herpetol.* 22:275–282.

WELDON, P.J., DEMETER, B.J., WALSH, T.S., and KLEISTER, J.S.E. 1992. Chemoreception in the feeding behavior of reptiles: Considerations for maintenance and management. *in* K. Adler, J.T. Collins, and J.B. Murphy (eds.). SSAR-HL Symposium Volume Honoring Roger Conant. In press.

ZALISKO, E.J., and KARDONG, K.V. 1992. Histology and histochemistry of the Duvernoy's gland of the brown tree snake *Boiga irregularis* (Colubridae). *Copeia* 1992:791–799.

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IMPACT OF ACIDIC DEPOSITION ON *Encelia farinosa* GRAY (COMPOSITAE: ASTERACEAE) AND FEEDING PREFERENCES OF *Trirhabda geminata* HORN (COLEOPTERA: CHRYSOMELIDAE)

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Abstract—Container grown *Encelia farinosa* were exposed to three 3-hr episodes of acidic fog (pH 2.5) typical of events in southern California. Adults and larvae of the specialist leaf-feeding herbivore, *Trirhabda geminata*, preferred to feed on the acidic-treated foliage compared to control fogged (pH 6.3–6.5) foliage. Previous feeding damage on the plants did not affect feeding preference. The acidic-fogged foliage was significantly higher in total nitrogen and soluble protein but not different from control-treated tissue in water content. Stress on native populations of this drought-deciduous shrub caused by atmospheric pollutants may also result in altered feeding ecology of the beetle.

Key Words—*Encelia farinosa*, Asteraceae, *Trirhabda geminata*, Coleoptera, Chrysomelidae, feeding preferences, acidic deposition, plant stress.

INTRODUCTION

The impact of urban expansion on agricultural and natural ecosystems can be direct (e.g., the development of land for urban uses) or indirect (e.g., the effect of pollutants from urban sources on plants and their herbivores). The southwestern United States continues to be one of the fastest growing population centers in the country. An increase in the number of individuals is accompanied by increases in industries and automobiles. Consequently, there has been an increase in anthropogenic air pollution, including ozone, NO_x, SO_x, peroxyace-

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tyl nitrate (PAN), and acidic deposition. Unlike the sulfate acidic rains typical in eastern North America, the more common form of wet acidic deposition in southern California is fog containing both nitric acid and sulfuric acid (Johnson and Siccama, 1983; Granett and Musselman, 1984). These fog events typically have durations of 4–12 hr with a pH of 2.0–3.0, although events of pH 1.7 have been recorded (Hoffman, 1984).

The acidic fogs that typically occur during the winter and early spring have the potential to damage both agricultural crops (Musselman and McCool, 1989) and native plant communities. *Encelia farinosa* Gray (Compositae: Asteraceae) is a dominant drought-deciduous component of the creosote bush scrub and coastal sage scrub plant communities of arid southern California, southern Nevada, central Arizona, and Baja California (Munz and Keck, 1968; Shreve and Wiggins, 1964; Wisdom, 1988). New foliage is flushed following the annual winter rains. Plants can retain foliage throughout the year, but will defoliate if summer drought stress becomes severe.

Trirhabda geminata Horn (Coleoptera: Chrysomelidae) is the primary insect herbivore on *E. farinosa* foliage (Wisdom, 1985). Estivating eggs hatch following winter rains, and the developing larvae feed on the newly flushed leaves (Redak et al., unpublished). Young larvae skeletonize the upper leaf surface, but mature larvae consume all but the main veins. Adults also feed on the leaves. Mating occurs on the host plant, but eggs are laid in the soil and litter at the base of the plant. There is normally a single generation each year, but rare early summer rains may trigger a second generation (Paine, personal observation). Large local populations of the beetle can defoliate plants over a considerable area.

The objective of this study was to determine if acidic fogs common to southern California affect host plant quality and feeding preference of herbivorous insects. The *T. geminata/E. farinosa* system is an excellent model to study the influence of anthropogenic pollution (acidic fogs) on foliage quality, in part because both adults and larvae feed on plant tissue directly exposed to the pollutant. In addition, the system can be used to investigate the potential role of prior insect feeding as a predisposing stress on the leaf tissue. The results have potential implications for the impact of urban expansion on endangered natural and agricultural systems in the southwestern United States.

METHODS AND MATERIALS

Acidic (pH 2.5, reagent-grade nitric and sulfuric acids at a 2.5:1 ratio, v/v in distilled water) or control (pH 6.3–6.5) fog treatments (Dercks et al., 1990) were applied for 3 hr every other day (three total applications) to 1-year-old *E. farinosa* grown in 4-liter containers. Ionic components typical of southern

California acidic fogs (Waldman et al., 1982; Musselman and McCool, 1989) were also added to the treatment solutions. Plants were treated inside 1-m^3 plastic fogging chambers (Musselman et al., 1985) set up in a greenhouse room (22–26°C, 300 $\mu E/m^2/sec$ light), then transferred to a shaded greenhouse room to dry. Acidic and control treatments were alternated in individual chambers on different days to minimize any chamber effect. Following the final fog application, the plants were left in the greenhouse for seven days before use in the bioassay tests or harvested for chemical analyses.

Feeding preferences based on the amount of leaf tissue consumed by adult or larval *T. geminata* were assessed in paper cup arenas (9 cm diameter) containing disks (2 cm^2) of leaf tissue (Jermy et al., 1968; Yoshida and Parrella, 1991). The disks were cut from the young fully expanded leaves with a cork borer seven days after the final treatment with either acidic or control fogs (40 plants per treatment). Plants were individually numbered; assignment of leaf disks cut from these plants to arenas (20 arena replicates per test) was by random draw with replacement. Leaf disks from the same plants were used in more than one arena (no plant was drawn more than twice), but because of the randomization, the same combination of plants was never repeated. Disk size was measured with a leaf area meter (LI 3000; Li-cor, Inc., Lincoln, Nebraska), and two disks of each treatment were randomly positioned within a circle around the bottom of the arena. Moist blotter paper covered with Saran screening lined the bottom of the feeding arena, and the test disks were held in place with map pins. Tight-fitting translucent lids covered each arena. Adult and third-instar larval *T. geminata* were field-collected from endemic populations on the University of California, Riverside campus. Feeding preferences of the two insect life stages were separately tested. Two individuals were placed in the arena and allowed to feed for 48 hr at ambient laboratory light and temperature conditions. The feeding interval did not allow the insects to consume more than 50% of any treatment type. Consumption of more than 50% of any treatment could bias the preference evaluation (Jermy et al., 1968; Jones and Coleman, 1988b). Disk areas were again measured after the feeding exposure to determine the area of tissue consumed. Mean values for consumption of the two acid-treatment or two control disks was determined within arenas. If there had been no feeding within the arena, the replicate was disgarded. Differences between treatments ($P < 0.05$) were determined using two-tailed Wilcoxon signed-rank tests, an approach utilizing a nonparametric paired comparison with arena as the replicate (Sokal and Rohlf, 1981).

In addition to testing the effect of acidic deposition on feeding preference, the effect of previous feeding damage on subsequent preference was also tested. A set of 40 plants each for acid and control fog treatments was exposed to feeding larvae seven days before treatment with the fogs. Leaves exhibiting previous feeding damage were included in the bioassay in addition to leaves

from plants that had not been previously damaged. Differences between treatments were determined as previously described.

An additional set of plants, 10 receiving the acidic deposition treatment and 10 receiving the control fog treatment, was used for chemical analyses of the foliage. Leaves were removed seven days after the third fogging treatment. Percent water content was calculated from leaf fresh and dry weights [(fresh weight − dry weight)/fresh weight] (×100). Tissue not analyzed immediately was stored in an ultracold freezer (−65°C) for later use. Total nitrogen composition (percent) was determined (10 plants/treatment, four tissue samples/plant) using standard microkjeldahl techniques (McKenzie and Wallace, 1954) as modified by Trumble and Hare (1989). Soluble protein content (milligrams per gram) representative of more easily assimilated nitrogen was assessed (10 plants/treatment, four tissue samples/plant) with the Bradford (1976) reagent using the technique of Jones et al. (1988). Difference in water content between treatments was determined using Student's t test (Sokal and Rohlf, 1981). Differences between treatments in total nitrogen and soluble protein were determined with General Linear Models procedure (SAS Institute, 1988) using type III sums of squares with samples nested within plants as the error term.

RESULTS

Adult beetles consumed significantly more leaf tissue (Wilcoxon signed rank $Z = -2.512$, $N = 14$, $P = 0.0120$) from acidic-fog-treated plants than from the control-fogged plants (Figure 1). Because there were no differences in larval preferences among acidic-fogged leaves that had been previously damaged by feeding or were uninjured (Wilcoxon signed rank $Z = -1.161$, $N = 20$, $P = 0.2458$), or among the same two damage classes of control-treated plants (Wilcoxon signed rank $Z = -0.756$, $N = 20$, $P = 0.4496$) (Figure 2), larval consumption data from the two damage classes within each fogging treatment were combined to test for differences between fog treatments. Larval feeding preference followed the same pattern as adults. There was a significant preference (Wilcoxon signed rank $Z = -2.767$, $N = 40$, $P = 0.0057$) for feeding on acidic-fogged leaves compared to the controls (Figure 3).

There were no significant differences ($t = -0.2060$, $df = 17$, $P = 0.8391$) in moisture content (percent) of acid-fogged ($\bar{x} = 75.00$, SE = 0.882, $N = 10$) or control-fogged ($\bar{x} = 74.78$, SE = 0.572, $N = 9$) leaves. However, there were differences in the amounts of nitrogen between treatments. Total nitrogen (percent) was significantly higher (analysis of variance, $F = 5.98$, $df = 1, 18$, $P = 0.0213$) in acidic-fogged leaves ($\bar{x} = 2.33$, SE = 0.152, $N = 10$) than control leaves ($\bar{x} = 1.94$, SE = 0.160, $N = 9$). Similarly, soluble protein (milligrams per gram) was also significantly higher (analysis of variance, $F = 9.51$, $df = 1, 18$, $P = 0.0064$) in acidic-fogged leaves ($\bar{x} = 4.96$, SE = 0.371, $N = 10$) than control leaves ($\bar{x} = 3.94$, SE = 0.319, $N = 10$).

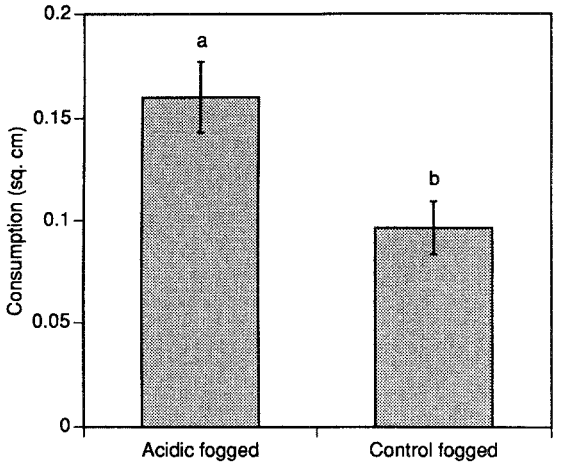

FIG. 1. Amount of acidic or control fogged *Encelia farinosa* tissue consumed by adult *Trirhabda geminata* in a leaf-disk feeding bioassay. Standard errors shown by lines through bars. Significant differences ($P = 0.0120$) indicated by different lowercase letters.

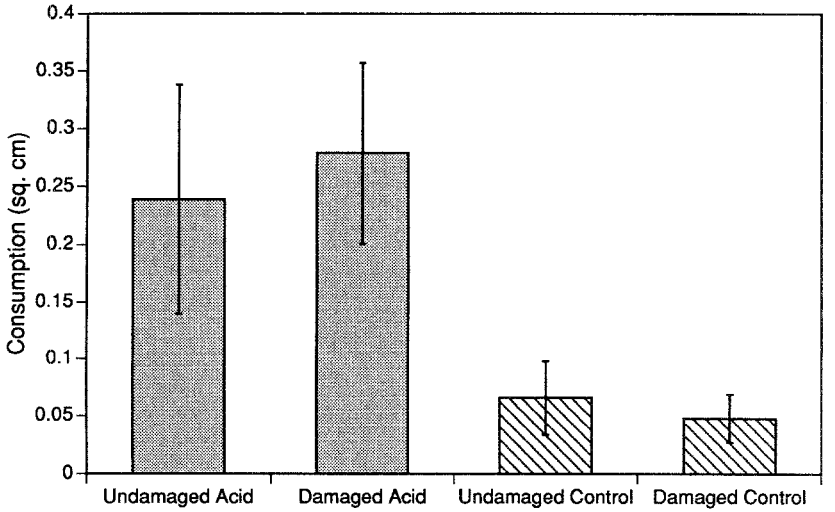

FIG. 2. Amount of tissue consumed by larval *Trirhabda geminata* from undamaged plants or plants damaged by prior insect feeding in a leaf-disk feeding bioassay. Standard errors shown by lines through bars. There were no significant differences between damage classes for either acidic fogged ($P = 0.2458$) or control fogged ($P = 0.4496$) plants.

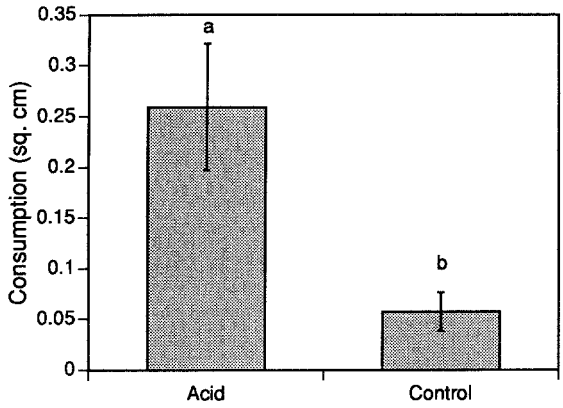

FIG. 3. Amount of acidic or control fogged *Encelia farinosa* tissue consumed by larval *Trirhabda geminata* in a leaf-disk feeding bioassay. Standard errors shown by lines through bars. Significant differences ($P = 0.0057$) indicated by different lowercase letters.

DISCUSSION

In previous studies of *T. geminata* feeding on *E. farinosa*, Wisdom (1985) demonstrated that the larvae move around on plants and aggregate to feed on particular plants or plant parts. He suggested that the larval feeding pattern was related to variation in chemical composition of plant tissues. *Encelia farinosa* shows high levels of seasonal, individual, and population variation in the concentrations of a sesquiterpene lactone, farinosin, and two chromenes, encecalin and euparin, and it has been suggested that the high concentrations found in leaves of some populations are maintained in response to herbivore pressure (Wisdom and Rodriguez, 1982). In fact, *T. geminata* develop at slower rates on diets containing high concentrations of these secondary chemicals and consequently may suffer higher rates of parasitism because of prolonged development time (Wisdom, 1985, 1988).

Wisdom and Rodriguez (1983) found that encecalin and euparin concentrations were greater in a population of *E. farinosa* subject to herbivory by *T. geminata* as compared to a population without significant herbivory, but nitrogen content did not differ between populations. In feeding preference trials, the larvae were unable to distinguish between diets containing high or low concentrations of the secondary chemicals but did prefer to feed on leaves with high nitrogen contents (Wisdom, 1985). Thus, although the sesquiterpene lactone and chromenes may affect the survivorship of the leaf beetle through delayed development and the action of natural enemies (Wisdom, 1985, 1988), differences in nitrogen content rather than secondary chemicals appear to affect the

feeding preferences of the beetle. Acidic deposition increases both total nitrogen and soluble protein in treated leaves. Although variation in plant chemistry may result in aggregated feeding patterns and prolonged insect development on foliage characterized by high concentrations of the sesquiterpene lactone and chromenes under endemic conditions, acidic deposition on plants growing adjacent to urban environments may result in greater levels of foliar nitrogen and thus alter the nature of the interactions between herbivore and host. Specifically, if preference for treated foliage leads to increased herbivory in areas exposed to acidic fogs, plant distributions and local or regional ecologies may be altered.

Preference of a chrysomelid leaf beetle for host foliage exposed to an atmospheric pollutant is not limited to the *T. geminata/E. farinosa*/acid deposition system (Hughes et al., 1982; Endress and Post, 1985). Jones and Coleman (1988a) found that adults and larvae of *Plagiodera versicolora* Laich. preferred to feed on leaf disks cut from *Populus deltoides* Bartr. that had been treated with two 5-hr ozone (0.20 ppm) fumigations. Although both adults and larvae consumed more ozone-fumigated tissue, adult fecundity was lower than in those insects feeding on foliage that had not received the ozone exposure (Coleman and Jones, 1988). The investigators suggested that the ozone treatment reduced the quality of the host. In contrast, acidic deposition results in an increase in foliar nitrogen in *E. farinosa*. Not only do *T. geminata* adults and larvae prefer to feed on the treated leaf tissue, but larvae feeding on treated plants are significantly larger than those feeding on control plants [larval biomass gain: acid $\bar{x} = 2.98 \pm 0.14$ mg, control $\bar{x} = 2.23 \pm 0.14$ mg (unpublished data)].

The results demonstrate that the acidic fogs typical of southern California can alter the primary nutritive value of *E. farinosa* foliage. Both adults and larvae of *T. geminata* prefer to feed on leaves exposed to the acidic fogs. While arena bioassays on excised tissue may not completely reflect the natural system (Barnes, 1963; Jones and Coleman, 1988b; Risch, 1985), the results presented in this study are supported by increases in *T. geminata* larval growth rates (acid $\bar{x} = 0.21 \pm 0.01$ mg/mg/day, control $\bar{x} = 0.16 \pm 0.01$ mg/mg/day) and larval biomass gains in whole plant no-choice tests (Redak, unpublished data). Previous studies in other systems have described the physical changes in leaf tissue, increased stomatal resistance with a decrease in CO_2 assimilation, and changes in leaf chemistry following acidic fog events (Musselman and McCool, 1989; Trumble and Hare, 1989; Dercks et al., 1990; Trumble and Walker, 1991). Many of these studies examined the impact on agricultural plants, and, consequently, effects of anthropogenic pollution on agricultural productivity have been relatively well documented (Takemoto et al., 1988a, b; Musselman and McCool, 1989; McCool et al., 1990). Less well understood are the subtle impacts of these pollutants on noncultivated plant–insect systems. Although there is increasing interest in preserving tracts of open space, massive development of urban areas may indirectly have a great impact on the remaining remnants of the fragile

arid ecosystems by altering the interactions among plants, herbivores, and, potentially, their natural enemies.

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REFERENCES

BARNES, O.L. 1963. Food-plant tests with the differential grasshopper. *J. Econ. Entomol.* 56:396–399.

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein binding. *Anal. Biochem.* 72:248–254.

COLEMAN, J.S., and JONES, C.G. 1988. Plant stress and insect performance: Cottonwood, ozone and a leaf beetle. *Oecologia* 76:57–61.

DERCKS, W., TRUMBLE, J.T., and WINTER, C. 1990. Impact of atmospheric pollution on linear furanocoumarin content in celery. *J. Chem. Ecol.* 16:443–454.

ENDRESS, A.G., and POST, S.L. 1985. Altered feeding preference of Mexican bean beetle, *Epilachna varivestis*, for ozonated soybean foliage. *Environ. Pollut.* 39:9–16.

GRANETT, A.L., and MUSSELMAN, R.C. 1984. Simulated acidic fog injures lettuce. *Atmos. Environ.* 18:887–890.

HOFFMAN, M.R. 1984. Comment on acidic fog. *Environ. Sci. Technol.* 18:61–64.

HUGHES, P.R., POTTER, J.E., and WEINSTEIN, L.H. 1982. Effects of air pollution on plant-insect interactions: Reactions of the Mexican bean beetle to sulfur dioxide fumigated pinto beans. *J. Environ. Qual.* 2:365–368.

JERMY, T., HANSON, F.E., and DETHIER, V.G. 1968. Induction of specific food preference in lepidopterous larvae. *Entomol. Exp. Appl.* 11:211–230.

JOHNSON, A.H., and SICCAMA, T.G. 1983. Acid deposition and forest decline. *Environ. Sci. Technol.* 17:299–304.

JONES, C.G., and COLEMAN, J.S. 1988a. Plant stress and insect behavior: Cottonwood, ozone and the feeding and oviposition preference of a beetle. *Oecologia* 76:51–56.

JONES, C.G., and COLEMAN, J.S. 1988b. Leaf disk size and insect feeding preference: Implications for assays and studies on induction of plant defenses. *Environ. Exp. Appl.* 47:167–172.

JONES, C.G., HARE, J.D., and COMPTON, S.J. 1988. Measuring plant protein with the Bradford assay: I. Evaluation and a standard method. *J. Chem. Ecol.* 15:979–992.

MCCOOL, P.M., MUSSELMAN, R.C., and STERRETT, J.L. 1990. Injury of three flower crops from simulated acidic fog. *Plant Dis.* 74:310–312.

MCKENZIE, H.A., and WALLACE, H.S. 1954. The Kjeldahl determination of nitrogen: A critical study of digestion conditions, temperature, catalyst, and oxidizing agent. *Aust. J. Chem.* 7:55–70.

MUNZ, P.A., and KECK, D.D. 1968. A California Flora and Supplement. University of California Press, Los Angeles.

MUSSELMAN, R.C., and MCCOOL, P.M. 1989. Effects of acidic fog on productivity of celery and lettuce and impact on incidence and severity of diseases. *Ann. Appl. Biol.* 114:559–565.

MUSSELMAN, R.C., STERRETT, J.L., and GRANETT, A.L. 1985. A portable fogging apparatus for field or greenhouse use. *HortScience* 20:1127–1129.

RISCH, S.J. 1985. Effects of induced chemical changes on interpretation of feeding preference trials. *Entomol. Exp. Appl.* 39:81–84.

SAS INSTITUTE. 1988. SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina.

SHREVE, F., and WIGGINS, I.L. 1964. Vegetation and Flora of the Sonoran Desert. Stanford University Press, Stanford, California.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry, 2nd ed. Freeman and Co., New York.

TAKEMOTO, B.K., BYTNEROWICZ, A., and OLSZYK, D.M. 1988a. Depression of photosynthesis, growth, and yield in field-grown green pepper (*Capsicum annuum* L.) exposed to acidic fog and ambient ozone. *Plant Physiol.* 88:477–482.

TAKEMOTO, B.K., OLSZYK, D.M., JOHNSON, A.G., and PARADA, C.R. 1988b. Yield responses of field-grown crops to acidic fog and ambient ozone. *J. Environ. Qual.* 17:192–197.

TRUMBLE, J.T., and HARE, J.D. 1989. Acidic fog-induced changes in host-plant suitability. *J. Chem. Ecol.* 15:2379–2390.

TRUMBLE, J.T., and WALKER, G.P. 1991. Acute effects of acidic fog on photosynthetic activity and morphology of *Phaseolus lunatus*. *HortScience* 20:1531–1534.

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.

WISDOM, C.S. 1985. Use of chemical variation and predation as plant defenses by *Encelia farinosa* against a specialist herbivore. *J. Chem. Ecol.* 11:1553–1565.

WISDOM, C.S. 1988. Comparisons of insect use and chemical defense patterns of two Sonoran desert shrubs, pp. 36–49, *in* R.G. Zahary (ed.). Desert Ecology 1986: A Research Symposium. Southern California Academy of Sciences and the Southern California Desert Studies Consortium.

WISDOM, C.S., and RODRIGUEZ, E. 1982. Quantitative variation of the sesquiterpene lactones and chromenes of *Encelia farinosa*. *Biochem. Syst. Ecol.* 10:43–48.

WISDOM, C.S., and RODRIGUEZ, E. 1983. Seasonal age-specific measurements of the sesquiterpene lactones and chromenes of *Encelia farinosa*. *Biochem. Syst. Ecol.* 11:345–352.

YOSHIDA, H.A., and PARRELLA, M.P. 1991. Chrysanthemum cultivar preferences exhibited by *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environ. Entomol.* 20:160–165.

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MALE-PRODUCED AGGREGATION PHEROMONE OF *Carpophilus mutilatus* (COLEOPTERA: NITIDULIDAE)

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Abstract—Males of *Carpophilus mutilatus* Erichson produce an aggregation pheromone to which both sexes respond. The pheromone includes two hydrocarbon components, (3*E*,5*E*,7*E*)-5-ethyl-7-methyl-3,5,7-undecatriene **(1)** and (3*E*,5*E*,7*E*)-6-ethyl-4-methyl-3,5,7-decatriene **(2).** These were emitted in a 10:1 ratio and in a total amount of ca. 5 ng per feeding male per day. All tested doses of **1** and **2,** from 0.03 to 30 ng, were more attractive than controls in wind-tunnel tests, but there was no evidence of synergism between these trienes. Dramatic synergism between the pheromone and a food-type coattractant occurred in the field, however. In a date garden in southern California, traps with a combination of synthetic **1** and fermenting whole-wheat bread dough attracted 22 times more beetles than dough by itself and 295 times more than **1** by itself. Volatile collections from males also contained three oxygenated compounds that were absent from females. One of these was tetradecanal (ca. 5 ng per male per day), but the structures of the other two are presently undetermined (0.8 and 1.1 ng per male per day). No function for these was demonstrated. One compound originating in the artificial diet, 2-phenylethanol, was particularly attractive in the wind-tunnel bioassay, as was the chromatographic solvent, methanol.

Key Words—*Carpophilus mutilatus*, sap beetle, Coleoptera, Nitidulidae, aggregation pheromone, hydrocarbon, triene, date, host volatiles.

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INTRODUCTION

The confused sap beetle, *Carpophilus mutilatus* Erichson (Coleoptera: Nitidulidae), is a small (<4 mm) brown sap beetle that occurs throughout the tropical, subtropical, and milder temperate regions of the world. In North America it ranges as far north as California (Tehama County) and Virginia (Connell, 1991). *C. mutilatus* belongs to a complex of five very similar species, along with *C. dimidiatus* (Say), *C. freemani* Dobson, *C. fumatus* Boheman, and *C. pilosellus* Motschulsky (Connell, 1975). Until *C. mutilatus* was redescribed by Dobson (1954), it was confused in the literature with *C. dimidiatus*.

C. mutilatus feeds primarily on fallen and decomposing fruits, but it will also infest fruit as it ripens on the plant, especially if it has been damaged. The species is a pest in crops such as dates (Lindgren and Vincent, 1953; Warner et al., 1990; Kehat et al., 1983); figs (Hall et al., 1978; Smilanick, 1979); peaches and plums (Gaven, 1964; Tate and Ogawa, 1975); and corn (Connell, 1975). Like other nitidulids, *C. mutilatus* can transmit fruit-degrading microorganisms, such as brown rot [*Monilinia fructicola* (Wint.) Honey] in stone fruits (Tate and Ogawa, 1975).

Male-produced aggregation pheromones have been identified in three other *Carpophilus* species: *C. hemipterus* (L.), *C. lugubris* Murray, and *C. freemani* Dobson. In each case, the pheromone is a blend of tetraene or tetraene plus triene hydrocarbons (Bartelt et al., 1990a,b, 1991, 1992b). The obvious abundance of *C. mutilatus* in a date garden in southern California (Bartelt et al., 1992a) prompted us to investigate its pheromone also. The resulting pheromone identification was guided by wind-tunnel bioassays and was later corroborated by a field test.

METHODS AND MATERIALS

Beetles. The *C. mutilatus* culture was started from insects captured in a date garden near Oasis, California. The beetles were reared on the diet reported by Dowd (1987), except that additional brewer's yeast replaced the pinto beans. Insects from the culture were used both for pheromone production and for wind-tunnel pheromone bioassays.

Pheromone Collection and Analysis. Beetles were separated by sex and placed with diet medium in volatile-collection flasks as described earlier for *C. hemipterus* (Bartelt et al., 1990a). Volatiles were adsorbed onto Tenax. Each flask contained ca. 70 beetles, but twice as many collectors were set up for males because all of the previously studied, related species had male-produced pheromones. Counts were kept so that amounts of volatiles could be expressed in "beetle-days" (the average amount of material collected per beetle per day).

The pooled collections over a three-week period amounted to 8800 beetle-days from males and 4400 from females.

Except for small bioassay aliquots, these collections were fractionated on open columns of silica gel (1 × 5 cm, column void volume = 3.8 ml). Ten-milliliter fractions were collected for each of the following elution solvents: hexane; 5%, 10%, and 50% ether in hexane (by volume); and 10% methanol in methylene chloride.

After these fractions were bioassayed, some were fractionated further by HPLC. For the hexane fractions (hydrocarbons), a $AgNO_3$-coated silica column was used (eluted with 10% toluene in hexane). For the active 5% and 50% ether–hexane fractions, a 50 Å size-exclusion column was used (eluted with hexane).

All fractions were analyzed by GC (15 m × 0.25 mm ID DB-1 capillary column with 1.0 μm film thickness). Kovats indices (KI), relative to *n*-alkanes, were determined for some GC peaks. The indices were calculated by linear interpolation for GC runs beginning at 100°C and temperature programming at 10°/min.

Positive ion, electron impact mass spectra were obtained for compounds of interest on a Hewlett-Packard 5970 MSD instrument, with sample introduction through a DB-1 capillary GC column. Proton NMR spectra were obtained at 300 MHz for synthetic compounds and for one beetle-derived hydrocarbon, which was isolated in sufficient quantity. The samples were dissolved in deuterobenzene. The chromatographic and spectroscopic instrumentation were as described previously (Bartelt et al., 1990a,b).

Wind-Tunnel Bioassay. The wind-tunnel methods were reported earlier (Bartelt et al., 1990a). Briefly, ca. 1000 beetles were placed into the wind tunnel without food. After 1–2 hr, they became active and began to fly. Bioassays were begun when ca. 100 beetles were in flight at any instant and were continued for as long as the flight activity persisted (typically 3–4 hr). For each bioassay test, two baits (pieces of filter paper treated with extracts, fractions, or solvents) were hung in the upwind end of the tunnel for 3 min, and the numbers of beetles flying to the baits and landing were recorded. The baits were then removed, and after a pause of 2–3 min, the next pair of test baits was put in. When chromatographic fractions were being screened against controls, an appropriate, active, parent material was employed every fourth or fifth test to confirm that the beetles remained responsive. Disposable rubber gloves were worn while handling test baits because the beetles usually responded to any filter papers (including controls) touched directly. Responses to some preparations such as the 50% ether–hexane fractions were inconsistent from day to day; addition of a drop of water to each filter paper (including controls) improved reproducibility without unduly increasing control responses.

Synthetic Hydrocarbons. Two synthetic hydrocarbons (Figure 1) were used

FIG. 1. Hydrocarbons used in this research and assigned structure numbers.

in this project: (3*E*,5*E*,7*E*)-5-ethyl-7-methyl-3,5,7-undecatriene **(1)** and (3*E*,5*E*,7*E*)-6-ethyl-4-methyl-3,5,7-decatriene **(2).** The synthesis of **1** involved seven steps. Butanal was the starting material. Wittig-Horner olefinations, first with triethyl 2-phosphonopropionate and second with triethyl 2-phosphonobutyrate, were the key reactions; these introduced both side chains and created the trisubstituted double bonds. In preparation for subsequent olefinations, the ester product of each Wittig-Horner reaction was reduced to the corresponding alcohol with $LiAlH_4$ and then converted to the aldehyde with MnO_2. A final Wittig reaction using propyl(triphenyl)phosphonium iodide formed the third double bond and completed the hydrocarbon.

The synthesis of **2** required only four steps because the aldehyde, (*E*)-2-methyl-2-pentenal, is commercially available as a starting material. The same final four steps used in the preparation of **1,** beginning with the olefination with triethyl 2-phosphonobutyrate, produced **2** from this starting material. Conditions for these reactions were as described previously for analogous hydrocarbons (Bartelt et al., 1990c). The mass and NMR spectra of these compounds were published earlier (Bartelt et al., 1990b).

The synthetic hydrocarbons were purified on silica gel and, for wind-tunnel tests, by $AgNO_3$ HPLC as well. Purity was 95% for both **1** and **2.** Impurities were primarily geometrical isomers. Aliquots were then diluted to between 0.015 and 15 ng/μl with hexane, the final concentration depending on the nature of the experiment. Only the silica gel purification was used in preparation for the field bioassay. Compound **1** was applied to rubber septa (200 μg/septum) with 300 μl of CH_2Cl_2.

Field Bioassay. Enough synthetic **1** was on hand late in 1990 so that an initial field test could be conducted. This was done in a date garden near Oasis, California. The location and methodology were as described previously for an experiment with *C. hemipterus* (Bartelt et al., 1992a). The wind-directed pipe traps (Dowd et al., 1992) were hung 1 m above the ground, and trap spacing was ca. 20 m. The traps were baited either with pheromone **(1)** only, fermenting whole-wheat dough only, a combination of pheromone plus dough, or were left unbaited as controls. There were two replications of each treatment in a completely randomized design. Beetles were collected from the traps weekly. The pheromone septa were replaced every two weeks, and the dough baits were replaced weekly. The test ran from August 31, 1990, until February 14, 1991. Whole-wheat bread dough is a commonly used nitidulid attractant, and its major volatile emissions have been characterized (Lin and Phelan, 1991).

RESULTS AND DISCUSSION

Chemical Analysis and Wind-Tunnel Bioassays. Volatile collections from both males and females of *C. mutilatus* were attractive in wind-tunnel tests, although the female-derived samples were less so (Table 1). The activity was distributed over several silica gel fractions (Table 1), suggesting the attractive compounds spanned a broad range of polarities. Further analysis was concentrated on the fractions eluted with hexane and with 5% and 50% ether in hexane. These fractions are discussed below in order of increasing polarity. Both 10% MeOH–CH_2Cl_2 fractions also attracted many beetles, but this activity was essentially due to the methanol in the solvent (note control, Table 1); methylene chloride was not attractive in the wind tunnel.

Hexane Fraction. The hydrocarbons from both sexes were further fractionated by $AgNO_3$ HPLC, and the bioassay results for these fractions are listed in Table 2. For both males and females, the eluant between 7 and 9 ml after injection contained essentially all of the activity. By GC, both the 7- to 8- and 8- to 9-ml fractions from males contained a compound (KI = 1393) that was not initially detected in the female-derived fractions (a total of 4.5 ng per beetle day). By GC retention, MS, and proton NMR spectrum (15-μg sample), the compound from the male beetles was identical with **1** (Figure 1). Compound **1** had been encountered previously as a minor volatile constituent from *C. freemani*, but it was behaviorally inactive in that species (Bartelt et al., 1990b).

Two additional hydrocarbons were present in the male-derived fractions

TABLE 1. WIND-TUNNEL ACTIVITY OF VOLATILE COLLECTIONS FROM FEEDING MALE AND FEMALE *C. mutilatus* AND SILICA GEL FRACTIONS DERIVED FROM THESE COLLECTIONS

Bioassay treatment[a]	Mean bioassay count[b]			*N*
	Male-derived	Female-derived	Control	
Whole collection	22.5 a	9.6 b	0.1 c	8
Silica gel fractions				
Hexane	25.0 a	8.9 b	2.1 c	24
5% Ether–hexane	23.6 a	4.3 b	2.1 c	8
10% Ether–hexane	2.2 a	0.9 a	1.6 a	8
50% Ether–hexane	30.3 a	15.1 b	1.5 c	16
10% MeOH–CH_2Cl_2	28.5 a	40.2 b	29.4 ab	12

[a] Treatments bioassayed at 7 beetle-days per test.

[b] Bioassay counts are the numbers of beetles flying upwind to the filter paper baits and alighting during the 3-min tests. In each row, means followed by the same letter are not significantly different [LSD, 0.05 level, balanced incomplete block analysis, log (X + 1) scale].

TABLE 2. WIND-TUNNEL ACTIVITY OF $AgNO_3$ HPLC HYDROCARBON FRACTIONS DERIVED FROM MALE AND FEMALE *C. mutilatus*[a]

HPLC retention volume (ml)	Mean bioassay count[b]		Mean bioassay count	
	Male-derived fraction	Control	Female-derived fraction	Control
3–4	0.4	0.7	1.5	0.9
4–5	1.1*	0.2	1.8	0.7
5–6	0.6	0.1	1.3	0.9
6–7	0.7	0.3	1.1	0.4
7–8	17.2***	0.1	15.1***	0.4
8–9	17.2***	0.9	10.3***	0.4
9–10	1.2**	0.1	1.6*	0.3
10–11	0.5	0.2	0.7	0.5
11–12	0.6	0.2	0.6	0.4
12–13	0.6	0.0	2.8***	0.2
13–14	0.2	0.2	1.4	0.8
14–15	0.2	0.1	0.8	0.2

[a]Fractions tested at 7 beetle-days per trial; $N = 8$ for each fraction. The male-derived and female-derived fractions were tested on different days; thus the data cannot be compared quantitatively between sexes.

[b]Bioassay counts are the numbers of beetles flying upwind to the filter paper baits and alighting during the 3-min tests. Differences from the control at the 0.05, 0.01, and 0.001 levels denoted by *, **, and ***, respectively [t tests in log $(X + 1)$ scale, using pooled error for each sex].

that were not evident in the corresponding female fractions (KI = 1300 and KI = 1352). These were less abundant than **1** (0.4 and 0.3 ng per beetle-day, respectively). The former occurred along with **1** in the two most active HPLC fractions (Table 2). It was identical by GC retention and MS to **2** (Figure 1), another compound found previously in *C. freemani* (Bartelt et al., 1990b). The other minor hydrocarbon in *C. mutilatus* males (KI = 1352) was found only in an inactive HPLC fraction 6-7 ml after injection. The mass spectrum of this compound was indistinguishable from that of **1.** From previous experience (Bartelt et al., 1992b), this compound was believed to be a geometrical isomer of **1** with a *Z* configuration at one of the trisubstituted double bonds. Due to the bioassay inactivity, structure determination was not pursued further.

Compounds **1** and **2** fully accounted for the activity of the 7- to 9-ml $AgNO_3$ HPLC fractions. By GC, the 7- to 8-ml fraction contained 3.6 ng of **1** and 0.14 ng of **2** per beetle-day. This fraction (7 beetle-days per test), a solution of synthetic **1** and **2** in corresponding amounts, and controls were compared in the wind tunnel, and the resulting mean responses were 15.4, 15.7, and 1.2 beetles per test, respectively ($N = 12$). For the 8- to 9-ml fraction, having 0.94 ng of

1 and 0.26 ng of **2** per beetle-day, the wind-tunnel results for the fraction (7 beetle-days per test), the corresponding synthetic mixture, and controls were 16.2, 20.6, and 0.3, respectively ($N = 12$). For both experiments, the natural and synthetic mixtures did not differ from each other ($P > 0.4$), but all mixtures differed from the control ($P << 0.01$).

Both synthetic **1** and **2** were active by themselves in wind-tunnel tests (Table 3). The beetles responded to the lowest dose, 30 pg, of either **1** or **2** at levels significantly above controls, and the responses increased with higher doses for both compounds. At the lower doses (≤ 3 ng), **1** appeared somewhat more active than **2.** [In the table, the activity of each dose is expressed as a percentage of the standard blend (3 ng of **1** plus 0.3 ng of **2**), corrected for controls. Overall means for the standard and the control in this experiment were 35.7 and 1.1 landings per test]. There was no evidence for synergistic activity of **1** and **2.** Three treatments, in bold type in the table, had **1** and **2** in the natural ratio, ca. 10:1, but these were not superior to adjacent treatments with similar amounts of triene in other ratios. The main feature of the table is that at the higher doses, **1** and **2** and their blends appeared interchangeable. No treatments containing 3–30 ng of **1** and/or 30 ng of **2** were significantly different from the standard.

No female-specific hydrocarbons were detected, based upon careful GC analysis of the fractions. However, these analyses did reveal minute amounts

TABLE 3. RELATIVE ACTIVITY[a] OF DOSES AND COMBINATIONS OF **1** AND **2** IN WIND TUNNEL

	Dose of **1** (ng)				
Dose of **2** (ng)	0	0.03	0.3	3	30
0	0 c[b]	7 b	46 b	81 a	98 a
0.03	7 b	9 b	**39**[c] b	107 a	113 a
0.3	21 b	18 b	64 b	**100** a	91 a
3.0	58 b	69 a	69 b	88 a	**89** a
30	106 a	140 a	99 a	87 a	108 a

[a] Each dose treatment was compared with the control and the standard dose (3 ng **1** plus 0.3 ng **2**) in a balanced incomplete block experiment ($N = 8$, two baits per test). Numbers of beetles flying upwind and alighting on the filter paper baits during the test periods were recorded. Relative activity, R, of each treatment is expressed as a percentage of the standard dose, corrected for responses to controls: $R = [(\text{treatment} - \text{control})/(\text{standard} - \text{control})] \times 100$. By definition, R for the standard dose = 100, and R for the control = 0.

[b] Each incomplete block experiment was analyzed separately [log $(X + 1)$ scale]. Treatments followed by "a" were not significantly different from the standard. Treatments followed by "b" were different from both the standard and the control. All treatments were significantly more attractive than the control, marked "c" (t tests, 0.05 level).

[c] Responses in bold type represent the natural proportions of **1** and **2** (ca. 10:1).

(ca. 50 pg per beetle-day) of **1** in the two most active fractions (7–9 ml after injection). Compound **2** may have been present also, but it was below the limits of detection in the female-derived fractions. While it is possible that female beetles produce a pheromone, the presence of **1** in the fractions was more likely due to errors in sorting the beetles by sex under the microscope when the volatile collectors were set up. An error rate of about 1% could have accounted for the observed amount of male-specific compound in the female sample. Thus, there is presently no compelling evidence for a potent, female-produced, hydrocarbon attractant, despite the initial bioassay tests (Table 1). The minor activity in the 12- to 13-ml $AgNO_3$ fraction remains unexplained.

5% Ether–Hexane Fraction. By GC, the active, 5% ether–hexane fraction from males contained over 100 compounds. Three of these (KI = 1546, 1551, and 1598 in amounts of 1.1, 0.8, and 5.0 ng per beetle day, respectively) were absent from females. These compounds were relatively abundant, the KI = 1598 peak representing 12% of the material in the 5% ether–hexane fraction. On the size-exclusion HPLC column, all three compounds occurred in a fraction 11.2–12.4 ml after injection. The major compound (KI = 1598) was also detected in the next earlier fraction, 10.6–11.2 ml after injection.

Surprisingly, the HPLC fractions at 10.6–11.2 and 11.2–12.4 ml were essentially inactive in the wind tunnel (20 beetle-days per test). The mean bioassay counts were 0.8 and 1.5 for the fractions and 0.5 and 0.4 for the respective controls ($N = 4$, $P > 0.05$). The activity eluted entirely in the 8.4- to 9.5- and 9.5- to 10.2-ml fractions, which is where hydrocarbon **1** elutes. The mean bioassay counts were 29.2 and 19.7, and for the respective controls, 0.6 and 1.4 ($N = 4$, $P < 0.001$). After rechromatography of the active fractions on silica gel, the hexane fractions were very active in the wind tunnel at 20 beetle-days per test (means of 13.0 for both and 0.0 and 0.5 for their respective controls), but the subsequent, 5% ether–hexane fractions were not (no beetles alighted on either the fractions or the controls). Careful GC revealed a minute amount of **1** in the hexane fraction (ca. 60 pg per beetle-day). The original 5% ether–hexane silica gel fraction was probably active in the wind tunnel only because the hydrocarbon **1** tailed slightly in the silica gel chromatography.

The oxygenated compound at KI = 1598 was identified as tetradecanal by a GC and MS comparison to an authentic standard. The other two compounds remain unidentified. The mass spectrum of the compound at KI = 1546 was obtained [*m/z* (% of base peak): 206(M^+, 23%), 192(15%), 191(7%), 177(56%), 167(8%), 163(26%), 149(23%), 135(10%), 125(19%), 123(17%), 121(42%), 109(19%), 107(17%), 105(16%), 95(23%), 93(29%), 91(20%), 83(53%), 77(21%), 71(21%), 69(22%), 67(28%), 57(79%), 55(100%), 43(79%), 41(84%)]. The compound at KI = 1551 had an almost identical spectrum and is probably a geometrical isomer. The spectra suggest a molecular weight of 206, which probably corresponds to $C_{14}H_{22}O$. The similarity of these spectra

to that of **1** (Bartelt et al., 1990b) suggests these are oxygenated analogs of **1.** Several branched, 14-carbon, triene aldehydes prepared during the course of this project had similar, but not identical, mass spectra and GC retentions.

Although no pheromonal activity was demonstrated for the male-specific, oxygenated compounds, it is possible that a function would be revealed with a different type of bioassay or in combination with other chemicals. The existence of oxygenated, sex-specific compounds in *Carpophilus* beetles raises new questions to be investigated.

50% Ether–Hexane Fraction. The 50% ether–hexane fractions derived from males and females were both quite active in the wind tunnel (Table 1). After fractionation of the male-drived material by size exclusion HPLC, the activity was located primarily in an HPLC peak 30–35 ml after injection (73.6 beetles per test and 0.9 for controls, $N = 4$; no other fractions exceeded 3.7). For females, the peak eluted slightly earlier and was contained in two fractions, 20–30 and 30–35 ml after injection. Both of these fractions were active (21.1 and 31.7 beetles per test and 1.3 and 0.3 for respective controls, $N = 4$; no other fractions exceeded 3.1). By GC, all of these fractions shared one obvious, common peak (KI = 1095), and in the male-derived HPLC fraction, it represented 85% of the total material (ca. 40 ng per beetle-day). From the mass spectrum and a subsequent library search, the compound was determined to be 2-phenylethanol [*m/z* (% of base peak): 122(M^+, 24%), 104(3%), 103(4%), 92(60%), 91(100%), 78(5%), 77(6%), 65(24%), 63(7%), 51(11%), 50(6%)]. The sample matched an authentic standard in GC and MS properties. Subsequent volatile collection from a diet blank revealed that the compound originated in the artificial diet medium, and the compound is, therefore, not believed to be a pheromone component. In wind-tunnel comparisons such that weights of 2-phenylethanol were equal, the synthetic alcohol accounted for between 49% and 59% of the activity of the HPLC fractions. Thus, other, less abundant compounds in the HPLC fractions probably have activity as well. Food-related attractants were not pursued further here. No sex specific compounds were detected in these HPLC fractions.

Initial Field Study with 1. Synthetic **1** was very active in the test in the date garden, but as with the other *Carpophilus* species we have studied, the activity was obvious only when a food-type coattractant was present with the pheromone (Table 4). The pheromone alone attracted only 16 *C. mutilatus* during the study, but in combination with fermenting whole-wheat bread dough, it attracted 4722 *C. mutilatus*. The dough by itself attracted only 211 beetles. For all treatments, both sexes were captured, and the sex ratios favored females slightly. Trap catches were variable (see ranges in Table 4) primarily because of seasonal fluctuations in flight activity. Activity peaked in February, but moderate catches were also recorded during November and January (Figure 2).

Summary. C. mutilatus is like the three previously studied *Carpophilus*

TABLE 4. INITIAL FIELD TEST WITH SYNTHETIC PHEROMONE FOR *C. mutilatus* IN DATE GARDEN IN SOUTHERN CALIFORNIA, AUGUST 31, 1990 TO FEBRUARY 14, 1991[a]

Treatment	Overall total catch	Mean catch per week per trap[b]	Range in trap catch
1 plus dough	4722 (65% females)	45.8 a	0–668
Dough only	211 (53% females)	2.8 b	0–20
1 only	16 (69% females)	0.2 c	0–5
Control	0	0.0 c	0–0

[a]There were two traps per treatment; weekly trap catches were analyzed in log (X + 1) scale. Factors in two-way ANOVA were treatment and week.
[b]Means followed by the same letter not significantly different (LSD, 0.05 level). Means calculated in log (X + 1) scale and returned to numerical scale for presentation.

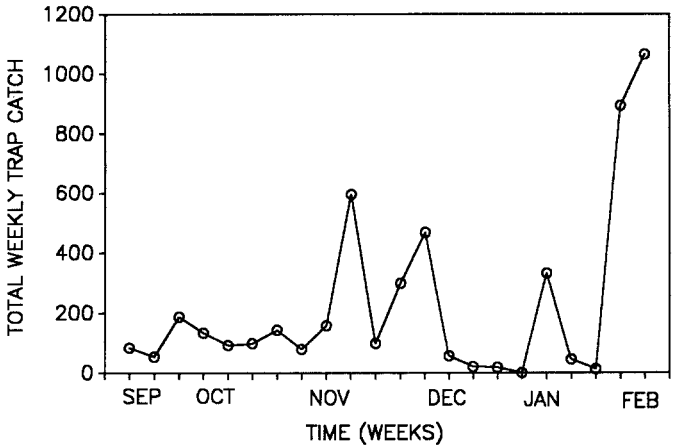

FIG. 2. Trap catch pattern over time for *C. mutilatus* in a date garden near Oasis, California, August 31, 1990, to February 14, 1991. Each point is a weekly catch, totaled over all traps and treatments.

species in having a male-produced, hydrocarbon pheromone to which both sexes respond. The pheromone includes two conjugated trienes (**1** and **2**), which were previously identified in the volatile emissions from *C. freemani*.

As with the other species, *C. mutilatus* responds best to a combination of food odors and pheromone. One especially attractive compound, 2-phenylethanol, was isolated from the artificial diet, and the chromatographic solvent, methanol, is also attractive. Additional work remains to be done on food-type attractants for this species. Male-specific, oxygenated compounds were also

isolated from *C. mutilatus* volatiles, but no pheromonal function has yet been demonstrated for these.

An initial field bioassay established that **1** is very active under natural conditions, as long as a food-type coattractant is also present. Further field studies involving pheromone dose, component blends of **1** and **2**, and trap height are under way and will be reported later.

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REFERENCES

BARTELT, R.J., DOWD, P.F., PLATTNER, R.D., and WEISLEDER, D. 1990a. Aggregation pheromone of driedfruit beetle, *Carpophilus hemipterus:* Wind-tunnel bioassay and identification of two novel tetraene hydrocarbons. *J. Chem. Ecol.* 16:1015–1039.

BARTELT, R.J., DOWD, P.F., SHOREY, H.H., and WEISLEDER, D. 1990b. Aggregation pheromone of *Carpophilus freemani* (Coleoptera: Nitidulidae): A blend of conjugated triene and tetraene hydrocarbons. *Chemoecology* 1:105–113.

BARTELT, R.J., WEISLEDER, D., and PLATTNER, R.D. 1990c. Synthesis of nitidulid beetle pheromones: Alkyl-branched tetraene hydrocarbons. *J. Agric. Food Chem.* 38:2192–2196.

BARTELT, R.J., DOWD, P.F., and PLATTNER, R.D. 1991. Aggregation pheromone of *Carpophilus lugubris:* New pest management tools for the nitidulid beetles, pp. 27–40, *in* P.A. Hedin (ed.). Naturally Occurring Pest Bioregulators. ACS Symposium Series No. 449, American Chemical Society, Washington, D.C.

BARTELT, R.J., DOWD, P.F., VETTER, R.S., SHOREY, H.H., and BAKER, T.C. 1992a. Responses of *Carpophilus hemipterus* (Coleoptera: Nitidulidae) and other sap beetles to the pheromone of *C. hemipterus* and host-related coattractants in California field tests. *Environ. Entomol.* 21:1143–1153.

BARTELT, R.J., WEISLEDER, D., DOWD, P.F., and PLATTNER, R.D. 1992b. Male-specific tetraene and triene hydrocarbons of *Carpophilus hemipterus:* Structure and pheromonal activity. *J. Chem. Ecol.* 18:379–402.

CONNELL, W.A. 1975. Hosts of *Carpophilus dimidiatus. J. Econ. Entomol.* 68:279–280.

CONNELL, W.A. 1991. Sap beetles (Nitidulidae, Coleoptera), pp. 151–174, *in* J.R. Gorham (ed.). Insect and Mite Pests in Food: An Illustrated Key, Vol. 1. USDA and USDHHS Agric. Handbook No. 655, U.S. Government Printing Office, Washington, D.C.

DOBSON, R.M. 1954. The species of *Carpophilus* Stephens (Col. Nitidulidae) associated with stored products. *Bull. Entomol. Res.* 45:389–402.

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.

DOWD, P.F., BARTELT, R.J., and WICKLOW, D.T. 1992. Novel insect trap useful in capturing sap beetles (Coleoptera: Nitidulidae) and other flying insects. *J. Econ. Entomol.* 85:772–778.

GAVEN, M.J. 1964. *Carpophilus* beetles as a pest of peaches. *J. Entomol. Soc. Austr. (N.S.W.)* 1:59–62.

HALL, R.W., SMILANICK, J.M., and EHLER, L.E. 1978. Laboratory rearing and field observations on *Carpophilus mutilatus*. *Ann. Entomol. Soc. Am.* 71:408–410.

KEHAT, M., BLUMBERG, D., and WILLIAMS, R.N. 1983. Seasonal abundance of sap beetles (Coleoptera: Nitidulidae) in date plantations in Israel. *Phytoparasitica* 11:109–112.

LIN, H., and PHELAN, P.L. 1991. Identification of food volatiles attractive to dusky sap beetle, *Carpophilus lugubris* (Coleoptera: Nitidulidae). *J. Chem. Ecol.* 17:1273–1287.

LINDGREN, D.L., and VINCENT, L.E. 1953. Nitidulid beetles infesting California dates. *Hilgardia* 22:97–118.

SMILANICK, J.M. 1979. Colonization of ripening figs by *Carpophilus* spp. *J. Econ. Entomol.* 72:557–559.

TATE, K.G., and OGAWA, J.M. 1975. Nitidulid beetles as vectors of *Monolinia fructicola* in California stone fruits. *Phytopathology* 65:977–983.

WARNER, R.L., BARNES, M.M., and LAIRD, E.F. 1990. Reduction of insect infestation and fungal infection by cultural practice in date gardens. *Environ. Entomol.* 19:1618–1623.

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SEX PHEROMONE CANDIDATES WITH A CONJUGATED TRIENE SYSTEM: SYNTHESIS AND CHEMICAL CHARACTERIZATION

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Abstract—Sex pheromone candidates with a conjugated triene system, 8,10,12-, 9,11,13- and 11,13,15-hexadecatrienyl acetates (double bond positional isomers of the *Glyphodes pyloalis* pheromone, 10,12,14-triene) were synthesized by introducing an *E* configuration stereospecifically to two of three double bonds and rather nonspecifically to another double bond, so as to obtain two geometrical isomers; *E,E,E* and *E,E,Z* or *E,Z,E* isomers. The two geometrical isomers of each triene acetate were separated on a reverse-phase HPLC column and characterized by ^{1}H NMR analysis. The ^{13}C NMR signals in the olefinic region of each isomer were assigned by two-dimensional NMR techniques and also by an empirical rule based on the changes of the chemical shifts by converting the configuration. Based on the assignments, substituent parameters for calculating the chemical shifts of 1,6-dialkyl conjugated trienes were generated. Electron impact mass spectrometry showed characteristic fragment ions that enabled the double bond positional isomers to be distinguished from each other.

Key Words—Sex pheromone, lepidopterous pheromone, conjugated triene, hexadecatrienyl acetate, synthesis, ^{13}C NMR shift parameters.

INTRODUCTION

Lepidopterous sex pheromones have hitherto been identified from females belonging to more than three hundred species. Analysis of their chemical struc-

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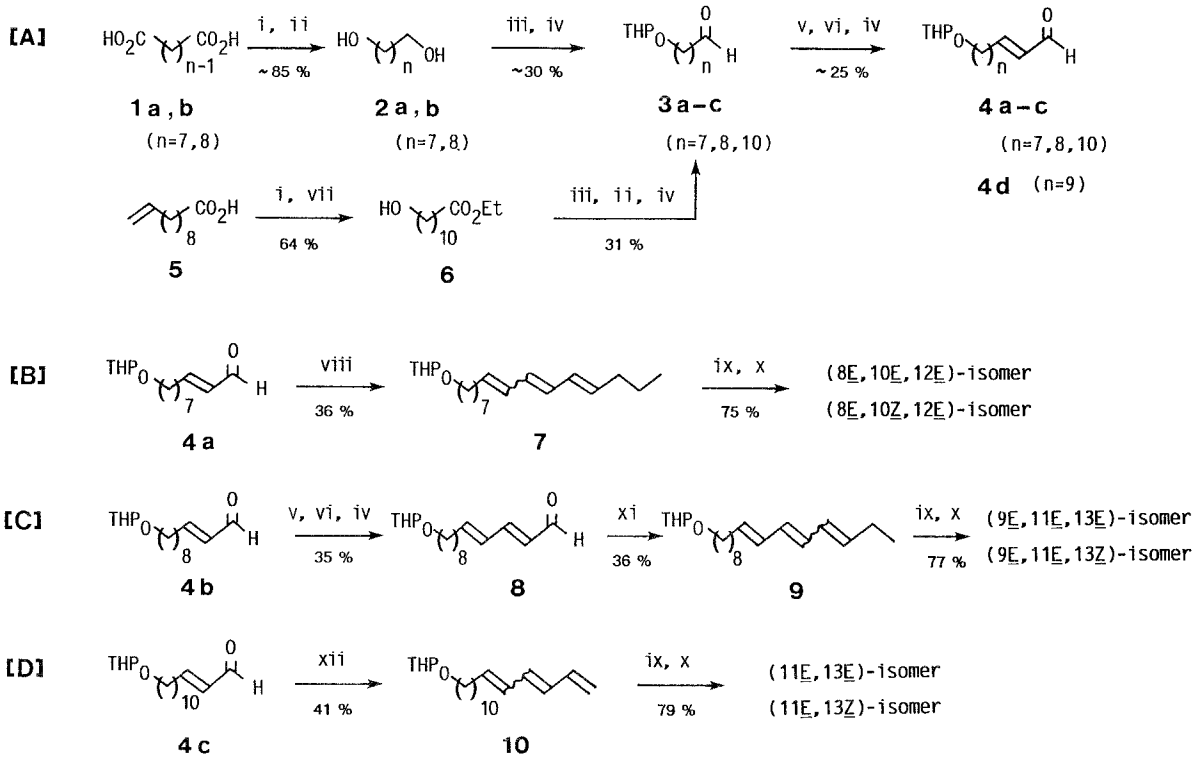
tures (Arn et al., 1986; Ando, unpublished) showed that ca. 85% of them were composed of C_{10}–C_{18} fatty alcohols, acetates, and aldehydes with one or two C=C double bond(s), and ca. 10% consisted of C_{17}–C_{23} hydrocarbons with a homoconjugated triene system and their monoepoxy analogs, possibly biosynthesized from linolenic acid, one of the essential fatty acids (Roelofs and Bjostad, 1984). Recently, sex pheromone components with a conjugated triene system have been identified from three lepidopterous species: (10*E*,12*E*,14*Z*)-10,12,14-hexadecatrienyl acetate from *Glyphodes pyloalis* Walker (Seol et al., 1987; Honda et al., 1990), the aldehyde derivative from *Manduca sexta* L. (Tumlinson et al., 1989), and (9*Z*,11*E*)-9,11,13-tetradecatrienal from *Ectomyelois ceratoniae* Zeller (Baker et al., 1989). These findings indicate that one group of lepidopterous sex pheromones consists of conjugated triene compounds and that new pheromone components with a conjugated triene system are likely to be identified in future. Since it is difficult to collect a sufficient amount of a natural pheromone for chemical analysis, particularly in the case of unstable polyunsaturated compounds, it is important to synthesize standards. This paper deals with the synthesis and chemical characterization of several hexadecatrienyl acetates, consisting of double-bond positional isomers of the pheromone of *G. pyloalis*, which are considered to be lepidopterous pheromone candidates. We also propose substituent parameters for calculating the chemical shifts of olefinic ^{13}C NMR signals and characteristic MS fragment ions that are useful for the identification of the conjugated trienes.

METHODS AND MATERIALS

Synthesis of (E)*-Alkenal (**4a–c**,* n = *7, 8, and 10, Scheme 1A).* Suberic acid **1a** was converted to an ethyl ester and reduced to a diol **2a** with $LiAlH_4$ in dry ether. After one hydroxy group of the diol **2a** was protected as a tetrahydropyranyl (THP) ether, the other hydroxy group was oxidized with pyridinium chlorochromate (PCC) to produce aldehyde **3a,** which was converted into (*E*)-2-alkenal **4a** ($n = 7$) in three steps; a coupling reaction with methoxycarbonylmethylenetriphenylphosphorane, reduction with $LiAl(OEt)_2H_2$, and oxidation with PCC. Starting from azelaic acid **1b,** homologous (*E*)-2-alkenal **4b** ($n = 8$) was synthesized by the same procedure.

Undecenoic acid **5** was esterified and converted into an alcohol **6** by hydroboration and successive protonolysis. After protecting the hydroxy group as a THP ether, the ester group of **6** was reduced with $LiAlH_4$ and further oxidized with PCC to produce an aldehyde **3c,** which was converted to (*E*)-2-alkenal **4c** with the longest chain ($n = 10$) in the above three steps. Experimental conditions were almost the same as those for the synthesis of **4d** ($n = 9$), a key intermediate in the preparation of 10,12,14-hexadecatrienyl acetate (Ando et al., 1988b). The



SCHEME 1. Synthetic routes for (*E*)-2-alkenals (**4a–c**) (A) and 8,10,12-, 9,11,13-, and 11,13,15-hexadecatrienyl acetates (B–D): i, EtOH/H_2SO_4; ii, $LiAlH_4$/ether; iii, 2,3-dihydropyran/*p*-TsOH; iv, PCC/CH_2Cl_2; v, $Ph_3P{=}CHCO_2Me$/benzene; vi, $LiAl(OEt)_2H_2$/ether; vii, BH_3/THF, then H_2O_2/aq.NaOH; viii, $Ph_3P{=}CHC{=}C(CH_2)_2CH_3$/THF; ix, *p*-TsOH/EtOH; x, Ac_2O/pyridine; xi, $Ph_3P{=}CHCH_2CH_3$/THF; xii, $Ph_3P{=}CHCH{=}CH_2$/THF.

^1H and ^{13}C NMR data of **4a–c** corresponded well with those of **4d** except for the signal intensity of the overlapping methylene protons at 1.31 ppm and carbons at 29.4 ppm.

Synthesis of 8,10,12-Hexadecatrienyl Acetate (ω4,ω6,ω8-Triene, Scheme 1B). (*E*)-2-Hexen-1-ol was treated with phosphorus tribromide in ether. Distillation of the crude products yielded 1-bromo-(*E*)-2-hexene, which was found to be contaminated with the *Z* isomer (ca. 15%) and 3-bromo-1-hexene (ca. 15%) by NMR analysis. Without further purification, the bromide was converted into a triphenylphosphonium salt by treatment with triphenylphosphine under reflux conditions in benzene and coupled with (*E*)-2-alkenal **4a** in dry THF using *n*-butyllithium as a base to obtain the 8,10,12-triene compound **7.** After removing the THP group by heating with catalytic *p*-toluenesulfonic acid in ethanol, the free alcohol was acetylated with acetic anhydride in pyridine. The HPLC analysis with a reverse-phase column revealed that the 8*E*,10*E*,12*E* and 8*E*,10*Z*,12*E* isomers were produced in a ratio of 44:56 in addition to some trienes derived from by-products of the bromination step. From the geometrical mixture (10 mg) the projected two isomers were separately collected by the preparative HPLC. After evaporation of the solvent and usual TLC purification, 8*E*,10*E*,12*E* isomer (3 mg, >98% purity on HPLC and GC analyses) and 8*E*,10*Z*,12*E* isomer (4 mg, 90% purity) were obtained. Their chemical structures were confirmed by NMR analyses.

Synthesis of 9,11,13-Hexadecatrienyl Acetate (ω3,ω5,ω7-Triene, Scheme 1C). The chain of (*E*)-2-alkenal **4b** was extended by one more conjugated double bond to obtain (*E*,*E*)-2,4-alkadienal **8** by three successive reactions used for its own preparation, namely a coupling reaction, reduction, and oxidation. The ^1H and ^{13}C NMR data of **8** corresponded to those of the C_{14} homolog, another intermediate of the previous 10,12,14-triene synthesis (Ando et al., 1988b). By linking **8** to propylidenetriphenylphosphorane, 9,11,13-triene THP ether **9** was obtained. The THP group was removed and the deprotected hydroxy group was acetylated as already described. The HPLC analysis revealed the formation of 9*E*,11*E*,13*E* and 9*E*,11*E*,13*Z* isomers of 9,11,13-hexadecatrienyl acetate in a ratio of 58:42. From the mixture (10 mg) each geometrical isomer (ca. 3 mg, >98% purity) was obtained by the successive purification of HPLC and TLC, and the structure was confirmed by the NMR analysis.

Synthesis of 11,13,15-Hexadecatrienyl Acetate (ω1,ω3,ω5-Triene, Scheme 1D). By the Wittig reaction between (*E*)-2-alkenal **4c** and 2-(*E*)-propenylidenetriphenylphosphorane, the 11,13,15-triene compound **10** was obtained in a manner similar to that of the 8,10,12-triene synthesis, and the THP ether was converted into acetate to yield a mixture of 11*E*,13*E* and 11*E*,13*Z* isomers of 11,13,15-hexadecatrienyl acetate in a ratio of 45:55. These geometrical isomers (ca. 3 mg, >98% purity) were separated from the mixture (10 mg) in the same way to that for the other trienes.

Other Chemicals. 1,3-Butadiene and 1,3,5-hexatriene were purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin). The triene compound was a mixture of *E* and *Z* isomers in a ratio of ca. 2:1 as indicated by the following NMR data (ppm from TMS in $CDCl_3$); (*E*)-1,3,5-hexatriene, ^1H NMR: H^1 5.10, $H^{1'}$ 5.23, H^2 6.36, H^3 6.22 ($J_{1,2}$ = 10 Hz, $J_{1',2}$ = 17 Hz, $J_{2,3}$ = 10 Hz), ^{13}C NMR: C^1 117.7, C^2 136.9, C^3 133.7, (*Z*)-1,3,5-hexatriene, ^1H NMR: H^1 5.15, $H^{1'}$ 5.24, H^2 6.80, H^3 6.00 ($J_{1,2}$ = 10 Hz, $J_{1',2}$ = 17 Hz, $J_{2,3}$ = 10 Hz), ^{13}C NMR: C^1 118.4, C^2 132.0, C^3 130.5. The configuration of the two geometrical isomers was determined by the calculation of the chemical shift values of C^2 signals. Carbons attached to an *E* double bond resonated at a lower field by ca. 5 ppm than those attached to a *Z* double bond (Ando et al., 1988b).

Chromatography. HPLC analyses were carried out with a Shimadzu LC-6A system equipped with a UV detector (SPD-6A) operated at 265 nm and an integrator (Shimadzu C-R3A Chromatopac) and fitted with a Senshu Pak ODS column (8 mm ID × 15 cm, 5 μm) packed by Senshu Kagaku Co. (Tokyo, Japan). The mobile phase consisted of 10% water in acetonitrile (flow rate: 2 ml/min). Preparative HPLC was conducted using another Senshu Pak ODS column (20 mm ID × 25 cm, 5 μm) with acetonitrile (100%) as the mobile phase (flow rate: 7 ml/min) in a recycling mode (three cycles). GC analysis was carried out using a Shimadzu GC-4BM chromatograph equipped with a PEG-20M capillary column (0.25 mm ID × 25 m, Gasukuro Kogyo Inc., Tokyo, Japan). Carrier gas was nitrogen, and the effluent was detected by FID. The column temperature was programmed from 70°C to 230°C at a rate of 5°C/min.

Spectroscopy. The NMR spectrum of each compound in $CDCl_3$ was measured with a JEOL GX 270 Fourier transform spectrometer (270.2 MHz for ^1H and 67.9 MHz for ^{13}C) using TMS as an internal standard. Two-dimensional (2D) spectra of ^1H–^1H correlation spectroscopy (COSY) and ^{13}C–^1H COSY were measured with the same spectrometer using ordinal pulse sequences (Ando et al., 1993). Electron impact (EI) GC-MS was accomplished using a JEOL JNM DX-300 mass spectrometer with a OV-1 capillary column (0.25 mm ID × 25 m, Gasukuro Kogyo Inc.). Ionization voltage was 70 eV and ion source temperature was 240°C.

RESULTS AND DISCUSSION

Synthesis. Some synthetic routes for conjugated triene pheromones have been reported (Millar, 1990; Doolittle et al., 1990; Tellier and Descoins, 1990; Tellier, 1991; Tellier et al., 1991). In this study three kinds of double-bond positional isomers, 8,10,12-, 9,11,13-, and 11,13,15-hexadecatrienyl acetates, were prepared by referring to the previous synthetic route described for the

pheromone of *G. pyloalis*, 10,12,14-triene compound (Ando et al., 1988b), in which two of three double bonds were introduced stereospecifically and another nonspecifically utilizing the Wittig reaction. Since a C_6 alkenyl halide for the Wittig reaction was available, the C_{16} straight chain and the central double bond in the 8,10,12-triene compound were constructed by the coupling reaction with a C_{10} 2-alkenal unit **4a,** which was derived from a C_8 dicarboxylic acid **1a,** to yield the 8*E*,10*E*,12*E* and 8*E*,10*Z*,12*E* isomers (Scheme 1A,B). Similarly the 11*E*,13*E* and 11*E*,13*Z* isomers of the 11,13,15-triene compound were obtained from a C_3 alkenyl halide and a C_{13} 2-alkenal unit **4c** derived from a C_{11} acid **5** (Scheme 1A,D). The triene system of 9,11,13-hexadecatrienyl acetate was built by the reaction between a C_3 alkyl unit and C_{13} 2,4-alkadienal unit **8,** which was derived from a C_9 dicarboxylic acid **1b** by twice C_2 extension, to yield the 9*E*,11*E*,13*E* and 9*E*,11*E*,13*Z* isomers (Scheme 1A, C). The coupling reactions with alkenylidenephosphorane were performed by the slightly dominant introduction of the *Z* configuration, while the *E* configuration was preferred to some extent to the reaction with alkylidenephosphorane.

If the coupling reaction is carried out with appropriate (*E*)-alkenals in Scheme 1B, other pheromone analogs may be produced, such as 6,8,10-tetradeca- and 4,6,8-dodecatriene derivatives and so on, which are considered to be ω4,ω6,ω8-trienes based on the double-bond positions from the methyl terminal. Schemes 1C and 1D are considered to correspond to the synthetic routes for ω3,ω5,ω7-trienes and ω1,ω3,ω5-trienes, respectively.

Chromatographic Characteristics. Table 1 shows the HPLC and GC R_t values of the hexadecatrienyl acetates synthesized in this experiment compared with the 10*E*,12*E*,14*E* isomer. On a reverse-phase HPLC column (ODS) the two geometrical isomers of each triene were completely separated. Isomers with all *E* configurations always eluted more slowly than isomers with the *Z* configuration at one double bond. Among the double-bond positional isomers with all *E* configurations, 10,12,14-triene showed the largest R_t value. The separation of the two geometric isomers by GC was not adequate even when a capillary column with a rather strong polarity (PEG-20M) was used. Double-bond positional isomers also showed almost the same R_t values by GC except for the 11,13,15-triene.

NMR Analysis. Olefinic proton signals (H^n to H^{n+5}, 8,10,12-triene: n = 8; 9,11,13-triene: n = 9; 11,13,15-triene: n = 11) of the synthetic conjugated trienes were analyzed based on ^1H–^1H COSY experiments, and the results are listed in Table 2. Outer protons (H^n and H^{n+5}) and inner protons (H^{n+1} to H^{n+4}) of the conjugated systems in 8*E*,10*E*,12*E* and 9*E*,11*E*,13*E* isomers could be distinguished, but detailed chemical shift values were not obtained due to their overlapping. The signal patterns were the same as those of the 10*E*,12*E*,14*E* isomer (Ando et al., 1988b), indicating that the triene system displayed all the *E* configurations. Since the 8*E*,10*Z*,12*E* isomer showed a symmetri-

TABLE 1. CHROMATOGRAPHIC CHARACTERISTICS OF HEXADECATRIENYL ACETATES SYNTHESIZED IN EXPERIMENT

Compound	HPLC R_t^a, min (relative value)	GC R_t^b, min (relative value)
8,10,12-Triene (ω4, ω6, ω8-triene)		
8*E*, 10*E*, 12*E* isomer	14.6 (92)	29.5 (100)
8*E*, 10*Z*, 12*E* isomer	13.1 (82)	29.3 (99)
9,11,13-Triene (ω3, ω5, ω7-triene)		
9*E*, 11*E*, 13*E* isomer	14.6 (92)	29.5 (100)
9*E*, 11*E*, 13*Z* isomer	13.2 (83)	29.8 (101)
10,12,14-Triene (ω2, ω4, ω6-triene)		
10*E*, 12*E*, 14*E* isomer[c]	15.9 (100)	29.5 (100)
11,13,15-Triene (ω1, ω3, ω5-triene)		
11*E*, 13*E* isomer	15.1 (95)	28.6 (97)
11*E*, 13*Z* isomer	13.9 (87)	28.3 (96)

[a] Chromatography on a ODS column (8 mm ID × 15 cm) with an acetonitrile–water (90 : 10) solvent system at a flow rate of 2.0 ml/min.
[b] Chromatography on a PEG-20M capillary column (0.25 mm ID × 25 m) under programed temperature (70°C to 230°C at 5°C/min).
[c] Synthesized in a previous experiment (Ando et al., 1988b).

TABLE 2. PARTIAL ^{1}H PEAK ASSIGNMENTS OF HEXADECATRIENYL ACETATES SYNTHESIZED IN EXPERIMENT

Compound	$-CH^n$	CH^{n+1}	CH^{n+2}	CH^{n+3}	CH^{n+4}	$CH^{n+5}-$
Position	*n*	*n* + 1	*n* + 2	*n* + 3	*n* + 4	*n* + 5
8,10,12-Triene (*n* = 8, ω4, ω6, ω8-triene)						
8*E*, 10*E*, 12*E* isomer	~5.7	~6.1	~6.1	~6.1	~6.1	~5.7
8*E*, 10*Z*, 12*E* isomer	5.69	6.48	5.84	5.84	6.48	5.69
9,11,13-Triene (*n* = 9, ω3, ω5, ω7-triene)						
9*E*, 11*E*, 13*E* isomer	~5.7	~6.1	~6.1	~6.1	~6.1	~5.7
9*E*, 11*E*, 13*Z* isomer	5.70	6.08	6.17	6.39	5.97	5.40
11,13,15-Triene (*n* = 11, ω1, ω3, ω5-triene)						
11*E*, 13*E* isomer	5.73	6.06	6.21	6.11	6.35	5.03, 5.16
11*E*, 13*Z* isomer	5.75	6.49	5.97	5.88	6.81	5.10, 5.20

cal shape in the conjugated triene part, only three kinds of proton signals (H^8 and H^{13}, H^9 and H^{12}, and H^{10} and H^{11}) were observed. Each of the other three isomers showed six well-resolved olefinic proton signals and the projected *E* and *Z* configuration was confirmed by the coupling constants of ca. 15 and 11

Hz, respectively. The ^1H signal pattern of (11*E*, 13*Z*)-11,13,15-hexadecatrienyl acetate corresponded well to that of (9*E*, 11*Z*)-9,11,13-tetradecatrienyl acetate (Tellier et al., 1991).

Olefinic carbon signals (C^n to C^{n+5}) of the above three 9*E*,11*E*,13*Z*, 11*E*,13*E*, and 11*E*,13*Z* isomers, for which the resonance of each olefinic proton appeared separately, were readily assigned based on ^{13}C–^1H COSY experiments. Since some proton signals of the 8*E*,10*Z*,12*E* isomer overlapped, it was difficult to differentiate C^8 from C^{13} and C^9 from C^{12} even by analyzing the ^{13}C–^1H COSY spectrum. In the case of the 8*E*,10*E*,12*E* and 9*E*,11*E*,13*E* isomers, the differentiation of two outer carbons (C^n and C^{n+5}) and of four inner carbons (C^{n+1} to C^{n+4}) was also difficult. For the assignment of the carbon signals, therefore, we utilized an empirical rule based on the changes of the chemical shifts of olefinic carbons by converting the configuration of the conjugated triene system (Ando et al., 1988b). According to this rule, predicted chemical shift values for the isomers with the all *E* configurations were calculated from the values of their geometrical isomers. Table 3 shows the partial ^{13}C peak assignments of the conjugated trienes and chemical shift differences between two geometrical isomers.

Shift Parameters for ^{13}C NMR Signal Calculation. Dorman et al. (1971), who analyzed approximately 50 alkenes, reported an empirical method whereby the value of the chemical shift for an olefinic carbon of monoene compounds can be calculated with ethylene as a reference (Levy et al., 1980). For the 1,4-dialkyl conjugated diene compounds, a similar method was applied to the ^{13}C NMR data of 18 diene pheromone analogs synthesized systematically (Ando et al., 1985) and of 1,3-butadiene (C^1 117.6 and C^2 137.8 ppm) as a reference. Chemical shift values of the outer carbon (C^{*1}) and the inner carbon (C^{*2}) of (*E*,*E*)-diene were separately calculated using different substituent parameters (α, β, γ, and α') and correction factors for other geometrical isomers, *Z*,*E*, *E*,*Z*, and *Z*,*Z* isomers, as shown in Table 4A. For example, the values of the chemical shifts for the olefinic carbons of bombykol were calculated as follows; C^{10} 134.1, C^{11} 125.3, C^{12} 128.3, and C^{13} 130.1 ppm. These values were in good agreement with the observed values (134.6, 125.7, 128.8, and 129.8 ppm), which were confirmed by the ^{13}C–^1H COSY experiment.

It was interesting to determine whether the values of the ^{13}C chemical shifts for conjugated triene compounds could also be calculated. Differences of the chemical shifts for olefinic carbons between two geometrical isomers of 10,12,14-hexadecatrienyl acetate showed specific values (Ando et al., 1988b), indicating the presence of correction factors for the calculation of the conjugated triene compounds. Using the olefinic carbon signal assignments of the 10,12,14-triene and three double-bond positional isomers synthesized in this experiment, substituent parameters were tentatively estimated for 1,6-dialkyl conjugated trienes with all the *E* configurations as shown in Table 4B. (*E*)-1,3,5-Hexatriene (C^1

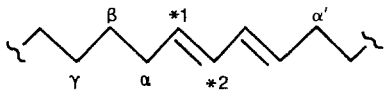
TABLE 3. PARTIAL ^{13}C PEAK ASSIGNMENTS OF HEXADECATRIENYL ACETATES SYNTHESIZED IN EXPERIMENT AND CHEMICAL SHIFT DIFFERENCES ($\Delta\delta$) BETWEEN TWO GEOMETRICAL ISOMERS

Compound	$-C^nH = C^{n+1}H - C^{n+2}H = C^{n+3}H - C^{n+4}H = C^{n+5}H-$									
Position	$n-1$	n	$n+1$	$n+2$	$n+3$	$n+4$	$n+5$	$n+6$	$n+7$	$n+8$
8,10,12-Triene (n = 8, ω4, ω6, ω8-triene)										
8*E*, 10*E*, 12*E* isomer	32.8	134.3	130.5[a]	130.9	130.9	130.6[a]	134.3	34.9	22.6	13.7
8*E*, 10*Z*, 12*E* isomer	33.0	135.2[b]	125.9[c]	127.7	127.7	126.0[c]	135.4[b]	35.0	22.6	13.7
$\Delta\delta$	−0.2	−0.9	4.6	3.2	3.2	4.6	−1.1	−0.1	0.0	0.0
9,11,13-Triene (n = 9, ω3, ω5, ω7-triene)										
9*E*, 11*E*, 13*E* isomer	32.8	134.4	130.5	130.9	130.9	129.5	135.9	25.9	13.7	
9*E*, 11*E*, 13*Z* isomer	32.8	135.2	130.6	132.8	125.9	128.1	133.5	21.2	14.3	
$\Delta\delta$	0.0	−0.8	−0.1	−1.9	5.0	1.4	2.4	4.7	−0.6	
10,12,14-Triene (n = 10, ω2, ω4, ω6-triene)										
10*E*, 12*E*, 14*E* isomer[d]	32.8	134.4	130.6	130.7	130.5	131.8	128.7	18.2		
11,13,15-Triene (n = 11, ω1, ω3, ω5-triene)										
11*E*, 13*E* isomer	32.8	136.1	130.2	133.6	131.0	137.2	116.2			
11*E*, 13*Z* isomer	33.0	136.9	125.6	130.4	127.8	132.3	117.1			
$\Delta\delta$	−0.2	−0.8	4.6	3.2	3.2	4.9	−0.9			

[a-c]Chemical shift values may be reversed.
[d]Data from the previous paper (Ando et al., 1988b).

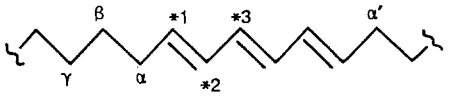
TABLE 4. ^{13}C CHEMICAL SHIFT PARAMETERS FOR OLEFINIC COMPOUNDS

A. Conjugated dienes (ppm)[a]



	Substituent parameter				Configuration factor[b]		
Base value	α	β	γ	α'	*ZE*	*EZ*	*ZZ*
C^{*1} 117.6	12.0	7.5	−1.5	−3.5	−2.5	2.0	−0.5
C^{*2} 137.8	−5.5	−2.5	1.0	−0.5	−2.0	−5.0	−7.0

B. Conjugated trienes (ppm)[c]



	Substituent parameter				Configuration factor[b]		
Base value	α	β	γ	α'	*ZE*	*EZ*	*ZZ*
C^{*1} 117.7	12.5	7.0	−1.5	−1.5	−3.0	1.0	1.0
C^{*2} 136.9	−5.5	−2.0	1.0	0.5	−2.5	−5.0	0.0
C^{*3} 133.7	−0.5	0.5	0.0	−3.0	−5.0	−3.5	2.0

[a] C^{10} chemical shift of bombykol, (10*E*,12*Z*)-10,12-hexadecadien-1-ol, is calculated as follows: 117.6 + 12.0 + 7.5 − 1.5 − 3.5 + 2.0 = 134.1 ppm (obs. 134.6 ppm).
[b] *ZE* refers to *Z,E* isomer.
[c] C^6 chemical shift of (2*Z*, 4*Z*, 6*E*)-2,4,6-undecatriene is calculated as follows: 136.9 − 5.5 − 2.0 + 1.0 + 0.5 − 5.0 + 0.0 = 125.9 ppm (obs. 125.8 ppm).
[d] *ZEE* refers to *Z,E,E* isomer. The factor for *Z,Z,E* isomer is obtained by the addition of *ZEE* and *EZE* as follows: C^{*1} −3.0 + 1.0 = −2.0, C^{*2} − 2.5 −5.0 = −7.5, C^{*3} −5.0 −3.5 = −8.5

117.7, C^2 136.9, and C^3 133.7 ppm) is a reference compound, and for each of the three carbons in a triene (C^{*1}, C^{*2}, and C^{*3}) substituent parameters and configuration factors with different magnitudes were established. While the configuration factors for only three geometrical isomers with one *Z* double bond, *Z,E,E*, *E,Z,E*, and *E,E,Z* isomers, were listed in this table, those for other geometrical isomers could be obtained by adding the values of the three isomers. Namely, the factor for the *Z,Z,E* isomer is the sum of that of the *Z,E,E* and *E,Z,E* isomers, and the factor for the *Z,Z,Z* isomer is obtained by further addition of the factor for the *E,E,Z* isomer. Utilizing the parameters in Table 4B, the values of the chemical shifts of (2*Z*,4*Z*,6*E*)-2,4,6-undecatriene were estimated as follows: C^2 126.7, C^3 124.4, C^4 121.7, C^5 129.2, C^6 125.9, and C^7 136.2 ppm. These values were in agreement with the observed data (126.3, 124.8, 122.2, 129.3, 125.8, and 136.2 ppm; Gaudin and Morel, 1990). As indicated

by these parameters, an alkyl substitution located far from the δ position or β' position did not affect the shielding of olefinic carbons. Chemical shifts in Table 3 could be generalized to other straight-chain compounds with a conjugated triene system at the same position by counting from the terminal methyl group, i.e., $\omega 1,\omega 3,\omega 5$-trienes, $\omega 2,\omega 4,\omega 6$-trienes, and so on. Compounds with the triene system at a higher ω position than the $\omega 4,\omega 6,\omega 8$-trienes may show almost the same olefinic signals if they posses the same configuration.

MS Analysis. Table 5 shows the relative intensity of the molecular ion M^+ and some fragment ions in mass spectra of the synthetic hexadecatrienyl acetates with an *E* configuration at all the double bonds. All of eight geometrical isomers of 10,12,14-hexadecatrienyl acetate showed very similar EI-MS spectra (Ando et al., 1988b). The geometrical isomers synthesized in this experiment also gave almost identical mass spectra. Abundant M^+ at *m/z* 278 was observed in each EI mass spectrum, but the relative intensity of $[M - CH_3CO_2H]^+$ at *m/z* 218 was very low in the acetates. The spectra of these double-bond positional isomers can be easily distinguished from each other by the presence of some characteristic fragment ions. In the case of pheromone analogs with a conjugated diene system in the terminal methyl side, two representative fragment ions including the terminal methyl group were produced (Ando et al., 1988a). Dienes including C=C double bonds at the ωn and $\omega(n+2)$ positions [$\omega n,\omega(n+2)$-diene] were fragmented predominantly due to the cleavage of bonds between $C^{\omega(n+4)}$ and $C^{\omega(n+5)}$ (α-cleavage) and between $C^{\omega(n+3)}$ and $C^{\omega(n+4)}$ after H rearrangement to yield $[C_{n+4}H_{2n+5}]^+$ and $[C_{n+3}H_{2n+4}]^+$ ions, respectively. The length of the carbon chain and functional group did not affect appreciably these fragmentations. Similar fragmentation pathways were predicted for a conjugated triene [$\omega n,\omega(n+2)$, $\omega(n+4)$-triene] to yield $[C_{n+6}H_{2n+7}]^+$ and $[C_{n+5}H_{2n+6}]^+$ ions. The former fragment ion was produced by α-cleavage and the latter ion by the cleavage of a C—C bond immediately adjacent to the triene system after H rearrangement; namely, $C_7H_9^+$ (*m/z* 93) and $C_6H_8^+$ (*m/z* 80) for the $\omega 1,\omega 3,\omega 5$-triene, $C_8H_{11}^+$ (*m/z* 107) and $C_7H_{10}^+$ (*m/z* 94) for the $\omega 2,\omega 4,\omega 6$-triene, $C_9H_{13}^+$ (*m/z* 121) and $C_8H_{12}^+$ (*m/z* 108) for the $\omega 3,\omega 5,\omega 7$-triene, and $C_{10}H_{15}^+$ (*m/z* 135) and $C_9H_{14}^+$ (*m/z* 122) for the $\omega 4,\omega 6,\omega 8$-triene were predicted. Relative intensity values of these ions recorded for hexadecatrienyl acetates are indicated by asterisks in Table 5. The marked intensities are larger than those at the same *m/z* of the other double-bond positional isomers except for *m/z* 93 of $\omega 1,\omega 3,\omega 5$-triene.

Since insects contain a limited amount of sex pheromones, it is not easy to identify the double-bond positions. To examine the possibility of utilizing these fragment ions in the study of a natural pheromone, the spectra of synthetic trienes were compared with those of natural pheromones analyzed under other instrumental conditions. Recently Kuwahara et al. (1986) proposed an identification method for a double-bond position using a series of synthetic isomers

TABLE 5. RELATIVE INTENSITY OF MOLECULAR ION (M^+) AND SOME FRAGMENT IONS IN MASS SPECTRA OF HEXADECATRIENYL ACETATES AND NATURAL PHEROMONES

	Relative intensity (%)										
	$C_6H_7^+$	$C_6H_8^+$	$C_7H_9^+$	$C_7H_{10}^+$	$C_8H_{11}^+$	$C_8H_{12}^+$	$C_9H_{13}^+$	$C_9H_{14}^+$	$C_{10}H_{15}^+$	$[M\text{-AcOH}]^+$	M^+
m/z	79	80	93	94	107	108	121	122	135	218	278
ω4, ω6, ω8-Triene											
8,10,12-Triene[a]	100	58	80	30	29	11	25	23*[b]	8*	2	45
ω3, ω5, ω7-Triene											
9,11,13-Triene[a]	100	36	55	32	34	19*	28*	10	7	3	22
ω2, ω4, ω6-Triene											
10,12,14-Triene[a]	100	32	44	51*	43*	18	17	10	7	2	26
Natural pheromone[c]	100	55	90	100	84	43	30	22	18	5	48
ω1, ω3, ω5-Triene											
11,13,15-Triene[a]	100	89*	52*	31	32	4	5	2	2	1	30
Natural pheromone[d]	100	75	38	18	8	8	5	3	3		24[e]

[a] Spectra of hexadecatrienyl acetates with *E* configuration at all double bonds were analyzed with a JEOL DX-300 mass spectrometer.
[b] Fragment ions with asterisks were assumed to be produced by the similar pathways proposed for conjugated diene compounds (Ando et al., 1988a).
[c] Sex pheromone of *G. pyloalis*, (10*E*, 12*E*, 14*Z*)-10,12,14-hexadecatrienyl acetate, analyzed with a JEOL DX-303 mass spectrometer (Seol et al., 1987).
[d] Sex pheromone of *E. ceratoniae*, (9*Z*, 11*E*)-9,11,13-tetradecatrienal, analyzed with a Hewlett-Packard 5970 mass spectrometer coupled with a Hewlett-Packard 5890 gas chromatograph (Baker et al., 1989).
[e] M^+ at m/z 206.

TABLE 6. AVERAGE FITNESS INDEXES OF NATURAL SEX PHEROMONES TO SYNTHETIC CONJUGATED TRIENES[a]

Species and sex pheromone	ω1, ω3, ω5–Triene	ω2, ω4, ω6–Triene	ω3, ω5, ω7–Triene	ω4, ω6, ω8–Triene
Glyphodes pyloalis (10*E*, 12*E*, 14*Z*)-10,12,14-Hexadecatrienyl acetate	62.5	14.9[b]	42.7	75.2
Ectomyelois ceratoniae (9*Z*, 11*E*)-9,11,13-Tetradecatrienal	67.6[b]	201	190	170

[a] The indexes (Kuwahara et al., 1986) were calculated using the relative intensities of eight fragment ions (m/z 80, 93, 94, 107, 108, 121, 122 and 135) in the spectra of hexadecatrienyl acetates (Table 5).
[b] Significant at the 0.1% level by the t test.

and the newly devised "average fitness index." The smallest value of the index among isomers indicated adequately the best fit to a natural pheromone. Based on the reported spectra of *G. pyloalis* and *E. ceratoniae*, the smallest values of the indexes for the two pheromones were obtained with our synthetic ω2,ω4,ω6- and ω1,ω3,ω5-trienes, respectively (Table 6). These results agree with the double-bond positions already reported. It should be emphasized that the spectral data of C_{16} acetates can also be used to determine the location of the triene system in a C_{14} aldehyde sex pheromone of the latter species.

REFERENCES

ANDO, T., KUROTSU, Y., KAIYA, M., and UCHIYAMA, M. 1985. Systematic syntheses and characterization of dodecadien-1-ols with conjugated double bond, lepidopterous sex pheromones. *Agric. Biol. Chem.* 49:141–148.

ANDO, T., OGURA, Y., and UCHIYAMA, M. 1988a. Mass spectra of lepidopterous sex pheromones with a conjugated diene system. *Agric. Biol. Chem*, 52:1415–1423.

ANDO, T., OGURA, Y., KOYAMA, M., KURANE, M., UCHIYAMA, M., and SEOL, K.Y. 1988b. 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.

ANDO, T., ABURATANI, M., KOSEKI, N., ASAKAWA, S., MOURI, T., and ABE, H. 1993. ^{13}C NMR assignments of brassinosteroids by two-dimensional techniques. *Magn. Reson. Chem.* 31: In press.

ARN, H., TOTH, M., and PRIESNER, E. 1986. List of sex pheromones of Lepidoptera and related attractants. OILB-SROP/IOBC-WPRS, Secretariat General, 149 Rue de Bercy, F-75595 Paris cedex 12; ISBN 92-9067-002-9, 123 pp.

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.

DOOLITTLE, R.E., BRABHAM, A., and TUMLINSON, J.H. 1990. Sex pheromone of *Manduca sexta* (L). Stereoselective synthesis of (10*E*,12*E*,14*Z*)-10,12,14-hexadecatrienal and isomers. *J. Chem. Ecol.* 16:1131–1153.

DORMAN, D.E., JAUTELAT, M., and ROBERTS, J.D. 1971. Carbon-13 nuclear magnetic resonance spectroscopy. Quantitative correlations of the carbon chemical shifts of acyclic alkenes. *J. Org. Chem.* 36:2757–2766.

GAUDIN, J.-M., and MOREL, C. 1990. Two new stereoselective syntheses of (3*E*,5*Z*)-1,3,5-undecatriene. *Tetrahedron Lett.* 31:5749–5752.

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.

KUWAHARA, Y., YONEKAWA, Y., KAMIKIHARA, T., and SUZUKI, T. 1986. Identification of the double bond position in insect sex pheromones by mass spectrometry; trial comparison of methyl undecenoates with the natural pheromone of the varied carpet beetle. *Agric. Biol. Chem.* 50:2017–2024.

LEVY, G.C., LICHTER, R.L., and NELSON, G.L. 1980. Carbon-13 Nuclear Magnetic Resonance Spectroscopy, 2nd ed., John Wiley & Sons, New York, pp. 78–79.

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., and BJOSTAD, L. 1984. Biosynthesis of lepidopteran pheromones. *Bioorg. Chem.* 12:279–298.

SEOL, K.Y., HONDA, H., USUI, K., ANDO, T., and MATSUMOTO, Y. 1987. 10,12,14-Hexadecatrienyl acetate: Sex pheromone of the mulberry pyralid, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae). *Agric. Biol. Chem.* 51:2285–2287.

TELLIER, F., and DESCOINS, C. 1990. Stereospecific synthesis of (*Z*,*E*)-9,11,13-tetradecatrien-1-yl acetate and aldehyde sex pheromone components of *Stenoma cecropia* and *Ectomyelois ceratoninae*. *Tetrahedron Lett.* 31:2295–2298.

TELLIER, F. 1991. Stereospecific synthesis of (*E*,*E*)-9,11,13-tetradecatrienyl acetate and aldehyde (geometrical isomers of the major components of two sex pheromone of Lepidoptera). *Synth. Commun.* 21:2287–2294.

TELLIER, F., DESCOINS, C., and SAUVETRE, R. 1991. Stereospecific synthesis of 1,5-dien-3-ynes and 1,3,5-trienes application to the stereochemical identification of trienic sex pheromones. *Tetrahedron* 47:7767–7774.

TUMLINSON, J.H., BRENNAN, M.M., DOOLITTLE, R.E., MITCHELL, E.R., BRABHAM, A., MAZOMENOS, B.E., BAUMHOVER, A.H., and JACKSON, D.M. 1989. Identification of a pheromone blend attractive to *Manduca sexta* (L.) males in a wind tunnel. *Arch. Insect Biochem. Physiol.* 10:255–271.

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MATURATION OF TERGAL GLAND ALKENE PROFILES IN EUROPEAN HONEY BEE QUEENS, *Apis mellifera* L.

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Abstract—In a series of husbandry and stop-time chemical experiments with honey bee queens, the production of tergal gland alkenes was found to be stimulated by natural mating and not by instrumental insemination. Carbon dioxide, physical manipulation of the sting chamber and vagina, presence of sperm in the spermatheca, egg production, and chemicals transferred via drone semen are demonstrated to not initiate the synthesis of the tergal gland alkenes. The compounds probably do not function as sex pheromones. However, the circumstances and timing of the initiation of production of the tergal gland alkenes strongly suggests a communication role for the compounds within the hive.

Key Words—Hydrocarbons, honey bees, insects, Hymenoptera, mating, natural mating, instrumental insemination, communication, pheromones, exocrine glands, Apidae, gas chromatography, chemical communication

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INTRODUCTION

Researchers have long recognized the ability of the queen honey bee to regulate hive functions through her glandular exudates; however, in many cases the exact chemicals involved have yet to be elucidated. Evidence that suggests that queens chemically mark rival queen cells for destruction has been published (Caron and Greve, 1979). Kin recognition is believed to be based partly upon cuticular hydrocarbons and other compounds (Page et al., 1991). It has been suggested that Varroa mites chemically cloak themselves in the hive with cuticular hydrocarbons (Nation et al., 1991). Secretions from the tergal gland, located on the dorsal surface of the abdomen, are highly attractive to worker bees in the court surrounding the queen, much more so than the head, the source of the queen pheromone (De Hazan et al., 1989). These secretions are believed to mediate hive cohesiveness. Queens are also thought to produce a close-range sex attractant from the tergal glands. The active compound has been proposed to be decyl decanoate (Espelie et al., 1990).

In 1987, we described a series of mono-and diunsaturated straight-chain hydrocarbons that were found in hexane extracts from queen honey bees, but not workers. The monounsaturated alkenes formed a homologous series of odd-chain compounds from C_{23} to C_{37} and higher, all unsaturated 15 carbons from one end of the chain (Smith, 1987). Further studies of these compounds (Smith and Taylor, 1990) found a concentration gradient of the compounds on the cuticle of the queen centered on the dorsal part of the abdomen, suggesting that their point of origin was the tergal glands. Other information suggested that their production was controlled independently from the synthesis of the other cuticular alkenes. Recently we have studied the alkene patterns of queens to distinguish Africanized from European queens (Smith and Taylor, 1988; Smith et al., 1992). Part of the last paper described experiments examining the maturation of alkene profiles in queens. These same experiments provided preliminary information about the development of the tergal gland alkene patterns, which we decided to explore in greater detail.

METHODS AND MATERIALS

Queens used in this study were obtained from stocks at the Carl Hayden Bee Research Laboratory, Tucson, Arizona, or the apiary of R.-K. Smith, Douglasville, Georgia. The Smith stock was originally obtained from the Rossman Apiaries, Moultrie, Georgia. The Mackensen technique (Laidlaw, 1977) was used for all instrumental insemination procedures. Four breeding and rearing experiments provided the data for this study. They were conducted as follows and are summarized in Table 1.

Experiment 1. One-day-old sister larvae from a line called GL at the Tucson

TABLE 1. NUMBERS AND TREATMENT OF BRED EUROPEAN QUEENS OF KNOWN AGE POSTEMERGENCE

Age			II[a]			Naturally mated[b]	
Hours	Days	Virgin	+ Sperm[c]	− Sperm[d]	Saline[e]	No eggs	Eggs
0	0	2[f]					
12	0.5	4					
24	1	4					
36	1.5	7					
48	2	12					
60	2.5	4					
72	3	6					
84	3.5	4					
96	4	8					
120	5	7					
144	6	4					
168	7	4					
180	7.5			2	2		
192	8	3		2	2		
216	9	4	2[g]	2	2	3	
240	10	9	2[g]	2	2	4	
264	11	3	2[g]	2		1	2
288	12	4	1*	1*	2	1	2
312	13		1[g] + 2*	1*	1[g] + 1		3
336	14	3					3
372	15.5	4					
384	16						3
408	17	2					
432	18						3
480	20	2					3
552	23	5 + 8[h]	3				4
720	30			2[i]			
840	35	5					
1008	42			2[i]			

[a] II at 7 days in all cases except those marked with an asterisk.
[b] Allowed to fly free at day seven; the lack or presence of eggs in the hive is indicated by "No eggs" or "Eggs," respectively.
[c] Sperm found in spermatheca after dissection.
[d] No sperm in spermatheca after dissection.
[e] Insect saline used in place of sperm during the II.
[f] Swarm cell queens raised naturally by bees in a hive, captured as they emerged from the cells.
[g] LQ line of queens.
[h] Treated with carbon dioxide at 7 and 10 days. Four were allowed to lay drone eggs while the other four were confined in queen cages and prevented from laying eggs.
[i] Other Q lines from CHBRC USDA-ARS.

lab were grafted into queen cups, then the cups placed into queen rearing colonies for rearing and capping. The capped cells were transferred to a 34–35°C, 75–85% relative humidity incubator until the queens emerged. All queens that emerged during a 12-hr shift were assigned the age of 12 hr postemergence. The queens were transferred to holding cages in a queen bank. Queens were removed from the holding cages at intervals of time and terminated by freezing.

Experiment 2. A second group of 1-day-old sister larvae from the GL line were grafted and reared and the newly emerged queens stored in a queen bank as in experiment 1, then divided into two groups. The first group ($N = 8$) was treated with carbon dioxide at seven days postemergence and again at 10 days, then four were returned to the queen bank with the control group, and the other four treated queens were confined and allowed to lay drone eggs in mating nucs. The treated and control groups were terminated at 23 days postemergence by freezing.

Experiment 3. A third group of 1-day-old sister larvae from the GL line were grafted and reared as in experiments 1 and 2. The newly emerged queens were stored in a queen bank and divided into a control group and four treatment groups. Two treatment groups were subjected to the instrumental insemination technique at day 7 with one group ($N = 10$) receiving drone semen and the other group ($N = 8$) insect saline. Both groups were exposed to carbon dioxide again at day 10. The second set of treatment groups was subjected to the instrumental insemination technique at day 13, with one group ($N = 5$) receiving semen and the other ($N = 3$) saline. Samples of the treatment groups and controls were terminated at intervals of time after treatment by freezing. This experiment was repeated with queens reared from a different stock called LQ.

Experiment 4. A group of 1-day-old sister larvae from the GL line were grafted and reared as in the above experiments. The newly emerged queens were transferred to mating nucs and confined to them with queen excluders. The queens were divided into two groups: one treatment ($N = 26$) and the other control ($N = 13$). The queen excluders were removed from the treatment nucs at day 7 to allow free mating. By day 9, two days after the queens were allowed free flight, all the queens sampled had sperm in the spermatheca, indicating the queens had mated between days 7 and 9. Samples from the treatment and control groups were removed at time intervals and terminated by freezing.

In addition, a number of queens of known mating histories and ages were examined and included as data in the study. All queens were terminated by freezing and preserved by air drying, then the hydrocarbons extracted and assayed by already described methods (Smith et al., 1992). Gas chromatographic analyses were performed on an Hewlett-Packard 5890A FIDGC instrument with a 50-m Ultra-2 column as described previously (Smith et al., 1992). The data were processed through a Macintosh SE 40/40 (courtesy of Apichemical Consultants) or a Macintosh SE/30 40/80 (Southern College of Technology) with

the software program StatView SE^{+}™ (Abacus Concepts, 1984 Bonita Ave, Berkeley, California 94704).

The presence of the TG alkenes was expressed as a decimal fraction of the total extractable alkenes. For queens not expressing the TG alkenes the mean of the TG/total alkene ratio was 0.053 (±0.020 SD, $N = 88$) and an observed range of 0.018–0.135. For queens displaying the TG alkenes a mean of the TG/total alkene ratio was 0.500 (±0.188 SD, $N = 27$) and an observed range of 0.195–0.780.

RESULTS

Experiment 1. If tergal gland alkenes were produced and present on the queen by the onset of normal mating flights, the expected results of experiment 1 would be substantial development of the TG alkenes by five days (120 hr) postemergence. The results indicated that there was no observed development of the TG alkenes before day 8 (192 hr) postemergence in any sample. The compounds were observed sporadically in virgins ages 8–17 days (192–408 hr), and then with increasing frequency, until by day 35 (840 hr) all virgins exhibited TG alkenes. These data are illustrated in Figure 1.

Experiment 2. Carbon dioxide is commonly used for queen anesthesia dur-

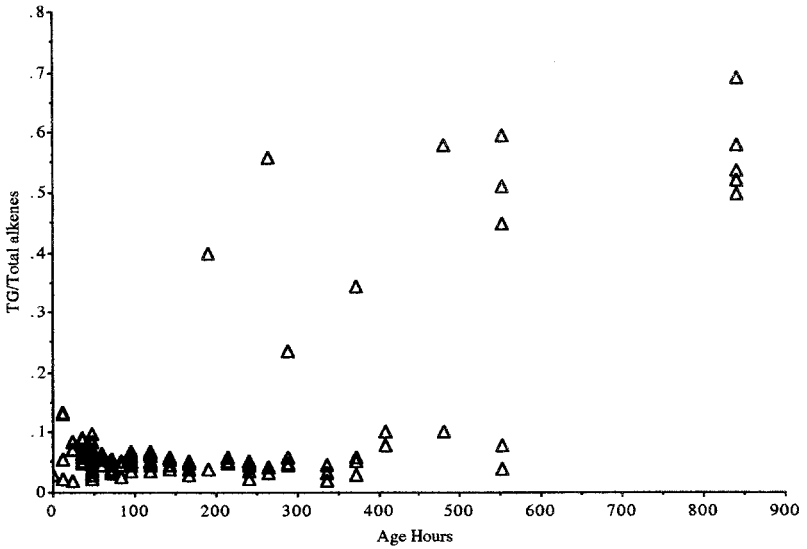

FIG. 1. Ratio of TG alkenes to total alkenes found in extracts from virgin queens of known age postemergence.

ing the instrumental insemination process and acts as a trigger for initiation of egg laying by inseminated queens (Laidlaw, 1977). Experiment 2 was performed to determine if carbon dioxide was acting also as a trigger for production of the TG alkenes in queens. The carbon dioxide-treated queens were divided into two groups of four each to verify that the treated queens had received sufficient gas to initiate egg laying. The fraction of TG alkenes in the total alkene extract per queen at 23 days (552 hr) postemergence is presented in Table 2. The carbon dioxide-treated queens and the untreated queens were not shown to be different by the Mann-Whitney U test ($P > 0.9999$). It is noteworthy that two of the eight carbon dioxide-treated queens exhibited essentially no TG alkenes, which would weigh heavily against the gas acting as a trigger for production of the compounds.

Experiment 3. The objective of experiment 3 was to determine if there was some chemical agent in drone semen that initiated the production of the TG alkenes or if physical manipulation of the queen's sting chamber or vagina was a sufficient trigger. The amount of TG alkenes produced was expressed as a fraction of the total extracted alkenes and plotted against time, then compared with control virgins. The results are presented in Figure 2. The two early producers of TG alkenes, ages 192 and 216 hr were both treated with insect saline. The older queen (336 hr) exhibiting the TG alkenes had her spermatheca only partially filled with semen. These data qualitatively indicate that neither the instrumental insemination procedure nor physical stimulation of the vagina is a sufficient stimulus for production of TG alkenes.

Experiment 4. The objective of experiment 4 was to monitor the production

TABLE 2. RATIO OF TG ALKENES TO TOTAL ALKENES EXTRACTED FROM 552-HR POSTEMERGENCE VIRGIN QUEENS, UNTREATED OR EXPOSED TO CARBON DIOXIDE

Treatment	TG/total alkenes
None	0.040
None	0.447
None	0.511
None	0.594
None	0.080
Carbon dioxide	0.033
Carbon dioxide	0.378
Carbon dioxide	0.479
Carbon dioxide	0.681
Carbon dioxide	0.228
Carbon dioxide	0.470
Carbon dioxide	0.033
Carbon dioxide	0.641

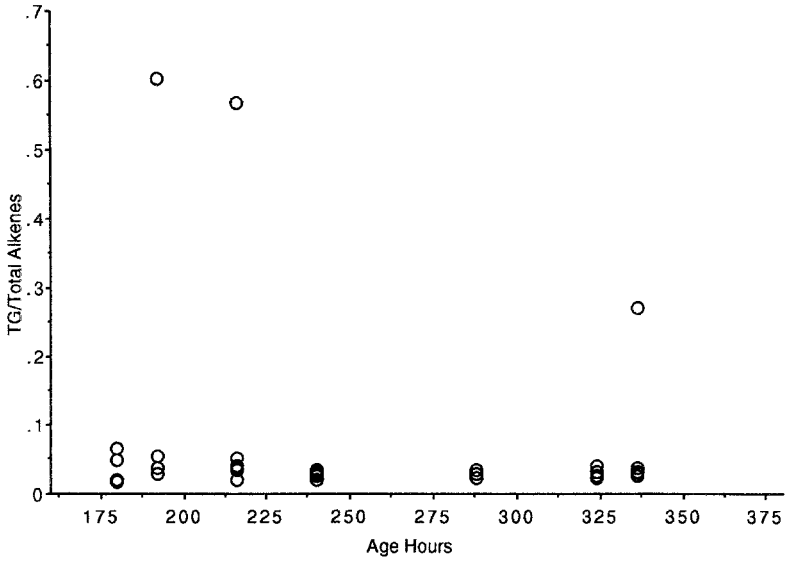

FIG. 2. TG/total alkenes ratio for queens subjected to the instrumental insemination procedure with either semen or insect saline. Insemination performed at either 7 days or 13 days. Age in hours postemergence. The two queens at 192 and 216 hr exhibiting high ratio of TG/total alkenes are saline treated. The one queen at 336 hr with a high TG/total alkene ratio is the GL queen with the spermatheca partially filled with semen.

of TG alkenes over time for a group of naturally mated queens and to compare the results against a sister group of queens that remained virgins, yet were allowed to roam free within the hive. Queen excluders served to isolate the queens until they were released on day 7 for mating. The results are plotted as the ratio of TG alkenes in the total extracted alkenes from each queen against the time of termination and are presented in Figure 3. Ignoring any time variation, the Mann-Whitney U test indicated a significant difference between the naturally mated queens and both the virgin queen controls and the instrumentally inseminated queens from the previous experiment ($P = 0.0001$). The experiment also served to eliminate conclusively the sting glands as the source of the TG alkenes as these were removed from all the mated queens to check for the presence of sperm in the spermatheca. The TG alkenes were still found in large quantities in all of the free mated queens.

DISCUSSION

The first experiment was conducted with the twofold purpose of: (1) determining the minimum age for identification and/or certification of queen honey bees via extracted unsaturated hydrocarbons, and (2) gaining some insight on

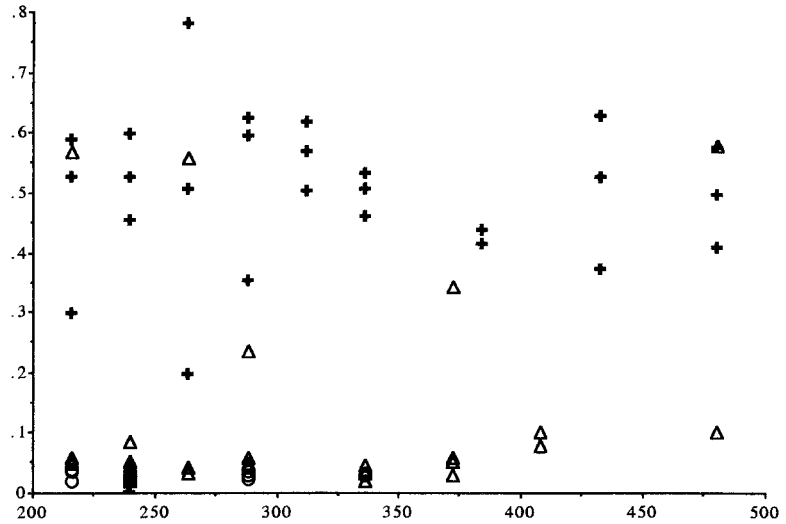

FIG. 3. Ratio of TG alkenes to total alkenes extracted from virgin (△), instrumentally inseminated with drone semen (○) and naturally mated (+) queens vs. age in hours postemergence.

the development of the TG alkenes with a possible sex pheromone function in mind. The first objective has been reported (Smith et al., 1992). Queens are ready to mate naturally by days 5–7 postemergence, requiring that any chemicals participating in the mating process be present by that time. The absence of significant amounts of the TG alkenes within this time window indicates that the compounds lack any sex pheromone function. We extended the observation time of the first experiment and found that virgin queens do not have a particular point in time for initiation of syntheses of the TG alkenes, but that by day 35 postemergence, all seem to exhibit the compounds.

We had examined a very large number of queens (over 700) of European, Africanized, and pure African origin, most of them mated naturally, and had observed the presence of the TG alkenes in all but a very few of the queens. Many of the European queens in those we had examined were known to be less than 20 days old. This suggested that the TG alkenes may be linked to the mated or egg laying status of the queen.

Experiments 2 and 3 probed for specific stimuli for the production of the TG alkenes. Based on the results of these tests, exposure to carbon dioxide, physical manipulation of the sting chamber or vagina, the presence of sperm in the spermatheca, transferred chemicals from the drone to the queen via drone semen, and the production or laying of eggs were eliminated as appropriate

triggers. Experiment 4 verifies that natural mating of queens is a suitable stimulus for the production of the TG alkenes.

These experiments establish the presence or absence of the TG alkenes as a quantifiable chemical difference between the virgin and naturally mated state of a queen. Instrumentally inseminated queens are no different than virgins by this test. It has long been recognized within both the research community and the beekeeping industry that instrumentally inseminated (II) queens are not as productive as naturally mated queens. Observable differences are problems with initial introduction and acceptance of the II queens, rapid replacement of the introduced II queen by a queen raised from her eggs and decreased brood production by II queens (Harbo and Szabo, 1984; S. Cobey and J. Thomas, personal communications). These problems make utilization of II queens for maintenance of European stocks in areas subject to Africanization quite difficult. We suggest that the TG alkenes may play a pivotal role in the care and acceptance of the queen and her eggs by workers in the hive.

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REFERENCES

CARON, D.M., and GREVE, C.W. 1979. Destruction of queen cells placed in queenright *Apis mellifera* colonies. *Ann. Entomol. Soc. Am.* 72(3):405–407.

DE HAZAN, M.Y., LENSKY, Y., and CASSIER, P. 1989. Effects of queen honeybee (*Apis mellifera* L.) ageing on her attractiveness to workers. *Comp. Biochem. Physiol.* 93A(4):777–783.

ESPELIE, K.E., BUTZ, V.M., and DIETZ, A. 1990. Decyl decanoate: A major component of the tergite glands of honeybee queens (*Apis mellifera* L.). *J. Apic. Res.* 29(1): 15–19.

HARBO, J., and SZABO, T. 1984. A comparison of instrumentally inseminated and naturally mated queens. *J. Apic. Res.* 23(1):31–36.

LAIDLAW, H.H., JR. 1977. Instrumental Insemination of Honey Bee Queens. Dadant & Sons, Hamilton, Illinois.

NATION, J.L., SANFORD, M.T., and MILNE, K. 1991. Comparison of cuticular hydrocarbons from Varroa mites and honey bees. *Am. Bee J.* 131(12):778–779.

PAGE, R.E., JR., METCALF, R.A., METCALF, R.L., ERICKSON, E.H., JR., and LAMPMAN, R.L. 1991. Extractable hydrocarbons and kin recognition in honeybee (*Apis mellifera* L.). *J. Chem. Ecol.* 17(4):745–756.

SMITH, R.-K. 1987. Identification of Africanization in honey bees based on extracted hydrocarbons assay. Presented at the Second International Conference on Africanized Honey Bees and Bee Mites, Ohio State University, Columbus, Ohio, March 30–April 1.

SMITH, R.-K., ROSSMAN, F.R., YORK, H.F. JR., and TAYLOR, O.R. JR., 1988. Stock certification of European Queens. *Am Bee J.* 128:676–678.

SMITH, R.-K., and TAYLOR, O.R. JR. 1988. Determination of Africanization in Honey Bee Queens. Am. Bee J. 128(12):808–809.

SMITH, R.-K., and TAYLOR, O.R., JR. 1990. Unsaturated extracted hydrocarbon caste differences between European queen and worker honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). *J. Kans. Entomol. Soc.* 63(3):369–374.

SMITH, R.-K., SPIVAK, M., TAYLOR, O.R., JR., BENNETT, C., and SMITH, M.L. 1992. Chemotaxonomy of honey bees (*Apis mellifera* L.) Part 3: Identification of Africanization in honey bee queens (Hymenoptera: Apidae). *Bee Sci.* 2(2):93–105.

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ELECTROPHYSIOLOGICAL AND BEHAVIORAL RESPONSES OF TURNIP MOTH MALES, *Agrotis segetum*[1] TO FLUORINATED PHEROMONE ANALOGS

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Abstract—The electrophysiological and behavioral responses of male *Agrotis segetum* to fluorinated analogs of (Z)-5-decenyl acetate (Z5-10:OAc) were investigated. The single sensillum recordings showed that 4,4-difluoro-(Z)-5-decenyl acetate (4,4-F2), 7,7-difluoro-(Z)-5-decenyl acetate (7,7-F2), 10,10,10-trifluoro-(Z)-5-decenyl acetate (10,10,10-F3) and 7,7,8,8-tetrafluoro-(Z)-5-decenyl acetate (7,7,8,8-F4) were each 100-fold less active than the natural Z5-10:OAc, whereas the 7,7,8,8,9,9,10,10,10-nonafluoro-(Z)-5-decenyl acetate (F9) analog was essentially inactive. A mixture of Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc on a filter paper dispenser was as attractive as female gland extracts when tested in a flight tunnel. With Z5-10:OAc omitted, the two-component mixture elicited a significantly lower male response. Four analogs, 7,7-F2, 10,10,10-F3, 7,7,8,8-F4, and F9, were added separately to the two-component mixture to replace Z5-10:OAc. The responses elicited by the mixtures containing the 7,7-F2, 10,10,10-F3, and 7,7,8,8-F4

[1] Schiff, Lepidoptera: Noctuidae.

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analogs did not differ significantly from that of the natural three-component mixture and the two-component mixture, whereas the mixture containing F9 elicited a significantly lower male response, as low as the response to the two-component mixture. In a field test the mixtures containing 10,10,10-F3 and 7,7,8,8-F4 were significantly more active than the two-component mixture, but still less active than the natural three-component mixture. It appears that field tests provided greater discrimination among pheromone analogs in assessing their behavioral activity than the flight-tunnel test did. Structure-activity analyses demonstrate the importance of the lipophilic interaction between the terminal alkyl chain and the receptor site for the activity of the stimulus. The lipophobicity of the fluorinated analogs impedes a productive receptor interaction.

Key Words—*Agrotis segetum*, Lepidoptera, Noctuidae, (Z)-5-decenyl acetate, fluorinated analogs, behavioral activity, electrophysiological activity, flight tunnel, single sensillum recording, field test, lipophobicity, lipid solubility, structure-activity analysis, pheromone.

INTRODUCTION

Fluorine has been widely used as an isosteric replacement for hydrogen in the study of the molecular basis of pheromone perception and metabolism (Prestwich, 1987a,b, 1991). The biological activity of fluorinated pheromone analogs has been studied in several insect species. The studies show that these analogs can exhibit a variety of biological activities including attractancy, synergism, or inhibition, depending on the position and extent of fluorination (Bengtsson et al., 1990a; Briggs et al., 1986; Camps et al., 1984; Dickens et al., 1991a,b; Masnyk et al., 1989; McLean et al., 1989; Prestwich et al., 1986, 1988, 1990).

The sex pheromone of *Agrotis segetum* consists of three major monounsaturated acetates: (Z)-5-decenyl acetate (Z5-10:OAc), (Z)-7-dodecenyl acetate (Z7-12:OAc) and (Z)-9-tetradecenyl acetate (Z9-14:OAc) in a 1:5:2.5 ratio (Löfstedt et al., 1982; Arn et al., 1983). This species has become a model for studying structure-activity relationships in pheromone communication focusing on the properties of the Z5-10:OAc receptor cell on the male antenna. More than 50 analogs of Z5-10:OAc have been synthesized and their activities have been investigated by single-cell recordings at the Lund University and a detailed model of the receptor has been developed (Bengtsson, 1988; Bengtsson et al., 1987, 1990b; Jönsson, 1991; Jönsson et al., 1991a,b; Liljefors et al., 1985, 1987). To expand our knowledge about the relation between structure and activity for pheromone analogs in this moth and to compare data on *A. segetum* with former fluoromone studies on other species, we tested the single-cell activities of fluorinated analogs of Z5-10:OAc and investigated their behavioral activities in the flight tunnel and in the field.

METHODS AND MATERIALS

Insect Material. Turnip moths used in this study were F_1, F_2, or F_3 progeny obtained from a cross-mating of a laboratory culture maintained at the Department of Ecology, Lund University, for more than 20 generations and recently established cultures of wild insects from southern Sweden and Denmark. Larvae were reared on a Hinks and Byers (1976) diet, using potato instead of pea beans and kept at 25°C on a reversed 18-hr light–6-hr dark cycle. Pupae were sexed and males intended for behavioral work were put in a separate room at 20°C with a 14-hr light–10 hr dark photoperiod.

Chemicals. Z5–10:OAc, Z7–12:OAc, and Z9–14:OAc were purchased from the Institute for Pesticide Research, Wageningen, The Netherlands, and were >99.5% pure with respect to positional and geometrical isomers as determined by gas chromatography. The fluorinated analogs (Figure 1) were synthesized at Stony Brook (Sun et al., 1992). The 7,7-F2 contained <0.5% natural Z5–10:OAc and the rest contained <0.1% natural Z5–10:OAc (if present) as determined by GC.

Stimulus Amounts Released From Odor Sources. The relative amounts released of Z5–10:OAc, 7,7-F2, 10,10,10-F3, 7,7,8,8-F4, and F9 from filter paper (Munktell, technical, 1002), were investigated according to a technique earlier described (Bengtsson et al., 1990b). One hundred micrograms of each compound was applied as a hexane solution to a piece of filter paper, which was loaded into a disposable syringe. After 20 min equilibration at room temperature, headspace samples were analyzed on capillary GC (25 m × 0.32 mm ID SE 54 column, Kupper + CO., Bonaduz, Switzerland, initial temperature

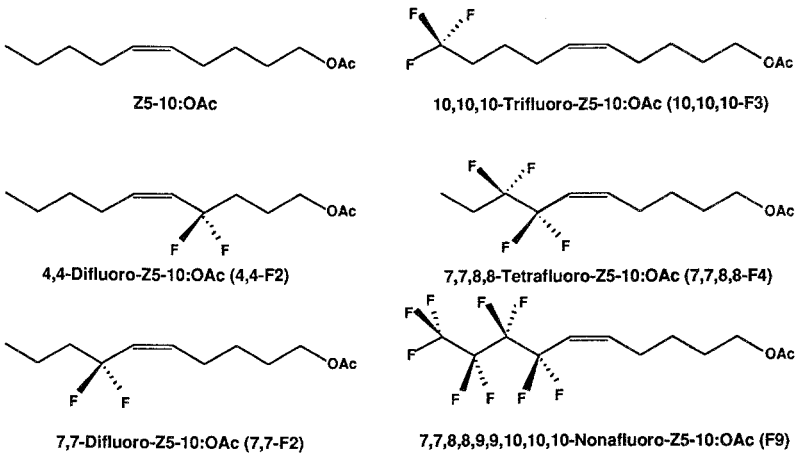

FIG. 1. Structures of Z5–10:OAc and five fluorinated analogs.

was 35°C, splitless injection during 2 min). Three different syringes were prepared and six consecutive injections were made from each syringe.

Electrophysiological Recordings. Single-cell recordings were performed with the tip-cutting technique (Kaissling, 1974; Van Der Pers and Den Otter, 1978). The stimulus was delivered in a charcoal-filtered and humidified airstream, flushing continuously over the preparation at 0.5 m/sec. The stimulus duration was 1 sec during which 2 ml of the atmosphere from a Pasteur pipet, containing a filter paper with stimulus, was injected into the continuous airstream.

Behavioral Tests. Flight-tunnel experiments were performed in a 2.5-m-long × 0.9-m-wide × 0.9-m-high Plexiglas flight tunnel as described by Löfstedt and Herrebout (1988). The flight-tunnel conditions used were: 21–23°C, 40–60% relative humidity, 0.3 m/sec wind velocity, and 1 lux light intensity.

Blends to be tested in the first experiment contained 0.45 ng Z5–10:OAc or its analogs + 2.25 ng Z7–12:OAc + 1.12 ng Z9–14:OAc in 10-μl hexane solutions. More than 50 males were tested with each blend. In the second experiment, blends containing different doses of Z5–10:OAc or its three fluorinated analogs + 2.25 ng Z7–12:OAc + 1.12 ng Z9–14:OAc were tested. Sources were prepared immediately before the test and renewed every 15 min because of the high volatilities of the 10-carbon acetates.

Two- to 3-day-old male moths were transferred individually to 250-ml cylindrical screen cages in plastic cups before the initiation of the dark period and allowed to acclimate to the conditions in the tunnel room for 1 hr before they were tested 3–5 hr into the scotophase. Males were released individually into the plume from a cylindrical screen cage with the open end facing upwind. Six behavioral steps were typically exhibited in the flight tunnel: orientation (Or), upwind flight 30 cm from the release cage in the plume (30 cm), flying half the distance between the source and the release cage in the plume (HW), close approach within 10 cm to the source (190 cm), source contact (SC), and copulation attempts (Cop) were recorded. A male was considered to have contacted the source as soon as its antennae touched the source. The time required to perform a behavioral response was recorded with a stopwatch. Males were tested once and then discarded. After testing, the release cages were heated at 200°C for 1 hr and the insect needles holding the filter paper dispensers were rinsed in acetone.

Field tests were conducted in sugar beet fields in southern Sweden, using red rubber septa (Thomas Scientific) as dispensers and Lund II sticky traps (Anderbrant et al., 1989). In the first experiment, July 2–12, 1991, six different blends containing 3-μg doses of Z5–10:OAc or its analogs (if present) + 15 μg Z7–12:OAc + 7.5 μg Z9–14:OAc (Figure 7 below) were tested. A second experiment was performed, July 15–24, 1991, using 0.09 μg, 3μg, or 90 μg (dosage referred to is the amount of Z5–10:OAc or its analogs) of four selected

blends. Five replicate series were placed in five separated fields. Within a replicate series, traps were at least 10 m apart and in a row at a right angle to the predominant wind direction. Trapped moths were counted and removed every second day.

Molecular Mechanics Calculations. Calculations of geometries and conformational energies were done using the molecular mechanics program MM2(91) (Burkert and Allinger, 1982). Starting structures for the molecular mechanics program were obtained from the molecular modeling system, MacMimic (Instar Software AB, Ideon Research Park, Lund, Sweden).

RESULTS AND DISCUSSION

Release Rates of Fluorinated Analogs. As expected, the relative amounts of the fluorinated analogs 7,7-F2, 10,10,10-F3, 7,7,8,8-F4, and F9 released from the filter papers were found to vary with position and degree of fluorination (Table 1). With equal amounts of the five components added to the filter paper, the vapor content showed a ratio of Z5–10:OAc, 7,7-F2, 10,10,10-F3, 7,7,8,8-F4 and F9 of 1.0:0.8:2.2:2.0:28. This is in agreement with the limited existing vapor pressure data on fluorinated compounds in the literature. Data are only available for smaller molecules from other structural classes, but they show an increase in volatility for a high degree of fluorination (Dykyj and Repás, 1979). The result is also in line with our earlier study on fluorinated analogs of Z9–12:OAc, where 11,11,12,12,12-pentafluoro-Z9–12:OAc was seven times more volatile than Z9–12:OAc, while 11,11-difluoro-Z9–12:OAc and 12,12,12-trifluoro-Z9–12:OAc did not differ much in volatility compared with Z9–12:OAc (Bengtsson et al., 1990a). This implies that, only in the case of the F9 analog, corrections for differences in volatility need to be taken into account

TABLE 1. RELATIVE AMOUNTS OF FLUORINATED Z5–10:OAc ANALOGS RELEASED FROM FILTER PAPER[a]

	Substance			
	7,7-F2 and 4,4-F2	10,10,10-F3	7,7,8,8-F4	F9
Test 1	0.7 ± 0.1	2.2 ± 0.1	2.0 ± 0.1	25.2 ± 5
Test 2	0.8 ± 0.2	2.3 ± 0.3	2.0 ± 0.3	30.1 ± 4
Test 3	0.8 ± 0.1	2.2 ± 0.3	1.9 ± 0.3	28.7 ± 5
Mean	0.8	2.2	2.0	28

[a]Z5–10:OAc = 1. Each test is the average from six consecutive GC injections of headspace samples.

when interpreting the electrophysiological single-cell data and the results of the behavioral tests.

Electrophysiological Recordings. The results of the single sensillum recordings are shown in Figure 2. The di-, tri-, and tetrafluoro compounds (4,4-F2, 7,7-F2, 10,10,10-F3, and 7,7,8,8-F4) elicit very similar activities and are less active than Z5–10:OAc by a factor of ca. 100. The nonafluoro analog F9 is essentially inactive, in spite of its high volatility. The strongly reduced activity of the F9 analog is consistent with the results obtained by Prestwich et al. (1990). They investigated the electrophysiological activities of various pheromone analogs perfluorinated in the terminal alkyl chain on three insect species and found that all these fluorinated analogs had a 100-to 10,000-fold lower single-cell activity than the pheromone stimulus.

Conformational and steric effects are too small to rationalize the reduced

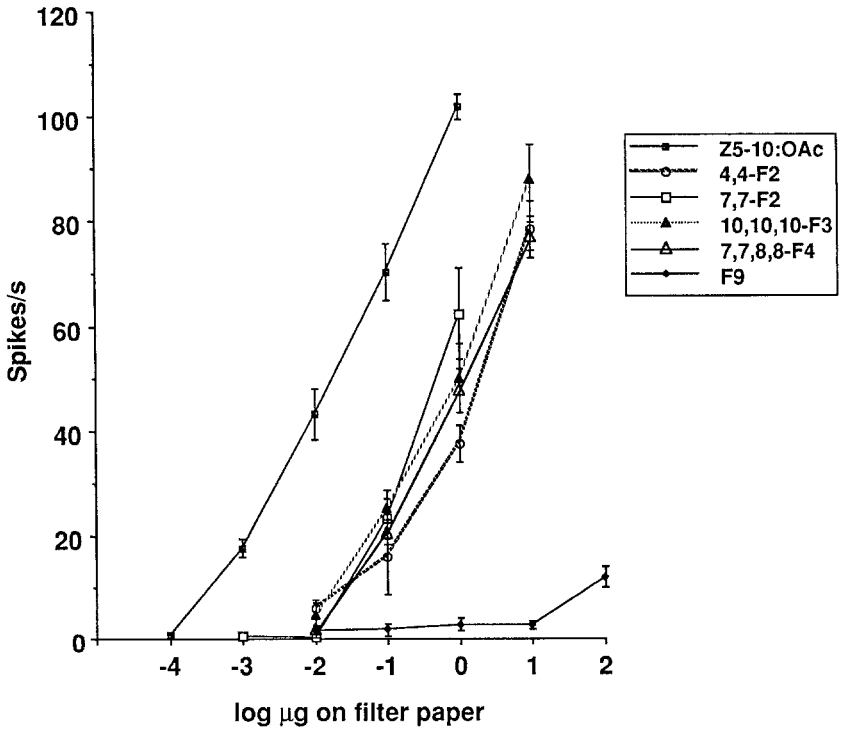

FIG. 2. Single sensillum responses (mean ± SEM, $N = 10$) of the Z5–10:OAc receptor in male *Agrotis segetum* to Z5–10:OAc and five fluorinated analogs: 4,4-difluoro-(Z)-5-decenyl acetate (4,4-F2), 7,7-difluoro(Z)-5-decenyl acetate (7,7-F2), 10,10,10-trifluoro-(Z)-5-decenyl acetate (10,10,10-F3), 7,7,8,8-tetrafluoro-(Z)-5-decenyl acetate (7,7,8,8-F4), and 7,7,8,8,9,9,10,10,10-nonafluoro-(Z)-5-decenyl acetate (F9).

electrophysiological activities of the fluoro analogs. Using molecular mechanics calculations [MM2(91)], we have computed the energies required by each of the analogs to adopt their proposed biologically active conformation according to our previously reported receptor–interaction model (Liljefors et al., 1985, 1987; Bengtsson et al., 1987; Jönsson et al., 1991a,b). The required energies are calculated to be 1.5 kcal/mol for 10,10,10-F3 and 0.4–0.6 kcal/mol for the other analogs. These energies are too small to significantly reduce the activities due to a conformational effect (Liljefors et al., 1987; Bengtsson et al., 1987).

According to the calculated energy-optimized geometries of the fluoro analogs and Z5–10:OAcs the C—F bond is 0.24 Å longer than the C—H bond (1.36 and 1.12 Å, respectively). The van der Waals (vdW) radius of fluorine is ca. 10% larger than that of hydrogen (vdW(F) = 1.65 Å; vdW(H) = 1.50 Å, according to the MM2(91) force-field). Thus, the vdW size [(C—*X* + vdW(*X*)] of the C—F group in the C—F bond direction is only 0.39 Å larger than that of the C—H group. This indicates that even in the case of a high steric complementarity between the natural pheromone component and its receptor (Bengtsson et al., 1990b; Jönsson et al., 1991a,b), steric repulsive interactions between the fluoro analogs and the receptor should be too small to rationalize the observed reductions of the electrophysiological activities.

The part of the receptor interacting with the terminal alkyl chain is most probably highly lipophilic. The most likely reason for the reduced electrophysiological activity of the fluorinated analogs, especially the highly fluorinated ones, is the low affinity of fluorinated hydrocarbons for a lipophilic environment as has been discussed previously by Prestwich et al. (1990). As an example, heptane and perfluoroheptane are only partially miscible at room temperature (Hildebrand et al., 1950). Furthermore, semifluorinated hydrocarbons form micelles in organic solvents including alkane media and reversed micelles in perfluorinated hydrocarbon solvents (Turberg and Brady, 1988). In addition to a low affinity between a fluorinated analog and the pheromone component receptor, the low lipid solubility of especially highly fluorinated analogs may decrease their adsorption on the lipophilic antennal surface and their solubilization in the sensillum lymph via complex equilibria involving lipophilic pheromone binding proteins (Prestwich, 1991). This may lead to a reduction of the number of molecules reaching the receptor.

Although the steric effects of fluorine substituents in the allylic positions (4,4-F2 and 7,7-F2) are small, the strong permanent dipole of the C—F bond may interfere with the proper binding of the double bond to its receptor binding site. This may be an additional activity-decreasing factor for such fluoro analogs.

Flight-Tunnel Assay. Initially, *Agrotis segetum* female gland extracts and synthetic pheromone compounds in different dosages were tested (Figure 3). There were no significant differences eliciting any of the behavioral sequences from 1–10 female equivalents (FE) of gland extract and 0.1–100 FE of synthetic

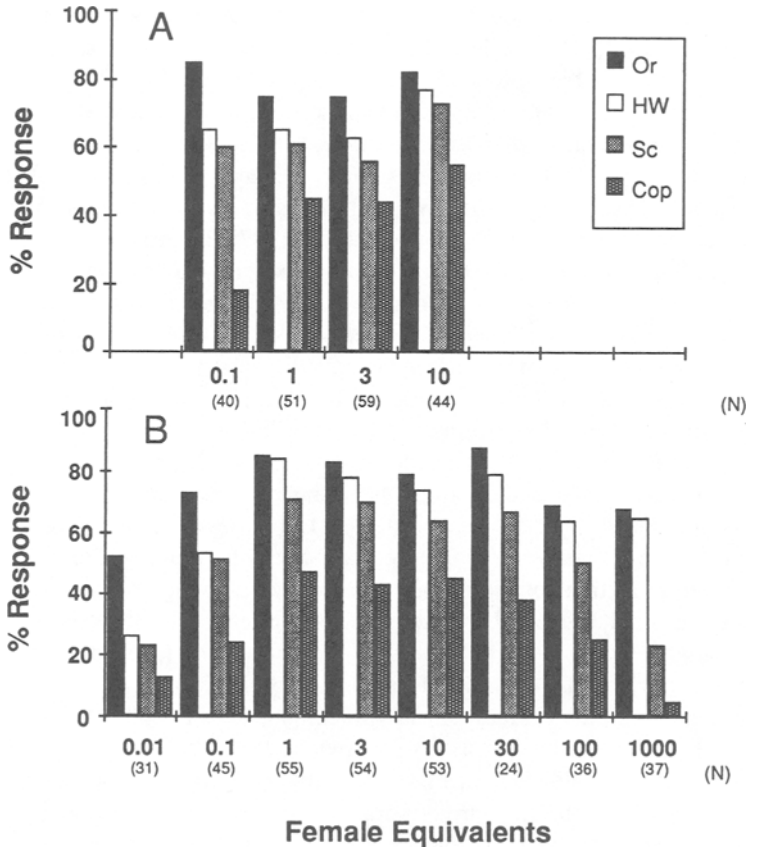

FIG. 3. Behavioral responses of male *Agrotis segetum* to different doses of female gland extracts (A) and a synthetic three-component pheromone (B) in a flight tunnel. (N) represents the number of males tested for each blend. Or: orientation; HW: flying half the distance between the source and the release cage in the plume; Sc: source contact; Cop: copulation attempts.

pheromone compounds, according to the method of adjusted significance levels for proportions ($P < 0.05$) (Ryan, 1960). Earlier studies on the Oriental fruit moth *Grapholita molesta*, for instance, suggest that the pheromone dose–response curve exhibits a normal distribution pattern (Baker and Roelofs, 1981). Therefore, we chose the intermediary 3 FE (equivalent to 0.45 μg Z5–10:OAc + 2.25 μg Z7–12:OAc + 1.12 μg Z9–14:OAc) as an optimal dosage in the later experiments.

The flight-tunnel activities of the mixtures containing the 7,7-F2, 10,10,10-F3, and 7,7,8,8-F4 analogs did not differ significantly from that of the natural

three-component mixture and the two-component mixture. Thus, behaviorally these analogs appeared more active than could be expected from their approximately 100-fold lower electrophysiological activity relative to the Z5–10:OAc. The mixture containing F9 elicited a significantly lower male response, as low as the response to the two-component mixture (Figure 4).

The times required for male moths to perform some major behavioral responses are summarized in Figure 5. The times taken for males to be activated by the 7,7-F2-and F9-containing mixtures were as long as for their response to the two-component mixture, according to the Mann-Whitney U test ($P < 0.05$). In contrast, 10,10,10-F3 appeared to be as an effective stimulus as the pheromone component, which holds also for the other two behavioral steps examined.

Considering the higher dose needed for all the fluoro analogs in eliciting the electrophysiological activity and the higher volatility of F9, different doses of Z5–10:OAc and three fluorinated analogs in blends were tested in the flight tunnel (Figure 6). The differences in the proportion of males making source contacts in response to the different treatments were tested for statistical significance according to the method of adjusted significance levels for proportions ($P < 0.05$). For the natural pheromone blend, the male responses were significantly lower when the dose of Z5–10:OAc was 10 times higher or 100 times

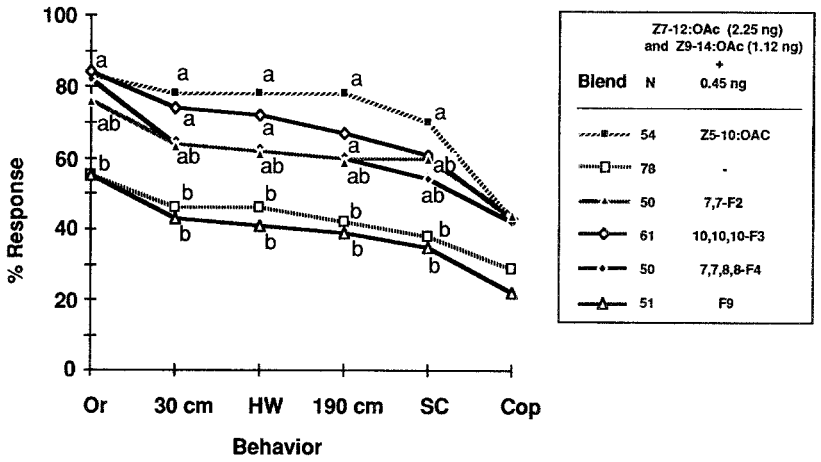

FIG. 4. Behavioral responses of male *Agrotis segetum* to mixtures containing Z5–10:OAc or one of four fluorinated analogs in a flight tunnel. Different letters indicate values significantly different at the 95% confidence level, according to the method of adjusted significance levels for proportions. Or: orientation; 30 cm: upwind flight 30 cm from the release cage in the plume; HW: flying half the distance between the source and the release cage in the plume; 190 cm: close approach within 10 cm to the source; SC: source contact; Cop: copulation attempts.

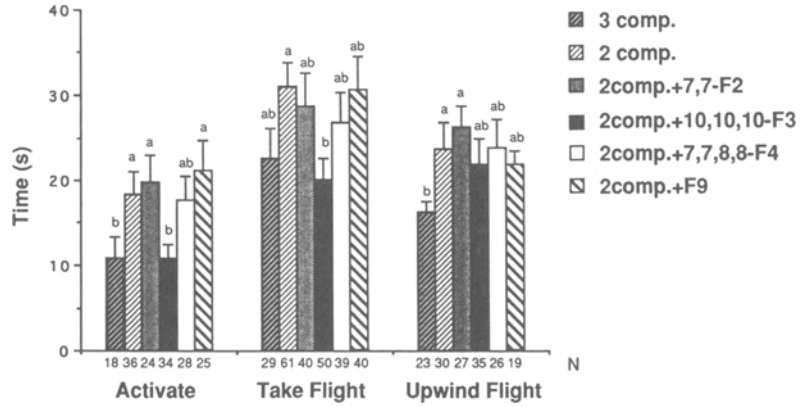

FIG. 5. Time taken by male *Agrotis segetum* to activate, take flight, and to fly upwind from the release cage until touching the source, in response to treatments as in Figure 4. Values are means ± SEM. Same letters within each behavior indicate values that are not significantly different, according to the Mann-Whitney U test ($P < 0.05$). N represents the number of males recorded for each behavior.

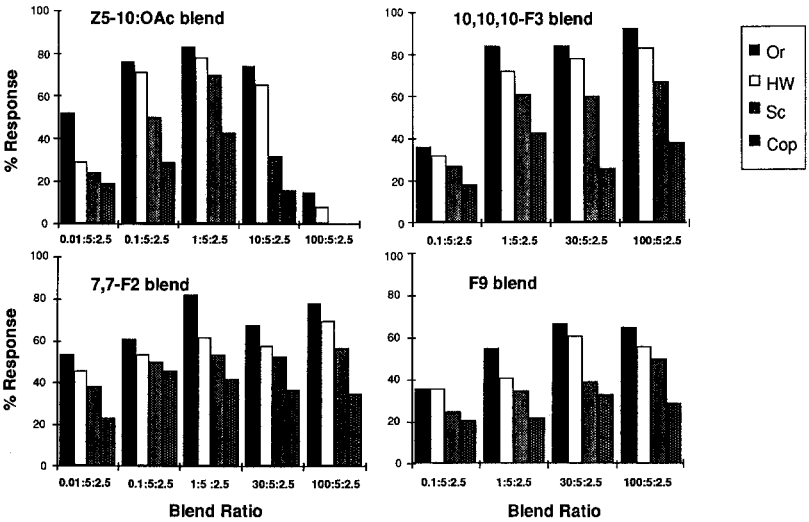

FIG. 6. Behavioral responses of male *Agrotis segetum* to dose series of three-component pheromone blend containing Z5–10 : OAc or a fluorinated analogs plus Z7 –12 : OAc and Z9–14 : OAc.

lower than its natural ratio, which was found in female pheromone gland extracts (Z5–10:OAc/Z7–12:OAc/Z9–14:OAc is 1:5:2.5), but for all the fluorinated analogs tested there was no statistical difference among those different ratios. One interesting thing observed here is that at the 100:5:2.5 ratio, all three fluorinated analogs were significantly more active than the natural pheromone blend at this ratio. Our interpretation is that as the fluorinated analogs are less effective stimuli at the receptor level, higher doses of the analogs are necessary to elicit the same input to the CNS, whereas native Z5–10:OAc at the 100-μg level may cause adaptation of the peripheral receptors, and thereby produce arrestment of upwind flight (Baker et al., 1988).

Field-Trapping Test. The number of moths trapped by the different blends in the field are shown in Figure 7. The 10,10,10-F3-and 7,7,8,8-F4-containing mixtures have significantly higher activity than the two-component mixture (Figure 7A). With increasing amounts of F2 and F3 in the blends, there seemed to be slightly more moths caught but these differences were not statistically significant (Figure 7B), which is consistent with the flight-tunnel results. However, a clear difference between the flight-tunnel and the field results is that traps baited with the natural three-component mixture caught significantly more moths than any of the fluorinated analog mixtures. Thus, it appears that the field test is more discriminatory with respect to testing the activity of different treatments than the flight-tunnel assay. However, different dispensers were used in the two assays, which may account for some of the differences. Since the expected relative release rates from the rubber septa and filter paper are different: half-lives of 10:OAc and 14:OAc on rubber septa differ approximately by a factor 56 (McDonough, 1991), while the release rates of Z5-10:OAc and Z9-14:OAc from filter paper differ approximately by a factor 256 (Bengtsson et al., 1990b).

Among the fluorinated analogs, the F9 compound is behaviorally inactive, as a mixture containing this analog was no more attractive than the two-component mixture. The inactivity of a pheromone component analog with a perfluorobutyl alkyl moiety was also reported in both *Heliothis zea* and *Diatraea grandiosella* at the receptor neuron level (Prestwich et al., 1990) and in *Trichoplusia ni* both at the receptor neuron (Prestwich et al., 1990) and behavioral levels (Linn et al., 1992). The low activity of 7,7-F2 observed in the field is, on the other hand, contrary to earlier studies in *Eupoecilia ambiguella* moth and *Grapholita molesta*, which showed that fluorination at the allylic position can maintain the pheromone activity (Bengtsson et al., 1990a; Masnyk et al., 1989).

With the techniques currently available, single sensillum recordings give the most relevant information about interactions between the stimulus and the receptor. Behavioral results and even electrophysiological recordings using the electroantennogram technique may be obscured by interaction between different receptors and processing of signals in the CNS. Taking these cautions into consideration, we think that the relative activity of the pheromone stimulus and

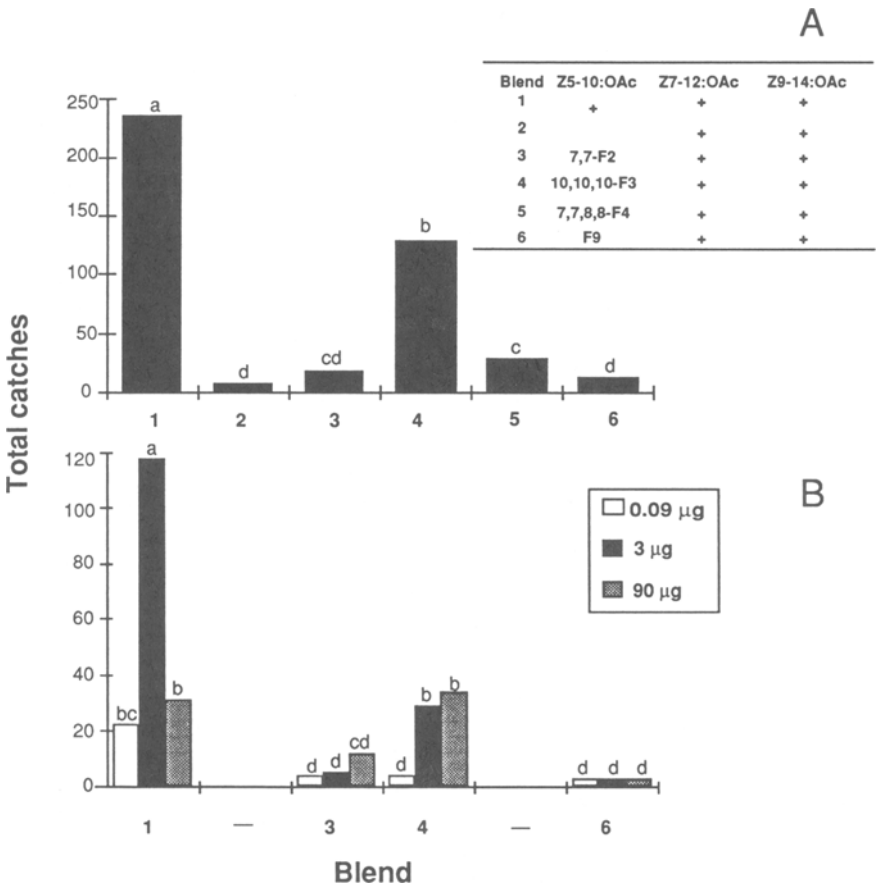

FIG. 7. Field trapping of male *Agrotis segetum* with six (A) and three (B) blends, respectively, containing Z5–10:OAc or one of its fluorinated analogs. Same letters within each series ($N = 5$), indicate values that are not significantly different, according to the Mann-Whitney U test ($P \leq 0.05$).

the fluorinated analogs, as estimated by the single sensillum recordings, the flight-tunnel tests, and the trapping study, are in general agreement. The F9 analog was found to be inactive in all the tests. With respect to the activity of the other fluorinated analogs, the single sensillum recordings provided little resolution. All are less active than the native Z5–10:OAc and more active than the F9 analog. In the flight-tunnel test the 10,10,10-F3 analog appears more active than the di- and tetrafluorinated analogs, which were not significantly different from the two-component mixture. Finally, the field tests allow the recognition of the 7,7,8,8-F4 analog as having low but significant activity.

The conclusion to be drawn from the three assays is that the 10,10,10-F3, 7,7-F2, and 7,7,8,8-F4 analogs do have significant but reduced biological activity, whereas the F9 analog is inactive. The replacement of hydrogen atoms with fluorine atoms in pheromone components will change physical properties of the pheromone molecule, including polarity, flexibility, and hydrophobicity, but most important, the lipophobicity of the fluorinated analogs prohibits a productive receptor interaction.

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REFERENCES

ANDERBRANT, O., LÖFQVIST, J., JÖNSSON, J., and MARLING, E. 1989. Effects of pheromone trap type, position and colour on the catch of the pine sawfly *Neodiprion sertifer* (Geoff.) (Hym., Diprionidae). *J. Appl. Entomol.* 107:365–369.

ARN, H., ESBJERG, P., BUES, R., TÒTH, M., SZÖCS, G., GUERIN, P., and RAUSCHER, S. 1983. Field attraction of *Agrotis segetum* males in four European countries to mixtures containing three homologous acetates. *J. Chem. Ecol.* 9:267–276.

BAKER, T.C., and ROELOFS, W.L. 1981. Initiation and termination of oriental fruit moth male response to pheromone concentrations in the field. *Environ. Entomol.* 10:211–218.

BAKER, T.C., HANSSON, B.S., LÖFSTEDT, C., and LÖFQVIST, J. 1988. Adaptation of antennal neurons in moths is associated with cessation of pheromone-mediated upwind flight. *Proc. Natl. Acad. Sci. U.S.A.* 85:9826–9830.

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 acetates, a pheromone component of the turnip moth, *Agrotis segetum*: Synthesis, conformational analysis and structure–activity relationships. *Bioorg. Chem.* 15:409–422.

BENGTSSON, M., RAUSCHER, S., ARN, H., SUN, W.-C., and PRESTWICH, G.D. 1990a. Fluorine-substituted pheromone components affect the behaviour of the grape berry moth. *Experientia* 46:1211–1213.

BENGTSSON, M., LILJEFORS, T., HANSSON, B.S., LÖFSTEDT, C., and COPAJA, S.V. 1990b. 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.

BRIGGS, G.G., CAYLEY, G.R., GRIFFITHS, D.C., MACAULAY, E.D., PICKETT, J.A., PILE, M.M., WADHAMS, L.J., and WOODCOCK, C.K. 1986. Some fluorine containing pheromone analogues. *Pestic. Sci.* 17:441–448.

BURKERT, U., and ALLINGER, N.L. 1982. Molecular Mechanics. American Chemical Society, Washington, D.C. The MM2(91) program is available from the Quantum Chemistry Program Exchange, University of Indiana, Bloomington, Indiana 47405, and from Molecular Design Ltd., 2132 Farallon Drive, San Leandro, California 94577. A Macintosh version is available from InStar Software AB, IDEON Research Park, S-223 70 Lund, Sweden.

CAMPS, F., COLL, J., GUERRERO, A., and RIBA, M. 1984. Fluorinated analogs of insect sex pheromones. *Experientia* 40:933–934.

DICKENS, J.C., PRESTWICH, G.D., and SUN, W.-C. 1991a. Behavioral and neurosensory responses of the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae), to fluorinated analogs of aldehyde components of its pheromone. *J. Chem. Ecol.* 17:1007–1020.

DICKENS, J.C., PRESTWICH, G.D., SUN, W.-C., and MORI, K. 1991b. Receptor site analysis using neurosensory responses of the boll weevil to analogs of the cyclohexylidene ethanol of its aggregation pheromone. *Chem. Senses* 16:239–250.

DYKYJ, J., and REPÁS, M. 1979. The Vapour Pressure of Organic Compounds (in Slovak). Veda, Bratislava.

HILDEBRAND, J.H., FISHER, B.B., and BENESI, H.A. 1950. Solubility of perfluoro-*n*-heptane with benzene, carbon tetrachloride, chloroform, *n*-heptane and 2,2,4-trimethylpentane. *J. Am. Chem. Soc.* 72:4348–4351.

HINKS, C.F., and BYERS, J.R. 1976. Biosystematics of the genus *Euxoa* (Lepidoptera: Noctuidae) V. Rearing procedures and life cycles of 36 species. *Can. Entomol.* 108:1345–1357.

JÖNSSON, S. 1991. Studies on the complementarity between a moth sex pheromone component and its receptor. Synthesis and structure–activity relationships. PhD thesis. University of Lund, Sweden.

JÖNSSON, S., LILJEFORS, T., and HANSSON, B.S. 1991a. Alkyl substitution in terminal chain of (*Z*)-5-decenyl acetate, a pheromone component of turnip moth, *Agrotis segetum*. Synthesis, single-sensillum recordings, and structure–activity relationships. *J. Chem. Ecol.* 17:103–122.

JÖNSSON, S., LILJEFORS, T., and HANSSON, B.S. 1991b. Replacement of the terminal methyl group in a moth sex pheromone component by a halogen atom. Hydrophobicity and size effects on the electrophysiological single-cell activities. *J. Chem. Ecol.* 17:1381–1397.

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

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.

LINN, C., ROELOFS, W.L., SUN, W.-C., and PRESTWICH, G.D. 1992. Activity of perfluorobutyl-containing pheromone components in pheromone blend of cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 18:737–748.

LÖFSTEDT, C., and HERREBOUT, W.M. 1988. Sex pheromones of ermine moths on European spindle tree. *Entomol. Exp. Appl.* 46:29–38.

LÖFSTEDT, C., VAN DER PERS, J.N.C., LÖFQVIST, 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 behavioural activity. *J. Chem. Ecol.* 8:1305–1321.

MASNYK, M., FRIED, J., and ROELOFS, W.L. 1989. Synthesis and pheromonal properties of (*Z*)-7,7-difluoro-8-dodecenyl acetate, a difluoro derivative of the sex pheromone of the oriental fruit moth. *Tetrahedron Lett.* 25:3243–3245.

MCDONOUGH, L.M. 1991. Controlled release of insect sex pheromones from a natural rubber substrate, pp. 106–124, *in* P.A. Hedin (ed.). Naturally Occurring Pest Bioregulators. ACS symposium series 449, ACS, Washington, D.C.

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.

PRESTWICH, G.D. 1987a. Chemistry of pheromone and hormone metabolism in insects. *Science* 237:999–1006.

PRESTWICH, G.D. 1987b. 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. 1992. Chemical studies of pheromone and hormone receptors in insects. *Arch. Insect Biochem. Physiol.* In press.

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 the aldehyde components of the 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.* 6:1761–1778.

RYAN, J.A. 1960. Significance tests for multiple comparison of proportions, variances and other statistics. *Psychol. Bull.* 57:318–328.

SUN, W.-C., NG, C.-S., and PRESTWICH, G.D. 1992. Synthesis of partially fluorinated analogues of (*Z*)-5-decenyl acetate: Probes for hydrophobic interaction in pheromone reception. *J. Org. Chem.* 57:132–137.

TURBERG, M.P., and BRADY, J.E. 1988. Semifluorinated hydrocarbons: Primitive surfactant molecules. *J. Am. Chem. Soc.* 110:7797–7801.

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.

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A NOVEL *Streptomyces* SPECIES FOR CONTROLLING PLANT-PARASITIC NEMATODES

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Abstract—A novel species of *Steptromyces* isolated from nematode suppressive soils in Costa Rica was evaluated for efficacy in controlling plant-parasitic nematodes. This isolate, designated CR-43, was shown to inhibit reproduction of *Caenorhabditis elegans* in a laboratory assay. Greenhouse trials utilizing three different methods of treatment with CR-43 gave significant reductions of tomato root galling due to *Meloidogyne incognita*. In a field experiment in Puerto Rico, in soil naturally infested with *M. incognita*, CR-43-treated pepper showed significant reductions in root galling and significant increases in yield as compared to untreated controls. In a second experiment in Puerto Rico, a significant reduction in tomato root galling and a slight reduction in root galling on pepper occurred. In this trial, yields on both tomato and pepper were higher in CR-43 treatments, but these differences were not statistically significant. In both experiments populations of *Rotylenchulus reniformis* were reduced by CR-43 treatment. In a field trial on strawberry in Massachusetts, CR-43-treated plants had lower numbers of *Pratylenchus penetrans* within roots and showed a significant decrease in black root rot disease. Studies on sterile filtrates from CR-43 cultures indicated that a major determinant of CR-43 antinematodal activity was mostly thermostable macromolecules of molecular weight higher than 6000. Culture filtrates of CR-43 exhibited antifungal activity in vitro.

Key Words—*Streptomyces* sp., biological control, antinematodal protein(s), *Meloidogyne incognita*, *Rotylenchulus reniformis*, *Pratylenchus penetrans*, plant-parasitic nematodes, *Caenorhabditis elegans*, *Rhizoctonia fragariae*.

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INTRODUCTION

Soils that are naturally suppressive to plant diseases incited by pathogenic fungi (Lumsden et al., 1987) and plant-parasitic nematodes (Zuckerman et al., 1989) represent an ideal source of potential biocontrol organisms. Examination of suppressive soils in Central America by our research team resulted in the isolation of several microorganisms that exhibit a spectrum of antinematodal properties. Two of the isolates were different strains of *Bacillus thuringiensis*, whose effectiveness as a biocontrol agent for plant-parasitic nematodes was later demonstrated in field experiments (Zuckerman et al., 1992). Another promising isolate was a novel *Streptomyces* sp. The current paper reports on the results of laboratory, greenhouse, and field studies on the potential of this *Streptomyces* sp. as a biological control agent.

METHODS AND MATERIALS

Organisms. A *Streptomyces* sp., designated CR-43, was isolated from nematode suppressive soils in Costa Rica and sent to the American Type Culture Collection (ATCC) for identification. The ATCC analysis concluded that this isolate was a novel species of *Streptomyces*. A paper naming the new species is in preparation. Cryopreserved samples of CR-43 have been deposited with the ATCC and an application for a United States patient has been filed by Research Corporation Technologies, Tucson, Arizona 85710, under license from the University of Massachusetts, Amherst, Massachusetts 01003. Inoculum for the current experiments was stored in cryopreservation buffer (Brenner, 1974) at −80°C. When required for experiments, the organism was grown on potato dextrose agar (PDA) at 25°C or potato dextrose broth (PDB) at ambient room temperature on a rotary shaker at 100 rpm.

Meloidogyne incognita race 3 for greenhouse studies were from Dr. M. McClure, University of Arizona. Inoculum for experiments consisted of eggs obtained from root-knot-infected tomato roots, by the method of Hussey and Barker (1973). *Caenorhabditis elegans* for the in vitro assay was from axenic cultures on liver extract medium (Sayre et al., 1963) from our laboratory. Field studies were performed on soils heavily infested with naturally occurring populations of *M. incognita* and *Rotylenchulus reniformis* at the University of Puerto Rico Isabela Experiment Station, Puerto Rico. In Massachusetts, a field experiment was run in a field with a high population of *Pratylenchus penetrans* and an infestation of *Rhizoctonia fragariae* located on the University of Massachusetts Experimental Farm, Deerfield, Massachusetts.

An in vitro assay for antifungal activity by CR-43 proceeded with pure cultures of *Rhizoctonia solani* and *Pythium aphanidermatum* supplied by Dr.

W. Manning, Department of Plant Pathology, University of Massachusetts, Amherst.

Tomato for the greenhouse assay was *Lycopersicon esculentum* cv. Rutgers. Tomato cv. Flora Dade and pepper, *Capsicum frutescens* cv. Cubanelle were used in the Isabela field trials. The Massachusetts strawberry field trial was performed with *Fragaria vesca* cv. Early Glow.

Greenhouse Trials. Control of *M. incognita*-induced root galling was evaluated in greenhouse trials using three different methods of application: liquid drench, infested oatmeal, or seed coat. Liquid cultures for drench applications were prepared by inoculating 100 μl of cryopreserved CR-43 into 50 ml PDB held in a 250-ml Erlenmeyer flask. The cultures were incubated at ambient room temperature on a rotary shaker at 100 rpm for 7–10 days. Controls were medium + *M. incognita*. Medium alone was tested and shown to be nonphytotoxic.

Oatmeal cultures were prepared by adding 15 ml sterile, distilled water to 25 g autoclaved oatmeal in 250-ml Erlenmeyer flasks and adding a 5-mm plug from a CR-43 PDA culture. Flasks were incubated at 25°C for 7–10 days, during which period they were shaken by hand once each day to ensure even colonization of the oatmeal. Each pot received 25 g infested oatmeal. Controls were 25 g oatmeal + *M. incognita*. There were five replicates for each treatment.

For seed coats, CR-43 was combined with methyl cellulose + carrier by the methods of Townshend et al. (1989). For this experiment, coated seed was planted directly into a mixture of 1 part sterile potting soil and 1 part sand in 1-liter pots previously infested with 10,000 eggs of *M. incognita*. There were five replicates per treatment. At 21 days, seedlings were thinned to 1/pot. The experiment was terminated after 10 weeks (Table 1). Controls with seed coat alone demonstrated that the coating was not phytotoxic.

In a drench trial, CR-43 inoculum was added to 1-liter pots containing 1 part sterilized potting soil and 1 part washed sand. A 2- to 3-week-old tomato seedling was transplanted to each pot and grown for one week: 5000–10,000 *M. incognita* eggs (numbers of eggs varied between experiments but not within an experiment) were then added to each pot where appropriate (Table 1). Media controls were included. There were five replicates per treatment, with pots being randomized on the greenhouse bench. Experiments were terminated after six weeks and data taken on the fresh weights of roots and plant tops. The degree of galling was evaluated by counting the number of galls on each root system.

Statistical analyses of greenhouse and field data were by ANOVA supplemented by paired *t* tests (Minitab, Pennsylvania) or by ANOVA and means separated by Duncan's multiple range test (SAS Institute, Inc., Cary, North Carolina).

Field Trials. Two one-half-acre replicated field experiments were performed at the University of Puerto Rico Isabela Experiment Station, Puerto Rico,

during 1990–1991. These fields contained high populations of *M. incognita*. The soil type was described by the University of Puerto Rico Mayaguez soil testing laboratory as a coto clay (72.5% clay, 20.0% sand, 6.5% silt). Several nematode biocontrol microorganisms were evaluated in these trials, but only those treatments pertinent to evaluating CR-43 are reported.

In the first trial, three of the 11 treatments were relevant to the current report: (1) an untreated control, (2) plots treated with the chemical nematicide Fenamiphos (15% active ingredient) at the rate of 7.94 g/20 ft-row of plants, and (3) CR-43 applied as a seed coat (Table 2). The preparation of the seed

TABLE 1. GREENHOUSE TRIALS EVALUATING THREE METHODS OF APPLYING CR-43 TO CONTROL *Meloidogyne incognita* (MI) ON TOMATO

Treatment[a]	Fresh weight (g)		Galls (*N*)	Control (%)
	Roots	Tops		
Trial 1[b]				
Oat medium + MI	18 ± 3.1	69 ± 15.1	125 ± 59a	
CR-43 + oat medium + MI	17 ± 3.0	68 ± 6.1	68 ± 36b	45
Trial 2[c]				
Drench medium (PDB) + MI	12 ± 3.2	58 ± 6.8	148 ± 20a	
CR-43 + medium + MI	14 ± 1.5	57 ± 8.1	74 ± 16b	50
Trial 3[c]				
Seed coat ± MI	16 ± 3.7ab	62 ± 13.2	283 ± 155a	
Seed coat + MI + CR-43	12 ± 3.2a	59 ± 8.4	65 ± 23.5b	77

[a] Each treatment was replicated five times. Numbers followed by different letters differ at $P = 0.05$.
[b] Means separated by *t* tests (Minitab).
[c] Means separated by Duncan's multiple range test (SAS).

TABLE 2. FIELD TRIAL EVALUATING CR-43 SEED COAT IN CONTROL OF *Meloidogyne incognita* ON PEPPER, UNIVERSITY OF PUERTO RICO ISABELA EXPERIMENT STATION[a]

Treatment[b]	Yield (kg)	Yield increase (%)	Gall index
Untreated control	11 ± 2b[c]		2.0 ± 1.2a[d]
Fenamiphos	19 ± 2a	70	0.5 ± 0.4b
CR-43 seed coat	18 ± 4a	62	0.7 ± 0.6b

[a] Numbers followed by different letters differ at $P = 0.05$.
[b] Each treatment was replicated four times.
[c] Means separated by *t* tests (Minitab).
[d] Means separated by Duncan's multiple range test (SAS).

coat was as described for the greenhouse trials. For these experiments, coated seeds were germinated and grown to 4–5 in. height in 2-in. pots prior to transplantation.

In the second trial, CR-43 was applied as a drench (Table 3). The inoculum was first grown for five days in 1 liter of PDB, then added to 10 liters of PDB and fermented for 24 hr to a final concentration of $1 \times 10^{5-6}$ colony forming units (CFU)/ml. Fermentations were performed in Massachusetts and inoculum then packed in ice and shipped to Puerto Rico by overnight carrier. Prior to shipping, the inoculum was concentrated by centrifugation and the supernatant medium removed. In the field, water was added to bring the inoculum back to concentration, which was then added to the planting hole at the time of transplant at 50 ml/plant. During the first period of the 12-week experiment, CR-43 was applied four times at two-week intervals. There were 40 tomato plants and 40 pepper plants in each plot, with four replicates per treatment. The total yields of five tomato harvests and five pepper harvests were combined for analysis (Table 3). Nematode soil populations were sampled when the experiment was initiated, six weeks later, and at 53 days (during harvest). Population estimates, including levels of *R. reniformis*, were obtained using a combined sieving and Baermann funnel extraction (Hooper, 1986).

A field trial evaluating control by CR-43 of black root rot of strawberry was performed in 1990–1991 at the University of Massachusetts Experimental Farm, Deerfield, Massachusetts (Table 4). Black root rot of strawberry is caused by interaction between the fungus *R. fragariae* and the nematode *P. penetrans* (Lamondia and Martin, 1989). The soil type, as determined by the University of Massachusetts Soil Analysis Laboratory, was a fine sandy loam, pH 7.1.

TABLE 3. FIELD TRIAL EVALUATING CR-43 DRENCH IN CONTROLLING *Meloidogyne incognita* ON PEPPER AND TOMATO, UNIVERSITY OF PUERTO RICO ISABELA EXPERIMENT STATION[a]

	Yield (kg)[c]		Yield increase (%)[d]		Gall index[e]	
Treatment[b]	Pepper	Tomato	Pepper	Tomato	Pepper	Tomato
Control	18 ± 5	115 ± 11			0.9 ± 0.6	4.3 ± 1.6a
Fenamiphos	19 ± 3	133 ± 9	9	11	0.8 ± 0.5	4.4 ± 1.3a
CR-43 (drench)	23 ± 2	139 ± 17	26	12	0.8 ± 0.6	2.3 ± 1.5b

[a] Number followed by different letters differ at $P = 0.05$
[b] Each treatment was replicated four times.
[c] Means separated by t tests (Minitab).
[d] Yields given as percent increase over untreated controls.
[e] Means separated by Duncan's multiple range test (SAS).

TABLE 4. FIELD EVALUATION OF CR-43 FOR CONTROL OF *Pratylenchus penetrans* AND BLACK ROOT ROT OF STRAWBERRY, UNIVERSITY OF MASSACHUSETTS EXPERIMENTAL FARM, DEERFIELD, MASSACHUSETTS[a]

Treatment[b]	Yield (g/m)	Berries (N) per meter, X	$\bar{X}$ berry weight (g)	Black root rot index		*P. penetrans* (N/g root $\bar{X}$)	
				Fall 1990	Spring 1991	Fall 1990	Spring 1991
Untreated control	446 ± 225	201 ± 42	2.2 ± 0.8	3.4 ± 0.7	3.6 ± 1.1a	192 ± 115	82 ± 85.1
Rice control	555 ± 332	223 ± 52	2.4 ± 0.9	3.5 ± 1.1	3.7 ± 1.1a	182 ± 115	50 ± 25.4
CR-43	695 ± 315	265 ± 38	2.6 ± 1.0	3.4 ± 0.7	2.7 ± 0.7b	144 ± 67	30 ± 27.1

[a]Numbers followed by different letters differ at $P = 0.05$. Means separated by t tests (Minitab).
[b]Each treatment was replicated four times.

Tissue culture grown disease-free strawberry plants (*Fragaria vesca* cv. Early Glow) were obtained from Nourse Farms, South Deerfield, Massachusetts. No artificial inoculum was applied to the site; natural infestations accounted for disease observed. CR-43 was grown on rice and 25 g/plant of rice/CR-43 inoculum applied in June 1990. This was followed by three drenches (50 ml/plant) at 2, 4, and 12 months after planting. Each replicate plot consisted of 25 plants, five of which were destructively sampled in the fall 1990 and assayed for black root rot and for *P. penetrans* populations within roots. Each treatment was replicated five times. Strawberries were harvested in the spring 1991. One meter of each replicate was harvested and data taken on the number of berries per meter, yield in grams per meter, and mean weight of berries in grams. Five more plants were dug up and assayed for black root rot. Each root was given a black root rot index on a scale where: 1 = no significant deterioration, rot, or discoloration; 2 = up to 25% discoloration, lesions on perennial roots; 3 = 25–50% discoloration, blackened perennial roots, significant deterioration of fibrous roots; 4 = 50–75% damage, most perennial roots blackened, most fibrous roots damaged; 5 = 75–100% damage, all perennial and fibrous roots rotted and blackened. To assay for *P. penetrans*, 2-g subsamples of roots were shaken vigorously on a rotary shaker for 48 hr in 40 ml of sterile tap water at ambient room temperature. Roots and liquid were poured onto modified Baermann funnels for an additional 24 hr. *P. penetrans* present in 2-ml subsamples were counted and data expressed as number of nematodes per gram of root. Data were analyzed by ANOVA and Duncan's multiple range test (SAS Institute, Inc., Cary, North Carolina).

Assay of CR-43 for Antifungal Activity. Four 5-mm plugs of CR-43 on PDA were placed equidistant and 3 cm from the center of a 9-cm Petri plate and a 5-mm plug of a challenge plant-pathogenic fungus placed in the center of the plate. Activity of CR-43 exometabolites was evaluated against two plant-pathogenic fungi, *R. solani* and *P. aphanidermatum*. The plates were incubated at 25°C and assayed at 72 hr for zones of inhibition. Controls were PDA plugs.

Biological Characterization of CR-43 Exometabolites. For study of the effects of the culture filtrate (CF), *C. elegans* was added to wells of a dilution series of CF, and after a period of time the numbers of living worms determined. The specific protocol for this bioassay was a follows. CF was diluted with liver extract medium at concentrations of 1:1, 2:1, 4:1 and 8:1. Fifty age-synchronized second-stage larvae of *C. elegans* were added to each well (Zuckerman and Geist, 1983), the plates sealed with parafilm, and held at 22°C for seven days. Controls were equal dilutions of PDB/liver extract. After seven days, the effect on reproduction was measured by counting the *C. elegans* from three 50-μl aliquots from each well. The effect of the CF test solution on growth was assayed by measuring the lengths of five nematodes from each replicate. The test procedure influenced growth of the nematodes, for the lower CF concentration contained more liver extract (LE) than did the higher CF concentration, as follows: 1 part CF: 1 part LE; 2 parts CF: 1 part LE; 4 parts CF: 1 part LE; or 8 parts CF: 1 part LE. Since liver provides a factor necessary for the growth of *C. elegans*, the higher dilutions of LE served to limit nematode growth. Therefore, a separate control was run for each medium dilution tested (Table 5).

Another experiment was performed to assay for the time of production of a reproduction inhibiting factor previously reported from CR-43 culture filtrates (Zuckerman et al., 1991). CR-43 in PDB was grown for one to seven days, and each day sterile culture filtrate collected and stored at 4°C. There were three replicate wells for each day and three controls containing medium alone, giving a total of 24 wells/plate evaluated. This test was replicated for each extract, and the entire extract series repeated twice. To evaluate inhibition of egg-laying, 50 newly hatched *C. elegans* were added to 2.5 ml in each well, and the cultures incubated at 22°C for seven days. The numbers of nematodes were then counted in three 50-μl aliquots from each sample (Table 6).

Observations were made under the light microscope at 100× to determine if the egg-laying inhibiting factor affected egg and gonad formation.

To examine for the failure of the egg-laying mechanism, ultrathin sections were taken from *C. elegans* treated with culture filtrates from a *Bacillus* spp. from Costa Rica, which produced a reproduction inhibiting factor similar to that produced by CR-43. The objective here was to examine for morphological defects in the dilator and sphincter muscles associated with the vagina, thereby giving a possible explanation for the inhibition of egg laying. Nematodes were

TABLE 5. EFFECT OF DIFFERENT CONCENTRATIONS OF CR-43 CULTURE FILTRATE (CF) ON GROWTH AND EGG-LAYING OF *C. elegans*[a]

Treatment[b]	Concentration CF	Reproduction[c]	Length (μm)[d]
Medium control	1:1[e]	116 ± 33a	992 ± 20
	2:1	125 ± 39a	1,088 ± 14
	4:1	135 ± 12a	821 ± 12
	8:1	59 ± 10a	624 ± 21
CF	1:1	13 ± 4b	1,009 ± 11
	2:1	4 ± 2b	1,056 ± 13
	4:1	4 ± 2b	1,120 ± 8
	8:1	1 ± 1b	400 ± 30
CF boiled	1:1	20 ± 5b	–
	2:1	3 ± 2b	–
	4:1	1 ± 1b	–
	8:1	0 ± 0c	–
CF frozen	1:1	5 ± 3b	–
	2:1	3 ± 2b	–
	4:1	4 ± 3b	–
	8:1	2 ± 2b	–

[a] Numbers followed by different letters differ at $P = 0.05$. Length measurements were taken at seven days.
[b] Each treatment was replicated nine times.
[c] Average number of nematodes in a 50-μl subsample from a 2500-μl sample. Means separated by *t* tests (Minitab).
[d] – = no measurements taken.
[e] Parts CF:parts PDB.

TABLE 6. DEVELOPMENT OF *C. elegans* EGG-LAYING INHIBITING FACTOR IN CULTURE FILTRATES OF CR-43[a]

	PDB Control	Culture filtrate (day) 1	2	3	4	5	6	7
C. elegans (*N*/ml)	63 ± 14	64 ± 14	55 ± 15	29 ± 19	25 ± 16	15 ± 8	5 ± 4	3 ± 4
Reproduction inhibition (%)		0	14	55	62	77	92	96

[a] Data give the average number of nematodes from three 50-μl subsamples taken from 2.5 ml test solution in each well after six days culture. Filtrate concentration 4 parts CF: 1 part LE. Percent inhibition on day 1 served as a baseline for sampling on the succeeding days. Means separated by *t* tests (Minitab). Each treatment was replicated three times.

sectioned and processed for transmission electron microscopy as described by Himmelhoch and Zuckerman (1978). Micrographs were taken on a Philips 300 Electron Microscope operated at 100 KV.

Molecular and Biochemical Characterization. To study physical factors, CF stored at 100°C for 5 min or frozen at −80°C was assayed to determine if the filtrate retained antinematodal activity. The bioassay was as previously described.

Molecular weight estimates of the active antinematodal fractions in CF were obtained in dialysis studies. CF from 7-day-old cultures were dialyzed against distilled water for 48 hr with three changes of water, and then a bioassay run with *C. elegans*. Molecular weight cutoffs of the dialysis membranes were 6000–8000, and 12,000–14,000. Each dialysis level was tested 3 times.

CF from the time course study and dialysis extracts were examined by SDS gel electrophoresis in an attempt to correlate the occurrence of protein in CF with antinematodal activity against *C. elegans*. Extracts were treated with SDS–mercaptoethanol and proteins separated on 7.6% polyacrylamide gels on a Hoefer Scientific SE600 vertical slab unit with discontinuous buffer, as described by Laemmli (1970). Gels were stained with silver nitrate to enhance the sensitivity for detection of protein (Merril et al., 1980).

RESULTS

Greenhouse Trials. In the greenhouse, when CR-43 was applied in oat medium as a drench in PDB or as a seed coat, a significant reduction in root-knot symptoms on tomato occurred ($P = 0.05$). However, the greatest degree of control was attained when CR-43 was incorporated in the seed coat (Table 1). Comparative plant growth data from treated and untreated plants indicated that CR-43 was not phytotoxic. The slightly lesser growth of CR-43 seed-coat-treated plants as compared to seed coat alone, was not considered indicative of phytotoxicity in view of the enhanced yield of pepper in CR-43 seed coat treatments in the field. In the trials reported in Table 1, infection of roots by *M. incognita* may have been affected by the medium used to deliver the biocontrol organism, but in each case the significant reduction in galling demonstrated the efficacy of CR-43 in controlling root-knot.

Field Trials. Significant reductions in root-knot infection ($P = 0.05$) and increases in yield ($P = 0.05$) occurred in plots to which seed coat incorporated CR-43 was applied to pepper in the first Isabela field trial (Table 2). Yields and root-knot nematode control approximated those attained by nematicide treatment. Nematode sampling showed an 85% reduction in soil populations of the reniform nematode, *R. reniformis*, in CR-43-treated plots as compared to untreated controls at 12 weeks, when the experiment was concluded.

In the second Isabela Experiment Station Trial, in which the carrier medium was removed and water was used to deliver the biocontrol organism, CR-43 applied as a drench resulted in a significant reduction in tomato root galling ($P = 0.05$) due to *M. incognita* (Table 3). Root galling on treated pepper was less than on untreated pepper, and CR-43 applications were associated with yield increases on both tomato and pepper, but these differences were not statistically significant. At harvest, there was a 15% decrease of *R. reniformis* in CR-43 drench-treated plots as compared to untreated controls.

CR-43-treated strawberry plants contained lower root populations of *P. penetrans*, showed greater growth, and gave higher yields than did untreated plants (Table 4), although these differences were not statistically significant. However, treatment significantly reduced black root rot ($P = 0.05$), suggesting that the antifungal–antinematodal properties of CR-43 functioned in the overall successful performance of the biocontrol agent.

Antifungal Activity. Growth of *R. solani* and *P. aphanidermatum* was restricted by CR-43 as shown by a zone of inhibition surrounding the CR-43 plugs (Figure 1), thereby demonstrating antibiotic activity by CR-43 exometabolites.

Biological Characterization of Egg-Laying Inhibiting Factor. Egg-laying of *C. elegans* was inhibited at concentrations of CF that did not affect growth, specifically 1, 2, and 4 parts CF:1 part LE (Table 5). Nematodes exposed to these concentrations of CF underwent normal maturation, including formation of the vulva, vagina, and gonads in adults. At 100×, larvae were observed to develop within eggs (Figure 2). Occasionally eggs hatched within the gonad, and live larvae emerged into the uterus. Normally, in axenic, liquid culture, *C.*

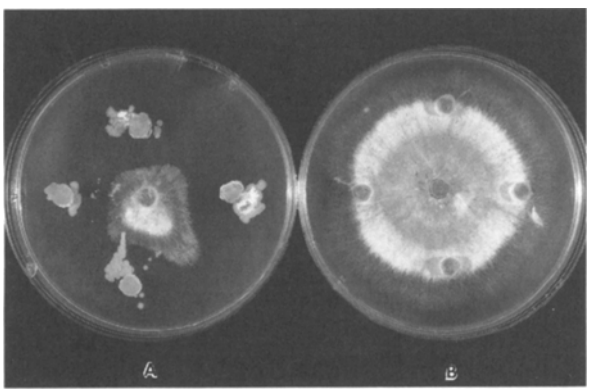

FIG. 1. (A) Zones of inhibition of growth of *Rhizoctonia solani* surrounding agar plugs containing CR-43. (B) PDA plug controls showing no inhibition of fungus growth.

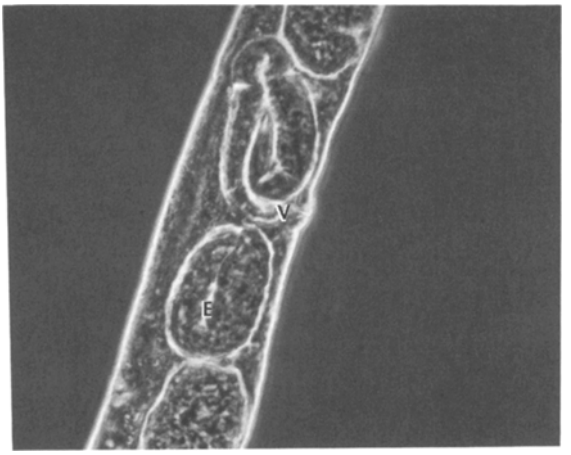

FIG. 2. *Caenorhabditis elegans*: Eggs (E) with developing larvae within the gonad of a nematode grown for six days in a sterile reproduction inhibiting culture filtrate. Development of the vulva and vagina (V) occurred normally, but egg-laying was completely inhibited.

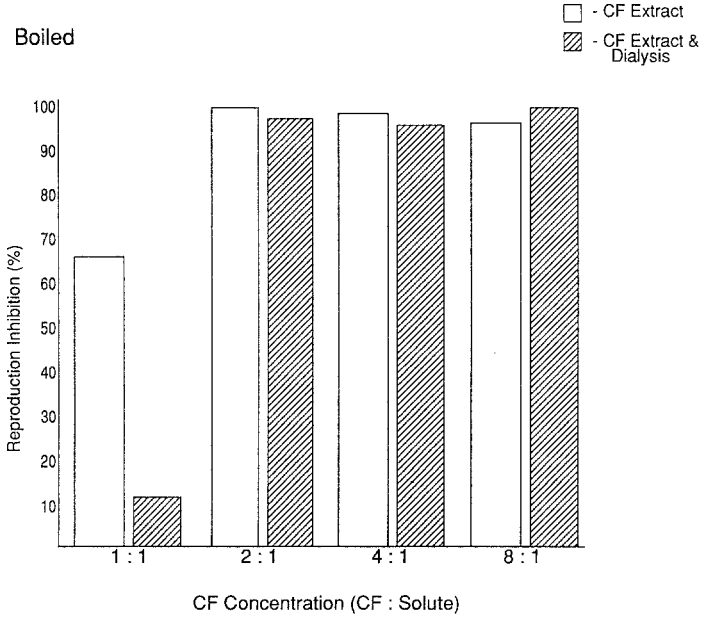

FIG. 3. The amount of inhibition of egg-laying in *Caenorhabditis elegans* grown for six days in several concentrations of boiled culture filtrate of CR-43.

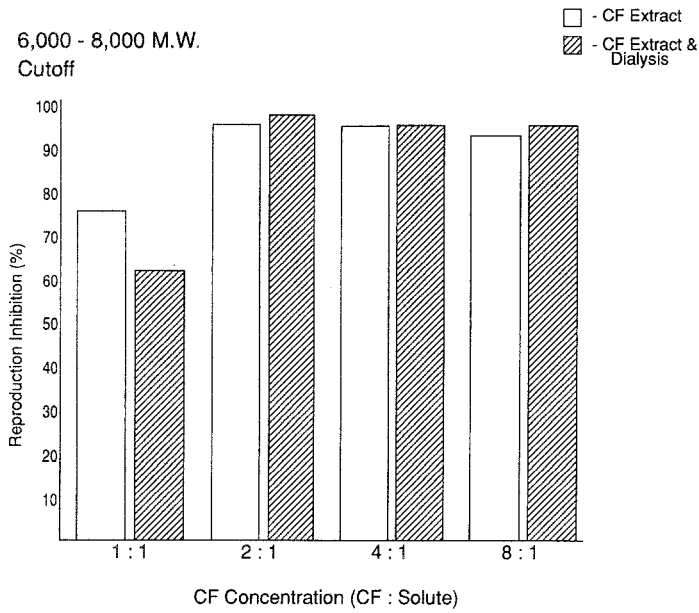

FIG. 4. Dialysis through a membrane with 6000–8000 mol wt cutoff resulted in a slight degree in reproduction inhibition at only the lowest concentration of culture filtrate, indicating that most egg-laying inhibiting factors were greater than 8000 mol wt.

elegans lay eggs into the medium and hatch occurs there. Our interpretation of this observation is that egg-laying inhibition was complete, with the small population increase at 1, 2, and 4 parts CF : 1 part LE (Table 5) due to endotokia matricida (a state in which larvae hatch within the uterus, resulting in death of the female) and the subsequent escape of larvae from the nematode corpus. The results in Table 5 are more graphically illustrated by conversion of the nematode populations to reflect the entire 2.5-ml sample. For example, at 2 parts CF : 1 part LE the total nematode population at seven days was 200, whereas comparable control populations were 7250 nematodes.

Examination of ultrathin sections of treated *C. elegans* by TEM revealed normal-appearing dilator and sphincter muscles associated with the vagina. Function of these muscles is requisite to egg-laying.

Biochemical, Physical, and Molecular Characterization of Egg-Laying Inhibiting Factor. Storage of CF for six months at −80°C had no effect on the antinematodal or antifungal properties of the extract. When boiled at 100°C for 5 min., the 1 : 1 concentration showed a large decrease in the reproduction inhibiting factor, but this decrease did not occur at 2 : 1, 4 : 1, and 8 : 1 concentrations (Figure 3). Our interpretation of these results is that at the lower con-

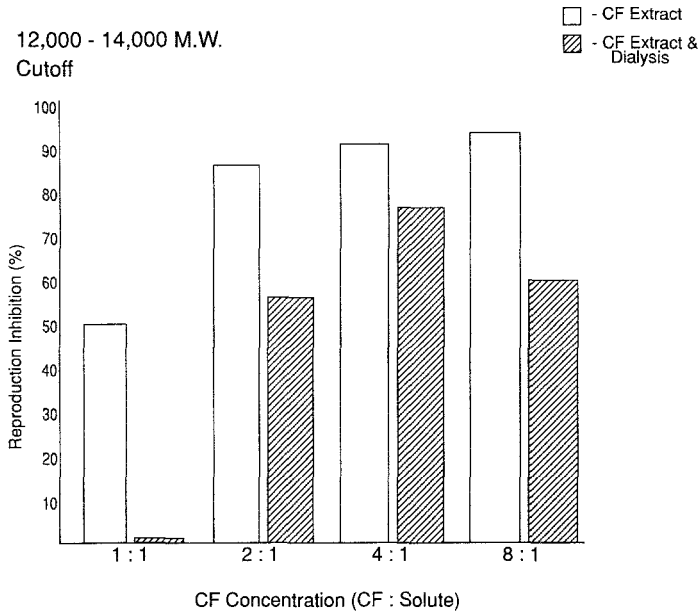

FIG. 5. Dialysis through a membrane with 12,000–14,000 mol wt cutoff resulted in a partial loss of the reproduction inhibiting factor at all concentrations of culture filtrate tested, indicating that more than one molecule in the filtrate is involved in the inhibition phenomenon.

centration at least one compound that acts to inhibit egg-laying was denatured, but at higher concentrations sufficient amounts of egg-laying inhibiting factors were present to mask the loss of the denatured compound(s). This observation provided evidence in support of our conclusion that more than one compound produced by CR-43 acts to inhibit egg-laying of *C. elegans*. Dialysis against a membrane with a 6000–8000 mol wt cutoff resulted only on a slight reduction in egg-laying capacity at the 1 : 1 concentration, indicating that the major constituents of CF responsible for inhibition of egg-laying were greater than 8000 molecular weight (Figure 4). Again, at the 2 : 1, 4 : 1, and 8 : 1 concentrations, there was no loss of egg-laying inhibition capacity, suggesting that concentration of the CF factors is of importance to the inhibition phenomenon. Dialysis against membranes with 12,000–14,000 mol wt cutoffs resulted in a partial loss of egg-laying inhibition at all concentrations, again providing evidence in support of the conclusion that two or more proteins present in CF were responsible for the observed antinematodal effect (Figure 5). CF showed no antinematodal activity against *C. elegans* at 24 hr, increasing to 55% egg-laying inhibition at three days and 96% by seven days (Table 6). SDS gel electrophoresis of CF at

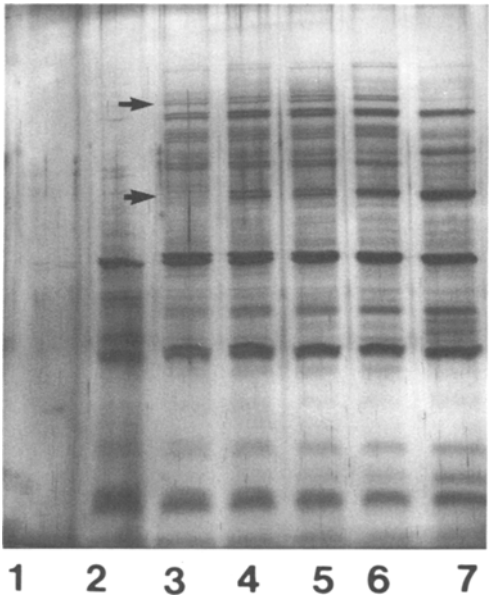

FIG. 6. SDS gel protein spectra produced by culture filtrates of CR-43 for one to seven days. At three days substantial amounts of protein in the range of 55–160 kDa are produced (→). Our interpretation of these results is that most of the antinematodal factors are high-molecular-weight proteins. A few of these proteins begin to appear by day 2.

24-hr intervals for seven days showed that a number of proteins, between 55 and 160 kD, appeared by three days in CF (Figure 6). These protein bands increased in intensity to seven days. Correlation of these results with those of the dialysis experiments suggest that one or more proteins are the determinants for the observed antinematodal activity.

DISCUSSION

The experiments described in this paper demonstrate the potential of CR-43 as a bionematicide. Promising field results were obtained with three plant-parasitic nematodes, *M. incognita*, *P. penetrans*, and *R. reniformis*. In addition, antifungal characteristics demonstrated in vitro successfully translated in the field into partial control of the fungus–nematode complex black root rot disease of strawberry. Of three different methods of delivery of the biocontrol organism under field conditions, incorporation of CR-43 into the seed coat showed the greatest promise. Most encouraging was that CR-43 yields approximated those of a commercial chemical nematicide in field trials.

The current report also indicates that the nematicidal properties of CR-43 reside in one or more proteinaceous compounds that are produced as exometabolites. Further characterization and purification of these proteins is in progress in our laboratory. These steps logically precede isolation of the genes responsible for production of these proteins and the sequential cascade of recombinant potentials.

REFERENCES

BRENNER, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* 77:71–94.

HIMMELHOCH, S., and ZUCKERMAN, B.M. 1978. *Caenorhabditis briggsae:* Aging and the structural turnover of the outer cuticular surface and the intestine. *Exp. Parasitol.* 45:208–214.

HOOPER, D.J. 1986. Extraction of free-living stages from soil, pp. 5–30, *in* J.F. Southey (ed.). Laboratory Methods for Work with Plant and Soil Nematodes. Ministry of Agriculture, Fisheries and Food Reference Book 402.

HUSSEY, R.S., and BARKER, K.R. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Dis. Rep.* 57:1025–1028.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.

LAMONDIA, J.A., and MARTIN, S.G. 1989. The influence of *Pratylenchus penetrans* and temperature on black root rot of strawberry by binucleate *Rhizoctonia* spp. *Plant Dis.* 73:107–110.

LUMSDEN, R.D., GARCIA-E., R., LEWIS, J.A., and FRIES-T., G.A. 1987. Suppression of damping-off caused by *Pythium* spp. in soil from the indigenous Mexican chinampa agricultural system. *Soil Biol. Biochem.* 19:501–508.

MERRIL, C.R., GOLDMAN, D., SEDMAN, S.A., and EBERT, M.H. 1980. Ultrasensitive stains for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* 211:1437–1438.

SAYRE, F.W., HANSEN, E.L., and YARWOOD, E.A. 1963. Biochemical aspects of the nutrition of *Caenorhabditis elegans*. *Exp. Parasitol.* 13:98–107.

TOWNSHEND, J.L., MESKINE, M., and BARRON, G.L. 1989. Biological control of *Meloidogyne hapla* on alfalfa and tomato with the fungus *Meria coniospora*. *J. Nematol.* 21:179–183.

ZUCKERMAN, B.M., and GEIST, M.A. 1983. Effects of vitamin E on the nematode *Caenorhabditis elegans*. *J. Am. Aging Assoc.* 6:1–4.

ZUCKERMAN, B. M., DICKLOW, M. B. and ACOSTA, N. 1992. Two strains of *Bacillus thuringiensis* for control of plant-parasitic nematodes. *Biocon. Sci. and Tech.* (in press).

ZUCKERMAN, B.M., DICKLOW, M.G., COLES, G.C., GARCIA-E., R., and MARBÁN-MENDOZA, N. 1989. Suppression of plant parasitic nematodes in the chinampa agricultural soils. *J. Chem. Ecol.* 15:1947–1955.

ZUCKERMAN, B.M., DICKLOW, M.B., and WILLETT, J.D. 1991. A microbial egg-laying inhibiting factor for *Caenorhabditis elegans*. *Caenorhabditis elegans* Genetics Conference, June 1–5, University of Wisconsin, Madison, p. 353 (abstract).

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INHIBITION OF RADISH GERMINATION AND ROOT GROWTH BY COUMARIN AND PHENYLPROPANOIDS

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Abstract—Thirteen natural and synthetic phenylpropanoids as well as coumarin (2×10^{-4}M) were tested for their biological activity on radish germination and subsequent root growth in light and darkness. Coumarin was the most potent inhibitor. With some exceptions, phenylpropanoids with a carboxylic group in the side chain inhibited root growth. Coumarin was formed spontaneously by photooxidation of 2-hydroxycinnamic acid. Microscopic observations of root treated with coumarin suggest that this substance inhibits the elongation of cells of the differentiating zone of the root.

Key Words—Coumarin, dormancy, light, phenylpropanoids, radish, *Raphanus sativus*, seed, root inhibition.

INTRODUCTION

It is well known that phenolic compounds such as simple phenols, phenylpropanoids, and coumarins may play an important role in allelopathy by either inhibiting germination or reducing growth of seedlings (Bewley and Black, 1985; Putnam and Tang, 1986). However, clear insight into the precise physiological perturbation caused by these substances has not been obtained because of the

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difficulty in determining the specific site of the action (Rice, 1984; Einhellig, 1986).

Recently, we have shown that some free forms of glucosylated phenylpropanoids possess antialgal activity *in vitro* (Aliotta et al., 1992a) and that in radish seeds coumarin inhibits germination in the presence of light (Aliotta et al., 1992b). These data have led us to investigate the structure–activity relationship of some synthetic and natural phenylpropanoids and coumarin upon germination and seedling growth of radish in light and in darkness. Moreover, the ultrastructure of the root of radish seedlings treated with coumarin was studied to identify the morphological aspects of growth reduction.

METHODS AND MATERIALS

Compounds **1, 2, 3, 4, 5, 6, 7, 8**, and **9** (Table 1) were purchased from Aldrich-Chemical Company (Milwaukee, Wisconsin); the remaining phenylpropanoids were synthesized.

To synthesize (*E*)-propenylbenzene **(10)** and (*Z*)-propenylbenzene **(11),** allylbenzene **(9)** (600 mg) was refluxed with ethanol saturated with KOH (3 ml). After 5 hr, the solution was neutralized with 2 N HCl and extracted with Et_2O. The organic material (450 mg) was then separated by argentation preparative TLC [petrol–Et_2O (9 : 1)] to give **10** (310 mg) and **11** (110 mg).

To synthesize 2-methoxycinnamic **(12),** 3-methoxycinnamic **(13),** and 4-methoxycinnamic **(14)** acids, a commercial sample (200 mg) of the corresponding hydroxy acid was treated with ethereal CH_2N_2 to give the permethylated derivative, which was refluxed in aqueous methanolic KOH for 3 hr to give, after acidification, the methoxy acid.

Bioassay. Seeds of *Raphanus sativus* L. Saxa, collected during 1990, were purchased from Imperatore Co., Naples. The seeds were surface-sterilized in 95% ethanol for 15 sec and germinated on 30-ml layers of Bacto-Agar gel (10 g/liter H_2O) in covered 9-cm sterilized Petri dishes. Germination conditions were 25 $\pm$ 1°C with a continuous irradiance of 25 $\mu E/m^2/sec$ or darkness. Different amounts of neutral substances **8**, **9**, **10**, and **11**, whose concentrations ranged from 5×10^{-5} to 2×10^{-4} were dissolved in acetone and adsorbed by Whatman filter paper disks (diameter 0.6 cm). Disks were inserted in the middle of Petri dishes after the solvent had evaporated. An acetone control filter disk was included in each treatment series. Concentrations corresponding to those of neutral compounds were tested for acidic compounds **(1, 2, 3, 4, 5, 6, 7, 12, 13,** and **14),** previously neutralized with $NaHCO_3$. Aliquots of these compounds were placed directly in Petri dishes with agar. Seeds were placed in Petri dishes only after uniform distribution of the substances in agar had been obtained by diffusion (three days). The actual time required varied from a few hours for

TABLE 1. INHIBITION OF PHENYLPROPANOIDS AND COUMARIN (2×10^{-4} M) ON GERMINATION AND ROOT GROWTH OF RADISH AFTER FIVE DAYS OF SOWING.

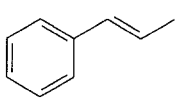
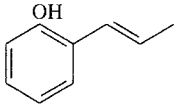
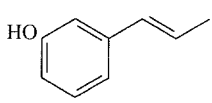
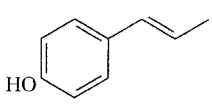
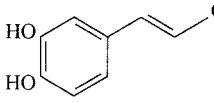
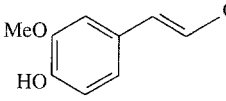
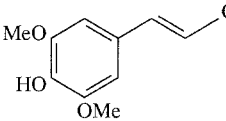
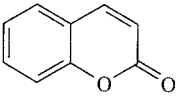
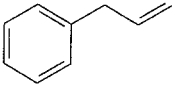
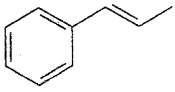
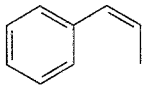
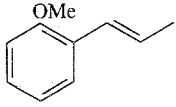
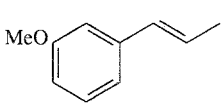
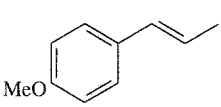
Compound	Germination (%)		Root Length (mm)	
	Light	Dark	Light	Dark
CONTROL	88 ± 3	99 ± 1	76 ± 8	97 ± 7
 cinnamic acid **1**	88 ± 5	95 ± 1	54 ± 6	41 ± 4
 2-hydroxycinnamic acid **2**	40 ± 5	99 ± 1	12 ± 8	42 ± 4
 3-hydroxycinnamic acid **3**	90 ± 4	99 ± 1	75 ± 10	96 ± 9
 4-hydroxycinnamic acid **4**	80 ± 3	99 ± 1	65 ± 9	42 ± 6
 3,4-dihydroxycinnamic acid **5**	91 ± 4	99 ± 1	54 ± 8	62 ± 8
 4-hydroxy-3-methoxycinnamic acid **6**	87 ± 6	99 ± 1	65 ± 10	64 ± 6
 4-hydroxy-3,5-dimethoxycinnamic acid **7**	88 ± 5	99 ± 1	75 ± 8	95 ± 4
 coumarin **8**	50 ± 6	90 ± 5	6 ± 3	8 ± 4
 allylbenzene **9**	85 ± 6	99 ± 1	75 ± 7	92 ± 5

TABLE 1. CONTINUED

Compound	Germination (%)		Root Length (mm)	
	Light	Dark	Light	Dark
 (*E*)-propenylbenzene **10**	89 ± 7	99 ± 1	79 ± 8	94 ± 6
 (*Z*)-propenylbenzene **11**	90 ± 2	99 ± 1	78 ± 7	95 ± 7
 2-methoxycinnamic acid **12**	89 ± 4	99 ± 1	45 ± 6	85 ± 7
 3-methoxycinnamic acid **13**	89 ± 5	99 ± 1	46 ± 5	61 ± 9
 4-methoxycinnamic acid **14**	89 ± 3	99 ± 1	45 ± 5	63 ± 8

[a]Data are expressed as percentages of germination ± SD and root length ± SD.

neutralized phenylpropanoids to three days for **8, 9, 10,** and **11**. The time of this equilibrium distribution was established by assaying the substances over time in the filter paper disk as well as in three cylinders of agar having the same volume (0.132 ml, diameter 0.6 cm) and the distances of the centers from the middle of the filter paper disks of 1.2, 2.4 and 3.6 cm, respectively (Aliotta et al., 1992b). For each substance, concentrations corresponding to those used were observed in agar cylinders after the times indicated above. Germination and root growth were followed in light and in darkness. Seeds were considered germinated when the protrusion of the radicle became evident. Effects on root elongation were determined by measuring, to the nearest millimeter, the length of the radicle of each seedling five days after placing the seeds on the agar medium. Each determination was replicated three times using five Petri dishes containing 20 seeds each. Data are expressed as mean ± standard deviation (SD).

Estimation of Coumarin Formed by 2-Hydroxycinnamic Acid. The agar containing 2-hydroxycinnamic acid was dissolved at 70°C for 3 min and extracted

for 6 hr with ethyl acetate in a Soxhlet apparatus. The extract was dried and chromatographed on preparative TLC [$CHCl_3$–AcOEt (3:1)] to separate 2-hydroxycinnamic acid from coumarin. The coumarin was identified from NMR spectral data. To determine the rate of transformation, a neutralized solution (120 ml) of 2-hydroxycinnamic acid (360 mg) was placed into 12 flasks (10 ml each). Six flasks were kept at 25°C with a continuous irradiance of 25 $\mu E/m^2/$ sec and six flasks were kept in darkness. Every day, one flask from the light and one from the dark treatments were acidified with 2 N HCl and extracted with diethyl ether. The extract was dried and chromatographed on TLC [$CHCl_3$–AcOEt (3:1)]. Coumarin and unchanged 2-hydroxycinnamic acid were identified by comparison with authentic samples.

Light and Electron Microscopy. Control and coumarin-treated root tips were excised 96 and 170 hr after sowing, respectively. The difference in time was due to a delay in germination of seeds treated with coumarin (2×10^{-4} M). In this respect, control and coumarin-treated root tips were observed 48 hr after their protrusion. Root tips were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 2 hr at room temperature. The root tips were then placed for 1 hr into 2% OsO_4 in 0.1 M phosphate buffer (pH 7.3) before being dehydrated in a graded series of ethanol solutions and embedded in Epon 812 resin (Luft, 1961).

Thick sections (ca. 1 μm each) were stained with 0.1% toluidine blue and observed with a Zeiss light photomicroscope.

Thin sections, obtained with a diamond knife on a Supernova microtome, were sequentially stained at room temperature with 2% uranyl acetate (aqueous) for 5 min and by lead citrate for 10 min (Reynolds, 1963). Ultrastructural studies were made using a Philips CM12 transmission electron microscope operated at 80 kV.

Some root tips, after fixation and ethanol dehydration, were also critical-point dried and finally coated with carbon and gold in a sputter-coater. These specimens were observed at 20 kV with a Cambridge 250 Mark3 scanning electron microscope.

The observations were carried out at Centro Interdipartimentale di Ricerca sulle Ultrastrutture Biologiche (Faculty of Sciences, University of Naples).

RESULTS AND DISCUSSION

As can be seen, germination and root growth of radish were slightly inhibited by light; moreover, coumarin and 2-hydroxycinnamic acid were the most potent inhibitors of both germination and subsequent root growth (Table 1). Radish seed inhibition induced by coumarin was higher in light than in darkness, which differs from what has been seen with lettuce (Berrie et al., 1968). Percent

inhibition of germination of radish seeds by coumarin in light and darkness, however, was less than its inhibition of root length. For the phenylpropanoids tested, it appears that those with a carboxylic function in the side chain and only a hydroxyl or methoxyl group in the ring were most active on root growth, except for 3-hydroxycinnamic acid. 4-Hydroxy-3, 5-dimethoxycinnamic acid and the neutral compounds were inactive. No clear pattern of root growth inhibition for light vs dark was observed. In particular, 4-hydroxycinnamic acid is more active in darkness; in contrast 2-methoxycinnamic acid is more active in light.

It must be noted that 2-hydroxycinnamic acid is closely related to coumarin. Indeed, the presence of coumarin in Petri dishes containing neutralized 2-hydroxycinnamic acid was first noted by its aroma of mown hay and then confirmed by its spectral data. This is consistent with the report of Murray et al. (1982). Figure 1 shows that 2-hydroxycinnamic acid is transformed to coumarin in the presence of light. In this respect, the inhibition observed with 2-hydroxycinnamic acid may be due to the formation of coumarin.

It must be emphasized that light, an important ecological and morphogenetic factor (Fenner, 1985), influences both the biological effects of coumarin and its formation from 2-hydroxycinnamic acid.

Root Anatomy. Figure 2 shows a comparison of longitudinal median sections of a control radish root and one chosen among the few seedlings grown in light in presence of coumarin. These roots were excised 48 hr after their germination. As can be seen, the pattern of radish root development in the

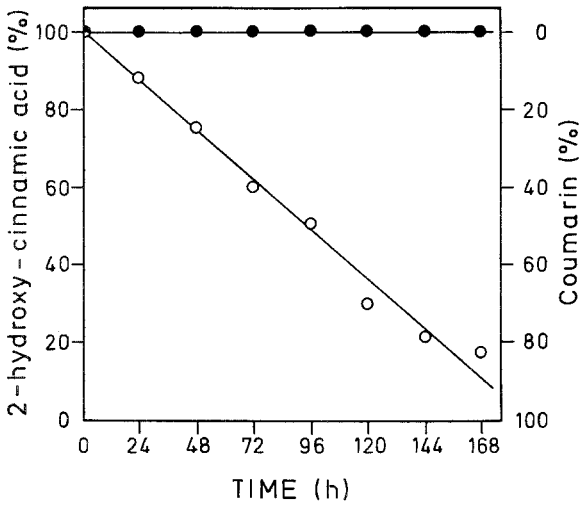


FIG. 1. Time-course of oxidation of 2-hydroxycinnamic acid to coumarin in presence of continuous light (○) and dark (●).

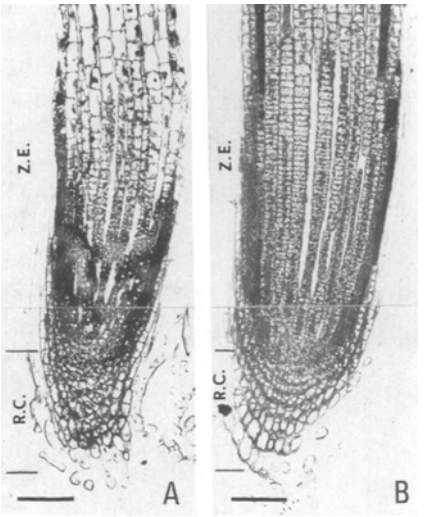


FIG. 2. Anatomy of radish root tips. (A) control; (B) treated with coumarin. Bar = 100 μm. R.C. = root cap; Z.E. = zone of elongation.

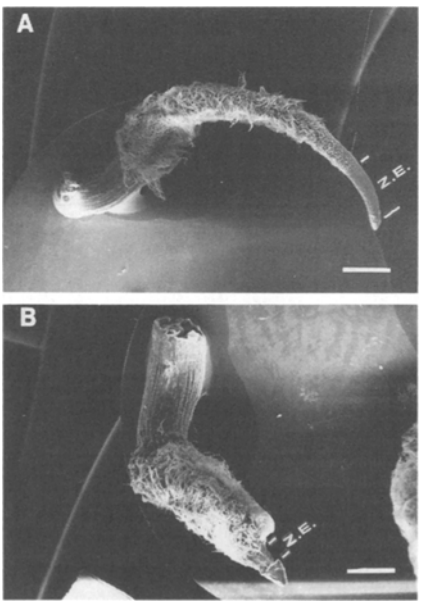


FIG. 3. Scanning electron micrographs of radish root tips. (A) control; (B) treated with coumarin. Bar = 1 mm. Z.E. = zone of elongation.

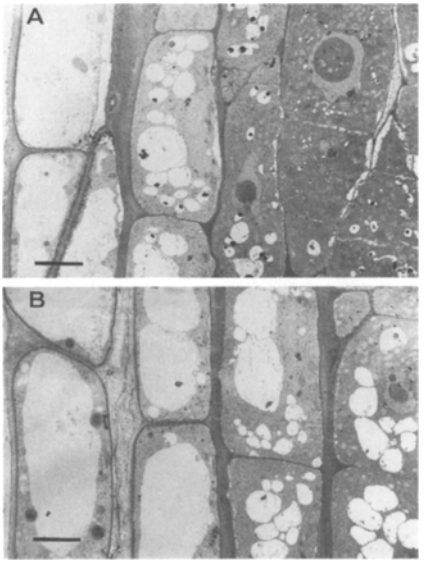


FIG. 4. Transmission electron micrographs of the meristematic zone. (A) control; (B) treated with coumarin. Bar = 0.5 μm.

presence of coumarin (Figure 2B) differs from that of the control (Figure 2A). In fact, the architecture of the control root system is elongated, unlike the coumarin-treated root system, which is more compact in growth. According to Avers and Goodwin (1956) and Jankay and Muller (1976), coumarin appears to inhibit the elongation of cells of the differentiating zone of the root. Scanning electronic micrographs of the same roots confirmed that the elongation zone of the root is shorter for the treated coumarin root (Figure 3B) than for the control (Figure 3A). Moreover, in the coumarin root system, there was an apical shift of root hair differentiation to form a tuft (Figure 3B) not observed in the control (Figure 3A).

A low-magnification view of a transverse cross-cut of the meristematic areas obtained with transmission electron micrographs shows that cells treated with coumarin (Figure 4B) are highly vacuolated compared with the control (Figure 4A). Further studies are in progress to identify the sequence of cellular events causing growth reduction.

REFERENCES

ALIOTTA, G., MOLINARO, A., MONACO, P., PINTO, G., POLLIO, A., and PREVITERA, L. 1992a. Three biologically active phenylpropanoid glucosides from *Myriophyllum verticillatum*. *Phytochemistry* 31(1):109–111.

ALIOTTA, G., FUGGI, A., and STRUMIA, S. 1992b. Coat-imposed dormancy by coumarin in radish seeds: the influence of light. *Giorn. Bot. Ital.* In press.

AVERS, C.J., and GOODWIN, R.H. 1956. Studies on roots. IV. Effects of coumarin and scopoletin on the standard root growth pattern of *Phleum pratense*. *Am. J. Bot.* 43:612–619.

BERRIE, A.M.M., PARKER, W., KNIGHTS, B.A., and HENDRIE, M.R. 1968. Studies on lettuce seed germination. *Phytochemistry* 7:567–573.

BEWLEY, J.D., and BLACK, M. 1985. Seeds, Physiology of Development and Germination. Plenum Press, New York.

EINHELLIG, F.A. 1986. Mechanisms and modes of action of allelochemicals. In A. Putnam and C.S. Tang (eds.). The Science of Allelopathy. 171–188 Wiley-Interscience, New York.

FENNER, M. 1985. Seed Ecology. Chapman and Hall, London.

JANKAY, P., and MULLER, W.H. 1976. The relationships among umbelliferone growth and peroxidase levels in cucumber roots. *Am. J. Bot.* 63:126–132.

LUFT, J.H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* 9:409–414.

MURRAY, R.D.M., MENDEZ, J., and BROWN, S.A. 1982. The Natural Coumarins: Occurrence, Chemistry and Biochemistry. Wiley, Chichester, U.K.

PUTNAM, A., and TANG, C.S. (eds.). 1986. The Science of Allelopathy. Wiley-Interscience, New York.

REYNOLDS, E.S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17:208–213.

RICE, E.L. 1984. Allelopathy. Academic Press, Orlando, Florida.

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MONOTERPENE CONCENTRATIONS IN FRESH, SENESCENT, AND DECAYING FOLIAGE OF SINGLE-LEAF PINYON (*Pinus monophylla* TORR. & FREM.: PINACEAE) FROM THE WESTERN GREAT BASIN

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Abstract—Senescent foliage from pines is potentially a large contributor to the total monoterpene content of the litter layer, and the availability of these compounds as phytotoxins may result from release of these compounds into the vapor phase. In order to determine the fate of several monoterpene hydrocarbons in the natural environment, we examined their concentrations in fresh, senescent, and decaying needles from 32 single-leaf pinyon pine (*Pinus monophylla* Torr. & Frem.: Pinaceae) trees growing at two different locations. Total monoterpene content was highest in the fresh needles (mean = 5.6 ± 2.2 mg/g extracted air dry weight), but also remained relatively high in senescent needles (mean = 3.6 ± 1.8 mg/g extracted air dry weight), either still attached to the tree or forming the freshest layer of understory litter. Decaying needles within a dark decomposing layer of litter material 5–20 cm from the surface were found to contain much lower amounts of total monoterpenes (average: = 0.12 ± 0.06 mg/g extracted air dry weight). Further investigation of the fate of these compounds in the pinyon understory is required to determine if these hydrocarbons are indeed exerting phytotoxic characteristics.

Key Words—*Pinus monophylla*, single-leaf pinyon pine, allelopathy, monoterpene, senescent, tree litter, decaying, vapor phase, volatile hydrocarbons, phytotoxin.

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INTRODUCTION

The environmental fate and transport of monoterpene hydrocarbons characteristic of the Coniferales has been little studied. These compounds have previously been implicated as being capable of causing allelopathy (Muller 1968; Rice 1984; Elmore 1985; Groves and Anderson, 1981; Putnam 1985). More recently, their potential role as allelopathic chemicals in the single-leaf pinyon understory has been discussed (Everett 1987; Jameson 1966). The current study was initiated to describe the changes in monoterpene hydrocarbon concentrations during the processes of senescence, needle abscission, and decay within the single-leaf pinyon forest.

In a previous investigation, Wilt et al. (1988) found that monoterpene hydrocarbon levels are at least 50 times higher in pinyon litter than in the upper 2 cm of mineral soil beneath the pinyon litter was not allelopathic to selected native understory grasses, whereas the litter itself exhibited inhibitory effects to these same grass species.

Litter under the single-leaf pinyon forest canopy is heterogenous in composition and may contain foliage, large and small branches, cones, seeds, bark, and decaying boles. Foliage comprises a major proportion of the pinyon litter layer. Although single-leaf pinyon needles may live longer, senescence usually starts during the sixth or seventh year. Foliage abscision exceeds decomposition rates in pinyon woodlands, thus an accumulation of needle litter under the tree crown develops. This litter layer may also become quite thick and covered with large amounts of gum oleoresin exudates.

By carefully documenting the presence and amounts of monoterpene hydrocarbons in the pinyon understory, we hope to develop a better understanding of how these compounds accumulate to toxic levels and exert their effects on "target" plant species. In order to help understand the process of allelopathy in single-leaf pinyon forests, our goal was to quantify the changes in monoterpene hydrocarbons from living foliage to its decline in the decaying foliage of the litter. To accomplish this, we analyzed monoterpene hydrocarbon levels in fresh, senescent, and decaying pinyon foliage.

METHODS AND MATERIALS

Samples of fresh, senescent, and dead decaying needles were collected in June 1989 from a total of 32 individual single-leaf pinyon trees growing at two sites 113 km apart along the extreme western edge of the Great Basin. Sixteen trees were sampled from each site. Population 1 is located on an eastern exposure in the Virginia Mountains, T17N, R21E, sect. 6, se 1/4 at an elevation of 1990 m and on a Duco soil series (Lithic Argixeroll; Soil Survey Staff, 1975). Pop-

ulation 2 is located on an eastern exposure at 2219 m in the Wellington Hills on a Roloc soil series (Aridic Argixeroll) T9N, R23E, sect. 34, se 1/4.

Fresh pinyon needles were obtained by hand stripping the foliage three to four whorls (years) back from the terminal shoot to about five to six whorls. Foliage near terminal shoots was avoided since monoterpene production is generally lower in young tissues (Fahn, 1970). The freshly harvested needles were placed in 25-ml glass screw-cap sample vials, which were sealed and immediately placed in a chest with ice.

Collections of the yellowed or tan-colored senescent needles consisted of both those still attached to a branch and of recently fallen needles forming the topmost layer of litter. The senescent needles that had recently abscised were collected off the top of the litter layer from the east side of each tree. Alternatively, senescent foliage still attached to branches was collected, after vigorously shaking the limbs, off a plastic tarp placed on the ground under the east side of each tree. Both collections were kept separate in sealed 25-ml glass vials and also stored on ice.

The decaying needles were collected by gently removing the top 10-cm layer of fresher litter to expose the decaying foliage mat 10–25 cm below. Samples of this dark "cakelike" litter were put in paper bags and placed on ice for transport to the laboratory, where they were placed in a −70°C freezer.

Needles were prepared for analysis by placing in a precooled mortar and pestle and pulverizing under liquid nitrogen. A 5.00 ± 0.01 g (wet weight) aliquot of the powdered fresh foliage from each tree was placed into 25-ml sample vials and allowed to equilibrate in 20 ml of pentane for 48 hr at room temperature. Senescent and decaying needles samples were prepared in the same manner; however, 2.50 g of the powdered recently fallen needles were combined with 2.50 ± 0.01 g of the senescing foliage shaken off a tree to yield the senescent needle extracts. Decaying needle extracts consisted of 10.00 ± 0.01 g of the pulverized foliage litter.

After extraction, each sample was filtered and rinsed with 50 ml of pentane. The filtered from each sample by cooling the concentrate in an ice bath, causing them to precipitate, and the remaining solution was transferred to a calibrated 15-ml glass centrifuge tube. Fresh and senescent foliage extracts were further concentrated to 8 ml under nitrogen; decaying needle samples were concentrated to 0.5 ml. In order to express the concentrations of monoterpenes on an extracted air dry weight basis, the remains of the pulverized foliage from each sample were allowed to air dry to constant weight and then reweighed.

Each sample was analyzed by injection of 2-μl aliquots onto a Varian Acrograph 1700 series gas chromatograph. Monoterpene hydrocarbons were separated using a 0.75-mm-iD × 30-m Supelco methylsilicone-coated wide-bore glass capillary column with oven temperature programmed from 55° to 155°C at 10°C/min. Individual peaks were integrated using a Hewlett-Packard

3393 reporting integrator. Monoterpenes were identified by GC-MS. Several of the foliage extracts from each population were further analyzed on the GC so that compounds that coeluted on the methylsilicone-coated wide-bore column were resolved and identified on an Alltech 30-m × 0.32-mm carbowax 20 M coated capillary column. Although these coeluting compounds were not individually quantified, their ratios to each other were calculated in order to determine relative concentrations.

Monoterpene hydrocarbons that were quantified include α-pinene, camphene, β-pinene, myrcene, α-phellandrene, 3-carene, limonene, β-phellandrene, and γ-terpinene. Monoterpene quantitation was performed using external standards, and results were expressed on an extracted air dry weight basis. Because limonene coeluted with β-phelandrene, and myrcene was only partially resolved from β-pinene, these two pairs of compounds were quantified as single peaks. However, at the lower concentrations found in the decaying needles, myrcene was sufficiently resolved from β-pinene to allow quantitation of each compound separately. Total monoterpene content was estimated by summing concentrations only for the compounds listed above. Other monoterpene hydrocarbons present in the extracts were not quantified due to their relatively low concentrations. The average percent composition of the monoterpenes was also determined for both sites.

A discriminant analysis was performed on the monoterpene contents in fresh foliage from both sites in order to help assess their contributions to monoterpene variation present in senescent needles and litter. The resulting frequency histogram provides a visual representation of the variability of monoterpene content within and between the Virginia Highlands and the Wellington Hills population. A Wilks' Lambda (μ-statistic) and univariate F-ratio test was performed within the discriminant analysis in order to test individual monoterpenes for significant differences.

RESULTS

Quantification of Monoterpenes in Foliage. The limit of detection for the monoterpenes on the analytical GC was approximately 10 ng. Compounds identified in the extracts of single-leaf pinyon foliage included tricyclene, thujene, α-pinene, α-fenchene, camphene, β-pinene, myrcene, α-phellandrene, 3-carene, α-terpinene, β-phellandrene, limonene, *cis*-ocimene, *trans*-ocimene, γ-terpinene, and terpinolene. Results for both sites reveal similar trends and show that five of these compounds (α-pinene, β-pinene, myrcene, β-phellandrene, and limonene) comprise the majority of monoterpene hydrocarbons in fresh and senescent foliage, whereas other monoterpenes are found only in relatively small amounts (Table 1). In the fresh and senescent needles of both populations,

TABLE 1. AVERAGE MONOTERPRENE CONCENTRATIONS PRESENT IN NEEDLES OF SINGLE-LEAF PINYON[a]

Compound	Fresh			Senescent			Decaying		
	X	SD	CV	*X*	SD	CV	*X*	SD	CV
Population 1, mg/g extracted adw)									
α-Pinene	2.98	1.67	56	1.91	0.92	48	0.019	0.019	100
Camphene	0.05	0.03	60	0.03	0.02	67	0.004	0.006	150
β-Pinene/myrcene	2.32	1.46	63	1.05	0.72	69	R		
β-Pinene	NR			NR			0.100	0.031	31
Myrcene	NR			NR			0.006	0.006	100
α-Phellandrene	0.62	0.54	87	0.17	0.08	47	ND		
3-Carene	0.03	0.02	67	0.02	0.02	100	0.007	0.004	57
β-Phellandrene/limonene	1.63	0.88	54	0.69	0.33	48	0.004	0.002	50
γ-Terpinene	0.03	0.01	33	0.02	0.01	50	0.003	0.002	67
Total monoterpene	6.16	2.95	48	3.79	1.81	48	0.143	0.050	35
Population 2, mg/g extracted adw)									
α-Pinene	2.01	0.92	46	1.59	0.97	61	0.014	0.018	129
Camphene	0.06	0.04	67	0.04	0.03	75	0.002	0.001	50
β-Pinene/myrcene	1.87	1.19	64	1.13	0.58	51	R		
β-Pinene	NR			NR			0.065	0.034	52
Myrcene	NR			NR			0.003	0.002	67
α-Phellandrene	0.21	0.16	76	0.11	0.07	64	ND		
3-Carene	0.02	0.01	5	0.01	0.004	40	0.006	0.005	83
β-Phellandrene/limonene	1.27	0.65	51	0.66	0.26	39	0.003	0.002	67
γ-Terpinene	0.04	0.02	50	0.02	0.01	50	0.003	0.001	33
Total monoterpene	4.95	1.54	31	3.52	1.71	49	0.096	0.063	66
Population 1, % of monoterpene present									
α-Pinene	41.9	8.9	21	50.7	10.0	20	11.3	8.2	73
Camphene	0.6	0.4	67	0.6	0.3	50	2.6	3.3	127
β-Pinene/myrcene	3.16	11.6	37	19.0	13.5	71	R		
β-Pinene	NR			NR			72.4	12.1	17
Myrcene	NR			NR			4.7	3.4	72
α-Phellandrene	2.1	3.4	162	2.9	4.4	152	ND		
3-Carene	0.5	0.3	60	0.7	0.6	86	3.5	1.6	46
β-Phellandrene/limonene	23.0	6.2	27	18.7	5.0	27	3.2	1.4	44
γ-Teripinene	0.3	0.2	67	0.4	0.2	50	1.8	0.7	39
Total % monoterpene	100			100			100		
Population 2, % of monoterpene present									
α-Pinene	38.2	9.8	26	43.3	7.1	16	10.8	9.8	91
Camphene	1.0	0.7	70	1.0	0.9	90	2.1	0.7	33
β-Pinene/myrcene	33.2	9.6	29	30.8	8.9	29	R		
β-Pinene	NR			NR			70.2	14.0	20
Myrcene	NR			NR			4.7	3.7	79
α-Phellandrene	4.1	4.2	102	3.3	3.3	100	ND		
3-Carene	0.3	0.2	67	0.3	0.2	67	3.5	3.0	86
β-Phellandrene/limonene	22.3	10.5	47	20.7	7.6	37	3.2	2.0	63
γ-Terpinene	0.6	0.3	50	0.5	0.2	40	2.9	2.0	69
Total % monoterpene	100			100			100		

[a] *X* = average, SD = standard deviation, CV = coefficient of variation, adw = air dry weight, R = resolved, NR = not resolved, ND = not detected.

α-pinene was always present in the highest amounts. The decaying needles also contained these same monoterpenes, but at substantially lower concentrations. In the few samples that were analyzed on the Carbowax column, we found β-phellandrene present in five times greater amounts than limonene.

Although β-pinene and myrcene were not individually quantified in the fresh and senescent foliage due to inadequate resolution, β-pinene was present at higher concentrations than myrcene in all samples. Subsequent analysis of several samples from both sites on a Carbowax-coated column determined the ratio of β-pinene to myrcene to be about 7 : 1 in both fresh and senescent foliage.

Senescent needles from populations 1 and 2 contained 39% and 29% less, respectively, total monoterpenes than fresh needles. In sharp contrast, the decaying needles from both sites showed a 98% reduction in total monoterpene content from fresh needles. Thus, total monoterpene content in the decaying foliage at both sites was an average of 47 times less than in the fresh needles and 30 times less than in the senescent foliage.

Variability of Monoterpene Content in Fresh Foliage. The stacked frequency histogram for the discriminant analysis between the two populations of fresh pinyon needles is presented in Figure 1. Group 1 represent the Virginia Highlands population and group 2 the Wellington Hills population. Each stacked group of four numbers indicates an individual sample and the y axis shows the frequency of samples occurring at any point along the x axis. Figure 1 reveals that two trees (13%) from group 1 belong to group 2 and four trees (25%) from group 2 fell into group 1. Results for the Wilks' Lambda (μ-statistic) and univariate F-ratio test performed within the discriminant analysis revealed only one compound (3-carene) to show significant variation between the two groups at $p \leq 0.05$.

DISCUSSION

Contribution of Monoterpene Content in Fresh Foliage to Variability of Litter. The frequency histogram shows the intertree variability of monoterpene content in fresh needles to be relatively wide, with each population substantially intergrading with one another. Thus, the results suggest that intertree variation for monoterpene content within the living needles is quite high and at least partially determines the heterogeneity of monoterpene content in the litter at each site. Furthermore, the discriminant analysis results from fresh foliage may help to explain the wide range of monoterpene contents we previously observed (Wilt et al., 1988) in the bulk and foliage litter from both sites.

The significant amounts of β-phellandrene we observed in single-leaf pinyon foliage may be due to chemical variation among populations. However, a portion of the variability of these compounds in pinyon litter may also be

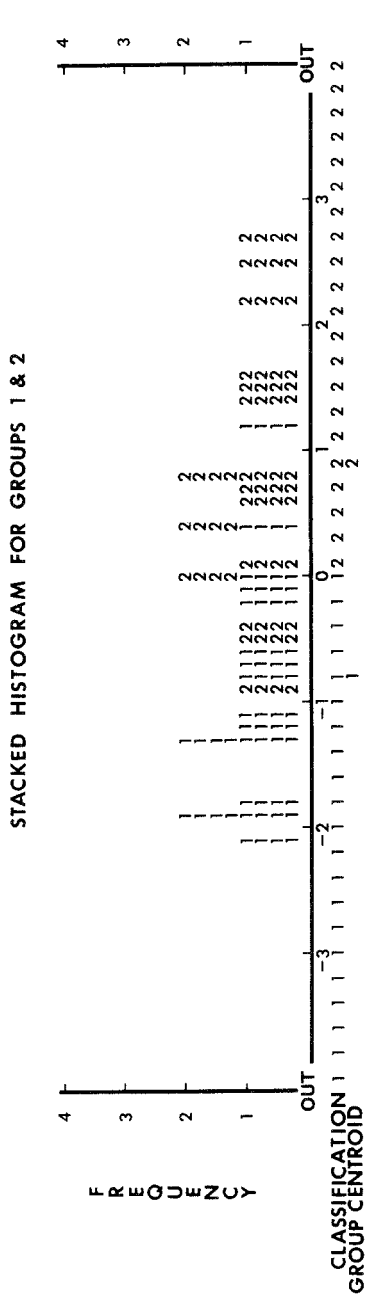

FIG. 1. Stacked frequency histogram for single-leaf pinyon populations 1 and 2 based on the variation in concentrations of eight monoterpenes present in the fresh foliage.

explained by the observations of Mirov (1967) and Zavarin (1968), who found that the quantitative and qualitative composition of monoterpenes in taxa belonging to the genus *Pinus* is highly dependent upon the tissue or organ analyzed. Furthermore, other studies (Zavarin and Snajberk, 1980; Zavarin et al., 1990) suggest that the presence of β-phelladrene in foliage most likely results from chemical variability among the parts of these trees that have been investigated since this compound has not been found in gum and wood turpentines of single-leaf pinyon.

Differences in amounts of total monoterpenes probably contributes to the weak separation of populations 1 and 2 more than the variability of 3-carene alone. For example, population 2 contained 19.6% lower total monoterpenes than population 1. Thus, a gradient from higher total monoterpene concentration (population 1) to lower total monoterpene concentration (population 2) is suggested from left to right along the x axis in Figure 1.

Environmental conditions were different at each site and were certainly another important contributor to the observed amounts of monoterpenes in the fresh foliage as well as the litter. For instance, trees at site 1 are growing in a partially sheltered exposure and tend to have a deep litter layer; trees at site 2 occupy a more open canopied east-facing exposure with a comparatively shallow litter layer. Therefore, production of monoterpenes may be greater at site 1 due to more favorable growing conditions. Furthermore, monoterpenes would be expected to volatilize more rapidly at site 2 considering the more exposed and shallow litter layer. To what extent the variation in monoterpene contents of living tissues within and between these two pinyon stands is environmentally or genetically influenced is not known.

Fate of Monoterpenes in Pinyon Foliage. The data presented in Table 1 show that monoterpene levels declined drastically by the time needles become part of the dark, partially decayed mat immediately above the mineral soil. Thus, although quite variable depending on the degree of weathering and stage of decay, monoterpene hydrocarbons can initially be present at relatively high concentrations in recently accumulated litter. However, these compounds are particularly volatile (vapor pressure of α-pinene = 10 torr at 37.3°C; Weast et al., 1986) and their disappearance over time from pinyon litter is expected, especially during the warm conditions that are frequent in the pinyon understory.

In living pinyon, monoterpenes are produced within epithelial secretory cells that line schizogenously formed resin ducts in needles, bark, roots, reproductive structures, and wood (Esau 1965, 1977; Fahn 1970, 1979; Gifford and Foster 1989). These compounds accumulate within the resin duct lumina of the needles and apparently are released gradually to the atmosphere through diffusion. This process is probably further facilitated as the needles are weathered, decayed by fungi and bacteria, or eaten by insects and other invertebrates over the several-year period that the needles undergo decomposition.

Within the litter layer of the pinyon understory, temperatures well over 38°C, often reached in the spring and summer months, contribute to rapid volatilization of these low-molecular-weight hydrocarbons from the litter source. Under appropriate conditions, this rapid loss of hydrocarbon leads to saturation of the interstitial air within the litter layer with monoterpene vapors. The rates at which individual monoterpenes volatilize from pinyon litter should be correlated to their vapor pressures and also related to their boiling points. Thus, relative vapor pressures of monoterpenes should be a major determinant of their vapor phase concentrations within the litter layer. Furthermore, the concentrations of individual monoterpenes in the source tissues, environmental conditions, and the physical attributes of the woody materials in which these compounds are contained are also important factors determining the rate of hydrocarbon loss from the source tissues.

It is now well established that volatile seed germination and growth inhibitors can emanate from living or decaying plant materials (Whittaker and Feeny, 1971; Putnam, 1983; Bradow and Connick, 1990). In order to document whether or not monoterpene hydrocarbons actually cause allelopathy in the pinyon understory, an in-depth study of their release and movement is necessary. Such a study should include the contributions of abiotic transformations such as vaporization losses and oxidation, as well biotic processes such as fungal and microbial catabolism. However, we believe that under most circumstances the majority of these compounds are passed directly to the vapor phase through volatilization, and their loss rates are primarily related to the physical properties of the monoterpenes themselves and to the permeability of the woody tissues in which they are contained. As monoterpenes accumulate within the vapor phase of the litter layer, it is conceivable that they would then become equilibrated within lipophilic membranes and fats or oils contained in nearby seeds or seedlings.

Determination of monoterpene concentrations in the vapor phase surrounding pinyon litter and the ability for these hydrocarbons to be reequilibrated (replaced) following dilution with the surrounding air would help determine if toxic levels in understory seeds and seedlings could be achieved by such a process. Experiments would also be necessary to reveal if monoterpenes are absorbed by the "target" plants, the rate of accumulation, and their effects described in a dose-dependent manner.

In conclusion, our results indicated that senescent pinyon foliage in the uppermost layer of litter still retains relatively high concentrations of monoterpenes and are potentially available as phytotoxins. By the time the pinyon needles weather and decay to form a dark "cakelike" litter mat just below the surface layer, monoterpene levels are substantially reduced, and thus having a greatly decreased ability to exert allelopathic effects.

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REFERENCES

BRADOW, J.M., and CONNICK, JR., W.J. 1990. Volatile seed germination inhibitors from plant residues. *J. Chem. Ecol.* 16(3):645–666.

ELMORE, C.D. 1985. Assessment of the allelopathic effects of weeds on field crops in the humid Midsouth, pp. 21–32, *in* A.C. Thompson (ed.). The Chemistry of Allelopathy. American Chemical Society Symposium Series No. 268. American Chemical Society, Washington, D.C.

ESAU, K. 1965. Plant Anatomy, 2nd ed. John Wiley & Sons, New York.

ESAU, K. 1977. Anatomy of Seed Plants, 2nd ed. John Wiley & Sons, New York.

EVERETT, R.L. 1987. Allelopathic effects of pinyon and juniper litter on emergence and growth of herbaceous species, pp. 62–67, *in* G.W. Frasier and R.A. Evans (eds.). Proceedings, Symposium on Seed and Seedbed Ecology of Rangeland Plants. USDA-ARS, Tucson, Arizona, 21–23 April 1987.

FAHN, A. 1970. Morphological and anatomical changes related to resin stimulation. The Hebrew University of Jerusalem, Jerusalem, Israel. USDA final report, Department of Botany Project A10-FS-15.

FAHN, A. 1979. Secretory Tissues in Plants. Academic Press, New York.

GIFFORD, E.M., and FOSTER, A.S. 1989. Morphology and Evolution of Vascular Plants, 3rd ed. W.H. Freeman, and Co., New York.

GROVES, C.R., and ANDERSON, J.E. 1981. Allelopathic effects of *Artemisia tridentata* leaves on germination and growth of two grass species. *Am. Midl. Nat.* 106:73–79.

JAMESON, D.A. 1966. Pinyon–juniper litter reduces growth of blue grama. *J. Range Manage.* 19:214–217.

MIROV, N.T. 1967. The Genus *Pinus*. The Ronald Press, New York.

MULLER, C.H. 1968. Allelopathic control of herb growth in the fire cycle of California chaparral. *Bull. Torrey Bot. Club* 95:225–231.

PUTNAM, A.R. 1983. Allelopathic chemicals: Nature's herbicides in action. *Chem. Eng. News* 61(14):35–45.

PUTNAM, A.R. 1985. Allelopathic research in agriculture: past highlights and potential, pp. 1–8, *In* A.C. Thompson (ed.). The Chemistry of Allelopathy. American Chemical Society Symposium Series No. 268. American Chemical Society, Washington, D.C.

RICE, E.L. 1984. Allelopathy, 2nd ed. Academic Press, New York.

SOIL SURVEY STAFF. 1975. Soil Taxonomy: A Basic System of Soil Classification for Making and Interpreting Soil Surveys. USDA-SCS Agric. Handb. 436. U.S. Government Printing Office, Washington, D.C.

WEAST, R.C., ASTLE, M.J., and BEYER, W.H. 1986. Handbook of Chemistry and Physics. Chemical Rubber Co. Press, Boca Raton, Florida.

WHITTAKER, R.H., and FEENY, P.P. 1971. Allelochemics: chemical interactions between species. *Science* 171(3973):757–770.

WILT, F.M., MILLER, G.C., and EVERETT, R.L. 1988. Monoterpene concentrations in litter and soil of single-leaf pinyon woodlands of the western great basin. *Great Basin Nat.* 48(2):228–231.

ZAVARIN, E. 1968. Monoterpenoids of Coniferales. *L.A.W.A. Bull.* 1:3–12.

ZAVARIN, E., and SNAJBERK, K. 1980. Oleoresins of pinyons. *J. Agric. Food Chem.* 28:829–834.

ZAVARIN, E., SNAJBERK, K., and COOL, L. 1990. Chemical differentiation in relation to the morphology of the single-needle pinyons. *Biochem. Syst. Ecol.* 18:125–137.

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A CHEMICAL BASIS FOR DIFFERENTIAL ACCEPTANCE OF *Erysimum cheiranthoides* BY TWO *Pieris* SPECIES

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Abstract—Wormseed mustard, *Erysimum cheiranthoides*, is unacceptable as a host for the cabbage butterfly, *Pieris rapae*. However, it is preferred for oviposition by *Pieris napi oleracea* in the greenhouse. Isolation and identification of the oviposition stimulants to *P. napi oleracea* were accomplished by C_{18} open-column chromatography, TLC, ion-exchange chromatography, HPLC, UV, and NMR spectroscopy. Glucoiberin and glucocheirolin were identified as the most active stimulants. The extracted glucoiberin was as stimulatory as glucocheirolin, although its concentration in the *Erysimum* plants was about 10 times lower than that of glucocheirolin. These glucosinolates were only weak stimulants to *P. rapae*. Furthermore, *P. rapae* was strongly deterred by the cardenolides, erysimoside and erychroside, from *E. cheiranthoides*, and *P. napi oleracea* was less sensitive to these compounds. No other deterrent to *P. napi oleracea* was detected in this plant species. The results explain the differential acceptance of *E. cheiranthoides* by these two *Pieris* species.

Key Words—*Pieris rapae*, *Pieris napi oleracea*, Lepidoptera, Pieridae, *Erysimum cheiranthoides*, oviposition, stimulants, deterrents, glucosinolates, glucoiberin, glucocheirolin, cardenolides.

INTRODUCTION

The cabbage butterflies, *Pieris rapae* L. and *P. napi oleracea* Harris, have overlapping but distinct host ranges (Richards, 1940; Chew, 1977a,b). Previous comparative studies using 11 potential host-plant species under controlled con-

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ditions (Huang and Renwick, 1993) showed that some plant species such as cabbage were acceptable as host plants for *Pieris* species, but that preferences for other plants were quite different. Wormseed mustard, *Erysimum cheiranthoides*, was unacceptable to *P. rapae*, but was strongly preferred by ovipositing *P. napi oleracea*. In a 10-day no-choice oviposition bioassay, *P. rapae* refused to oviposit on *E. cheiranthoides* plants throughout their life, whereas *P. napi oleracea* laid a large number of eggs. *P. napi oleracea* laid significantly more eggs on *E. cheiranthoides* than on cabbage plants in two-choice bioassays (Huang and Renwick, 1993). These observations suggest that the oviposition stimulants to *P. napi oleracea* in *E. cheiranthoides* are much more potent than those in cabbage and that the acceptance or rejection of *E. cheiranthoides* by the two *Pieris* species is probably influenced by different classes of chemicals. However, we still do not know to what extent plant chemistry controls host specificity (Bernays and Graham, 1988; Thompson, 1988). Investigation into the chemical basis of differential selection of host plants by the two *Pieris* species may provide useful information on the mechanism of host specificity of herbivorous insects.

Studies have been made to document the chemical factors regulating the avoidance of *E. cheiranthoides* by *P. rapae*. Renwick and Radke (1985,1987) found that a polar extract of *E. cheiranthoides* contained oviposition stimulant(s) as well as deterrents to *P. rapae*. The deterrents were subsequently identified as the cardenolides, erysimoside and erychroside (Renwick et al., 1989; Sachdev-Gupta et al., 1990), but the stimulants have yet to be identified. Other plant secondary compounds such as coumarin and rutin also deter oviposition by *P. rapae* (Tabashnik, 1987). No study has been conducted to determine how *P. napi oleracea* responds to the cardenolides or if there are other deterrents in the plants. Furthermore, the oviposition stimulants to *P. napi oleracea* from *E. cheiranthoides* are unknown.

The study reported here was designed to determine whether known cardenolides from *E. cheiranthoides* act as oviposition deterrents to *P. napi oleracea*, to identify the oviposition stimulants from the same plant species, to measure the relative sensitivities of *P. rapae* and *P. napi oleracea* to the deterrents and the stimulants, and finally to explain chemically the differential acceptance of the plants by the two *Pieris* species.

METHODS AND MATERIALS

Insects and Plants. P. rapae and *P. napi oleracea* butterflies for behavioral assays were obtained from colonies started from field-collected insects each summer and maintained in the laboratory at ca. 22°C under fluorescent lights providing a photoperiod of 16:8 hr light–dark. Oviposition occurred in the greenhouse, with supplementary lighting, at ca. 25°C. *P. rapae* larvae were

reared on cabbage (*Brassica oleracea* L. var. Golden Acre) and *P. napi oleracea* on *Conringia orientalis* plants. Pupae were separated by sex (Richards, 1940) and kept in screen cylinders until eclosion. *E. cheiranthoides* and cabbage plants (4–6 weeks old) for extraction were grown in an air-conditioned greenhouse at ca. 25°C. Supplemental light was provided by 400-W multivapor high-intensity discharge lamps.

Extraction of Plant Materials. Fresh foliage was extracted in boiling ethanol for 5 min, cooled, homogenized, and filtered. The ethanolic extract was evaporated to dryness under reduced pressure and then defatted with *n*-hexane. The defatted extract was dissolved in water and extracted three times with *n*-butanol. The butanol extract and the postbutanol water extract were concentrated under reduced pressure at ca. 50°C and kept in the refrigerator.

Isolation of Active Compounds. The postbutanol water extract was found to contain strong oviposition stimulant(s) to *P. napi oleracea*. The active material was therefore separated by atmospheric-pressure chromatography using a 45 × 2-cm reversed-phase column packed with 30 g 55 to 105-μm preparative C_{18} (Millipore Corporation, Milford, Massachusetts). After loading the sample (200 gram leaf equivalents), the column was sequentially eluted with 0.5% potassium sulfate (150 ml), water (150 ml), 25% (15 ml), 33% (15 ml), 50% (15 ml), and 100% (45 ml) methanol in water, and 26 fractions (15 ml each) were collected. Different combinations of these fractions were tested for stimulatory activity. Preparative thin-layer chromatography (TLC) of active fractions (fractions 6–8) was carried out on 20 × 20-cm, 0.5-mm-thick, Merck silica gel 60 plates. Twenty gram leaf equivalents of the sample was loaded on each plate in a line, and the plate was developed in a solvent system consisting of ethyl acetate–methanol–acetic acid–water (4 : 1 : 1 : 0.5). The plates were dried with a hair-drier immediately after development. A small portion of the plate was cut off and bands were visualized by spraying with 1% ceric sulfate solution followed by heating at 110°C for ca. 15 min. Corresponding bands on the untreated portion were individually collected and washed with methanol. The eluted material was used for oviposition bioassays or glucosinolate analyses.

Cardenolides (erysimoside, erychroside, and erycordin) were isolated as a combination from the butanol extract by HPLC as previously described (Renwick et al., 1989; Sachdev-Gupta et al., 1990) using a water–acetonitrile gradient. A semipreparative reversed-phase C_{18} column (50 cm × 8 mm) was used and the flow rate was maintained at 3.3 ml/min. The solvent composition increased from the initial pure water to 23% CH_3CN at 10 min, 35% CH_3CN at 40 min and 100% CH_3CN at 45 min. The eluate was monitored at 219 nm.

Bioassays. Oviposition bioassays were conducted in screen cages (48 × 48 × 48 cm) in a greenhouse as described by Renwick and Radke (1988). Eight pairs of newly emerged butterflies were transferred to each cage in the greenhouse. Each cage was supplied with a vial of 10% sucrose solution containing

yellow food coloring and a cotton wick to facilitate feeding. During the preoviposition period, a cabbage plant was placed in each cage. When more than 50 eggs were observed, the plant was removed and the butterflies were used for testing the next day. Treated and control plants were placed in opposite corners of the cage. Positions of plants were alternated in each cage to control for possible position effects. However, the plants were randomly arranged in a circle when three or more test plants were included in each cage, as when the activities of fractions from a column were compared. Bioassays were started at 9:30 a.m. and the eggs laid were counted at 3:30 p.m. When a comparison between the two *Pieris* species was necessary, both species were tested at the same time to minimize the possible effects of differences between plant batches, intensity of sunlight, and other factors on oviposition behavior. Treated plants were sprayed with samples dissolved in a methanol–water combination (70 or 80% methanol depending on solubility of the samples). Control plants were sprayed with cabbage postbutanol water extract (as a standard of stimulant) or solvent alone. The solutions were applied in a fine mist with chromatographic sprayer to both upper and lower leaf surfaces. The concentrations applied were expressed as gram leaf equivalents of the original plant foliage. Deterrent activity was monitored by applying test solution on cabbage plants grown individually in 10-cm cord pots. In separate bioassays, stimulatory effects were tested using neutral (stimulants were not present) bean (*Phaseolis vulgaris* var. Sieva) plants as the oviposition substrate. The bean plants used for bioassays were grown under the same conditions as for cabbage plants and presented as single plants at the two-leaf stage (ca. 20 days old) in plastic pots (6.25 × 6.25 cm).

Design and Analysis. A randomized complete block design was used in the bioassays. A replication consisted of one cage with eight pairs of butterflies, and four to nine replications were performed for each bioassay. When cabbage postbutanol water extracts were used as controls and when comparisons between bioassays were necessary, the relative stimulatory activities were presented as an oviposition stimulant index (OSI), where OSI = 100 (treated − control)/(treated + control).

Deterrent activities were compared by calculating an oviposition deterrent index (ODI), i.e., ODI = 100 (control − treated)/(control + treated).

A paired t test or a Waller-Duncan K-ratio t test was used to assess significance of differences between treatments and controls or among treatments.

HPLC of Desulfoglucosinolates. Desulfated samples were prepared to test for the presence of glucosinolates, according to the method of Minchinton et al. (1982). A column was packed in a 12.7-cm Pasteur pipet with 200 mg DEAE Sephadex A-25 in 0.5 mol pyridine–acetate buffer. The column was conditioned with the buffer (6 ml) followed by water (6 ml). After loading the samples (5–10 gram leaf equivalents in 0.5–1.0 ml water), the column was eluted with water (10 ml or until the eluate was colorless), and 1 ml 0.25% aqueous solution

of sulfatase (Sigma Chemical Co.) was applied. The column was kept at room temperature overnight and then eluted with 2 ml water. The desulfated products were filtered and analyzed by HPLC on a reversed-phase C_{18} column (25 × 0.46 cm) using a gradient program as follows: 0% CH_3CN in water at 0 min, 10% at 35 min, 20% at 60 min, and 100% at 70 min. The flow rate was maintained at 1 ml/min. A diode array detector (Hewlett Packard model 1040A) was used to monitor the eluate at 219 nm.

Identification. UV spectra of isolated compounds were obtained from the diode array detector in water-acetonitrile.

Based on the TLC, HPLC, and UV spectra of the desulfated samples, the two stimulants were found to be aliphatic glucosinolates with similar polarities. To readily obtain a sufficient sample of these stimulants for NMR spectroscopy, desulfation of the postbutanol water extract was performed. The desulfation procedure was similar to that described above. However, larger amounts of DEAE Sephadex A-25 (4 g), plant extract (200 gram leaf equivalents), and sulfatase (20 ml 0.25% aqueous solution) were used. The desulfated glucosinolates were individually collected by HPLC on a semipreparative reversed-phase C_{18} column (50 cm × 8 mm) using a linear water–acetonitrile gradient from pure water to 15% CH_3CN in 30 min at a flow rate of 3.3 ml/min. Solvent was evaporated from collected material under reduced pressure at ca. 55°C, and the desulfoglucosinolates were analyzed by ^{13}C NMR spectroscopy. The samples were dissolved in methanol-d_4–pyridine-d_5 (9:1). The spectra were recorded at ambient temperature on a Varian XL-400 instrument. The chemical shifts were indirectly referenced to tetramethylsilane by using methanol-d_4 as an internal reference.

RESULTS

P. napi oleracea laid significantly more eggs on bean plants treated with the *E. cheiranthoides* postbutanol water extract than on control bean plants treated with solvent (70% MeOH) alone (Figure 1). The number of eggs laid by *P. rapae* on the test plants was not significantly different from that on control plants. This indicates the presence of strong oviposition stimulant(s) for *P. napi oleracea* in the extract and a relative insensitivity of *P. rapae* to the extract components. Therefore, subsequent bioassays of fractions and purified compounds were carried out using only *P. napi oleracea*.

Oviposition bioassays of five combinations of fractions from the atmospheric-pressure column revealed that fractions 6–10 were most stimulatory to *P. napi oleracea* (Figure 2). Fractions 1–5 were also stimulatory, but significantly fewer eggs were laid when compared with fraction 6–10. All the other combinations were not significantly stimulatory to *P. napi oleracea* when com-

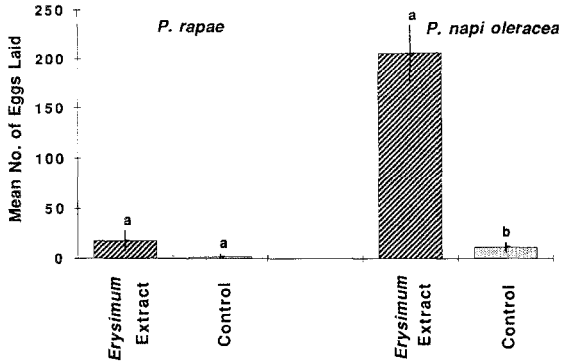


FIG. 1. Oviposition by *P. rapae* and *P. napi oleracea* on a choice of bean plants treated with postbutanol water extract of *E. cheiranthoides* or with 70% MeOH in H_2O (control). Five gram leaf equivalents were used for each replication. Replicated eight times. A replication consists of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters in each series are not significantly different according to a paired *t* test ($P < 0.05$).

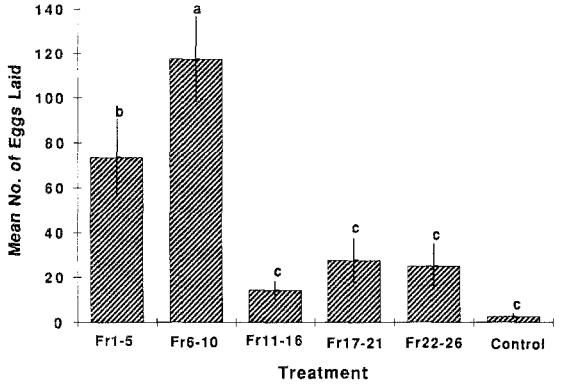


FIG. 2. Oviposition by *P. napi oleracea* on bean plants treated with different combinations of atmospheric-pressure column fractions from the postbutanol water extract of *E. cheiranthoides* or with 70% MeOH in H_2O (control). Five gram leaf equivalents were used for each replication. Replicated nine times. A replication consists of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters are not significantly different according to a Waller-Duncan *K*-ratio *t* test ($K = 100$).

pared with the control (Figure 2). The results demonstrated that the most important stimulants were limited to fractions 1–10. These active fractions were therefore further tested in smaller combinations in subsequent bioassays. As shown in Figure 3, fractions 6–8 were the most stimulatory, and minor activity

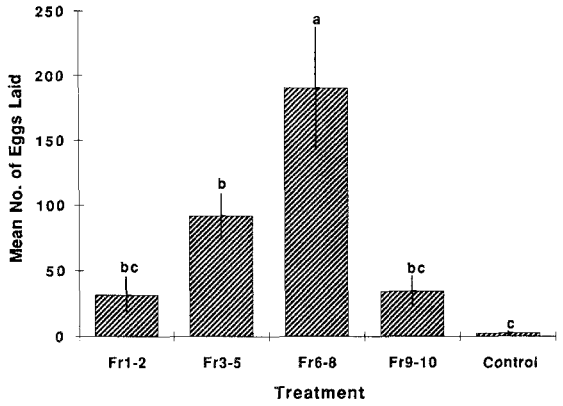


FIG. 3. Oviposition by *P. napi oleracea* on bean plants treated with combinations of selected atmospheric-pressure column fractions (1–10) from the postbutanol water extract of *E. cheiranthoides* or with 70% MeOH in H_2O (control). Five gram leaf equivalents were used for each replication. Replicated seven times. A replication consists of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters are not significantly different according to a Waller-Duncan *K*-ratio *t* test ($K = 100$).

was detected from fractions 3–5. Fractions 1 and 2 or 9 and 10 were not significantly different from the control.

Each of these 10 fractions was subjected to glucosinolate analysis by HPLC of the desulfated products. Two glucosinolates were detected in fractions 5–8 from the postbutanol water extract, and the highest concentration of these glucosinolates was found in fractions 6 and 7 (Figure 4A). When total glucosinolates in *E. cheiranthoides* were analyzed in the same way, these two glucosinolates were the most abundant (Figure 4B). When fractions 6–8 were combined and developed on the preparative TLC plates, three prominent bands were individually collected. These compounds, with hR_fs of 10.0, 23.8 and 41.5, were named **1, 2,** and **3,** respectively. Desulfation foilowed by HPLC analyses of these compounds showed that **1** and **2** were glucosinolates (Figure 5A and B) and the retention times of their desulfated products were identical to those of the two compounds in the desulfated samples of the active fractions (Figure 4A). Compound **3** was not a glucosinolate. In bioassays using cabbage postbutanol water extracts (containing oviposition stimulants) as controls (Figure 6), significantly more eggs were laid by *P. napi oleracea* on bean plants treated with either **1** or **2** than on the control plants (OSI = 22.2 and 13.1, respectively), indicating that both compounds are highly stimulatory to the insects. Compound **3** was not active as a stimulant when compared with cabbage extract (OSI = −64.4).

The UV spectra of both compounds **1** and **2** were characteristic of aliphatic

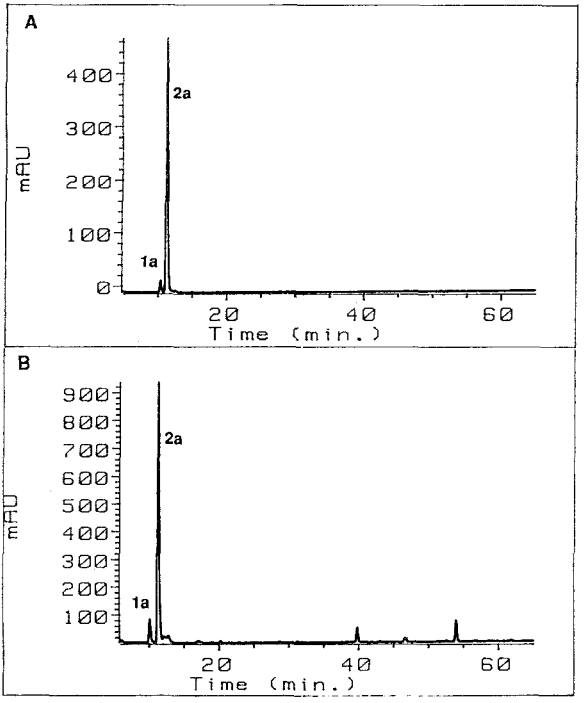


FIG. 4. HPLC separation of desulfoglucosinolates from: (A) the active open-column fractions (only fraction 7 is shown); (B) the postbutanol water extract of *E. cheiranthoides*. UV monitoring at 219 nm.

glucosinolates, with the maximum absorption at ca. 230 nm. The retention time on HPLC, and the hR_f on TLC of desulfated or nondesulfated compound **1** were identical to those of standard glucoiberin (Carl Roth, D75 Karlsruhe). Identification of **1** was further confirmed by ^{13}C NMR data of its desulfated product **1a** (Figure 7, Table 1).

Compound **2** was identified as glucocheirolin solely on the basis of ^{13}C NMR data of the desulfated product **2a** (Figure 7, Table 1). Products **1a** and **2a** are structurally related. The sulfinyl group in **1a** is replaced by a sulfonyl group in **2a**. In **2a**, the methyl carbon (C-4), which is attached to a sulfonyl group, resonates 2.5 ppm downfield compared to that of **1a**. A similar difference is observed in the ^{13}C NMR data of **1** and **2** reported by Cox et al. (1984).

Oviposition by both *P. napi oleracea* and *P. rapae* was stimulated by the commercial glucoiberin at a dose of 1 mg/plant, but the relative response of the two species differed dramatically when offered a choice of glucosinolate or cabbage extracts (Figure 8, OSI = 35.0 for *napi* and −57.7 for *rapae*).

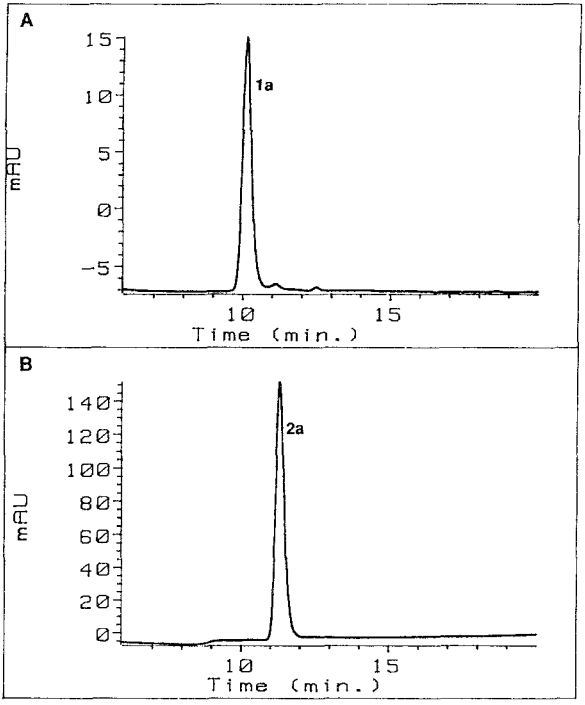


FIG. 5. HPLC of the desulfated products **1a** (A) and **2a** (B), respectively, from compounds **1** and **2** isolated by TLC of the active open-column fractions (Figure 4). UV monitoring at 219 nm.

To test the relative importance of these two glucosinolates in stimulating oviposition by either *Pieris* species, a bioassay was performed using the natural glucoiberin and glucocheirolin collected from *E. cheiranthoides* by TLC (Figure 9). The two compounds were equally stimulatory to *P. napi oleracea*, although the concentration of glucoiberin was about 10 times lower than that of glucocheirolin (as determined by HPLC, Figure 4). At the test concentration used, isolated glucoiberin did not stimulate oviposition by *P. rapae*. *P. rapae* laid more eggs on plants treated with glucocheirolin than on plants treated with solvent alone. However, the stimulatory effect of glucocheirolin on *P. rapae* was quite weak, based on the relatively low numbers of eggs laid in each cage. This observation was confirmed when glucocheirolin from *E. cheiranthoides* was compared with an equivalent amount of cabbage postbutanol water extract. The average number of eggs laid in each replication by *P. rapae* on the glucocheirolin-treated plant was 45, while that on the cabbage-treated plant was 217 (OSI $= -64.5$, paired t test $P < 0.038$). Furthermore, cabbage plants

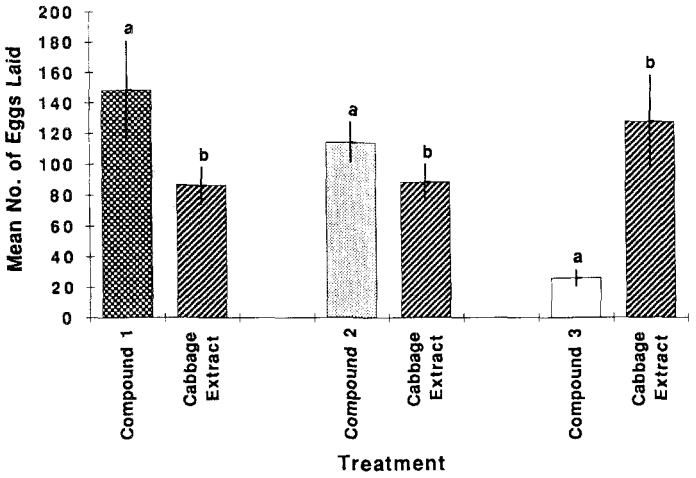
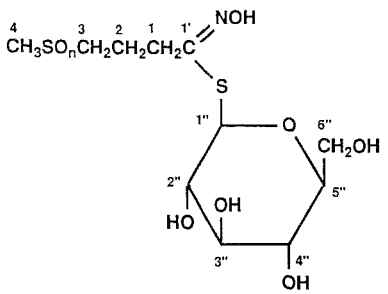


FIG. 6. Oviposition by *P. napi oleracea* on a choice of bean plants treated with compound **1**, **2**, or **3** isolated by TLC of the active atmospheric-pressure column fractions from the postbutanol water extract of *E. cheiranthoides*, or with postbutanol water extract of cabbage (control). Three gram leaf equivalents from each compound or extract were used for each replication. Replicated five times. A replication consists of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters in each series are not significantly different according to a paired *t* test ($P < 0.05$).



1a. n=1

2a. n=2

FIG. 7. Structures of desulfated products **1a** and **2a** from compounds **1** and **2**, respectively.

TABLE 1. ^{13}C NMR DATA OF DESULFATED PRODUCTS **1a** AND **2a** FROM COMPOUNDS **1** AND **2**, RESPECTIVELY

Carbon	Chemical shift (ppm)	
	1a	**2a**
1	31.94	31.61
2	21.57	21.38
3	54.03	54.53
4	38.21	40.71
1′	152.86	152.09
1″	83.45	83.42
2″	74.63	74.60
3″	79.91	79.93
4″	71.51	71.45
5″	82.53	82.50
6″	62.90	62.84

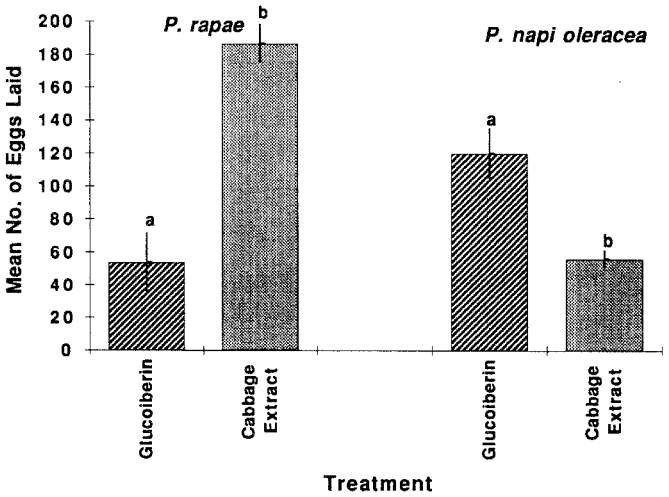


FIG. 8. Oviposition by *P. rapae* and *P. napi oleracea* on a choice of bean plants treated with commercial glucoiberin (1 mg/plant) or with postbutanol water extract of cabbage (control, 4 gram leaf equivalents/plant). Replicated four times. A replication consists of one bioassay cage with eight pairs of butterflies. Means ($\pm$SE) with the same letters in each series are not significantly different according to a paired t test ($P < 0.05$).

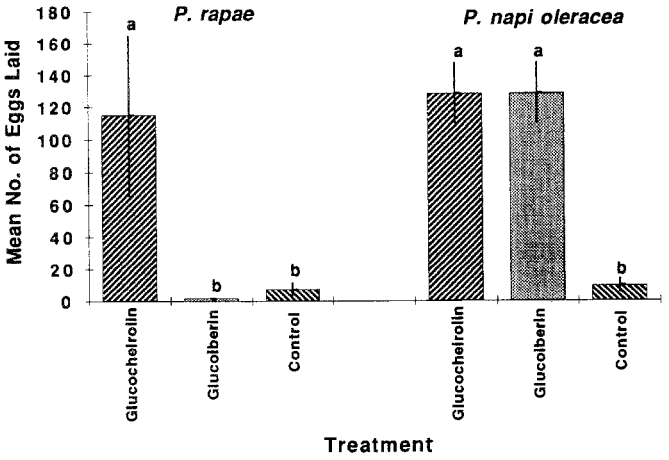


FIG. 9. Oviposition by *P. rapae* and *P. napi oleracea* in response to a choice of glucoiberin or glucocheirolin, collected by TLC of the active atmospheric-pressure column fractions from the postbutanol water extract of *E. cheiranthoides*. Three gram leaf equivalents from each compound were used for each replication. Control = 70% MeOH in H_2O. Replicated four times. A replication consists of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters in each series are not significantly different according to a Waller-Duncan *K*-ratio *t* test ($K = 100$).

treated with the postbutanol water extract of *E. cheiranthoides* were much less preferred by *P. rapae* than those sprayed with solvent alone. An ODI of 45.8 (paired *t* test $P < 0.0118$) indicated the presence of deterrent(s) in the extract. HPLC confirmed that the extract contained a small amount of the known oviposition deterrents (cardenolides). This finding explains the fact that the postbutanol water extract was not stimulatory to *P. rapae*, although the equivalent amount of glucocheirolin isolated from it was active. The results indicate that glucoiberin and glucocheirolin can account for stimulation of oviposition by *P. napi oleracea* on *E. cheiranthoides*. Furthermore, glucocheirolin in this plant can stimulate oviposition by *P. rapae*, but the effect of the glucocheirolin is readily outweighed by the deterrents.

A previous study (Huang and Renwick, 1993) showed that the butanol extract of *E. cheiranthoides* was strongly deterrent to *P. rapae*, with an ODI of 91.5 (paired *t* test $P < 0.0009$), but it had little effect on *P. napi oleracea* (ODI = 17.2, paired *t* test $P < 0.26$). In this study, the combination of cardenolides (erysimoside, erychroside, and erycordin) isolated from the butanol extract by HPLC was highly deterrent to *P. rapae* (Figure 10, ODI = 75.4). However, *P. napi oleracea* was much less sensitive to these deterrents, although significantly fewer eggs were laid on test plants than on control plants (Figure

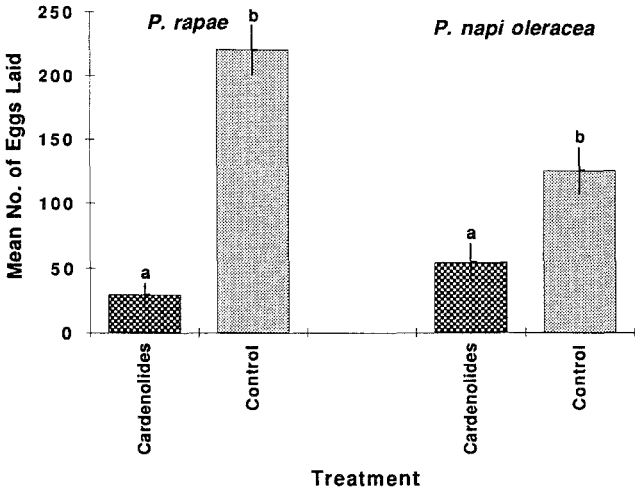


FIG. 10. Oviposition by *P. rapae* and *P. napi oleracea* on a choice of cabbage plants treated with cardenolides (erysimoside, erychroside, and erycordin) isolated by HPLC from the butanol extract of *E. cheiranthoides*, or with 80% MeOH in H_2O (control). Five gram leaf equivalents were used for each replication. Replicated eight times. A replication consists of one bioassay cage with eight pairs of butterflies. Means ($\pm$SE) with the same letters in each series are not significantly different according to a paired *t* test ($P < 0.05$).

10, ODI = 43.4). No additional deterrents to *P. napi oleracea* were found in the butanol extract. Instead, the remaining portion (the cardenolides had been removed) of the butanol extract was stimulatory to *P. napi oleracea*, and HPLC of the desulfated sample of this portion showed the presence of some glucocheirolin and glucoiberin, which had been partially extracted from the water into butanol. This explains why the deterrence of the whole butanol extract was not as strong as that of the cardenolides isolated from the extract.

DISCUSSION

Previous studies suggest that landing by ovipositing females of *P. rapae* is guided mainly by visual cues and that acceptance or avoidance is then controlled by contact chemoreception of semiochemicals on the plant surface (Traynier, 1979; Renwick and Radke, 1988). In this study we have confirmed and clarified the key role of plant chemistry in host-plant selection by both *P. rapae* and *P. napi oleracea*. Two different classes of plant secondary substances, glucosinolates and cardenolides, determine the acceptance or rejection of *E. cheiranthoides* by these two *Pieris* species. The isolation and identification

of glucoiberin and glucocheirolin as the most active stimulatory compounds in *E. cheiranthoides* clearly indicates that these glucosinolates can account for recognition of this plant as a suitable host for *P. napi oleracea*. Similarly, the cardenolides in *E. cheiranthoides* act as deterrents, which prevent *P. rapae* from ovipositing on this plant species.

The experimental procedures employed in this study have provided us with a way to explain the differential acceptance of *E. cheiranthoides* by the two *Pieris* species. The separation of stimulants from deterrents is based on their relative solubilities in water and butanol. However, this separation is obviously not absolute, and traces of opposing stimuli may have a distinct effect on the insect behavior. The small amounts of glucoiberin and glucocheirolin present in the butanol fraction from *E. cheiranthoides* were sufficient to weaken the deterrent effect of the cardenolides on *P. napi oleracea*. In the case of *P. rapae*, the slight stimulatory effect of glucocheirolin was outweighed by the deterrent effect of traces of cardenolides in the postbutanol water extract. These observations point to the possibility of a delicate balance of positive and negative stimuli affecting the insect behavior.

P. rapae and *P. napi oleracea* are specialists whose host ranges are restricted to members of the Cruciferae and a few related plant families that contain glucosinolates (Verschaffelt, 1911). Glucosinolates have been shown to be important larval feeding stimulants (Hovanitz and Chang, 1963) or oviposition stimulants for *Pieris brassicae* (van Loon et al., 1992), *P. napi mocdunnoughii* (Rodman and Chew, 1980), and several other crucifer specialists (reviewed by Schoonhoven, 1972). However, the idea that glucosinolates are responsible for host recognition by all crucifer-feeding insects has been challenged (Nielsen, 1978; Chew, 1988). In this study, we find that the presence of glucosinolates does not always result in the acceptance of a crucifer by a *Pieris* species. Stimulatory effects of glucosinolates on *Pieris* butterflies may differ among insect species or among chemical classes of the glucosinolates on a particular insect species. For example, *P. rapae* refuses to oviposit on *E. cheiranthoides* despite the presence of large amounts of glucocheirolin and some other glucosinolates including glucoiberin. The aliphatic glucocheirolin from *E. cheiranthoides* was only weakly stimulatory to *P. rapae*, but oviposition of this insect species is strongly stimulated by the indole glucosinolate, glucobrassicin, in cabbage plants (Traynier and Truscott, 1991; Renwick et al., 1993). On the other hand, *P. napi oleracea* prefers the aliphatic glucosinolates in *E. cheiranthoides* over the indole glucosinolate in cabbage (Figures 6 and 8). *P. napi oleracea* laid many more eggs on bean plants treated with commercial glucoiberin (1 mg/plant) than on bean plants treated with cabbage extract (4 gram leaf equivalents/plant). Because cabbage plants contain the indole glucosinolate at an average of 50 mg/100 g (reviewed by van Loon et al., 1992), it is safe to conclude that the amount of glucobrassicin used in this bioassay was equal to or more than that of glu-

coiberin. Thus, glucoiberin is more stimulatory to *P. napi oleracea* than is the indole glucosinolate. These results indicate that differential sensitivities to glucosinolates have evolved in the indigenous *P. napi oleracea* and the naturalized *P. rapae*.

The structural requirements for activity of glucosinolates may be quite complex. The two major glucosinolates in *E. cheiranthoides* are closely related structurally, but *P. napi oleracea* is apparently more sensitive to glucoiberin than to glucocheirolin. The response of this species to the natural concentration of the two glucosinolates was approximately equal, but glucocheirolin is present at about 10 times the level of glucoiberin.

Chemical deterrents may frequently co-occur with stimulants in a potential host plant. The final step of rejection or acceptance of *E. cheiranthoides* by ovipositing *P. rapae* or *P. napi oleracea* is mediated by more than one chemical stimulus in the plant. *E. cheiranthoides* is highly preferred by *P. napi oleracea*, although cardenolides (including erysimoside and erychroside) in the plant are, to a certain extent, deterrent to this insect. In contrast, *P. rapae* avoids *E. cheiranthoides* as a host, even though the glucosinolates in the plant are somewhat stimulatory. These results suggest that the relative importance of the stimulants and the deterrents in host-plant recognition by the two *Pieris* species may differ considerably. In *P. napi oleracea*, the negative effect is outweighed by the positive effects, leading to acceptance of *E. cheiranthoides*. However, in *P. rapae*, the minor stimulatory activity of glucosinolates is masked by the potent deterrent activity of the cardenolides, resulting in rejection of the plant. The significance of such a balance between negative and positive chemical input in host selection by herbivorous insects (Dethier, 1982; Miller and Strickler, 1984; Renwick and Radke, 1987) is clearly demonstrated.

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REFERENCES

BERNAYS, E., and GRAHAM, M. 1988. On the evolution of host specificity in phytophagous arthropods. *Ecology* 69:886–892.

CHEW, F.S. 1977a. Coevolution of pierid butterflies and their cruciferous foodplants. II. The distribution of eggs on potential foodplants. *Evolution* 31:568–579.

CHEW, F.S. 1977b. The effects of introduced mustards (Cruciferae) on some native north American cabbage butterflies (Lepidoptera: Pieridae). *J. Xerces Soc.* 5:13–19.

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!, pp. 81–112, *in* K.C. Spencer (ed.). Chemical Mediation of Coevolution. Plenum Press, New York.

COX, I.J., HANLEY, A.B., BELTON, P.S., and FENWICK, G.R. 1984. N.m.r. spectra (^{1}H, ^{13}C) of glucosinolates. *Carbohydr. Res.* 132:323–329.

DETHIER, V.G. 1982. Mechanisms of host-plant recognition. *Entomol. Exp. Appl.* 31:49–56.

HOVANITZ, W., and CHANG, V.C.S. 1963. Comparison of two mustard oils and their glucosides to *Pieris* larvae. *J. Res. Lepid.* 2:281–288.

HUANG, XINPEI, and RENWICK, J.A.A. 1993. Differential utilization of host plants by two Pieris species: the role of oviposition stimulants and deterrents. *Entomol. Exp. Appl.* In press.

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. Sinauer Associates, Sunderland, Massachusetts.

MINCHINTON, I., SANG, J., BURKE, D., and TRUSCOTT, R.J.W. 1982. Separation of desulfoglucosinolates by reversed-phase high performance liquid chromatography. *J. Chromatogr.* 247:141–148.

NIELSEN, J.K. 1978. Host plant discrimination within the Cruciferae: Feeding responses of four leaf beetles (Coleoptera: Chrysomelidae) to glucosinolates, cucurbitacins and cardenolides. *Entomol. Exp. Appl.* 24:41–54.

RENWICK, J.A.A., and RADKE, C.D. 1985. Constituents of host and nonhost 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., and RADKE, C.D. 1988. Sensory cues in host selection for oviposition by the cabbage butterfly, *Pieris rapae. J. Insect Physiol.* 34:251–257.

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.

RENWICK, J.A.A., RADKE, C.D., SACHDEV-GUPTA, K., and STÄDLER, E. 1992. Leaf surface chemicals stimulating oviposition by *Pieris rapae* (Lepidoptera: Pieridae) on cabbage. *Chemoecology* 3: 33–38.

RICHARDS, O.W. 1940. The biology of the small white butterfly (*Pieris rapae*), with special reference to the factors controlling its abundance. *J. Anim. Ecol.* 9:243–288.

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.

SACHDEV-GUPTA, K., RENWICK, J.A.A., and RADKE, C.D. 1990. Isolation and identification of oviposition deterrents to cabbage butterfly, *Pieris rapae*, from *Erysimum cheiranthoides. J. Chem. Ecol.* 16:1059–1067.

SCHOONHOVEN, L.M. 1972. Secondary plant substances and insects. *Recent Adv. Phytochem.* 5:197–224.

TABASHNIK, B.E. 1987. Plant secondary compounds as oviposition deterrents for cabbage butterfly, *Pieris rapae* (Lepidoptera: Pieridae). *J. Chem. Ecol.* 13:309–316.

THOMPSON, J.N. 1988. Coevolution and alternative hypothesis on insect/plant interactions. *Ecology* 69:893–895.

TRAYNIER, R.M.M. 1979. Long term changes in the oviposition behavior of the cabbage butterfly *Pieris rapae* induced by contact with plants. *Physiol. Entomol.* 4:87–96.

TRAYNIER, R.M.M., and TRUSCOTT, R.J.W. 1991. Potent natural egg-laying stimulant for cabbage butterfly *Pieris rapae. J. Chem. Ecol.* 17:1371–1380.

VAN LOON, J.J.A., BLAAKMEER, A., GRIEPINK, F.C., VAN BEEK, T.A., SCHOONHOVEN, L.M., and DE GROOT, A. 1992. Leaf surface compound from *Brassica oleracea* induces oviposition by *Pieris brassicae*. Chemoecology 3: 39–44.

VERSCHAFFELT, E. 1911. The cause determining the selection of food in some herbivorous insects. *Proc. Acad. Sci. Wet., Amsterdam* 13:536–542.

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QUANTITATIVE AND TEMPORAL ANALYSIS OF EFFECTS OF TWOSPOTTED SPIDER MITE (ACARI: TETRANYCHIDAE) FEMALE SEX PHEROMONE ON MALE GUARDING BEHAVIOR

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Abstract—Responses of male twospotted spider mite, *Tetranychus urticae* (Koch), to female sex pheromone were described by a glass slide bioassay and computerized pathway digitizer. Pheromone was extracted from quiescent deutonymphs and fractionated by HPLC, and responses of guarding males to each fraction, all fractions combined, unfractionated extract, and a hexane control were bioassayed for 6 min. Mean angular velocities, linear velocities, percent time stationary, and distance from the 3-mm-diameter treatment circle were calculated for each mite at 20-sec intervals and these behavioral parameters regressed on time. Analysis of variance of regression-equation intercepts showed that no differences in initial male angular velocities were observed among treatments, but initial linear velocities were greater in response to all fractions combined and to extract than to individual fractions, and greater in response to individual fractions than to the control. Angular velocities decreased and linear velocities increased more rapidly in response to individual fractions than to all fractions combined and extract, while males turned preferentially in one direction and were stationary (no displacement) more often in response to all-fraction combinations than to individual fractions and the control. Significant differences were observed among the parameter values elicited by individual fractions, suggesting that active fractions differed qualitatively. The described changes in movement parameters show that male *T. urticae* response to presence of pheromone occurs almost immediately, and that multiple pheromonal components are necessary to elicit maximum male response. The

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overall effect of pheromonal components was to decrease the rate at which males moved away from the treatment cycle, thereby causing male arrestment.

Key Words—*Tetranychus urticae*, twospotted spider mite, Acari, Tetranychidae, female sex pheromone, quiescent deutonymphs, quantified male behavior, temporal analysis, active components.

INTRODUCTION

The mating behavior of the twospotted spider mite, *Tetranychus urticae* Koch, has been described extensively (Ewing, 1914; Lehr and Smith, 1957; Cone et al., 1971b; Potter et al., 1976; Potter and Wrensch, 1978; Cone, 1985). Female deutonymphs deposit webbing on the leaf surface and become quiescent. Males encountering quiescent deutonymphs can become arrested locally near the female; the probability of arrestment increases as ecdysis approaches (Cone et al., 1971b; Potter et al., 1976). Arrested male behavior has been described as "guarding" (Ewing, 1914; Lehr and Smith, 1957; Potter et al., 1976; Cone, 1979, 1985; Gerson, 1985); arrested males may remain immobile, walk in small circles in the vicinity of the female, touch the idiosoma of the female with their forelegs and palps, cover the female with thin filaments of webbing, and attack other males that encounter the quiescent female. Males assist females in emergence from the exuviae and immediately attempt mating (Laing, 1969; Potter et al., 1976; Cone, 1985).

Cone et al. (1971a) demonstrated that quiescent deutonymphs contained chemicals that arrested males. Subsequent studies suggested that short-range orientation to volatile pheromone enables males to locate quiescent deutonymphs (Penman and Cone, 1972, 1974) and that pheromone also initiates and prolongs arrestment (Cone et al., 1971a; Penman and Cone, 1972, 1974). However, two more recent studies (Royalty et al., 1992, in press) have shown that males perceive pheromone by contact chemoreception, but are unable to perceive pheromone volatiles and therefore do not orient to quiescent deutonymphs via olfaction. In addition, presentation of pheromone to males in the absence of other stimuli seldom initiates arrestment; the principal role of pheromone is to prolong the localized searching behavior initiated by other cues.

Royalty et al. (1992) also bioassayed HPLC-separated quiescent deutonymph extract, isolating four active fractions. Subtractive bioassay showed that the pheromonal components contained in three of the fractions were essential to elicit maximum male response, while components in the fourth fraction were not. Effects of these pheromonal components on male mate-location and guarding behavior were not quantitatively described, however. Changes in movement parameters (angular velocity, linear velocity, turn magnitude, etc.) in response to contact chemoreception of semiochemicals have been quantified for many

walking arthropods (Waage, 1978; Bell, 1984; Visser, 1988, and respective references therein), but specific effects of such chemicals on mite behavior have not been studied. Furthermore, quantification over time of chemically medicated arthropod behavior has not been attempted, although factors such as starvation, experience, and semiochemical concentration have been shown to affect displacement rate and arrestment duration. Our studies were done to quantify the effects of *T. urticae* female sex pheromone on the various component parameters of male guarding behavior and to describe changes in these parameters over time.

METHODS AND MATERIALS

Pheromone Extraction and Fractionation. T. urticae were reared on Henderson lima beans, *Phaseolus lunatus* L., at 25°C, 60% relative humidity, and on a 14:10 hr light–dark photoperiod. Pheromone was extracted by immersing 2500 quiescent deutonymphs in 250 μl of HPLC-grade hexane (Mallinckrodt, Inc., Paris, Kentucky) for 120 hr. All extractions used in our studies were stored under nitrogen in the dark at 0–5°C to preserve potential activity.

A 100-μl sample of extract was fractionated using a Hewlett-Packard (Avondale, Pennsylvania) model 1090 liquid chromatograph with an HP Hypersil silica column (5-μm particle size; 200 mm × 2.1 mm internal diameter). The mobile-phase program (flow rate = 1 ml/min) consisted of 2 ml hexane, and 8-ml linear conversion from hexane to diethyl ether, a 6-ml linear conversion to CH_2Cl_2, and a 4-ml CH_2Cl_2 column flush. Eighteen 1-ml fractions were collected.

Bioassay. Male response to an individual treatment was bioassayed by placing one quiescent deutonymph equivalent (QDE) (1.0 μl) of the treatment on a glass microscope slide using a 1-μl syringe (Hamilton Co., Reno, Nevada). The treatment was placed in a 3-mm-diameter circle, and the slide dried in air for 20 min to allow the solvent to evaporate. A 15-mm × 15-mm lanolin arena was drawn around the treatment circle to prevent mites from escaping.

Previous studies showed that the primary role of *T. urticae* female sex pheromone is to prolong male arrestment; initiation of arrestment seldom occurs (Royalty et al., 1992, in press). Therefore, only males that were actively guarding quiescent deutonymphs were used in our experiments. One randomly selected guarding *T. urticae* male was placed on the edge of the circle using a camel-hair brush. Care was taken to transfer the mite as quickly and gently as possible; the mite was discarded if it stuck to the brush or was damaged during the transfer process.

Seven treatments were tested: the three individual active fractions that were deemed essential in our previous studies (fractions 2, 4, and 9), the nonessential

active fraction (12), the four active fractions combined (A), unfractionated extract (E), and a hexane control (✓). Each treatment was tested by the response of 20 males; all males tested in each replicate were collected from the same plant. Bioassays were done at 23–28°C, 50–60% relative humidity, and 3–8 hr after the beginning of the 14-hr photophase (from 0900 to 1400 hr).

A male's response to the treatment circle was tracked by a pathway-digitizing computer, and the position of the mite on the glass slide recorded at 0.2-sec intervals. The accompanying software translated the position of the mite into Cartesian coordinates and filtered out background noise. The digitizer and software were obtained from W.J. Bell's laboratory (Department of Entomology, University of Kansas, Lawrence, Kansas).

Pathways were described by a pathway-analysis program. Three parameters were used to describe responses of individual males: angular velocity (deg/sec) and linear velocity (mm/sec) of the male while moving, and percent of time either stationary or pivoting in one place (no displacement), hereafter referred to as the stationary index. In addition, the pathways of all 20 males were used to calculate a fourth parameter, the turn bias. Turn bias is the absolute value of the sum of signed turn magnitudes elicited by each treatment; this quantity described males' tendencies to turn more in one direction (left or right) than the other (Bell, 1990; Spivak et al., 1991). The summary effects of these four parameters on duration of male arrestment were described by measuring the mean distance of the male from the center of the 3-mm treatment circle over time.

Bioassay duration was 6 min or until the male touched the edge of the lanolin arena. The pathway for each mite was divided into 20-sec intervals, and parameters for each interval calculated. The interval parameters for linear and angular velocities, stationary index, and mean distance from the center of the circle for each mite were regressed on time, and mean regression-equation coefficients for each treatment compared by analysis of variance with a randomized complete-block design and orthogonal-contrast comparisons (df = 1, 114 for each comparison) (Snedecor and Cochran, 1967). Because turn bias describes a population's response to a particular treatment, regressions of individual male behavior over time were not used to estimate the effects of treatments on this parameter. Instead, pathways for all 20 males were used to calculate turn bias for each 20-sec interval; these values were used to calculate mean turn bias for the entire 6-min observational period. Differences in turn bias among treatments were described by analysis of variance (blocked on interval) with orthogonal-contrast comparisons (df = 1, 102 for each comparison). SYSTAT MGLH (Wilkinson, 1990) was used for all data analyses.

RESULTS

Figure 1 shows the typical response of a male *T. urticae* to a female sex pheromone fraction and to the control over time. In spite of general increases over time in linear velocity, and decreases over time in angular velocity and the

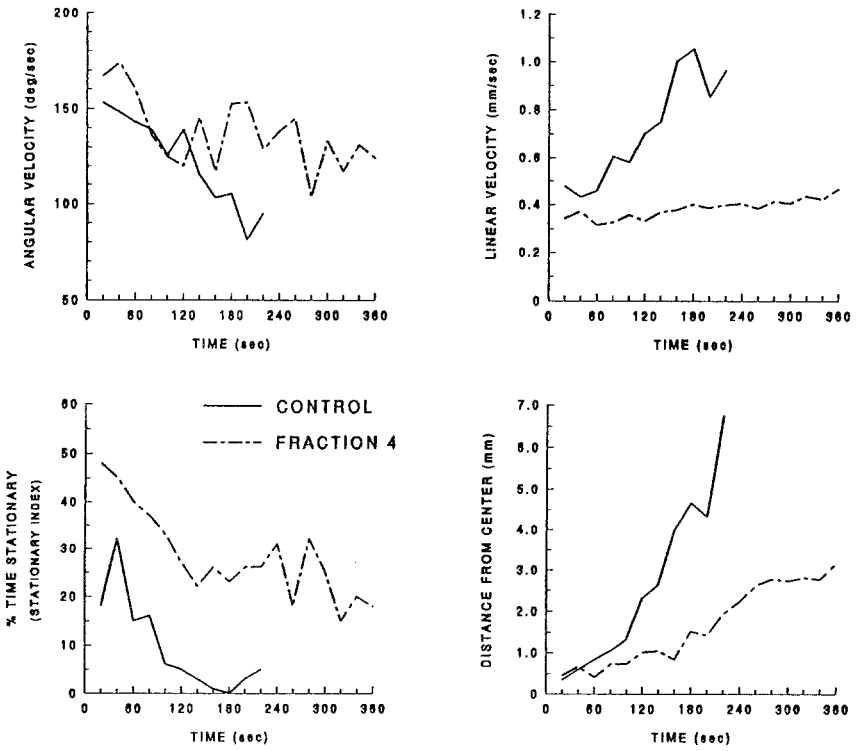
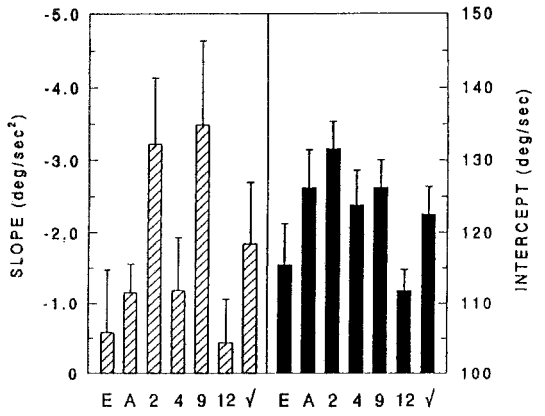


FIG. 1. Typical response of one male *T. urticae* to a female sex pheromone fraction and to the control over time: angular velocity; linear velocity; stationary index; and distance from the treatment circle.

stationary index, behaviors of individual males were highly variable from one 20-sec interval to the next. This erratic male response necessitated the use of first-order polynomial regressions to describe treatment effects over time for these three parameters. Mean intercept from the 20 individual regression equations predicted a treatment's initial effect (time = 0) on the parameter, while the mean slope predicted the change in the parameter over time elicited by the treatment.

The ratio of angular velocity to linear velocity often is used to measure a pheromone's capacity to elicit arrestment; a large ratio usually results in a high turning rate and no linear displacement and therefore is characteristic of arrestment and/or long-term localized searching behavior. While individual active fractions had no significant effect on either initial (time = 0) or subsequent angular velocities of male *T. urticae* with respect to the control (Figure 2), linear velocities were lower at all 20-sec intervals in the presence of pheromonal



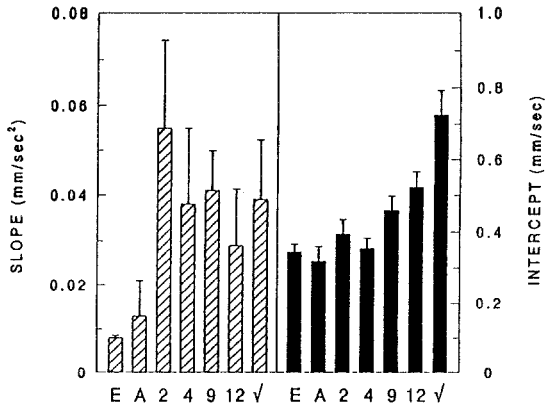
	Coefficient F - Ratios	
Contrast	Slope	Intercept
Individual Fractions vs. Control	0.01 ns	0.03 ns
Fractions + Control vs. All + Extract	4.03 *	0.42 ns
Essentials vs. Non-essential	5.63 *	9.99 ***
2 + 4 vs. 9	1.73 ns	0.07 ns
2 vs. 4	3.27 ns	0.78 ns
Extract vs. All	0.25 ns	3.28 ns

FIG. 2. Regression equation coefficients describing changes in male *T. urticae* angular velocities caused by female sex pheromone fractions over time. Error bars are standard errors of means ($N = 20$; $ANOVA_{slope}$: $F_{fraction} = 2.08$, $P = 0.061$; $ANOVA_{intercept}$:$F_{fraction} = 2.50$, $P = 0.026$; $df = 1, 114$ for all comparisons).

components (Figure 3). These results suggest that the principal effect of *T. urticae* female sex pheromone is to reduce linear velocity of males.

Steeper slopes for linear and angular velocity were observed in response to individual fractions than to treatments with all fractions, and linear velocity intercepts also were significantly different. These data show that longer arrestment durations were observed in response to treatments containing all fractions combined than to individual fractions, and therefore confirm our previous conclusion (Royalty et al., 1992a) that multiple pheromonal components are necessary to elicit maximum male *T. urticae* response.

Although few significant differences were observed among the angular velocities elicited by various treatments, the tendency for males to turn preferentially in one direction was significantly greater in response to all active fractions combined than to individual fractions and the control ($df = 1, 102$ for all comparisons) (Figure 4). Reduced frequency of change in turn direction can cause a decrease in displacement rate (Bell, 1990); therefore the greater turn



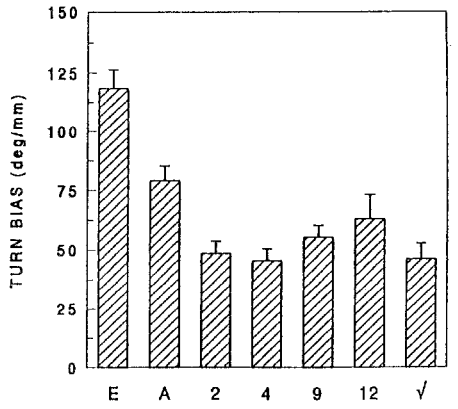
	Coefficient F - Ratios	
Contrast	Slope	Intercept
Individual Fractions vs. Control	0.02 ns	41.06 ****
Fractions + Control vs. All + Extract	8.52 **	21.94 ****
Essentials vs. Non-essential	1.23 ns	6.39 *
2 + 4 vs. 9	0.13 ns	2.82 ns
2 vs. 4	0.97 ns	0.45 ns
Extract vs. All	0.08 ns	0.21 ns

FIG. 3. Regression equation coefficients describing changes in male *T. urticae* linear velocities caused by female sex pheromone fractions over time. Error bars are standard errors of means ($N = 20$; $ANOVA_{slope}$: $F_{fraction} = 2.12$, $P = 0.057$; $ANOVA_{intercept}$:$F_{fraction} = 9.88$, $P < 0.001$; $df = 1, 114$ for all comparisons).

bias elicited by treatments containing all active fractions may have prolonged male arrestment duration.

Arrestment can be described further by comparing time spent moving (displacement across the glass slide) with time spent in one place (male *T. urticae* rarely remained immobile; when males were not walking across the glass slide, they usually pivoted in a stationary position). Regressing stationary index on time shows that males spent more time pivoting in response to treatments that contained all active fractions than to individual fractions and the control throughout the 6-min observation period (Figure 5). The stationary indices elicited by individual fractions and the control did not differ; these data are further evidence that multiple fractions are essential to elicit maximum male response.

Significant differences in slopes and intercepts of angular-velocity and intercepts of linear-velocity regression lines were observed between the essential and nonessential fractions. Differences in these parameters and in turn bias were not observed among essential fractions 2, 4, and 9, but the stationary index regression line for fraction 9 has a significantly lower intercept than did regression

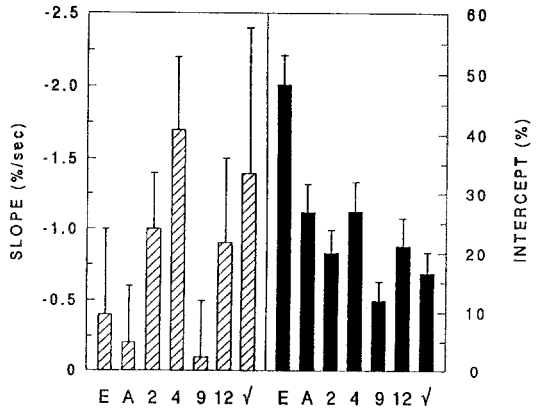


Contrast	F - Ratio
Individual Fractions vs. Control	0.79 ns
Fractions + Control vs. All + Extract	66.87 ****
Essentials vs. Non-essential	2.78 ns
2 + 4 vs. 9	0.97 ns
2 vs. 4	0.01 ns
Extract vs. All	16.10 ***

FIG. 4. Effects of female sex pheromone on male *T. urticae* turn bias over a 6-min interval. Error bars are standard errors of means ($N = 20$; ANOVA: $F_{\text{fraction}} = 14.60$, $P < 0.001$; $df = 1, 102$ for all comparisons).

lines for fractions 2 and 4. The varying effects on male *T. urticae* movement elicited by the four individual fractions and the fact that the active fractions were eluted from the column by solvents of differing polarities suggests that each fraction contained qualitatively different pheromonal components.

The differing effects of treatments on angular and linear velocities, stationary index, and turn bias had significant effects on male *T. urticae* arrestment duration. Relationships between effects of treatments on mean distance from the center of the 3-mm-diameter circle ($\hat{Y}$) and time (x) were best described by the equation $\hat{Y} = x/\alpha x + \beta)$, where α estimates the maximum distance a male could move from the circle (defined by the edge of the lanolin arena), and β the rate at which the male approached this asymptote. This equation is similar to the Michaelis-Menten enzyme kinetics equation, but with better parameter estimation properties (Ratkowsky, 1990). No differences were observed among treatments in estimated maximum distances from the circle (Figure 6); these results show that at least some of the males reached the edge of the arena even in the presence of extract or all fractions combined. However, treatment differences were observed in the rates at which males moved away from the treatment circle. These results show that males were arrested longer by active fractions than by



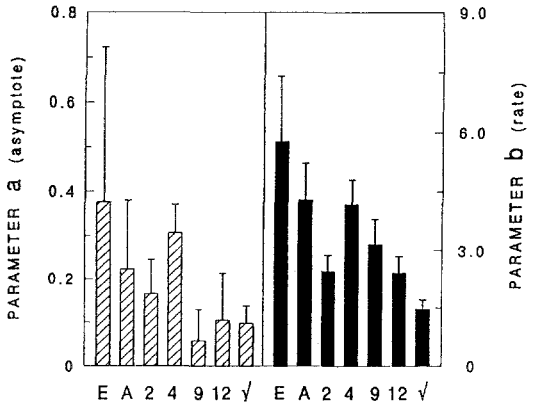
Contrast	Coefficient F - Ratios: Slope	Intercept
Indivdual Fractions vs. Control	0.20 ns	0.51 ns
Fractions + Control vs. All + Extract	1.10 ns	37.93 ****
Essentials vs. Non-essential	0.11 ns	0.55 ns
2 + 4 vs. 9	3.99 *	7.13 **
2 vs. 4	0.58 ns	1.65 ns
Extract vs. All	0.52 ns	7.91 **

FIG. 5. Regression equation coefficients describing changes in male *T. urticae* stationary indices caused by female sex pheromone fractions over time. Error bars are standard errors of means ($N = 20$; $\text{ANOVA}_{\text{slope}}$: $F_{\text{fraction}} = 1.14$, $P = 0.343$; $\text{ANOVA}_{\text{intercept}}$:$F_{\text{fraction}} = 9.29$, $P < 0.001$; $df = 1, 114$ for all comparisons).

the control and that extract and all fractions combined elicited longer arrestment durations than did individual fractions (Figure 7). Essential fractions 2, 4, and 9 did not prolong male arrestment longer than nonessential fraction 12.

DISCUSSION

Individual extract fractions arrested male *T. urticae* for significantly longer periods than did the control; this effect was amplified for treatments containing all the fractions. Arrestment resulted primarily from an almost immediate decrease in male linear velocity, and although linear velocity increased over time for all treatments, the rate of velocity increase was significantly less in response to all active fractions than to the control or to individual fractions. Males presented with the entire pheromone blend also pivoted or remained immobile more often than did control males; the relatively flat slopes of stationary-index regression lines suggest that this pivoting behavior continued



Contrast	Coefficient F - Ratios	
	a (Asymptote)	b (Rate)
Individual Fractions vs. Control	0.02 ns	3.92 *
Fractions + Control vs. All + Extract	2.19 ns	30.88 ****
Essentials vs. Non-essential	0.82 ns	1.55 ns
2 + 4 vs. 9	0.01 ns	0.15 ns
2 vs. 4	0.26 ns	1.36 ns
Extract vs. All	1.28 ns	1.79 ns

FIG. 6. Regression equation coefficients describing changes in distances of male *T. urticae* from 3-mm-diameter circles of female sex pheromone fractions over time. Error bars are standard errors of means ($N = 20$; $\text{ANOVA}_{\text{asymptote}}$: $F_{\text{fraction}} = 0.77$, $P > 0.1$; $\text{ANOVA}_{\text{rate}}$:$F_{\text{fraction}} = 6.56$, $P < 0.001$; $df = 1, 114$ for all comparisons).

throughout the 6-min observation period. Individual fractions had no effect on angular velocity with respect to the control, but male angular velocities decreased less rapidly in response to all fractions than to individual fractions and the control. Males also tended to turn more in one direction than the other in the presence of all fractions combined. These results confirm that *T. urticae* quiescent deutonymphs contain a pheromone that locally arrests adult males, and that this pheromone contains multiple essential components.

It is possible that the differences in movement parameters elicited by treatments containing all fractions and those elicited by individual fractions were quantitative, and that 4 QDE of any individual fraction would have elicited behaviors indistinguishable from behaviors elicited by extract or all fractions combined. However, significantly different parameters of male movement were observed in response to the different individual fractions. This suggests that the components of each individual fraction are qualitatively different from those contained in the other fractions.

Our previous studies showed that mean durations of male arrestment elicited

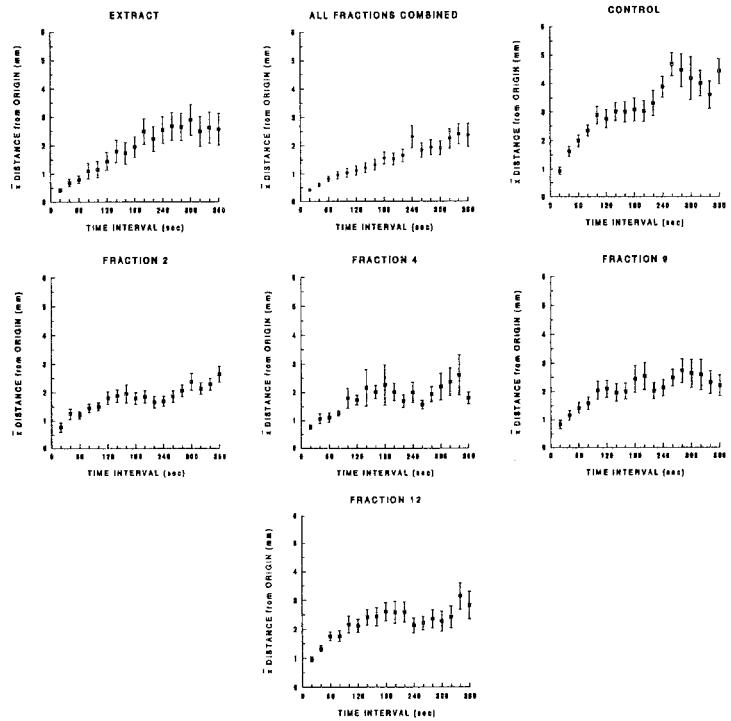


FIG. 7. Arrestment of male *T. urticae* caused by female sex pheromone fractions over time. Error bars are standard errors of means ($N = 20$).

by the four active fractions combined and by unfractionated extract did not differ (Royalty et al., 1992); therefore we concluded that all essential pheromonal components were contained in the four fractions. No differences in angular and linear velocity regression equation coefficients were observed between male response to all fractions combined and to unfractionated extract, but the stationary index was greater throughout the experiment in response to extract. In addition, turn bias was greater in response to extract than to the combination. These results suggest that a pheromonal component may be missing from the four-fraction blend.

One source of the possible missing pheromonal component(s) could be fractions 3 and/or 16 of the original HPLC-separated extract. These fractions elicited slightly longer arrestment durations than did the control in our initial bioassays (Royalty et al., 1992), but differences were not significant. Subtractive bioassay data suggested that neither fraction contained essential components, either alone or in combination with other fractions, while contrast comparisons of preliminary digitizer data showed that no parameter differences were observed

between male responses to fraction 16 and the control over a 2-min observation period. We therefore concluded that fraction 3 probably contained small quantities of active components found in fractions 2 and 4, while fraction 16 contained no activity. Failure to include fraction 3 in the all-fraction combination used in this study may have resulted in slight quantitative differences in the amounts of pheromone contained in the extract and in the all-fraction treatment, while omission of fraction 16 may have caused qualitative differences.

Early work suggested that male *T. urticae* locate quiescent deutonymphs through short-range orientation to volatile pheromone and chemo- or mechanoperception of the webbing spun by the deutonymph prior to becoming quiescent (Penman and Cone, 1972, 1974). Pheromone and tactile perception of the form of the female were thought to elicit and prolong arrestment (Cone et al., 1971a; Penman and Cone, 1972, 1974). However, we have shown that males perceive pheromone by contact chemoreception but are unable to perceive pheromone volatiles (Royalty et al., 1992). Males probably locate quiescent deutonymphs by short-range orientation to the yellow color of the female, perception of webbing, or by chance (Royalty et al., 1992 in press). Localized searching and guarding behaviors are initiated primarily by tactile perception of the quiescent deutonymph; pheromone seldom initiates arrestment in the absence of other quiescent-deutonymph stimuli. The quantified male behaviors observed in the current study have shown that male perception of the complete blend of active pheromonal components reduce linear velocity and directional change, increase angular velocity, and increase pivoting of the arrested male. These behavioral alterations result in reduced displacement, thus prolonging area-restricted searching behavior and enabling the male to maintain close proximity with the quiescent deutonymph.

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REFERENCES

BELL, W.J. 1984. Chemo-orientation in walking insects, pp. 93–124, *in* W.J. Bell and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, New York.

BELL, W.J. 1990. Searching behavior patterns in insects. *Annu. Rev. Entomol.* 35:447–467.

CONE, W.W. 1979. Pheromones of Tetranychidae, pp. 309–317, *in* J.G. Rodriguez (ed.). Recent Advances in Acarology, Vol. 2. Academic Press, London.

CONE, W.W. 1985. Mating and chemical communication, pp. 243–252, *in* W. Helle and M.W. Sabelis (eds.). Spider Mites, Their Biology, Natural Enemies and Control, Vol. 1A, World Crop Pests. Elsevier, Amsterdam.

CONE, W.W., MCDONOUGH, L.M., MAITLEN, J.C., and BURDAJEWICZ, S. 1971a. Pheromone studies of the two-spotted spider mite. 1. Evidence of a sex pheromone. *J. Econ. Entomol.* 64:355–358.

CONE, W.W., PREDSKI, S., and KLOSTERMEYER, E.C. 1971b. Pheromone studies of the two-spotted spider mite. 2. Behavioral response of males to quiescent deutonymphs. *J. Econ. Entomol.* 64:379–382.

EWING, H.E. 1914. The common red spider or spider mite. *Oreg. Agric. Exp. Stn. Bull.* 121:1–95.

GERSON, U. 1985. Webbing, pp. 223–232, *in* W. Helle and M.W. Sabelis (eds.). Spider Mites, Their Biology, Natural Enemies and Control, Vol. 1A, World Crop Pests. Elsevier, Amsterdam.

LAING, J.E. 1969. Life history and life table of *Tetranychus urticae* Koch. *Acarologia* 11:32–42.

LEHR, R., and F.F. SMITH. 1957. The reproductive capability of three strains of the two-spotted spider mite complex. *J. Econ. Entomol.* 50:634–636.

PENMAN, D.R., and CONE, W.W. 1972. Behavior of male two-spotted spider mites in response to quiescent female deutonymphs and to web. *Ann. Entomol. Soc. Am.* 65:1289–1293.

PENMAN, D.R., and CONE, W.W. 1974. Role of web, tactile stimuli, and female sex pheromone in attraction of male two-spotted spider mites to quiescent female deutonymphs. *Ann. Entomol. Soc. Am.* 67:179–182.

POTTER, D.A., and WRENSCH, D.L. 1978. Interrupted matings and the effectiveness of second inseminations in the two-spotted spider mite. *Ann. Entomol. Soc. Am.* 71:882–885.

POTTER, D.A., WRENSCH, D.L., and JOHNSTON, D.E. 1976. Guarding, aggressive behavior and mating success in male two-spotted spider mites. *Ann. Entomol. Soc. Am.* 69:707–711.

RATKOWSKY, D.A. 1990. Handbook of Non-linear Regression Models. Marcel Dekker, New York.

ROYALTY, R.N., PHELAN, P.L., and HALL, F.R. 1992. Arrestment of male two-spotted spider mite caused by female sex pheromone. *J. Chem. Ecol.* 18:137–153.

ROYALTY, R.N., PHELAN, P.L., and HALL, F.R. 1992. Comparative effects of form, color, and pheromone of two-spotted spider mite (Acari: Tetranychidae) quiescent deutonymphs on male guarding behavior. *Physiol. Entomol.* In press.

SNEDECOR, G.W., and COCHRAN, W.G. 1967. Statistical Methods, 6th ed. The Iowa State University Press, Ames, Iowa.

SPIVAK, M., CONLON, D., and BELL, W.J. 1991. Wind-guided landing an search behavior in fleshflies and blowflies exploiting a resource patch (Diptera: Sarcophagidae, Calliphoridae). *Ann. Entomol. Soc. Am.* 84:447–452.

VISSER, J.H. 1988. Host-plant finding by insects: Orientation, sensory input and search patterns. *J. Insect Physiol.* 34:259–268.

WAAGE, J.K. 1978. Arrestment response of the parasitoid, *Nemeritis canescens*, to a contact chemical produced by its host, *Plodia interpunctella*. *Physiol. Entomol.* 3:135–146.

WILKINSON, J. 1990. SYSTAT: The System for Statistics. SYSTAT, Inc., Evanston, Illinois.

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SHEEP FOOD REPELLENTS: EFFICACY OF VARIOUS PRODUCTS, HABITUATION, AND SOCIAL FACILITATION

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Abstract—The study of food repulsion in ungulates is of practical importance for controlling browsing damage to forest trees and agricultural crops. Experiments with domestic sheep assessed the repulsive value of three natural and two synthetic products, using a food-choice situation. Animals were individually presented with two troughs, each containing 30 g of maize, one treated with a chemical product, and the other a control. The odor of domestic dog feces appeared to be highly repulsive, as none of the treated food was eaten, whereas 3 g ± 9 and 6 g ± 10 were eaten, respectively, of food treated with fetal fluids from sheep and odor of pig feces. Synthetic odors of lion feces and a commercial deer repellent were less efficient as repellents with 11 g ± 13 and 14 g ± 15 of food eaten, respectively. Habituation to three of the repellents was tested for a minimum of seven successive days. With dog feces, no habituation to the odor was observed. On the contrary, significant habituation ($P < 0.05$) was observed as early as the third trial for sheep fetal fluids and the fourth trial for the commercial repellent. To test for the effects of social facilitation, groups of four sheep including no, one, or two anosmic animals were presented with food treated with the odor of dog feces. No social facilitation was observed as none of the intact sheep ate any of the food, although the anosmic animals actively ate it throughout the test. In individual food choice tests, the odor of dog feces appeared to be an especially efficient repellent when compared with the other natural or synthetic products. Such repulsion was nearly complete, and neither habituation nor social facilitation could be observed.

Key Words—Sheep, *Ovis aries*, repellent, fecal odor, predator odor, food choice, social facilitation, habituation, domestic dog, *Canis familiaris*.

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INTRODUCTION

Rodents as well as ungulates inflict serious feeding damage to fruit and forest trees and to agricultural crops. The use of chemical repellents could protect this vegetation.

The odor of excretions or secretions of predators induce avoidance in those animals that could be potential prey. Compounds from anal glands of stoats (*Mustela erminea*) and/or mink (*M. vison*) have been reported to protect trees and crops from voles (*Microtus* spp.) and hares (*Lepus americanus*) (Sullivan and Crump, 1984; Sullivan et al., 1988a) and urine odors from the wolverine (*Gulo gulo*) reduce damage to trees by hares (Sullivan et al., 1985a). The 2,5-dihydro-2,4,5-trimethylthiazoline identified in fox (*Vulpes vulpes*) feces (Vernet-Maury, 1980; Vernet-Maury et al., 1984) as well as a synthetic fox urine mixture inhibit feeding by voles (Sullivan et al., 1988a). Moreover, feces from Felidae or Canidae, or urine from Canidae reduced feeding damage by hares (Sullivan et al., 1985a) and deer (*Odocoileus* spp. and *Cervus elaphus*) (Van Haaften, 1963; Müller-Schwarze, 1972; Melchiors and Leslie, 1985; Sullivan et al., 1985b; Abbott et al., 1990; Swihart et al., 1991). Odors of Canidae and Felidae feces are also rejected by sheep (*Ovis aries*) and cattle (*Bos taurus*) (Poindron, 1974; Pfister et al., 1990). Other animal products that appear to be repulsive include putrefied egg or fish, which have an effect on deer (Oita et al., 1976). Moreover, a synthetic product prepared from compounds in putrefied eggs is commercially available as a deer repellent: the Big Game Repellent (BGR). Fetal fluids from sheep, goats (*Capra hircus*), and cows are repulsive for sheep except in parturient females (Lévy et al., 1983; Arnould et al., 1991).

Most of these results have been obtained on wild animals in field experiments or on captive animals in enclosures. Thus, parameters such as grass availability, rainfall, or previous experience are difficult or impossible to control. An analysis of food repulsion under controlled conditions would be necessary for understanding the basis of this olfactory-based avoidance, making it possible to design effective products. Controlled experiments are only feasible with laboratory or domestic animals, and sheep would be a good model for such studies.

The aim of this study was, first, to test in sheep a series of products known, or presumed, to have a repellent effect on wild or domestic ungulates. The products were as follows: domestic dog (*Canis familiaris*) feces, previously used as a repellent in sheep by Poindron (1974); feces from pigs (*Sus scrofa*), an animal that is not considered a sheep predator; fetal fluids from sheep, described as a sheep repellent by Lévy et al. (1983); and two synthetic products that are supposedly effective on deer: BGR and a synthetic odor of lion (*Panthera leo*) feces.

The repellent action of these products was first assessed in a series of short trials. However, the presentation of a new stimulus could by itself reduce food

intake. In fact, Chapple et al. (1987a) have observed in sheep an increase of food consumption resulting from habituation to a new food and a new trough. Consequently, the hypothesis of a decreasing effectiveness (habituation) of a repellent odor, resulting from the lack of negative stimuli associated with it, has to be tested.

Finally, social facilitation of food intake cannot be excluded, especially in ungulates in which feeding behavior is a group activity. Therefore, it is necessary to verify whether the presence of animals eating actively in a group could stimulate others to do so, despite the presence of a repellent. Thus, intact ewes have been tested together with anosmic ones, which do not react to the repellent odor, to test the hypothesis of the possibility that such a social facilitation may mitigate the olfactory repulsion.

METHODS AND MATERIALS

Experiment 1: Repellent Effectiveness of Various Products

Animals and Methods. Eleven ovariectomized Ile-de-France ewes, 5 to 6 years old were used. Animals were kept indoors and fed 650 g of a mixture of maize, wheat, dehydrated alfalfa and a complement of vitamins and minerals, and straw ad libitum.

In a first series of experiments, the repulsive value of the following substances was tested: domestic dog feces, pig feces, fetal fluids from sheep, a commercial deer repellent [Big Game Repellent (BGR); M.G.K., Minneapolis, Minnesota], and a product based on synthetic odor of lion feces (formulation F′; kindly provided by Imperial Chemical Industries Public Health, UK). Furthermore, the possible neophobic reaction to a novel odor was measured using amyl acetate (Aldrich-Chemie, Steinheim, Germany). In a second series of experiments, habituation to dog feces, sheep fetal fluids, and BGR was tested. Fecal matter was collected 1–7 hr before the test from animals always fed with the same commercial food and from a single animal—an adult female of the French Epagneul Breton breed—in the case of the dog. As described by Lévy et al. (1983), fetal fluids were collected from the coat of Ile-de-France lambs at birth, frozen at −18°C, and thawed at 37°C for the tests. The two commercial repellents and amyl acetate were used pure.

Experimental Schedules. The effectiveness of these products as repellents was measured using a food-choice test adapted from Lévy et al. (1983). Each animal was individually tested for 3 min in a 3.80 × 2-m pen after 1 min isolation in a starting box. In the testing pen, two stainless steel troughs (control and experimental) were placed 50 cm apart. The top of each trough consisted of a pan or a grid (20 × 20 cm) that contained 30 g of maize. The amount of maize eaten from each trough after 3 min was recorded. Odorous products were

placed 10 cm under the grid containing the food in the experimental trough. In the case where no repulsion was observed in preliminary studies (i.e., fetal fluids, BGR, or formulation F′), the odorous liquid products were mixed with the maize and the same amount of water was spread over the food in the control trough. In this case, the animals were trained to eat moistened food as readily as dry maize before the beginning of the testing procedure. Feces (50–100 g for dog and 500–1000 g for pig) were placed in the trough 10 cm under the grid containing maize. Fetal fluids (10 ml) and commercial repellents (2 ml) were mixed with the maize. Amyl acetate was used to test the reaction of neophobia; 0.5 ml of pure compound was deposited on a filter-paper placed 2 cm below the grid. It was changed for each animal to avoid a decreasing amount of vapor as a consequence of evaporation.

Before each experiment, the floor of the testing pen was cleaned to remove any bedding material that could be eaten. Ewes were fed once a day after the end of the tests. Having received no food for a minimum of 16 hr, their motivation to eat maize was very important as it was the only food available during the tests. Animals had been trained in this test situation for one month before the beginning of the experiments, until they always ate all the maize from the two troughs in less than 30 sec/trough. Such a long training period minimized possible anxiety induced by the test situation (social isolation, human presence). Temperature at the level of the troughs was recorded since it might affect evaporation of products tested.

In the first series of experiments, the efficacy of each product was tested twice on each animal. To avoid any effect of lateralization, the position of the experimental and control troughs was reversed between the two tests. The order of products tested was chosen at random.

In a second series of experiments, habituation of the ewes to each of the three repellents was tested consecutively. Individual food-choice tests were repeated seven (fetal fluids) to nine times (BGR and dog feces).

Statistical Analysis. In the first series of experiments, the weight of maize eaten by each ewe during the two tests performed with the same product was recorded. The mean amounts of treated vs. control maize that had been eaten were then compared for each product with a Wilcoxon signed ranks test. In the second series of experiments, the influence of successive tests on maize consumption was analyzed with Friedman's two-way analysis of variance. Wilcoxon signed ranks test was then used to compare the amount of maize eaten during the first trial with that eaten during each of the following ones for each product. In all cases, the P value for statistical reliability = 0.05.

Experiment 2: Social Facilitation

Animals. Twenty-two adult Ile-de-France ewes were used to test the hypothesis that the olfactory repulsion could be overcome by social facilitation. Eighteen ewes were intact and four were anosmic, following surgical removal of the olfactory bulbs, to eliminate their reaction to odorous substances. They

were kept indoors and fed as in experiment 1. Animals were assigned to eight groups of four ewes: two groups with four anosmic ewes (A and E; the same four ewes were tested twice), two groups with four intact ewes (B and F), two groups with one anosmic and three intact ewes (C and G) and two groups with two anosmic and two intact ewes (D and H). Anosmic ewes were assigned to different groups and then tested several times. Each group of four ewes was penned separately 24 hr before the test.

Because dog feces was the most effective product in experiment 1, it was used in this experiment. Feces were collected, frozen at $-18°C$ about 2 hr after their excretion, and thawed for 2.5 hr before the experiment.

Experimental Schedules. Groups of four animals were tested in a 3.70 $\times$ 3.70-m pen. A 120 $\times$ 25-cm trough, with a grid top containing 800 g of maize, was placed in the center of the test pen. Ten centimeters under this grid, a pan contained either a suspension of 180 g of feces in 360 ml of demineralized water (experiment tests), or 540 ml of demineralized water (control tests). The test was stopped when the maize was completely eaten or at the latest after 15 min. Each group was tested once a day. All experimental tests were conducted first, followed by all the control tests on the following days.

The feeding time of each ewe was measured with a stopwatch. The number of sniffs before feeding (or during the duration of the test when no food was eaten) and total amount of maize eaten were recorded.

RESULTS

Experiment 1: Repellent Effectiveness of Various Products. For all of the presumed repellent products tested in the first series of experiments (Table 1), the mean amount of maize eaten was always significantly higher in the control trough than in the experimental one ($P < 0.05$). On the contrary, ewes were not disturbed by amyl acetate as each of them ate all the maize from control and experimental troughs.

Mean amounts of maize eaten in the experimental trough were compared according to the product tested. There was no significant difference in the amount of food eaten in the tests performed with dog feces and with fetal fluids ($P = 0.11$). The latter two products were the most active repellents: i.e., the consumption of dog feces-treated food was significantly lower than for the BGR or formulation F′ ($P = 0.03$ and $P = 0.01$). Likewise animals ate less food treated with fetal fluids than with either BGR or formulation F′ ($P = 0.04$ and $P = 0.03$). Dog feces had the greatest effect as the mean amount of maize eaten was significantly lower than with pig feces ($P = 0.02$), but the comparison with fetal fluids was not statistically significant ($P = 0.11$). Between the BGR and the formulation F′ there was no significant difference ($P = 0.35$). The formulation F′ was the least effective substance, i.e., more food was eaten than with

TABLE 1. REPELLENT EFFECTS OF VARIOUS PRODUCTS[a] ON CONSUMPTION OF FOOD IN CONTROL AND EXPERIMENTAL TROUGHS (EACH CONTAINING 30 g OF MAIZE) BY 11 EWES.

Product	Mean amount of maize eaten (g + SD)		P^b
	Control trough	Experimental trough	
Dog feces	22 ± 14	0	<0.02
Fetal fluids	27 ± 6	3 ± 9	<0.01
Pig feces	26 ± 10	6 ± 10	<0.01
BGR (putrescent eggs)	27 ± 6	11 ± 13	<0.01
Formulation F' (lion feces)	30 ± 1	14 ± 15	<0.03
Amyl acetate	30 ± 0	30 ± 0	NS

[a]Products are arranged in decreasing order of their repulsive value.
[b]Wilcoxon test.

pig feces ($P = 0.06$), whereas there was no difference between the effect of the BGR and pig feces ($P = 0.16$).

In the test performed with dog feces, the mean amount of maize eaten in the control trough was only 22 g ± 14 as three ewes refused to eat from both troughs during this test. The diffusion of the odor could account for such an extended repulsion. The same was observed with pig feces but for only one of these three ewes.

In the second series of experiments, the ewes increased significantly the amount of maize eaten from the experimental trough during successive tests with the BGR ($P = 0.005$, analysis of variance), and a similar, although non-significant, tendency appeared with the fetal fluids ($P = 0.08$, analysis of variance). The mean amounts were 9 g ± 14 and 27 g ± 9, respectively, for the first and the last trial with the BGR (Figure 1a), and 4 g ± 9 and 18 g ± 14 for fetal fluids (Figure 1b). Compared with the first trial, the amount of maize eaten at the fourth test for the BGR and at the third test for the fetal fluids had already significantly increased ($P = 0.04$ and $P = 0.04$, respectively). By contrast, with dog feces, the amount of maize eaten did not change significantly over successive tests ($P = 0.63$, analysis of variance). There was no significant increase of maize eaten when compared with the first test (except at T3, $P = 0.04$), even though the consumption of maize fluctuated from 0 to 5 g according to the trial (Figure 1c).

Great individual variation was observed in the reaction of the ewes to the products tested. One and four ewes out of 11 never ate from the experimental trough with the BGR and the fetal fluids, respectively, whereas three and one

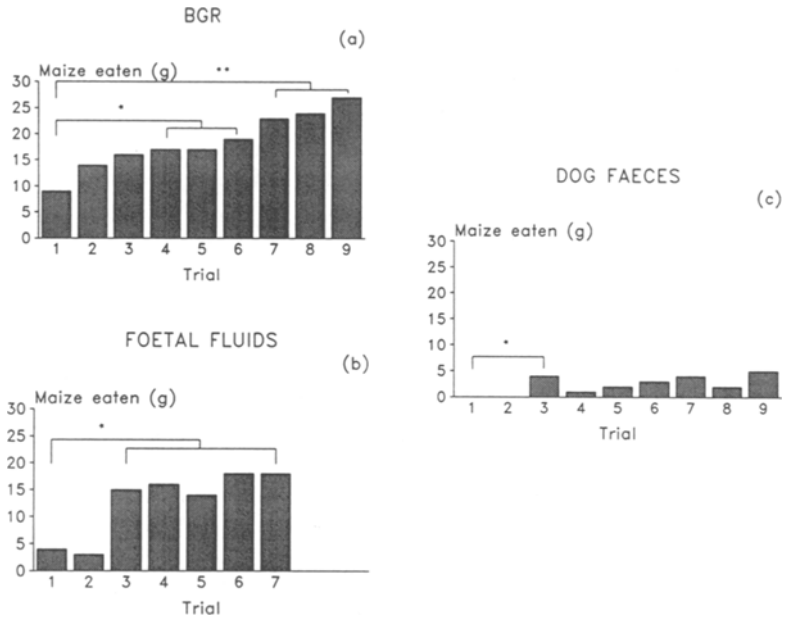

FIG. 1. Mean amount of maize eaten in the experimental trough during successive trials with three repellents. Trials in which significantly more food was consumed than in trial 1 are indicated (Wilcoxon test): $*P < 0.05$; $**P = 0.01$.

ewes, respectively, ate all the maize from the experimental trough throughout the trials. Seven ewes with the BGR and six with the fetal fluids increased their food consumption over successive trials. This increase was not as regular with the latter product as with the first one. With the odor of dog feces, eight females never ate from the experimental trough and two ate once at the third test, but only 4 g and 3 g, respectively. The amount of maize eaten by two other ewes varied between trials, but did not increase systematically.

The results of the trials in experiment 1 stress the effectiveness of dog feces as a repellent, as this substance has nearly totally prevented ewes from eating maize, and no habituation could be observed within the limited series of repetitions.

Experiment 2: Social Facilitation. When tested under control conditions, all four animals in each group ate the maize, and the trough was empty at the end of the test.

The four anosmic ewes (groups A and E) ate from the trough with dog feces dilution without previously sniffing it. On the other hand, in the groups of four intact ewes (groups B and F), no animals fed in the trough with repellent even if they all had sniffed it 1–10 times.

In the four groups containing both anosmic and intact ewes, the anosmic female(s) fed from the trough in which the maize was adulterated with the odor of dog feces suspension until it was empty. Even though all intact ewes sniffed the trough 5–19 times, only one ate any food. Indeed, in group C, one of three intact females fed at the odorized trough twice at the beginning of the test but only for 4 sec (this ewe sniffed the trough 13 times). Consequently, no evidence of social facilitation could be observed.

DISCUSSION

Suppression of ungulates' feeding by odors of predators has often been mentioned (Van Haaften, 1963; Müller-Schwarze, 1972; Melchiors and Leslie, 1985; Sullivan et al., 1985b; Abbott et al., 1990; Pfister et al., 1990; Swihart et al., 1991). Our results indicate that a similar phenomenon is observed in sheep in response to the odor of dog feces and illustrate its specific repellent properties. This odor is highly effective as a repellent compared to other products assumed to be aversive. Furthermore, no habituation to this odor is observed under our experimental conditions, and the presence of an animal eating actively does not induce eating by others, i.e., social facilitation.

The food repulsion of the ewes does not appear to reflect a neophobic reaction as their feeding behavior was not disturbed by amyl acetate, an odor unfamiliar to them. Among the repellents tested, it is possible to distinguish two types of products: those effective from a distance without necessity of contact with the food (placed under the grid containing maize) and those effective only when mixed with maize. In the first case, the volatile components of the products have a repulsive value, acting only via the sense of smell, whereas in the second, the necessity of direct contact suggest a more complex situation. The repellent molecules may be less volatile in the second than in the first case or could be more diluted. Furthermore, when direct contact occurs at the oral level, both the main and the accessory olfactory pathways could be stimulated. The sense of taste does not appear to be involved in such a repulsion, as anosmic ewes are not disturbed by such products.

Fecal products are repellent and dog feces have the strongest effect as the amount required to elicit repulsion is lower than for pig feces. Dog feces are coated with secretions from anal glands, and these secretions, used for intra- and interspecific communication, could explain the different potency of these two fecal products. Some ewes never ate in the control trough when tested with dog or pig feces. This reaction could be explained by the diffusion of the odor outside the trough as some ewes seemed to have perceived the odor far from its source and did not approach it up to the end of the test. This could also reflect some kind of "fear" reaction of the ewes, because when ewes were tested with

two troughs containing only maize (or in a choice situation with no repellent odor in the experimental trough) they always ate all the maize available. On the other hand, the rejection of the control trough food disappeared after several weeks of trials with unpleasant odors. According to Sullivan et al. (1988b), "predator odors originating from feces . . . elicit a 'fear' response when detected by prey species" and Vernet-Maury et al. (1968) described a fear reaction of laboratory rats when air odorized with a fox dropping extract was introduced inside their box. When dog feces are given repetitively during nine days (experiment 2), the repellent effect remained stable. Similar results were obtained by Sullivan et al. (1985b): leaves dipped in cougar (*Felis concolor*), coyote (*Canis latrans*), or wolf (*Canis lupus*) feces were not eaten by black-tailed deer (*Odocoileus hemionus*) after 20 days. In our habituation experiment, only two out of 11 ewes ate some maize with the odor of dog feces, but they showed no tendency to increase their consumption. There were only some fluctuations in the amount of maize eaten in the experimental trough according to the day. These fluctuations probably are not the result of a variable volatility of active compounds, as no correlation with temperature inside the test pen was observed. The analysis of anal gland secretions of the red fox by Albone and Fox (1971) shows that its composition varies in quality and in the relative ratios of constituents, even when collected from the same animal. Similar variations are likely for dog fecal matter.

The other products tested were effective only when mixed with the maize. Lévy et al. (1983) mentioned a repellent effect of the fetal fluids from sheep on ewes, except around parturition. Similar results were obtained in our first experiment, but two ewes ate maize adulterated with these fluids. Such a discrepancy could be explained by a greater habituation of our animals to the experimental conditions and to repeated testing with novel odors. A difference in the amount of fluids used by these authors could also account for the differences observed in the repellency. During the successive tests performed on the ewes with the fetal fluids, the mean amount of food eaten increased regularly, except at the fifth test, where the amount eaten was slightly lower, but not significantly so, than at the third and fourth tests. In fact, this "higher" repellency at the fifth test reflects the behavior of two ewes who were repelled more than during the two previous tests.

The BGR and formulation F′ are effective as wild ungulate repellents (Cervidae), but they had never been tested on domestic ungulates. The BGR has been reported to be very effective as a deer (*Odocoileus* spp.) repellent (Harris et al., 1983; Melchiors and Leslie, 1985), but it had little effect on sheep. Similar results have been observed with formulation F′, although it was effective in red deer (*Cervus elaphus*) according to Abbott et al. (1990). Formulation F′ elicits a weak response in sheep, even though it resembles the odor of predator fecal matter. This product reconstitutes the odor of lion feces at least as per-

ceived by humans. However, what makes it repulsive for ungulates may be different from what allows humans to recognize this odor as that of lion feces. The repellent action could be due to minor components that do not account for human identification of this odor. Furthermore, in this study the motivation of the ewes for eating the food from the trough was very high. Motivation of the deer was probably lower, as they were tested in pens where a variety of grass was available. Only a limited number of studies are available concerning the repellent effect of BGR, and effects on deer are variable. According to Sullivan et al. (1985b), salal leaves that had been treated with BGR were not consumed by deer (*Odocoileus* spp.) for a period of 20 days, but Melchiors and Leslie (1985) observed a decrease in the protection of salal branches by this repellent after 35 days. In these studies, deer were in an enclosure and were fed a commercial food *ad libitum*. Results on sheep deprived of food are similar to those obtained by Melchiors and Leslie (1985); sheep increased their consumption of maize mixed with the BGR over successive trials.

In sheep a modification of feeding behavior has been observed in animals enclosed with conspecifics with different food preferences. In fact, when naive sheep are introduced to a novel food in the presence of experienced animals, a rapid social transmission from experienced to naive sheep is observed (Chapple et al., 1987b). However, in the conditions of experiment 2, the lack of social facilitation is clear: intact females grouped with anosmic females never ate food mixed with the odor of dog feces. During these trials with both intact and anosmic ewes, the content of the trough was rapidly eaten by the anosmic ewes, thus limiting the duration of the experiments. It would be interesting to do further experiments in which intact ewes are housed together for a longer time (several days) with anosmic ones.

The effect of the odor of dog feces on sheep could be explained by predator–prey relations existing between these two species (see above). Indeed, domestic dogs, and closely related wolves, are potential predators for sheep. However, for several generations, sheep of the laboratory flock have had no contact with dogs. Therefore, previous learning of this odor cannot explain the observed repulsion. This effect seems more complex than a simple fear reaction for a potential predator. In spite of the lack of danger (predation), habituation to the odor has not been observed, and there was no social facilitation. A phenomenon more general than a reaction towards a predator odor seems to exist. This raises the question of the origin and the developmental mechanisms accounting for such an avoidance that appears to be "inborn" in adult sheep. The characteristics of this avoidance led us to hypothesize a genetic basis for this behavior, as has been previously demonstrated to mediate voles' (*Microtus arvalis*) avoidance of stoat (*Mustela erminea*) scent (Gorman, 1984) and suggested for specific food repulsions in young deer (*Odocoileus hemionus*) (Müller-Schwarze, 1972).

Finally, a high level of control of the testing environment is possible with

domestic animals such as sheep, which in turn allows a reliable measure of repellency. Detailed investigations dealing with repellency itself, and especially the chemicals involved, are planned. The high effectiveness of the odor of dog feces compared with other repellents makes it potentially interesting in this context. The identification of the active chemical components could contribute to the development of practical repellents designed to protect agricultural crops or plantations.

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REFERENCES

ABBOTT, D.H., BAINES, D.A., FAULKES, C.G., LEWIS, E., NING, P.C.Y.K., and TOMLIMSON, A.J. 1990. A natural deer repellent: Chemistry and behaviour, pp. 599–609, *in* D.W. Macdonald, D. Müller-Schwarze, and S.E. Natynczuk (eds.). Chemical Signals in Vertebrates 5. Oxford University Press, Oxford.

ALBONE, E.S., and FOX, M.W. 1971. Anal gland secretion of the red fox. *Nature* 233:569–566.

ARNOULD, C., PIKETTY, V., and LÉVY, F. 1991. Behaviour of ewes at parturition toward amniotic fluids from sheep, cows and goats. *Appl. Anim. Behav. Sci.* 32:191–196.

CHAPPLE, R.S., WODZICKA-TOMASZEWSKA, M., and LYNCH, J.J. 1987a. The learning behaviour of sheep when introduced to wheat. I. Wheat acceptance by sheep and the effect of trough familiarity. *Appl. Anim. Behav. Sci.* 18:157–162.

CHAPPLE, R.S., WODZICKA-TOMASZEWSKA, M., and LYNCH, J.J. 1987b. The learning behaviour of sheep when introduced to wheat. II. Social transmission of wheat feeding and the role of the senses. *Appl. Anim. Behav. Sci.* 18:157–162.

GORMAN, M.L. 1984. The response of prey to Stoat (*Mustela erminea*) scent. *J. Zool. London* 202:419–423.

HARRIS, M.T., PALMER, W.L., and GEORGE, J.L. 1983. Preliminary screening of white-tailed deer repellents. *J. Wildl. Manage.* 47:516–519.

LÉVY, F., POINDRON, P., and LE NEINDRE, P. 1983. Attraction and repulsion by amniotic fluids and their olfactory control in the ewe around parturition. *Physiol. Behav.* 31:687–692.

MELCHIORS, 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.

OITA, K., OH, J.H., and TIEDEMAN, G.T. 1976. Wildlife damage control I: Development of deer repellents. 172nd National Meeting of the American Chemical Society, San Francisco. Abstract No. 67.

PFISTER, J.A., MÜLLER-SCHWARZE, D., and BALPH, D.F. 1990. Effects of predator fecal odors on feed selection by sheep and cattle. *J. Chem. Ecol.* 16:573–583.

POINDRON, P. 1974. Méthode de suppression réversible de l'odorat chez la brebis et vérification de l'anosmie au moyen d'une épreuve comportementale. *Ann. Biol. Anim. Biochim. Biophys.* 14:411–415.

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.* 12:1809–1821.

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., SULLIVAN, D.S., CRUMP, D.R., WEISER, H., and DIXON, E.A. 1988b. Predator odors and their potential role in managing pest rodents and rabbits. *Proc. Vertebr. Pest Conf.* 13:145–150.

SWIHART, R.K., PIGNATELLO, J.J., and MATTINA, MJ.I. 1991. Aversive responses of white-tailed deer, *Odocoileus virginianus*, to predator urines. *J. Chem. Ecol.* 17:767–777.

VAN HAAFTEN, J.L. 1963. A natural repellent, pp. 389–392, *in* International Union of Game Biologists, Transactions VI Congress, 7–12 October. The Nature Conservancy, London.

VERNET-MAURY, E. 1980. Trimethyl-thiazoline in fox feces: A natural alarming substance for the rat, p. 407, *in* H. van der Starre (ed.). Proceeding of the VII International Symposium on Olfaction and Taste VII.

VERNET-MAURY, E., LE MAGNEN, J., and CHANEL, J. 1968. Comportement émotif chez le rat; influence de l'odeur d'un predateur et d'un non-prédateur. *C. R. Acad. Sci. Paris (Ser. D).* 267:331–334.

VERNET-MAURY, E., POLAK, E.H., and DEMAEL, A. 1984. Structure-activity relationships of stress-inducing odorants in the rat. *J. Chem. Ecol.* 10:1007–1018.

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EFFECTS OF SALICYLIC ACID ON PLANT–WATER RELATIONSHIPS[1]

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Abstract—Soybean seedlings [*Glycine max* (L.) Merr.] were used as the test species to study the allelopathic influence of salicylic acid (SA) on short- and long-term plant water status. Plants were grown in greenhouse conditions in nutrient culture medium amended with SA. Treatments were initiated 10 days after germination and continued for either 14 or 28 days. The threshold for inhibition of seedling growth over a 28-day treatment was 0.15 mM SA. Seedlings grown with 0.3 mM SA consistently had higher leaf diffusive resistance and lower transpiration and water potentials than control plants. The stable carbon isotope ratio ($^{13}C:^{12}C$) in tissue from both the 0.15 and 0.30 mM SA-treated plants was significantly higher than control seedlings, indicating SA caused a chronic water stress during the 28-day treatment. These data show that an interference with plant–water relationships is one mechanism whereby this allelochemical inhibits plant growth.

Key Words—Salicylic acid, allelopathy, plant–water status, water stress, ^{13}C, carbon isotopes, soybean, *Glycine max*.

INTRODUCTION

Benzoic and cinnamic acid derivatives and related compounds are among the most commonly identified allelopathic compounds produced by higher plants (Rice, 1984). Salicylic acid (2-hydroxybenzoic acid) has been isolated from donor plants, the decomposition of plant residue, and the soil (DeBell, 1971;

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Pareck and Gaur, 1973; Chou and Patrick, 1976; Jain et al., 1989; Singh et al., 1989; Siqueira et al., 1991). In bioassays, it is one of the most phytotoxic of the phenolic compounds (Einhellig et al., 1985a; Jain et al., 1989).

Several physiological effects have been documented as possible actions that may explain the inhibitory effects phenolic acids have on receiving species (Einhellig, 1986). The effects of salicylic acid (SA) include impacts on plant functions such as the activity of nitrate reductase, ethylene biosynthesis, ion uptake, membrane permeability, level of cellular ATP, and mitochondrial respiration (Glass, 1973; Jain and Srivastava, 1981; Harper and Balke, 1981; Balke, 1985; Leslie and Romani, 1988; Quah, 1992). Einhellig (1986, 1989) suggested that perturbations of cell membranes by phenolic allelochemicals may be a primary event that results in a cascade of physiological changes that reduce plant growth. Any effect on membranes would likely alter plant water balance and directly lead to growth inhibition. Glass and Dunlop (1974) reported that SA causes membrane depolarization. In studies on other phenolic acids, Einhellig et al. (1985b) showed that *p*-coumaric and ferulic acids increased diffusive leaf resistance and lowered water potential in grain sorghum [*Sorghum bicolor* (L.) Moench.]. Ferulic acid treatment of cucumber (*Cucumis sativus*) in a split-root system depressed net uptake of water (Lyu and Blum, 1990). Additional work indicated that the extent to which ferulic acid interfered with water utilization depended on the species (Holappa and Blum, 1991).

Transient or short-term fluctuations in water balance can be ascertained by standard techniques, such as monitoring leaf transpiration, resistance, and water potential. Now, determination of integrated plant water-use efficiency over an extended period of time can be achieved by measuring the relative amounts of carbon-12 (^{12}C) and carbon-13 (^{13}C) incorporated into tissue. The theory of this analysis is based on the fact that several plant factors, as well as environmental conditions such as water stress, influence the extent to which plants discriminate against the heavier isotope (Berry, 1989; Farquhar et al., 1982; Roeske and O'Leary, 1984; O'Leary, 1988; Tieszen, 1991). Water stress reduces the magnitude of discrimination against ^{13}C (Farquhar et al., 1982, 1989; Ehleringer, 1989; Guy et al., 1989).

The aim of this investigation was to establish both momentary and long-term water relationships of plants subjected to allelopathic stress from SA and to determine how any perturbations in these parameters related with growth effects.

METHODS AND MATERIALS

Soybean [*Glycine max* (L.) var. Wells II] seedlings were used as the test species in this investigation. Soybean has previously been shown to be sensitive to allelopathic stress from certain phenolic acids (Einhellig and Rasmussen,

1979; Patterson, 1981; Einhellig and Eckrich, 1984). The effects of salicylic acid (SA) on soybean seedling growth and water relationships were determined during 14- and 28-day treatment periods.

Soybean seeds were germinated in vermiculite flats in a greenhouse. After seven days, the seedlings were individually transplanted to opaque plastic vials containing 120 ml of nutrient medium. The seedlings were supported through a hole in the lid of the vial. The nutrient medium was a modified Hoagland's (Hoagland and Arnon, 1950) solution containing 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2mM $MgSO_4$, 0.9 mM $NH_4H_2PO_4$, 0.1 mM $(NH_4)_2HPO_4$, standard Hoagland's micronutrients, 72 μM iron supplied as sodium ferric diethylene-triamine pentaacetate (Sequestrene 330), and the pH adjusted to 5.8. The nutrient medium was not aerated. Seedlings were allowed to acclimate in the greenhouse three days before they were treated with SA. On the day of treatment (day 10), a subset of seedlings was harvested to establish leaf area and dry weight baseline data.

SA treatments were initiated by replacing the nutrient medium in which the soybean seedling roots were suspended with nutrient medium amended with SA. Thereafter, the treatment solution was replaced every three days to ensure the plants had a renewed nutrient supply and allelochemical exposure. It was not necessary to add any solution to the containers between the three-day replacement schedule. Plants in the first experiment were treated for 14 days with either zero, 0.1, 0.3, and 0.5 mM SA. The initial pH of these solutions was 5.8, 5.7, 3.9, and 3.6, respectively. The results of this experiment showed the threshold concentration at which SA inhibited soybean seedling growth.

The second experiment was designed to subject seedlings to a longer-term (28-day) exposure to SA at concentrations near the growth-inhibition threshold. SA levels employed in the second experiment were zero, 0.15, and 0.30 mM SA. Eighteen soybean seedlings were used per treatment group, thus providing a minimum of six plants per group at the final harvest after the sacrifice of some seedlings for water potential determinations during the treatment period. Procedures were the same as in the first experiment except that two weeks into the treatment period the plants were transferred to 400-ml containers. The shift to larger containers was to allow for the increased size of the seedlings and to ensure ample solution was maintained during the three-day intervals between each change of solution.

The growth environment during the investigations had the normal variation of summer greenhouse conditions. Diffusive leaf resistance and transpiration were measured every second day throughout the experiments on six, randomly chosen plants per treatment group. Measurements were obtained from the abaxial surface of the unifoliate leaves using a Li-Cor LI-1600 steady-state porometer. In the second experiment, leaf water potential was obtained once a week from four plants in each treatment group. This was accomplished by punching

a 7-mm-diameter disk from the center of a leaflet in the most fully developed trifoliate, allowing these leaf samples to reach equilibrium (2 hr) in Wescor C-52 sample chambers, and then determining water potential using a Wescor HR 33 dewpoint microvoltmeter. Soybean plants from which a leaf disk was cut were discarded. Diffusive resistance, transpiration, and water potential were obtained on sunny days between 1300 and 1500 hr.

At the termination of each experiment, plants were harvested by separating the leaves from the plant, obtaining the leaf area, and oven drying all tissue at 104°C for 48 hr. These data were used to compute the leaf-to-plant weight ratio (LWR; mg/mg), specific leaf weight (SLW; mg/cm^2), and relative growth rate (RGR; mg/mg/day) (Evans, 1972; Bhowmik and Doll, 1983). Calculation of RGR over the treatment period utilized the initial plant weights from the subsample harvested at the time of treatment (T_1; W_1), compared to weights at harvest (T_2; W_2), using equation 1:

$$RGR = \frac{(\ln W_2 - \ln W_1)}{(T_2 - T_1)} \quad (1)$$

Leaf tissue from plants harvested at the end of the 28-day experiment was analyzed to determine the carbon isotope ratio, $^{13}C : {}^{12}C$. The second and third trifoliate leaves were ground in a Cyclone sample mill fitted with a 0.4-mm screen. The carbon isotope ratio of this tissue from each plant was measured with an isotope-ratio mass spectrometer, and the ratio of the two masses was compared to a standard (Dr. Larry Tieszen's laboratory, Augustana College, Sioux Falls, South Dakota). The results of this analysis are expressed as the delta (δ) ^{13}C value, which is obtained from equation 2 and has units of ‰ (O'Leary, 1988).

$$\delta^{13}C = \frac{{}^{13}C/{}^{12}C_{(\text{sample})} - {}^{13}C/{}^{12}C_{(\text{standard})}}{{}^{13}C/{}^{12}C_{(\text{standard})}} \times 1000 \quad (2)$$

All the data were analyzed using one-way analysis of variance (ANOVA) with means separated by Duncan's multiple-range test using the Statistical Analysis System (SAS).

RESULTS

Soybean seedlings treated with 0.3 mM and 0.5 mM SA in the 14-day preliminary experiment exhibited observable stunting of growth by the third day of treatment, and several 0.5 mM SA-treated soybeans eventually died. At harvest, plants in these two treatment regimes had significantly lower plant dry weight, leaf weight, leaf area, and RGR than controls (Table 1). However, the SA-treated plants had higher SLW. In all of the growth parameters, 0.5 mM SA-treated plants were more severely affected.

TABLE 1. EFFECTS OF SALICYLIC ACID (SA) ON GROWTH OF SOYBEANS AFTER 14 DAYS OF TREATMENT[a]

Plant parameters	SA treatment			
	Control	0.1 mM	0.3 mM	0.5 mM
Leaf area (cm^2)	155.0 A	153.7 A	110.0 B	31.4 C
Leaf wt. (mg)	552 A	570 A	471 B	148 C
Plant wt. (mg)	1262 A	1278 A	1062 B	389 C
SLW (mg/cm^2)	3.56 A	3.71 A	4.28 B	4.71 C
LWR (mg/mg)	438.2 A	444.1 A	442.6 A	380.5 B
RGR (mg/mg/day)	131.2 A	132.1 A	118.8 B	47.0 C

[a] Values ($N = 6$) within a row not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test. SLW = specific leaf wt.; LWR = leaf wt. ratio; RGR = relative growth.

The only treatment in the 14-day experiment that consistently altered soybean water status was 0.5 mM SA (Figure 1). Although day-to-day variations occurred, these plants had diffusive resistances and transpiration rates that were significantly different from controls on the days monitored.

Data from the first experiment established the growth-inhibition threshold for soybean between 0.1 and 0.3 mM SA. Hence, the second experiment focused in this mild-stress range, employing 0.15 and 0.3 mM SA treatments during the 28-day experiment. Both treatments resulted in significant reductions in growth when compared with controls. Plant dry weight, leaf weight, leaf area, and RGR were all significantly lower than controls, and the treatment groups differed from each other (Table 2). In contrast to the results in the first experiment, SLW of 0.3 mM SA-treated plants was lower than controls.

Soybean seedlings treated for 28 days with 0.3 mM SA had leaf diffusive resistances that were significantly higher than controls throughout most of the experiment, and their transpiration was significantly lower (Figure 2). One week into the treatment period, seedlings in both SA treatments had significantly lower leaf water potentials than those grown with no SA. The data show that lower water potentials continued in the 0.3 mM SA-treated plants throughout the experiment.

At the end of the 28-day experiment, carbon isotope ratio mass spectrometry showed that leaf tissue samples from the three treatment groups were distinctive. The $\delta\ ^{13}C$ values establish that plants grown with SA in the medium had significantly less discrimination against ^{13}C (Table 3). The extent of these differences depended on the treatment level, but the tissue from 0.3 mM salicylic acid plants had an enhancement of 1.6‰ of $\delta\ ^{13}C$.

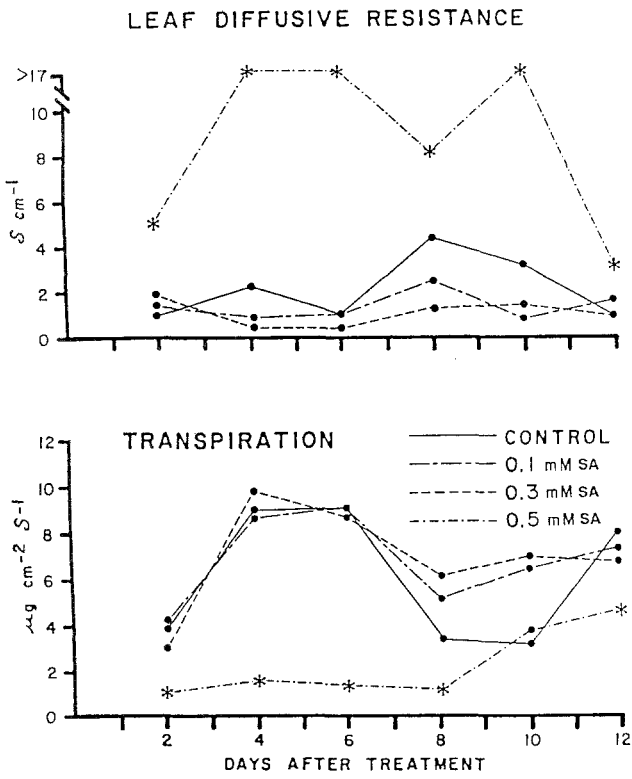

FIG. 1. Effects of salicylic acid (SA) on water status of soybean during 14 days of treatment. Each value is the mean of six plants. *Significantly different from the control, $P < 0.05$.

DISCUSSION

These experiments demonstrate that prolonged exposure to 0.15 mM SA, or greater concentrations, inhibits soybean seedling growth. This growth-inhibition threshold will probably vary according to stresses from other environmental conditions (Einhellig and Eckrich, 1984; Einhellig, 1989). However, by comparison to other allelopathic phenolic acids, SA is one of the more inhibitory compounds of this class (Einhellig and Rasmussen, 1979; Einhellig et al., 1982, 1985a). In our experience, and as shown by Patterson (1981) for several other phenolic acids, the growth reductions that resulted from SA were not due to pH differences in the treatment solutions. However, the relatively low pH of the 0.3 and 0.5 mM SA treatments probably resulted in more rapid uptake of SA than in other treatments (Harper and Balke, 1981).

TABLE 2. EFFECTS OF SALICYLIC (SA) ACID ON GROWTH OF SOYBEANS AFTER 28 DAYS OF TREATMENT[a]

Plant parameters	SA treatment		
	Control	0.15 mM	0.30 mM
Leaf area (cm^2)	1058.9 A	854.1 B	287.4 C
Leaf wt. (mg)	4178 A	3520 B	559 C
Plant wt. (mg)	9330 A	7176 B	2073 C
SLW (mg/cm^2)	3.94 A	4.12 A	1.95 B
LWR (mg/mg)	449.2 A	491.7 A	268.7 B
RGR (mg/mg/day)	139.4 A	130.0 B	85.0 C

[a]Values ($N = 6$) within a row not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test. SLW = specific leaf wt.; LWR = leaf wt. ratio; RGR = relative growth rate.

The whole-plant growth reductions from SA appear to be casually related to effects on plant–water balance. The overall changes in the water potential, diffusive resistance, transpiration, and carbon isotope fractionation patterns provide evidence for this conclusion. The extent of discrimination against ^{13}C was a sensitive indicator of SA effects on water relationships, with the δ ^{13}C value of 0.15 mM SA-treated plants showing that they had significantly less discrimination against ^{13}C than occurred in untreated plants. The carbon isotope ratios of SA-treated soybean indicate there was an enhancement of their integrated water-use efficiency, a result that is consistent with some stomatal diffusion limitations (O'Leary, 1988; Farquhar et al., 1982, 1989). Farquhar and Richards (1984) reported experimental observations of the strong correlation between water-use efficiency of entire plants and their carbon isotope ratio. Admittedly, there are additional factors that affect carbon isotope discrimination, and the theory of how they interrelate is imperfect. The conclusion that effects on stomatal conductance are the significant factors in salicylic acid-induced changes in carbon isotope discrimination is reinforced by the fact that plants subjected to 0.3 mM SA also had consistently higher diffusive resistances and lower leaf transpiration and water potentials.

The decrease in leaf water potential associated with SA treatment may have resulted from a reduction in root uptake of water. In short-term experiments, Booker et al. (1992) reported such an effect from ferulic acid. Even seedlings grown with a SA level at the threshold for growth inhibition, 0.15 mM SA, had water potentials 0.6 MPa below control plant values at the end of the first week of treatment, and a general trend toward lower water potentials was found in the last two weeks of treatment. The decline in the magnitude of SA effects on

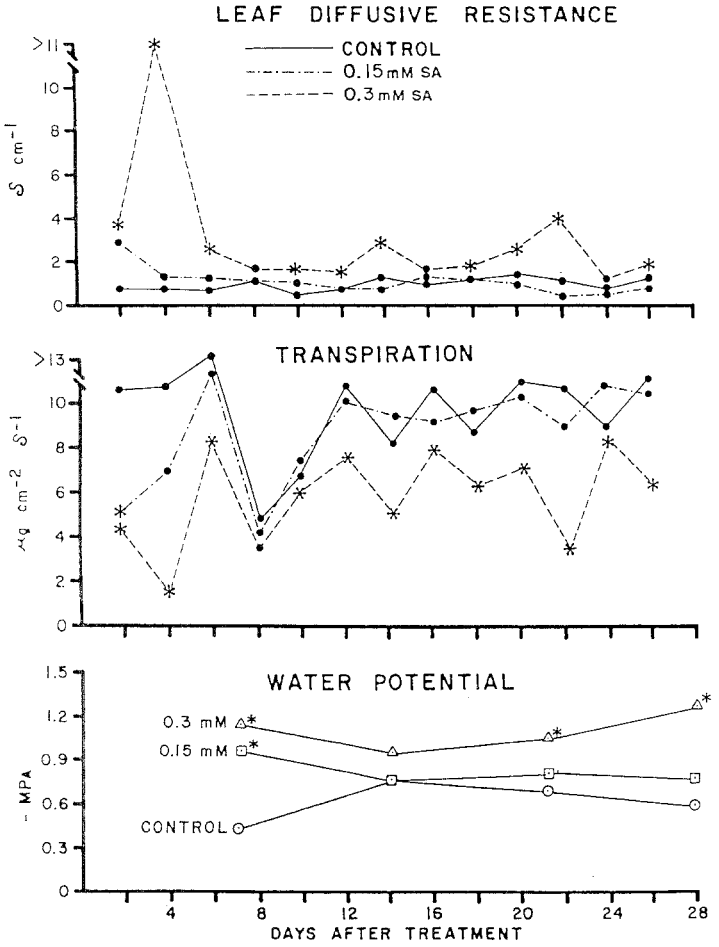

FIG. 2. Effects of salicylic acid (SA) on water status of soybeans during 28 days of treatment. Each resistance and transpiration value is the mean of six plants; water potentials are the mean of four. *Differs significantly from the control, $P < 0.05$.

water potential after the first week may have been due to an increase in microbial activity or to SA detoxification mechanisms. Shafer and Blum (1991) found that additions of phenolic acids to soil altered the microbial ecology of the rhizosphere. Balke et al. (1987) reported that root tissue was able to metabolize SA by glycosylation.

The collective data on effects of SA support the inference that disruption of water status is related to the growth reductions that occurred. In previous work, we have found that one of the physiological effects of two cinnamic acid

TABLE 3. EFFECTS OF SALICYLIC ACID (SA) ON RELATIVE CONTENT OF STABLE CARBON ISOTOPES IN SOYBEAN LEAF TISSUE[a]

Leaf tissue carbon isotopes	SA treatment level		
	Control	0.15 mM	0.3 mM
$\delta\ ^{13}C(‰)$	−29.08 A	−28.63 B	−27.48 C

[a] Data are expressed as the magnitude of discrimination against ^{13}C. Values ($N = 6$) not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.

derivatives, ferulic and *p*-coumaric acids, was related to water stress (Einhellig et al., 1985b). The present data on effects of SA suggests that the benzoic and cinnamic acid allelochemicals are acting in similar ways. Disturbance of water relations was reported as an allelopathic effect of *Helianthus annuus*, *H. tuberosus*, *Xanthium pensylvanicum*, and *Kochia scoparia* (Schon and Einhellig, 1982; Einhellig and Schon, 1982; Einhellig et al., 1985b). Hence, an early action causing allelopathic growth interference in a field situation may be through subtle changes in plant water status.

In conclusion, this investigation shows that one mechanism of action of SA is through disruption of plant-water relationships. The work on SA also extends the analytical use of stable carbon isotope fractionation patterns and illustrates that the stable carbon isotope ratio is a sensitive indicator of allelopathic water stress.

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. ACS Symposium 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 glycosylation of salicylic acid by *Avena sativa*, pp. 214–227, *in* G.R. Waller (ed.). Symposium on Allelochemicals: Role in Agriculture and Forestry. ACS Symposium Series 330, American Chemical Society, Washington, D.C.

BERRY, J.A. 1989. Studies of mechanisms affecting the fractionation of carbon isotopes in photosynthesis, pp. 82–94, *in* P.W. Rundel, J.R. Ehleringer, and K.A. Nagy (eds.). Stable Isotopes in Ecological Research. Springer-Verlag, New York.

BHOWMIK, P.C., and DOLL, J.D. 1983. Growth analysis of corn and soybeans response to allelopathic effects of weed residues at various temperatures and photosynthetic flux densities. *J. Chem. Ecol.* 9:1263–1280.

BOOKER, F.L., BLUM, U., and FISCUS, E.L. 1992. Short-term effects of ferulic acid on ion uptake and water relations in cucumber seedlings. *J. Exp. Bot.* 43:649–655.

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.

DEBELL, D.S. 1971. Phytotoxic effects from cherry-bark oak. *For. Sci.* 17:180–185.

EHLERINGER, J.R. 1989. Carbon isotope ratios and physiological processes in aridland plants, pp. 41–54, *in* P.W. Rundel, J.R. Ehleringer, and K.A. Nagy (eds.). Stable Isotopes in Ecological Research. Springer-Verlag, New York.

EINHELLIG, F.A. 1986. Mechanisms and modes of action of allelochemicals, pp. 171–188, *in* A.R. Putnam and C.S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

EINHELLIG, F.A. 1989. Interactive effects of allelochemicals and environmental stress, pp. 101–118, *in* C.H. Chou and G.R. Waller (eds.). Phytochemical Ecology: Allelochemicals, Mycotoxins and Insect Pheromones and Allomones. Institute of Botany, Academia Sinica Monograph Series No. 9, Taipei, Taiwan.

EINHELLIG, F.A., and ECKRICH, P.C. 1984. Interactions of temperature and ferulic acid stress on grain sorghum and soybeans. *J. Chem. Ecol.* 10:161–170.

EINHELLIG, F.A., and RASMUSSEN, J.A. 1979. Effects of three phenolic acids on chlorophyll content and growth of soybean and grain sorghum seedling. *J. Chem. Ecol.* 5:815–824.

EINHELLIG, F.A., and SCHON, M.K. 1982. Noncompetitive effects of *Kochia scoparia* on grain sorghum and soybean. *Can. J. Bot.* 60:2923–2930.

EINHELLIG, F.A., SCHON, M.K., and RASMUSSEN, J.A. 1982. Synergistic inhibitory effects of four cinnamic acid compounds on grain sorghum. *J. Plant Growth Reg.* 1:251–258.

EINHELLIG, F.A., LEATHER, G.R., and HOBBS, L.L. 1985a. Use of *Lemna minor* L. as a bioassay in allelopathy. *J. Chem. Ecol.* 11:65–72.

EINHELLIG, F.A., STILLE MUTH, M. and SCHON, M.K. 1985b. Effects of allelochemicals on plant-water relationships, pp. 179–195, *in* A.C. Thompson (ed.). The Chemistry of Allelopathy: Biochemical Interactions Among Plants. ACS Symposium Series 268, American Chemical Society, Washington, D.C.

EVANS, G.C. 1972. The Quantitative Analysis of Plant Growth. Blackwell Scientific, Oxford Press, 197 pp.

FARQUHAR, G.D., and RICHARDS, R.A. 1984. Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. *Aust. J. Plant Physiol.* 11:539–552.

FARQUHAR, G.D., O'LEARY, M.H., and BERRY, J.A. 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Aust. J. Plant Physiol.* 9:121–137.

FARQUHAR, G.D., HUBICK, K.T., CONDON, A.G., and RICHARDS, R.A. 1989. Carbon isotope fractionation and plant water-use efficiency, pp. 21–40, *in* P.W. Rundel, J.R. Ehleringer, and K.A. Nagy (eds.). Stable Isotopes in Ecological Research. Springer-Verlag, New York.

GLASS, A.D.M. 1973. Influence of phenolic acids on ion uptake I. Inhibition of phosphate uptake. *Plant Physiol.* 51:1037–1041.

GLASS, A.D.M., and DUNLOP, J. 1974. Influence of phenolic acids on ion uptake IV. Depolarization of membrance potentials. *J. Plant Physiol.* 54:855–858.

GUY, R.D., WARNE, P.G., and REID, D.M. 1989. Stable carbon isotope ratio as an index of water-use efficiency in C_3 halophytes—possible relationship to strategies for osmotic adjustment, pp. 55–75, *in* P.W. Rundel, J.R. Ehleringer, and K.A. Nagy (eds.). Stable Isotopes in Ecological Research. Springer-Verlag, New York.

HARPER, J.R., and BALKE, N.E. 1981. Characterization of the inhibition of K^+ absorption in oat roots by salicylic acid. *Plant Physiol.* 68:1349–1353.

HOAGLAND, D.R., and ARNON, D.I. 1950. The water-culture method for growing plants without soil. Calif. Agric. Ext. Serv. Circ. No. 347.

HOLAPPA, L.D., and BLUM, U. 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. *J. Chem. Ecol.* 17:865–886.

JAIN, A., and SRIVASTAVA, H.S. 1981. Effect of salicylic acid on nitrate reductase activity in maize seedlings. *Physiol. Plant.* 51:339–342.

JAIN, R., SINGH, M., and DEZMAN, D.J. 1989. Qualitative and quantitative characterization of phenolic compounds from Lantana (*Lantana camara*) leaves. *Weed Sci.* 37:302–307.

LESLIE, C.A., and ROMANI, R.J. 1988. Inhibition of ethylene biosynthesis by salicylic acid. *Plant Physiol.* 88:833–837.

LYU, S.-W. and BLUM, U. 1990. Effects of ferulic acid, an allelopathic compound, on net P, K, and water uptake by cucumber seedlings in a split-root system. *J. Chem. Ecol.* 16:2429–2439.

O'LEARY, M.H. 1988. Carbon isotopes in photosynthesis. *Bioscience* 38:328–336.

PARECK, R.P., and GAUR, A.C. 1973. Organic acids in the rhizosphere of *Zea mays* and *Phaseolus aureus* plants. *Plant Soil* 39:441–444.

PATTERSON, D.T. 1981. Effects of allelopathic chemicals on growth and physiological responses of soybean (*Glycine max*). *Weed Sci.* 29:53–59.

QUAH, S.G.H. 1992. Effects of six allelochemicals on respiration of soybean mitochondria. MA thesis. University of South Dakota, Vermillion, 43 pp.

RICE, E.L. 1984. Allelopathy. Academic Press, Orlando, Florida, 422 pp.

ROESKE, C.A., and O'LEARY, M.H. 1984. Carbon isotopes effects on the enzyme catalyzed carboxylation of ribulose bisphosphate. *Biochemistry* 23:6275–6284.

SCHON, M.K., and EINHELLIG, F.A. 1982. Allelopathic effects of cultivated sunflower on grain sorghum. *Bot. Gaz.* 143:505–510.

SHAFER, S.R., and BLUM, U. 1991. Influence of phenolic acids on microbial populations in the rhizosphere of cucumber. *J. Chem. Ecol.* 17:369–389.

SINGH, M., TAMMA, R.V., and NIGG, H.N. 1989. HPLC identification of allelopathic compounds from *Lantana camara*. *J. Chem. Ecol.* 15:81–89.

SIQUEIRA, J.O., NAIR, M.G., HAMMERSCHMIDT, R., and SAFIR, G.R. 1991. Significance of phenolic compounds in plant-soil-microbial systems. *Crit. Rev. Plant Sci.* 10:63–121.

TIESZEN, L.L. 1991. Natural variations in the carbon isotope values of plants: Implications for archaeology, ecology, and paleoecology. *J. Archaeol. Sci.* 18:227–248.

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INFLUENCE OF FOLIAR GLUCOSINOLATES IN OILSEED RAPE AND MUSTARD ON FEEDING AND GROWTH OF THE BERTHA ARMYWORM, *Mamestra configurata* WALKER

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Abstract—The relationship between host plant glucosinolate profile and feeding and growth of the Bertha armyworm, *Mamestra configurata* Walker was investigated using eight cultivated rape and mustard varieties. Mean larval weights of neonates reared on intact rosette-stage plants were significantly different on the different species in the order *Brassica juncea* < *Sinapis alba* < *B. napus* < *B. campestris*. While *B. juncea* was least preferred, *S. alba* was significantly more attractive to neonate larvae in choice tests. Relative consumption and growth rates of fourth-instar larvae were also reduced on *B. juncea* foliage. Other differences were dependent on the plant growth stage. Neonate preference was not correlated to total glucosinolate levels, but rather to the concentrations of isothiocyanate-releasing glucosinolates. However, the relationship between consumption and glucosinolate levels was inconsistent. Relative growth rate was negatively correlated to total glucosinolate content for stage 3 and 4 foliage—mainly due to the concentration of isothiocyanate-releasing glucosinolates. The relative importance of isothiocyanate-releasing glucosinolates was verified by rearing neonates on meridic diets containing equimolar concentrations of sinigrin, its metabolite, allyl isothiocyanate, and indole-3-carbinol, metabolite of 3-indolylmethyl glucosinolate. Sinigrin and allyl isothiocyanate in the diet produced virtually identical negative weight vs. concentration regression lines. No such dose–response effect was observed with indole-3-carbinol. The data suggest that foliar isothiocyanate-releasing glucosinolates may provide some degree of plant protection from polyphagous insects.

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Key Words—*Brassica campestris*, *B. juncea*, *B. napus*, Bertha armyworm, canola, glucosinolate, insect-plant interactions, isothiocyanate, *Mamestra configurata*, Lepidoptera, Noctuidae, mustard, *Sinapis alba*, thiocyanate.

INTRODUCTION

Glucosinolates and their volatile degradation products have historically been implicated as kairomones for insects that specialize on cruciferous plants (Verschaffelt, 1911; Nayar and Thorsteinson, 1963; Traynier, 1965, David and Gardiner, 1966; Read et al., 1970; Feeny et al., 1970; Nault and Styler, 1972; Rygg and Somme, 1972; Hicks, 1974; Nair and McEwen, 1976; Finch, 1978; Reed et al., 1989; Traynier and Truscott, 1991). While the presence of these compounds undoubtedly contributes to host plant recognition, recent laboratory studies have revealed that the host selection responses of some crucifer specialists are not necessarily side-chain- or dose-dependent (Reed et al., 1989; Bodnaryk and Palaniswamy, 1990). Field experiments comparing high- and low-glucosinolate rape cultivars have shown that infestation levels of certain crucifer pests are not related to total glucosinolates (Ahman, 1982; Lamb, 1988; Williams, 1989; Butts and Lamb, 1990). Moreover, the widespread cultivation of low-glucosinolate rape varieties (canola) on the Canadian prairies has not led to differences in crucifer pest infestation levels (Lamb, 1989).

Allelochemicals in plants may also affect the palatability of the substrate as well as the growth of the herbivore, two important parameters of insect resistance. In this mode, glucosinolates have been shown to function as allomones for some nonspecialist insects (Nault and Styler, 1972; Blau et al., 1978). Hypothetically, the presence of glucosinolates in the foliage of the crop may be advantageous if the consumption and growth of foliage-feeding insects can be inhibited. In the present study we have examined foliar glucosinolates qualitatively and quantitatively, and investigated their relationship to food consumption and growth of a Canadian canola pest, the Bertha armyworm, *Mamestra configurata* Walker. *M. configurata* is a nonspecialist insect that includes brassicaceous plants in its host range.

Eight cultivars representing four species of cultivated oilseed rape and mustard were chosen for our study, the main criteria for their selection being their differences in glucosinolate profile. Essentially two types of glucosinolates, based on their metabolic products following the action of endogenous myrosinase (EC 3.2.3.1), dominate in the foliage of the plants studied. Unsubstituted alkenyl and aryl glucosinolates produce isothiocyanates that are generally insecticidal (Lichtenstein et al., 1962, 1964; Nayar and Throsteinson, 1963; Lowe et al., 1971; Ahman, 1986; Bartelt and Mikolajczak, 1989), while those possessing an indolyl or hydroxy-aryl side chain ultimately degrade to form

R-alcohols and thiocyanate ion (at the pH range of the lepidopteran alimentary tract), whose biological activity is less well known. This study examines the relationship of biological activity in *M. configurata* to foliar concentrations of total glucosinolates, isothiocyanate-releasing glucosinolates, and thiocyanate-releasing glucosinolates. In addition, we attempt to verify the plant foliage effects with feeding studies using pure compounds augmented to meridic diet. Allyl isothiocyanate, the metabolite of allyl glucosinolate (sinigrin), and indole-3-carbinol, the metabolite of 3-indolylmethyl glucosinolate, were commercially available examples of the two types of glucosinolate metabolites of interest. Sinigrin, also commercially available, was used as an example of an intact glucosinolate.

METHODS AND MATERIALS

Insect Culture. A laboratory culture of *Mamestra configurata* was maintained at 20°C with a 16:8 (hr light–dark) photoperiod on an agar-based meridic diet (Velvetbean caterpillar diet, BioServ No. F9795, BioServ, Frenchtown, N.J.) augmented with 1.5% Vanderzant vitamin mixture and 1% alfalfa meal. Lamb's quarters (*Chenopodium album* L.) were presented to moths for oviposition (Bucher and Bracken, 1976). Eggs were removed from the leaves prior to hatching so that insects used for experiments had no previous experience with plant material.

Insect Growth on Greenhouse-Grown Plants. Seeds of *Brassica napus* cv. Westar, Regent, and Midas, *B. campestris* cv. Candle and Tobin, *B. juncea* cv. Lethbridge 22A, and commercial brown mustard and *Sinapis alba* cv. Gisilba were sown in vermiculite. Cotyledon stage plants were individually transplanted into 10-cm pots of a homogenous, sterilized soil mix and grown to the four-leaf rosette stage under high-intensity sodium vapor lamps (40,000 lux at plant level). Soluble 20–20–20 (N:P:K) fertilizer was applied weekly.

Five neonate *M. configurata* larvae were placed on each of 10 plants per variety. A screen-bottomed, clear plastic beverage cup was inverted over each plant and embedded into the soil to confine the insects. The plants and insects were transferred to a controlled environment growth chamber with a 16:8 (hr light–dark) photoperiod (16,000 lux) and a constant temperature of 20°C for seven days, after which time the insects were weighed. The data were subjected to ANOVA with individual degree of freedom (idf) tests (orthogonal contrasts) for differences based on species and cultivar within species.

Biological Assays with Field-Grown Foliage. Seeds of the previously described varieties were sown in the field at the Plant Science Field Laboratory, University of British Columbia campus, Vancouver. Blocks that included plots of each cultivar were sequentially planted to ensure that foliage of the desired

plant growth stages were available for testing when insect stocks were available. The preemergent herbicide trifluralin (Treflan E.C.) was applied to control weeds. Soluble 20–20–20 (N : P : K) fertilizer was applied weekly. Plots were subdivided such that individual plants were sampled once only.

Three broad plant growth stages (Harper, 1973) were examined in the study: rosette (stage 2), stem elongation (stage 3), and flowering (stage 4). Two blocks (= replicates in time) were sampled for each growth stage, except the flowering stage due to limitations in the insect culture. For each sampling interval, the following biological assays were carried out within a period of three days:

1. Neonate choice test. Leaf disks were punched from foliage using a 1.6-cm cork borer. Disks of each cultivar (eight in total) were arranged randomly and evenly spaced around the perimeter of a Petri dish (14 cm) on a moist filter paper disk. (12.5 cm, Whatman No. 1). One hundred neonate *M. configurata* larvae were introduced into the center of each dish, which was covered and left in darkness at 20°C for 16 hr, after which time the numbers of larvae on each leaf disk were recorded. Ten replicate Petri dishes (1000 insects) were set up for each sampling interval. Because the treatment variances were proportional to the means, the data were square-root transformed $(x + 0.5)^{0.5}$ prior to analysis.

2. Fourth-Instar Nutritional Indices. Individual leaves were excised from the plants and placed in a 10-cm Petri dish on a moist filter paper disk. A fourth-instar *M. configurata* larva, within a weight range of 13–22 mg, was placed on the leaf and allowed to feed for two days at 20°C with a 16 : 8 (hr light–dark) photoperiod. The following nutritional indices (Waldbauer, 1968) were calculated on a dry weight basis using the insect's weight at the start of the feeding period as the reference weight (Farrar et al., 1989):

$$\text{Relative consumption rate (RCRi)} = \frac{\text{food ingested/insect initial weight}}{\text{no. of days}}$$

$$\text{Relative growth rate (RGRi)} = \frac{\text{weight gained/initial weight}}{\text{no. of days}}$$

Sixteen insects per treatment were tested at each sampling date (replicate).

For the two described assays, ANOVA with idf tests was performed for each plant growth stage, and the treatment (cultivar) means were regressed against glucosinolate concentration for each block.

Glucosinolate Analysis. Bulk samples of foliage (a minimum of 20 plants) from each sampling interval were collected for analysis of glucosinolates. Foliage was quick-frozen with liquid N_2, lyophilized, and ground to a homogenous powder in a blender (maximum particle size 1 mm). Freeze-dried material was stored at −20°C prior to analysis.

Samples (100 mg) of powdered plant material were heated for 2 min in a boiling water bath before being extracted thrice with 3 ml of boiling 70% methanol for 2 min per extraction at the solvent boiling point. Methanol was removed from the pooled extracts by heating in a 40°C water bath under a stream of N_2. The aqueous solution was made up to 6 ml with water, and 125 μl of 0.6 M barium-lead acetate solution [0.3 M $Ba(CH_3COO)_2$ and 0.3 M $Pb(CH_3COO)_2$] was added to precipitate phenolics and free sulfate (McGregor, 1985). Following centrifugation, the supernatant was used for glucosinolate determination. Extraction efficiency averaged 83% as determined by HPLC of recovered desulfosinigrin from sinigrin-spiked Westar canola foliar slurries. Reported quantifications are the means of two separate extractions and analyses.

Total Glucosinolates. Total glucosinolates were quantified by the thymol method (Brzezinski and Mendelewski, 1984; Tholen et al., 1989). Glucosinolates were further purified from the supernatant by ion exchange on DEAE-Sephadex A-25 minicolumns regenerated with water. After washing with 2 × 1 ml of 30% w/v formic acid and 4 × 1 ml water, glucosinolates were eluted with 5 × 1 ml 0.3 M potassium sulfate. Aliquots (0.5 ml) were mixed with 100 μl of ethanolic 6% thymol and 2 ml 78% v/v sulfuric acid, and incubated for 35 min in a 93°C water bath. The absorbance of the solution was measured at 505 nm, and the linear regression equation of a standard curve of sinigrin was used for quantification.

Individual Glucosinolates. These were determined by HPLC of their desulfo derivatives combining the methods of McGregor (1985) and Bjerg and Sorensen (1987). The supernatant was applied to a 100-mg (dry weight) minicolumn of DEAE-Sephadex A-25 regenerated in 0.02 M sodium acetate buffer (pH 5.0). After washing with 4 × 1 ml of water and 4 × 1 ml of the sodium acetate buffer, a 0.5-ml aliquot of aryl sulfatase (EC 3.1.6.1) (Sigma, H-1) solution, prepared as per McGregor (1985) was applied to the column. After 20 hr in darkness, desulfoglucosinolates were eluted with 4 × 1 ml water, concentrated to dryness using a freeze-drier, and taken up in 400 μl water in an ultrasonic bath. Twenty-microliter aliquots were analyzed by HPLC.

The analytical column used was either a Waters Nova-Pak or a Phenomenex Bondclone (both C-18, 3.9 × 150 mm). This was preceded by a 2 × 20-mm guard column packed with C-18 pellicular packing (Perisorb, Upchurch Scientific). The solvent program consisted of water and acetonitrile at the following ratios: 99:1 (water–acetonitrile) for 8 min, followed by a linear gradient to 76:24 at 34 min, and held at this ratio until 36 min. The flow rate was 0.6 ml/min, and the absorbance of desulfoglucosinolates was monitored at 226 nm. Quantifications were based on response factors relative to an internal standard of *O*-nitrophenyl-β-D-galactopyranoside (Sigma) as described by McGregor (1985). Identification of major peaks (Figures 1 and 2 below) was confirmed by HPLC-MS as per the methods of Hogge et al. (1988a, b). The quantities of

isothiocyanate- or thiocyanate-producing gsls from individual peaks were added and these values used in the correlation analyses with the biological data.

Bioassay of Pure Compounds in Meridic Diet. Sinigrin monohydrate (Sigma), allyl isothiocyanate (Eastman), and indole-3-carbinol (Sigma) were tested against *M. configurata* in meridic diets at concentrations of 0.5, 1.0, and 1.5 μmol/g wet weight. Sinigrin and indole-3-carbinol were dissolved in methanol and added to the dry mix of the previously described standard diet and the methanol allowed to evaporate for 5 hr prior to mixing with the agar–water portion. A control diet was prepared by adding methanol only. Allyl isothiocyanate, which is extremely volatile, was added directly to the molten diet prior to gelling. The solidified diets were divided into 30-ml plastic cups and a single neonate larva was introduced into each cup. The tightly capped cups were placed in a humidified plastic box inside a controlled environment chamber at 23°C with a 16:8 hr light–dark photoperiod for eight days, after which time the insects were weighed. Forty insects were used for each treatment, and the mean larval weights for each treatment were regressed against concentration for each compound.

RESULTS

HPLC glucosinolate profiles of our plants are qualitatively similar to those published for *B. juncea* and *B. napus* cv. Midas (Sang et al., 1984). The dominant glucosinolate in *B. juncea* is allyl glucosinolate (sinigrin, **1**); in *S. alba*, OH-benzyl glucosinolate (sinalbin, **2**) dominates (Figure 1). Among the rape species, the indolyl glucosinolates predominate, particularly 3-indolylmethyl glucosinolate **(8)** and its 4-hydroxy analog **(4)** (Figure 2).

The growth rate of neonate *M. configurata* as measured by larval weight after seven days was influenced by the cultivar upon which the larvae fed (Figure 3). Orthogonal contrasts revealed significant differences between mean larval weights by plant species in the order: *B. juncea* < *S. alba* < *B. napus* < *B. campestris*. There were no significant differences between cultivars within *B. juncea* or *B. napus*. However, within *B. campestris*, larvae reared on Tobin were significantly larger than those reared on Candle.

The relative acceptability of the various host plants to neonate larvae was indicated by the choice tests with leaf disks (Figure 4). Larval distribution on host plants of all growth stages tested was similar to the ranking of larval weights in the growth experiment (Table 1). The number of larvae found on the *B. juncea* leaf disks was significantly lower than on the other species. However, *S. alba* consistently attracted significantly more insects than the other species. In agreement with the results of the growth experiment, *B. campestris* was more acceptable than *B. napus* rosette stage foliage. This ranking was reversed for

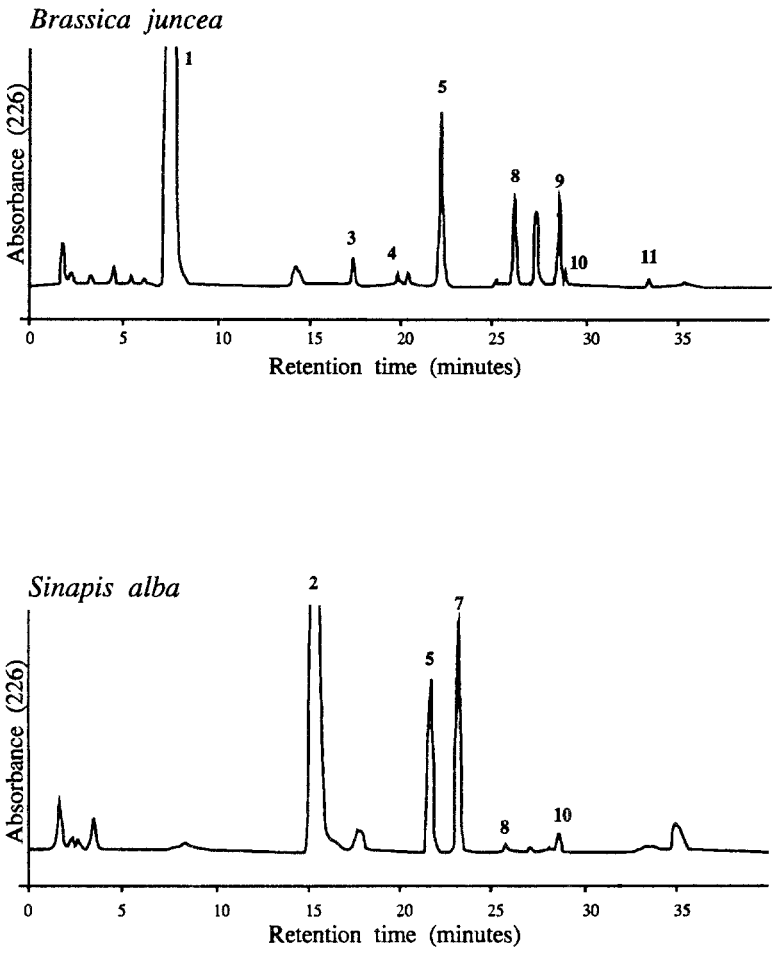


FIG. 1. Representative HLPC chromatograms of glucosinolate profiles for host plant species and/or cultivars used in the study. Peak numbers represent the following desulfoglucosinolates: **1,** allyl (sinigrin); **2,** OH-benzyl (sinalbin); **3,** 3-butenyl; **4,** 4-OH-3-indolylmethyl; **5,** ONPGal (internal standard); **6,** 4-pentenyl; **7,** benzyl; **8,** 3-indolylmethyl; **9,** 2-phenylethyl; **10,** 4-methoxy-3-indolylmethyl; **11,** 1-methoxy-3-indolylmethyl.

stage three foliage, and there was no significant difference between the attractiveness of the two species for flowering stage foliage. Within *B. napus*, the numbers of larvae on the high-glucosinolate cultivar Midas did not differ significantly from the low-glucosinolate cultivars, Regent and Westar. However, with stage 3 foliage, Westar attracted significantly more larvae than Regent.

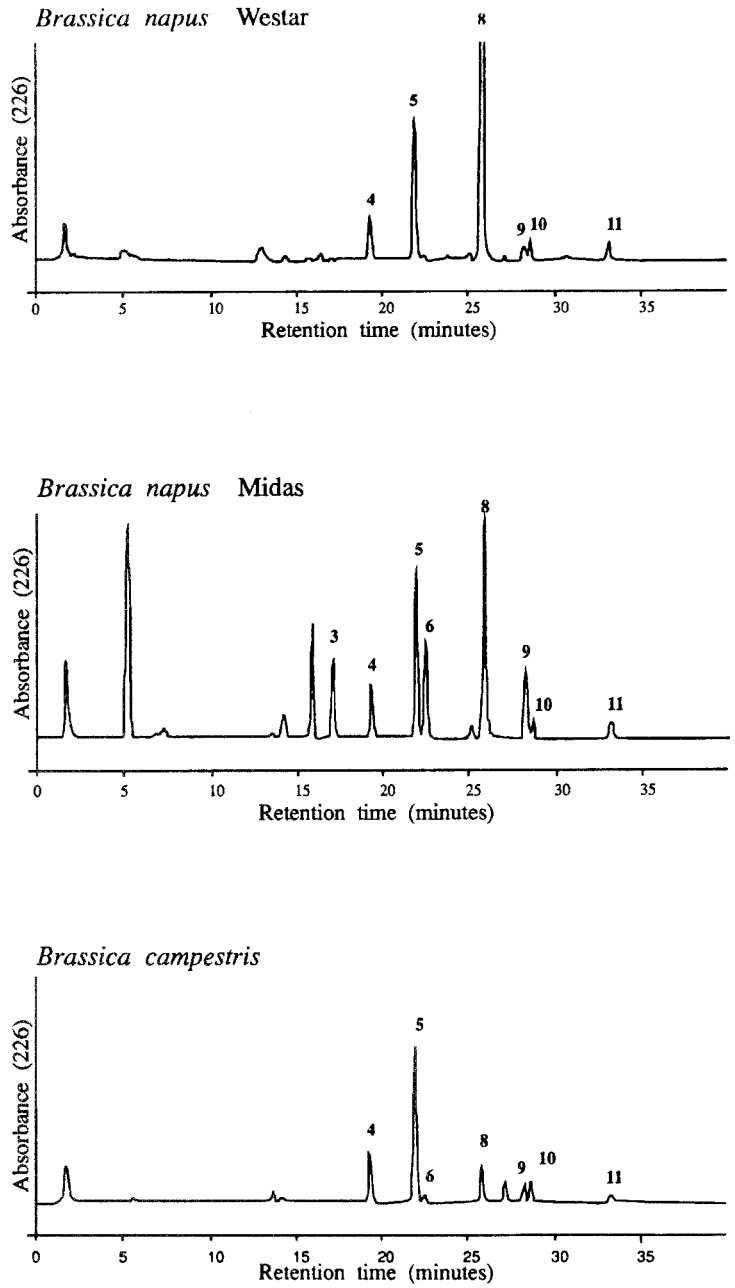
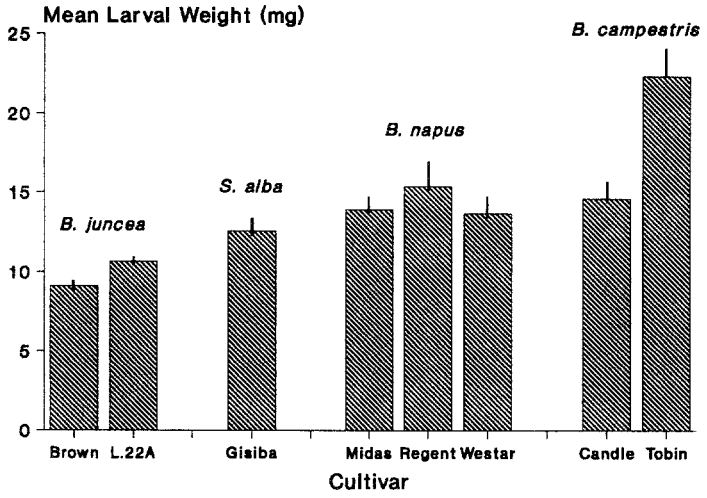


FIG. 2. Representative HPLC chromatograms of glucosinolate profiles for rape species used in the study. Peak numbers as in Figure 1.



I.D.F. Tests

Contrast	F	Pr>F
B. juncea vs others	35.57	.0001
S. alba vs *B. napus* + *B. campestris*	7.72	.0058
B. napus vs *B. campestris*	13.99	.0002
B. juncea: Brown vs Lethbridge 22A	0.96	.3288
B. napus: Midas vs Regent + Westar	0.10	.7526
B. napus: Regent vs Westar	0.89	.3458
B. campestris: Candle vs Tobin	21.63	.0001

FIG. 3. Mean final weights of *M. configurata* larvae reared for seven days on intact rosette-stage plants. Vertical lines above bars represent SEM.

Acceptability to neonates was not correlated to foliar concentrations of total glucosinolates (Table 2). In general, the isothiocyanate-releasing glucosinolates had a negative influence on acceptability, while the thiocyanate-releasing glucosinolates had a positive influence. The influence of glucosinolates on acceptability to neonates was more obvious with stage 3 and stage 4 foliage than with rosette foliage. For stage 3 plants, the numbers of larvae attracted to the leaf disks was negatively correlated to the isothiocyanate-releasing glucosinolate concentration in the foliage, and this was consistent over both blocks of the experiment.

The consumption and growth rates of fourth-instar *M. configurata* were

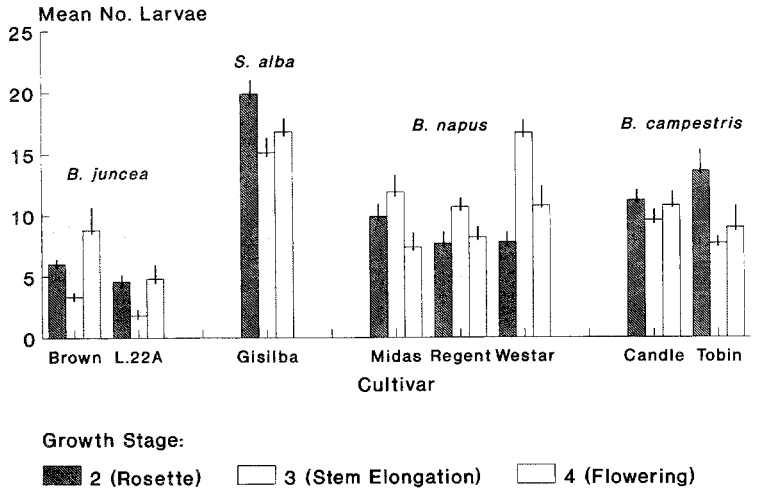


FIG. 4. Numbers of neonate *M. configurata* on leaf disks in choice tests with field-grown foliage, presented as weighted means of square-root transformed data. Vertical lines above bars represent SEM.

TABLE 1. EFFECT OF HOST SPECIES AND CULTIVAR ON NEONATE *M. configurata* HOST SELECTION (BASED ON LEAF DISC CHOICE TESTS), AND RELATIVE CONSUMPTION RATE (RCRi), AND RELATIVE GROWTH RATE (RGRi) OF FOURTH INSTAR LARVAE AS DETERMINED BY ANOVA AND ORTHOGONAL CONTRASTS

	Growth stage								
	Host choice			RCRi			RGRi		
Contrast	2	3	4	2	3	4	2	3	4
B. juncea vs. others	**[a]	**	*	nsd	**	*	nsd	**	**
S. alba vs. *B. napus* + *B. campestris*	**	*	**	nsd	**	nsd	nsd	nsd	nsd
B. napus vs. *B. campestris*	**	**	nsd	nsd	nsd	nsd	nsd	nsd	nsd
B. juncea: Brown vs. Lethbridge 22A	nsd	nsd	nsd	*	nsd	nsd	nsd	nsd	nsd
B. napus									
Midas vs. Regent + Westar	nsd	nsd	nsd	*	nsd	nsd	nsd	*	nsd
Regent vs. Westar	nsd	**	nsd	nsd	nsd	nsd	nsd	nsd	*
B. campestris: Candle vs. Tobin	nsd	nsd	nsd	nsd	nsd	nsd	nsd	nsd	nsd

[a] * = significant at $P < 0.05$; ** = significant at $P < 0.01$; nsd = no significant difference.

TABLE 2. LINEAR CORRELATION COEFFICIENTS FOR RELATIONSHIP OF FOLIAR GLUCOSINOLATES (μmol/g dry wt) TO *M. configurata* NEONATE CHOICE (WEIGHTED MEANS OF SQUARE ROOT-TRANSFORMED COUNTS) AND TO RELATIVE CONSUMPTION RATE (RCRi) AND RELATIVE GROWTH RATE (RGRi) OF FOURTH-NSTAR LARVAE

	Neonate host choice			Fourth-instar RCRi			Fourth-instar RGRi		
Growth stage	Total	ITC type[a]	SCN type[b]	Total	ITC type	SCN type	Total	ITC type	SCN type
2, Rosette									
Block 1	$r = -0.300$	$r = -0.409$	$r = 0.840$	$r = -0.611$	$r = -0.642$	$r = 0.123$	$r = -0.909$	$r = -0.60$	$r = -0.032$
	$P = 0.470$	$P = 0.314$	$P = 0.009$	$P = 0.108$	$P = 0.086$	$P = 0.773$	$P = 0.002$	$P = 0.116$	$P = 0.953$
Block 2	$r = 0.063$	$r = -0.524$	$r = 0.476$	$r = -0.669$	$r = 0$	$r = 0.176$	$r = 0$	$r = 0.095$	$r = -0.434$
	$P = 0.893$	$P = 0.183$	$P = 0.232$	$P = 0.100$	$P = 0.966$	$P = 0.675$	$P = 0.991$	$P = 0.827$	$P = 0.284$
3, Stem elongation									
Block 1	$r = -0.557$	$r = -0.787$	$r = 0.634$	$r = -0.580$	$r = -0.266$	$r = -0.804$	$r = -0.817$	$r = -0.887$	$r = 0.253$
	$P = 0.151$	$P = 0.020$	$P = 0.091$	$P = 0.132$	$P = 0.524$	$P = 0.016$	$P = 0.013$	$P = 0.003$	$P = 0.545$
Block 2	$r = -0.692$	$r = -0.766$	$r = 0.383$	$r = -0.840$	$r = -0.75$	$r = -0.173$	$r = -0.857$	$r = -0.778$	$r = -0.063$
	$P = 0.057$	$P = 0.027$	$P = 0.348$	$P = 0.009$	$P = 0.032$	$P = 0.682$	$P = 0.007$	$P = 0.023$	$P = 0.886$
4, Flowering									
Block 1	$r = -0.265$	$r = -0.533$	$r = 0.801$	$r = -0.574$	$r = -0.530$	$r = 0.207$	$r = -0.807$	$r = -0.743$	$r = 0.145$
	$P = 0.528$	$P = 0.174$	$P = 0.017$	$P = 0.136$	$P = 0.176$	$P = 0.620$	$P = 0.016$	$P = 0.035$	$P = 0.731$

[a] Isothiocyanate (ITC) releasing glucosinolates.
[b] Thiocyanate-releasing glucosinolates.

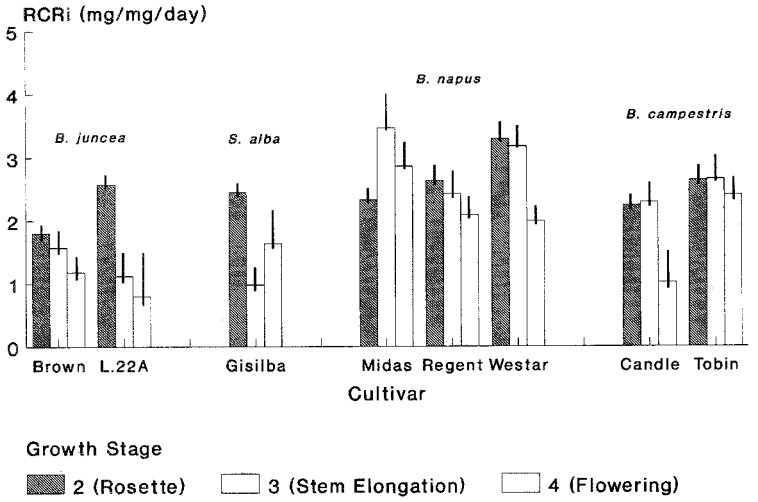


FIG. 5. Mean consumption indices for fourth-instar *M. configurata* larvae on field-grown foliage. Vertical lines above bars represent SEM.

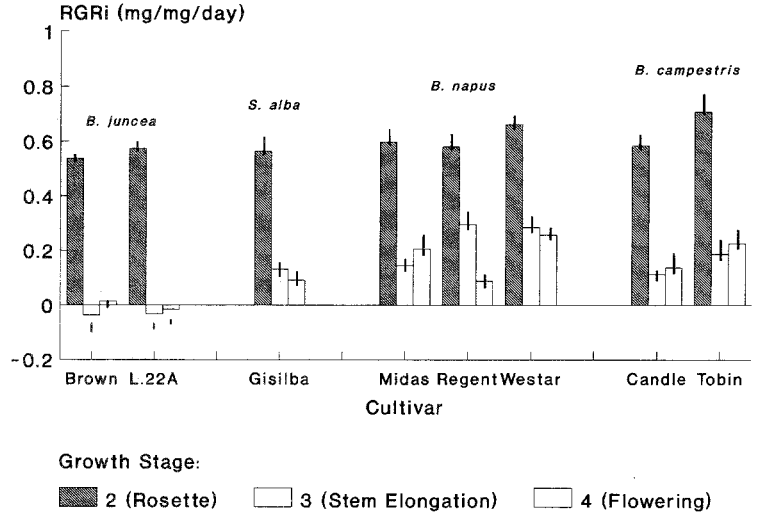


FIG. 6. Mean relative growth rates of fourth-instar *M. configurata* larvae on field-grown foliage. Vertical lines above bars represent SEM.

also influenced by the host species and cultivar (Figures 5 and 6). Predictably, lower consumption rates (RCRi) were observed on *B. juncea* than on the other host plants (Table 1), even though the difference was of borderline significance ($P = 0.0552$) for rosette stage foliage. However, the attractiveness of *S. alba* observed in the neonate choice tests was not reflected by the fourth-instar consumption rates. In fact, the mean RCRi on *S. alba* was significantly lower than those on *B. napus* and *B. campestris* stage 3 foliage. There were no significant differences in mean RCRi between *B. napus* and *B. campestris* for foliage of any growth stage.

Relative growth rates (RGRi) of fourth-instar larvae were reduced on *B. juncea* compared to the other plant species for stage 3 and 4 foliage, and these differences were highly significant (Table 1). However, no significant differences were detected for the rosette stage foliage in any of the contrasts. *S. alba* did not appear to inhibit the growth of fourth-instar larvae as observed with neonates. The comparison of *B. napus* with *B. campestris* also produced no significant differences, although with stage 3 foliage the growth rate of larvae on *B. napus* was somewhat reduced compared to that on *B. campestris* ($P = 0.06$). This was most likely due to the influence of the high-glucosinolate *B. napus* cv. Midas, which yielded a significantly reduced RGRi compared with the low-glucosinolate cultivars Regent and Westar.

These effects on fourth-instar *M. configurata* can be linked to the glucosinolate composition of the plants in some cases. However, a relationship between RCRi and glucosinolate content is not well-defined. Consumption rate tends to be inversely related to the concentration of total glucosinolates, but there was no significant linear correlation except in the second block of the stem elongation stage foliage (Table 2). Simple linear correlations between RCRi and isothiocyanate- and thiocyanate-releasing glucosinolates were also equivocal.

The relationship of RGRi and glucosinolate content appears to be relatively straightforward. RGRi is significantly negatively correlated to total glucosinolate content in the more mature plant growth stages, although the results are inconsistent in the rosette stage (Table 2). These correlations appear to be mainly due to the concentration of isothiocyanate-releasing glucosinolates, as RGRi is also significantly negatively correlated to the levels of these glucosinolates, but not to thiocyanate-releasing glucosinolates.

The data generated by feeding studies using intact plant tissue is partially supported by the results of the feeding study using pure compounds in meridic diet (Figure 7). The negative growth responses of neonate *M. configurata* larvae to equimolar concentrations of sinigrin and its metabolite, allyl isothiocyanate are similar, in that significant linear relationships between larval weight and dietary allelochemical concentration were obtained. However, allyl isothiocyanate in the diets resulted in abnormally high mortality rates, which was partially due to the fumigation effect of this volatile compound (unpublished data). No

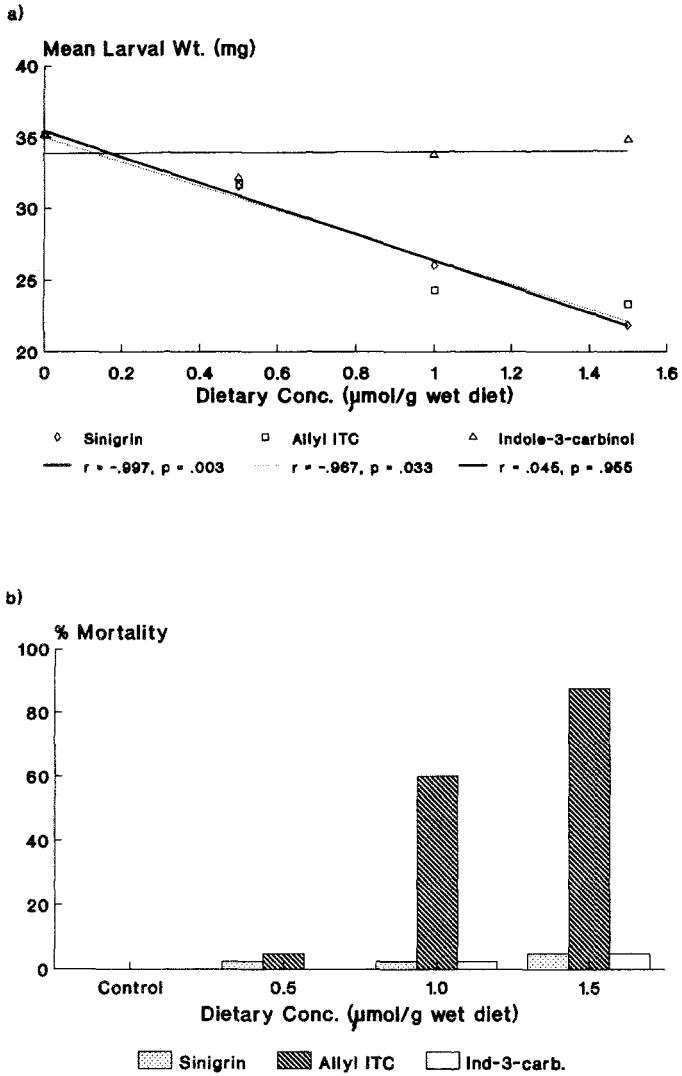


FIG. 7. Results of eight-day feeding assay with pure compounds augmented to meridic diet: (a) linear regression of final weight versus dietary concentration; (b) percent mortality versus dietary concentration.

such growth or mortality responses were observed with indole-3-carbinol, the degradation product of 3-indolylmethyl glucosinolate, which suggests that indolyl alcohols are not biologically active at the concentrations tested.

DISCUSSION

Our feeding studies using artificial diets and various rapes and mustards have established clearly that sinigrin and its metabolite, allyl isothiocyanate, adversely affect the growth of neonate and fourth-instar *M. configurata* larvae. These effects may be due in part to reduced rates of feeding on substrates that contain sinigrin. *B. juncea*, which contains very high levels of sinigrin, is relatively resistant to this polyphagous insect. This mustard was also found to be less preferred by the flea beetle, *Phyllotreta striolata*, than *B. oleracea*, *B. napus*, and *B. campestris* (Lamb and Palaniswamy, 1990). Currently, *B. juncea* is being considered for development as an oilseed because of its superior agronomic performance compared to the current canola species (Woods et al., 1991). The expression of high levels of sinigrin in the foliage may be viewed as another desirable trait of this mustard species.

The effects of thiocyanate-releasing glucosinolates are not as well defined as the effects of isothiocyanate-releasing glucosinolates. The artificial diet test of indole-3-carbinol suggests that this type of glucosinolate metabolite is relatively innocuous to neonate *M. configurata* larvae. However, the leaf disk choice tests suggest that OH-benzyl glucosinolate (sinalbin) may be a feeding stimulant for neonate larvae. Yet, larval growth on intact *S. alba* plants, which produce predominantly sinalbin, was relatively inhibited. Sinalbin previously has been shown to be a factor of antixenotic and antibiotic resistance to Bertha armyworm and flea beetles (Bodnaryk, 1991). However, it should be noted that benzyl glucosinolate occurs in significant amounts (0.7–3.9 μmol/g dry wt, depending on growth stage) in *S. alba* cv. Gisilba. Benzyl isothiocyanate has been shown to be toxic to European corn borer and fall armyworm (Bartlet and Mikolajczak, 1989). It is possible that benzyl glucosinolate may be partially responsible for the growth inhibitory effects observed with *M. configurata* on intact plants. The feeding stimulant effect we observed with *S. alba* in the neonate choice test may simply be due to the presence of relatively high glucoside concentrations with lower levels of associated isothiocyanates.

Thiocyanate-releasing glucosinolates in foliage do not appear to influence consumption by fourth-instar *M. configurata*. Rather, their relative consumption rate is actually reduced on stage 3 foliage of *S. alba*. Since there was no corresponding reduction in larval growth rate attributable to thiocyanate-releasing glucosinolates, it is probable that other factors also affect the consumption of foliage. In the Brassicaceae, glucosinolates are the dominating group of sec-

ondary plant substances and must be considered in questions of insect–plant interactions. However, substrate texture, nutrients, and other types of allelochemicals can also affect the response of insects to these plants. The data from this study suggest that plant growth stage can modify the relative expression of allelochemical effects. This will be the subject of a future paper.

Presently, rapeseed plant breeding efforts are directed towards eliminating glucosinolates due to their antinutritional effects on livestock that consume the seed meal. Although the canola cultivars are nutritionally superior to the mustards in this regard, our study suggests that isothiocyanate-releasing glucosinolates in the foliage may benefit the crop by providing a degree of protection from polyphagous insects like the Bertha armyworm. To this end, a useful target for genetic engineering may be the expression of isothiocyanate-releasing glucosinolates specifically in the foliage coupled with the elimination of glucosinolates from the seed.

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REFERENCES

AHMAN, I. 1982. A comparison between high and low glucosinolate cultivars of summer oilseed rape (*Brassica napus* L.) with regard to their levels of infestation by the Brassica pod midge (*Dasineura brassicae* Winn). *Z. Angew. Entomol.* 94:103–109.

AHMAN, I. 1986. Toxicities of host secondary compounds to eggs of the *Brassica* specialist *Dasineura brassicae*. *J. Chem. Ecol.* 12:1481–1488.

BARTELT, R.J., and MIKOLAJCZAK, K.L. 1989. Toxicity of compounds derived from *Limnanthes alba* seed to fall armyworm (Lepidoptera: Noctuidae) and European corn borer (Lepidoptera: Pyralidae) larvae. *J. Econ. Entomol.* 82:1054–1060.

BJERG, B., and SORENSEN, H. 1987. Quantitative analysis of glucosinolates in oilseed rape based on HPLC of desulfoglucosinolates and HPLC of intact glucosinolates, pp. 125–150, *in* J.-P. Wathelet (ed.). Glucosinolates in Rapeseeds: Analytical Aspects. Martinus Nijhoff, Dordrecht.

BLAU, P.A., FEENY, P., CONTARDO, L., and ROBINSON, D.S. 1978. Allylglucosinolate and herbivorous caterpillars: A contrast in toxicity and tolerance. *Science* 200:1296–1298.

BODNARYK, R.P. 1991. Developmental profile of sinalbin (*p*-hydroxybenzyl glucosinolate) in mustard seedlings, *Sinapis alba* L., and its relationship to insect resistance. *J. Chem. Ecol.* 17:1543–1556.

BODNARYK, R.P., and PALANISWAMY, P. 1990. Glucosinolate levels in cotyledons of mustard, *Brassica juncea* L. and rape, *Brassica napus* L. do not determine feeding rates of flea beetle, *Phyllotreta cruciferae* (Goeze). *J. Chem. Ecol.* 16:2735–2746.

BRZEZINSKI, W. and MENDELEWSKI, P. 1984. Determination of total glucosinolate content in rapeseed meal with thymol reagent. *Z. Pflanzenzuecht.* 93:177–183.

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.

BUTTS, R.A. and LAMB, R.J. 1990. Comparison of oilseed *Brassica* crops with high or low levels of glucosinolates and alfalfa as hosts for three species of *Lygus* (Hemiptera: Heteroptera: Miridae). *J. Econ. Entomol.* 83:2258–2262.

DAVID, W.A.L., and GARDINER, B.O.C. 1966. Mustard oil glucosides as feeding stimulants for *Pieris brassicae* larvae in a semisynthetic diet. *Entomol. Exp. Appl.* 9:247–255.

FARRAR, R., JR., BARBOUR, J., and KENNEDY, G.G. 1989. Quantifying food consumption in insects. *Ann. Entomol. Soc. Am.* 82:593–598.

FEENY, P., PAAUWE, K.L., and DEMONG, N.J. 1970. Flea beetles and mustard oil: Host plant specificity of *Phyllotreta cruciferae* and *P. striolata* adults (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 63:832–841.

FINCH, S. 1978. Volatile plant chemicals and their effect on host plant finding by the cabbage root fly (*Delia brassicae*). *Entomol. Exp. Appl.* 24:350–359.

HARPER, F.R. 1973. A key to standardize the description of growth stages in turnip rape, *Brassica campestris*. *Can. Plant Dis. Surv.* 53:93–95.

HICKS, K.L. 1974. Mustard oil glucosides: Feeding stimulants for adult cabbage flea beetle, *Phyllotreta cruciferae* (Coleoptera: Chrysonalidae). *Ann. Entomol. Soc. Am.* 67:261–264.

HOGGE, L.R., REED, D.W., and UNDERHILL, E.W. 1988a. The identification of desulfoglucosinolates using thermospray liquid chromatography/mass spectrometry. *J. Chromatogr. Sci.* 26:348–351.

HOGGE, L.R., REED, D.W., UNDERHILL, E.W., and HAUGHN G.W. 1988b. HPLC separation of glucosinolates from leaves and seeds of *Arabidopsis thaliana* and their identification using thermospray liquid chromatography/mass spectrometry. *J. Chromatogr. Sci.* 26:551–556.

LAMB, R.J. 1988. Susceptibility of low- and high-glucosinolate oilseed rapes to damage by flea beetles, *Phyllotreta* spp. (Coleoptera: Chrysomelidae). *Can. Entomol.* 120:195–196.

LAMB, R.J. 1989. Entomology of oilseed *Brassica* crops. *Annu. Rev. Entomol.* 34:211-229.

LAMB, R.J., and PALANISWAMY, P. 1990. Host discrimination by a crucifer-feeding flea beetle, *Phyllotreta striolata* (F.) (Coleoptera: Chrysomelidae). *Can. Entomol.* 122:817–824.

LICHTENSTEIN, E.P., STRONG, F.M. and MORGAN, D.G. 1962. Identification of 2-phenylethylisothiocyanate as an insecticide occurring naturally in the edible part of turnips. *J. Agric. Food Chem.* 10:30–33.

LICHTENSTEIN, E.P., MORGAN, D.G., and MUELLER, C.H. 1964. Naturally occurring insecticides in cruciferous crops. *J. Agric. Food Chem.* 12:158–161.

LOWE, M.D., HENZELL, R.F., and TAYLOR, H.J. 1971. Insecticidal activity to soldier fly larvae, *Inopus rubriceps* (Macq.) of isothiocyanates occurring in "choumoellier" (*Brassica oleracea* cv.). *N.Z. J. Sci.* 14:322–326.

MCGREGOR, D.I. 1985 Determination of glucosinolates in *Brassica* seed. *Eucarpia Cruciferae Newsl.* 10:132–136.

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.

NAULT, L.R., and STYLER, W.E. 1972. Effects of sinigrin on host selection by aphids. *Entomol. Exp. Appl.* 15:423–431.

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.* 41:923–929.

READ, D.P., FEENY, P.P., and ROOT, R.B. 1970. Habitat selection by the aphid parasite *Diaeretiella* rapae (Hymenoptera: Braconidae) and hyperparasite *Charips brassicae* (Hymenoptera: Cynipidae). *Can. Entomol.* 102:1567–1578.

REED, D.W., PIVNICK, K.A., and UNDERHILL, E.W. 1989. Identification of chemical oviposition stimulants for the diamondback moth, *Plutella xylostella*, present in three species of Brassicaceae. *Entomol. Exp. Appl.* 53:277–286.

RYGG, T. and SOMME, L. 1972. Oviposition and larval development of *Hylemya floralis* (Fallen) (Diptera, Anthomyidae) on various varieties of swedes (*B. napus*) and turnips (*B. rapa*). *Norsk. Entomol. Tidsskr.* 19:81–90.

SANG, J.P., MINCHINTON, I.R., JOHNSTONE, P.K., and TRUSCOTT, R.J.W. 1984. Glucosinolate profiles in seed, root and leaf tissue of cabbage, mustard, rapeseed, radish and swede. *Can. J. Plant Sci.* 64:77–93.

THOLEN, J.T., SHIFENG, S. and TRUSCOTT, R.J.W. 1989. The thymol method for glucosinolate determination. *J. Sci. Food Agric.* 49:157–165.

TRAYNIER, R.M.M. 1965. Chemostimulation of oviposition by the cabbage root fly, *Erioischia brassicae* (Bouche). *Nature* 207:218–219.

TRAYNIER, R.M.M. and TRUSCOTT, R.J.W. 1991. Potent natural egg-laying stimulant for cabbage butterfly *Pieris rapae*. *J. Chem. Ecol.* 17:1371–1380.

VERSCHAFFELT, E. 1911. The cause determining the selection of food in some herbivorous insects. *K. Acad. Van Wetenschappen Proc. Sect. Sci.* 13:536–542.

WALDBAUER, G.P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol.* 5:229–288.

WILLIAMS, I.H. 1989. Pest incidence on single low and double low oilseed rape cultivars. *Aspects Appl. Biol.* 23:277.

WOODS, D.L., CAPCARA, J.J., and DOWNEY, R.K. 1991. The potential of mustard [*Brassica juncea* (L.) Coss] as an edible oil crop on the Canadian prairies. *Can. J. Plant Sci.* 71:195–198.

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BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSES OF THE BANANA WEEVIL *Cosmopolites sordidus* TO HOST PLANT VOLATILES

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Abstract—Male and female *Cosmopolites sordidus* were attracted to freshly cut banana rhizome and pseudostem in a still-air olfactometer. Females responded similarly to odors from a comparatively resistant and from a susceptible cultivar of banana, when presented as either freshly cut tissue or as Porapak-trapped volatiles. Females were also attracted to rotting banana pseudostem and to volatiles collected from it. Males and females gave similar responses to host tissue in both the behavioral bioassay and to collected volatiles in EAG recordings. Weevils did not respond, either behaviorally or electrophysiologically, to a synthetic mixture of mono- and sesqiterpenes, which made up over 9% of the volatiles collected from pseudostem.

Key Words—Coleoptera, Curculionidae, *Cosmopolites sordidus*, banana weevil, *Musa* sp., pseudostem, rhizome, EAG, olfactometer.

INTRODUCTION

The banana weevil, *Cosmopolites sordidus* (Germar), is a pest in all major banana (*Musa* sp.) growing areas of the world (Ostmark, 1974; Waterhouse and Norris, 1987), but particularly on cooking bananas (Sikora et al., 1989) and plantains (Jones, 1986). The weevil lays eggs in the rhizome of the plant, and

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the larvae tunnel and feed in the rhizome, weakening the plant, reducing bunch weight and, in serious cases, leading to snapping of the rhizome at ground level before the bunch is ripe. Adult weevils cause no significant damage, and mainly feed on rotting banana tissue.

Populations of banana weevils may be monitored in the field (Mitchell, 1978), and even possibly controlled (Wallace, 1938; Yaringano and van der Meer, 1975), by using split pseudostem or rhizome traps. It is probable that weevils are attracted to these traps by volatiles released at the cut surfaces. Fresh traps are more effective than old ones (Delattre, 1980; Simmonds and Simmonds, 1953; Hord and Flippin, 1956). Laboratory experiments by both Cuille (1950) and Mitchell (1980) report attraction of the weevil by volatiles from the rhizome. Here we report the initial stages of a study aimed at identifying attractive kairomones from banana pseudostems and rhizomes. We have used two cultivars that are widely grown in the Nairobi area, namely (in Kikuyu), Githumo, a triploid (AAA) East African highland cooking type highly susceptible to weevil attack, and Wangae, a diploid (AB) desert type that appears comparatively resistant to weevil attack.

METHODS AND MATERIALS

Insects. C. sordidus used in the behavioral bioassays were collected from split pseudostem traps in a banana plantation in Nairobi. The weevils were sexed, and the sexes were subsequently kept separately. About 120 individuals were kept with 0- to 10-day-old Githumo pseudostem in 4.5-liter plastic jars at 24 ± 2°C, 12 hr light–12 hr dark, with the scotophase starting at 0900 hr. Before use in a bioassay, weevils were removed from the pseudostem between 0800 and 0900 hr and put in groups of five in small tubes with damp tissue paper. Bioassays were run between 1030 and 1630 hr, and weevils were then returned to their holding jars. The same weevils were never used in bioassays on consecutive days. *C. sordidus* used for electrophysiology were also field caught and were kept with pseudostem. They were removed from the pseudostem and put with moist tissue paper overnight before use to ensure that sensillae on the antennae were not covered by any deposit of material.

Plant Material and Collection of Volatiles. Pseudostems and rhizomes of the two banana cultivars (Githumo and Wangae) were collected immediately after harvest of the bunch from plants uninfested with banana weevil. Volatiles were collected from the headspace of a 5-liter flask filled with chopped pieces of plant material. Air was drawn through the flask (400 ml/min for 24 hr) via two columns (8 × 2 cm), containing 7.1 g of Porapak Q, 80–100 mesh (Chrompack). The Porapak was prepared by eluting with dichloromethane (Aldrich, HPLC grade) before activation at 150°C in a stream of N_2 (white spot). The

column at the inlet cleaned the air on entry into the flask while the one at the outlet trapped volatiles from the banana tissue. Odor collections were made simultaneously from the two cultivars for each tissue type, on two separate occasions. Volatiles were then eluted from the traps with 15 ml dichloromethane (Aldrich, HPLC grade), and the eluant was evaporated under a gentle stream of N_2 (white spot) to 3 ml, 1.5 ml of which was used for the bioassay and the remainder for electrophysiology.

Pseudostem was allowed to rot in the 5-liter flasks, and bioassays were conducted with material from these after four days. Volatiles from the 4-day-old rotting pseudostem were collected continuously for the next three days using activated charcoal traps (0.1 g, prepared in a similar manner to the Porapak) and a flow rate of 22 ml/min. Volatiles were eluted from the charcoal traps with 0.5 ml of dichloromethane. A collection of volatiles from fresh Githumo pseudostem was made similarly using charcoal traps and a flow rate of 22 ml/min for 24 hr. The major components of these volatiles had been previously identified (Ndiege et al., 1991), and the unidentified compound has now been identified as ocimene. These were mixed together to form a solution similar in concentration to the collected volatiles (Table 4 below).

Behavioral Bioassay. A still-air olfactometer (Figure 1, top), modified from Phillips and Burkholder (1981), was chosen for this work because adult *C. sordidus* live in rotting vegetation in or on the soil where there is little air movement. The apparatus consisted of a round ground glass plate (21 cm diam.) with two holes (13 mm diam.) drilled in it lying on one diameter, 5 cm either side of the center. A 10-cm-high Perspex ring (19 cm diam.) was placed on top of the plate and covered with an intact glass disk. Odor sources were put in jars (Figure 1, bottom), which were placed directly underneath the holes in the lower plate. All bioassays involved testing an odor source in one jar against a blank control in the other jar. Two series of experiments were done, one using chopped pieces (0.5–2 cm) of plant material that were put in the jars ($\approx$50 ml) and covered with a thin piece of dry cotton wool (to try to reduce any possible visual stimulus). The control jar contained wet cotton wool in place of the plant material. The second series employed the collected volatiles that were tested by treating filter papers (5 cm diam.) with 1.5 ml of the odor sample. Control papers were treated with 1.5 ml dichloromethane alone. After the dichloromethane had evaporated, the papers were folded to fit into the top of the jars, which had damp cotton wool at the base (Figure 1, bottom). Bioassays comparing the same material from the two cultivars were carried out concurrently. Observers of bioassays involving collected volatiles were unaware of the identity of the jars. For the bioassay with the volatiles collected from rotting pseudostem, two thirds of the collection was used, and the entire collection (0.4 ml) was used for the comparison of the natural and synthetic blend of volatiles from Githumo pseudostem.

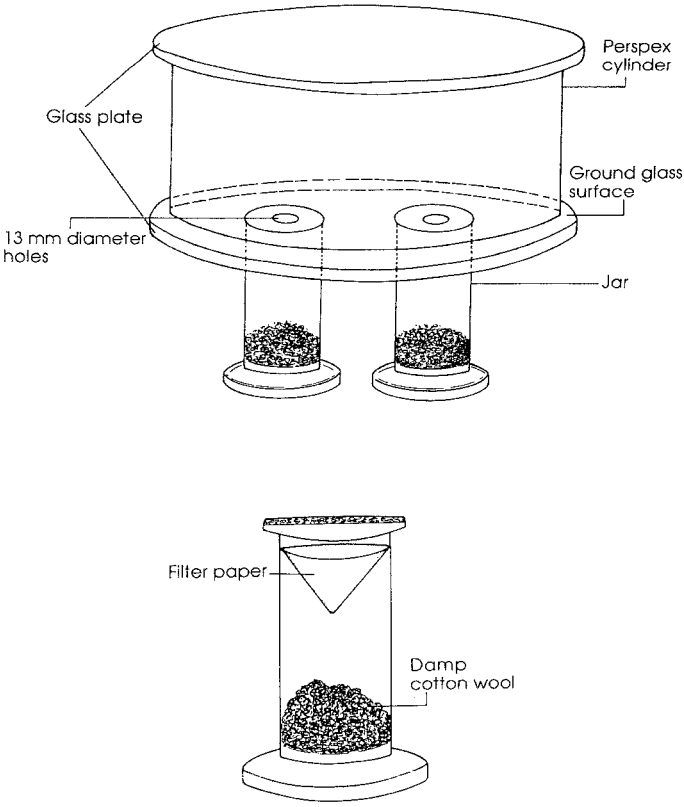

FIG. 1. Top: The bioassay apparatus; bottom: a jar with filter paper.

Bioassays were conducted in a small fan-ventilated room (25 ± 1°C, ≈80% relative humidity). The only light was provided by a light box covered with a deep red filter (Kodak Wratten No. 70, transmitting only wavelengths greater than 640 nm). Two bioassays were observed concurrently. Bioassays were started by putting five weevils approximately equidistant from the holes on the moist plate (humidity in the chamber was 100%). The weevils moved slowly and very rarely fell into the jars. Continuous observations were made and each visit to a hole and its duration was recorded. A visit was scored when any part of the weevil came within 5 mm of the edge of the hole and was terminated when all parts of the weevil left this area. Each bioassay lasted for 10 min and glass plates were washed with soap and water after each run.

Electroantennogram (EAG) Recordings. Sensillae on the antenna of *C. sordidus* are concentrated on a cone that forms the end of the last segment of the antenna. The flagellum was cut from the pedicel and its proximal end

was mounted in the reference electrode so that only the distal segment was exposed. The recording electrode was inserted into a small hole that had been made in the cone on the distal segment using an electrolytically etched tungsten needle. Electrodes were made of extruded glass micropipetes filled with Beadles' Ringer solution and were connected to the probe (amplifier) with chlorinated silver wires. The output from the probe was connected directly to an interface card in a personal computer, and recordings were saved onto the hard disk. The probe, interface, and software were supplied by Syntech Ltd.

The extracts containing the volatile odors to be tested were applied to pieces of filter paper (6 × 1 cm). The dichloromethane was allowed to evaporate, and the papers were then put individually into a disposable Pasteur pipete. Odor stimulation was performed by a puff of air (0.45 liters/min for 0.2 sec) through the pipete into a humidified airstream (2.1 liters/min) blowing over the antenna. Each antenna was stimulated with three doses (1, 10, and 100 μl) of the volatiles from the rhizome and pseudostem of both Githumo and Wangae. Eight male and eight female antennae were tested against collections from each tissue type. A reference stimulus of 10 μl of 10^{-1} dilution of nonanol in paraffin oil was applied after every two stimulations with test odors. Responses to the test odors were expressed relative to the reference stimulus (=100), using linear interpolation between the preceding and subsequent reference stimuli. EAGs to 10 μl of the natural and synthetic blends were recorded using a slightly different technique; the flagellum of the antenna was mounted in a glass electrode, and the recording electrode (tip diameter $\approx$ 0.5 ml) was placed over the tip of the intact cone of the antenna.

Statistical Analysis. For each run of the bioassay the number of excess visits to the treatment was calculated (i.e., number of visits to the treatment hole less the number of visits to the control hole). These numbers were then tested, using the nonparametric signed rank statistic, to see if they were different from zero (the expected number if there were no response to the treatment). Comparisons were made between the numbers of excess visits by males and females to a particular tissue, and by females to odors from the two cultivars, using the Wilcoxon rank-sum test. (These comparisons were only made when experiments had been carried out simultaneously, as the responsiveness of the weevils varied between days). The duration of visits to treatments and controls were compared using t tests on log-transformed data. In all cases differences were deemed to be significant at $P < 0.05$.

RESULTS

Both male and female *C. sordidus* visited chopped Githumo rhizome and pseudostem more frequently than they did the control (Table 1). There were, however, no significant differences between the numbers of excess visits made by males and females to the two tissues.

Female *C. sordidus* made excess visits to fresh pseudostem and rhizome from Wangae and Githumo, to rotting Githumo pseudostem, and to Porapak-trapped volatiles collected from these banana materials (Table 2). There were no significant differences between the numbers of excess visits made to Wangae and Githumo for any particular material (e.g., collected volatiles from pseudostem), although overall (i.e., including fresh tissue and collected volatiles from both pseudostem and rhizome) Githumo had significantly ($P < 0.05$) more excess visits than Wangae. Visits to the chopped material of pseudostem and rhizome of Wangae and Githumo were significantly ($P < 0.001$) longer than to control holes (Table 3). However, visits to collected volatiles were signifi-

TABLE 1. RESPONSES OF MALE AND FEMALE *C. sordidus* TO CHOPPED MATERIAL FROM CULTIVAR GITHUMO

	Pseudostem[a]		Rhizome[a]	
	Replicates (*N*)	(Excess visits mean ± SE)	Replicates (*N*)	(Excess visits mean ± SE)
Males	20	1.35 ± 0.30***	20	1.95 ± 0.49***
Females	20	2.05 ± 0.33***	20	3.20 ± 0.54***

[a] $P < 0.05$, $**P < 0.01$, $***P < 0.001$. Probability that the observed mean is significantly different from zero, using non-parametric signed rank statistic.

TABLE 2. RESPONSES OF FEMALE *C. sordidus* TO CHOPPED MATERIAL AND COLLECTED VOLATILES FROM SUSCEPTIBLE GITHUMO AND PARTIALLY RESISTANT WANGAE

	Pseudostem[a]		Rhizome[a]	
	Replicates (*N*)	(Excess visits mean ± SE)	Replicates (*N*)	(Excess visits mean ± SE)
Chopped material				
Fresh Githumo	40	2.18 ± 0.33***	40	1.50 ± 0.24***
Fresh Wangae	40	1.80 ± 0.34***	40	1.10 ± 0.27***
Rotting Githumo	40	1.03 ± 0.25***		
Collected volatiles				
Fresh Githumo	40	1.50 ± 0.33***	40	1.08 ± 0.31**
Fresh Wangae	40	0.80 ± 0.26**	40	0.73 ± 0.34*
Rotting Githumo	40	1.40 ± 0.25***		

[a] $P < 0.05$, $**P < 0.01$, $***P < 0.001$. Probability that the observed mean is significantly different from zero, using non-parametric signed rank statistic.

TABLE 3. DURATION (MEANS OF LOG_{10} NUMBER OF SECONDS) OF VISITS BY *C. sordidus* TO CONTROLS, CHOPPED MATERIAL AND COLLECTED VOLATILES FROM SUSCEPTIBLE GITHUMO AND PARTIALLY RESISTANT WANGAE[a]

	Pseudostem		Rhizome	
	N	Mean ± SEM	*N*	Mean ± SEM
Chopped material				
Fresh Githumo	120	1.18 ± 0.03***	103	1.31 ± 0.04***
Control	33	0.68 ± 0.08	43	0.73 ± 0.05
Fresh Wange	111	1.13 ± 0.04***	92	1.15 ± 0.04***
Control	39	0.65 ± 0.04	48	0.65 ± 0.06
Rotting Githumo	66	1.77 ± 0.07***		
Control	25	1.07 ± 0.09		
Collected volatiles				
Fresh Githumo	109	0.97 ± 0.03	77	1.07 ± 0.04***
Control	49	0.91 ± 0.06	34	0.77 ± 0.06
Fresh Wangae	61	0.95 ± 0.04***	71	0.99 ± 0.04
Control	29	0.67 ± 0.07	42	0.98 ± 0.06
Rotting Githumo	76	1.43 ± 0.05**		
Control	20	1.07 ± 0.12		

[a] N = number of visits. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Probability that the visits to the treated and control holes are of different mean duration, t test.

cantly longer than those to the control only for fresh Githumo rhizome, fresh Wangae pseudostem, and rotting Githumo pseudostem. There were no significant differences in the duration of visits to any particular material between Wangae and Githumo, but overall (i.e., including fresh material and collected volatiles from both pseudostem and rhizome) significantly longer visits were made to Githumo.

Antennal preparations gave responses to 10 μl of 10^{-1} nonanol of up to 1 mV depolarization. EAG responses to all the collections of volatiles increased as the volume of solution applied to the filter paper was increased (Figure 2). There were no significant differences in either relative or absolute responses between male and female antennae. Due to relatively large differences in response to the two collections from the same material, it is not possible to conclude that responses were higher to Wangae than Githumo.

Female weevils did not respond behaviorally or electrophysiologically to a synthetic blend of volatiles matching that of the natural volatiles (Tables 4 and 5).

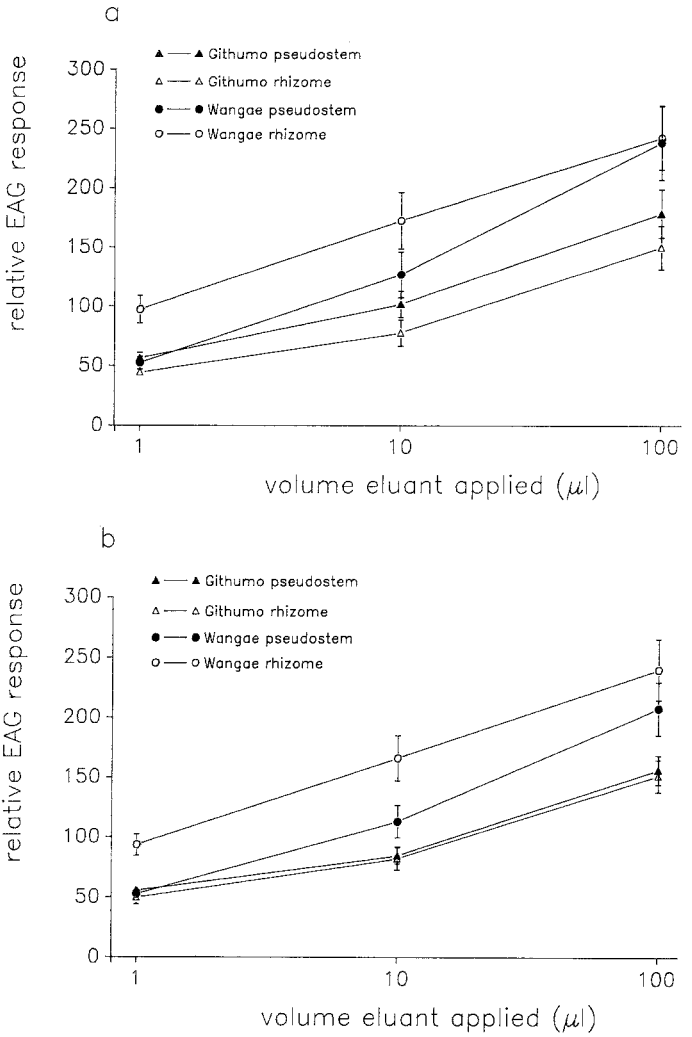

FIG. 2. The mean (± measurement SE) relative EAG responses (10 μl of 10^{-1} dilution of nonanol in paraffin oil = 100) to different amounts of Porapak-trapped volatiles from banana material from the susceptible cultivar Githumo and the partially resistant cultivar Wangae: (a) by females; (b) by males.

DISCUSSION

Male and female banana weevils responded in a similar manner to cut pieces of Githumo rhizome and pseudostem in the behavioral bioassay, and there were no differences in their EAG responses to the collected volatiles. This

TABLE 4. COMPONENTS OF VOLATILES TRAPPED FROM GITHUMO PSEUDOSTEM AND OF A SYNTHETIC BLEND

	Natural blend		Synthetic blend	
	Relative abundance (%)[a]	Conc. (μg/ml)	Relative abundance (%)[a]	Conc. (μg/ml)
α-Pinene	45.9	42.1	47.4	43.5
β-Pinene	23.1	21.2	24.5	22.5
Myrecene	3.9	8.9	4.4	10.0
Limonene	5.6	5.1	6.2	5.5
Ocimene	1.2	1.4	1.2	1.4
α-Cubebene	2.2	0.5	2.4	0.6
α-Copaene	5.2	3.5	5.1	3.4
α-Cedrene	0.7	0.7	0.6	0.6
β-Caryophyllene	4.4	3.0	4.8	3.3
α-Humulene	1.4	1.3	1.0	0.9
Unidentified minor peaks	6.6		2.6	

[a]Based on area under curve from FID output.

TABLE 5. BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSES TO NATURAL VOLATILES TRAPPED FROM GITHUMO PSEUDOSTEM AND SYNTHETIC BLEND OF MAJOR COMPONENTS

	Replicates (*N*)	(Excess visits mean ± SE)	Mean duration of visit ($\log_{10}$ time in sec)	Relative EAG response
Natural blend	20	1.15 ± 0.42**	trt 1.49 ± 0.05**	123 ± 3
			con 1.13 ± 0.11	
Synthetic blend	20	−0.05 ± 0.29	trt 1.21 ± 0.07	23 ± 2
			con 1.21 ± 0.13	
Untreated control				22 ± 2

[a]$P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Probability that the observed mean is significantly different from zero, using nonparametric signed rank statistic, or that the visits to the treated and control holes are of different mean duration, *t* test.

suggests that there are no differences, quantitative or qualitative, in sensillae sensitive to host plant compounds between the sexes and that the orientation responses observed are probably related to finding food, not to finding oviposition sites.

Responses by weevils in the behavioral bioassay appeared stronger to the susceptible cultivar Githumo than to the comparatively resistant Wangae, although not markedly so. It is unlikely that the lower response to Wangae is sufficient to explain its comparative resistance to attack by the weevil, but it might contribute to it.

The results suggest that rotting pseudostem is as good an attractant as fresh material for weevils, yet field results show that more weevils are caught using fresh traps. However, this could be because field trap catches reflect both the attractiveness of the traps and their retentive ability, and fresher material might be a preferred food source to rotting material.

A synthetic blend of the major components of a volatile collection from Githumo pseudostem failed to elicit any behavioral or electrophysiological response when presented in the same quantity and at the same time as a collection that did elicit responses. Thus, it is likely that the major components of the volatiles from the pseudostem are not involved in the attraction of weevils and that the biological activity is due to minor component(s) present, but not yet identified. This is in contrast to many insects that respond to major components of the host plant volatiles on their own or in combinations (Hopkins and Young 1990; Nordlander et al., 1986), although absolute release rates of the synthetic compounds and mixtures were not matched exactly with those of the original material.

Further work, utilizing gas-chromatography linked to an electroantennogram detector (GC-EAD), will attempt to identify these minor components from the pseudostem, and the behaviorally active compounds from the rhizome and rotting tissue. A blend of these compounds, possibly in conjunction with the aggregation pheromone of the weevil (Budenberg and Ndiege, unpublished results), could be used as a bait for the weevil. This bait could be useful in the development of standardized monitoring systems, mass-trapping control programs, and novel application methods for biocontrol agents (such as fungal pathogens and nematodes) or insecticides.

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REFERENCES

CUILLE, J. 1950. Recherches sur le charancon du bananier *Cosmopolites sordidus*, Germar: Monographie de l'insecte et recherche de ses chimiotropismes. Paris. IFAC Servic. Techn. 4. 225 pp.

DELATTRE, P. 1980. Recherche d'une methode d'estimation des populations du charancon du bananier, *Cosmopolites sordidus* Germar (Col., Curculionidae). *Acta Oecol. Oecol. Appl.*, 1:83-92.

HOPKINS, T.L., and YOUNG, H. 1990. Attraction of the grasshopper, *Melanoplus sanguinipes*, to host plant odors and volatile components. *Entomol. Exp. Appl.* 56:249-258.

HORD, H.H.V. and FLIPPIN, R.S. 1956. Studies of banana weevils in Honduras. *J. Econ. Entomol.*, 49:296-300.

JONES, M.T. 1986. Pests and diseases of bananas and plantains of Trinidad and Tobago. *J. Agric. Soc. Trin. Tobago* 86:18-33.

MITCHELL, G.A. 1978. The estimation of banana borer population and resistance levels. WINBAN Research and Development Technical Bulletin No. 2. WINBAN, Winward Islands.

MITCHELL, G.A. 1980. Banana entomology in the Windward Islands. Final report 1974-1978. Center for Overseas Pest Research, London.

NDIEGE, I.O., BUDENBERG, W.J., LWANDE, W. and HASSANALI, A. 1991. Volatile components of banana pseudostem of a cultivar susceptible to the banana weevil. *Phytochemistry* 30:3929-3930.

NORDLANDER, G., EIDMANN, H.H., JACOBSSON, U., NORDENHEIM, H. and SJODIN, K. 1986. Orientation of the pine weevil *Hylobius abietis* to underground sources of host volatiles. *Entmol. Exp. Appl.* 41:91-100.

OSTMARK, H.E. 1974. Economic insect pests of bananas. *Annu. Rev. Entomol.* 19:161-176.

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.

SIKORA, R.A., BAFOKUZAKA, N.D., MBWANA, A.S.S., OLOO, G.W., URONU, B., and SESHU REDDY, K.V. 1989. Interrelationship between banana weevil, root lesion nematode and agronomic practices, and their importance for banana decline in the United Republic of Tanzania. *F.A.O. Plant Prot. Bull.* 37:151-157.

SIMMONDS, N.W., and SIMMONDS, F.J. 1953. Experiments on the banana borer *Cosmopolites sordidus* in Trinidad, B.W.I. *Trop. Agric.* 30:216-223.

WALLACE, C.R. 1938. Unpoisoned baits for reducing populations of banana beetle borer (*Cosmopolites sordidus*). *J. Aust. Inst. Agric. Sci.* 4:157-160.

WATERHOUSE, D.F. and NORRIS, K.R. 1987. Biological Control, Pacific Prospects. Inkata Press, Melbourne.

YARINGANO, C.V.M., and van der MEER, F. 1975 Control del "gorgonjo del platano" *Cosmopolites sordidus*, mediante trampas diversas pesticidas granulados. *Rev. Peru. Entomol. Agric.* 18:112-116.

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THE DUFOUR GLAND AND THE SECRETION PLACED ON EGGS OF TWO SPECIES OF SOCIAL WASPS, *Liostenogaster flavolineata* and *Parischnogaster jacobsoni* (VESPIDAE: STENOGASTRINAE)

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Abstract—The secretion placed on eggs and fed to larvae and the "ant guard" placed on the nest stalk of *Parischnogaster jacobsoni* contain the same hydrocarbons and in approximately the same proportions as is found in the Dufour gland. The secretion on eggs is a mixture of the contents of the Dufour gland and nectar. The emulsifying agent is a palmitic acid salt. Similarly, in *Liostenogaster flavolineata*, the egg secretion is an emulsion of nectar and Dufour gland secretion, which contains alkoxyethanol emulsifiers, found in nature for the first time.

Key Words—Hymenoptera, Vespidae, Stenogastrinae, Dufour gland, Larval food, egg secretion, ant guard, emulsifier, eicosyloxyethanol, hydrocarbons.

INTRODUCTION

The Stenogastrinae, small streamlined East Asian tropical wasps known as "hover wasps" (Turillazzi, 1991), are the most primitive of social Vespids

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(Carpenter, 1991). *Liostenogaster flavolineata* builds heavy mud nests formed by a single comb that contains up to 100 cells and places its nests especially under the vaults of small bridges, in caves and culverts, sometimes in dense aggregations of colonies (Hansell et al., 1982; Samuel, 1987). *Parischnogaster jacobsoni* build nests, formed by a single row of cells defended by an ant guard, along threadlike substrata sheltered from the rain (Turillazzi, 1988). One of the main characteristics of their biology, which clearly differentiates them from the other subfamilies of social wasps (Polistinae and Vespinae), is the production by the females of a whitish gelatinous substance ("abdominal substance"), which has various functions in the brood rearing and colonial life of species belonging to all the six genera of the subfamily. The presence of egg-size drops of this substance on eggs and small larvae of various species was reported by the very first authors who studied these wasps: Williams (1919), Jacobson (1935), Pagden (1958), Iwata (1967), and Spradbery (1975). The first author thought it was of "vegetal origin," the second and third authors rather supposed it was of "insect origin," while Iwata (1967) and Spradbery (1975) suggested that it was secreted by the wasps themselves. This opinion was then confirmed by more recent authors in species of five genera (Hansell, 1977, 1982; Turillazzi and Pardi, 1982; Sakagami and Yamane, 1983; for *Parischnogaster*; Samuel, 1987, for *Liostenogaster*; Hansell, 1987, for *Eustenogaster*; Turillazzi, 1990, for *Metischnogaster*; Turillazzi and Hansell, 1991, for *Anischnogaster*). The proposed functions of this secretion were examined by Turillazzi (1985a,b; 1989), who concluded that the substance "is used by the females to handle the egg at the moment of oviposition, it becomes the substratum and the protection for the young larva, it serves as a dish for the larval food and the place where reserves of liquid food for the colony are stored" (Turillazzi, 1985a). He found no good evidence to support the idea that the secretion functioned as a larval food, and concluded that a decisive answer could only be given after chemical analysis of the substance.

The organ from which the substance is produced was not clearly identified, only supposed. The Dufour gland of these wasps is considerably enlarged and contains a secretion similar in appearance to the "abdominal substance," so it was the more probable source according to Hansell (1982), Turillazzi (1985a), and Delfino et al., (1987); but decisive evidence was still missing.

Another substance that resembles the abdominal substance in appearance but is used in a different context from brood rearing in species of at least three genera is the so-called "ant guard." Tiny drops of this substance, which are secreted by the tip of the abdomen by females of *Parischnogaster nigricans serrei* (Turillazzi and Pardi, 1981) and *P. jacobsoni* (Turillazzi, 1988) are applied to the threadlike substratum of the nest to form viscid obstacles that prevent ants invading the cells. Even if there are good reasons to maintain that this substance could be, at least in part, similar to the abdominal substance and produced by the same organ, we lacked decisive evidence.

In order to discover the origin and the nature of these substances, we have carried out the chemical analysis of the contents of the Dufour gland and the secretions placed on eggs of *L. flavolineata* and of the Dufour gland, the egg secretion, and the ant guard of *P. jacobsoni*.

METHODS AND MATERIALS

Females from separate colonies of *Liostenogaster flavolineata* and *Parischnogaster jacobsoni* were collected at Ulu Gombak and at the Genting Tea Estate (Genting Sempah), two localities along the old road to Bentong and Kuantan, respectively, 25 and 40 km from Kuala Lumpur (Malaysia).

Five samples of each material, Dufour glands, abdominal secretion placed on eggs in brood cells and, for *P. jacobsoni*, the ant guard, were sealed in soft glass capillaries (2 × 20 mm) and transported to Keele where they were kept in a refrigerator until analysis.

A synthetic model of the secretion of *L. flavolineata* was prepared from 9-tricosene (15 mg), honey (150 mg), octadecyloxyethanol (5 mg), and water (50 μl). An emulsion resembling the ant guard of *P. jacobsoni* was prepared from tricosene (480 mg), tricosane (150 mg), heneicosane (150 mg), heptadecane (100 mg), sodium palmitate (100 mg), honey (150 mg), and water (500 μl). Infrared spectra of the abdominal substance, the ant guard, and the synthetic mixtures prepared to resemble them were obtained on a Bruker IFS48 Fourier-transform infrared spectrometer connected to a microscope.

Linked gas chromatography–mass spectrometry was carried out on a Hewlett Packard 5890 instrument linked to a 5970B Mass Selective Detector and controlled by a HP59970C ChemStation. 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 150°C, and the capillary tubes were heated in the injection area for 2 min before crushing. The oven temperature was initially set at 30°C for 2 min and then increased at a rate of 8°C/min to a maximum temperature of 250°C. In later work, a retention gap made of deactivated fused silica tubing (10 m × 0.2 mm) was inserted between column and mass spectrometer, which increased retention times. Quantification of peak areas was achieved by comparison with known amounts of commercial tricosane.

Derivatization to Sugar Trimethylsilyl Ether Oximes. Glucose, fructose, sucrose, lactose, and maltose (15 mg of each) were converted to their oxime derivates by treatment with hydroxylamine hydrogen chloride (0.25 g in 10 ml of pyridine) and heating the mixture to 70–75°C for 30 min. It was the cooled and treated with hexamethyldisilazane (1 ml) and trifluoroacetic acid (0.1 ml). Hexane and water were added and the organic and aqueous layers separated. A

portion of the organic layer (1 μl) was injected into the chromotograph. Samples of each of the materials collected from wasps were treated similarly.

Derivatization to Carboxylic Acid Methyl Esters. An ethereal solution of diazomethane was added to one crushed Dufour gland, a droplet of secretion or a piece of ant guard in a microreactor, the mixture warmed, part of the ether allowed to evaporate, and a portion of the remainder chromatographed in the usual way.

Derivatization to Alkylthiomethyl Ethers. To determine the position of the double bonds in the alkenes, alkylthiolation (Francis and Veland, 1981) was carried out. A crushed Dufour gland was extracted with hexane (100 μl) and the extract treated with 2 μl of iodine solution (60 mg of I_2 in 2 ml diethyl ether) and 100 μl of dimethyl disulfide in a Keele microreactor (Attygalle and Morgan, 1986). The reaction was carried out at 100°C overnight; then aqueous $Na_2S_2O_3$ (50 μl of 5% solution) was added. The mixture was allowed to separate, and 1 μl of the organic layer was gas chromatographed.

RESULTS

Gas chromatographic analysis of the Dufour gland secretion and of the abdominal substance showed that all samples contain a mixture of linear hydrocarbons, which were very similar for a species (Table 1). The Dufour gland of *L. flavolineata* was found to contain chiefly 9-tricosene, pentacosene, nonacosene, heneicosane, and tricosane in decreasing order of abundance. The Dufour gland of *P. jacobsoni* contained 9-tricosene, pentacosene, tricosane, heptacosane, and pentacosane. The two main volatile compounds in this gland were the same for both species, although the proportion of the other hydrocarbons present varied between the species.

Two unusual compounds, 2-eicosyloxyethanol and 2-docosyloxyethanol, readily identified by their characteristic mass spectra (Figure 1) (see Kramer et al., 1971) and confirmed by the synthesis of the homolog 2-octadecyloxyethanol (see Keegans et al., 1992), were found in the Dufour gland and in the secretion of *L. flavolineata*. They were not detected in any samples of *P. jacobsoni* analyzed even when sensitive selective ion monitoring was carried out for the characteristic ions at *m/z* 63 ($HOCH_2CH_2OH_2^+$) and 45 ($HOCH_2CH_2^+$) of these compounds. Other important diagnostic ions (Figure 1) are found at *m/z* 311 (M^+-CH_2OH), 280 ($C_{20}H_{40}^+$) 252 ($C_{18}H_{36}$), and 75 ($HOCH_2CH_2OCH_2^+$).

The hydrocarbons identified in the secretion on the eggs of *L. flavolineata* were very similar to those present in the Dufour gland of the species (Table 1, Figure 2). The only major difference was that, on average, the egg secretion samples analyzed contained more 9-tricosene (36.3%) than the Dufour glands. More 9-tricosene was also seen in the egg secretion of *P. jacobsoni* than in the Dufour gland (Table 1).

TABLE 1. PERCENTAGE COMPOSITION OF VOLATILE COMPONENTS OF DUFOUR GLAND, EGG SECRETION OF *Liostenogaster flavolineata* AND *Parischnogaster jacobsoni* AND ANTGUARD FOR *P. jacobsoni*[a]

Compound	*L. flavolineata*		*P. jacobsoni*		
	Dufour gland	Egg secretion	Dufour gland[b]	Egg secretion[b]	Ant guard[b]
Heneicosene	1.9	0.3	2.0	1.7	0.8
Heneicosane	9.3	4.2	1.1	3.9	1.7
Docosene	0.4	0.3	0.5	0.9	0.3
Docosane	0.2	0.4	0.5	0.4	0.5
Eicosanal	0.4	0.1			
Tricosene	1.6	t		7.7	t
9-Tricosene	31.9	36.0	23.9	27.9	18.4
Tricosane	10.5	12.4	15.7	13.6	10.1
11-Methyltricosane	0.2	0.8	0.6	0.8	t
Tetracosene	t	0.2			0.5
Docosanal	0.4	0.3			
Pentacosene I	0.2	0.4	0.6	0.6	0.6
Pentacosene II	10.0	10.5	23.2	11.1	36.3
Pentacosane	4.1	5.3	6.3	1.5	12.8
2-Eicosyloxyethanol	1.6	2.3			
Hexacosene I	0.1	1.6			
Hexacosene II	0.1	0.1	0.6		
Tetracosanal	0.2	t			
3-Methylpentacosane			1.4		
Heptacosene I	0.3	2.1	0.2	0.5	0.3
Heptacosene II	4.2	2.9	3.7	2.2	5.4
Heptacosane	4.7	2.1	8.0	4.6	5.9
2-Docosyloxyethanol	0.9	2.0			
15-Methylheptacosane			3.7		
7-Methylheptacosane			3.3	0.3	0.2
Nonacosene I	0.3	1.7	0.8	4.5	3.6
Nonacosene II	7.3	1.4	3.2	1.9	1.4
Nonacosane	2.5	8.2	0.6		
11-Methylnonacosane	t	0.2			
Hentriacontene	3.8	3.1		6.1	0.7
Hentriacontane	2.9	1.1		1.4	0.5
Tritriacontadiene				1.9	
Tritricontene				4.0	
Tritriacontane				2.5	
Total weight (μg) of each sample analyzed	1.63 1.94 2.96	1.06 1.13 1.33	3.24 2.12 1.16	1.83 1.96 1.78	5.06 0.53 0.35

[a] Results are the means for three individual samples of each substance.

[b] *Palmitic (hexadecanoic acid) was identified in each of the materials but could not be accurately quantified.

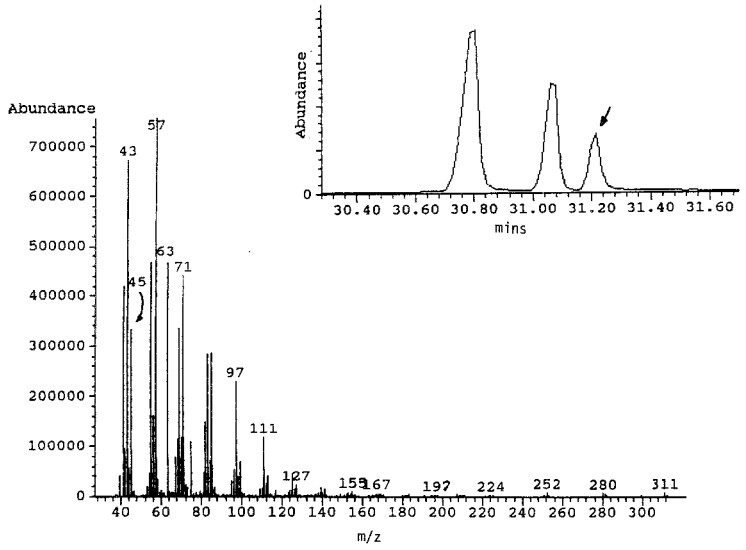

FIG. 1. Mass spectrum of eicosanyloxyethanol from *Liostenogaster flavolineata*, with inset of an expanded section of the total ion chromatogram, showing the C_{25} region in which it occurs. Retention times do not correspond to those in Figure 2.

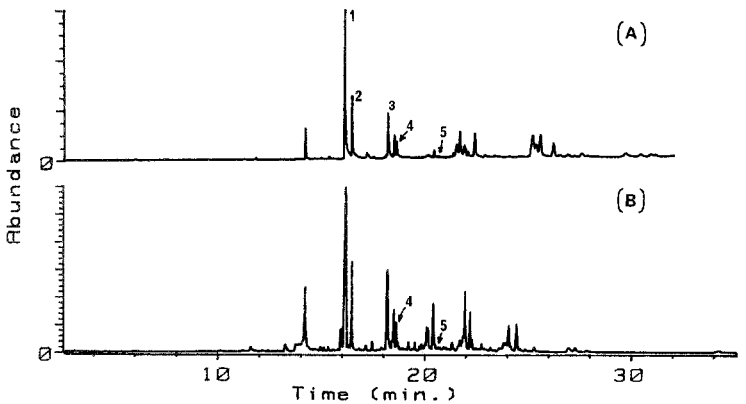

FIG. 2. Total ion chromatograms of (A) the Dufour gland contents and (B) the egg secretion of *Liostenogaster flavolineata* showing a similar pattern of hydrocarbons in both. Identified peaks are: 1, tricosene, 2, tricosane; 3, pentacosene; 4, eicosanyloxyethanol; 5, docosanyloxyethanol.

The ant guard and the Dufour gland of *P. jacobsoni* (Figure 3) were similar in percentage composition, but there was about twice the proportion of pentacosene and more pentacosane in the samples of ant guard than was found in the Dufour glands. By compound type, there was approximately twice as much alkenes as alkanes present in all samples. No alkoxyethanols or aldehydes were detected in *P. jacobsoni*, while 5% of the secretion on eggs and in Dufour glands of *L. flavolineata* were oxygenated compounds.

The characteristic cleavage ions at *m/z* 173 and 243 in the mass spectrum of the dimethyl disulfide adduct of the major component of these materials showed that it was 9-tricosene. The amounts of the other alkenes were too small for the double-bond positions to be determined with confidence, although two isomers for tricosene, pentacosene, heptacosene, and nonacosene were evident.

No evidence for free palmitic acid was found when any of the samples of gland, abdominal substance, and ant guard were chromatographed, although the quantity later identified (below) should have been readily visible. However, when the samples were treated with diazomethane solution, to convert any free carboxylic acids to methyl esters, and then chromatographed, the Dufour gland, abdominal substance, and ant guard of *P. jacobsoni* all showed methyl palmitate

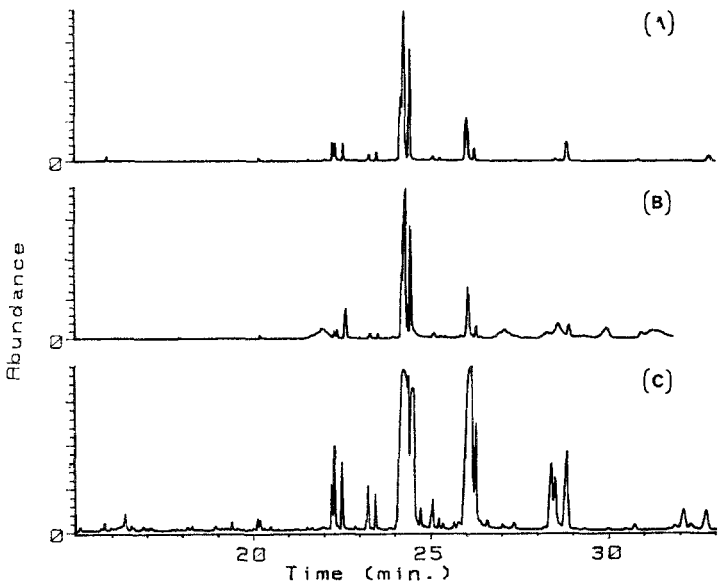

FIG. 3. Total ion chromatograms of (A) the Dufour gland contents, (B) the egg secretion, and (C) a portion of ant guard from the nest substrate of *Parischnogaster jacobsoni* showing a similar pattern of hydrocarbons in all three. The broad peaks in (C) are caused by overloading of the column.

(methyl hexadecanoate) (~ 500 ng in the gland or egg secretion). No esters were observed in the samples of the Dufour gland and abdominal substance of *L. flavolineata* analyzed in the same way.

The infrared spectrum of the secretion on eggs of both species gave absorptions at 3404 cm^{-1} (O—H), 2918 cm^{-1}, and 1099 cm^{-1} (C—O). The strong hydroxyl absorption suggested presence of carbohydrates and water in the secretion. An emulsion prepared from honey, tricosene, and alkoxyethanol gave an infrared spectrum very close to that of the natural mixture from *L. flavolineata* (see Keegans et al., 1992). The infrared spectrum of the ant guard of *P. jacobsoni* was very similar, with absorption maxima at 3369 cm^{-1} (broad, OH), 2916, 2849 (C—H), and 1032 (C—O), but there were additional weak but sharp absorptions at 3693 cm^{-1} and 3618 cm^{-1} attributed to non-hydrogen-bonded hydroxyls, a weak but sharp band at 1740 cm^{-1} (C=O of acid?), and a broad weak band at 1650 cm^{-1} (ionized carboxyl?). An emulsion prepared from hydrocarbons, honey, sodium palmitate, and water reproduced the general appearance of the infrared spectrum above, but without the hydroxyl bands at 3693 and 3618 cm^{-1} and the weak carbonyl at 1740 cm^{-1}. These differences may arise from the different degrees of dispersion of the natural and synthetic emulsions.

To identify the sugars present in the secretion on eggs, samples of the secretion and pure sugars were chromatographed as their trimethylsilyl ether oxime derivatives. The sugar in the secretion of both species was found to be almost entirely fructose with a small amount of glucose (Figure 4). Sugars were not detected in the Dufour glands. The broadening of the fructose peak is due to the presence of *syn* and *anti* trimethylsilyloxyoximes.

DISCUSSION

The Dufour gland and the secretion placed on eggs of *L. flavolineata* and the Dufour gland, secretion on eggs, and ant guard of *P. jacobsoni* all contain a mixture of hydrocarbons, different for each species, but in each case consisting chiefly of straight-chain alkenes (up to 65% of the total) in the range C_{21}–C_{33}. The Dufour glands of both species contain surface-active substances. This was first recognized in *L. flavolineata*, where 2-eicosyloxyethanol and 2-docosyloxyethanol were recognized to be present in small quantities by their mass spectra (Keegans et al., 1992). We looked carefully at the materials from *P. jacobsoni* for the same or similar compounds but found none. No evidence for free carboxylic acid was found either by gas chromatography or infrared spectroscopy. Considering that an alternative emulsifying agent would be a salt of a fatty acid, which would not be seen in our chromatograms, we treated portions of each material with diazomethane to convert any fatty acids to their methyl esters. Chromatography now showed the presence of methyl palmitate, surpris-

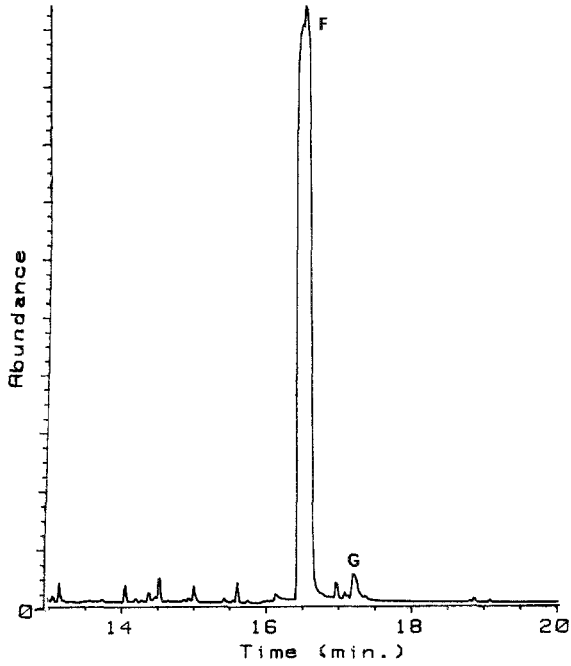

FIG. 4. Total ion chromatogram of the trimethylsilyl ether oxime derivatives prepared from the sugars in the egg secretion of *L. flavolineata*. The two labeled peaks correspond in retention time and mass spectra to the derivatives of fructose and glucose. Doubling of the fructose peak arises from *syn* and *anti* oximes.

ingly, not accompanied by any of its homologs, in all the materials from *P. jacobsoni* but not in *L. flavolineata*. We concluded the palmitic acid was present as a salt. The species therefore produce their own characteristic emulsifying agent in the Dufour gland. The 2-alkoxyethanols, long known as synthetic surface-active agents (Fieser et al., 1956) are found here in nature for the first time. Espelie and Hermann (1990) reported hexadecanoic acid and octadecenoic acid in the van der Vecht's (or sternal) gland and on the nest pedicel of the social wasp *Polistes annularis*. They reviewed the available literature on defensive secretions placed on nest pedicels. Methyl hexadecanoate has been found in the extract of the sternal gland of *P. fuscatus* and shown to act as a deterrent to ants (Post et al., 1984; Henderson and Jeanne, 1989). The C_{16} and C_{18} free fatty acids have been shown to elicit a necrophoric response in some ants (Wilson et al., 1958; Gordon, 1983). The palmitic acid salt in the ant guard of *P. jacobsoni* may act as a repellent to ants, which are the main predators on the nests of this species.

While the Dufour gland secretions of these two species are colorless trans-

parent oils, Turillazzi describes the egg secretion as a white substance, looking like mayonnaise (Turillazzi, 1985a). Evidently some substance is emulsified with the Dufour gland hydrocarbons in the egg secretion. Infrared spectra obtained on microscopic samples of the secretion showed strong hydroxyl absorption, as well as hydrocarbon bands; logical deduction suggested an aqueous sugar solution emulsified with the hydrocarbons. Identification of the sugars by thin-layer chromatography (not detailed here) and gas chromatography–mass spectrometry of trimethylsilyl ether-oximes showed large quantities of fructose with traces of glucose, typical of nectar. No sugars were detected in the Dufour gland of either species.

To confirm this identification of the egg secretion, a mixture of pure tricosene and smaller quantities of saturated hydrocarbons were emulsified with honey and water and stabilized with octadecyloxyethanol. The emulsion prepared from tricosene, honey, water, and octadecyloxyethanol had an infrared spectrum very similar to the egg secretion of *L. flavolineata*, which that prepared from hydrocarbons, honey, and sodium palmitate was similar to the ant guard but lacked some minor bands.

In conclusion, the ball of emulsion placed on eggs consists of Dufour gland contents (alkenes and alkoxyethanols for *L. flavolineata* and alkenes and a fatty acid salt for *P. jacobsoni*), as well as fructose and water. The fructose and water presumably originate from nectar collected by the female. Alkoxyethanols are particularly noted as useful in inhibiting evaporation of water by acting as a monomolecular surface film (cf. Deo et al. 1965; Trapeznikov and Zaozerskaya, 1972). They may have a similar function for *L. flavolineata* by reducing water loss from the ball of emulsified nectar and helping to keep the ball in a semiliquid state for the growing larva. Emulsions formed from a mixture of hydrocarbons (chiefly 9-tricosene), honey, water, and either octadecyloxyethanol or sodium palmitate, approximately in the proportions found in the egg secretions, gave very stable emulsions.

This chemical study provides a unique application of the alkoxyethanols as emulsifying substances in nature and, above all, it furnishes answers to the three questions we posed in the introduction: (1) The secretion plays an important role in larval feeding but it is not a larval food per se; rather it serves as protective substrate for sugary food (as well as protein food when supplied to small larvae; c.f. Turillazzi, 1985a) which adults collect from the environment. (2) The substances originates from the Dufour gland. (3) The ant guard substance (at least that of *Parischnogaster*) is the same as the abdominal substance, regardless of the slightly different ways in which females collect it from abdomen (Turillazzi and Pardi, 1981).

It is worth stressing that the Dufour gland secretion is used as a raw material in at least two different biological contexts, i.e., brood rearing and nest defense, in species of at least three genera (*Parischnogaster*, *Liostenogaster*, and *Eus-*

tenogaster; Turillazzi, 1991) and represents a unique example of the use of Dufour gland secretion in Vespidae.

The differences in composition of the principal components of the Dufour gland secretion of the two species probably make little difference in the physical properties of the emulsion. The difference in the emulsifying agents is much more important. *L. flavolineata* and *P. jacobsoni* belong to genera that are phylogenetically distant (Carpenter, 1991); they differ in their nesting places, in the prey that provides the solid food of larvae, and only *P. jacobsoni* constructs an ant guard (Samuel, 1987; Turillazzi, 1988). The chemical differences in the abdominal substances may be due to changes that occurred in the course of speciation or they may reflect differences in larval feeding and nest formation. Considering that in the same two genera, species with a more similar biology and nest defense exist, this question could be easily solved by future comparative studies and extended to species belonging to other genera.

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REFERENCES

ATTYGALLE, A.B., and MORGAN, E.D. 1986. A versatile microreactor and extractor. *Anal. Chem.* 56:1530–1533.

CARPENTER, J.M. 1991. Phylogenetic relationships and the origin of social behavior in the Vespidae, pp. 7–32. *in* K.G. Ross and R.W. Matthews (eds.). The Social Biology of Wasps. Cornell University Press, Ithaca, New York.

DELFINO, G., TURILLAZZI, S., and CALLONI, C. 1987. Structure and ultrastructure of the Dufour's gland in *Parischnogaster* (Hymenoptera, Stenogastrinae). 10th International Congress IUSSI, München, pp. 444–445.

DEO, A.V., KULKARNI, S.B., GHARPUNY, M.K., and BISWAS, A.B. 1965. Specific resistance of the monolayers of n-long chain alcohols and n-long chain alkoxyethanols to the evaporation of substrate water molecules. *Indian J. Chem.* 3:200–204.

ESPELIE, K.E., and HERMANN, H.R. 1990. Surface lipids of the social wasps *Polistes annularis* (L.) and its nest and nest pedicel. *J. Chem. Ecol.* 16:1841–1852.

FIESER, M., FIESER, L.F., TOROMANOFF, E., HIRATA, Y., HEYMANN, H., TEFFT, M., and BHATTACHARYA, S. 1956. Synthetic emulsifying agents. *J. Am. Chem. Soc.* 78:2825–2832.

FRANCIS, G.W., and VELAND, K. 1981. Alkythiolation for the determination of double bond positions of linear alkenes. *J. Chromatogr.* 219:379–384.

GORDON, D.M. 1983. Dependence of necrophoric response to oleic acid on social content in the ant *Pogonomyrmex badius*. *J. Chem. Ecol.* 9:105–171.

HANSELL, M.H. 1977. Social behavior of a three wasp colony: Stenogastrinae Vespidae. Proceedings, 15th International Ethology Conference, Bielefeld, 23–31 August 1977, p. 30.

HANSELL, M.H. 1982. Brood development in the sub-social wasp *Parischnogaster mellyi* (Saussure), Stenogastrinae (Hymenoptera). *Insects Soc.* 28:208–216.

HANSELL, M.H. 1987. Elements of eusociality in colonies of *Eustenogaster calyptodoma* (Sakagami & Yoshikawa) (Stenogastrinae, Vespidae). *Anim. Behav.* 35:131–141.

HANSELL, M.H., SAMUEL, C., and FURTADO, J.I. 1982. *Liostenogaster flavolineata*: Social life in the small colonies of an Asian tropical wasp, pp. 192–195, *in* M.D. Breed, C.D. Michener, and H.E. Evans (eds.). The Biology of Social Insects, Westview Press, Boulder, Colorado.

HENDERSON, G., and JEANNE, R.L. 1989. Response of aphid-tending ants to repellent produced by wasps. *Ann. Ent. Soc. Am.* 82:516–519.

IWATA, K. 1967. Report of the fundamental research on the biological control of insect pests in Thailand. II. The report on the bionomics of aculeate wasps. Bionomics of subsocial wasps of Stenogastrinae (Hymenoptera, Vespidae). *Nat. Life S.E. Asia* 5:529–293.

JACOBSON, E. 1935. Aanteekeningen over Stenogastrinae (Hymenoptera, Vespidae) *Entomol. Meded. Ned. Indie* 1:15–19.

KEEGANS, S.J., MORGAN, E.D., JACKSON, B.D., BERRESFORD, G., TURILLAZZI, S., and BILLEN, J. 1992. The chemical nature and origin of the abdominal substance of the wasps *Liostenogaster flavolineata* (Vespidae: Stenogastrinae). pp. 217–221, *in* J. Billen, (ed.). Biology and Evolution of Social Insects. Leuven University Press, Leuven.

KRAMER, J.K.G., HOLMAN, R.T., and BAUMANN, W.J. 1971. Naturally occurring diol lipids. VIII. Mass spectrometric analysis of mono- and dialkyl ethers of diols. *Lipids* 6:727–733.

PAGDEN, H.T. 1958. Some Malayan social wasps. *Malay. Nat. J.* 12:131–148.

POST, D.C., MOHAMED, M.A., HOPPEL, H.C., and JEANNE, R.L. 1984. Identification of ant repellent allomone produced by social wasp *Polistes fuscatus* (Hymenoptera: Vespidae). *J. Chem. Ecol.* 10:1799–1807.

SAKAGAMI, S.F., and YAMANE, S. 1983. Behavior inventory of *Parischnogaster mellyi*: In ecological study of social insects in central Sumatra with special reference to wasps and bees. *Sumatra Nature Study* (Entomology):12–18.

SAMUEL, C.T. 1987. Factors affecting colony size in the stenogastrine wasp *Liostenogaster flavolineata*. PhD dissertation. University of Malaya, Kuala Lumpur.

SPRADBERY, J.P. 1975. The biology of *Stenogaster concinna* van der Vecht, with comments on the phylogeny of Stenogastriniae (Hymenoptera: Vespidae). *J. Aust. Entomol. Soc.* 14:309–318.

TRAPEZNIKOV, A.A., and ZAOZERSKAYA, L.A. 1972. Viscosity of hexadecanol and oxyethylated octadecanol monlayers and the effect of a wave on it. *Kolloid Zh.* 34:579–584 (*Chem. Abs.* 77:144064m, 1972).

TURILLAZZI, S. 1985a. Function and characteristics of the abdominal substance secreted by wasps of the genus *Parischnogaster* (Hymenoptera: Stenogastrinae). *Monitor Zool. Ital.* 19:91–99.

TURILLAZZI, S. 1985b. Egg deposition in the genus *Parischnogaster* (Hymenoptera: Stenogastrinae). *J. Kans. Entomol. Soc.* 58:749–752.

TURILLAZZI, S. 1988. Social biology of *Parischnogaster jacobsoni* (Du Buysson) (Hymenoptera Stenogastrinae). *Insectes Soc.* 35:133–143.

TURILLAZZI, S. 1989. The origin and evolution of social life in the Stenogastrinae (Hymenoptera, Vespidae). *J. Insect. Behav.* 2:649–661.

TURILLAZZI, S. 1990. Notes on the biology, social behavior and nest architecture of *Metischnogaster drewseni* (Saussure) (Hymenoptera, Stenogastrinae). *Boll. Zool.* 57:331–339.

TURILLAZZI, S. 1991. The Stenogastrinae, pp. 74–98, *in* K.G. Ross and R.W. Matthews (eds.). The Social Biology of Wasps. Cornell University Press, Ithaca.

TURILLAZZI, S., and HANSELL, M.H. 1991. Biology and behavior of the species of *Anischnogaster* (Vespidae, Stenogastrinae) in Papua-New Guinea. *Insectes Soc.* 38:423–437.

TURILLAZZI, S., and PARDI, L. 1981. Ant guard on nests of *Parischnogaster nigricans serrei* (Buysson) (Stenogastrinae). *Monitor Zool. Ital.* 15:1–17.

TURILLAZZI, S., and PARDI, L. 1982. Social behavior of *Parischnogaster nigricans serri* (Du Buysson) (Hymenoptera: Vespoidea) in Java. *Ann. Entomol. Soc. Am.* 75:657–664.

WILLIAMS, F.X. 1919. Philippine wasp studies. II. Descriptions of new species and life history studies. *Bull Exp. Stn. Hawaii Sugar Plant. Assoc. (Entomol.)* 14:19–186.

WILSON, E.O., DURLACH, N.I., and RUTH, L.M. 1958. Chemical releasers of necrophoric behavior in ants. *Psyche* 66:108–114.

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PREDATORY BEHAVIOR IN *Tupinambis teguixin* (SAURIA: TEIIDAE). I. TONGUE-FLICKING RESPONSES TO CHEMICAL FOOD STIMULI

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Abstract—Black tegu lizards (*Tupinambis teguixin*) have the ability to detect food odors and discriminate between them and nonfood odors. This was tested by offering chemical stimuli on cotton-tipped applicators to the animals. Stimuli were from two plant and two animal species known to be principal items in these lizards' diets, demineralized water as an odorless control, and eau-de-cologne as an odorous control lacking feeding or social importance. Tongue-flick attack score, latency to attack, preattack tongue-flicks, and number of attacks were analyzed. The results clearly demonstrated that this species responds to chemical food stimuli, but does not respond to odorless nonfood stimuli. Responses differed among food types. There were no sex differences. These results are in agreement with the prediction that lizards having forked tongues and an active foraging mode rely on chemical cues for feeding.

Key Words—Teiidae, *Tupinambis*, food odor, foraging mode, chemical cues.

INTRODUCTION

Squamate reptiles have a well-developed ability to chemically recognize prey. Teiid lizards appear to use chemical cues in predatory behavior (Burghardt,

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1970; Simon, 1983), but this has been demonstrated only for *Tupinambis rufescens* (Cooper, 1989). Burghardt's review (1990) on chemically mediated predation in vertebrates highlighted six aspects in reptiles: (1) genetically based abilities to detect prey cues just after hatching, (2) ontogenetic changes in food preference, (3) different chemically mediated prey capture methods in aquatic or terrestrial habitats, (4) intraspecific prey preference polymorphisms, (5) experience-based changes during life-span, and (6) grouping with conspecifics would favor growth. The black tegu, *Tupinambis tequixin* fits into this framework as follows:

1. It has been found that newly hatched tegus begin tongue-flicking related to pawing (ground scratching), while still consuming abdominal yolk prior to initial feeding (Mercolli and Yanosky, 1989; Yanosky and Mercolli, 1992).

2. Ontogenetic changes in dentition morphology related to feeding types have been noted (Donadio and Gallardo, 1984).

3. Black tegu lizards detect and capture prey both in terrestrial and aquatic habitats (Mercolli and Yanosky, 1989), but no differential aspects of prey detection have been reported.

4. The importance of experience and grouping can be studied in captive programs where specimens would have controlled experience with food items, and, finally, the effect of grouping on growth rates could be studied to provide management guidelines for the improvement of animal reproduction.

In this study, the ability of *Tupinambis tequixin* to detect chemical food stimuli and distinguish them from controls was tested. Because the species has forked lingual morphology and actively forages, it was predicted that prey stimuli detection by chemical cues is highly likely to occur (Regal, 1978).

METHODS AND MATERIALS

Nineteen tegu lizards (10 females and 9 males), toe-clipped for individual recognition were maintained in an outdoor enclosure (210 × 150 × 75 cm). They were long-term captive specimens (over two years) averaging 31.28 ± 1.98 cm snout–vent length and 1560 ± 321.9 g body mass for males, and 31.77 ± 2.9 cm and 1473.3 ± 442.6 g for females. These tegu lizards were thoroughly acclimated to captive conditions and experimenters' presence. The enclosure was provided with a 30-cm layer of wood-shavings that allowed animals to be sheltered. Food, offered daily to satiation, was composed of commercial dog food and cattle liver and heart (Yanosky and Mercolli, 1992). Water could be obtained from a bowl buried in the substrate, but lizards were fed on a well-hydrated diet.

To determine whether tegu lizards can detect chemical food stimuli or discriminate them from chemical stimuli of nonfood sources, responses of these

lizards to several chemical stimuli presented on cotton-tipped (20-cm-long bamboo) applicators were recorded. These stimuli were from pindo palm fruits (*Syagrus romanzoffianum*), tala fruits (*Celtis spinosa*), locust (Acrididae), toad (*Bufo paracnemis*), normal daily diet, demineralized water, and eau-de-cologne. Pindo and tala fruits constituted important plant foods, while toad and locust constituted important animal prey of this omnivorous lizard in the wild (Mercolli and Yanosky, personal observation). Demineralized water and eau-de-cologne were used as control stimuli, the first one as an odorless control for response to the applicator and experimental situation, and the cologne as a highly odorous pungency control (Cooper and Alberts, 1990) lacking dietary and/or social importance.

Stimuli were prepared by means of impregnation (Cooper and Alberts, 1990). Concentrations of odorants on swabs varied among stimuli, but presumably this same variability occurs in nature.

Prior to testing, a randomly chosen animal was isolated from the rest of the group in the same enclosure and separated by an installed wooden screen. This remained in the same location throughout the testing period. Individuals were tested on the substrate to which they were habituated, so as to avoid excessive investigatory tongue-flicking (Mercolli and Yanosky, 1989) due to a new environment. To begin a trial, the experimenter slowly approached a lizard and placed the cotton tip of an applicator bearing one of the stimuli 3 cm in front of the lizard's snout. Tongue-flicks to the swab were counted for 60 sec. Attacks (= food-grab; Mercolli and Yanosky, 1989) on the swab and their latencies were also recorded. As tegu lizards attack "food (swab)" and then proceed to food-throw and shake, and attempt to swallow it, we terminated the trial at the time of the initial attack and withdrew the swab before swallowing occurred.

All tests were carried out between 29 January and 5 February 1992 from 1100 to 1500 hr at an air temperature of 32.42 $\pm$ 1.27°C, which was previously reported to support full activity by tegu lizards (Yanosky and Mercolli, 1991). Lizards attempted to feed during the trials, thus indicating their responsiveness. Lizards were fed the day before the experiments began (28 January) and were not fed during the trials (deprived until 6 February). Thus, the hunger level may have increased during the sampling period, but examination of the raw data revealed no indication that this affected responses.

Stimuli were presented in the following order: tala, pindo, cologne, toad, water, daily diet, locust. One trial per day was conducted with each lizard. Data analyzed included the tongue-flick attack score, preattack tongue-flicks, latency to attack in seconds, and number of individuals biting a particular stimulus. Data were analyzed following Burghardt (1970), Cooper and Alberts (1990, 1991), and Cooper and Burghardt (1990). The dependent measures are defined as follows: tongue-flick attack score is the number of tongue-flicks with no bite,

or if a bite occurred, tongue-flick attack score is the maximum number of preattack tongue-flicks by any lizard in the experiment for any stimulus plus 60 sec (latency to attack).

Statistical analysis was carried out according to Sokal and Rohlf (1981). Homogeneity of variances was tested with Bartlett and Hartley's tests, which indicated the use of nonparametric Friedman's method for randomized blocks and, particularly for responses between sexes, a test of equality of means with variances unequal. For both methods, alpha was set at 0.05. Biting frequencies were compared by McNemar's and Cochran's Q tests. Due to the large number of comparisons, alpha was lowered to 0.01.

RESULTS

Except for one lizard that did not respond to tala and toad stimuli, all other lizards tongue-flicked at the applicators in all conditions. Eleven individuals (58%) bit applicators bearing pindo stimuli, six individuals (32%) bit locust applicators, six (32%) bit tala applicators, three individuals (16%) bit daily diet applicators, two (11%) bit in response to toad stimuli, one (6%) in response to demineralized water, and none bit the applicator bearing cologne stimuli.

Preliminary data on stimuli yielded variances that were heteroscedastic by Bartlett's tests ($\chi^2 = 86.45$; $dF = 6$; $P < 0.01$). Because logarithmic transformations did not remove heteroscedasticity ($F_{max} = 8.72$; $dF = 17$; $P < 0.001$), primary analyses were done by nonparametric Friedman's method.

Tongue-flick attack score differed greatly among stimuli (Figure 1), producing a highly significant effect ($\chi^2 = 179.54$; $dF = 6$; $P < 0.001$). Comparisons carried out among stimuli show that tegus respond more strongly to pindo and daily diet than to demineralized water ($0.01 < P < 0.001$; $P < 0.001$, respectively) and cologne ($0.05 < P < 0.01$ for both). Responses to locust, tala, and toad stimuli did not differ significantly when compared with demineralized water ($P > 0.1$; $P > 0.5$; $P > 0.1$, respectively) or cologne ($P > 0.05$). Responses given to the two control stimuli did not differ significantly from each other ($P = 0.5$).

Ranges and also variances of tongue-flick attack score were very broad for each stimulus, being 2–158 for pindo and locust, 0–151 for tala and toad, 1–156 for daily diet, 2–149 for demineralized water, and 4–29 for cologne.

Differences among conditions for preattack tongue-flicks were not as great as for tongue-flick attack scores (Table 1), but highly significant differences were found between conditions ($\chi^2 = 179.69$; $dF = 6$; $P < 0.001$). Pairwise comparisons between controls and food stimuli revealed only three significant differences (of a total of 11): a lower level of response to demineralized water than to toad and daily diet ($0.05 < P < 0.01$) and between toad and cologne stimuli ($P < 0.001$).

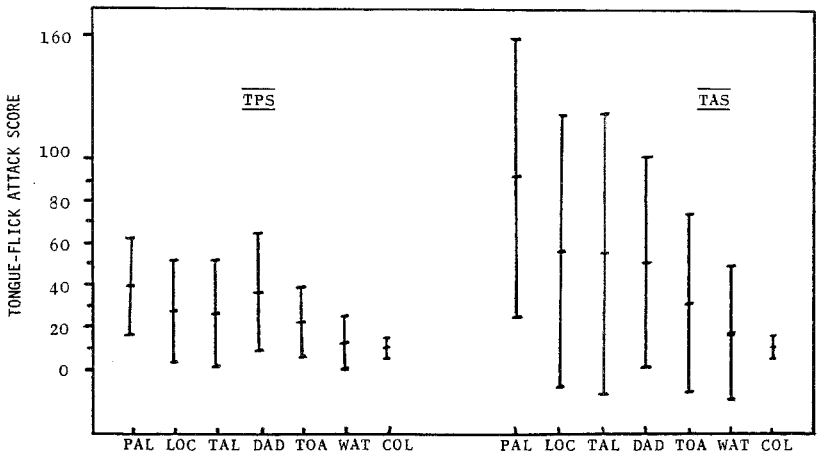

FIG. 1. Tongue-flick preattack scores (TPS) and attack scores (TAS) for *Tupinambis teguixin* in response to chemical stimuli derived from pindo palm fruit (PAL), locust (LOC), tala fruit (TAL), normal daily diet (DAD), toad (TOA), demineralized water (WAT), and eau-de-cologne (COL). Means are expressed by horizontal bars and the extremes represent standard deviations.

Latency to attack was lower in response to pindo and other food stimuli than to control stimuli (Table 2), resulting in a highly significant conditions effects ($\chi^2 = 280.36$; $dF = 6$; $P << 0.001$).

Attacks occurred in response to all types of food stimuli and in response to only one control stimulus (demineralized water). Pindo stimuli were the most frequently attacked followed by locust and tala (Figure 1). A Cochran's Q test among attacks for all stimulus conditions showed that there were highly significant differences among conditions ($Q = 33.77$; $dF = 5$; $P < 0.001$). Because the cologne control stimuli did not elicit an attack, the test was applied again without considering the pungency control, yielding a lower value of significance (a lower value of Q) but still highly significant ($Q = 25.37$; $dF = 5$; $P < 0.001$). Pairwise comparisons carried out with McNemar's test (Table 3) showed that pindo elicited more attacks than locust, tala, daily diet, toad, and demineralized water. Tala and locust stimuli only provoked more attacks than demineralized water and cologne. The remaining pairwise comparisons did not yield significant differences. Cologne did not provoke an attack response and was not included in pairwise comparisons.

Multiple comparisons given between females and males to total tongue-flick score, latency, and preattack score did not show any significant differences (Table 4). Differences among comparisons show that the nearest value to sig-

TABLE 1. *Tupinambis teguixin* RESPONSES TO CHEMICAL STIMULI FROM FIVE FOOD ITEMS AND TWO CONTROL STIMULI[a]

	Pindo		Locust		Tala		Daily diet		Toad		Water		Cologne	
	Pre	Tot	Pre	Tot	Pre	Tot	Pre	Tot	Pre	Tot	Pre	Tot	Pre	Tot
$\bar{X}$	39.94	90.89	27.47	55.26	26.42	54.21	36.15	50.05	22,31	31.57	12.84	17.47	10.78	10.78
SE	23.35	66.70	24.24	64.83	25.85	67.24	28.20	50.61	17.24	42.12	13.05	32.38	05.38	05.38
r	2–32	2–158	2–39	2–158	0–32	0–151	1–88	1–156	0–44	0–151	2–20	2–149	4–22	4–22

[a]Pre = number of preattack tongue-flicks; Tot = total tongue-flick score; $\bar{X}$ = average value among 19 individuals tested; SE = standard deviation of the mean; *r* = range of values obtained.

TABLE 2. LATENCY TO ATTACK (SECONDS) AND NUMBER OF *Tupinambis teguixin* ATTACKING COTTON-TIPPED APPLICATORS WITH CHEMICAL FOOD AND NONFOOD STIMULI

Stimulus	Latency to attack			Number of individuals attacking
	X	SE	Range	
Pindo fruit	34.36	24.41	5-60	11
Tala fruit	42.73	26.11	4-60	6
Locust	49.31	17.48	6-60	6
Daily diet	54.42	13.36	21-60	3
Toad	55.36	14.35	5-60	2
Water	57.10	12.61	5-60	1
Cologne	60.00	00.00	60-60	0

TABLE 3. G VALUE FOR MCNEMAR'S TEST IN PAIRED COMPARISONS BETWEEN STIMULI IN ATTACK RESPONSE[a]

	Pindo	Locust	Tala	Daily diet	Toad
Locust	6.79				
Tala	6.79	2.76[a]			
Daily diet	7.36	1.93[a]	1.93[a]		
Toad	12.47	2.91[a]	5.45	0.34[a]	
Water	13.86	6.93	6.93	2.77[a]	1.38[a]

[a]No significant difference ($P > 0.05$).

nificance for tongue-flick score was for toad responses (-1.71), and the furthest one was for locust responses (-14.88).

DISCUSSION

It is clear that tegu lizards (*Tupinambis teguixin*) are capable of detecting chemical food stimuli, thus confirming previous reports that lizards with the most forked tongues (Cooper, 1989; Cooper and Burghardt, 1990) and especially a congeneric species, *T. rufescens* (Cooper, 1990), discriminate prey chemically.

Our data show that tegus: (1) respond to stimuli as if they were food items, (2) do not respond in the same way to odorous and odorless nonfoods, and (3) respond differentially among food types.

TABLE 4. MULTIPLE PAIRWISE COMPARISONS WITH TEST OF EQUALITY OF MEANS FOR HETEROGENEOUS VARIANCES AMONG FOOD AND NONFOOD STIMULI, AND TONGUE-FLICK PREATTACK SCORE (PRE), LATENCY TO ATTACK (LAT), AND TONGUE-FLICK ATTACK SCORE (TOT)[a]

	PRE	LAT	TOT
Pindo	−3.12	−2.05	−2.21
Locust	−2.24	−1.76	**−14.88**
Tala	−2.03	−1.92	−1.88
Daily diet	−3.10	−2.16	−2.05
Toad	−1.99	−1.80	**−1.71**
Water	−2.10	−1.95	−1.96
Cologne	−2.05	—	−2.05

[a] (t_s' compared with $t_{0.05}$) between sexes. Numbers set in bold type are the nearest and furthest t_s' to the critical level of significance.

Values obtained for total tongue-flick score, tongue-flick preattack score, and latency consistently indicated stronger response to food stimuli than to nonfood stimuli. Evidence for differential response to chemical cues among food types can be seen in the pindo stimuli, which elicited more bites than any other food stimuli, and in tala stimuli, which elicited more bites than toad.

The two plant species may be preferred to animal items, and this is consistent with recent findings (Mercolli and Yanosky, personal observation) that a higher frequency and volume of wild fruits than animals are found in digestive analyses of *Tupinambis teguixin* and that tegus eat wild fruits when adults (Donadio and Gallardo, 1984). A less likely alternative is that stimulus concentrations were lower in animal swabs.

Our results agree with the prediction that widely foraging species, as shown by Mercolli and Yanosky (1989) for *Tupinambis teguixin*, which seems to find food by tongue-flicking, rely more on chemical senses (Regal, 1978; Cooper, 1990). Data presented here also show intraspecific variations in the response to particular food stimuli, called "chemical prey preference polymorphism," by Burghardt (1975) who considered it genetically based.

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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. 1. Communication by Chemical Signals. Appleton-Century-Crofts, New York.

BURGHARDT, G.M. 1975. Chemical prey preference polymorphism in newborn garter snakes *Thamnophis sirtalis*. *Behaviour* 25:202–225.

BURGHARDT, G.M. 1990. Chemically mediated predation in vertebrates: diversity, ontogeny, and information, pp. 475–499, *in* D.W. MacDonald, D. Müller-Schwarze, and S.E. Natvaczuk (eds.). Chemical Signals in Vertebrates 5. Oxford University Press, New York.

COOPER, W.E. 1989. 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. 1990. Prey odor detection by teiid and lacertid lizards and the relationship of prey odor detection to foraging mode in lizards families. *Copeia* 1990(1):237–242.

COOPER, W.E, and ALBERTS, A.C. 1990. Responses to chemical food stimuli by an herbivorous actively foraging lizard, *Dipsosaurus dorsalis*. *Herpetologica* 46(3):259–266.

COOPER, W.E., 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(1):135–146.

COOPER, W.E., and BURGHARDT, G.M. 1990. A comparative analysis of scoring methods for discrimination of chemical prey stimuli by squamate reptiles. *J. Chem. Ecol.* 16(1):45–65.

DONADIO, O.E., and GALLARDO, J.M. 1984. Biologia y conservacion de las especies del genero *Tupinambis* (Squamata, Sauria, Teiidae) en la Republica Argentina. *Rev. Mus. Arg. Cs. Nat. "B. Rivadavia," Zool.* 13(11):117–127.

MERCOLLI, C., and YANOSKY, A.A. 1989. Repertoire des comportements du Teju *Tupinambis tequixin*). Sauria: Teiidae. *Rev. Fr. Aquariol. Herpetol.* 16(1989)4:123–130.

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, US Department of Health, Education and Welfare, Washington, D.C.

SIMON, C.A. 1983. A review of chemoreception in lizards, pp. 119–133, *in* R.B. Huey, F.R. Pianka, and J.W. Schoener (eds.). Lizard Ecology: Studies on a Model Organism. Harvard University Press, Cambridge, Massachusetts.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry. W.H. Freeman and Co., New York.

YANOSKY, A.A., and MERCOLLI, C. 1991. Temperaturas internas y frecuencias de muda en crias de *Tupinambis teguixin* (Reptilia, Teiidae) bajo condiciones controladas. *Cuad. Herp.* 6(4):23–26.

YANOSKY, A.A., and MERCOLLI, C. 1992. Tegu lizard (*Tupinambis teguixin*) management in captivity at El Bagual Ecological Reserve, Argentina. *Arch. Zootec.* 41(151):41–51.

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ARRESTANT RESPONSES OF SOUTHWESTERN CORN BORER LARVAE TO FREE AMINO ACIDS: STRUCTURE–ACTIVITY RELATIONSHIPS

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Abstract—The leaf-feeding resistance of corn or maize *Zea mays* L. to the southwestern corn borer, SWCB, *Diatraea grandiosella* Dyar has been attributed at least in part to decreased protein, increased crude fiber, and increased hemicellulose in the whorls of resistant genotypes. In this study, individual amino acids and sugars were evaluated as arrestants, with the objective of identifying those that gave weak or negative responses. Several structure–activity relationships were identified. Larvae responded to three-carbon *n*-alkyl alpha amino acids more than to two-, four-, five-, and six-carbon compounds. Amino acids with terminal isopropyl functions gave decreased responses relative to their *n*-alkyl counterparts. Dicarboxylic acids and their amides gave the lowest responses of all classes of amino acids. The normally occurring basic amino acids were all good arrestants. The guanido [$HN{:}C(NH_2)NH-$] function was somewhat important to an arrestant response, as was the number of methylenes between the alpha and omega amino functions of diamino *n*-alkyl amino acids. Hydroxy amino acids were generally good arrestants unless the hydroxyl was located on a ring system. The two sugars present in expressed corn whorl juice, glucose and fructose, gave poor responses. However, two other sugars, mannose and arabinose, whose C-2 hydroxyls are conformationally in the axial position, were strongly arrestant. Formulated amino acid mixtures based on their content in whorl juice were as strong arrestants as whorl juice. However, the relative contributions of amino acids and sugars that are weak arrestants to the resistance of corn to SWCB larvae is uncertain because amino acid analyses did not reveal significantly higher contents of these amino acids in the whorl juices of resistant lines.

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Key Words—Southwestern corn borer, *Diatraea grandiosella* Dyar, Lepidoptera, Pyralidae, corn, *Zea mays* L., plant-insect interaction, amino acids, sugars, herbivory, feeding resistance.

INTRODUCTION

The southwestern corn borer (SWCB), *Diatraea grandiosella* Dyar, is a major pest of corn, *Zea mays* L., in the southern United States. First-generation SWCB larvae first feed in the corn whorl and later burrow into the stalk where they tunnel. Later generations attack reproductive-stage corn plants, where the larvae feed on leaf sheath, ear, and stem tissues (Davis et al., 1989).

A long-continuing corn breeding program at Mississippi State, Mississippi, has led to the release of eight germplasm lines with leaf-feeding resistance to the fall armyworm (FAW) [*Spodoptera frugiperda* (J.E. Smith)], the SWCB (Davis et al., 1988a,b), and several other insects. The resistance in corn to these insects appears to be operating by the same mechanisms (antibiosis and nonpreference), so there is the expectation that the same or similar chemical and physical factors may also be involved (Wiseman et al., 1981, 1983; Williams et al., 1985, 1987; Davis et al., 1988a,b).

Previous chemical studies showed that lines resistant to the SWCB were consistently higher in crude fiber, and the hemicellulose content of the fiber was higher while the cellulose content was unchanged. The susceptible lines were at least 25% higher in protein, lipid, total sugars, ash, and polyphenol oxidase activity (Hedin et al., 1984).

6-Methoxybenzoxazolinone was reported as a plant resistance factor to the ECB (Klun and Brindley, 1966), but we have found it to be only one third as toxic to the SWCB as to the ECB in larval feeding studies (Nicollier et al., 1982) and present in very low concentrations in corn lines with leaf-feeding resistance to the SWCB (Hedin et al., 1984, 1990). Maysin, a flavone glycoside, was isolated from corn silks of the line Zapalote Chico by Waiss et al. (1979), who reported it to have antibiotic activity toward the corn earworm *H. zea* (Boddie). However, we found only trace amounts of maysin in whorl tissue, the site of usual SWCB feeding (Hedin et al., 1984, 1990).

Recently (Hedin et al., 1990), the free amino acids were shown by isolational work and choice bioassays to be more important than all other factors evaluated in defining larval preference related to leaf-feeding resistance of corn to fall armyworm. In preliminary tests with SWCB larvae, a relatively strong response to amino acids and a weak response to sugars was observed, but the relative responses to individual amino acids were different from those of FAW larvae, and several structure–activity relationships were apparent. This report describes various tests conducted to further explore these observations.

METHODS AND MATERIALS

Corn Lines and Harvesting. Corn lines used in these studies were developed by the Corn Host Plant Resistance Research Unit at Mississippi State, Mississippi. They were selected for desirable resistance properties to the SWCB and grown using normal agronomic practices in the area. Whorls were excised on site from plants in the midwhorl V_{8-10} (Ritchie and Hanway, 1982) stage of growth, transported to the laboratory, dissected to obtain the inner green-yellow tissue that constitutes the feeding site of SWCB first-generation larvae, and frozen at −20°C or pressed to collect juices (see below). This section was later freeze-dried, ground in a Wiley mill, and the powder stored in a freezer until analyzed.

Processing and Chromatography of Cold-Pressed Corn Whorl Juice. Upon pressing green–yellow corn whorls (see above) containing about 85% moisture in a laboratory press at 10,000 psi, about 45–50% could be recovered as a juice, which yielded 2.5–4.0% total solids. After filtration of the juice to remove a small amount of remaining particulate material, 200-ml portions of the juice were chromatographed on a 5 × 70-cm Sephadex G-50-150 column with water as the eluant. Eight to ten 250-ml fractions were collected, of which 80–85% of the total solids eluted as a brown band, mostly sugars and amino acids, at about 1200–1500 ml. Quantitative recovery of total solids applied to the Sephadex G-50-150 column was demonstrated by weighing the fractions after freeze-drying.

The sugars were separated from the amino acids by chromatography on a 2 × 6-cm Dowex 50 column that was prepared by equilibration with HCl, followed by washing with water. The sample (1–2 g) was dissolved in water and introduced onto the column. The sugars were recovered by further elution with water until reaction of aliquots with anthrone demonstrated that all sugars had been eluted. The amino acids were subsequently eluted with 10% NH_4OH until reaction of aliquots with ninhydrin demonstrated that all amino acids had been eluted. There was a quantitative recovery (as determined by weighing of the freeze-dried eluates) of total solids in the two fractions; 75% of the total solids in the neutral fraction were mono- or disaccharides, and 92% of the total solids in the NH_4OH fraction were amino acids.

Analytical Procedures. Amino acids were determined by HPLC analyses before and/or after acid hydrolysis, employing their phenylthiocarbamyl derivatives (Cohen et al., 1986). Their content in corn whorl juice and freeze-dried whorls was published in a previous report (Hedin et al., 1990). Individual sugars were also determined by HPLC analysis; employing a Waters Bondapak NH_4 column and acetonitrile–water (80:20).

SWCB Choice Laboratory Bioassays. Bioassays were conducted as paired tests using extracts or known constituents applied in water solution to filter paper

disks (2.5 cm) placed in Petri dishes (15 cm in diameter, six replicates per compound), and approximately 40 neonate larvae were allowed to respond to the disks according to the procedure of Williams et al. (1987). Results were first recorded as the average number of larvae responding to the test (coming to the disk) minus those responding to the water check, or in confrontational (preference) tests, test A minus test B, unless otherwise described. In the study with the 54 amino acids and sugars, which was carried out on six occasions over a three-year period, responses were reported as "response, % of alanine" to which larvae were most attracted. In one other test, the response of larvae to the amino acids over a range of concentration levels was evaluated.

Statistical Methods. Means and standard deviations were calculated for the various extracts or constituents of extracts tested. The *t* test was also carried out on selected pairs. Because the evaluations were carried out as paired tests over an extended period, and all of the data was normalized and then averaged, multiple range tests were not considered feasible.

RESULTS

Preference Tests Comparing Whorl Juice, Amino Acids, and Sugars. A preliminary test was carried out with neonate larvae, 40 per test, to determine whether mixtures of amino acids that were based on their content in whorl juice (Hedin et al., 1990) were as stimulatory as whorl juice (Table 1). In two tests, glucose and fructose were added to the amino acids. All preference tests failed significance at the 0.5 probability level in the *t* test, confirming the conclusion

TABLE 1. CHOICE BIOASSAYS COMPARING RESPONSES OF SWCB NEONATE LARVAE TO S AND R WHORL JUICES WITH S AND R WHORL JUICE-BASED AMINO ACID (WJAA) MIXTURES AND SUGARS

Test	Formulations	Bioassay: insects responding to tests
A	WJAA (S × S)[a] vs juice (S × S)[b]	8.3 ± 3.7/5.5 ± 2.2[d]
B	WJAA (R × R)[b] vs juice (S × S)	9.2 ± 4.1/8.3 ± 2.8
C	WJAA (S × S) + FRU + GLU[c] vs juice (S × S)	6.2 ± 2.1/5.2 ± 3.2
D	WJAA (R × R) + FRU + GLU vs juice (S × S)	3.8 ± 2.9/4.9 ± 2.1
E	WJAA (S × S) vs WJAA (R × R)	6.8 ± 2.4/5.7 ± 3.0

[a] Mixtures based on the average of three S and R lines (see Hedin et al., 1990), all tests at 7 mg/disk.
[b] S × S juice (all tests) = T × 601 × T232, R × R juice (all tests) = Mp704 × Mp707.
[c] 7 mg fructose + 14 mg glucose/disk.
[d] $\bar{X}$ ± SD; approximately 40 neonate larvae were used per test.

that whorl juice amino acid mixtures were as preferred or even slightly more preferred than whorl juice. When sugars were added to the amino acids, preference was not increased. The juices were employed in these tests because they are encountered by neonate larvae in their feeding patterns on the whorl.

Responses of SWCB Larvae to Amino Acids and Sugars. To evaluate the relative arrestant responses of SWCB larvae to amino acids and sugars, 54 compounds were tested at 7 mg/disk against water checks as described in Methods and Materials. Responses of neonate larvae to amino acids and other components were observed to vary with time of year. It was noted that highest responses were observed in the late summer, coincident with the annual addition of freshly collected field insects to the colony. By the following spring, responses had decreased by approximately 50%. Therefore, responses in Table 2 were averages of three to six tests, each conducted as six replicates, and "normalized" by conversion to "response, %of alanine" to which larvae were most attracted. To facilitate comparisons, amino acids were ranked according to classes and according to molecular size. Standard deviations were calculated, and *t* tests were performed on selected pairs (Table 2). In comparison of some pairs by the *t* test, values higher than 0.05 probability were obtained. However, trends were evident that seemed structurally plausible.

Among the *n*-alkyl alpha amino acids, the response for the three-carbon alanine (1) was best, and responses decreased for glycine (2) and those with four to six carbons (8, 13, 16). Two *n*-alkyl beta amino acids (6 and 11) gave weaker responses than their alpha amino acid counterparts (1 and 8). The terminal isopropyl functions of valine and leucine (14 and 17) seem to be associated with the weaker responses of these amino acids relative to norvaline and norleucine (13 and 16). The peptides glycylglycine and alanylalanine (4 and 5) were intermediate in responses relative to alanine and glycine (1 and 2), but acetylation of glycine (3) totally abolished a response.

Dicarboxylic alpha amino acids and their amides (18–24) were, as a class, the weakest arrestants of all the compounds tested. Methylation of the beta carbon of aspartic acid (20) and of the alpha carbon of glutamic acid (23) improved responses to an intermediate level. Methylation of the polar terminus of the molecule also improved responses of these two compounds (20 and 23), while methylation to form an isopropyl group at the alkyl terminus (15 and 17) decreased the response.

The normally occurring basic amino acids (29, 30, 33, 34) were stronger arrestants in comparison with the normally occurring acidic amino acids (18, 19, 21, 22). The guanido function [HN : $C(NH_2)NH-$] of arginine (30) may be important to responses by the larvae, because as substitution of the function or adjoining carbons occurs, the response decreases [see citrulline (31), canavanine (32), and creatine (25)]. The number of *n*-alkyl carbons between the alpha and omega amino functions also appears to be important because the potencies of

TABLE 2. RESPONSES OF NEONATE SWCB LARVAE TO AMINO ACIDS

No.	Compound	Structure	Response % of alanine[a]
	Neutral amino acids		
1	L-Alanine	$CH_3CH(NH_2)COOH$	100
2	Glycine	$CH_2(NH_2)COOH$	18.7 ± 20.9
3	*N*-Acetylglycine	$CH_3CONHCH_2COOH$	−13.7 ± 14.4
4	Glyclyglycine	$NH_2CH_2CONHCH_2COOH$	61.0 ± 20.9
5	L-Alanyl-L-alanine	$CH_3CH(NH_2)CONHCH(CH_3)COOH$	53.5 ± 36.5
6	β-Alanine	$NH_2(CH_2)_2COOH$	83.0 ± 24.6
7	Sarcosine	CH_3NHCH_2COOH	56.7 ± 6.1
8	L-α-Aminobutyric acid	$CH_3CH_2CH(NH_2)COOH$	79.3 ± 3.7
9	DL-α-Amino-*n*-butyric acid	$CH_3CH_2CH(NH_2)COOH$	85.7 ± 19.4
10	γ-Amino-*n*-butyric acid	$NH_2(CH_2)_3COOH$	58.0 ± 23.5
11	DL-β-amino-*n*-butyric acid	$CH_3CH(NH_2)CH_2COOH$	65.7 ± 30.9
12	DL-β-Amino-isobutyric acid	$NH_2CH_2{-}CH(CH_3)COOH$	62.7 ± 42.8
13	L-Nor-valine	$CH_3(CH_2)_2CH(NH_2)COOH$	64.2 ± 17.5
14	L-Valine	$CH_3CH(CH_3)CH(NH_2)COOH$	45.8 ± 26.0
15	L-Isoleucine	$CH_3CH_2CH(CH_3)CH(NH_2)COOH$	43.8 ± 13.8
16	L-Norleucine	$CH_3(CH_2)_3CH(NH_2)COOH$	37.7 ± 32.8
17	L-Leucine	$CH_3{-}CH(CH_3)CH_2CH(NH_2)COOH$	16.5 ± 16.3
	Acidic amino acids and amides		
18	L-Aspartic acid	$HOOCCH_2CH(NH_2)COOH$	14.0 ± 9.6
19	L-Asparagine	$H_2NCOCH_2CH(NH_2)COOH$	11.7 ± 6.8
20	DL-β-Methyl aspartric acid	$HOOCCH(CH_3)CH(NH_2)COOH$	30.0 ± 20.6
21	L-Glutamic acid	$HOOC(CH_2)_2CH(NH_2)COOH$	23.4 ± 18.9
22	L-Glutamine	$H_2NCO(CH_2)_2CH(NH_2)COOH$	2.0 ± 1.7
23	DL-α-Methyl glutamic acid	$HOOC(CH_2)_2C(CH_3)(NH_2)COOH$	41.3 ± 11.3
24	L-α-Aminoadipic acid	$HOOC(CH_3)_3CH(NH_2)COOH$	17.3 ± 11.0
	Basic amino acids		
25	Creatine	$HN:C(NH_2)N(CH_3)CH_2COOH$	3.3 ± 3.9
26	Creatinine	2-Imino-*N*-methyl hydantoin	39.2 ± 24.2
27	L-α,β-Diaminopropionic acid	$H_2NCH_2CH(NH_2)COOH$	64.0 ± 21.0
28	L-Ornithine	$H_2N(CH_2)_3CH(NH_2)COOH$	53.0 ± 17.7
29	L-Lysine	$H_2N(CH_2)_4CH(NH_2)COOH$	77.8 ± 4.7
30	L-Arginine	$HN:C(NH_2)NH(CH_2)_3CH(NH_2)COOH$	49.7 ± 8.7
31	L-Citrulline	$HN:CONH(CH_2)_3CH(NH_2)COOH$	39.7 ± 9.0
32	L-Canavanine	$HN:C(NH_2)NHO(CH_2)_2CH(NH_2)COOH$	36.0 ± 15.0
33	L-Histidine	(*S*)-2-amino-3-(4-imidazolyl)propionic acid	51.2 ± 14.9
34	L-Tryptophan	(*S*)-2-amino-3-(3-indolyl)propionic acid	51.6 ± 14.3
	Hydroxylated amino acids		
35	L-Serine	$HOCH_2CH(NH_2)COOH$	64.3 ± 14.7
36	DL-Isoserine	$H_2NCH_2CH(OH)COOH$	18.0 ± 8.6
37	L-Homoserine	$HO(CH_2)_2CH(NH_2)COOH$	54.7 ± 25.5
38	L-Phosphoserine	$(HO)_2P(O)OCH_2CH(NH_2)COOH$	46.5 ± 32.6

TABLE 2. CONTINUED

No.	Compound	Structure	Response % of alanine[a]
	Neutral amino acids		
Hydroxylated amino acids			
39	L-Threonine	$CH_3CH(OH)CH(NH_2)COOH$	70.6 ± 19.7
40	(L-Proline)	(*S*)-Pyrrolidine-2-carboxylic acid	62.0 ± 23.4
41	L-4-Hydroxyproline	(2*S*, 4*R*)-4-hydroxypyrrolidine-2-carboxylic acid	38.3 ± 21.3
42	(*L*-Phenylalanine)	(*S*)-2-Amino-3-phenylpropionic acid	75.8 ± 21.6
43	L-Tyrosine	(*S*)-2-Amino-3-(4-hydroxyphenyl) propionic acid	13.3 ± 10.0
Sulfur-containing amino acids			
44	L-Cysteine	$HSCH_2CH(NH_2)COOH$	12.2 ± 10.6
45	L-Methionine	$CH_3S(CH_2)_2CH(NH_2)COOH$	72.2 ± 13.5

[a] $\bar{X}$ ± SD.

α, β-diamino propionic acid (27) and ornithine (28) are slightly decreased relative to lysine (29).

Hydroxyl alpha amino acids consistently evoked strong responses [see serine (35), homoserine (37), and threonine (39)]. However, location of the hydroxyl group at the aromatic/cyclic terminus, as in hydroxyproline (41) and tyrosine (43), decreased the response. This decrease was also observed relative to proline (40) and phenylalanine (42).

The alternative substitution pattern of isoserine (36) relative to serine caused a sharply decreased response, but phosphorylation of the serine hydroxyl function (38) had a lesser effect. Finally, cysteine (44), possessing an alkyl terminus sulfhydryl function, gave a much decreased response relative to methionine (45), which has the *S*-methyl function. The poor response of cysteine may be attributed to its pK_1 of 1.71, which is similar to the dicarboxyl amino acids.

Arrestant tests were also carried out with nine sugars (46–54) whose potencies ranged from 101 to 2.6 (Table 3). Of those tested, only glucose (49) and fructose (54) were found in corn whorl juice, although sucrose was also reported as present in corn plants (Dadd, 1985). These sugars appeared to be weaker arrestants than most of the normally occurring amino acids. The two sugars giving the highest responses were mannose (46) and arabinose (47). The only structural relationship to be identified was the conformation at the C-2 position in which the hydroxyls of mannose and arabinose were in the axial position, whereas the C-2 hydroxyl of all the other sugars was in the equatorial position.

Effects of Concentration on Arrestant Responses. Three amino acids—ala-

nine (1, strong arrestant), valine (14, intermediate), and aspartic acid (18, low)—were tested at five levels to determine whether concentrations based on that of whorl juice were optimal (Table 4). In this study, one concentration was threefold higher than normal and the others were 1/3, 1/10, and 1/30 of normal. Response of larvae at the threefold level was slightly poorer than at the normal level for all three amino acids. The response to alanine and valine was decreased at the three lower levels. In this study, alanine and aspartic acid performed as previously observed, while valine performed as well as alanine at the two highest levels. The results support the decision that was made to conduct tests with disks containing 7 mg of the test compounds.

TABLE 3. RESPONSES OF NEONATE SWCB LARVAE TO MONO- AND DISACCHARIDES

No.	Compound	Conformation at C-2, C-3, C-4	Response (% of alanine)[a]
46	D-(+)-Mannose	a,e,e[b]	101.0 ± 8.0
47	D-(+)-Arabinose	a,a,e	47.5 ± 19.5
48	D-(+)-Xylose	e,e,e	30.5 ± 6.5
49	D-(+)-Glucose	e,e,e	28.4 ± 5.3
50	D-(+)-Sucrose	e,e,e e,e,e	24.2 ± 17.6
51	D-(+)-Galactose	e,e,a	23.5 ± 10.5
52	L-(+)-Rhamnose	e,a,a,	8.5 ± 8.5
53	D-(−)-Ribose	e,a,e	6.0 ± 4.0
54	D-(−)-Fructose	e,e,e	2.6 ± 8.7

[a] $\bar{X}$ ± SD.
[b] a = axial, e = equatorial

TABLE 4. NEONATE SWCB LARVAE RESPONDING TO THREE AMINO ACIDS OVER A RANGE IN CONCENTRATIONS

		Insects responding (test minus check)				
No.	Compound	22 mg[a]	7 mg	2.2 mg	0.7 mg	0.22 mg
1	L-Alanine	9.3 ± 3.3[b]	9.5 ± 2.9	4.0 ± 2.4	3.0 ± 2.2	4.7 ± 4.9
14	L-Valine	9.7 ± 5.9	11.7 ± 2.4	2.5 ± 2.9	2.3 ± 1.0	(−)1.2 ± 3.7
18	L-Aspartic acid	(−)3.8 ± 3.9	(−)2.7 ± 4.8	2.0 ± 3.9	(−)1.0 ± 4.5	0.7 ± 2.6

[a] mg/disk; 7 mg is normal level based on solids in whorl juice.
[b] $\bar{X}$ ± SD.

DISCUSSION

Evidence has been developed that young SWCB larvae respond to amino acids to a greater extent than to sugars. They also respond more strongly to a blend of amino acids based on their content in juice than to the juice itself. The addition of glucose and fructose to the amino acid blend did not further improve the response by the larvae. Because the importance of free amino acids to stimulation of larval feeding had previously been established, a study was initiated to identify individual amino acids to which larvae responded. A study of 45 amino acids uncovered several structural relationships. Three-carbon *n*-alkyl alpha amino acids were stronger arrestants than two-, four-, five-, and six-carbon compounds. Terminal isopropyl functions decreased preference relative to their *n*-alkyl counterparts. Dicarboxylic acids and their amides were, as a class, the least preferred amino acids. The normally occurring basic amino acids were all preferred. The guanido function was shown to be somewhat important to preference, as was with the number of carbons between the alpha and omega amino groups (lysine, 4C; ornithine, 3C; etc.). Hydroxyl amino acids were generally preferred unless the hydroxy function was located on a cyclic function. Cysteine was nonpreferred.

The nutritional and stimulatory roles of amino acids for insects have been studied extensively. For example, Dadd (1985) reported that in addition to the 10 normally recognized essential amino acids for rats, proline, asparagine, and aspartic acid were found to be essential for some insects. Bernays (1985) reported that a number of sugars, particularly fructose, glucose, maltose, and sucrose, were phagostimulants for some insects. A number of amino acids, particularly glycine, alanine, serine, γ-aminobutyric acid, methionine, histidine, and proline, were found to be phagostimulants to one or several insects (Dadd, 1985).

Nine sugars were also tested in this preference study. Glucose and fructose, the only ones found in corn whorl juice, were not very stimulatory. However, two other sugars, mannose and arabinose, were highly preferred. These results are in apparent contrast to those of Chippendale and Reddy (1974), who evaluated a number of sugars as additives to diets for their effect on feeding and development of SWCB larvae. In their study, glucose, fructose, and sucrose were feeding stimulants, while arabinose, mannose, and several other sugars inhibited larval growth. Finally, in studies to determine the effects of concentration on preference, employment of quantities reflecting their content in corn whorl juice gave optimal responses.

These studies and previous work (Hedin et al., 1984, 1990) provide evidence that a number of constituents in corn plants, including amino acids, appear to modify preference of SWCB larvae. Although amino acid analyses of a number of insect-susceptible and -resistant corn varieties (Hedin et al., 1990) have identified some relatively minor differences, no one resistant variety exam-

ined to date has been found to contain large quantities of one or several of the amino acids that were shown in this study to be nonpreferred. However, it may eventually be possible and desirable to alter the corn plant genetically to biosynthesize higher quantities of one or more of these nonpreferred amino acids, thereby increasing their free amino acid levels.

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REFERENCES

BERNAYS, E.A. 1985. Regulation of feeding behavior, pp. 1–32, *in* G.A. Kerbut and L.I. Gilbert (eds.). Insect Physiology, Biochemistry, and Pharmacology, Vol. 4, Regulation, Digestion, Nutrition, Excretion. Pergamon Press, Elmsford, New York.

CHIPPENDALE, G.M., and REDDY, G.P.V. 1974. Dietary carbohydrates: Role in feeding behavior and growth of the southwestern corn borer, *Diatraea grandiosella*. *J. Insect Physiol.* 20:751–759.

COHEN, S.A., BIDLINGMEYER, D.A., and TARVIN, T.L. 1986. PITC derivatives in amino acid anlaysis. *Nature* 320:769–770.

DADD, R.H. 1985. Nutrition: Organisms, pp. 313–390, *in* G.A. Kerbut and L.I. Gilbert (eds.). Insect Physiology, Biochemistry, and Pharmacology, Vol. 4, Regulation, Digestion, Nutrition, Excretion. Pergamon Press, Elmsford, New York.

DAVIS, F.M., NG, S.S., and WILLIAMS, W.P. 1988a. Mechanisms of resistance in corn to leaf-feeding by southwestern corn borer and European corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 82:919–922.

DAVIS, F.M., WILLIAMS, W.P., MIHM, J.A., BARRY, B.D., OVERMAN, J.L., WISEMAN, B.R., and RILEY, T.J. 1988b. Resistance to multiple lepidopterous species in tropical derived corn germplasm. Miss. Agric. For. Exp. Stn. Tech. Bull. 157, 6 pp.

DAVIS, F.M., WILLIAMS, W.P., and WISEMAN, B.R. 1989. Methods used to screen maize for and to determine mechanisms of resistance to the southwestern corn borer and fall armyworm, pp. 101–108, *in* Toward Insect Resistant Maize for the Third World: Proceedings of the International Symposium on Methodologies for Developing Host Plant Resistance to Maize Insects, Mexico, D.F., CIMMYT.

HEDIN, P.A., DAVIS, F.M., WILLIAMS, W.P., and SALIN, M.L. 1984. Possible factors of leaf-feeding resistance in corn to the southwestern corn borer. *J. Agric. Food. Chem.* 32:262–267.

HEDIN, P.A., WILLLIAMS, 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.

KLUN, J.A., and BRINDLEY, T.A. 1966. Role of 6-methoxybenzoxazolinone in inbred resistance of host plant (maize) to first-brood larvae of European corn borer. *J. Econ. Entomol.* 59:711–718.

NICOLLIER, G.F., HEDIN, P.A., and DAVIS, F.M. 1982. 5-, 6-, and 7-methoxybenzoxazolinone: Carbon-13 nuclear magnetic resonance spectra and biological activity. *J. Agric. Food Chem.* 30:1133–1135.

RITCHIE, S.W., and HANWAY, J.J. 1982. How a corn plant develops. J. Iowa State Univ. of Sci. and Tech. Coop. Ext. Serv. Special Rpt. 48. 21 pp.

WAISS, A.C., JR., CHAN, B.G., ELLIGER, C.A., WISEMAN, B.R., MCMILLIAN, W.W., WIDSTROM, N.W., ZUBER, M.S., and KEASTER, A.J. 1979. Maysin, a flavone glycoside from corn silks with antibiotic activity. *J. Econ. Entomol.* 72:256–258.

WILLIAMS, W.P., BUCKLEY, P.M., and DAVIS, F.M. 1985. Larval growth and behavior of the fall armyworm (Lepidoptera: Noctuidae) on callus initiated from susceptible and resistant corn hybrids. *J. Econ. Entomol.* 78:951–954.

WILLIAMS, W.P., BUCKLEY, P.M., and DAVIS, F.M. 1987. Feeding responses of corn earworm (Lepidoptera: Noctuidae) to callus and extracts of corn in the laboratory. *Environ. Entomol.* 16:532–534.

WISEMAN, B.R., WILLIAMS, W.P., and DAVIS, F.M. 1981. Fall armyworm resistance mechanisms in selected corns. *J. Econ. Entomol.* 74:622–624.

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

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THE RELATIVE IMPORTANCE OF MOTHER AND TOXICOSIS IN THE SELECTION OF FOODS BY LAMBS

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Abstract—A lamb's mother and postingestive feedback both influence learning about foods, but their relative importance is unknown. We conducted a study to compare the ingestion of elm (1) by lambs whose mothers avoided elm because ingestion of elm by mother was previously paired with the toxin lithium chloride (LiCl) (M), (2) by lambs who received a mild dose of LiCl after they ingested elm, and whose mothers also avoided elm (M + L), and (3) by lambs who received LiCl after they ingested elm, but whose mothers ate elm avidly (M vs. L); in treatment (4) neither mothers nor lambs were given LiCl (C). In all four treatments, mothers and lambs ate poplar. Each lamb was exposed with its mother for 5 min/day to poplar on days 1, 2, 5, 7, 9, and 11 and to elm on days 3, 4, 6, 8, 10, and 12. Following exposures to elm on days 8, 10, and 12, lambs in treatments M + L and M vs. L were given a low dose of LiCl (100 mg/kg body weight orally in a capsule) when they ingested elm to produce an aversion to elm. During the first test after weaning, lambs could choose between elm and poplar. Lambs in C took more bites of elm than did lambs in M, M + L, and M vs. L (13, 2, 2, <1; $P > F = 0.003$), and they also took a higher percentage of bites from elm (42, 11, 6, 1; $P > F < 0.001$). The number of bites of poplar did not differ among treatments (M = 31, M + L = 26, M vs. L = 42, C = 27; $P > F = 0.458$). During the second test, when lambs were offered only elm, lambs in C took significantly more bites of elm than those in the other treatments, and lambs in M took more bites of elm than lambs in M + L, but not M vs.

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L (C = 38, M = 16, M + L = 3, M vs. L = 6; $P > F < 0.001$). During the third test, when lambs again could choose between elm and poplar, lambs in C again took more bites of elm (C = 14, M = 3, M + L = <1, M vs. L = <1; $P > F = 0.034$), and they also took a higher percentage of bites from elm (C = 26, M = 5, M + L = 2, M vs. L = 2; $P > F < 0.001$), than did lambs in the other three treatments. The number of bites of poplar did not differ among treatments (M = 47, M + L = 43, M vs. L = 62, C = 41; $P > F = 0.223$). We conclude that the response by lambs to the toxin LiCl was more important than was the mother as a social model because lambs that received LiCl avoided elm whether or not the mother ate it.

Key Words—Herbivory, elm, *Ulmus procera*, poplar, *Populus nigra*, secondary metabolite, learning, social facilitation, food aversion, food selection, ruminants, sheep, *ovis aries*.

INTRODUCTION

Chemical defenses have been categorized either as toxins or as digestion inhibitors, albeit some overlap between the divisions has been recognized (Feeny, 1976; Rhoades and Cates, 1976). However, there is growing evidence that food selection and ingestion are regulated by toxicity rather than by digestion inhibition. Studies of domesticated and wild mammals offered unpalatable plants normally available to them, or otherwise nutritious foods treated with extracts from these plants, verify the importance of toxicity. For example, ruminants purportedly avoid eating foods high in condensed tannins because tannins reduce the digestibility of proteins and carbohydrates (Van Soest, 1982; Robbins, 1983); however, goats apparently avoid foods that contain tannins because tannins are toxic, not because they inhibit digestion (Provenza et al., 1990; Distel and Provenza, 1991). In fact, most phytotoxins deter feeding by domestic herbivores, and the degree of deterrence depends upon the concentration of the toxin (Provenza et al., 1992a). Other animals such as snowshoe hares (*Lepus americanus*) (Reichardt et al., 1984, 1990a,b), microtine rodents (Batzli and Jung, 1980; Jung and Batzli, 1981), and bushy tailed woodrats (*Neotoma lepida*) (Meyer and Karazov, 1989) reduce intake to well below maintenance when offered plants containing high concentrations of secondary metabolites or when fed diets treated with extracts from these plants. Essentially all feeding deterrents, extracted from plants that potentially could be eaten by snowshoe hares, are toxic to mammals, insects, and microbes (Bryant et al., 1991b).

Young herbivores can learn from social models like their mothers and from postingestive feedback to avoid foods that contain toxins (Bryant et al., 1991b; Provenza et al., 1992a,b). For instance, lambs learn quickly to avoid a "harmful" novel food that their mothers were trained to avoid and to select a nutritious novel alternative selected by their mothers (Mirza and Provenza, 1990, 1992; Thorhallsdottir et al., 1990a,b). Lambs also learn to avoid foods that contain

toxins, without reference to their mothers, from aversive postingestive feedback (Provenza et al., 1992a,b). As toxicity increases and aversive feedback from a toxin increases, intake of the food decreases; conversely, as toxicity diminishes, intake of the food increases (Thorhallsdottir et al., 1987; Burritt and Provenza, 1989a; du Toit et al., 1991; Launchbaugh and Provenza, 1992).

Learning from both mother and aversive postingestive feedback are apparently important in determining the diet selected by the unweaned lamb, but their relative importance in unknown. Our study was designed to help assess which is more important.

METHODS AND MATERIALS

Study Site, Herbivore, and Plants. The experiment was conducted in Australia at CSIRO facilities near Armidale, NSW. Throughout the study, mature Merino ewes and their lambs foraged on grass pastures, except for the 2 hr (from about 0900 to 1100 hr daily) required each morning to conduct the experiment.

Thirty-two mature ewes were randomly assigned to one of two groups: 16 ewes were allowed to eat leaves of poplar (*Populus nigra* L.), but were conditioned with the toxin lithium chloride (LiCl) to avoid leaves of elm (*Ulmus procera* Salisb.); the other 16 ewes were allowed to eat both foods. Subsequently, the two groups of ewes and their lambs were exposed to poplar and elm, and half of the lambs in each group received a mild dose of LiCl on the last three occasions elm was offered, if any was eaten.

Poplar and elm branches were secured in a wooden frame between two boards held together with metal clamps. The frame was about 2 m in length, and held about 20 branches erect. Branches of elm and poplar were 1–1.5 cm in diameter and 20–30 cm in height. Branches were harvested daily throughout the experiment. Ewes and lambs ingested the leaves of poplar and elm, but generally did not ingest twigs from the branches.

Conditioning the Ewes. Conditioning of the ewes in the absence of their lambs began on October 22 and ended on October 27, 1991. Ewes were exposed in pairs to poplar on days 1, 3, and 4, and to elm on days 2, 5, and 6 (5 min/day in a 3-m × 4-m pen). When ewes from group 1 ingested elm, they were given LiCl (150 mg/kg body weight orally in a capsule). Ewes and lambs were reunited following daily exposures, and they were allowed to forage on grass pastures.

Conditioning the Lambs. Conditioning of the lambs with their mothers began on October 28 and ended on November 9, 1991. Lambs were placed in one of four treatments and exposed to poplar and elm with their mothers. The four treatments compared the ingestion of elm (1) by lambs whose mothers

avoided elm because its ingestion by mother was previously paired with LiCl (M), (2) by lambs who received a mild dose of LiCl after they ingested elm, and whose mothers also avoided elm (M + L), (3) by lambs who received LiCl after they ingested elm, but whose mothers ate elm avidly (M vs. L); and (4) by lambs that neither they nor their mothers were given LiCl (C). In all four treatments, mothers ate poplar. Thus, treatments M and M + L measured the effect of mother and mother plus LiCl, respectively, on intake of elm by lambs. Treatment M vs. L compared the net effect of opposing influences on the intake of elm by the lamb. Treatment C was the control. Ewes with different numbers of lambs were assigned equally to all treatments, as were male and female lambs. Only one lamb, selected at random, was used when ewes had twins.

Each lamb was exposed with its mother for 5 min/day to poplar on days 1, 2, 5, 7, 9, and 11 and to elm on days 3, 4, 6, 8, 10, and 12. Following the last three days of exposure to elm (days 8, 10, and 12), lambs in treatments 2 and 3 were given a low dose of LiCl (100 mg/kg body weight orally in a capsule; du Toit et al., 1991) if they ingested elm. We recorded the number of bites of each shrub taken by each ewe and lamb during exposure for 5 min/day.

Effects on Food Intake Associated with Process of Conditioning. We conditioned ewes and lambs to avoid elm by giving LiCl orally in a capsule. To do so, the animals were removed from the pen where the food was ingested and then given the capsule. There was minimal physical disturbance to the animals associated with giving the capsule. Data from other experiments show that the process of administering LiCl does not cause a reduction in food intake (Provenza et al., 1992b). Moreover, there is good reason to believe that any aversive effects from administering the capsule were associated not with the food, but rather with the person (F.D.P.) who administered the capsule, as Garcia et al. (1985) contend.

Testing the Lambs. The lambs were weaned on December 4, and a series of three tests began on December 9, 1991. During the first test, individual lambs were offered branches of poplar and elm simultaneously for 5 min/day for five consecutive days (December 9–13); poplar branches were placed on one side of the frame and elm branches on the other, and the location of the plant species was rotated daily. For the second test, individual lambs were offered only elm for 5 min/day for five consecutive days (December 14–18). The final test was conducted to determine if exposure to elm alone during the second test changed preference when lambs were offered a choice between poplar and elm; lambs were again offered poplar and elm simultaneously for 5 min/day for five consecutive days (December 19–23).

Data Recording during Conditioning and Testing. Animals were exposed simultaneously in two adjacent pens separated by a visual barrier. During conditioning of the lambs, four observers, one for each animal, recorded the number of bites taken by each ewe and lamb during exposure. During testing, two

observers, one for each animal, recorded the number of bites taken by each lamb. Animals were exposed and tested in random order within treatments, but treatments were selected systematically such that animals from all treatments were exposed and tested throughout daily trials.

Statistical Analysis. The analysis of variance had four treatments, with eight lambs per treatment. The exposures and the preference tests were repeated over a number of days (exposure six days; tests five days), so we used a repeated measures analysis (Winer, 1971). All data, including those for percentage of elm bites taken during the two-choice tests between elm and poplar, were normally distributed; therefore, none of the data were transformed (e.g., arc sine) prior to analysis. In tests 1 and 3, preference for elm was calculated as the percentage of total bites contributed by elm, which ranged from 0 to 100%. Low values represent avoidance of elm while high values represent preference for elm. Values near 50% represent equal preference for elm and poplar.

RESULTS

Conditioning the Lambs. During conditioning of the lambs, ewes and lambs in all treatments ingested poplar, but ewes that were conditioned to avoid elm (M and M + L) took somewhat fewer bites of poplar than did controls (M vs. L and C) (Table 1). Lambs in the different groups did not differ in the number of bites of poplar.

When offered elm, ewes that were conditioned to avoid elm (M and M + L) took significantly fewer bites of it than did controls (M vs. L and C), and

TABLE 1. MEAN NUMBER OF BITES PER DAY OF POPLAR AND ELM TAKEN BY EWES AND PREWEANED LAMBS DURING EXPOSURES OF MOTHER AND LAMB TOGETHER FOR 5 MIN/DAY FOR 6 DAYS[a]

	Ewes		Lambs	
Treatment	Poplar	Elm	Poplar	Elm
Mother (M)	48a	<1a	17a	4a
Mother + LiCl (M + L)	52a	<1a	8a	2a
Mother vs. LiCl (M vs. L)	64b	78b	21a	14b
Control (C)	73b	70b	18a	14b
$P > F$	0.017	<0.001	0.288	0.011
SEM	5.8	7.7	4.9	3.0
$LSD_{0.05}$	12	16		6

[a]Ewes were previously conditioned with LiCl, in the absence of their lambs, to avoid elm. Means followed by a different letter, within a column, differ significantly ($LSD_{0.05}$).

the same was true for their lambs (Table 1). However, there was an interaction between treatment and day for the lambs. The interaction occurred primarily because lambs in M vs. L ingested less elm on days 5 and 6 than they did on days 1–4 (Figure 1), because they received LiCl when they ingested elm on days 4, 5, and 6.

Testing the Lambs. In the first test after weaning, when lambs could choose between elm and poplar, lambs in C took significantly more bites of elm than did lambs in the other treatments (Table 2; $P > F = 0.003$). They also took a higher percentage of bites from elm than lambs in the other treatments (Table 2; $P > F < 0.001$). Bites of poplar did not differ among treatments (M = 31, M + L = 26, M vs. L = 42, C = 27; $P > F = 0.458$).

During the second test, lambs were offered only elm. Lambs in C took significantly more bites of elm than did those in the other treatments, and lambs in M took more bites of elm than did lambs in M + L ($P > F < 0.001$).

During the third test, lambs were again offered a choice between elm and poplar. Lambs in C took more bites of elm than did lambs in the other three treatments (Table 2; $P > F = 0.034$). They also took a higher percentage of

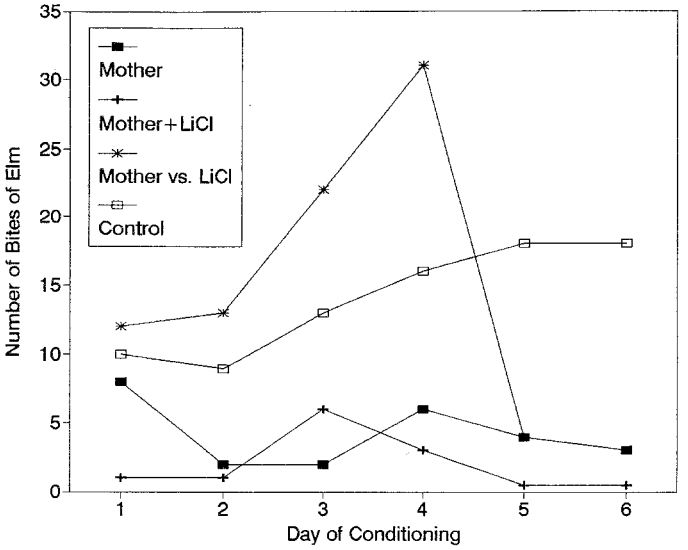

FIG. 1. Mean number of bites of elm taken daily by lambs during exposures of 5 min/day with their mothers (SEM = 4.0; $LSD_{0.05} = 5.7$; $P > F < 0.001$ for the interaction between treatment and day). In treatments M and M + L, mothers were previously conditioned with LiCl, in the absence of their lambs, to avoid elm. In treatments M + L and M vs. L lambs were given a mild dose of LiCl if they consumed elm on day 4 or thereafter.

TABLE 2. MEAN NUMBER OF BITES AND PERCENTAGE OF BITES OF ELM TAKEN BY WEANED LAMBS DURING TESTS OF 5 MIN/DAY[a]

	Number of bites of elm, test			Percentage of bites of elm, test	
Treatment	1	2	3	1	3
Mother (M)	2a	16a	3a	11a	5a
Mother + LiCl (M + L)	2a	3b	<1a	6a	2a
Mother vs. LiCl (M vs. L)	<1a	6ab	<1a	1a	2a
Control (C)	13b	38c	14b	42b	26b
$P > F$	0.003	<0.001	0.034	<0.001	<0.001
SEM	2.3	5.7	3.5	5.1	3.9
$LSD_{0.05}$	5	12	7	10	8

[a]The lambs had been conditioned, as described in the text, before weaning. During tests 1 and 3, lambs were given a choice between elm and popular; during test 2 they were offered only elm. Means followed by a different letter, within a column, differ significantly ($LSD_{0.05}$).

bites from elm than lambs in the other three treatments (Table 2; $P > F < 0.001$). There were no differences among treatments in the number of bites of poplar (M = 47, M + L = 43, M vs. L = 62, C = 41; $P > F = 0.223$).

DISCUSSION

Social vs. Individual Learning. According to Galef (1988), the term social learning "suggests a dichotomy between learning that is influenced socially and instances of individual learning in which behavior acquisition is not influenced by interactions with others," and he noted "in the final analysis it is always individuals that learn." Thus, Galef argued that while social interactions may facilitate acquisition of a behavior, continuation of the behavior depends on the consequences of the behavior to the individual.

Our experimental analyses suggests that such was the case for lambs. Lambs in M, whose mothers avoided elm, took fewer bites of elm than did lambs in C, which is consistent with the findings of others (Thorhallsdottir et al., 1990b; Mirza and Provenza, 1990, 1992), and supports the hypothesis that a lamb's food preferences are strongly influenced by its mother. Lambs ingested foods that the mother ingested and avoided foods that she avoided because of their proximity to and association with her. The mother was not actively involved, however, in teacher her offspring dietary habits. Rather, lambs apparently learned from the consequences associated with ingesting the food.

Aversive postingestive feedback increased lambs' avoidance of elm, as was

evident from the fact that lambs that received LiCl (M + L) took significantly fewer bites of elm than did lambs that did not (M), when only elm was offered in the second test (Table 2). Lambs that received LiCl, but whose mothers ate elm (M vs. L), also avoided elm altogether in tests 1 and 3, and they essentially avoided elm during test 2 when only elm was offered. Finally, seven of the eight lambs in treatment M vs. L received LiCl on two occasions, one lamb received LiCl only once, and none of the lambs received LiCl for three days. The administration of a low dosage of LiCl on only two occasions deterred lambs from ingesting elm during the 15 days of testing (Table 2), even though they had eaten elm avidly for four days with their mothers before they received LiCl (Figure 1). Thus, while it is clear that the mother had a strong influence on the diet selection of her lamb, nevertheless we conclude that the response by lambs to the toxin LiCl was more important than was the mother as a model because lambs that received LiCl avoided elm whether or not the mother ate it.

From a more general standpoint, what young herbivores learn initially from the mother is unlikely to be appropriate throughout the animal's entire lifetime because of the variable nature of forages and the variable requirements of herbivores. Both wild and domesticated herbivores select nutritious diets from rangelands that contain a diverse array of plant species, individuals, growth stages, and parts that vary in nutritional value and chemical and mechanical defenses (Provenza and Balph, 1990). These animals select nutritious diets even though their requirements vary with age, physiological state, and environmental conditions. Thus, we argue that the ability to select a diet does not depend on the mother alone, but is the result of postingestive feedback from nutrients and toxins as well. Thus, "palatability" is a function of the "nutrient content" of foods, and "unpalatibility" is related to the "toxin" concentration of foods (Provenza, 1991; Provenza et al., 1992a). As aversive feedback from a toxin (Thorhallsdottir et al., 1987; Burritt and Provenza, 1989a; du Toit et al., 1991; Launchbaugh and Provenza, 1992) or a nutrient deficiency (Rogers and Egan, 1975; Egan and Rogers, 1978) increases, intake of the food decreases. Conversely, as nutritional value improves and positive feedback from an energy- or nutrient-rich food increases, intake of the food increases (Burritt and Provenza, 1992).

Preference for Poplar vs. Elm. All lambs preferred poplar to elm, a result that was particularly evident in group C during test 3. The reason for the difference in preference is unknown, but at least two possibilities exist. First, even though the species were similar morphologically in leaf size and shape throughout the experiment, elm changed more phenologically than did poplar between exposure and testing. Poplar leaves were mature during both exposure and testing, but lambs were exposed to immature elm leaves and tested on mature elm leaves, which may also have lowered their acceptance of elm. Second, changes in leaf chemistry, perhaps caused by a secondary metabolite, may also have reduced the preference of lambs for elm as the season progressed.

Such seemingly subtle differences in leaf chemistry markedly influenced preference, as we found one day when elm branches were collected at a site 15 km from where the branches were always collected. All animals sniffed the elm leaves continuously throughout the 5-min test, but averaged only three bites of elm. We repeated the test immediately with elm from the usual location, and the average number of bites increased to 22 ($P > F < 0.001$).

Changes in environmental conditions (e.g., soil fertility, soil moisture content, amount of sunlight or shade) (Bryant et al., 1983, 1991, 1991a; Gershenzon, 1984) affect a plant's chemical composition, which can lead to subtle changes in flavor. Subtle changes in flavor cause herbivores to sample otherwise "familiar foods" cautiously (Burritt and Provenza, 1989b; Launchbaugh and Provenza, 1992), because lambs are reluctant to ingest novel foods (Lynch et al., 1983; Green et al., 1984; Chapple et al., 1987a,b; Thorhallsdottir et al., 1987, 1990a,b; Burritt and Provenza, 1989b, 1991).

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REFERENCES

BATZLI, G.O., and JUNG, H.G. 1980. Nutritional ecology of microtine rodents: Resource utilization near Atkasook, Alaska. *Arct. Alp. Res.* 12:483–499.

BRYANT, J.P., CHAPIN, F.S., III, KLEIN, D.R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

BRYANT, J.P., DANELL, K., PROVENZA, F.D., REICHARDT, P.B., CLAUSEN, T.P., and WERNER, R.A. 1991a. Effects of mammal browsing on the chemistry of deciduous woody plants, pp. 135–154, *in* M.J. Raupp and D.W. Tallamy (eds.). Phytochemical Induction by Herbivores. John Wiley & Sons, New York.

BRYANT, J.P., PROVENZA, F.D., PASTOR, J., REICHARDT, P.B., CLAUSEN, T.P., and DU TOIT, J.T. 1991b. Interactions between woody plants and browsing mammals mediated by secondary metabolites. *Annu. Rev. Ecol. Syst.* 22:431–446.

BURRITT, E.A., and PROVENZA, F.D. 1989a. Food aversion learning: Conditioning lambs to avoid a palatable shrub (*Cercocarpus montanus*). *J. Anim. Sci.* 67:650–653.

BURRITT, E.A., and PROVENZA, F.D. 1989b. Food aversion learning: Ability of lambs to distinguish safe from harmful foods. *J. Anim. Sci.* 67:1732–1739.

BURRITT, E.A., and PROVENZA, F.D. 1991. Ability of lambs to learn with a delay between food ingestion and consequences given meals containing novel and familiar foods. *Appl. Anim. Behav. Sci.* 32:179–189.

BURRITT, E.A., and PROVENZA, F.D. 1992. Lambs form preferences for non-nutritive flavors paired with glucose. *J. Anim. Sci.* 70:1133–1136.

CHAPPLE, R.S., WODZICKA-TOMASZEWSKA, M., and LYNCH, J.J. 1987a. The learning behavior of sheep when introduced to wheat. I. Wheat acceptance by sheep and the effect of trough familiarity. *Appl. Anim. Behav. Sci.* 18:157–162.

CHAPPLE, R.S., WODZICKA-TOMASZEWSKA, M., and LYNCH, J.J. 1987b. The learning behavior of sheep when introduced to wheat. II. Social transmission of wheat feeding and the role of the senses. *Appl. Anim. Behav. Sci.* 18:163–172.

DISTEL, R.A., and PROVENZA, F.D. 1991. Experience early in life affects voluntary intake of blackbrush by goats. *J. Chem. Ecol.* 17:431–450.

DU TOIT, J.T., PROVENZA, F.D., and NASTIS, A. 1991. Conditioned food aversions: How sick must a ruminant get before it learns about toxicity in foods? *Appl. Anim. Behav. Sci.* 30:35–46.

EGAN, A.R., and ROGERS, Q.R. 1978. Amino acid imbalance in ruminant lambs. *Aust. J. Agric. Res.* 29:1263–1279.

FEENY, P. 1976. Plant apparency and chemical defense, pp. 1–40, *in* J.W. Wallace and R. L. Mansell (eds.). Recent Advances in Phytochemistry, Vol. 10, Biochemical Interactions between Plants and Insects. Plenum, New York.

GALEF, B.G., JR. 1988. Imitation in animals: history, definition, and interpretation of data from the psychological laboratory, pp. 3–28, *in* T.R. Zentall and B.G. Galef, Jr. (eds.). Social Learning: Psychological and Biological Perspectives, Lawrence Erlbaum Associates, Hillsdale.

GARCIA, J., LASITER, P.A., BERMUDEZ-RATTONI, F., and DEEMS, D.A. 1985. A general theory of aversion learning, pp. 8–21, *in* N.S. Braveman and P. Bronstein (eds.). Experimental Assessments and Clinical Applications of Conditioned Food Aversions. New York Academy of Science, New York.

GERSHENZON, J. 1984. Changes in the levels of plant secondary metabolites under water and nutrient stress. *Recent Adv. Phytochem.* 18:273–320.

GREEN, G.C., ELWIN, R.L., MOTTERSHEAD, B.E., and LYNCH, J.J. 1984. Long-term effects of early experience to supplementary feeding in sheep. *Proc. Aust. Soc. Anim. Prod.* 15:373–375.

JUNG, H.G., and BATZLI, G.O. 1981. Nutritional ecology of microtine rodents: Effects of plant extracts in the growth of arctic microtines. *J. Mammal.* 62:286–292.

LAUNCHBAUGH, K.L., and PROVENZA, F.D. 1993. Can plants practice mimicry to avoid grazing by mammalian herbivores? *Oikos* 66: in press.

LYNCH, J.J., KEOGH, R.G., ELWIN, R.L., GREEN, G.C., and MOTTERSHEAD, B.E. 1983. Effects of early experience on the post-weaning acceptance of whole grain wheat by fine-wool Merino lambs. *Anim. Prod.* 36:175–183.

MEYER, M.W., and KARAZOV, W.H. 1989. Antiherbivore chemistry of *Larrea tridentata*: Effects of woodrat (*Neotoma lepida*) feeding and nutrition. *Ecology* 70:953–961.

MIRZA, S.N., and PROVENZA, F.D. 1990. Preference of the mother affects selection and avoidance of foods by lambs differing in age. *Appl. Anim. Behav. Sci.* 28:255–263.

MIRZA, S.N., and PROVENZA, F.D. 1992. Effects of age and conditions of exposure on maternally mediated food selection in lambs. *Appl. Anim. Behav. Sci.* 33:35–42.

PROVENZA, F.D. 1991. Behavior and nutrition are complementary endeavors, pp. 157–169, *in* Proceedings 2nd Grazing Livestock Nutrition Conference. Agric. Expt. Stn. MP-133. Oklahoma State University.

PROVENZA, F.D., and BALPH, D.F. 1990. Applicability of five diet-selection models to various foraging challenges ruminants encounter, pp. 423–459, *in* R.N. Hughes. Behavioral Mechanisms of Food Selection. NATO ASI Series G: Ecological Sciences, Vol. 20. Springer-Verlag, Heildelberg.

PROVENZA, F.D., BURRITT, E.A., CLAUSEN, T.P., BRYANT, J.P., and REICHARDT, P.B. 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. 1992a. Mechanisms of learning in diet selection with reference to phytotoxicosis in herbivores. *J. Range Manage.* 45:36–45.

PROVENZA, F.D., ORTEGA-REYES, L., SCOTT, C.B., LYNCH, J.J., and BURRITT, E.A. 1992b. Antiemetic drugs attenuate food aversions in sheep. *J. Anim. Sci.* Submitted.

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.

REICHARDT, P.B., BRYANT, J.P., ANDERSON, B.J., PHILLIPS, D., and CLAUSEN, T.P. 1990a. Germacrone defends Labrador tea from browsing by snowshoe hares. *J. Chem. Ecol.* 16:1961–1970.

REICHARDT, P.B., BRYANT, J.P., MATTES, B.R., CLAUSEN, T.P., and CHAPIN, F.S., III. 1990b. The winter chemical defense of balsam poplar against snowshoe hares. *J. Chem. Ecol.* 16:1941–1960.

RHOADES, D.F., and CATES, R.G. 1976. Toward a general theory of plant antiherbivore chemistry, pp. 168–213, *in* J.W. Wallace and R.L. Mansell (eds.). *in* Recent Advances in Phytochemistry, Vol. 10, Biochemical Interactions between Plants and Insects. Plenum, New York.

ROBBINS, C.T. 1983. Wildlife Nutrition. Academic Press, New York, 343 pp.

ROGERS, Q.R., and EGAN, A.R. 1975. Amino acid imbalance in the liquid-fed lamb. *Aust. J. Biol. Sci.* 28:169–181.

THORHALLSDOTTIR, A.G., PROVENZA, F.D., and BALPH, D.F. 1987. Food aversion learning in lambs with or without a mother: Discrimination, novelty and persistence. *Appl. Anim. Behav. Sci.* 18:327–340.

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.

VAN SOEST, P. 1982. Nutritional Ecology of the Ruminant. O & B Books, Corvalis, Oregon, 374 pp.

WINER, B.V. 1971. Statistical Principles in Experimental Design. McGraw-Hill, New York, 907 pp.

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PHENOLIC COMPOUNDS ISOLATED FROM BITTER LUPINE SEEDS AND THEIR INHIBITORY EFFECTS ON GERMINATION AND SEEDLING GROWTH OF LETTUCE

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Abstract—The phenolic acids, including 4-hydroxybenzoic and 4-hydroxycinnamic acids, and their derivatives, such as 6,7-dihydroxycoumarin and 1,2-dihydroxybenzene, were isolated from bitter lupine seeds and were identified using gas chromatography–mass spectrometry. These compounds inhibited lettuce seed germination in the first 24 hr after sowing, but after 72 hr germination was comparable with that of the control. However, very strong suppression of seedling growth, especially the roots, was observed for higher concentrations of the lupine seed fractions containing phenolic acids. Effects observed in the lettuce germination bioassays were compared with those produced by nine pure phenolic acids previously identified in the mixture. The ethyl acetate fraction from lupine seed extract inhibited seedling growth as effectively as pure 1,2-dihydroxybenzene, the strongest inhibitor of the pure phenolic compounds studied. The possible reason for this could be the synergistic effect created in the mixture of phenolic compounds isolated from the extract.

Key Words—Gas chromatography–mass spectrometry, germination, lupine extract, phenolic compounds, lettuce seedling.

INTRODUCTION

Lupine cultivation is very small among grain legumes grown in the world because of the adaptability problems of sweet lupine and because of high alkaloid content

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in bitter lupines. It is also known that bitter cultivars are more resistant to diseases and pests; they give a reasonable crop on poor, sandy soils; and produce proteins at low cost and without intensive management. In this situation, efficient large-scale debittering of bitter lupine seeds can offer an alternative source of protein. One of the procedures of debittering was developed by Gulewicz (1986).

Studies have been undertaken on the utilization of an extract, obtained as a waste during the debittering process of bitter lupine seeds from *Lupinus angustifolius* L. cv. Mirela, which were further used as fodder. The extract had a stimulating effect on the crop yield of wheat, barley, potato, sugar beet, and tomato (Cwojdzinski et al., 1989; Kahnt and Hijazi, 1987, 1991). The potatoes were also protected against the potato beetle (Krzymanska et al., 1988). Studies of the biological activity and identification of different groups of secondary metabolites present in the extract were undertaken, but the mechanism of action of the lupine seed extract on crops is not known.

One of the most abundant groups of secondary metabolites in plants are phenolics, which occur either in a free state or conjugated as esters or glycosides (Harborne, 1964; Harborne and Simmonds, 1964; Thakur, 1977). Many phenolics are inhibitory to germinating seeds or growing plants (Williams and Hoagland, 1982; Rice, 1984). The allelochemical activity of some phenolics has been related to plant competition, antagonism, and species distribution (Rice, 1984; Glass, 1976; Van Sumere et al., 1971). The influence of allelochemicals on agricultural production has been also described (Rice, 1979). On the basis of the above information, the objectives of our study were to identify phenolic compounds in crude lupine seed extract and to study the influence of these compounds on lettuce seed germination and seedling growth. In order to estimate the effectiveness of phenolics present in lupine extract on seedling growth, because of possible synergistic effects in the mixture, nine pure phenolic compounds identified in the extract were also studied for their activity.

METHODS AND MATERIALS

Chemicals. Solvents of analytical grade were obtained from POCh (Gliwice, Poland), and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% of trimethyl chlorosilane (TMS), used as the derivatizing reagent, was obtained from Fluka (Buchs, Switzerland). Silica gel H_{60} for column chromatography and silica gel 60 F_{254}) plates for thin layer chromatography (TLC) were purchased from E. Merck (Darmstadt, Germany). Pure phenolic compounds used as the standards in GC-MS analysis and in the biological tests were obtained from Sigma (St. Louis, Missouri): 1,2-dihydroxybenzene, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 4-hydroxycinnamic acid, 4-hydroxyphenylacetic acid. 1,4-Dihydroxybenzene

and 3-hydroxybenzoic acid were purchased from Aldrich (Milwaukee, Wisconsin). Samples of genistein and 2′-hydroxygenistein were a gift of Dr. S. Tahara, Hokkaido University.

Bitter Lupine Seed Extract. Lupine seeds were debittered in 48% ethanol according to the procedure patented by Gulewicz (1986).

Isolation of Free Phenolic Compounds from Lupine Seed Extract. Lupine seed extract was fractionated with organic solvents of increasing polarity: hexane, methylene chloride, and ethyl acetate. Further purification of the ethyl acetate fraction was performed on silica gel column eluted stepwise with ethyl acetate in hexane (0–100% v/v) and afterwards with methanol in chloroform (20–60% v/v). Fractions obtained after column chromatography were monitored by TLC in chloroform–methanol–water (20:5:0.5) and in ethyl acetate–methyl ethyl ketone–water–formic acid (5:3:1:1). Developed plates were inspected in UV light $\lambda = 254$ or 366 nm, and the characteristic colors formed by phenolic compounds with Gibbs reagent (Tahara et al., 1984) were examined.

Instrumentation. GC-MS analyses were carried out with Hewlett-Packard gas chromatograph model 5890 equipped with a mass selective detector model 5971A and an HP-1 fused silica capillary column (25 m × 0.25 mm ID). Nuclear magnetic resonance spectra were made in dimethyl sulfoxide (DMSO-d_6) on a Varian 300 MHz spectrometer, model Unity.

Seed Germination Tests. Lettuce [*Lactuca sativa* L. (cv. Rakowiecka)] seeds were purchased from CNOS (Poznan, Poland). Entire ethyl acetate fractions from lupine seed extract, three subfractions A, B, and C obtained after silica gel column chromatography, eight analytical-grade phenolic compounds, and 6,7-dihydroxycoumarin isolated from lupine seed extract were used in lettuce germination tests. Concentrations of fractions tested were expressed in milligrams per liter since the investigated fractions from the lupine extract being studied consisted of more than one compound, and thus molar expression of concentration was impossible.

Germination tests of the above-mentioned fractions were made in four dilutions (I–IV), differing from each other by one order of magnitude. The highest concentrations of tested fractions or pure compounds were prepared in methanol in concentrations of about 1 mg/ml^{-1}. For standards of phenolic compounds, three dilutions (I–III) were prepared in the concentration range 10^{-3} M–10^{-5} M.

To 11 cm Petri dishes with three layers of filter paper, purchased from POCh (Gliwice, Poland) 3 ml of methanolic solution, or 3 ml of pure methanol to the controls were pipetted. After evaporation of solvent at room temperature over 24 hr, 8 ml of redistilled water was added, and 30 seeds (surface sterilized with 5% H_2O_2) were placed in each dish. The dishes were incubated in darkness in 25°C for 24 hr, after which germination was assessed. The seeds were then exposed for 15 min to white light and placed back in the dark for 48 hr. The

seeds were assessed for germination again, and the lengths of hypocotyls and roots were measured. All treatments were replicated three times in each experiment, and the experiments were replicated twice. In the description of the germination bioassays, the percentages of germination are presented in relation to that of control. Germination of lettuce seeds used as controls was 80% of sown seeds after 24 hr and 95% after 72 hr. Differences between mean values of root and hypocotyl length were calculated using Student's t test, at a confidence level of $\alpha = 0.05$.

RESULTS

Identification of Phenolic Compounds in Ethyl Acetate Fraction. Ethyl acetate fraction from bitter lupine seed extracts contained many compounds, which were separated using a silica gel column eluted with a step gradient of ethyl acetate in hexane, followed by a gradient of methanol in chloroform. Fractions from the column were combined in three parts (subfractions A, B, and C) according to retention times of phenolic compounds on TLC plates. Most of free phenolic compounds were isolated in the first fractions eluted with up to 50% of ethyl acetate in hexane. The column eluate with 100% of ethyl acetate and up to 20% of methanol in chloroform (subfraction B) contained minor amounts of free phenolics and mainly phenolic conjugates. Subfraction C contained some polar phenolic conjugates, alkaloids, and sugars. Subfractions A and B were subjected to GC-MS analysis as trimethylsilyl (TMS) derivatives. Total ion current (TIC) chromatograms are presented in Figures 1 and 2. The compounds identified in both subfractions are shown in Table 1 according to their mass spectra and GC retention. Available standards of identified compounds were run in order to confirm proposed structures, and some of the compounds were identified by comparison with mass spectra in the library of the National Bureau of Standards-NBS49K in the GC-MS data system.

The percentage composition of free phenolic compounds was estimated on the basis of integrated TIC chromatograms generated during each GC-MS run. Only approximate quantification could be made this way. For accurate quantification, response curves of compound amounts injected against the total ion current must be constructed for each compound present in the mixture. We used the percentage area of the peaks in the total ion current for relative quantification of compounds in the analyzed subfractions. In subfraction A were found a series of benzoic, phenylacetic, and 3-phenyl-2-propenoic acids derivatives with hydroxy or methoxy substituents at different positions of the phenyl ring.

Identification of the compound in the most intense peak of subfraction A (Figures 1 and 2, peak 23) was not possible on the basis of its mass spectrum. The molecular weight of the TMS derivative was 322 daltons. Lack of distinctive

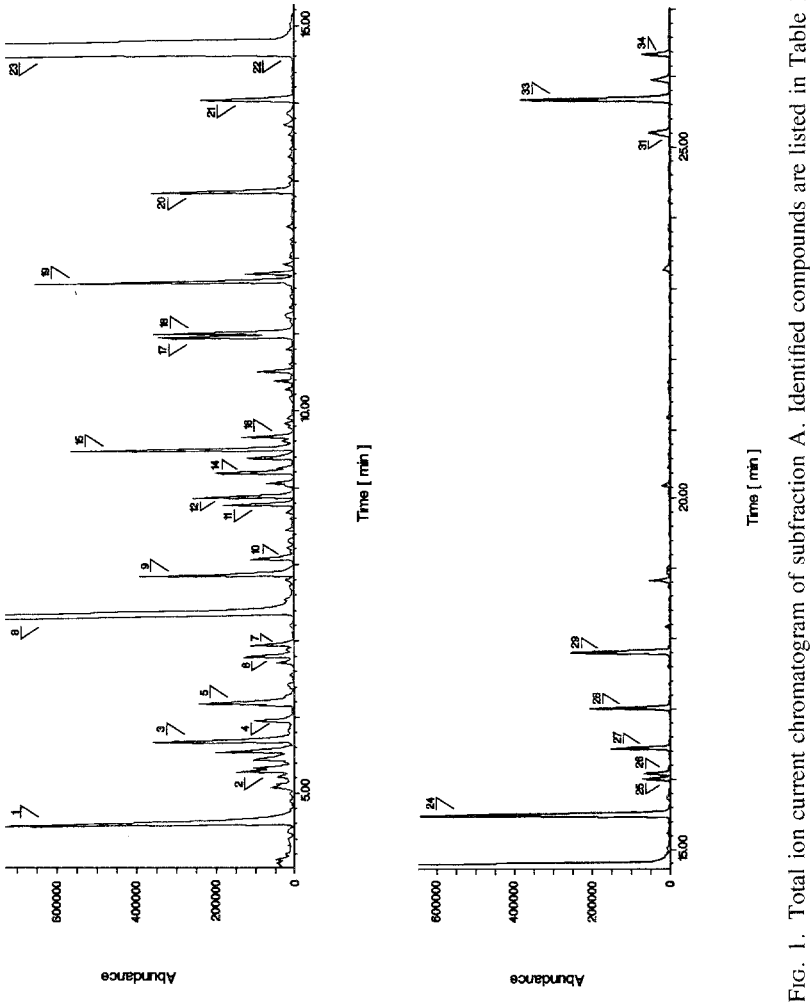

FIG. 1. Total ion current chromatogram of subfraction A. Identified compounds are listed in Table 1.

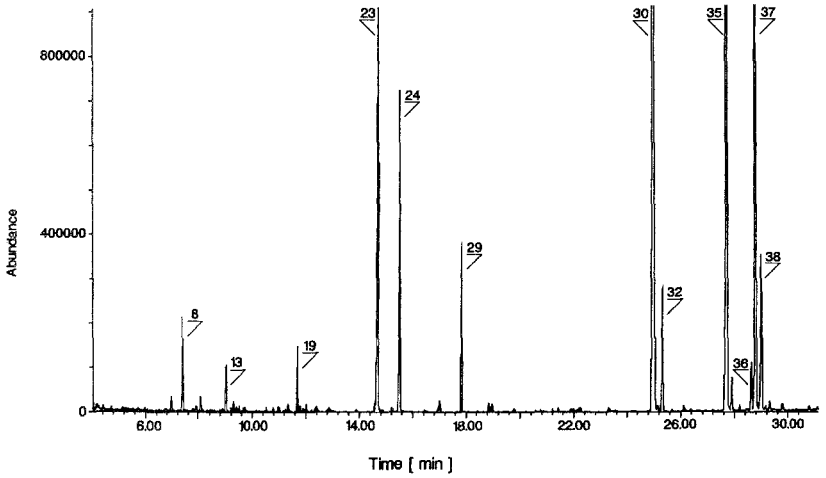

FIG. 2. Total ion current chromatogram of subfraction B. Identified compounds are listed in Table 1.

fragment ions in the mass spectrum made the assignment of structure impossible. The unidentified compound was isolated from the mixture after silica gel chromatography with a step gradient of ethyl acetate in hexane. Proton and carbon-13 nuclear magnetic resonance (NMR) spectra gave the structure as 6,7-dihydroxycoumarin. Chemical shifts of protons and carbons are presented in Table 2. In the TIC of subfractions A and B was recognized the peak of a trihydroxylated coumarin with one methylated hydroxyl group, but the position of methylation was not established (Figures 1 and 2, peak 24). In the range of GC retention time over 20 min, there were two isoflavones: genistein and 2′-hydroxygenistein; apigenin (5,7,4-trihydroxyflavone) was also identified.

Apart from phenolic compounds in subfraction A, we recognized six carboxylic acids containing 6–18 carbon atoms. These compounds were identified by a computer search of the reference mass spectral library.

In subfraction B, seven compounds identified in subfraction A were also recognized. However, in the TIC chromatogram, peaks related to conjugates of phenolic compounds were found in the region of the retention time above 24 min. Identification of these compounds was not possible on the basis of only mass spectra of TMS derivatives ionized in the electron impact mode.

Biological Tests. After fractionation of the bitter lupine extract with organic solvents, the fraction obtained after ethyl acetate extraction delayed seed germination and inhibited seedling growth. In dilution I of the ethyl acetate fraction, germination after 24 hr was 40% of control; after 72 hr it was comparable with that of the control (Table 3). In 10-, 100-, and 1000-fold dilutions germination

TABLE 1. SUBSTANCES IDENTIFIED IN SUBFRACTIONS A AND B AFTER GC-MS ANALYSIS

Peak	R_f	Compound	MW as TMS derivative	% area of peak in TIC, subfraction	
				A	B
1	4.62	Benzoic acid	194	4.22	
2	5.29	Phenylacetic acid	208	0.47	
3	5.68	1,2-Dihydroxybenzene	254	1.61	
4	5.94	Glyceric acid	322	0.52	
5	6.19	Unknown	?	1.51	
6	6.78	1,4-Dihydroxybenzene	254	0.62	
7	6.93	Unknown	272	0.56	
8	7.36	Hexenedioic acid[a]	288	21.33	1.20
9	7.86	Hexadienedioic acid[a]	286	1.38	
10	8.05	4-Hydroxybenzyl alcohol	268	0.52	
11	8.76	3-Hydroxybenzoic acid	282	0.64	
12	8.88	3, 4-Dihydroxybenzaldehyde	282	0.92	
13	8.99	Unknown	360		0.69
14	9.38	Unknown	282	0.46	
15	9.51	4-Hydroxybenzoic acid	282	1.95	
16	9.65	3,4,5-Trimethoxyphenylacetic acid[a]	298	0.59	
17	10.92	4-Hydroxphenylacetic acid	310	1.26	
18	11.08	4-Hydroxy,3-methoxybenzoic acid	312	1.18	
19	11.69	3,4-Dihydroxybenzoic acid	370	1.96	
20	12.84	4-Hydroxycinnamic acid	308	1.46	
21	14.03	Hexadecanoic acid[a]	338	0.87	
22	14.66	4-Hydroxy-3-methoxycinnamic acid	338	n.m.	
23	14.80	6,7-Dihydroxycoumarin	322	49.99	11.04
24	15.51	6,7-Dihydroxy-8-methoxycoumarin[a]	352	3.05	4.84
25	16.01	9,12-Octadecadienoic acid[a]	352	0.57	
26	16.09	Oleic acid[a]	354	0.46	
27	16.45	Octadecanoic acid[a]	356	0.92	
28	17.04	Unknown	394	1.77	
29	17.82	Unknown	468	2.31	2.29
30	25.05	Unknown	368[b]		40.54
31	25.24	2'-Hydroxygenistein	574	0.96	
32	25.31	Unknown	368[b]		5.24
33	25.72	Genistein	486	3.75	
34	26.36	Apigenin	486	0.56	
35	27.69	Unknown	368[b]		19.16
36	28.61	Unknown	368[b]		0.98
37	28.78	Unknown	368[b]		12.47
38	28.97	Unknown	368[b]		3.95

[a] Substances identified on the basis of library search.
[b] The most intense peak in the mass spectrum.

TABLE 2. ^{1}H AND ^{13}C NMR DATA FOR 6, 7-DIHYDROXYCOUMARIN

Chemical shifts for aromatic protons (δ ppm/TMS in DMSO-d_6)								
H-3		H-4		H-5		H-8		
7.80–7.91 d		6.10–6.21 d		6.98 s		6.74 s		
Carbon-13 NMR shifts (δ ppm/TMS in DMSO-d_6)								
C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
160.64	111.45	144.22	112.31	142.82	150.34	102.62	148.45	110.68

was not affected by the compounds present in the fraction. The strongest delay of germination, caused by subfractions obtained from the ethyl acetate fraction, was observed for subfraction A, consisting of free phenolic acid derivatives. In dilution I, complete inhibition of seed germination was observed after 24 hr, but after 72 hr germination was 90%. The effect of subfractions B and C on germination was similar. Only after 24 hr was a slight decrease of germination observed (to 76% of control) for subfraction B in dilution I. In the case of subsequent dilutions of subfractions B and C, there were insignificant differences in the germination when compared with the control.

Lettuce seedling growth was reduced in dilution I of all tested fractions from bitter lupine seed extract (Figure 3). The most active was subfraction A, containing free phenolic acids, where the lengths of roots and hypocotyls were not measurable. Other fractions affected growth of seedlings to a smaller degree; elongation of roots was reduced from 50% to 60% and hypocotyls from 20% to 40%, when compared with the control seedlings. In dilution II, growth inhibition was observed for subfractions A and B, where the length of hypocotyls and roots was reduced by about 20% in comparison with the control. However, in dilution IV of subfraction A, the length of roots exceeded that observed in the control, but the hypocotyl growth was not affected. In subfraction C were present distinct amounts of alkaloids and their derivatives (lupanin, 13-hydroxylupanin, and its esters), but their presence had no distinct effect on germination and lettuce seedling growth.

1,2-Dihydroxybenzene and 4-hydroxycinnamic acid were the most active standard compounds. They inhibited germination in dilution I after 24 hr, and germination of only 8% and 24% of control values, respectively, was observed after 72 hr (Table 3). Six compounds, 4-hydroxy-3-methoxycinnamic acid, 4-hydroxyphenylacetic acid, 4-hydroxy-3-methoxybenzoic acid, 4-hydroxybenzoic acid, 6,7-dihydroxycoumarin, and 1,4-dihydroxybenzene, delayed germination by about 50% after 24 hr. Two other compounds, 3,4-dihydroxybenzoic and 3-hydroxybenzoic acids, did not affect germination, regardless of concentration and time of action.

The effects of single phenolic compounds tested on seedling growth were

TABLE 3. EFFECTS OF ETHYL ACETATE FRACTION ISOLATED FROM LUPIN SEED EXTRACT, SUBFRACTIONS A–C, AND FREE PHENOLIC ACIDS AT DIFFERENT CONCENTRATIONS ON LETTUCE SEEDS GERMINATION AFTER 24 AND 72 HOURS

Compound	Time (hr)	Germination (% of control) at dilutions I	II	III	IV
Ethyl acetate fraction	24	40	90	100	90
	72	93	96	96	100
Subfraction A	24	0	90	95	100
	72	72	100	108	108
Subfraction B	24	76	81	100	86
	72	104	104	104	100
Subfraction C	24	104	114	95	91
	72	108	116	108	104
1,2-Dihydroxybenzene	24	0	42	79	
	72	8	85	85	
1,4-Dihydroxybenzene	24	80	120	150	
	72	76	96	96	
4-Hydroxybenzoic acid	24	44	56	72	
	72	52	72	88	
3-Hydroxybenzoic acid	24	80	120	150	
	72	76	96	96	
3,4-Dihydroxybenzoic acid	24	83	92	108	
	72	92	96	112	
4-Hydroxy-3-methoxybenzoic acid	24	56	111	106	
	72	84	100	96	
4-Hydroxyphenylacetic acid	24	50	106	94	
	72	80	104	104	
4-Hydroxycinnamic acid	24	5	100	100	
	72	24	100	100	
4-Hydroxy-3-methoxycinnamic acid	24	56	83	100	
	72	92	84	104	
6,7-Dihydroxycoumarin	24	39	78	89	
	72	83	86	90	

variable. The strongest suppression of growth at concentrations from 10^{-3} M to 10^{-5} M was noted for 1,2-dihydroxybenzene (Table 4). In dilution I, seedlings did not develop, and even in the least concentrated solution, significant reduction in root and hypocotyl growth were observed: 45% and 20%, respectively. 4-Hydroxybenzoic acid and 4-hydroxycinamic at 10^{-3} M concentration also completely inhibited seedling growth, but in more dilute solutions growth suppression was insignificant. Considerable reduction of seedling growth was found for all dilutions (I–IV) of 6,7-dihydroxycoumarin. At a concentration of

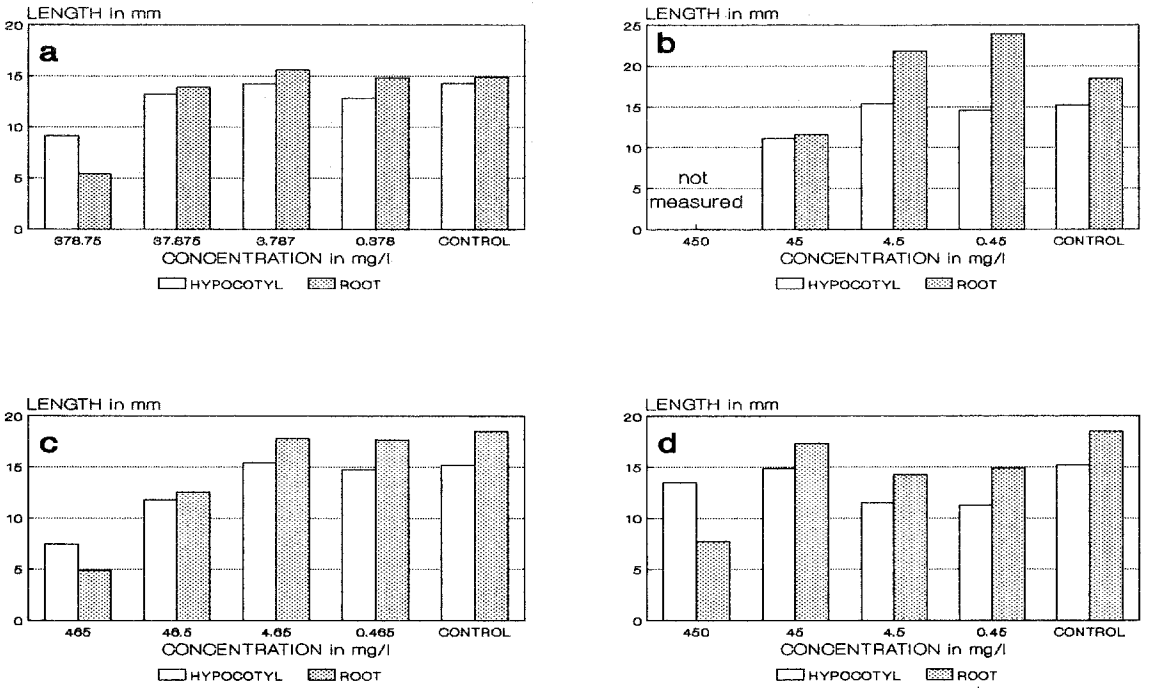

FIG. 3. Effect of the ethyl acetate fraction and its subfractions (A–C) from the lupine seed extract on lettuce seedlings growth. The mean length is of 30 roots and hypocotyls. (a) The entire ethyl acetate fraction from the lupine seed extract. (b) Subfraction A obtained after separation on the silica gel column, at the concentration 450 mg/ml, seedling growth was completely inhibited and not measured. (c) Subfraction B obtained after separation on the silica gel column. (d) Subfraction C obtained after separation on the silica gel column.

TABLE 4. EFFECTS OF SINGLE PHENOLIC ACIDS TESTED IN DIFFERENT CONCENTRATIONS ON LETTUCE SEEDLING GROWTH (72HR AFTER SOWING SEEDS)[a]

	Conc. 10^{-3} M				Conc. 10^{-4} M				Conc. 10^{-5} M				Control			
	Root		Hypocotyl		Root		Hypocotyl		Root		Hypocotyl		Root		Hypocotyl	
Compound	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
1,2-Dihydroxybenzene	n.m.		n.m.		2	± 1.58	5	± 1.62	11.19	± 1.60	12.67	± 1.65	20.80	± 2.70	14.94	± 1.92
1,4-Dihydroxybenzene	2.63	± 1.50	4.89	± 1.65	13.81	± 2.17	13.11	± 1.56	14.19	± 1.67	13.25	± 2.05	14.92	± 2.64	14.28	± 1.24
4-Hydroxybenzoic acid	n.m.		n.m.		14.37	± 2.27	15.40	± 1.97	20.05	± 1.64	16.44	± 2.42	18.25	± 2.32	15.53	± 2.26
3-Hydroxybenzoic acid	6.29	± 1.95	10.64	± 1.27	13.61	± 1.79	13.11	± 1.57	15.30	± 2.57	14.39	± 1.39	14.92	± 2.64	14.28	± 1.24
3,4-Dihydroxybenzoic acid	15.11	± 2.40	13.36	± 1.56	18.19	± 2.49	15.72	± 2.40	18.89	± 2.46	14.78	± 2.85	20.80	± 2.70	14.94	± 1.92
4-Hydroxy-3-methoxy-benzoic acid	6.25	± 1.86	7.28	± 2.71	10.64	± 1.59	15.25	± 1.24	20.01	± 1.97	15.94	± 2.41	18.25	± 2.32	15.53	± 2.26
4-Hydroxyphenylacetic acid	4.25	± 1.57	7.28	± 1.68	15.61	± 2.57	14.68	± 2.14	18.05	± 2.04	15.42	± 2.93	18.25	± 2.32	15.53	± 2.26
4-Hydroxycinnamic acid	n.m.		n.m.		14.44	± 1.75	14.68	± 1.44	18.50	± 2.90	15.42	± 2.81	18.55	± 2.32	15.53	± 2.26
4-Hydroxy-3-methoxy-cinnamic acid	9.28	± 2.61	13.78	± 2.48	19.28	± 2.11	16.83	± 2.68	16.11	± 2.60	16.06	± 1.50	18.55	± 2.32	15.53	± 2.26
6,7-Dihydroxycoumarin	8.19	± 0.75	9.97	± 1.28	10.24	± 1.44	11.22	± 0.96	13.70	± 2.05	12.63	± 1.26	16.41	± 1.50	12.94	± 1.58

[a] n.m.—after germination, seedling growth was completely inhibited and not measured. Mean root/hypocotyl length of 30 seedlings.

10^{-3} M, the decrease of root length exceeded 50%, while at the higher dilutions, there were decreases of 35% and 20%, respectively. Influence of this compound on hypocotyl growth was much less, and inhibition was 20% and 10% at dilutions I and II, respectively. At the highest dilution, the hypocotyl growth was not affected.

The remaining phenolic acids tested all strongly reduced growth of roots by 85–50%, and hypocotyl growth decreased up to 50% of control at a concentration of 10^{-3} M. In dilutions II and III, seedling development was not affected.

DISCUSSION

According to GC-MS analysis, the most abundant compound among isolated free phenolics from bitter lupine seed extract was 6,7-dihydroxycoumarin. It represented over 50% of the mass of isolated compounds. Another distinct group consists of benzoic acid itself and the benzoic acid analogs 4-hydroxybenzoic, 4-hydroxy-3-methoxybenzoic, and 3,4-dihydroxybenzoic acids. Genistein was also recognized as one of the most prominent compounds in the ethyl acetate fraction. Other identified free phenolic compounds in subfraction A were present in minor amounts.

4-Hydroxy-3-methoxycinnamic (ferulic) acid coeluted in one peak with 6,7-dihydroxycoumarin, and it was identified on the basis of mass chromatograms of the characteristic ions present in the mass spectrum and the retention time of the standard.

Identified phenolic compounds are known as inhibitors of plant development (Rice, 1984). Subfraction A showed a strong delay of germination in biological tests in relation to other fractions obtained from the ethyl acetate extract. Among the single compounds analyzed, only 1,2-dihydroxybenzene and 4-hydroxycinnamic acid had delaying and inhibiting effects on germination. Seedling growth was affected in the first dilutions of all samples tested from the ethyl acetate extract of bitter lupine seeds; subfraction A was also the most active. However, in dilution IV, a weak stimulation of the root growth was observed. This fact can be attributed to the presence of isoflavones and flavon in the mixture. Root length was increased by flavonoids at concentrations ranging from 10^{-5} M to 10^{-7} M, (Nandakumar and Rangaswamy, 1985). In the tests with single phenolic compounds, complete inhibition of growth was found for 1,2-dihydroxybenzene and 4-hydroxycinnamic acid in dilution I. In more dilute solutions, strong growth reduction was observed only for 1,2-dihydroxybenzene. 6,7-Dihydroxycoumarin also demonstrated clear inhibition of seedling growth in dilutions II and III, but at the highest concentration the decrease of root growth was only 50% and of hypocotyls 25%.

The comparison of bioassay results obtained for fractions from the ethyl acetate extract clearly showed that free phenolic acids were responsible for inhibition of seedling growth and delay of germination. Data presented here demonstrate that seedling growth was more affected by phenolics than was germination. They are consistent with the results of Rasmussen and Einhellig (1977) and Nandakumar and Rangaswamy (1985). A possible reason for seedling growth inhibition could be that phenolics are antagonistic to gibberellins (Green and Corcoran, 1975) and also some phenolics can decrease bound indole-3-acetic acid formation (Lee, 1980). Phenolic conjugates had only a rather small influence on lettuce seedling development in the present studies, but the above-mentioned conjugates can be decomposed in the rhizosphere by microorganisms and, after liberation, free phenolic acids can also be inhibitory to plants (Henderson, 1963; Turner and Rice, 1975; Rice, 1984).

Total amounts of free phenolics in subfraction A were comparable with the amounts of pure phenolic compounds used in the tests. There were no distinct differences in the delay of germination and suppression of seedling growth between subfraction A, containing phenolic acids, and the most active pure compound 1,2-dihydroxybenzene. The reason for this would be the synergistic effect of phenolic present in the mixture.

Phenolic compounds had no direct influence on the crop yield after spraying plants with the extract in the field experiments (Cwojdzinski et al., 1989; Kahnt and Hijazi, 1987, 1991). The extract was sprayed on the fields, on well-developed plants. Phenolic compounds could act in this situation as allelochemicals, decreasing weeds germination and growth by strong inhibition of plant growth regulators (gibberellins and auxins), and in this way influence crop yield. In the extract are present other unidentified compounds which are involved in crop yield stimulation, and their action is not inhibited by presence of phenolics.

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REFERENCES

CWOJDZINSKI, W., MICHALSKI, Z., NOWAK, K., and GULEWICZ, K. 1989. Studies on the influence of bitter lupin extract on the yield of different cultivated plants. *Lupin News Lett.* 13:46–54.

GLASS, A.D.M. 1976. The allelopathic potential of phenolic acids associated with the rhizosphere of *Pterodium aquilinum*. *Can. J. Bot.* 54:2440–2444.

GREEN, F.B., and CORCORAN, M.R. 1975. Inhibitory action of five tannins on growth induced by several gibberelins. *Plant Physiol.* 56:801–806.

GULEWICZ, K. 1986. Patent No. 152748 Pl, Poland.

HARBORNE, J.B. 1964. Phenolic glycosides and their natural distribution, pp. 129–169, *in* J.B. Harborne (ed.). Biochemistry of Phenolic Compounds. Academic Press, New York.

HARBORNE, J.B., and SIMMONDS, S.W. 1964. The natural distribution of the phenolic aglycones,

pp. 77–127, *in* J.B. Harborne (ed.). Biochemistry of Phenolic Compounds. Academic Press, New York.

HENDERSON, M.E.K. 1963. Fungal metabolism of certain aromatic compounds related to lignin. *Pure Appl. Chem.* 7:589–602.

KAHNT, G., and HIJAZI, L.A. 1987. Effect of bitter lupin extract on growth and yield of different crops. *J. Agron. Crop Sci.* 159:320–338.

KAHNT, G., and HIJAZI, L.A. 1991. Use of Lupinex to increase crop yield and improve harvest quality with lesser nitrogen fertilization. *J. Agron. Crop Sci.* 166:228–237.

KRZYMANSKA, J., WALIGORA, D., MICHALSKI, Z., and GULEWICZ, K. 1988. Observation on the influence of spraying potatoes with lupin extract on the feeding and development of potato beetle population (*Leptinotarsa decemlineata* Say.). *Bull. Pol. Acad. Sci. Biol. Sci.*, 36:45–51.

LEE, T.T. 1980. Effects of phenolic substances on metabolism of exogenous indole-3-acetic acid in maize stem. *Physiol. Plant.* 50:107–112.

NANDAKUMAR, L., and RANGASWAMY, N.S. 1985. Effect of some flavonoids and phenolic acids on seed germination and rooting. *J. Exp. Bot.* 36:1313–1319.

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

RICE, E.L. 1979. Allelopathy—an update. *Bot. Rev.* 45:15–109.

RICE, E.L. 1984. Allelopathy, 2nd ed. Academic Press, Orlando, Florida.

TAHARA, S., INGHAM, J.L., NAKAMURA, S., MIZUTANI, J., and HARBORNE, J.B. 1984. Fungitoxic dihydrofuranoisoflavons and related compounds in white lupin, *Lupinus albus*. *Phytochemistry* 23:1189–1900.

THAKUR, M.L. 1977. Phenolic growth inhibitors isolated from dormant buds of sugar maple, *Acer saccharum* Marsh. *J. Exp. Bot.* 28:795–803.

TURNER, J.A., and RICE, L.E. 1975. Microbial decomposition of ferulic acid in soil. *J. Chem. Ecol.* 1:41–78.

VAN SUMERE, C.F., COTTENIE, J., DEGREEF, J., and KINT, J. 1971. Biochemical studies in relation to the possible germination regulatory role of naturally occurring coumarin and phenolics. *Recent Adv. Phytochem.* 4:165–221.

WILLIAMS, R.D., and HOAGLAND, R.E. 1982. The effects of naturally occurring phenolics on seeds germination. *Weed Sci.* 30:206–212.

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DEFENSIVE ALLOMONES IN THREE SPECIES OF *Hypselodoris* (GASTROPODA: NUDIBRANCHIA) FROM THE CANTABRIAN SEA

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Abstract—Three *Hypselodoris* species, *H. villafranca, H. cantabrica*, and *H. tricolor*, have been studied in order to further investigate the defensive strategies of Chromodorididae mollusks from Iberian coasts. All animals possess large amounts of furanosesquiterpenoids, probably derived from their diet of sponges. The products are transferred and stored in specific sites of the mantle (mantle dermal formations, MDFs) to be used as chemical deterrents against predators. Chemical analysis of Cantabrian *Hypselodoris* species has led to the characterization of eight sesquiterpenoids. Among these, six have been found previously in both sponges and nudibranchs, while two are new natural products. *Hypselodoris* species cooccurring in the same area contained comparable mixtures of sesquiterpenoids. However, two populations of *H. cantabrica* from different sites of the Cantabrian sea showed different metabolic patterns. All sesquiterpenoids induce feeding deterrence in *Carassius auratus* and two are also toxic to *Gambusia affinis*.

Key Words—Opisthobranch, nudibranch, *Hypselodoris*, chemical defense, prey–predator relationship, mantle dermal formations, furanosesquiterpenoids, Dysideidae.

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INTRODUCTION

Nudibranchs have elaborated varied defensive strategies toward attack by predators. In spite of the lack of mechanical protection by a shell, these mollusks are seldom eaten (Edmunds, 1966; Ros, 1976, 1977; Thompson, 1960); very often they are protected by chemical compounds that are obtained from the food or by de novo biosynthesis (Cimino et al., 1983, 1985; Gustafson and Andersen, 1985). Many studies of this topic have been reviewed by Faulkner (1991) and Karuso (1987).

Continuing our research on the chemical ecology of European opisthobranch mollusks (Cimino et al., 1980, 1982, 1985, 1986; Cimino and Sodano, 1989), we have recently concerned ourselves with the family Chromodorididae (Avila et al., 1991; Cimino et al., 1990; Garcia-Gomez et al., 1990, 1991; Gavagnin et al., 1992). The systematics of the four genera (*Hypselodoris, Chromodoris, Glossodoris*, and *Cadlina*) belonging to the family Chromodorididae has recently been clarified by Rudman (1984), who has differentiated the first three genera on the basis of some anatomical differences.

Glossodoris spp. have a rachidian tooth: the first lateral bears denticles on both sides of its cusps, the second only the outer side. The vestibular gland in the genital system is small and never branched when present.

The genus *Hypselodoris* includes species with a radula lacking a median tooth: the lateral teeth have two cusps, with or without denticles under the second cusp. The vestibular gland is large and always branched.

The genus *Chromodoris* can have a median tooth in its radula: when present it is a triangular plate. The genital system has a little vestibular gland.

It is noteworthy that specimens belonging to closely related genera are able to select prey possessing different secondary metabolite patterns: furanosesquiterpenoids for *Hypselodoris* and spongian diterpenoids for *Chromodoris*. Although other field observations are necessary, chemical analysis of the secondary metabolites from two Mediterranean *Hypselodoris* species, *H. webbi* (Avila et al., 1991; Cimino et al., 1982; Garcia-Gomez et al., 1990) and *H. villafranca* (Avila et al., 1991), suggest that both animals graze upon sponges, such as *Dysidea fragilis*. Conversely, the metabolite pattern of *Chromodoris luteorosea* from the Iberian and Italian coasts (Cimino et al., 1990; Gavagnin et al., 1992) suggests that these mollusks prey only upon sponges containing spongian diterpenoids.

To evaluate this hypothesis, we have undertaken chemical research on three *Hypselodoris* species from the Cantabrian sea. The study has confirmed the ability of *Hypselodoris* species to select sponges containing furanosesquiterpenoids. Here we report the chemical characterization of eight terpenoids, two of them new natural products, and some laboratory bioassays aimed at supporting the defensive role of the terpenoids.

METHODS AND MATERIALS

General Experimental Procedure

GC-MS analysis was performed on a Hewlett-Packard instrument model 5890 with a MS 5970 spectrometer equipped with a cross-linked ME silicone capillary Hewlett-Packard column (25 m × 0.2 mm ID). The samples were injected at 290°C using an autosampler. The oven was programmed from 150°C to 220°C at 3°C/min. Helium was used as carrier. Transfer line was programmed at 280°C. The ions were detected in the *m/z* range of 50–400.

UV spectra were recorded on a Varian DMS 90 spectrometer.

Optical rotations were measured on a JASCO polarimeter equipped with a 10-cm cell.

One-dimensional and two-dimensional NMR spectra were performed in $CDCl_3$ or C_6D_6 at room temperature with a Bruker WM-500 spectrometer (^1H, 500.13 MHz; ^{13}C, 125.7 MHz), equipped with Aspect 2000 data system, using a 5-mm $^1H/^{13}C$ dual probe. Chemical shifts expressed in δ units are reported in parts per million downfield from the internal standard TMS ($\delta = 0$).

Mass spectra were recorded with AEI MS 30 and for HREIMS on Kratos MS 50 spectrometers.

Organic solvents were RP grade (Farmitalia Carlo Erba, Milan, Italy). 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.

Preparation of SiO_2–$AgNO_3$ (7.4%)

Silica gel (10 g) (Merck, 70–230 Mesh) was added to 100 ml of a solution of acetone–water (8:2 v/v) containing 0.8 g of $AgNO_3$ RPE (Farmitalia-Carlo Erba, Milan, Italy). The mixture was stirred vigorously for 10 min and then dried in vacuum on a rotary evaporator. The SiO_2–$AgNO_3$ was activated at 110°C for 2 hr.

Preparation of SiO_2–$AgNO_3$ (8%) TLC

$AgNO_3$ RPE (Farmitalia-Carlo Erba, Milan, Italy) (12 g) was dissolved in 150 ml of a solution of acetone–water (7:3 v/v). The solution was heated and vigorously stirred for 10 min. Ten precoated silica gel plates were immersed into the solution and then activated at 110°C for 2 hr.

Biological Material

H. cantabrica were collected at Asturias (Las Llanas, north Spain) during September 1989 (38 specimens) and December 1989 (14 specimens), and near Santander (north Spain) during March 1990 (24 specimens). Specimens of *H.*

villafranca (24) and of *H. tricolor* (32) were taken at Las Llanas (north Spain) during December 1989. The animals were collected in the intertidal area by using SCUBA. After collection, the samples were immediately stored at −20°C until analyzed. The dorsal pattern of the nudibranchs is reported in Figure 1A below.

Anatomical Dissection

Five specimens of each collection of the three *Hypselodoris* species were dissected into four parts: mantle dermal formations (MDFs), rest of mantle and foot, digestive gland, and the rest of the viscera (reproductive organs, digestive tract, etc.). Anatomical dissections were made while observing the animal through a Swift microscope (20–40×). The anatomical distribution of the MDFs is reported in Figure 1B below.

Annual Distribution of H. cantabrica, H. villafranca, *and* H. tricolor

The relative population density of the three nudibranchs in a selected area (Muros del Nalon) of the Cantabrian Sea was determined by direct observation every month from November 1989 to March 1991. The data are summarized in Figure 2 below.

Chromatographic Analysis of Lipid Material

TLC of Sections. Dissected parts of *H. cantabrica, H. villafranca,* and *H. tricolor* collected during December 1989 were separately extracted with acetone. The organic fractions were dried under vacuum and redissolved in diethyl ether. The ether extracts were analyzed by SiO_2 TLC in petroleum ether and by SiO_2–$AgNO_3$ (8%) TLC in C_6H_6–*n*-heptane (1 : 1 v/v). The plates were sprayed with Ehrlich's reagent and heated to detect spots.

Isolation of Nonpolar Metabolites of Whole Nudibranchs. H. cantabrica specimens collected in Las Llanas during September and December 1989 were separately extracted with acetone. The organic fractions were evaporated under vacuum, and the resulting aqueous suspension was partitioned between diethyl ether and water. The ether extracts were dried over Na_2SO_4 and the solvent evaporated to obtain 772 mg of brown oil from the sample collected in September, and 53 mg from the collection in December. The organic extracts were redissolved in petroleum ether and chromatographed on a column of silica gel using solvents of increasing polarity from petroleum ether to diethyl ether. Specifically, the petroleum ether-soluble fractions of both collections contained longifolin **(1),** tavacfuran **(2),** and a mixture of less polar products positive to Ehrlich's reagent. This latter material was ultimately fractionated on a SiO_2–$AgNO_3$ (7.4%) column using *n*-heptane–benzene (9 : 1 v/v) as eluant to yield

nakafuran-9 **(3),** dendrolasin **(4),** agassizin **(5),** *ent*-furodysinin **(6),** isonakafuran-9 **(7)** and isodehydrodendrolasin **(8).** Each of these compounds was identified by analysis of the NMR spectra.

The collection of *H. cantabrica* taken near Santander was similarly extracted to give 119 mg of oily residue. The fractionation on silica gel furnished a mixture of *ent*-furodysinin (**6,** 6.7 mg) and nakafuran-9 (**3,** 4 mg), which were fully purified on a SiO_2–$AgNO_3$ column eluting with *n*-heptane–benzene (9 : 1 v/v).

The smallest *H. villafranca* and *H. tricolor* were chromatographed in the same way, yielding, respectively, 30 and 51 mg of crude ether extracts. These oils were processed on silica gel using petroleum ether and yield longifolin **(1),** tavacfuran **(2),** and a mixture of Ehrlich-positive products. Direct GC-MS analysis of the latter fraction (Figures 3 and 4 below) gave qualitatively the same composition of furanosesquiterpenoids as *H. cantabrica* collected in same geographic area (Las Llanas, north Spain).

Bioassays

Ichthyotoxicity assays were performed using the mosquito fish *Gambusia affinis* (Coll et al., 1982; Gunthorpe and Cameron, 1987). Compounds were added to fresh water (50 ml) in pure acetone at 0.1, 1, and 10 ppm, whereas controls employed pure solvent. Toxicity was defined as death of fishes within 24 hr. Anomalies of behavior (loss of equilibrium and frequency of movement) were also observed.

Antifeedant activity (Cimino et al., 1982; Thompson et al., 1982) was assessed by observing the feeding response of goldfish *Carassius auratus* toward food pieces treated with the purified substances and applied to the pellets with diethyl ether. The feeding deterrence of allomones was measured in a range of 20–600 $\mu g/cm^2$ of fish food. Blanks were obtained by feeding untreated pellets to the fishes before and after the experiment with treated food. The biological data are reported in Table 1.

Spectral Data of Purified Compounds

Longifolin (1). Optically inactive; C_{15} H_{18} O_2; EI-MS *m/z*: 230 (M^+); ^1H NMR (C_6D_6): 1.60 (3H, s); 1.78 (3H, s); 1.81 (3H, s); 3.16 (2H, s); 3.22 (2H, d, J = 7.0 Hz); 5.46 (1H, t, J = 7.0 Hz); 5.77 (1H, bs); 6.00 (1H, d, J = 1.5 Hz); 6.92 (1H, bs); 7.07 (1H, d, J = 1.5 Hz). ^{13}C NMR (C_6D_6): 9.7 (q); 15.8 (q); 25.5 (t); 38.6 (t); 109.2 (d); 113.0 (d); 113.7 (s); 120.5 (s); 122.8 (d); 133.2 (s); 138.0 (d); 140.1 (d); 149.8 (s); 154.3 (s). The assignments are according to Avila et al. (1991).

Tavacfuran (2). Optically inactive; UV (diethyl ether) = 285 nm (ϵ = 7100), 274 nm (ϵ = 10800), 220 nm (ϵ = 7300); $C_{15}H_{18}O_2$; EI-MS *m/z*: 230 (M^+); ^1H NMR (C_6D_6): 1.63 (3H, bs); 1.78 (3H, s); 1.83 (3H, s); 2.76 (4H,

TABLE 1. ICHTHYOTOXICITY AND ANTIFEEDANT ACTIVITY OF PURIFIED METABOLITES

Compound	Dose				
	Toxicity[a]		Antifeedant activity[b]		
	10 ppm	1 ppm	300 mg/cm^2	150 mg/cm^2	30 mg/cm^2
Longifolin (**1**)	T[c]	I	A	I	I
Tavacfuran (**2**)	I	I	A	I	I
Nakafuran-9 (**3**)	I	I	A	I	I
Dendrolasin (**4**)	I	I	A	I	I
Agassizin (**5**)	N.T.	N.T.	N.T.	N.T.	N.T.
ent-Furodysinin (**6**)	VT	I	A	A	A
Isonakafuran-9 (**7**)	N.T.	N.T.	A	I	I
Isodehydrodendrolasin (**8**)	N.T.	N.T.	N.T.	N.T.	N.T.

[a]Tested on *Gambusia affinis*.
[b]Tested on *Carassius auratus*.
[c]T = toxic; VT = very toxic; A = active; I = inactive; N.T. = not tested.

m); 5.99 (1H, bs); 6.01 (1H, d, J = 1.5 Hz); 6.08 (1H, bs); 6.90 (1H, bs); 7.09 (1H, d, J = 1.5 Hz). Assignments are according to Guella et al., (1985).

Nakafuran-9 (3). $[\alpha]_D = -107.5°$ (c = 0,2 $CHCl_3$); UV (hexane) = 214 nm (ϵ = 6020); $C_{15}H_{20}O$; EI-MS *m/z* (%): 216 (M^+,100), 201 (55), 175 (35). ^1H NMR ($CDCl_3$): 1.08 (3H, s); 1.37 (1H, ddd, J = 4.1, 13.3, and 13.3 Hz); 1.57 (3H, s); 1.59 (3H, s); 1.72 (1H, ddd, J = 2.7, 2.7, and 13.0 Hz), 1.79 (1H, ddd, J = 1.8, 4.3, and 13.0 Hz); 1.92 (1H, bddd, J = 4.1, 4.1, and 13.3 Hz); 1.95 (1H, bd, J = 15.5 Hz); 2.25 (1H, ddd, J = 4.1, 13.3, and 16.0 Hz); 2.33 (1H, ddd, J = 4.1, 4.1, and 16.0 Hz); 2.37 (1H, bdd, J = 5.2 and 15.5); 3.17 (1H, m); 6.06 (1H, d, J = 1.7 Hz); 7.12 (1H, d, J = 1.7 Hz). ^{13}C NMR ($CDCl_3$): 13.2 (q); 19.8 (q); 23.0 (t); 30.7 (q); 31.8 (d); 37.6 (s); 38.2 (t); 38.6 (t); 41.3 (t); 113.1 (d); 118.3 (s); 126.3 (s); 129.5 (s); 138.4 (d); 156.2 (s). The assignments are according to Avila et al. (1991)〉

Dendrolasin (4). Optically inactive; UV (diethyl ether) 273 nm (ϵ = 6480); $C_{15}H_{22}O$; EI-MS *m/z* (%): 218 (5), 203 (15), 81 (90), 69 (100); ^1H NMR (C_6D_6 δ 1.52 (3H, s), 1.55 (3H, s), 1.67 (3H, s), 2.06 (2H, t, J = 7.3 Hz), 2.14 (2H, t, J = 7.3 Hz), 2.18 (2H, q, J = 7.3 Hz), 2.33 (2H, t, J = 7.5 Hz), 5.21 (2H, m), 6.08 (1H, s), 7.07 (1H, s), 7.12 (1H, s); ^{13}C NMR (C_6D_6) δ: 16.1 (q), 17.8 (q), 25.4 (t), 25.8 (q), 27.1 (t), 28.9 (t), 40.1 (t), 111.3 (d), 124.4 (d), 124.8 (d), 125.2 (s), 131.2 (s), 135.6 (s), 139.2 (d), 142.8 (d).

Agassizin (5). UV (diethyl ether) 223 nm (ϵ = 9430), 258 nm (ϵ = 3510; $C_{15}H_{18}O$; EI-MS *m/z* (%): 214 (90); ^1H NMR (C_6D_6) δ 0.77 (3H, s); 0.81 (3H, s), 1.63 (2H, m), 2.26 (1H, m), 2.45 (1H, m), 2.59 (1H, m), 3.25 (1H, m),

3.46 (1H, bd, J = 16 Hz), 5.40 (1H, bd), 5.50 (1H, bd, J = 5 Hz), 5.74 (1H, m), 5.98 (1H, d, J = 1.5 Hz), 7.05 (1H, d, J = 1.5 Hz). Assignments are according to Hochlowski et al. (1982).

ent-*Furodysinin* ***(6).*** $[\alpha]_d$ = −43.7°(c = 0.3 $CHCl_3$); UV ($CHCl_3$) 214 nm (ϵ = 8700); $C_{15}H_{20}O$; EI-MS *m/z* (%): 216 (15), 122 (100), 107 (15); ^1H NMR (C_6D_6) δ 1.12 (3H, s, CH_3-13 or CH_3-14), 1.14 (3H, s, CH_3-14 or CH_3-13), 1.17 (1H, m, H-10a), 1.44 (1H, ddd, J = 3, 3, and 13 Hz, H-11), 1.54 (1H, m, H-10b), 1.55 (3H, bs, CH_3-15), 1.79 (1H, bdd, J = 6.8 and 17.4 Hz, H-9a), 1.85 (1H, m, H-9b), 2.36 (1H, dd, J = 10.5 and 16.4, H-5a), 2.54 (1H, m, H-6), 2.70 (1H, dd, J = 6.9 and 16.4 Hz, H-5b), 5.46 (1H, bd, J = 6.9 Hz, H-7), 6.13 (1H, bs, H-2), 7.11 (1H, s, H-1). ^{13}C NMR (C_6D_6) δ 19.6 (C-10), 23.2 (C-15), 26.4 (C-13 or C-14), 28.0 (C-5), 31.6 (C-6), 31.8 (C-9), 33.0 (C-14 or C-13), 33.3 (C-12), 44.9 (C-11), 108.3 (C-2), 124.9 (C-8), 126.6 (C-7), 133.1 (C-3), 140.9 (C-1), 147.6 (C-4). The assignments have been supported by ^1H–^1H decoupling experiments, ^1H–^1H COSY, and ^1H–^{13}C HETCOR; they are according to Guella et al. (1985).

Isonakafuran-9 ***(7).*** $[\alpha]_d$ = +6° (c = 0.3 $CHCl_3$); UV ($CHCl_3$) = 214 nm (ϵ = 4240); $C_{15}H_{20}O$; EI-MS *m/z*: 216 (M^+, 80), 201 (40), 147 (100), 84 (85); ^1H NMR (C_6D_6) δ 0.72 (3H, s, CH_3-15), 0.92 (3H, d, J = 7.2, CH_3-14), 1.37 (1H, m, H-5), 1.44 (1H, dd, J = 5.1 and 14.6 Hz, H-12), 1.47 (1H, bd, J = 14.6 Hz, H-12), 1.83 (1H, ddd, J = 3.5, 9.8 and 16.5 Hz, H-5), 1.95 (1H, q, J = 7.2 Hz, H-7), 2.30 (2H, m, H-4 and H-9), 2.40 (1H, bd, J = 14.5 Hz, H-9), 2.43 (1H, ddd, J = 3.5, 8.6 and 15.7 Hz, H-4), 3.11 (1H, bm, H-10), 4.59 (1H, bs, J = 2 Hz, H-13a), 4.66 (1H, bs, J = 2 Hz, H-13b), 5.97 (1H, bs, J = 1.3, H-2), 7.03 (1H, bs, J = 1.3 Hz, H-1). Numbering and assignments are according to Tanis and Herrinton (1985). They were confirmed by ^1H–^1H COSY and ^1H–^1H decoupling.

Isodehydrodendrolasin ***(8).*** Optically inactive; UV (diethyl ether) 230 nm (ϵ = 9430), 224 nm (ϵ = 3510); $C_{15}H_{20}O$; EI-MS *m/z*: (%): 216 (5), 201 (20), 93 (100), 81 (90); ^1H NMR (C_6D_6) δ 1.50 (3H, s, CH_3-15), 1.76 (3H, s, CH_3-14), 2.17 (2H, t, J = 7.4 Hz, H-6), 2.31 (2H, t, J = 7.5 Hz, H-5), 2.70 (2H, d, J = 7.0, H-9), 4.89 (1H, bs, H-13a), 4.94 (1H, bs, H-13b), 5.22 (1H, bt, J = 7.0 Hz, H-7), 5.65 (1H, dt, J = 15.5, 7.0 and 7.0 Hz, H-10), 6.07 (1H, bs, H-3), 6.19 (1H, d, J = 15.5 Hz, H-11), 7.05 (1H, s, H-4), 7.12 (1H, s, H-1). ^{13}C NMR (C_6D_6) δ: 16.2 (q, C-15), 18.8 (q, C-14), 25.2 (t, C-5), 28.9 (t, C-6), 43.3 (t, C-9), 111.2 (d, C-3), 115 (t, C-13), 125.2 (s, C-2), 134.6 (s, C-8), 139.2 (d, C-1), 142.2 (s, C-12), 142.8 (d, C-4).

RESULTS AND DISCUSSION

Until now chemical studies of *Hypselodoris* nudibranchs from north Spain were limited to *H. villafranca* (Avila et al., 1991), although there are many reports of other species living in the Mediterranean Sea (Avila et al., 1991;

Cimino et al., 1982; Garcia-Gomez et al., 1990). In this paper, we report chemical evidence obtained by studying three *Hypselodoris* populations from the Cantabrian sea: *H. cantabrica*, *H. tricolor*, and *H. villafranca*.

The first reference to a blue chromodorid in northern Spain is that of Rioja (1917), who recorded *Chromodoris coerulea* (=*Hypselodoris villafranca*) off Gijon. In the Basque Country, almost 60 years later, Ros (1976) collected the three *Hypselodoris* species studied in this paper: *H. villafranca*, *H. cantabrica*, and *H. tricolor*, referring to them as *Glossodoris gracilis*, *G. valenciennesi*, and *G. tricolor*, respectively. These three species were, almost at the same time, cited by Bouchet and Tardy (1976) under the names of *Hypselodoris gracilis*, *H.* sp., and *H. tricolor*; the authors pointed out that *H.* sp. is a species related to the Mediterranean *H. valenciennesi*, but not identical with the latter. This *Hypselodoris* sp. was later described by Bouchet and Ortea (1980) as *H. cantabrica*.

Since 1980, references to these species have become more frequent, and many systematic, ecological and chemical aspects have been studied (Avila et al., 1991; Bachelet et al., 1980; Urgorri and Besteiro, 1983).

The *Hypselodoris* species belonging to the blue chromatic group living in the Cantabrian sea are: *H. villafranca*, *H. cantabrica*, and *H. tricolor*; any previous references to *gracilis* must be changed to *villafranca*, those to *valenciennesi*, *messinensis*, and *fontandraui* to *cantabrica*.

The three species can be clearly distinguished by their dorsal color pattern, when about 20–25 mm long (Figure 1): a single central yellow line in *H. tricolor*, a median yellow line expanded in front and behind rhinophores flanked by yellow spots in *H. cantabrica*, and three lines surrounding the rhinophores partially connected by oblique lines in *H. villafranca* (Figure 1A).

H. tricolor bears MDFs only behind the gills, whereas in the other two species they are present also near the rhinophores (Figure 1B). Comparing animals of similar size (more than 20 mm), one observes that the number of MDFs (behind the gills) in *H. cantabrica* is the greatest, namely, six; *H. villafranca* and *H. tricolor* have about four MDFs.

The three species have different temporal distribution. *H. cantabrica* is most abundant during summer, whereas *H. tricolor* is almost the only species that can be found in winter. Figure 2 shows the relative percentage of each species each month in the central part of the Cantabrian sea (Muros del Nalon–Asturias). This does not mean that their actual abundance is constant; in fact all of them appear in maximum numbers in the summer.

Distribution of *Hypselodoris* sp. along the north coast of Spain is a function of both temperature and depth. As one reaches Galician shores and cold water, only *H. villafranca* can be found. This species extends further to the north, reaching as far as the English Channel. Both *H. cantabrica* and *H. tricolor* are absent in this area, but appear again toward the Portuguese coast. The opposite

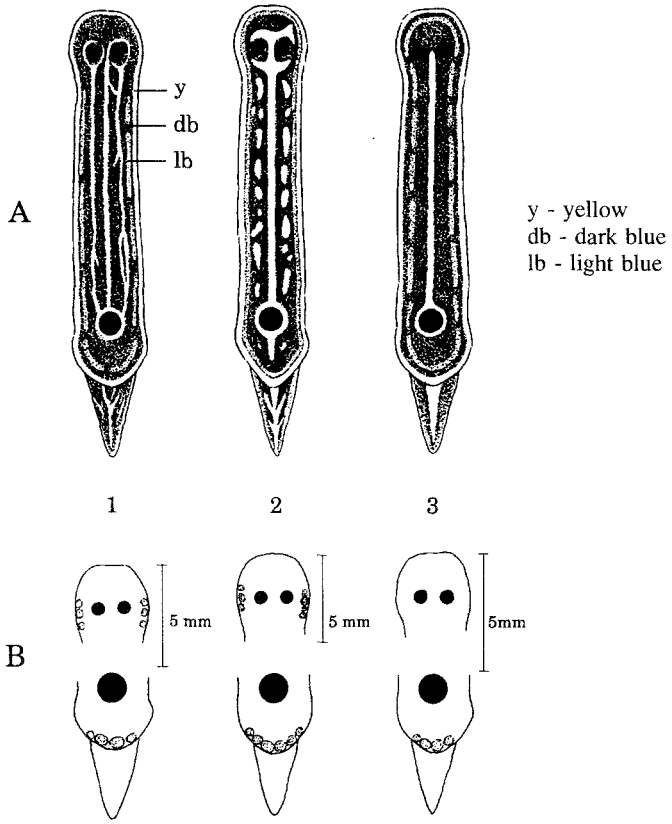


FIG. 1. (A) Dorsal pattern in Cantabrian H. villafranca (1), H. cantabrica (2) and H. tricolor (3); animal size about 20 mm; (B) position of the MDF's.

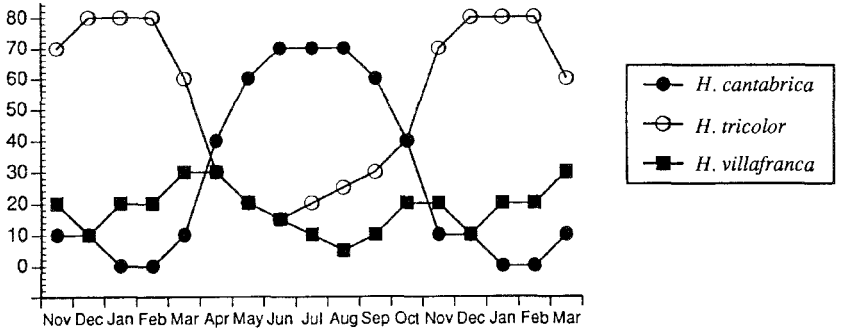


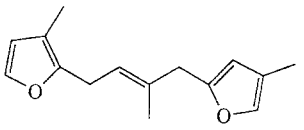
FIG. 2. Annual percentage variation of the three *Hypselodoris* species. Data referred to the central Cantabric area (see text).

happens reaching the Bay of Biscay; the latter two species increase in abundance going from west to east.

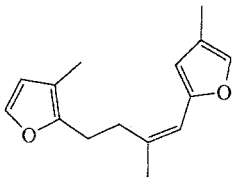
Vertically, all three species can be found as high as the upper infralittoral (the small pools and channels in the intertidal area, but always below sea level), where they are quite scarce, down to 40 m or more. However, the only species inhabiting depths greater than 4–5 m (below the lowest level at low tide) is *H. tricolor*.

All three species in our study were collected at Las Llanas (Asturias) during September and December 1989, while a population of *H. cantabrica* was found in Santander during March 1990. In order to confirm the storage sites of allomones, some specimens of every species were dissected into four parts: MDFs, the rest of the mantle, the digestive gland, and the rest of the viscera. TLC analysis of the ether extracts from the sections of the three *Hypselodoris* revealed very similar patterns of Ehrlich-positive compounds compartmentalized only in the MDFs and in the digestive gland.

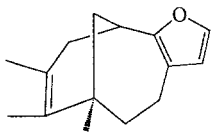
The remaining mollusk parts were subjected to the usual procedure of chemical analysis. Specifically, the diethyl ether-soluble fraction from *H. cantabrica* was processed on a silica gel column, yielding in order of increasing polarity a mixture of very nonpolar compounds (R_f = 0.8 in petroleum ether) along with two isomeric furanosesquiterpenoids identified by analysis of the spectral data as longifolin (**1,** 1.47 mg/animal) and travacfuran (**2**, 1.02 mg/animal). The less polar fraction was further separated by chromatography on silica gel treated with $AgNO_3$ (7.4%). Elution with *n*-heptane–benzene 9:1 led to the isolation of known sesquiterpenoids: nakafuran-9 (**3,** 0.5 mg/animal), dendrolasin (**4,** 0.6 mg/animal), agassizin (**5,** 0.03 mg/animal), *ent*-furodysinin (**6**, trace), along with two new compounds named isonakafuran-9 (**7**, 0.03 mg/animal) and isodehydrodendrolasin (**8**, 0.03 mg/animal).



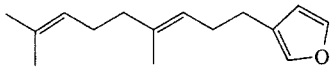
STRUCTURE 1. Longifolin.



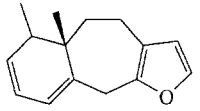
STRUCTURE 2. Tavacfuran.



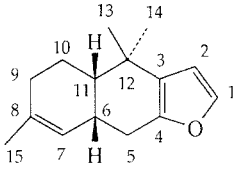
STRUCTURE 3. Nakafuran-9.



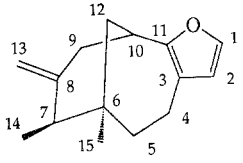
STRUCTURE 4. Dendrolasin.



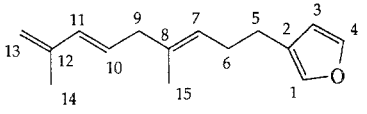
STRUCTURE 5. Agassizin.



STRUCTURE 6. *ent*-Furodysinin.



STRUCTURE 7. Isonakafuran-9.



STRUCTURE 8. Isodehydrodendrolasin.

To the best of our knowledge, this is the first time that the latter two compounds have been isolated from natural sources. In fact, only **7** is known as an intermediate in the synthesis of nakafuran-9 developed by Tanis and Herrinton (1985). The molecular formula, $C_{15}H_{20}O$, was inferred by HREI-MS from the molecular peak at *m/z* 216. The UV maximum at 214 nm along with the positive reaction to Ehrlich's reagent suggested the presence of a furan ring. The α and β substituents of the furan ring were easily deduced by analysis of the ^{1}H NMR spectrum, which showed two coupled broad singlets at δ 5.97 (H-2) and 7.03 (H-1). A broad multiplet at δ 3.11 (H-10) was highly diagnostic for structural analogy with nakafuran-9 **(3)**. In fact, the signal displayed vicinal couplings with the protons at C-9 and C-12. The spectrum was completed by an isolated four-proton system with resonances at δ 2.43 and 2.30 (H-4s) and at δ 1.83 and 1.37 (H-5s); by a quaternary methyl at δ 0.72 (CH_3-6), by a secondary methyl at δ 0.92 (CH_3-7) coupled with the allylic proton at δ 1.95 (H-7) and, finally, by an exomethylene moiety.

The ^{1}H NMR spectrum in C_6D_6 was in complete accord with the reported NMR data of synthetic **7**. The relative stereochemistry of natural isonakafuran at C-7 and C-6 was defined by positive n.O.e.s between CH_3-7 and H-12, and between CH_3-6 and H-7.

The second metabolite, isodehydrodendrolasin **(8)**, had the same molecular composition as **7**, $C_{15}H_{20}O$, deduced from HREI-MS on the molecular ion at *m/z* 216. A diene chromophore was suggested by the UV maximum at 230 nm. The ^{1}H signals at δ 7.12, δ 7.05, and δ 6.07 were attributed to the protons of a β-substituted furan ring. The nature of the alkyl chain was established by further analysis of the ^{1}H NMR spectrum, which showed an isolated A_2B_2M system with resonances at δ 2.31, 2.17, and 5.22. This latter proton (H-7) was also allylically coupled to a methyl resonating at δ 1.50 (CH_3-8). The ^{1}H NMR spectrum was completed by resonances attributed to the diene system and by those at δ 1.76 (3H) and 2.70 (2H) assigned to the protons of a vinyl methyl (CH_3-12) and a bisvinyl methylene (C-9). The *E* stereochemistry of the double bonds between C-7 and C-8 and between C-10 and C-11 was deduced from the coupling constant between H-10 and H-11 (J = 15.5 Hz) and the ^{13}C NMR chemical shift values of C-9 (δ 43.3) and C-15 (δ 16.2).

Analysis of a second collection of *H. cantabrica* from Santander confirmed the hypothesis that the furanosesquiterpenoids are the specific defensive allomones of *Hypselodoris* nudibranchs. In fact, the lipid extract (119 mg) from 14 specimens was mainly characterized by *ent*-furodysinin (**6**, 0.5 mg/animal) and nakafuran-9 (**3**, 0.3 mg/animal), whereas longifolin **(1)** and tavacfuran **(2)** were absent. *ent*-Furodysinin, which is enantiomeric with a metabolite from an Australian *Dysidea* sp. (Kazlauskas et al., 1978), was previously found in *Dysidea tupha* from the east Pyrenean area (Guella et al., 1985) and recently synthetized by Vaillancourt et al. (1991). It is interesting to note that furodysinin is also

known as the main metabolite in the ether extract of *Hypselodoris porterae* and *Hypselodoris californiensis* (Hochlowski et al., 1982) and cooccurs along with related structures in the prey–predator couple *Hypselodoris zebra* and *Dysidea etheria* from the Caribbean (Grode and Cardellina, 1984).

As already noted, furanosesquiterpenoids **1–6** were previously found in sponges and nudibranchs from different geographical areas. In fact, longifolin **(1)** (Hayashi et al., 1972), recently found in the Mediterranean sponge *Dysidea fragilis* (Avila et al., 1991), is the defensive allomone of *H. webbi* and *H. villafranca* (Avila et al., 1991; Cimino et al., 1982; Garcia-Gomez, et al., 1990).

Tavacfuran **(2)** was isolated from *Dysidea* sp. (Guella et al., 1985) and, more recently, its *E* isomer has been found in *H. webbi* from Blanes (eastern Spain) (Avila et al., 1991).

Nakafuran-9 **(3)** was first found in Hawaiian *Dysidea fragilis* and in the sponge predators *Hypselodoris godeffroyana* and *Hypselodoris* (=*Chromodoris*) *maridadilus* (Schulte et al., 1980), and then in *H. webbi* and *H. villafranca* (Avila et al., 1991), collected in the Mediterranean Sea.

Dendrolasin **(4)**, isolated from the ant *Dendrolasius fuliginosus* (Quilico et al., 1957), is a metabolite of *Chromodoris lochi* and its prey, *Spongia microfijensis* (Kakou et al., 1987), and of two nudibranchs, *Hypselodoris ghiselini* and *Hypselodoris californiensis* (Hochlowski et al., 1982).

Agassizin **(5)** is a component of the ether extract prepared from the Mexican *Hypselodoris agassizi* (Hochlowski et al., 1982).

Our results with *H. cantabrica* strongly confirm the predator–prey relationship between *Hypselodoris* species and sponges.

The study of *H. villafranca* and *H. tricolor* led to substantially analogous results. In fact, the chromatographic fraction of the lipid extract of *H. villafranca* yielded, in order of decreasing polarity, longifolin (**1**, 0.3 mg/animal), tavacfuran (**2**, 0.04 mg/animal) and a complex mixture (0.5 mg/animal) of Ehrlich-positive compounds that were directly analyzed by GC-MS, which revealed a pattern dominated by two peaks that were easily assigned to dendrolasin **(4)** and nakafuran-9 **(3)**, by comparison with the compounds previously purified from *H. cantabrica*. Analogously, isonakafuran-9 **(7)**, *ent*-furodysinin **(6)**, agassizin **(5)**, isodehydrodendrolasin **(8)** were recognized among the minor components of the GC profile (Figure 3).

H. tricolor exhibited a chromatographic pattern very similar to that of *H. villafranca* and *H. cantabrica* from Las Llanas. In fact, the SiO_2 column (petroleum ether) yielded longifolin (**1**, 0.05 mg/animal) and tavacfuran (**2**, 0.05 mg/animal) and a mixture of furanosesquiterpenoids (0.14 mg/animal) that GC-MS analysis resolved into nakafuran-9 **(3)**, dendrolasin **(4)**, isonakafuran-9 **(7)**, *ent*-furodysinin **(6)**, agassizin **(5)** and isodehydrodendrolasin **(8)**, along with some unidentified minor components exhibiting mass spectra related to those of the cooccurring furanosesquiterpenoids (Figure 4).

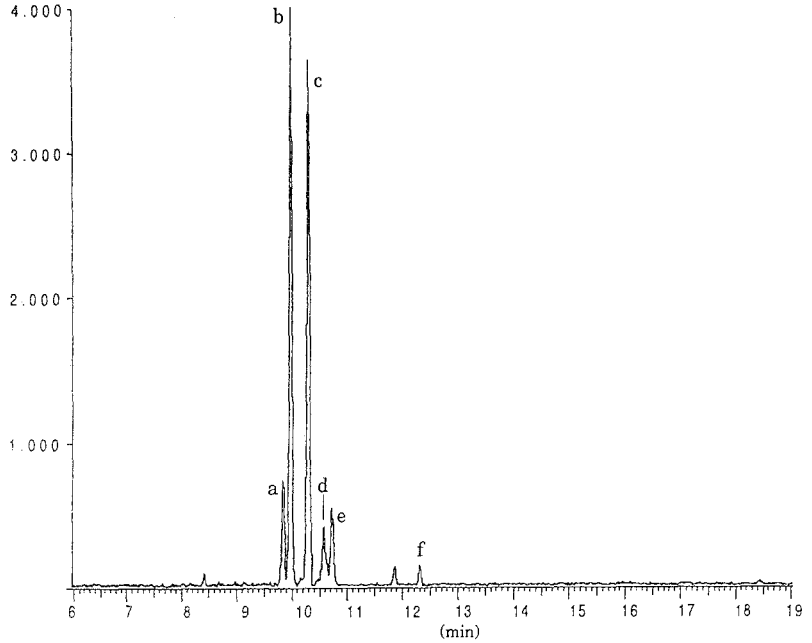


FIG. 3. Gas-chromatographic profile of sesquiterpenoids of ether extract from *H. villafranca*. (a) isonakafuran-9; (b) dendrolasin; (c) nakafuran-9; (d) isodehydrodendrolasin; (e) *ent*-furodysinin; (f) agassizin.

Ichthyotoxic and antifeedant assays were performed to determine whether the products isolated from *Hypselodoris* species were effective deterrents against predators. Toxicity was measured toward *Gambusia affinis*, and feeding deterrence was assessed by observing the feeding response of *Carassius auratus* (Table 1). Of course, more rigorous tests should be performed in the same habitat where the animals live and against their natural predators, but these simple laboratory bioassays, which avoid complex experimental procedures, rapidly offer useful clues with regard to potential bioactivities.

Almost all the molecules exhibited antifeedant properties in a concentration range between 300 and 400 $\mu g/cm^2$ of fish food. *ent*-Furodysinin **(6)** proved to be 10-fold more active than the other compounds. The same product resulted, analogously to longifolin **(1)**, in toxicity to *G. affinis* at a concentration of 10 ppm, while the others did not show any activity.

CONCLUSION

Examination of the mixture of furanosesquiterpenoids from three *Hypselodoris* species collected in the Cantabrian area revealed that the mollusks selectively prey upon the same sponges. It is also obvious that the absolute amounts

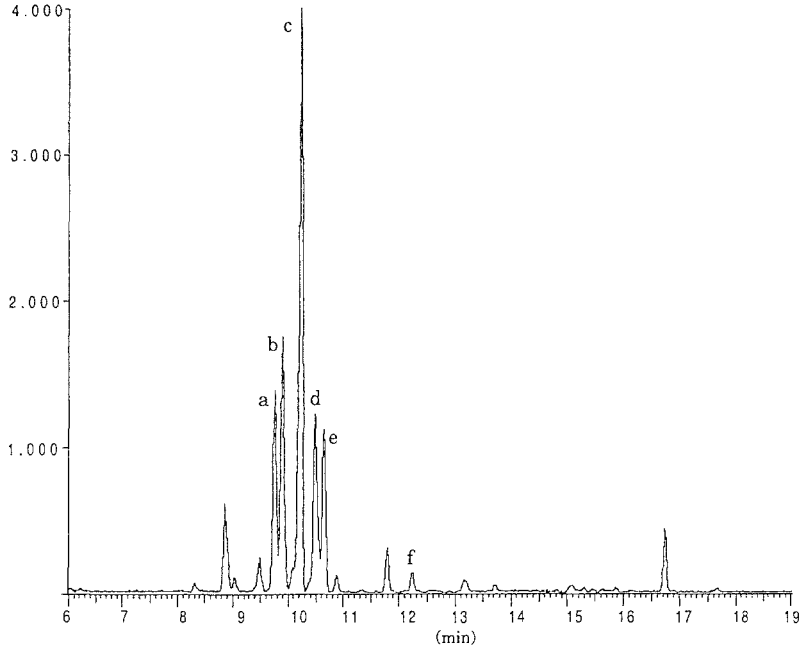


FIG. 4. Gas-chromatographic profile of sesquiterpenoids of ether extract from *H. tricolor*. (a) isonakafuran-9; (b) dendrolasin; (c) nakafuran-9; (d) isodehydrodendrolasin; (e) *ent*-furodysinin; (f) agassizin.

of metabolites isolated from each *Hypselodoris* species were directly related to the size of the animals.

The data support the specific relationship between *Hypselodoris* species and furanosesquiterpenoids. In fact, to the best of our knowledge, only *Hypselodoris orsinii* (Cimino et al., 1982) from the Italian coast selects sponges containing sesterterpenoids rather than sesquiterpenoids. This species has often been referred to *H. tricolor* and *H. coelestis* in both zoological (Ballesteros et al., 1986; e.g., Ros, 1978) and chemical (e.g., Cimino et al., 1982) studies.

The defensive strategy already described of Mediterranean *H. webbi* and *H. villafranca* (Avila et al., 1991; Garcia-Gomez et al., 1990) is confirmed by the compartmentalization of allomones in MDFs displaced near gills and rhinophores in all Cantabrian *Hypselodoris* species. Moreover, the results of our experiments with sections of *H. tricolor* and *H. cantabrica* clearly demonstrate that this strategy is also successfully adopted by these latter species.

At Las Llanas, other nudibranchs, belonging to the family Chromodorididae, were found in the same habitat populated by the three *Hypselodoris* described above. Chemical studies on *Chromodoris luteorosea*, the most abundant species, revealed (Gavagnin et al., 1992) metabolic patterns characterized by a series of

diterpenoids biogenetically derivable from spongian precursors. Most probably, *Chromodoris* and *Hypselodoris* nudibranchs are able to prey selectively upon sponges containing spongian diterpenoids or furanosesquiterpenoids, respectively. Both animals transfer the sequestered terpenoids into protected sections, edge and MDFs, of the mantle. There they are located in a strategically favorable position, where the deterrent effects of sesquiterpenoids and diterpenoids will have maximum effect against attacking predators.

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REFERENCES

AVILA, C., CIMINO, G., FONTANA, A., GAVAGNIN, M., ORTEA, J., and TRIVELLONE, E. 1991. Defensive strategy of two *Hypselodoris* nudibranchs from Italian and Spanish coasts. *J. Chem. Ecol.* 17(3):625–636.

BACHELET, G., CAZAU, C., GANTES, H., and JABOURG, P. 1980. Contribution a l'etude de la faune marine de la région d'Arcachon. *IX Bull. Cent. Etud. Rech. Sci. Biarritz* 13(1):45–46.

BALLESTEROS, M., ALVAREZ, C., and MATEO, B. 1986. Aproximacion a la fauna de opistobranquios de la isla de Menorca. *P. Dept. Zool. Barcelona* 12:93–106.

BOUCHET, P., and ORTEA, J. 1980. Quelques Chromodorididae bleus (Mollusca, Gastropoda, Nudibranchiata) de l'Atlantique Oriental. *Ann. Inst. Oceanogr. Paris* 56(2):117–125.

BOUCHET, P., and TARDY, J. 1976. Faunistique et biogeographie des nudibranches des cotes francaises de l'Atlantique et de la Manche. *Ann. Inst. Oceanogr.* 52(2):205–213.

CIMINO, G., and SODANO, G. 1989. The chemical ecology of Mediterranean opisthobranchs. *Chem. Scri.* 29:389–394.

CIMINO, G., CRISPINO, A., GAVAGNIN, M., and SODANO, G. 1990. Diterpenes from the nudibranch *Chromodoris luteorosea*. *J. Nat. Prod.* 53:102–106.

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., SODANO, G., and VILLANI, G. 1980. Novel metabolites from some predator–prey pairs. *Bull. Soc. Chim. Belg.* 89:1069–1073.

CIMINO, G., DE ROSA, S., DE STEFANO, S., SODANO, G., and VILLANI, G. 1983. Dorid nudibranch elaborates its own chemical defense. *Science* 219:1237–1238.

CIMINO, G., DE ROSA, S., DE STEFANO, S., SODANO, G., and TRIVELLONE, E. 1986. Marine natural products: New results from Mediterranean invertebrates. *Pure Appl. Chem.* 58:375–386.

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. 1992. Marine natural products. *Nat. Prod. Rep.* 9(4):323–364 (and previous reports in the same series).

GARCIA-GOMEZ, J.C., CIMINO, G., and MEDINA, A. 1990. Studies on the defensive behaviour of *Hypselodoris* (Gastropoda, Nudibranchia): Ultrastructure and chemical analysis of mantle dermal formations (MDFs). *Mar. Biol.* 106:245–250.

GARCIA-GOMEZ, J.C., MEDINA, A., and COVEÑAS, R. 1991. Study of the anatomy and histology of the mantle dermal formations (MDFs) of *Chromodoris* and *Hypselodoris* (Opisthobranchia: Chromodorididae). *Malacologia* 32:233–240.

GAVAGNIN, M. VARDARO, R.R., AVILA, C., ORTEA, J., and CIMINO, G. 1992. Ichthyotoxic diterpenoids from the Cantabrian nudibranch *Chromodoris luteorosea*. *J. Nat. Prod.* In press.

GRODE, S.H., and CARDELLINA, J.H. 1984. Sesquiterpenes from the sponge *Dysidea etheria* and the nudibranch *Hypselodoris zebra*. *J. Nat. Prod.* 47(1):76–83.

GUELLA, G., MANCINI, I., GUERRIERO, A., and PIETRA, F. 1985. 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.

GUSTAFSON, K., and ANDERSEN, R.J. 1985. Chemical studies of British Columbia nudibranchs. *Tetrahedron* 41:101–110.

HAYASHI, N., KOMAE, H., EGUCHI, E., NAKAYAMA, N., HAYASHI, S., and SAKAO, T. 1972. Two new furanosesquiterpenes from *Actinodaphne longifolia*. *Chem. Ind.* 14:572–473.

HOCHLOWSKI, J.E., WALKER, R.P., IRELAND, C., and FAULKNER, D.J. 1982. Metabolites of four nudibranch of the genus *Hypselodoris*. *J. Org. Chem.* 47:88–91.

KAKOU, Y., CREWS, P., and BAKUS, G.J. 1987. Dendrolasin and lantraculin A from the Fijian *Spongia mycofijiensis* and an associated nudibranch *Chromodoris lochi*. *J. Nat. Prod.* 50(3):482–484.

KARUSO, P. 1987. Chemical ecology of the nudibranchs, 1: pp. 31–60, *in* P.J. Scheuer (ed.). Bioorganic Marine Chemistry, Vol. 1. Springer-Verlag, New York.

KAZLAUSKAS, R., MURPHY, P.T., WELLS, R.J., DALY, J.J., and SCHONHOLZER, P. 1978. Two sesquiterpene furans with new carbocyclic ring systems and related thiol acetates from a species of the sponge genus *Dysidea*. *Tetrahedron Lett.* 49:4951–4954.

QUILICO, A., PIOZZI, F., and PAVAN, M. 1957. The structure of dendrolasin. *Tetrahedron* 1:177–185.

RIOJA, E. 1917. Notas sobre una excursion por las costas de Gijon. *Bol. Soc. Esp. Hist. Nat.* 17(8):488–494.

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.

ROS, J. 1978. Distribuciò en l'espai i en el temps dels opistobranquis iberics, amb especial referencia als del litoral català. *Bull. Inst. Cat. Hist. Nat.* 42(Sec. Zool. 3):181–183.

RUDMAN, W.B. 1984. The Chromodorididae (Opistobranchia, Mollusca) of the Indo-west Pacific: A review of the genera. *Zool. J. Linn. Soc.* 81:115–273.

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.

TANIS, S.P., and HERRINTON, P.M. 1985. Furans in synthesis. 5. Furan-terminated cationic cyclizations in the preparation of fused, spirocyclic and bridged ring systems. An application to the synthesis of nakafuran-9. *J. Org. Chem.* 50:3988–3996.

THOMPSON, J.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.

THOMPSON, T.E. 1960. Defensive adaptations in opisthobranchs, *J. Mar. Biol. Assoc. U.K.* 39:123–134.

URGORRI, V., and BESTEIRO, C. 1983. Iventario de los moluscos opistobranquios de Galicia. *Inv. Pesq.* 47(1):3–28.

VAILLANCOURT, V., AGHARAHIMI, M.R., SUNDRAM, U.N., RICHOU, O., FAULKNER, D.J., and ALBIZATI, K.F. 1991. Synthesis and Absolute Configuration of the Antiparasitic Furanosesquiterpenes (−)-Furodysin and (−)-Furodysinin. Camphor as a Six-Membered Ring Chiral Pool Template. *J. Org. Chem.* 56:378–387.

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DETOXICATION ACTIVITY IN THE GYPSY MOTH: EFFECTS OF HOST CO_2 AND NO_3^- AVAILABILITY

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Abstract—We investigated the effects of host species and resource (carbon dioxide, nitrate) availability on activity of detoxication enzymes in the gypsy moth, *Lymantria dispar*. Larvae were fed foliage from quaking aspen or sugar maple grown under ambient or elevated atmospheric CO_2, with low or high soil NO_3^- availability. Enzyme solutions were prepared from larval midguts and assayed for activity of cytochrome P-450 monooxygenase, esterase, glutathione transferase, and carbonyl reductase enzymes. Activity of each enzyme system was influenced by larval host species, CO_2 or NO_3^- availability, or an interaction of factors. Activity of all but glutathione transferases was highest in larvae reared on aspen. Elevated atmospheric CO_2 promoted all but transferase activity in larvae reared on aspen, but had little if any impact on enzyme activities of larvae reared on maple. High NO_3^- availability enhanced activity of most enzyme systems in gypsy moths fed high CO_2 foliage, but the effect was less consistent for insects fed ambient CO_2 foliage. This research shows that gypsy moths respond biochemically not only to interspecific differences in host chemistry, but also to resource-mediated, intraspecific changes in host chemistry. Such responses are likely to be important for the dynamics of plant–insect interactions as they occur now and as they will be altered by global atmospheric changes in the future.

Key Words—Carbonyl reductase, carbon dioxide, cytochrome P-450 monooxygenase, detoxication enzymes, esterase, global change, glutathione transferase, gypsy moth, *Lymantria dispar*, Lepidoptera, Lymantriidae, nitrate, phytochemistry, resource availability.

INTRODUCTION

Phytochemical variation *among* plant species is known to produce highly variable detoxication activity in insects (Yu et al., 1979; Yu, 1982; Lindroth,

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1989a,b). Changes in detoxication activity are generally attributed to differences in host allelochemical profiles (e.g., via induction and/or inhibition), although differences in nutrient content may also play a role (Lindroth, 1991). Such changes are of particular consequence to the nutritional ecology of generalist species such as the gypsy moth (*Lymantria dispar*), which may feed on multiple host species during larval development (Lance and Barbosa, 1982).

Phytochemical variation *within* plant species is also likely to effect changes in insect detoxication activity, although this possibility has received little attention by researchers. A host of environmental factors is known to alter individual plant chemistry and thereby to change insect performance (Denno and McClure, 1983; Mattson and Haack, 1987). Carbon–nutrient balance theory contends that such intraspecific variation results from differences in the relative availability of resources (e.g., carbon, nutrients) to plants (Bryant et al., 1983; Bazzaz et al., 1987; Tuomi et al., 1988). For example, environmental conditions that increase availability of carbon relative to nutrients may promote accumulation of carbon-based allelochemicals (Larsson et al., 1986; Bryant et al., 1987). Whether insect detoxication metabolism also changes in this context is poorly understood.

The purpose of this study was to evaluate the impact of host plant resource availability on insect detoxication capacity. We assessed the effects of carbon dioxide and nitrate availability on detoxication activity in gypsy moths fed quaking aspen (*Populus tremuloides*) and sugar maple (*Acer saccharum*).

We selected this experimental system for several reasons. First, larvae of the gypsy moth are highly polyphagous and are serious forest pests in the northeastern and, more recently, north central and eastern United States. Yet, apart from several studies of cytochrome P-450 monooxygenase enzymes (Ahmad, 1986; Sheppard and Friedman, 1989), little is known about host plant mediation of detoxication activity in this species. Second, both plant chemistry and larval detoxication capacity are known to influence host use by gypsy moths (Rossiter et al., 1988; Lindroth and Hemming, 1990; Lindroth et al., 1993; Hemming and Lindroth, unpublished data). Third, effects of carbon dioxide and CO_2 × nitrate interactions on trees are of increasing environmental importance, as atmospheric concentrations of CO_2 are expected to double by the latter half of the next century (Hansen, 1981; Gates et al., 1983) and to exert significant impacts on forest ecosystems (Eamus and Jarvis, 1989; Graham et al., 1990).

METHODS AND MATERIALS

Insects and Diets. We obtained gypsy moth egg masses from USDA-APHIS, Otis Air National Guard Base, Massachusetts. Larvae were reared from egg hatch through the third stadium on standard wheat germ diet (ODell et al., 1985, but without the preservative methyl paraben) at 25°C, on a 15:9 hr light–

dark cycle. All insect rearing was conducted in the gypsy moth quarantine facility of the Department of Entomology, University of Wisconsin, Madison.

The experimental design was a 2 × 2 × 2 factorial, with two species of trees and two levels each of carbon dioxide and nitrate. We grew 1-year-old seedlings of quaking aspen and sugar maple in environmental control rooms at the University of Wisconsin Biotron under ambient (350 ppm) and elevated (650 ppm) atmospheric CO_2 (see Kinney and Lindroth, 1993, for additional experimental details). Within each room, half the trees were watered with low nitrate (1.25 mM) and half with high nitrate (7.5 mM) nutrient solution (1/2 strength Hoagland's). Foliage collected for insect feeding was pooled from several rooms at each level of CO_2 and NO_3^-. Leaves of an intermediate age were collected from aspen, which has indeterminate growth. Leaves from the first two leaf flushes were collected from maple.

Each experimental replicate consisted of 20–25 freshly molted fourth instars fed excised leaves from one of the experimental treatments. New leaves were provided every one to two days until larvae were mid-fifth instars (9–15 days, depending on development rate).

Enzyme Assays. Larval midguts (15–20) from each group were dissected into ice-cold potassium phosphate buffer (0.2 M, pH 7.8, with 1 mM EDTA) and ground in a Ten Broeck tissue homogenizer. We centrifuged the homogenates at 10,000 *g* (10 min) to remove cellular debris, and the resulting supernatants at 100,000 *g* (1 hr) to separate soluble and microsomal protein fractions. Microsomal pellets were resuspended in phosphate buffer containing 50% glycerol. Both enzyme fractions were flash-frozen in liquid nitrogen and stored at −70°C prior to assessing enzyme activity.

We determined protein concentrations of the solutions by the Bradford (1976) assay, using bovine serum albumin as a standard. Midgut preparations were then subjected to a suite of enzyme assays chosen to represent a broad range of detoxication activity. These tests included assays of cytochrome P-450-dependent monooxygenases (polysubstrate monooxygenases), esterases, glutathione transferases, and quinone reductases. All activities were quantified spectrophotometrically using a Perkin-Elmer Lambda 3B. Chemicals and reagents were obtained from Sigma Chemical Company (St. Louis, Missouri). The assays used were adapted from several sources; full descriptions are provided by Lindroth et al. (1990). A brief description of each assay follows.

In comparison to other Lepidoptera, P-450 monooxygenase activity in gypsy moths is very difficult to measure by many of the common catalytic assays, most likely because of exceptionally low activity (Lindroth, unpublished data; C. Sheppard, personal communication). Consequently, we used NADPH oxidation and cytochrome *c* reductase assays as indices of monooxygenase activity (Brattsten et al., 1980, 1984). Endogenous oxidation of NADPH by cytochrome P-450 was quantified as the decrease in absorbance at 340 nm over 90 sec.

Cytochrome *c* reductase is a redox flavoprotein coupled to cytochrome P-450, the terminal oxidase of the monooxygenase system. Reductase activity was measured by the rate of reduction of cytochrome *c* by NADPH, as indicated by the increase in absorbance at 550 nm over 60 sec. We caution that use of these assays as indices of cytochrome P-450 activity is not without problems. For the NADPH oxidation assay, other enzyme systems (e.g., tryptophan 2,3-dioxygenase) may contribute to NADPH oxidation. For the cytochrome *c* reductase assay, the ratio between reductase activity and P-450 activity can vary greatly, depending in part upon the particular P-450 isozymes and substrates involved. Our results should be interpreted accordingly.

Cytosolic general esterase activity was measured as the hydrolytic release of 1-naphthol from 1-naphthylacetate. Cytosolic glutathione-*S*-transferase activity was measured via halide substitution of reduced glutathione (GSH) onto the substrate 1-chloro-2,4-dinitrobenzene (CDNB). The conjugate GS-DNB absorbs light at 340 nm; enzyme activity is indicated by the increase in A_{340} over 60 sec. Enzymatic reduction of carbonyl compounds (specifically quinones) in cytosolic and microsomal fractions was measured by the juglone-dependent NADPH oxidation method (Yu, 1987). NADPH provides reducing equivalents for reduction of juglone (5-hydroxy-1,4-naphthoquinone); activity is detected as the decrease in A_{340} over 60 sec. The assay automatically corrects for endogenous NADPH oxidation (e.g., via P-450s) because enzymes and NADPH occur in both sample and reference cuvettes. Previous studies (e.g., Lindroth et al., 1990) documented that the NADPH oxidation observed in this assay is not catalyzed by cytochrome P-450 enzymes.

Statistical Analysis. Results were analyzed by three-way analysis of variance (ANOVA) to determine the effects of host species, CO_2 and NO_3^- levels, and their interactions on gypsy moth enzyme activities.

RESULTS

Indices of P-450 monooxygenase activity were significantly affected by host species, CO_2 and NO_3^- availability, and their interactions (Figure 1a). Rates of NADPH oxidation were 71% higher overall in larvae fed aspen than in those fed maple. High CO_2 levels increased oxidation activity 61% in larvae fed aspen but had no effect on larvae fed maple, as indicated by the significant species × carbon interaction term. High NO_3^- availability increased oxidase activity for insects fed high CO_2, but not ambient CO_2, foliage. Not surprisingly, treatment effects on cytochrome *c* reductase activity paralleled those on NADPH oxidation activity (Figure 1b). Activity was higher in larvae fed aspen than in those fed maple, increased with enhanced CO_2 availability to aspen but not maple, and increased with enhanced NO_3^- availability to trees grown under elevated CO_2.

The main effects of species, CO_2 and NO_3^- all significantly altered gypsy

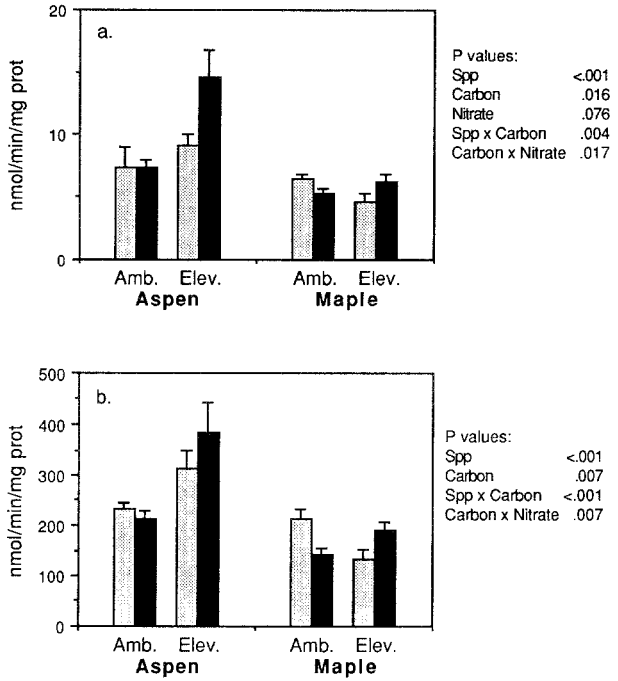

FIG. 1. Indices of cytochrome P-450 monooxygenase activity in gypsy moth larvae fed aspen and maple grown with low and high availability of CO_2 and NO_3^-. (a) NADPH oxidation activity; (b) cytochrome *c* reductase activity. Light- and dark-shaded bars represent low- and high-NO_3^- treatments, respectively. Vertical lines indicate 1 SE. Listed *P* values are from three-way ANOVAs; only values < 0.10 are shown.

moth esterase activities, but the magnitude of effect was large only for tree species (Figure 2). Larvae reared on aspen exhibited esterase activities 2.1-fold higher than those of larvae on maple. Activities increased for insects fed high CO_2 foliage, and more so for larvae on aspen than for those on maple. Improved host NO_3^- availability marginally increased (5–11%) insect esterase activity across all treatment combinations.

Glutathione transferase activities also responded to each of the main effects (Figure 3). Activities were 37% higher in larvae fed maple than in larvae fed aspen. Elevated CO_2 decreased transferase activity (37%) in larvae reared on aspen but had no effect in larvae reared on maple, as indicated by the significant interaction term. High NO_3^- availability promoted transferase activity (21–47%) in all treatment combinations.

Finally, carbonyl reductase activities also responded to host species and resource availability (Figure 4). Soluble reductase activity averaged slightly

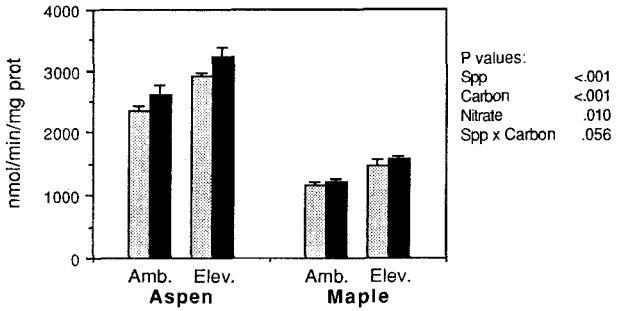

FIG. 2. Esterase activity in gypsy moth larvae fed aspen and maple grown with low and high availability of CO_2 and NO_3^-. See Figure 1 for description of figure components.

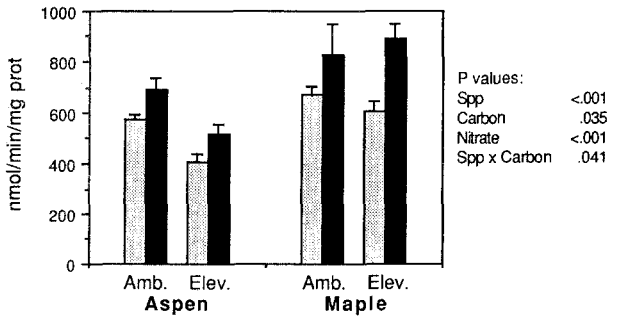

FIG. 3. Glutathione transferase activity in gypsy moth larvae fed aspen and maple grown with low and high availability of CO_2 and NO_3^-. See Figure 1 for description of figure components.

higher in larvae fed aspen than in larvae fed maple and was 25% higher in larvae fed elevated CO_2 foliage than in those fed ambient CO_2 foliage. The overall effect of high NO_3^- availability was a 16% increase in reductase activity. Treatment effects differed somewhat for microsomal reductase activities. Values were again higher in larvae fed aspen than in larvae fed maple, but the CO_2 effect was only significant for the former. We found no general NO_3^- effect but observed a $CO_2 \times NO_3^-$ interaction; high NO_3^- availability tended to reduce reductase activity in gypsy moths reared on low CO_2 leaves, but to increase activity in insects reared on high CO_2 leaves.

DISCUSSION

Our results show that gypsy moth larvae respond biochemically to changes in the chemical composition of their host plants, although specific cause-and-effect relationships cannot be ascertained from this study. (Increased activity of

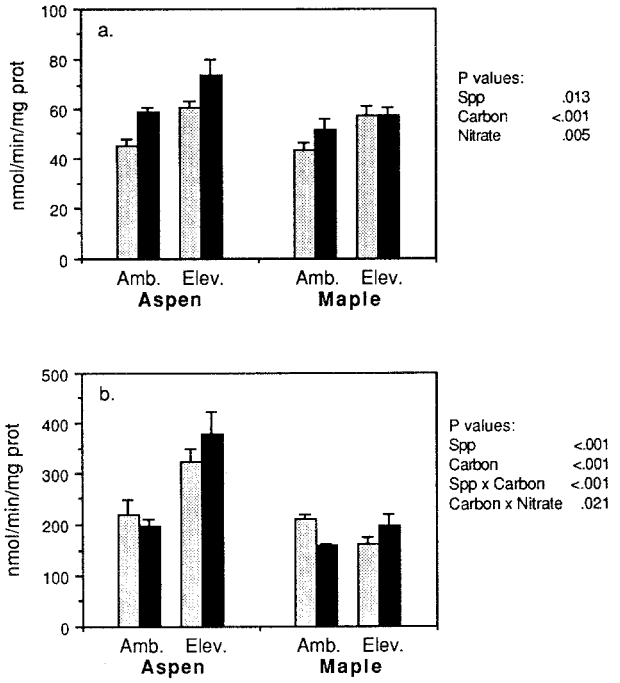

FIG. 4. Carbonyl reductase activity in gypsy moth larvae fed aspen and maple grown with low and high availability of CO_2 and NO_3^-. (a) Soluble enzyme fraction; (b) microsomal enzyme fraction. See Figure 1 for description of figure components.

an enzyme system may result from improved insect nutritional status or from consumption of inducing phytochemicals.)

Of the three factors investigated (species, CO_2, and NO_3^-), plant species generally had the greatest modulating effect on gypsy moth detoxication activity. This is as expected, because qualitative and quantitative differences in chemical composition were greater between species than within species (Kinney and Lindroth, 1993). Insects reared on aspen had higher activities than insects reared on maple for all enzyme systems but glutathione transferase. Aspen foliage contained higher concentrations of carbohydrates, but lower levels of nitrogen and condensed tannins, than did maple (Kinney and Lindroth, 1993). Maple had appreciable amounts of ellagitannins and gallotannins, which do not occur in aspen. In contrast, aspen contained phenolic glycosides (salicortin and tremulacin), which do not occur in maple. The latter compounds may be responsible for the high esterase activity in larvae reared on aspen, although earlier research on induction of gypsy moth esterases by dietary phenolic glycosides

has given conflicting results (Lindroth and Hemming, 1990; Lindroth and Weisbrod, 1991).

Several researchers have suggested that metabolic adaptations to different host species may exact a cost in terms of food processing efficiencies and, ultimately, larval performance (Schoonhoven and Meerman, 1978; Scriber, 1981). Thus, gypsy moth larvae that frequently switch host species (true generalists) may exhibit reduced performance in comparison to monophagous larvae, even though the hosts may be similarly "nutritious." This possibility has not been experimentally investigated using natural diets, but Sheppard and Friedman (1990) documented significant switching effects on gypsy moth food conversion efficiencies using a combination of artificial and natural diets. Alternatively, mixed diets may be less metabolically costly than are single-species diets that require maintenance of high titers of multiple detoxication enzymes. Larvae reared on aspen, for example, exhibit high enzyme activities across the board and are much less efficient at converting digested food into body mass than are larvae reared on maple (Lindroth et al., 1993, Kinney and Lindroth, 1993).

Gypsy moth detoxication activity also responded to changes in host chemistry as mediated by resource availability. We found significant CO_2 and CO_2 interaction effects, and NO_3^- and NO_3^- interaction effects, for every enzyme system assayed. Elevated CO_2 led to an increase in activity of all but glutathione transferases in larvae fed aspen, whereas no such trend was observed in larvae fed maple. High NO_3^- availability promoted activity of most enzyme systems in larvae reared on high CO_2 aspen and maple leaves, but the effect was less consistent in larvae reared on ambient CO_2 leaves.

Foliage of trees grown under conditions that shifted the carbon–nutrient balance in favor of carbon (high CO_2 and/or low NO_3^-) generally had increased concentrations of starch and tannin compounds but decreased concentrations of nitrogen (Kinney and Lindroth, 1993). Because multiple leaf chemical characteristics changed in concert, we cannot attribute specific enzymatic responses to particular chemicals. Nevertheless, results of this and earlier studies suggest some possibilities. Aspen phenolic glycosides are most likely metabolized via esterases in gypsy moths (Lindroth and Hemming, 1990), and increased consumption rates of larvae fed high CO_2 foliage compared to ambient CO_2 foliage may have led to induction of this enzyme system. Changes in larval glutathione transferase activity can be explained in part by shifts in host nitrogen (protein) concentrations. Transferase activity requires glutathione, a tripeptide, for conjugation. Thus transferase activity is likely reduced in insects feeding on nitrogen-deficient diets, as has been documented for gypsy moths fed artificial diets (Lindroth et al., 1990). Indeed, correlation analysis of mean transferase activity versus mean foliar nitrogen content of trees in our study revealed a strong positive relationship ($r = 0.81$, $P = 0.014$, $N = 8$).

Modulation of insect detoxication capacity in response to changes in host phytochemistry has been accorded importance in the dynamics of plant–insect interactions. Mattson and Haack (1987), for example, proposed that drought-induced changes in plant chemistry improve insect detoxication capacity, thereby contributing to the onset of insect outbreaks. Empirical evidence of such metabolic responses, however, has been virtually nonexistent. Our study illustrates that insect detoxication metabolism does change in response to intraspecific changes in host chemistry, but more research is needed to elucidate the mechanisms and biological importance of such responses. For example, to what extent are changes in detoxication capacity an active defensive response to host allelochemicals versus a passive response to changes in insect nutritional status? Are these metabolic changes of a magnitude great enough to influence insect fitness, and thus the evolution and ecology of plant–animal interactions? And if so, how will they influence the dynamics of plant–insect associations under global atmospheric conditions anticipated for the future?

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REFERENCES

AHMAD, S. 1986. Enzymatic adaptations of herbivorous insects and mites to phytochemicals. *J. Chem. Ecol.* 12:533–560.

BAZZAZ, F.A., CHIARIELLO, N.R., COLEY, P.D., and PITELKA, L.F. 1987. Allocating resources to reproduction and defense. *Bioscience* 37:58–67.

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.

BRATTSTEN, L.B., PRICE, S.L., and GUNDERSON, C.A. 1980. Microsomal oxidases in midgut and fatbody tissues of a broadly herbivorous insect larva, *Spodoptera eridania* Cramer (Noctuidae). *Comp. Biochem. Physiol.* 66C:231–237.

BRATTSTEN, L.B., EVANS, C.K., BONETTI, S., and ZALKOW, L.H. 1984. Induction by carrot allelochemicals of insecticide-metabolizing enzymes in the southern armyworm (*Spodoptera eridania*). *Comp. Biochem. Physiol.* 77C:29–37.

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.

BRYANT, J.P., CLAUSEN, T.P., REICHARDT, P.B., MCCARTHY, M.C., and WERNER, R.A. 1987. Effect of nitrogen fertilization upon the secondary chemistry and nutritional value of quaking aspen (*Populus tremuloides* Michx.) leaves for the large aspen tortrix [*Choristoneura conflictana* (Walker)]. *Oecologia* 73:513–517.

DENNO, R.F., and MCCLURE, M.S. (eds.). 1983. Variable Plants and Herbivores in Natural and Managed Ecosystems. Academic Press, New York.

EAMUS, D., and JARVIS, P.G. 1989. The direct effects of increase in the global atmospheric CO_2 concentration on natural and commercial temperate trees and forests. *Adv. Ecol. Res.* 19:1–55.

GATES, D.M., STRAIN, B.R., and WEBER, J.A. 1983. Ecophysiological effects of changing atmos-

pheric CO_2 concentration, pp. 503–526, *in* O.L. Lange, P.S. Nobel, C.B. Osmond, and H. Ziegler (eds.). Physiological Plant Ecology IV. Ecosystem Processes: Mineral Cycling, Productivity and Man's Influence. Springer-Verlag, New York.

GRAHAM, R.L., TURNER, M.G., and DALE, V.H. 1990. How increasing CO_2 and climate change affect forests. *Bioscience* 40:575–587.

HANSEN, J., JOHNSON, D., LEBEDEFF, S., LEE, P., RIND, D., and RUSSELL, F. 1981. Climatic impact of increasing atmospheric CO_2. *Science* 213:957–966.

KINNEY, K.K., LINDROTH, R.L., and JUNG, S.M. 1993. A test of carbon-nutrient balance theory with deciduous trees: effects of CO_2- and NO_3^--mediated changes, in foliar chemistry on gypsy moth performance. *Amer. Natur.* (submitted).

LANCE, D.L., and BARBOSA, P. 1982. Host tree influences on the dispersal of late instar gypsy moths, *Lymantria dispar*. *Oikos* 38:1–7.

LARSSON, S., WIRÉN, A., LUNDGREN, L., and ERICSSON, T. 1986. Effect of light and nutrient stress on leaf phenolic chemistry in *Salix dasyclados* and susceptibility to *Galerucella lineola* (Coleoptera). *Oikos* 47:205–210.

LINDROTH, R.L. 1989a. Chemical ecology of the luna moth: Effects of host plant on detoxification enzyme activity. *J. Chem. Ecol.* 15:2019–2029.

LINDROTH, R.L. 1989b. Host plant alteration of detoxication activity in *Papilio glaucus glaucus*. *Entomol. Exp. Appl.* 50:29–35.

LINDROTH, R.L. 1991. Differential toxicity of plant allelochemicals to insects: Roles of enzymatic detoxication systems, pp. 1–33, *in* E.A. Bernays (ed.). Insect–Plant Interactions, Vol. 3. CRC Press, Boca Raton, Florida.

LINDROTH, R.L., and HEMMING, J.D.C. 1990. Responses of the gypsy moth (Lepidoptera: Lymantriidae) to tremulacin, an aspen phenolic glycoside. *Environ. Entomol.* 19:842–847.

LINDROTH, R.L., and WEISBROD, A.V. 1991. Genetic variation in response of the gypsy moth to aspen phenolic glycosides. *Biochem. Syst. Ecol.* 19:97–103.

LINDROTH, R.L., ANSON, B.D., and WEISBROD, A.V. 1990. Effects of protein and juglone on gypsy moths: growth performance and detoxification enzyme activity. *J. Chem. Ecol.* 16:2533–2547.

LINDROTH, R.L., KINNEY, K.K., and PLATZ, C.L. 1993. Responses of deciduous trees to elevated atmospheric CO_2: productivity, phytochemistry and insect performance. *Ecology* In press.

MATTSON, W.J., and HAACK, R.A. 1987. The role of drought in outbreaks of plant-eating insects. *Bioscience* 37:110–118.

ODELL, T.M., BUTT, C.A., and BRIDGEFORTH, A.W. 1985. *Lymantria dispar*, pp. 355–367, *in* P. Singh and R.F. Moore, (eds.). Handbook of Insect Rearing, Vol. 2. Elsevier, New York.

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.

SCHOONHOVEN, L.M., and MEERMAN, J. 1978. Metabolic cost of changes in diet and neutralization of allelochemics. *Entomol. Exp. Appl.* 24:689–693.

SCRIBER, J.M. 1981. Sequential diets, metabolic costs, and growth of *Spodoptera eridania* (Lepidoptera: Noctuidae) feeding upon dill, lima bean, and cabbage. *Oecologia* 51:175–180.

SHEPPARD, C.A., and FRIEDMAN, S. 1989. Endogenous and induced monooxygenase activity in gypsy moth larvae feeding on natural and artificial diets. *Arch. Insect Biochem. Physiol.* 10:47–56.

SHEPPARD, C.A., and FRIEDMAN, S. 1990. Influence of host plant, foliar phenology and larval dietary history on *Lymantria dispar* larval nutritional indices. *Entomol. Exp. Appl.* 55:247–255.

TUOMI, J., NIEMALA, P., CHAPIN, F.S., III, BRYANT, J.P., and SIRIN, S. 1988. Defensive responses of trees in relation to their carbon/nutrient balance, pp. 57–72, *in* W.J. Mattson, J. Levieux, and C. Bernard-Dagan (eds.). Mechanisms of Woody Plant Defenses Against Insects. Search for Pattern. Springer-Verlag, New York.

YU, S.J. 1982. Induction of microsomal oxidases by host plants in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). *Pestic. Biochem. Physiol.* 17:59–67.

YU, S.J. 1987. Quinone reductase of phytophagous insects and its induction by allelochemicals. *Comp. Biochem. Physiol.* 87B:621–624.

YU, S.J., BERRY, R.E., and TERRIERE, L.C. 1979. Host plant stimulation of detoxifying enzymes in a phytophagous insect. *Pestic. Biochem. Physiol.* 12:280–284.

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EFFECTS OF ROOT EXUDATE SORGOLEONE ON PHOTOSYNTHESIS[1]

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Abstract—The aim of this investigation was to determine if sorgoleone (SGL), a *p*-benzoquinone in *Sorghum bicolor* root exudate, is a photosynthesis inhibitor. Assays using *Glycine max* leaf disks showed concentrations as low as 10 μM SGL inhibited oxygen evolution more than 50%. Tests conducted on chloroplasts isolated from *Pisum sativum* showed that SGL is a powerful inhibitor of CO_2-dependent oxygen evolution. Using a chloroplast suspension equivalent to 80–100 μg chlorophyll, the I_{50} was approximately 0.2 μM SGL. These data indicate inhibition of photosynthesis is part of the explanation for growth reduction caused by this allelochemical.

Key Words—Sorgoleone, allelochemical, allelopathy, photosynthesis, chloroplast, root exudate, *Sorghum bicolor*.

INTRODUCTION

The suppression of weed growth by *Sorghum* species has been reported by several investigators (Overland, 1966; Putnam et al., 1983; Putnam and DeFrank, 1983; Forney et al., 1985; Einhellig and Rasmussen, 1989). Both

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hydrophobic and hydrophilic allelopathic chemicals are exuded from the roots of grain sorghum [*Sorghum bicolor* (L.) Moench] (Panasiuk et al., 1986; Netzly and Butler, 1986; Schutt and Netzly, 1991; Einhellig and Souza, 1992). After oxidation, sorgoleone {2-hydroxy-5-methoxy-3-[(8′Z,11′Z)-8′,11′,14′-pentadicatriene]-*p*-benzoquinone, SGL} is the major hydrophobic phytotoxic component that has been identified in the root exudate (Netzly et al., 1988). Hess et al. (1992) checked 12 *Sorghum* genotypes and found they all produced about the same amount of sorgoleone. Einhellig and Souza (1992) showed that SGL is a potent inhibitor of growth of several broadleaf and grass weed seedlings.

We found SGL inhibited oxygen uptake in isolated mitochondria (Rasmussen et al., 1992). It appeared to act by blocking electron flow in the mitochondrial electron transport system (ETS). The hydrophobic nature of SGL and its action on mitochondria suggested it might also interfere with photosynthesis. Hence, the aim of this investigation was to determine if part of the allelopathic effect of SGL was through an interference with the processes of photosynthesis.

METHODS AND MATERIALS

Source of Sorgoleone. Sorghum root exudate was collected by dipping roots of grain sorghum seedlings [*Sorghum bicolor* (L.) Moench., Dekalb Hybrid DK 28] in acidified methylene chloride according to a procedure modified from Netzly et al. (1988) and described in Rasmussen et al. (1992). The exudate collected in this way is essentially pure sorgoleone and its dihydroquinone (Fate et al., 1990; Rasmussen et al., 1992).

Leaf Photosynthesis. The unifoliate leaf tissue from 3-week-old soybean [*Glycine max* (L.) Merr. var. Corsoy] seedlings was used in these experiments. Soybeans were grown in vermiculite in the greenhouse with periodic watering with one-half strength Hoagland's solution (Hoagland and Arnon, 1950). Plants were placed in low light (10 $\mu E/m^2/sec$) for 1 hr prior to cutting the 3-mm leaf disks used in the assays. Following procedures of Hensley (1981), leaf disks were vacuum infiltrated with 0, 10, 50, or 100 μM SGL solutions by alternating the pressure to as low as -93 kPa for 1.5 min. The solution containing SGL was a 10 mM potassium phosphate buffer, pH 6.8, with 0.015% Triton-X. The vacuum-infiltrated leaf disks were placed in the dark for 30 min before effects on photosynthesis were monitored.

The photosynthetic rate of leaf disks after SGL infiltration was studied in two ways: a floating leaf disk assay (Hensley, 1981) and quantification of oxygen evolution using a Hansatech LD-2 system, which has a Clark-type oxygen electrode (King's Lynn, England). The rate of photosynthesis was measured at 100 $\mu E/m^2/sec$ and 25°C in both procedures. The procedure in the floating leaf disk assay was to monitor the elapsed time for disks to float after being placed

in the light. Twenty 3-mm disks were used per treatment with three replications per treatment. The experiment was run several times with data recorded as a percent of control. A second determination of photosynthetic activity was obtained by measuring oxygen evolution with the gas-phase oxygen electrode system. Forty leaf disks were placed in the Hansatech chamber with an atmosphere of 5% carbon dioxide, and oxygen evolution was monitored for 10 min. Data from three replications were obtained for each treatment and the experiment was duplicated. After subjecting the data to analysis of variance (ANOVA) and Duncan's multiple-range test, the data were reported as a percent of the control rate of photosynthesis.

Experimentation on Chloroplasts. Little Marvel pea (*Pisum sativum* L.) seedlings were the source of chloroplasts used in this experiment. Peas were germinated and grown for 10–12 days in vermiculite with a 12 : 12 hr light–dark cycle, at 20°/16°C and 250 $\mu E/m^2/sec$ (Berkowitz and Gibbs, 1982; Burton et al., 1987). Approximately 1 hr into the light cycle on the day of an experiment, 50–80 g of leaf and stem tissue were cut for chloroplast isolation. The tissue was homogenized in a Waring blender in 200 ml of partially frozen (slushlike) grind mix containing 50 μM HEPES–NaOH (pH 7.5), 330 mM sorbitol, 0.1% w/v bovine serum albumin (BSA), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM isoascorbate, and 1.3 mM glutathione. Blending was accomplished by three 3- to 5-sec bursts of the blender set at high speed.

The homogenate was filtered through six layers of cheesecloth between two layers of miracloth, and the filtrate divided into four 45-ml glass centrifuge tubes. It was centrifuged (Sorvall RC-5 HB-4 rotor) at 750*g* for 2 min at 4°C. The supernatant was discarded, and each pellet was gently resuspended in 15 ml of chilled grind mix using a soft camel's hair brush. This resuspended material was layered by pipetting onto 15 ml of grind mix containing 33% Percoll and then centrifuged at 2500*g* for 3 min at 4°C. The supernatant was aspirated and each pellet was gently resuspended by brush in 0.3–0.4 ml of the grind mix.

The chloroplast suspension was stored in the dark on ice until it was used within the next 2–3 hr. The amount of suspension to be used in a test was based on chlorophyll concentration of the suspension, which was determined according to the methods of Arnon (1949). Percent chloroplast intactness was estimated using ferricyanide as a Hill oxidant according to a procedure described by Lilley et al. (1975).

Aqueous phase CO_2-dependent oxygen evolution was measured polarographically with the Hansatech instrument at 25°C and approximately 1300 $\mu E/m^2/sec$. Oxygen evolution was assayed in 2 ml of a reaction medium containing 50 mM HEPES–NaOH (pH 7.5), 330 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 10 mM $NaHCO_3$, 5 mM Na pyrophosphate, 1.5 mM ATP, and a chloroplast suspension equivalent to 80–100 μg chlorophyll.

SGL was dissolved in ethanol for injection into the reaction medium. The amount of ethanol carrier solution never exceeded 20 μl, a concentration that had no effect on chloroplast function. SGL was injected 1–2 min after the optimal oxygen evolution rate was established. Percentage inhibition was calculated as the rate of O_2 evolution before injection of SGL minus the rate after injection, divided by the initial rate. I_{50} is defined as the concentration of SGL causing 50% inhibition of oxygen evolution. In the results reported, each data point is the mean of four or more trials from different chloroplast isolations.

RESULTS AND DISCUSSION

The floating leaf disk bioassay established that 50 and 100 μM SGL effectively blocked photosynthesis (Figure 1). SGL, 10 μM, appeared to slow photosynthetic activity based on this test. Use of the Hansatech system to measure oxygen evolution confirmed that all levels of SGL tested significantly inhibited photosynthesis in vacuum-infiltrated leaf disks (Table 1). This inhibition was not a stomatal effect, since the level of CO_2 in the Hansatech chamber precludes stomatal interference. Even 10 μM SGL reduced photosynthesis to less than 50% of the control rate of oxygen evolution. As reported previously, this is the threshold level for growth inhibition of several weed species (Einhellig and Souza, 1992).

The experiments conducted on effects of SGL on isolated chloroplasts also

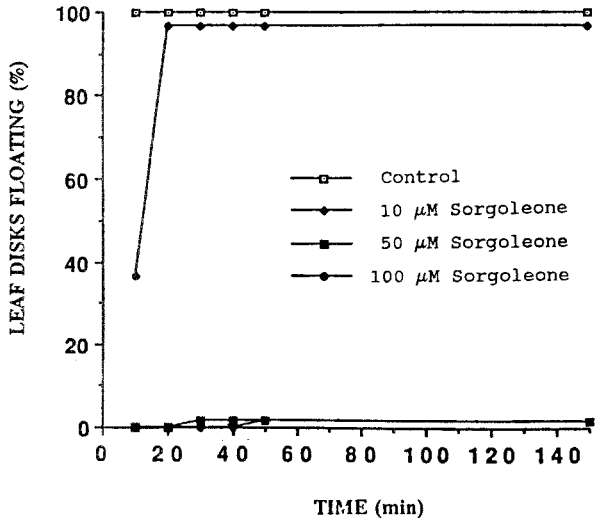

FIG. 1. Effects of sorgoleone on soybean leaf disk photosynthesis under 100 $\mu E/m^2/sec$ irradiance as indicated by the flotation time after vacuum infiltration.

showed that this allelochemical acts directly on the photosynthetic mechanism. Isolated pea chloroplasts used in this test typically averaged 125 μM O_2/mg chlorophyll/hr with an average intactness of 80%, values which are considered very good (Walker, 1980). SGL application between 0.1 and 1.0 μM reduced the chloroplast CO_2-dependent oxygen evolution with an I_{50} of approximately 0.2 μM SGL (Figure 2). Comparable tests with atrazine were done in our laboratory, since it is a powerful photosynthesis inhibitor in broadleaf plants. The concentration of atrazine required to achieve 50% inhibition of

TABLE 1. EFFECTS OF SORGOLEONE ON SOYBEAN LEAF DISK OXYGEN EVOLUTION

Sorgoleone treatment (μM)	Oxygen evolved (% of Control)[a]
0	100 a
10	44 b
50	11 bc
100	0 c

[a] Values in a column not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.

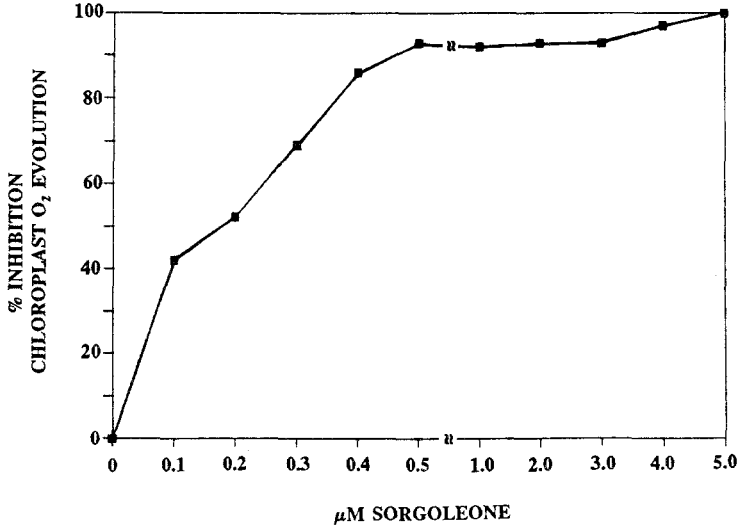

FIG. 2. Effects of sorgoleone on isolated pea chloroplast oxygen evolution, approximately 90 μg chlorophyll.

CO_2-dependent oxygen evolution of pea chloroplasts in these tests was the same as that found for SGL (data not shown).

The data of these experiments establish that SGL impairs photosynthesis and this disruption is a probable mechanism of action of this allelochemical. Since SGL also inhibits mitochondrial function (Rasmussen et al., 1992), its combined action on energy metabolism of photosynthesis and respiration must be considered a part of the explanation for the inhibitory effects this *Sorghum* root-exudate compound has on seedling growth.

REFERENCES

ARNON, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24:1–15.

BERKOWITZ, G.A., and GIBBS, M. 1982. Effect of osmotic stress on photosynthesis studies with the isolated spinach chloroplast. *Plant Physiol.* 70:1143–1148.

BURTON, J.D., GRONWALD, J.W., SOMERS, D.A., CONNELLY, J.A., GENGENBACH, B.G., and WYSE, D.L. 1987. Inhibition of plant acetyl-coenzyme A carboxylase by the herbicides Sethoxydim and Haloxyfop. *Biochem. Biophys. Res. Commun.* 148:1039–1044.

EINHELLIG, F.A., and RASMUSSEN, J.A. 1989. Prior cropping with grain sorghum inhibits weeds. *J. Chem. Ecol.* 15:951–960.

EINHELLIG, F.A., and SOUZA, I.F. 1992. Phototoxicity of sorgoleone found in grain sorghum root exudates. *J. Chem. Ecol.* 18:1–11.

FATE, G., CHANG, M., and LYNN, D.G. 1990. Control of germination in *Striga asiatica*: Chemistry of spatial definition. *Plant Physiol.* 93:201–207.

FORNEY, D.R., FOY, C.L., and WOLF, D.D. 1985. Weed suppression in no-till alfalfa (*Medicago sativa*) by prior cropping of summer-annual forage grasses. *Weed Sci.* 33:490–497.

HENSLEY, J.R. 1981. A method for identification of triazine resistant and susceptible biotypes of several weeds. *Weed Sci.* 29:70–73.

HESS, D.E., EJETA, G., and BUTLER, L.G. 1992. Selecting sorghum genotypes expressing a quantitative biosynthetic trait that confers resistance to *Striga*. *Phytochemistry* 31:493–497.

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

LILLEY, R.McC., FITZGERALD, M.P., REINITS, K.G., and WALKER, D.A. 1975. Criteria of intactness and the photosynthetic activity of spinach chloroplast preparations. *New Phytol.* 75:1–10.

NETZLY, D.H., and BUTLER, L.G. 1986. Roots of *Sorghum* exude hydrophobic droplets containing biologically active components. *Crop Sci.* 26:775–778.

NETZLY, D.H., RIOPEL, J.L., EJETA, G., and BUTLER, L.G. 1988. Germination stimulants of witchweed (*Striga asiatica*) from hydrophobic root exudate of sorghum (*Sorghum bicolor*). *Weed Sci.* 36:441–446.

OVERLAND, L. 1966. The role of allelopathic substances in "smother crop" barley. *Am. J. Bot.* 53:423–432.

PANASIUK, O., BILLS, D.D., and LEATHER, G.R. 1986. Allelopathic influence of *Sorghum bicolor* on weeds during germination and early development of seedlings. *J. Chem. Ecol.* 12:1533–1543.

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

PUTNAM, A.R., DEFRANK, J., and BARNES, J.P. 1983. Exploitation of allelopathy for weed control in annual and perennial cropping systems. *J. Chem. Ecol.* 8:1001–1010.

RASMUSSEN, J.A., HEJL, A.M., EINHELLIG, F.A., and THOMAS, J.A. 1992. Sorgoleone from root exudate inhibits mitochondrial functions. *J. Chem. Ecol.* 18:197–207.

SCHUTT, C., and NETZLY, D. 1991. Effect of apiforol and apigeninidin on growth of selected fungi. *J. Chem. Ecol.* 17:2261–2266.

WALKER, D.A. 1980. Preparation of higher plant chloroplasts. *Methods Enzymol.* 69:94–104.

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BIOLOGICAL ACTIVITY OF THE SHRUB *Boscia senegalensis* (PERS.) LAM. EX POIR. (CAPPARACEAE) ON STORED GRAIN INSECTS

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Abstract—Biological activity of leaves, fruits and extract of the African shrub *Boscia senegalensis* (PERS.) LAM. ex Poir. was evaluated against five stored-grain insects. When added to cowpeas at 2–4% (w/w), fresh ground fruits and leaves caused 80–100% mortality in *Callosobruchus maculatus* (F.) adults and significantly reduced both emergence and damage of the F_1 progeny. Acetone fruit extract exhibited a potent fumigant effect on *Prostephanus truncatus* HORN., *C. maculatus*, and *Sitotroga cerealella* OLIV.; with LT_{50} values of 3.8, 2.3, and below 1.5 hr, respectively. LC_{50} determination for *B. senegalensis* fruits and leaves as well as pure methylisothiocyanate (MITC) on *Tribolium castaneum* HERBST, *Sitophilus zeamais* MOTSCH. and *C. maculatus* showed a differential response of the insects to plant parts or MITC. Quantitative dosage of *Boscia* active components and LC_{50} values obtained for the plant tissues, compared to those of pure molecules, indicate that the biological activity of *B. senegalensis* is due to the liberation of MITC from a glucosinolate precursor glucocapparin contained in *Boscia* fruits and leaves.

Key Words—*Boscia senegalensis* (PERS.) LAM. ex Poir., glucosinolates, methylisothiocyanate, *Vigna unguiculata* (L.) WALP., *Callosobruchus maculatus* (F.), *Prostephanus truncatus* HORN., *Sitophilus zeamais* (MOTSCH.), *Sitotroga cerealella* (OLIV.), *Tribolium castaneum* HERBST coleoptera.

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INTRODUCTION

Insect infestation of stored grain causes weight and quality losses that lead to a reduction of commercial value and seed germination. To reduce this damage, several control measures have been taken, which mostly involve chemical methods. Synthetic insecticides are not only a drain on the farmer's meager resources but are often frequently used beyond permissible safe limits. The incidence of insecticide resistance is also a growing problem. Resistance to one or more insecticide(s) has been reported in at least 500 species of insects and mites (Georghiou, 1990). This situation has increased the need for alternatives to synthetic pesticides. Botanical insecticides are a less expensive and biodegradable option. The effectiveness of raw parts and plant extracts against stored-grain insects has been described by many authors (Jotwani and Sircar, 1965; Singh et al., 1978; Jacobson, 1983; Golob and Webley, 1980; Grainge et al., 1985; Lognay et al., 1991; Seck et al., 1991; Haubruge et al., 1989).

We investigated the biological activity of *Boscia senegalensis* (PERS.) LAM. (Capparaceae), a plant that is found throughout north-central and northern Senegal (Bille and Poupon, 1972), where it is traditionally used by farmers against stored grain insects. In addition, we isolated and identified biologically active components of this plant.

METHODS AND MATERIALS

Plant material was randomly harvested in the region of Thies (Senegal). Our material has been identified and deposited at the Jardin Botanique National de Belgique (BR) as *B. senegalensis*. Leaves used for trial 1 were harvested in November 1990. Fruits and leaves used for trials 2 and 3 were collected in May 1991.

Four experiments were conducted with plant parts (trials 1 and 2), extracts (trial 3), and pure molecules (trial 4) using five stored-grain insect species. The test insects, *Callosobruchus maculatus* (F.) (Col., Bruchidae), *Sitotroga cerealella* OLIV. (Lep., Gelechiidae), *Prostephanus truncatus* HORN. (Col. Bostrychidae), *Tribolium castaneum* HERBST. (Col., Tenebrionidae), *Sitophilus zeamais* MOTSCH. (Col., Curculionidae), were reared under controlled conditions (32° $\pm$ 2°C and 80 $\pm$ 10% relative humidity). Bioassays were also carried out under these conditions. All chemicals and solvents were of analytical grade.

Trials 1 and 2. These first two trials were performed with fresh ground leaves (FGL), fresh entire leaves (FEL), dry leaf powder (DLP), and fresh ground fruits (FGF). The test materials were added to cowpea seeds, *Vigna unguiculata* (L) WALP (var. Black Eyed) at 1–32% for FGL, FEL, and DLP in trial 1; and 0.5–2% for FGF and FGL in trial 2. FGL and FGF were prepared

using a small laboratory mill (Moulinex) for 5 min. DLP was similarly ground and then passed through a 500-mesh laboratory sieve. Following this procedure, 20 g of cowpeas were thoroughly mixed with plant material at the desired concentrations in Petri dishes (ϕ 90 mm) and infested with 10 unsexed 1-day-old *C. maculatus* adults. Untreated cowpea seeds were similarly infested with 10 insects. Each treatment, including the control, was replicated five times. Mortality assessment was made after one to two days. Twenty-two days later when F_1 adults started to emerge, insects were removed daily and counted for 10 days. Numbers of holed and unholed seeds were also counted and percent damage was calculated.

Data were subjected to analysis of variance followed by a comparison of the means (Duncan's multiple-range test) at $P = 0.05$.

Trial 3. Fifty grams of *Boscia* fruits were ground with 200 ml acetone in a Waring blender and kept for 2 hr at room temperature. The supernatants were then filtered and concentrated under vacuum to a final volume of 50 ml to obtain a *B. senegalensis* acetone fruit extract (BAFE) containing approximately 30% by volume of water.

Bioassays were carried out in 825-ml sealed glass desiccators in which 5 ml of BAFE were deposited. Five milliliters of acetone were similarly pipetted in another desiccator used as control. After 3 hr at room temperature, the solvent evaporated completely in the control. One hundred adults of different species were then introduced in the desiccators, which were closed and placed under experimental conditions. After various time exposures, from 1.5 to 12 hr, mortality readings were recorded for each treatment. The time required to kill 50% (LT_{50}) of the insects was then determined by transforming mortality data to probits and calculating the lethal times (LT) (Snedecor and Cochran, 1967).

Analysis of volatiles from Boscia senegalensis *leaves.* Freshly ground leaves (100 g) were steam-distilled for 45 min and the aqueous distillate (900 ml) extracted three times with 100 ml diethylether. The ether solution was dehydrated with anhydrous sodium sulfate, concentrated to 4 ml by distillation of the solvent at 38°C, and finally analyzed by gas–liquid chromatography (GLC) using two types of columns under the following conditions: The polar column was a CP-Wax 52CB (50 m long, 0.32 mm ID, 0.2 μm film thickness) from Chrompack; carrier gas was helium at 100 kPa; temperature program: from 30 to 240°C at 10°C/min; cold "on-column" injector and FID detector maintained at 250°C; apparatus: Hewlett Packard HP 5880. The apolar column was a CP-Sil 8 CB (25 m long; 0.32 mm ID, 0.2 μm film thickness) from Chrompack; carrier gas was helium at 50 kPa; temperature program: from 30 to 240°C at 5°C/min; cold "on-column" injector and FID detector maintained at 300°C; apparatus: Carlo Erba Mega 5160.

Analysis of glucosinolates. The glucosinolates were analyzed in *B. senegalensis* FGF, FGL, and BAFE by reverse-phase HPLC after enzymatic desul-

fation according to the official EEC method (European Economic Community, 1990). Identification of BAFE glucosinolates was performed by GC-MS analyses of trimethylsilylated molecules and by examination of their degradation products liberated enzymatically under controlled conditions. For GC-MS investigations, glucosinolates were enzymatically transformed to desulfoglucosinolates and trimethyl-silylated for 20 min at 110°C with 50 μl of a reagent containing *N*-methyl-*N*-trimethylsilyltrifluorobutyramide, 5% methylimidazole in acetone and trimethylchlorosilane 30:15:3 (v/v/v). The chromatographic conditions were as follows: Column SE-52 (25 m long, 0.35 mm ID, 0.2 μm film thickness) was from Macherly-Nagel; carrier: helium at 50 kPa; temperature program: 50–280°C at 20°C/min; cold "on-column" injection. The mass spectra were recorded in the EI mode on a Nermag R10-10C spectrometer (70 eV, source at 130°C, interface at 280°C, mass range scanned from 100 to 800 amu) coupled to a Delsi DI-700 gas chromatograph.

For enzymatic degradation of *B. senegalensis* glucosinolates, 0.1 ml acetate buffer (pH 4.5) and 50 μl of 10 mg/ml buffered solution of thioglucosidase (EC 3.2.3.1), purified from *Sinapis alba* L. according to Appelqvist and Josefson (1967), were added to 0.1 ml of residual aqueous phase from BAFE or to a solution of pure methylglucosinolate (glucocapparin, Roth ref. 7485). After 24 hr, the degradation products were extracted with 2 ml diethylether and analyzed by GLC on polar and apolar stationary phases. Methylisothiocyanate (MITC) from BAFE and glucocapparin were identified by comparison of their retention times with that of a pure reference (Sigma ref. M8632).

Detection of MITC in Volatiles Liberated during Biotests. Acetone solutions of BAFE (5 ml) were introduced into conical flasks. After evaporation of acetone, the vessels were hermetically closed and left for 24 hr at 32 ± 2°C. Headspace sampling was performed by purging the apparatus with nitrogen for 30 min. The volatiles were collected in 3 ml diethylether maintained in a cryogenic trap at −20°C. Ether solutions were submitted to GC-MS in the aforementioned conditions except that the temperature program rate was fixed at 5°C/min. MITC was identified on the basis of retention and mass spectral data. The mass spectra were compared with EPA-NIH and Wiley libraries.

Trial 4. To study the dose–mortality responses to *B. senegalensis* tissues and MITC, various amounts of FGF and FGL from 0 to 8 g/liter (w/v) and pure MITC from 0 to 3 mg/liter (w/v) were deposited in 750-ml glass desiccators containing 25 adults of each insect species, in four replications. After 24 hr under the experimental climatic conditions, insects were transferred to clean Petri dishes and maintained in controlled conditions until the next day for mortality readings as indicated by Busvine (1981). Data were subjected to probit analysis (Finney, 1964). Log dose–probit line was analyzed for goodness of fit by the chi-square test (Busvine, 1981), followed by computation of LC_{50} values for each material.

RESULTS

Trial 1

Mortality. FGL at a concentration of 4% (w/w) caused 100% mortality after 24 hr. At 2%, 55.6% of the insects died within 48 hr, and at 1%, mortality ranged from 7.4 to 11.1% in one to two days. FEL scored 0–18.5% mortality and DLP 3.7–40.7% within 24–48 hr (Table 1).

Progeny. FGL completely inhibited the production of *C. maculatus* progeny at 2%, but at 1%, 21.6 adults emerged. At the same time, progeny ranged from 36.2 to 87.2 adults for FEL and from 40.2 to 53.2 for DLP (Table 1).

TABLE 1. BIOLOGICAL ACTIVITY OF *B. senegalensis* FRESH GROUND LEAVES (FGL), FRESH ENTIRE LEAVES (FEL) AND DRY LEAF POWDER (DLP) TO *C. maculatus* ADULTS, F_1 PROGENY, AND DAMAGE (MEANS)[a]

Treatment	Conc (%, w/w)	Corrected mortality (%)[b] 24 hr	Corrected mortality (%)[b] 48 hr	F_1 progeny	Damage (%)
FGL	1	7.4c	11.1c	21.6ab	22.0b
	2	63.0b	55.6b	0.0b	0.0b
	4	100.0a	100a	0.0b	0.0b
	8	100.0a	100a	0.0b	0.0b
	16	100.0a	100a	0.0b	0.0b
	32	100.0a	100a	0.0b	0.0b
	Control			61.6a	70.3a
FEL	1	7.4a	18.5a	36.2b	49.1a
	2	0.0a	7.4ab	43.6ab	36.9a
	4	0.0a	7.4ab	69.0ab	59.8a
	8	0.0a	0.0b	87.2a	64.8a
	16	0.0a	0.0b	62.4ab	52.9a
	32	0.0a	0.0b	71.8ab	60.5a
	Control			61.6ab	56.3a
DLP	1	11.1a	37.0a	53.2a	67.5a
	2	3.7a	18.5a	47.6a	44.4abc
	4	11.1a	25.9a	51.0a	52.4abc
	8	11.1a	33.3a	49.8a	33.6bc
	16	14.8a	33.3a	40.2a	39.7abc
	32	11.1a	40.7a	45.8a	30.8c
	Control			45.0a	65.1ab

[a] Means followed by the same letter within a column of each treatment are not significantly different at the 5% level (Duncan's multiple-range test).
[b] By Abbott's (1925) formula.

FGL significantly reduced *C. maculatus* progeny, compared to FEL and DLP (Figure 1).

Damage. FGL gave 100% protection at 2% and 22% damage at 1%. In the same conditions, damage was 36.9–64.8% for FEL and 30.8–67.5% for DLP (Table 1).

Trial 2

Mortality. After 72 hr at concentration of 2%, mortality was 93.6% for FGF and 24.8% for FGL. After 48 hr, it ranged from 3.8 to 79.8% for FGF compared to 0–24.2% for FGL. After 24 hr, mortality ranged from 3.8 to 73.8% for FGF and from 0 to 8% for FGL (Table 2).

Progeny. At 2%, FGF showed no adult emergence while 7.8 and 133.8 adults emerged, respectively, in FGL and the control treatment. At 1%, 12 adults emerged from FGF compared to 104 for FGL (Table 3).

Damage. At 1% concentration, damage was 8.7% for FGF and 63% for FGL. At 2% concentration, FGF gave 100% protection, while 6.1 damage was noted in the FGL treatment (Table 3).

Trial 3

LT_{50} values were 2.3 hr for *C. maculatus* and 3.8 hr for *P. truncatus* (Table 4). For *S. cerealella* all adults died within 1.5 hr.

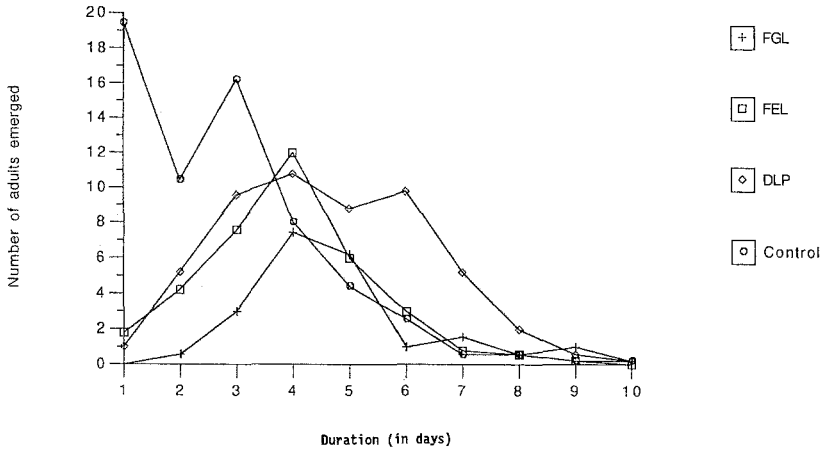

FIG. 1. Emergence pattern of *C. maculatus* from cowpea seeds treated with fresh ground leaves (FGL), fresh entire leaves (FEL), or dry leaf powder (DLP) of *Boscia senegalensis* at 1% conc. (w/w).

TABLE 2. COMPARATIVE TOXICITY OF *B. senegalensis* FRESH GROUND FRUITS (FGF) AND LEAVES (FGL) TO *C. maculatus* (MEANS)[a]

Treatment	Conc (%, w/w)	Corrected mortality after (%)[b]		
		24 hr	48 hr	72 hr
FGF	0.5	3.8b	3.8c	1.9c
	1	6.4b	24.9b	35.9b
	2	73.8a	79.8a	93.6a
FGL	0.5	0.0a	0.0b	23.4a
	1	3.8a	5.8ab	13.0a
	2	8.0a	24.2a	24.8a

[a]Means followed by the same letter within a column of each treatment are not significantly different at the 5% level (Duncan's multiple-range test).
[b]By Abbott's (1925) formula.

TABLE 3. EFFECT OF COWPEA TREATMENT WITH *B. senegalensis* FRESH GROUND FRUITS (FGF) OR LEAVES (FGL) ON *C. maculatus* EMERGENCE AND DAMAGE IN COWPEAS (MEANS)[a]

Treatment	Conc. (%, w/w)	Number of emerged adults[b]	Percentage Damage (%)	Reduction (%)	
				Emergence	Damage
FGF	0.5	3.4a	22.7a	68.2a	73.0a
	1	1.6b	8.7a	90.7a	89.6a
	2	0.0c	0.0a	100a	100a
FGL	0.5	4.2a	46.0a	41.7b	45.2b
	1	4.6a	63.0a	22.3b	25.0b
	2	1.8b	6.1b	94.5a	92.7a
Control		4.9	84.0		

[a]Means followed by the same letter within a column of each treatment are not significantly different at the 5% level (Duncan's multiple-range test).
[b]Log (numbers).

Trial 4

Acute Toxicity of B. senegalensis *Fresh Ground Tissues and Pure MITC.* FGF, FGL, and MITC exhibited a differential acute toxicity against three stored-grain beetle species. LC_{50} values (in grams ground matter per liter volume) ranged from 1 to 4.23 for FGL and from 0.42 to 1.75 for FGF (Table 5). LC_{50}

TABLE 4. LETHAL TIME (LT) VALUES OF TWO STORED-GRAIN INSECTS EXPOSED TO VAPORS OF *B. senegalensis* FRUIT EXTRACT (BAFE)

Species[a]	Slope ± SE	LT_{50} (95% FL[b]), hr
C. maculatus	4.47 ± 1.71	2.31 (1.01–5.28)
P. truncatus	4.21 ± 1.16	3.80 (1.92–7.54)

[a] One-day-old *C. maculatus*; 2-week-old *P. truncatus*.
[b] Fiducial limits.

TABLE 5. ACUTE TOXICITY OF *B. senegalensis* FRESH GROUND FRUITS (FGF) AND LEAVES (FGL) TO ADULTS OF THREE STORED-GRAIN INSECT SPECIES

	FGF		FGL	
Species[a]	Slope ± SE	LC_{50} (95% FL)[b]	Slope ± SE	LC_{50} (95% FL)[b]
Tribolium castaneum	8.60 ± 1.00	1.75 (1.63–1.86)	6.14 ± 1.38	4.23 (1.11–16.15)
Sitophilus zeamais	7.12 ± 0.79	0.87 (0.80–0.94)		
Callosobruchus maculatus	4.91 ± 0.75	0.42 (0.36–0.47)	6.15 ± 0.93	1.00 (0.88–1.09)

[a] Four replications of 20 insects were exposed to plant materials for 24 hr before they were transfered to Petri dishes and placed in controlled conditions (30°C, 70% relative humidity). Mortality was counted after 24 hr of pest exposure (Busvine, 1981).
[b] Grams per liter volume, with Fiducial limits.

for MITC ranged from 0.73 to 2.38 ppm according to insect species, with *C. maculatus* being the most susceptible (Table 6).

Chemical Investigations

GLC analysis of the volatiles from FGL showed one major peak (92% of the total area) at retention times of 9.3 min and 6.5 min, respectively, on polar and apolar columns, which corresponded to those of an authentic sample of MITC. The detection of MITC from *B. senegalensis* leaves (Figure 2) suggested that methylglucosinolate (glucocapparin) may be the main precursor of the insecticidal compound. Glucosinolate enzymatic degradation leads to several by-products among which isothiocyanates predominate (Tookey et al., 1980). To test the aforementioned hypothesis, we analyzed the residual water phase of BAFE following a three-step procedure (HPLC, identification of glucosinolate by-product, and GC-MS). The HPLC retention time of pure desulfomethylglucosinolate (2 min) corresponded to that of the major peak detected in BAFE. On the other hand, the elution profiles liberated enzymatically from BAFE

TABLE 6. ACUTE TOXICITY OF METHYL ISOTHIOCYANATE TO ADULTS OF THREE STORED-GRAIN INSECT SPECIES

Species[a]	Slope ± SE	LC_{50} (95% FL)[b]
Tribolium castaneum	7.05 ± 0.58	2.38 (2.24–2.52)
Sitophilus zeamais	7.22 ± 1.18	1.25 (1.14–1.34)
Callosobruchus maculatus	4.81 ± 0.59	0.73 (0.64–0.82)

[a] Four replications of 20 insects were exposed to plant materials for 24 hr before they were transfered to petri dishes and placed in controlled conditions (30°C, 70% relative humidity). Mortality was counted after 24 hr of pest exposure (Busvine, 1981).
[b] ppm, with Fiducial limits.

residual aqueous phase and methylglucosinolate are practically identical; MITC is distinguishable on the two chromatograms but is absent on the blank. The identification of glucocapparin in BAFE was finally validated by GC-MS. A typical mass spectra is shown in Figure 3. The mass fragments at m/e = 103, 117, 147, 169, 204, 243, 271, 361 (base peak), and 451 were generated by the glucidic moiety of the molecule and were not of interest for the identification of the aglycone. Nevertheless, the ions recorded at m/e = 613 $(M)^+$, 598 $(M\text{-}CH_3)^+$, 524 $(M\text{-}CH_3\text{-}TMS)^+$ and 508 $(M\text{-}CH_3TMSOH)^+$ indicated clearly a glucosinolate bearing a methyl radical. As for all other alkylglucosinolates, the intensities of these characteristic mass fragments were low. The methylglucosinolate content of *B. senegalensis* material tested in trial 4 represented 23.6 ± 0.8 μmol/g fresh leaves and 38 ± 1.2 μmol/g fresh fruits (HPLC determination with sinigrin as internal standard). Headspace sampling conducted under the same conditions as trial 3 and trapping the volatiles in diethyl ether at −20°C led to detection of MITC from the vapor phase. The total ion current (Figure 4) showed several peaks, among which was MITC (R_t = 16.6 min). The molecule was unambiguously identified by comparison of its mass spectrometric pattern with EPA-NIH and Wiley libraries and also on the basis of GLC retention time.

DISCUSSION

This research has demonstrated a significant biological effect of *B. senegalensis* plant parts and extracts. The evidence in support of these results was obtained from four experiments. First, *B. senegalensis* FGL (when added to cowpeas at 4% w/w) completely killed *C. maculatus* adults within 24 hr, inhibited the production of F_1, progeny and prevented bruchid damage. Under the same conditions, FEL and DLP had almost no effect. Second, comparative

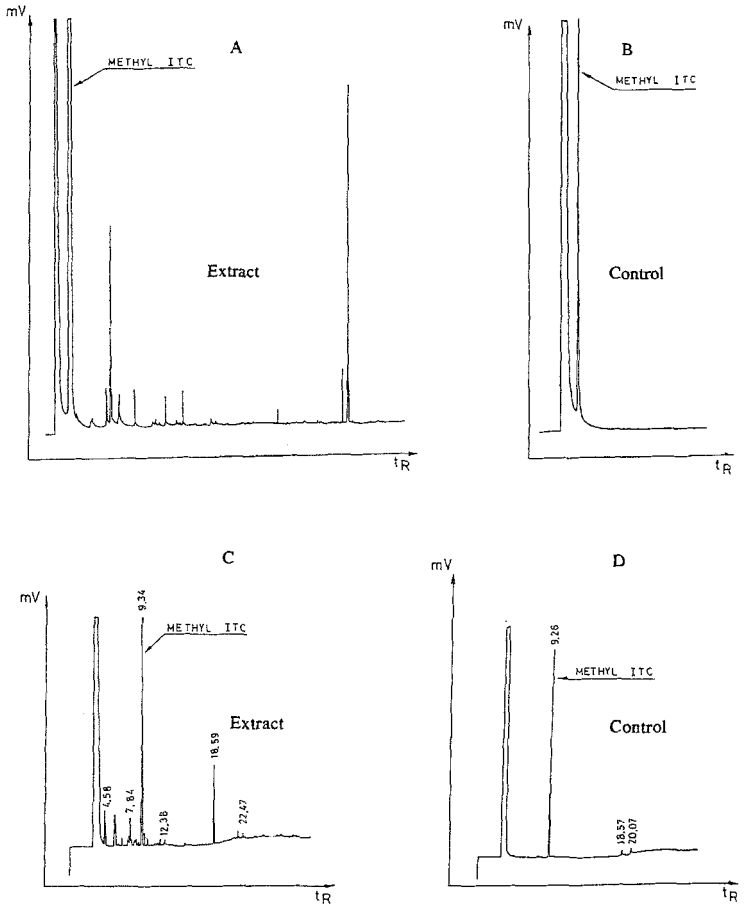

FIG. 2. Identification of methylisothiocyanate (MITC) from *Boscia senegalensis* leaves with apolar column (A and B) and polar column (C and D).

evaluation of FGF and FGL revealed that fruits were more toxic to *C. maculatus* and reduced both progeny and damage to a greater extent, than did leaves. Third, BAFE exhibited a high fumigant effect on three stored-grain insect species, which had a differential time-mortality response. Finally, we quantified the acute toxicity of *B. senegalensis* fresh ground fruits and leaves as well as pure MITC and obtained dose-mortality responses for three stored-grain beetles.

B. senegalensis is a shrub, growing up to 3 m high, that is frequently found on abandoned termite mounds and on barren and fire-scorched soil of the Sahel. It is distributed from Mauritania to Niger, northern Nigeria, the northwest Cameroons, and across Africa to Sudan and Ethiopia (Booth and Wickens, 1988).

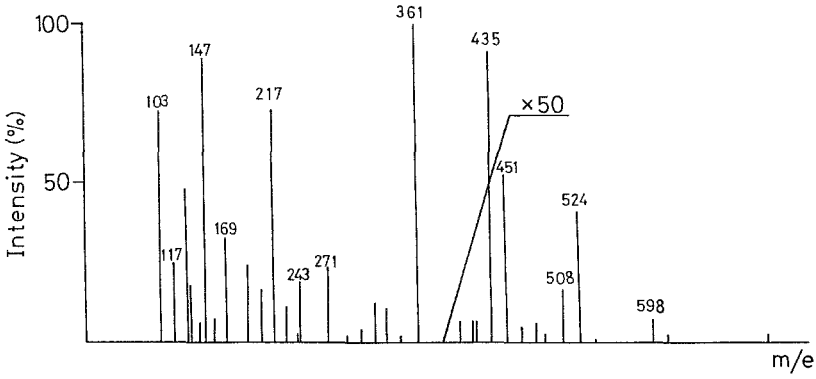

FIG. 3. Mass spectrum of silylated desulfoglucocapparin from *Boscia senegalensis* fruit extract.

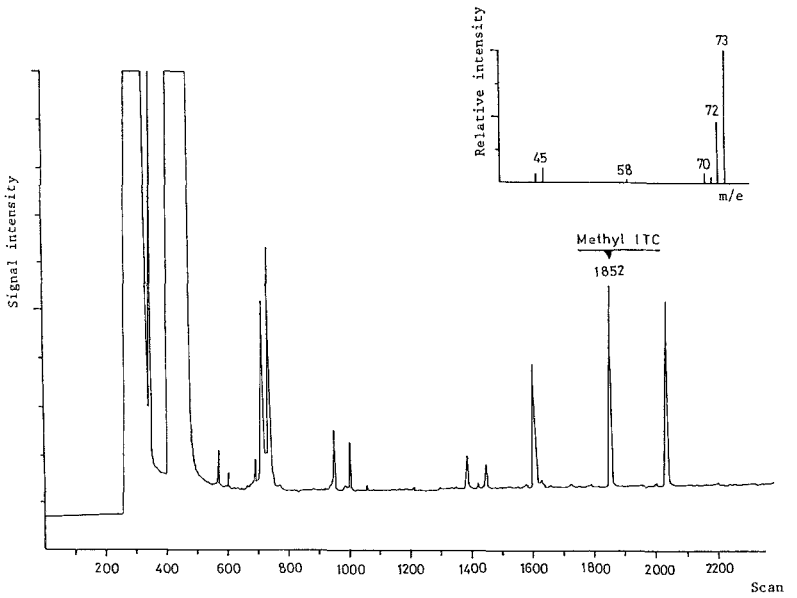

FIG. 4. GC-MS total ion current of the volatiles liberated during the bioassays of *Boscia senegalensis* fruit extract.

Leaves and fruits are used as human food and animal fodder (Bernus, 1979; Baumer, 1981; Maydell, 1983; Burkill, 1985; Becker, 1983). The leaves, bark, and roots are widely used in northern Senegal for their medicinal properties (Kerharo and Adam, 1974; Booth and Wickens, 1988).

Alzouma and Boubacar (1985) reported on the toxicity of *B. senegalensis* leaves from Niger, which also reduced *Bruchidius atrolineatus* Pic. and *C. maculatus* oviposition, but they gave no details about the active components. Kjaer et al. (1973) reported that *B. senegalensis* leafless twigs contained methyl and isopropyl glucosinolates.

Our bioassays, performed by comparing *Boscia* tissues and MITC, indicate that *B. senegalensis* fruits, leaves, and MITC were toxic to insects at various levels, according to the species and the plant tissue. They also indicate, considering LC_{50} values for FGF, FGL, and pure MITC on the one hand, and the amounts of glucocapparin found in the plant tissues on the other hand, that *Boscia* fruits and leaves contained sufficient glucocapparin to liberate MITC at levels comparable to the LC_{50} of the pure molecule.

These results indicate that in addition to its medicinal properties (Dalziel, 1948) and utilization as a famine food (Becker, 1986; Salih et al., 1991), *B. senegalensis* also has potential in stored-grain protection due to a potent fumigant effect on different insect species. *B. senegalensis* has been traditionally used by African farmers as a grain protectant, but the basis of its effectiveness has never been explained. We have shown that *B. senegalensis* biological activity is linked to the liberation of methyl isothiocyanate from a glucosinolate precursor, glucocapparin, contained in its fruits and leaves. As the plant grows spontaneously in some of the poorest areas of the world (mainly in the arid sahelian regions), this research suggests a natural insecticide from *B. senegalensis* as an alternative to synthetic pesticides in developing countries.

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REFERENCES

ABBOTT, W.S. 1925. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18:265–267.

ALZOUMA, I., and BOUBACAR, A. 1985. Effet des feuilles vertes de *Boscia senegalensis* (Capparidaceae) sur certains aspects de la biologie de *Bruchidius atrolineatus* et de *Callosobruchus maculatus*. Comm. Colloque sur les Légumineuses Alimentaires. Niamey, Niger.

ANONYMOUS, 1990. Règlement CEE No. 1864/90. Graines oléagineuses—détermination des glucosinolates par chromatographie liquide à haute performance. *J. Off. Commun. Eur.* L170:27.

APPELQVIST, L.A., and JOSEFSON, E. 1967. Method for the quantitative determination of isothiocyanates and oxazolidinethiones in digests of seed meals of rape and turnip rape. *J. Sci. Food Agric.* 18:510.

BAUMER, M. 1981. Rôle de *Boscia senegalensis* (PERS.) LAM. dans l'économie rurale africaine: Sa consommation par le bétail. *Rev. Elev. Med. Vet. Pays Trop.* 34(3):325–328.

BECKER, B. 1983. The contribution of wild plants to human nutrition in the Ferlo (northern Senegal). *Agrofor. Syst.* 1:257–267.

BECKER, B. 1986. Wild plants for human nutrition in the Sahelian zone. *J. Arid Environ.* 11:61–64.

BERNUS, E. 1979. L'arbre et le nomade. *J. Agric. Trad. Bot. Appl.* 26(2):103–128.

BILLE, J.C., and POUPON, H. 1972. Recherches écologiques sur une savane sahélienne du Ferlo septentrional, Sénégal: Description de la végétation. *Terre Vie* 26:351–365.

BOOTH, F.E.M., and WICKENS, G.E. 1988. Non-timber uses of selected arid zone trees and shrubs in Africa. F.A.O.-Conservation-Guide No. 19, 176 pp.

BURKILL, H.M. 1985. The useful plants of west tropical Africa. Vol. 1, Families A–D. Kew, Royal Botanic Gardens, London.

BUSVINE, J.R. 1981. Methodes recommandées pour la mesure de la résistance des ravageurs aux pesticides. Etude FAO: Production Végétale et Protection des Plantes No. 21.

DALZIEL, J.M. 1948. The Useful Plants of West Tropical Africa. The Crown Agents for the Colonies, London, 612 pp.

FINNEY, M.A. 1964. Probit Analysis. Cambridge University Press, Cambridge.

GEORGHIOU, P.G. 1990. Overview of insecticide resistance. pp. 19–41 *in* M.B. Green, H.M. Lebaron, and W.K. Moberg (eds.). ACS Symposium Series 421. American Chemical Society, Washington, D.C.

GOLOB, P., and WEBLEY, D.J. 1980. The use of plants and minerals as traditional protectants of stored product. *Reg. Trop. Prod. Inst.* 138:32.

GRAINGE, M., AHMED, S., MITCHEL, W.C., and HYLIN, J.W. 1985. Plant species reportedly possessing pest-control properties—an EWC/UH database. Resource Systems Institute, East-West Center, Honolulu, Hawaii, 249 pp.

HAUBRUGE, E., LOGNAY, G., MARLIER, M., DANHIER, P., GILSON J.-C., and GASPAR, C. 1989. Etude de la toxicité de cinq huiles essentielles extraites de *Citrus* ps. à l'égard de *Sitophilus zeamais* MOTSCH. (Col., Curculionidae), *Prostephanus truncatus* (HORN) (Col., Bostrychidae) et *Tribolium castaneum* HERBST. (Col Tenebrionidae). *Med. Fac. Landbouww, Rijksuniv. Gent* 54(3b):1083–1093.

JACOBSON, M. 1983. Control of stored product insects with phytochemicals. 3rd. International Working Conference on Stored Product Entomology. October 23–28. Manhattan, Kansas.

JOTWANI, M.G., and SIRCAR, P. 1965. Neem seed as a protectant against bruchid *Callosobruchus maculatus* (Fabricius) infesting some leguminous seeds. *Indian J. Entomol.* 29(1):21–24.

KERHARO, J., and ADAM, J.G. 1974. La pharmocopée sénégalaise traditionnelle. Plantes médicinales et toxiques. Vigot Frère Ed., Paris.

KJAER, A., SCHUTTER, A., DELAVEAU, P., and KOUDOGBO, B. 1973. Glucosinolates in *Boscia senegalensis. Phytochemistry* 12:725.

LOGNAY, G., MARLIER, M., SEVERIN, M., HAUBRUGE, E., GIBON V., and TREVEJO, E. 1991. On the characterization of some terpenes from *Renealmia alpinia* ROTT. (Maas) oleoresin. *Flavour Fragrance J.* 6:87–91.

MAYDELL, H.J. 1983. Arbres et arbustes du Sahel. Leurs caractéristiques et leurs utilisations. Eschborn, Germany, *Deutsche Gesellschaft für Technische zusammenarbeit* (G.T.Z.).

SALIH, O.M., NOUR, A.M., and HARPER, D.B. 1991. Chemical and nutritional composition of two famine food sources used in Sudan, mukheit (*Boscia senegalensis*) and maikah (*Dobera roxburghi*). *J. Sci. Food. Agric.* 57:367–377.

SECK, D., SIDIBE, B., HAUBRUGE, E., and GASPAR, C. 1991. La protection des stocks de niébé [*Vigna unguiculata* (L) WALP] en milieu rural: Utilisation de différentes formulations à base de neem (*Azadirachta indica*) A. JUSS provenant du Sénégal. *Med. Fac. Landbouww. Rijksuniv. Gent* 56(3b):1217–1224.

SINGH, S.R., LUSE, R.A., LENSCHNER, K., and NANGJU, D. 1978. Groundnut oil treatment for the control of *Callosobruchus maculatus* F. during cowpeas storage. *J. Stored Prod.* 14:77–80.

SNEDECOR, G.W., and COCHRAN, W.G. 1967. Statistical Methods, 6th ed. Iowa State University Press, Ames, 593 pp.

TOOKEY, H.L., VAN ETTEN, C.H., and DAXENBICHLER, M.E. 1980. Glucosinolates, p. 103, *in* Liener, I.E. (ed.). Toxic Constituents of Plant Foodstuffs, 2nd ed. Academic Press, New York.

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ANNOUNCEMENT

BIOLOGY OF COMMUNICATION AWARD

The Jean-Marie Delwart Foundation will support a fundamental original research project in the field of Chemical Communication by awarding for the first time a Prize to a study dealing with Primates and with Man in particular.

This Prize, $10,000 in amount, will be awarded in 1993 to a study or a series of studies, individual or collective, already published (or accepted for publication by the 14th of August, 1992), corresponding to the objectives mentioned in the previous paragraph. The text (in English or French) should be sent to the following address by the first of July, 1993 latest:

Fondation Jean-Marie Delwart
Château de Pellenberg
B-3212 Pellenberg
Belgique

The jury is composed of the members of the scientific committee of the Jean-Marie Delwart Foundation.

The prize will be awarded on the 15th December 1993 under the patronage of the Académie Royale de Belgique.

The experimental study of chemical communication in animals and especially in invertebrates constitutes the most important development of ethology in the last few years, partially in link with neuro-ethology.

This field is broadening presently on the side of vertebrates and particularly in mammals. This kind of work develops at three levels, viz. behavior patterns down to the species, chemical substances involved in pheromones and ecological conditions in which these phenomena occur.

The next development concerns chemical communication in man and in primates in general. Research in this field includes ethological as well as ecological phenomena and to analysis and synthesis of the substances involved.

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ANNOUNCEMENT

The 10th annual meeting of the International Society of Chemical Ecology will take place August 1–4, 1993 at the Sheraton Sand Key Resort on Clearwater Beach, Florida. The meeting site is 25 miles by car from Tampa International Airport. Taxi and limo service are readily available. The Sheraton is on the Gulf of Mexico with one of the nicest beaches in the U.S.A. All scientific events will be in the hotel conference rooms. Special group rates of $76 per room (single or double) have been arranged. A double room contains two good size beds. There is no charge for up to two children under 18. Consider bringing your families. This rate will be extended three days prior to and three days following the conference. Food is available at the hotel or at a variety of local restaurants. Reassurance–A major hurricane has not hit Tampa Bay in over 50 years. Peak hurricane season for the Gulf Coast of Florida is September.

A Symposium Committee consisting of John Romeo, William Fenical, Jean Langenheim, Lincoln Brower, Wittko Francke, and David Carlson is finalizing the program. The following is a tentative schedule:

Saturday, July 31	Registration and Welcome Reception (PM)
Sunday, August 1	Symposium–Marine Chemical Ecology (AM)
	Contributed Papers–General Subjects (PM)
	Poster Session–General Subjects (Evening)
Monday, August 2	Symposium–Chemical Ecology of Terpenoids (AM)
	Contributed Papers–General Subjects (PM)
Tuesday, August 3	Symposium–Physical Stress and Chemical Ecology (AM)
	Mini Symposium & Contributed Papers–Insect Biochemistry (PM)
	Banquet (Evening)
Wednesday, August 4	Symposium–Tropical Chemical Ecology (AM)

A post conference trip to Costa Rica has been organized. Members should

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have already received details of this by direct mailing. The trip, August 5–12, includes roundtrip flight from Miami, double rooms with private baths, and all meals. Cost will be $987 US. We do not know how great the demand for this excursion will be, but space is limited. To reserve a place, write directly as soon as possible to Holbrook Travel, 3540 NW 13 Street, Gainesville, FL 32609. Telephone (904) 377-7111 or (800) 451-7111.

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PHYSIOLOGICAL SOURCES OF VARIATION IN CHEMICAL DEFENSE OF *Oreina gloriosa* (COLEOPTERA: CHRYSOMELIDAE)

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Abstract—The defensive secretion of the alpine chrysomelid *Oreina gloriosa* is a complex mixture of mainly cardenolides and tyrosine betaine. Individually sampled secretions of adult laboratory-reared and field-collected beetles were analyzed by reverse-phase HPLC; 16 secretion components were quantified. Quantities and concentrations of different components were significantly affected by the age, sex, and reproductive status of individual beetles. Aging was correlated with marked increases (up to 4.4-fold) and decreases (up to 2.7-fold) of quantities and concentrations of several components. Differences between the sexes were smaller, but quantities of all components and concentrations of several components were larger in laboratory-reared females than in males. There was less of one component of the secretion in mated than unmated females, but the concentrations of four secretion components were higher (up to 1.6-fold) in mated females.

Key Words—*Oreina gloriosa*, Coleoptera, Chrysomelidae, chemical defense, cardenolides, quantitative variation, aging, HPLC.

INTRODUCTION

Chemical defense of adult *Oreina gloriosa* is based mainly on the exocrine secretion of a variety of de novo synthesized cardenolides, tyrosine betaine, and ethanolamine at the surface of pronotum and elytra (Van Oycke et al., 1988; Eggenberger et al., 1992). As is typical of arthropod defensive secretions (Blum, 1981), the constituents of the secretion of *O. gloriosa* display quantitative variation among populations and, to a smaller extent, within populations (Eggenberger and Rowell-Rahier, 1991).

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Quantitative variation in the composition of exocrine secretions is probably based on variation in the rate of enzymatic reactions involved in biosynthesis and degradation and may be affected by genetic, physiological, and environmental factors.

Genetic sources of variation were found in the pheromonal secretion of the butterfly *Colias eurytheme* (Sappington and Taylor, 1990). In the defensive secretion of the leaf beetle *O. gloriosa*, genetic differences explained a considerable part of the total variation (Eggenberger and Rowell-Rahier, 1992).

Physiological sources of variation in secretion composition have been frequently described (refs. in Blum, 1981). Jackson and Bartelt (1986) found a dramatic change in quantity and composition of cuticular hydrocarbons of adult *Drosophila virilis* between eclosion and day 8. Quantitative changes in hydrocarbons with aging were also observed in the milkweed bug *Oncopeltus fasciatus* (Jackson, 1983) and in the spruce sawfly *Pikonema alaskensis* (Bartelt et al., 1984). Aphid sex pheromones were reported to increase in quantity until day 6 while changing in composition (Hardie et al., 1990). Kuwahara (1979) found an increasing ratio of neral to geraniol in the courtship pheromone of aging male *Pieris melete* (Lepidoptera).

Seasonal variation in the composition of the defensive secretion of *Oreina gloriosa* was reported by Eggenberger et al. (1992). Such variation could reflect seasonal variation in environmental factors (e.g., predation pressure or nutritional quality) or could be due to a seasonally changing age structure of the population, provided that the composition of the secretion is affected by the age of the individual beetle.

We recently reported on the genetic sources of variation in the concentration of 16 secretion components of *O. gloriosa* (Eggenberger and Rowell-Rahier, 1992). The average genetic variance component proved to account for 31% of total variation, leaving 69% of unexplained variation that could potentially be accounted for by physiological factors. In this paper we examine the effects of age, sex, and mating on the defensive secretion of *O. gloriosa* using both field-collected and laboratory-reared specimens.

METHODS AND MATERIALS

Field-collected beetles were all derived from the same locality in Saas Grund (Wallis, Swiss Alps) at 1860 m above sea level. To prevent premature secretion release, beetles were transported from the site of capture in cooled containers. In the laboratory, the beetles were maintained individually in separate plastic containers randomly distributed in cooled incubators at constant temperature (17°C) and a variable photoperiod matching seasonal change under natural conditions. The beetles were regularly provided with fresh leaves of their

food plant *Peucedanum ostruthium* (Apiaceae), which were shipped weekly from the original locality. Release of the defensive secretion was induced by mechanical irritation of each beetle held with forceps under the binocular microscope. Pronotal secretions were taken up individually in capillary tubes and quantified. The collected secretions were each dissolved in 50 μl acetonitrile–water 1 : 10, plus 2 μg ouabain (Merck) as internal standard, filtered (0.2 μm pore size) and stored individually in microtubes (Treff) at $-70°C$.

The effects of age and sex on secretion composition were studied in both field-collected and laboratory-reared beetles. Rearing of beetles was started in July 1989 with the offspring of 48 field-collected females, which had already mated in the field with unknown males. The temperature of incubators was 17°C in summer and 2°C in winter. The development of 553 adult offspring was completed in June 1990. Details of the rearing methods may be found in Eggenberger and Rowell-Rahier (1992). The secretions of a total of 236 offspring belonging to 27 families were sampled either two weeks after emergence or 10 weeks after emergence. Adult offspring were sexed on the basis of body size (female *O. gloriosa* are larger than the males). They were maintained individually, except for 24 females, which, for the purpose of investigating the effects of mating, were maintained in pairs with males collected in the field. We used males that had overwintered in the field as adults, since males do not mate in their first summer (personal observation). Pairing was started in August, six weeks after emergence of laboratory-reared beetles. Females are referred to as "mated" when the male was seen in mating position on top of the female. In order to investigate the effect of mating on secretion regeneration, the pronotal glands of 18 females were depleted before pairing.

Definitive sex determination of reared beetles was accomplished by dissection in winter 1990–1991. The second experiment to investigate the effects of age and sex on secretion composition was carried out with beetles collected in August 1991 in the field. They were aged approximately on the basis of cuticle hardness the day after capture. Individual secretions were collected from 20 newly emerged beetles, which are characterized by a soft cuticle until about one week after eclosion. Another 40 newly emerged beetles, which had not been provoked to release secretion during age classification, were maintained separately in incubators at 17°C. The secretions of 20 of them were collected individually after one week and of the remainder after two weeks. After removal of the secretion, the beetles were killed by freezing and sexed by dissection.

Separation and quantification of the constituents of the secretion were performed by reverse-phase HPLC. A 20-μl sample of each secretion was manually injected and analyzed using a Waters HPLC system (Waters 510 pumps; Waters 994 photodiode array detector; Maxima 820 data analysis system). Solvents were acetonitrile (Baker) and water (Merck). Separation was performed using a

Macherey-Nagel cartridge system (C-18; 3 μm particle size; 4 $\times$ 130 mm; 0.45 ml/min; 15–42% acetonitrile in 36 min). The separated components were quantified by measuring UV absorbance at 220 nm. The quantity of the components (microgram ouabain equivalents) was twice the respective peak area divided by the area of the internal standard peak (ouabain). Concentration (microgram oua-

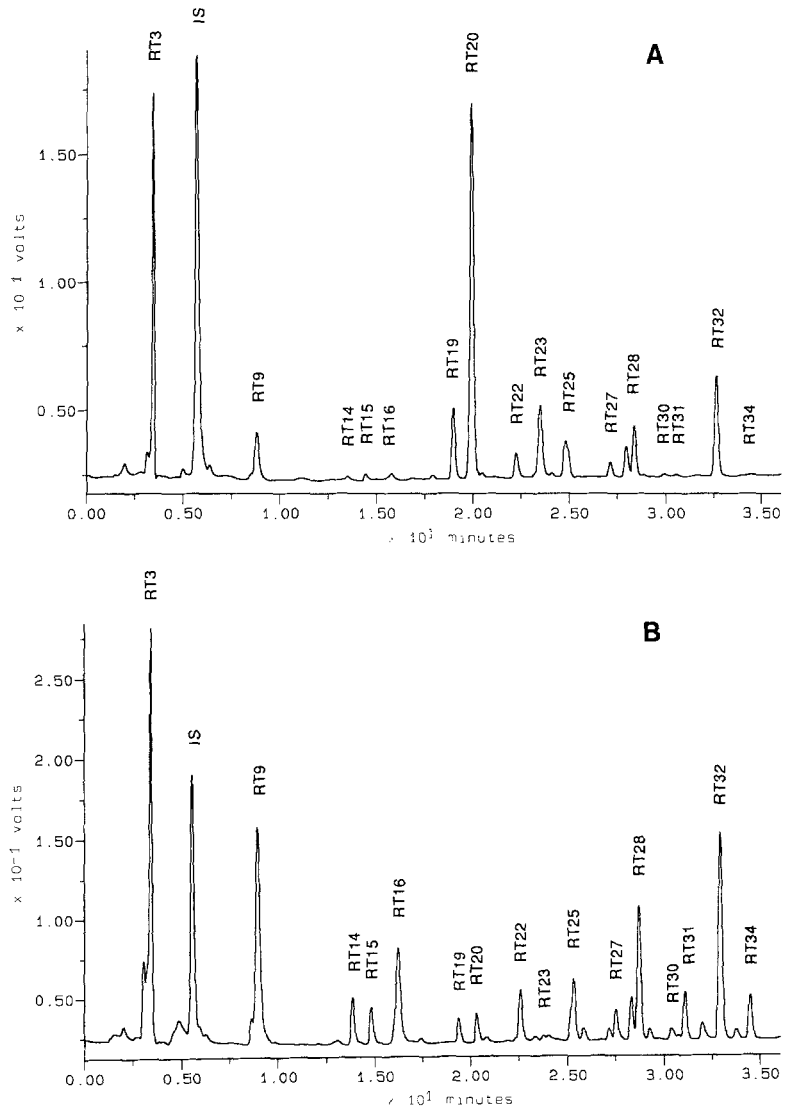

FIG. 1. HPLC trace of the defensive secretion emitted by an individual beetle at age 1 week (A) and 3 weeks (B).

TABLE 1. CHEMICAL STRUCTURE OF COMPOUNDS IN DEFENSIVE SECRETION OF *Oreina gloriosa*

RT3	*N,N,N*-trimethyltyrosine (tyrosine betaine)
RT9	sarmentogenin-3-*O*-β-D-allopyranoside
RT14	monoacetyl derivative of RT9
RT15	cardenolide
RT16	sarmentogenin-3-*O*-6′-*O*-acetyl-β-D-allopyranoside
RT19	periplogenin-3-*O*-[β-D-xylopyranosyl-(1 → 4)-β-D-allopyranoside]
RT20	periplogenin-3-*O*-β-D-allopyranoside
RT22	digitoxigenin-3-*O*-[β-D-xylopyranosyl-(1 → 4)-β-D-allopyranoside]
RT25	mono- or diacetyl derivative of RT22
RT28	mono- or diacetyl derivatives of RT22 and RT20
RT23	digitoxigenin-3-*O*-β-D-allopyranoside
RT27	mono- or diacetyl derivative of RT23
RT30	cardenolide
RT32	digitoxigenin-3-*O*-[β-D-xylopyranosyl (1 → 4)-2′, 3′-di-*O*-acetyl-β-D-allopyranoside]
RT31	cardenolide
RT34	cardenolide

bain equivalents per microliter secretion) of secretion components was calculated by dividing the respective quantity by the secretion volume. Quantified components are named RT3 to RT34 according to their retention time (Figure 1). The chemical structure of the compounds identified to date are given in Table 1 (see Eggenberger et al., 1992, for identification).

Data analysis was performed using SAS (SAS Institute Inc., 1990) on a VAX 8840. Multivariate (MANOVA) and univariate (ANOVA) two-way factorial analyses of variance were carried out with PROC GLM using type III sums of squares. Multiple comparisons of means were performed by Tukey's range tests. Data were square-root transformed prior to analyses of variance for the reasons detailed in Eggenberger and Rowell-Rahier (1992).

RESULTS

In the experiments on laboratory-reared beetles data were obtained from 138 2-week-old and 92 10-week-old offspring of 27 female *O. gloriosa*. Quantities and concentrations of 16 secretion components were tested for differences between age groups and sexes by two-way analyses of variance (Tables 2 and 3). Total quantity was significantly larger in females than in males and significantly smaller in 2-week-old beetles than in 10-week-old ones. The effect of the interaction between age and sex on total quantity was significant since the increase of total quantity with age was more distinct in females than in males.

TABLE 2. EFFECTS OF AGE AND SEX ON COMPOSITION OF SECRETION OF LABORATORY-REARED BEETLES[a]

	μg per female		μg per male		Effect[b]		
	Age 2 (N = 78)	Age 10 (N = 38)	Age 2 (N = 51)	Age 10 (N = 45)	Age	Sex	Age * Sex
RT3	3.39 ± 0.16	5.74 ± 0.28	2.75 ± 0.14	3.55 ± 0.24	***	***	**
RT9	2.79 ± 0.16	3.52 ± 0.39	1.92 ± 0.13	1.97 ± 0.17	NS	***	NS
RT14	0.26 ± 0.02	0.94 ± 0.06	0.19 ± 0.01	0.54 ± 0.03	***	***	***
RT15	0.20 ± 0.01	0.48 ± 0.03	0.16 ± 0.01	0.28 ± 0.02	***	***	**
RT16	0.72 ± 0.05	3.57 ± 0.26	0.51 ± 0.04	1.96 ± 0.13	***	***	***
RT19	0.28 ± 0.02	0.19 ± 0.02	0.21 ± 0.02	0.08 ± 0.01	***	***	*
RT20	0.53 ± 0.05	0.28 ± 0.04	0.38 ± 0.03	0.16 ± 0.02	***	**	NS
RT22	0.46 ± 0.02	0.63 ± 0.05	0.37 ± 0.03	0.41 ± 0.03	**	***	NS
RT23	0.39 ± 0.03	0.43 ± 0.04	0.31 ± 0.02	0.27 ± 0.02	NS	***	NS
RT25	0.78 ± 0.04	0.87 ± 0.07	0.62 ± 0.03	0.50 ± 0.03	NS	***	*
RT27	0.37 ± 0.03	0.43 ± 0.04	0.32 ± 0.03	0.28 ± 0.02	NS	**	NS
RT28	1.26 ± 0.07	2.02 ± 0.14	0.94 ± 0.05	1.22 ± 0.08	***	***	*
RT30	0.15 ± 0.01	0.27 ± 0.03	0.09 ± 0.01	0.18 ± 0.02	***	***	NS
RT31	0.28 ± 0.02	0.70 ± 0.07	0.18 ± 0.02	0.48 ± 0.04	***	***	NS
RT32	1.38 ± 0.07	2.45 ± 0.15	0.88 ± 0.07	1.89 ± 0.11	***	***	NS
RT34	0.31 ± 0.02	0.46 ± 0.04	0.22 ± 0.02	0.32 ± 0.03	***	***	NS
Sum	13.55 ± 0.55	22.96 ± 1.14	10.07 ± 0.46	14.09 ± 0.67	***	***	**
MANOVA					***	***	***

[a]Mean quantity (±SE) of secretion components of 2-week-old (age 2) and 10-week-old (age 10) beetles.
[b]$***P < 0.001$; $**P < 0.01$; $*P < 0.05$.

TABLE 3. EFFECTS OF AGE AND SEX ON COMPOSITION OF SECRETION OF LABORATORY-REARED BEETLES[a]

	μg/μl per female		μg/μl per male		Effect[b]		
	Age 2 (N = 72)	Age 10 (N = 37)	Age 2 (N = 50)	Age 10 (N = 44)	Age	Sex	Age * sex
RT3	53.91 ± 1.64	70.81 ± 3.40	50.18 ± 1.87	61.53 ± 3.07	***	**	NS
RT9	43.54 ± 2.03	40.61 ± 3.41	33.75 ± 1.73	34.74 ± 2.66	NS	**	NS
RT14	4.23 ± 0.28	11.43 ± 0.71	3.54 ± 0.22	9.66 ± 0.50	***	**	NS
RT15	3.30 ± 0.25	5.83 ± 0.43	3.08 ± 0.24	5.08 ± 0.30	***	NS	NS
RT16	12.13 ± 0.88	43.29 ± 2.91	9.66 ± 0.69	34.69 ± 2.09	***	**	NS
RT19	4.91 ± 0.54	2.24 ± 0.23	4.15 ± 0.44	1.37 ± 0.20	***	*	NS
RT20	8.43 ± 0.81	3.08 ± 0.33	6.90 ± 0.55	2.77 ± 0.32	***	NS	NS
RT22	7.46 ± 0.39	7.60 ± 0.53	6.88 ± 0.44	7.19 ± 0.64	NS	NS	NS
RT23	5.96 ± 0.36	5.14 ± 0.43	5.71 ± 0.43	4.93 ± 0.50	*	NS	NS
RT25	12.86 ± 0.70	10.46 ± 0.74	11.68 ± 0.70	8.72 ± 0.55	***	*	NS
RT27	5.72 ± 0.33	5.16 ± 0.43	5.77 ± 0.49	5.16 ± 0.49	NS	NS	NS
RT28	21.02 ± 1.20	24.51 ± 1.82	18.06 ± 1.11	21.29 ± 1.38	*	*	NS
RT30	2.40 ± 0.15	3.22 ± 0.31	1.69 ± 0.13	3.36 ± 0.32	***	NS	*
RT31	4.48 ± 0.37	8.74 ± 0.86	3.42 ± 0.33	8.52 ± 0.79	***	NS	NS
RT32	23.05 ± 1.20	29.90 ± 1.79	15.86 ± 1.03	33.69 ± 1.87	***	NS	***
RT34	5.11 ± 0.40	5.71 ± 0.56	3.98 ± 0.36	5.82 ± 0.54	*	NS	NS
Sum	218.5 ± 6.7	277.7 ± 10.4	184.3 ± 6.3	248.5 ± 9.6	***	***	NS
MANOVA					***	***	***

[a] Mean concentration (±SE) of secretion components of 2-week-old (age 2) and 10-week-old (age 10) beetles.
[b] ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

The overall effects of age, sex, and of the interaction between age and sex on the quantities of 16 secretion components were significant using MANOVA. Quantities of all components of the secretion were significantly larger in females than in males. There was significantly less of 10 components (RT3, RT14, RT15, RT16, RT22, RT28, RT30, RT31, RT32, and RT34) and significantly more of two others (RT19 and RT20) in 2-week-old beetles than in 10-week-old ones. The change in mean quantity with age was most pronounced in components RT16 (4.4-fold increase), RT14 (3.3-fold increase), RT31 (2.5-fold increase), RT20 (2.1-fold decrease), and RT19 (1.9-fold decrease). The effect of the interaction between age and sex was significant for seven components (RT3, RT14, RT15, RT16, RT19, RT25, and RT28), attributable to a different change in quantity with age in female and male *O. gloriosa*. Total concentration was significantly higher in females than in males and significantly lower in 2-week-old beetles than in 10-week-old ones. The overall effects of age, sex, and of the interaction between age and sex on the concentrations of 16 components of the secretion were significant. Concentrations of seven components (RT3, RT9, RT14, RT16, RT19, RT25, and RT28) were significantly higher in females than in males. Concentrations of nine components (RT3, RT14, RT15, RT16, RT28, RT30, RT31, RT32, and RT34) were significantly lower in 2-week-old beetles than in 10-week-old ones, whereas the concentrations of four other components (RT19, RT20, RT23, and RT25) were significantly higher in 2-week-old beetles. The change in mean concentration with age (Figure 2)

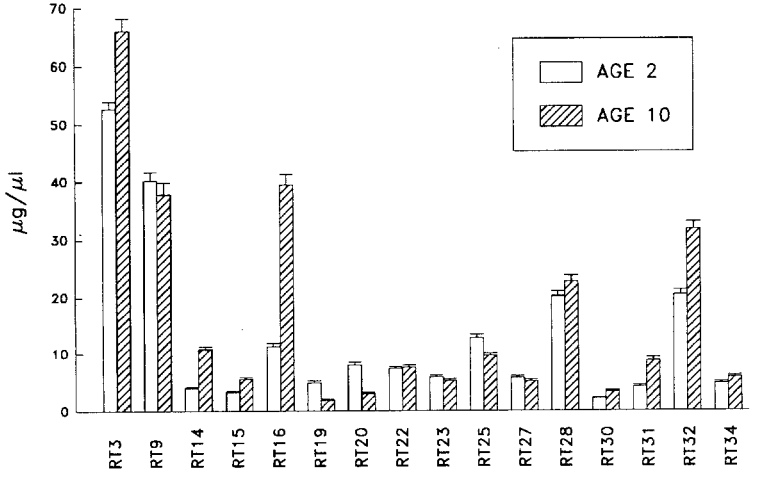

FIG. 2. Effects of age on the composition of the secretion of laboratory-reared beetles. Mean concentration (±SE) of secretion components of 2-week-old (age 2) and 10-week-old (age 10) beetles (see Table 3 for statistics).

was most pronounced for RT16 (3.5-fold increase), RT14 (2.7-fold increase), RT31 (2.1-fold increase), RT20 (2.7-fold increase), and RT19 (2.7-fold decrease). The effect of the interaction between age and sex was significant for two components (RT30 and RT32), where the increase of concentration with age was more distinct in female *O. gloriosa* than in males.

The effects of age and sex on quantity and concentration of 16 components in the secretion of field-collected beetles were investigated using 19 approximately 1-week-old individuals, 18 approximately 2-week-old beetles, and 18 approximately 3-week-old beetles. Differences between age groups and sexes were tested by two-way analyses of variance followed by multiple comparisons of age-group means (Table 4). The total quantity of secretion was significantly larger in females (7.40 $\pm$ 0.78 μg) than in males (5.96 $\pm$ 0.58 μg) and significantly different among age groups. The overall effect of age on quantity of 16 secretion components was significant, whereas overall effects of sex and of the interaction between age and sex were not significant. The quantities of 13 components (RT3, RT9, RT14, RT15, RT16, RT22, RT25, RT27, RT28, RT30, RT31, RT32, and RT34) increased significantly with age, whereas the quantity of one component (RT20) was significantly larger in 1-week-old beetles than in either 2- or 3-week-old beetles. Total concentration was significantly different among age groups, but not significantly different between the sexes. The overall effect of age on the concentrations of 16 components of the secretion was significant, but the overall effects of sex and of the interaction between age and sex were not significant. Concentrations of 12 components (RT3, RT9, RT14, RT15, RT16, RT22, RT27, RT28, RT30, RT31, RT32, and RT34) increased significantly with age, whereas the concentrations of two components (RT19 and RT20) were significantly higher in 1-week-old beetles than in 3-week-old ones (Figure 3).

The effects of mating and of the interaction between mating and secretion regeneration on quantity and concentration of 16 secretion components were tested by two-way analyses of variance on data of mated and unmated laboratory-reared 10-week-old females (Table 5). There was no overall significant effect of the interaction between mating and secretion regeneration on either quantity or concentration, showing that regeneration of secretion does not affect mating-related changes in composition of the secretion. The overall effect of mating on quantity was significant since the quantity of one component (RT9) was significantly smaller in mated than in unmated females. Total concentration was significantly higher in mated female *O. gloriosa* than in unmated ones. The overall effect of mating on concentration was significant. Concentrations of four components (RT3, RT22, RT25, and RT28) were significantly higher in mated females than in unmated ones. The mating-related change of mean concentration (Figure 4) was most pronounced in RT28 (1.6-fold).

TABLE 4. EFFECTS OF AGE ON COMPOSITION OF SECRETION OF FIELD-COLLECTED BEETLES[a]

	Quantity (μg)			Concentration (μg/μl)		
	Age 1 (N = 19)	Age 2 (N = 18)	Age 3 (N = 18)	Age 1 (N = 19)	Age 2 (N = 18)	Age 3 (N = 18)
RT3	1.17 ± 0.10a	1.77 ± 0.19b	3.03 ± 0.31c	30.3 ± 1.7a	43.7 ± 3.8b	50.8 ± 2.5b
RT9	0.46 ± 0.06a	1.12 ± 0.12b	1.68 ± 0.15c	11.3 ± 1.3a	27.9 ± 2.7b	29.5 ± 2.4b
RT14	0.03 ± 0.01a	0.11 ± 0.02b	0.29 ± 0.04c	0.7 ± 0.1a	2.5 ± 0.4b	5.1 ± 0.5c
RT15	0.04 ± 0.01a	0.13 ± 0.03b	0.25 ± 0.02c	0.9 ± 0.2a	3.0 ± 0.6b	4.6 ± 0.6c
RT16	0.06 ± 0.02a	0.25 ± 0.05b	0.83 ± 0.13c	1.5 ± 0.3a	5.6 ± 1.1b	14.1 ± 1.5c
RT19	0.10 ± 0.02a	0.07 ± 0.01a	0.06 ± 0.01a	2.4 ± 0.4a	2.2 ± 0.5ab	1.1 ± 0.2b
RT20	0.45 ± 0.09a	0.17 ± 0.03b	0.16 ± 0.02b	11.1 ± 2.3a	4.4 ± 0.7b	3.1 ± 0.5b
RT22	0.13 ± 0.02a	0.18 ± 0.02a	0.29 ± 0.03b	3.1 ± 0.4a	4.4 ± 0.5ab	5.0 ± 0.4b
RT23	0.23 ± 0.03a	0.20 ± 0.03a	0.20 ± 0.02a	5.7 ± 0.7a	4.7 ± 0.9a	3.9 ± 0.5a
RT25	0.27 ± 0.03a	0.33 ± 0.04a	0.46 ± 0.03b	6.7 ± 0.7a	8.6 ± 1.2a	8.6 ± 0.8a
RT27	0.12 ± 0.02a	0.21 ± 0.03b	0.33 ± 0.04c	3.0 ± 0.4a	5.1 ± 0.6b	5.9 ± 0.7b
RT28	0.35 ± 0.06a	0.43 ± 0.05a	0.86 + 0.08b	8.5 ± 1.2a	10.9 ± 1.5a	15.4 ± 1.3b
RT30	0.02 ± 0.01a	0.06 ± 0.01b	0.14 ± 0.02c	0.6 ± 0.1a	1.4 ± 0.2b	2.5 ± 0.3c
RT31	0.02 ± 0.01a	0.09 ± 0.02b	0.21 ± 0.03c	0.6 ± 0.2a	2.0 ± 0.3b	3.5 ± 0.4c
RT32	0.43 ± 0.08a	0.52 ± 0.06a	1.17 ± 0.10 b	10.2 ± 1.5a	13.6 ± 1.8a	20.7 ± 2.3b
RT34	0.04 ± 0.01a	0.11 ± 0.02b	0.23 ± 0.03c	0.9 ± 0.2a	2.7 ± 0.5b	3.9 ± 0.5b
Sum	3.92 ± 0.42a	5.72 ± 0.55b	10.19 ± 0.64c	97.5 ± 6.8a	142.7 ± 12.5b	177.6 ± 8.5c
		P = 0.0001 (MANOVA)			P = 0.0001 (MANOVA)	

[a] Means that do not share a letter in common are significantly ($P < 0.05$) different.

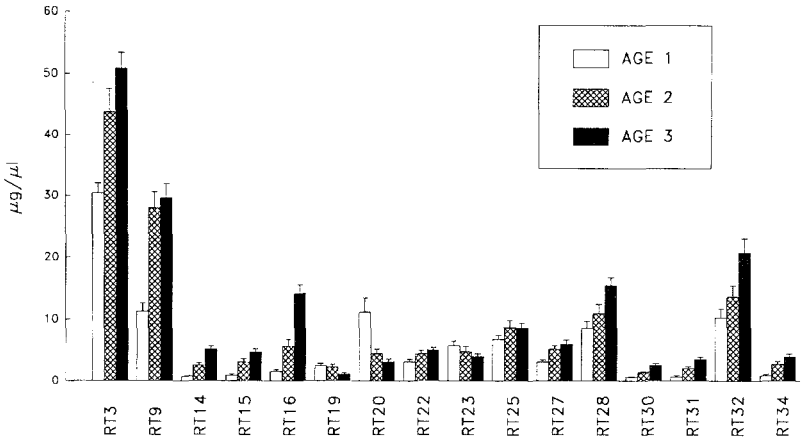

FIG. 3. Effects of age on the composition of the secretion of field-collected beetles. Mean concentration (±SE) of secretion components of 1-week-old (age 1), 2-week-old (age 2), and 3-week-old (age 3) beetles (see Table 4 for statistics).

DISCUSSION

The biological significance of the complexity of most defensive secretions is still unclear. Pasteels et al. (1983) suggested that the complexity of defensive blends may reduce counteradaptation by predators, analogous to the situation in plants, or could be due to the presence of precursors of the active compounds in the secretion, suggesting the composition of secretion to be the inevitable consequence of biosynthesis rather than of significance for chemical defense. Synergism between different components of defensive secretions has been demonstrated by Dettner and Grümmer (1986), who showed the ratio of components to correlate with penetration rate through the integument of predatory arthropods. In pheromones, the composition of secretory mixtures has been proven to be adaptive (refs. in Baker, 1989). Pheromonal activity of secretory compounds consequently may be an additional source of the complexity of defensive secretion blends. The use of defensive secretions as pheromones has been suggested to be highly adaptive, because no additional metabolic pathway would be required for pheromone biosynthesis and receptor proteins would be already present in the form of enzymes of the biosynthetic pathway (Blum, 1981). The pheromonal function of defensive compounds stored in glands has been suggested to be based on the slow leakage of gland contents, assuming defensive glands are not hermetically sealed (Attygalle et al., 1991).

In an attempt to clarify the biological significance of the secretory mixture

TABLE 5. EFFECTS OF MATING ON COMPOSITION OF SECRETION OF LABORATORY-REARED FEMALES[a]

	Quantity (μg)			Concentration (μg/μl)		
	Mated (N = 24)	Unmated (N = 94)	Effect[b] of mating	Mated (N = 23)	Unmated (N = 93)	Effect[b] of mating
RT3	5.31 ± 0.56	5.90 ± 0.19	NS	90.66 ± 7.58	73.42 ± 1.96	*
RT9	1.82 ± 0.25	2.86 ± 0.22	*	30.57 ± 4.27	33.20 ± 2.02	NS
RT14	0.63 ± 0.06	0.76 ± 0.04	NS	10.80 ± 1.06	9.56 ± 0.44	NS
RT15	0.33 ± 0.04	0.40 ± 0.02	NS	5.77 ± 0.70	5.07 ± 0.27	NS
RT16	2.28 ± 0.24	2.72 ± 0.15	NS	39.19 ± 4.16	34.14 ± 1.80	NS
RT19	0.12 ± 0.04	0.12 ± 0.01	NS	1.83 ± 0.51	1.51 ± 0.18	NS
RT20	0.13 ± 0.03	0.20 ± 0.02	NS	2.08 ± 0.39	2.28 ± 0.22	NS
RT22	0.40 ± 0.06	0.46 ± 0.03	NS	6.90 ± 0.96	5.74 ± 0.35	*
RT23	0.29 ± 0.03	0.38 ± 0.02	NS	4.87 ± 0.66	4.69 ± 0.27	NS
RT25	0.56 ± 0.08	0.64 ± 0.04	NS	10.01 ± 1.30	7.79 ± 0.50	*
RT27	0.36 ± 0.03	0.44 ± 0.03	NS	6.30 ± 0.55	5.37 ± 0.28	NS
RT28	1.60 ± 0.18	1.43 ± 0.09	NS	28.23 ± 3.03	17.59 ± 1.14	***
RT30	0.20 ± 0.02	0.24 ± 0.02	NS	3.39 ± 0.33	2.94 ± 0.18	NS
RT31	0.35 ± 0.06	0.45 ± 0.04	NS	6.46 ± 1.31	5.67 ± 0.49	NS
RT32	1.63 ± 0.14	1.99 ± 0.09	NS	28.72 ± 2.39	24.95 ± 1.13	NS
RT34	0.25 ± 0.05	0.31 ± 0.03	NS	4.65 ± 0.98	3.93 ± 0.33	NS
Sum	16.25 ± 1.25	19.29 ± 0.67	NS	280.4 ± 17.9	237.8 ± 6.6	**
	P = 0.0104 (MANOVA)			P = 0.0003 (MANOVA)		

[a]Mean quantity (±SE) and mean concentration (±SE) of secretion components.
[b]***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

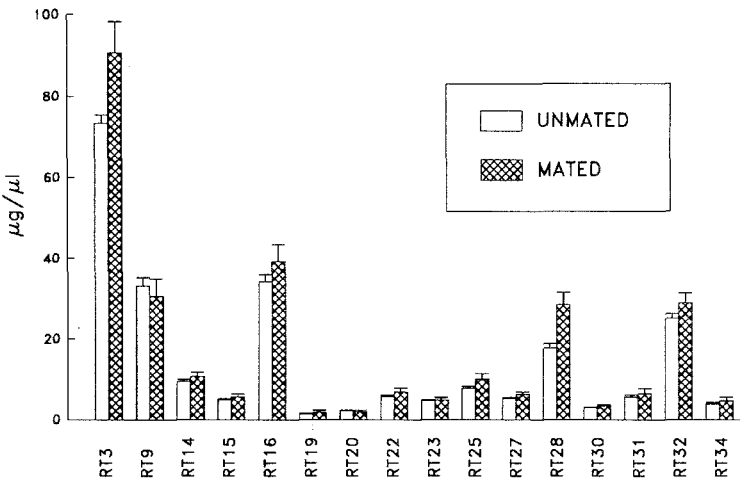

FIG. 4. Effects of mating on the composition of the secretion of laboratory-reared beetles. Mean concentration (±SE) of secretion components of mated and unmated females (see Table 5 for statistics).

of *Oreina gloriosa*, we assessed the effects of age, sex, and mating on quantity and concentration of 16 secretion components. Seasonal effects on the same components were reported in Eggenberger et al. (1992). Comparing both studies, both the season of capture of beetles in the field and the age of laboratory-reared beetles mainly affect the same components of the secretion, producing the most pronounced changes in both quantity and concentration in the same five components (RT14, RT16, RT19, RT20, and RT31). Regarding season, however, the changes go in opposite directions in field-collected and laboratory-reared beetles. This may be explained by the fact that field-collected beetles were sampled in June, just after overwintering, and in August, at the end of the season, when the population consisted of both overwintered and newly emerged beetles, resulting in a distinctly higher mean age in June than in August. Laboratory-reared beetles, on the other hand, were 2 weeks old when secretions were sampled in July and 10 weeks old in September, showing increasing mean age over the course of the season. Seasonal differences of components of the secretion are therefore thought to be for the most part due to seasonal differences in the age structure of the population, rather than seasonal variation in environmental factors such as predation pressure or nutritional quality.

Newly emerged beetles have less and more dilute secretions and consequently are less well protected chemically against predators than older ones.

This appears not to be ecologically beneficial, since newly emerged beetles are already more vulnerable to predation than older ones by virtue of their softer cuticle. The increase of total quantity and total concentration in both sexes may therefore reflect the time necessary to synthesize defensive compounds from dietary input rather than having adaptive significance.

The quantities of all components of the secretion, as well as the concentrations of several of them, are larger in laboratory-reared females than in males. However, in the secretion of field-collected beetles, the differences between the sexes are not as distinct as in laboratory-reared beetles (Eggenberger et al., 1992). This inconsistency may reflect the fact that several factors that affect the defensive secretion are under the investigator's control in the laboratory, but not in the field. Sexual differences between field-collected beetles may consequently be blurred by interactions with such factors such as regeneration of the secretion (Eggenberger and Rowell-Rahier, in preparation), reproductive status, and age.

Considering the effects of age and sex and of the interaction between them on the constituents of the secretion (Figure 5), it seems conceivable that beetles of different sexes and ages are distinguishable on the basis of their secretion. The ecological implications that follow from this may prove interesting, since it would enable a beetle to get information on the sex and age of another beetle, provided that an adequate sensory apparatus exists. Considering the life history of *O. gloriosa*, this would be beneficial. Development from larva to adult takes

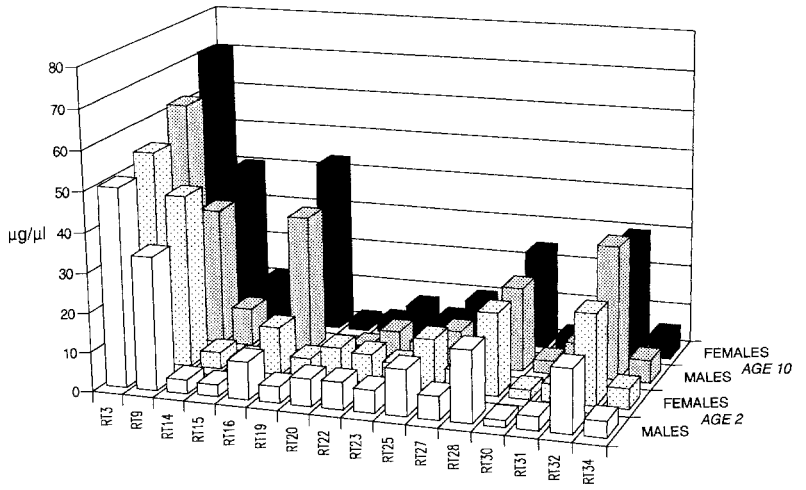

FIG. 5. Effects of age and sex on the composition of the secretion of laboratory-reared beetles. Mean concentration of secretion components of 2-week-old (age 2) and 10-week-old (age 10) beetles (see Table 3 for statistics).

about one year. Overwintered male and female beetles appear in June and decrease during August when the new generation appears, resulting in overlapping generations of overwintered and newly emerged beetles. Newly emerged females mate a few days after eclosion (Rowell-Rahier, personal observation), producing larvae after overwintering. Although adult *O. gloriosa* can live as long as three years, most beetles are likely to die in the second summer. Mating with newly emerged females would consequently be more advantageous than mating with overwintered females, suggesting that a mechanism by which male *O. gloriosa* can distinguish between newly emerged and overwintered females may exist.

With one exception only, the quantities of individual constituents were not affected by mating. On the other hand, total concentration as well as the concentrations of four components are higher in the secretions of mated females than in those of unmated ones. Although mated females may be heavier than unmated ones, the effects of mating are not thought to be based merely on the dependence of secretion concentration on body weight (Eggenberger et al., 1992), since secretion quantity, which also is dependent on body weight, should then also be larger in mated than in unmated females. This is not the case. In view of the higher concentration of the components, mated females seem to be better protected against predators than unmated ones. This would be advantageous. However, it is not clear whether the concentration of the components is more important than the quantity for chemical defense. Regeneration of the secretion following gland depletion does not affect mating-related changes in secretion composition. This specificity of secretion composition in relation to the reproductive status of the individual beetle may again indicate a potential role of secretion components in intraspecific communication.

In summary, the age of the individual beetle is shown to be an important factor affecting the composition of the secretion, and it may also account for most of the seasonal differences observed. Although the quantitative differences in the components of the secretion of males and females as well as mated and unmated females seem to be rather small, a pheromonal function of the secretion cannot be excluded.

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REFERENCES

ATTYGALLE, A.B., MEINWALD, J., LIEBHERR, J.K., and EISNER, T. 1991. Sexual dimorphism in the defensive secretion of a carabid beetle. *Experientia* 47:296–299.

BAKER, T.C. 1989. Sex pheromone communication in the Lepidoptera: New research progress. *Experientia* 45:248–262.

BARTELT, R.J., KRICK, T.P., and JONES, R.L. 1984. Cuticular hydrocarbons of the yellowheaded spruce sawfly, *Pikonema alaskensis*. *Insect Biochem.* 14:209–213.

BLUM, M.S. 1981. Chemical Defenses of Arthropods. Academic Press, New York.

DETTNER, K., and GRÜMMER, R. 1986. Quasisynergism as evolutionary advance to increase repellency of beetle defensive secretion. *Z. Naturforsch.* 41c:493–496.

EGGENBERGER, F., and ROWELL-RAHIER, M. 1991. Chemical defense and genetic variation. Interpopulational study of *Oriena gloriosa* (Coleoptera: Chrysomelidae). *Naturwissenschaften* 78:317–320.

EGGENBERGER, F., and ROWELL-RAHIER, M. 1992. Genetic component of variation in chemical defense of *Oriena gloriosa* (Coleoptera: Chrysomelidae). *J. Chem. Ecol.* 18:1375–1387.

EGGENBERGER, F., DALOZE, D., PASTEELS, J.M., and ROWELL-RAHIER, M. 1992. Identification and seasonal quantification of defensive secretion components of *Oreina gloriosa* (Coleoptera: Chrysomelidae). *Experientia* 48:1173 – 1179.

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.

JACKSON, L.L. 1983. Cuticular hydrocarbons of the milkweed bug, *Oncopeltus fasciatus* by age and sex. *Insect Biochem.* 13:19–25.

JACKSON, L.L., and BARTELT, R.J. 1986. Cuticular hydrocarbons of *Drosophila virilis*. Comparison by age and sex. *Insect Biochem.*. 16:433–439.

KUWAHARA, Y. 1979. Scent scale substances of male *Pieris melete* Ménétriès (Pieridae: Lepidoptera). *Appl. Entomol. Zool.* 14:350–355.

PASTEELS, J.M., GRÉGOIRE, J.-C., and ROWELL-RAHIER, M. 1983. The chemical defense in arthropods. *Annu. Rev. Entomol.* 28:263–289.

SAPPINGTON, T.W., and TAYLOR, O.R. 1990. Genetic sources of pheromone variation in *Colias eurytheme* butterflies, *J. Chem. Ecol.* 16:2755–2770.

SAS INSTITUTE INC. 1990. SAS/STAT User's Guide, Version 6. SAS Institute Inc., Cary, North Carolina.

VAN OYCKE, S., RANDOUX, T., BRAEKMAN, J.C., DALOZE, D., and PASTEELS, J.M. 1988. New cardenolide glycosides from defense glands of Chrysolinina beetles (Coleoptera: Chrysomelidae). *Bull. Soc. Chim. Belg.* 97:297–311.

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AN ELICITOR IN CATERPILLAR ORAL SECRETIONS THAT INDUCES CORN SEEDLINGS TO EMIT CHEMICAL SIGNALS ATTRACTIVE TO PARASITIC WASPS

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Abstract—Regurgitate of corn-fed beet armyworm (BAW) caterpillars, *Spodoptera exigua*, when applied to damaged sites of corn (*Zea mays*) seedlings, causes the release of relatively large amounts of terpenes by the seedlings several hours later. This plant response could be induced by merely placing the cut stem of seedlings in a solution of BAW regurgitate for 12 hr, a response that could not be induced by placing seedlings in water only. Regurgitate of BAW fed various diets, including a minimal diet of filter paper, were all active. However, seedlings placed in corn leaf juice, BAW hemolymph, or BAW feces extract released significantly smaller amounts of terpenes than did seedlings placed in BAW regurgitate. These results indicate that the active components are present in relatively large concentrations in regurgitate and that they are not related to the food source. Furthermore, regurgitate from several other species of caterpillars (*Spodoptera frugiperda*, *Helicoverpa zea*, *Trichoplusia ni*, and *Anticarsia gemmatalis*) as well as from the grasshopper *Schistocerca americana* induced the release of significant amounts of terpenes in corn seedlings. The release of these volatiles, therefore, appears to be a general response to attack by phytophagous insects. The terpene-releasing corn seedlings were highly attractive to the generalist parasitoid *Cotesia marginiventris* and to the specialized parasitoid *Microplitis croceipes*. This study confirms a systemic herbivore-elicited release of terpenes in corn. It is pro-

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posed that such chemicals serve multifunctional purposes that directly and indirectly protect plants against herbivorous arthropods and pathogens.

Key Words—Elicitor, corn, *Zea mays*, caterpillar regurgitate, plant volatiles, synomones, semiochemicals, parasitoids, *Cotesia marginiventris*, *Microplitis croceipes*, host searching.

INTRODUCTION

Plant odors play an important role in the foraging behavior of predators and parasitoids that attack herbivorous insects (Vinson et al., 1987; Nordlund et al., 1988; Williams et al., 1988; Tumlinson et al., 1992). In fact, previous studies show that plants under attack by herbivores may actually initiate the release of chemical signals that indicate the presence of prey or hosts to natural enemies. For instance, detailed studies on the tritrophic interaction between plants, spider mites, and predatory mites have revealed that spider mite-infested plants initiate the release of several terpenes and terpenoids that are exploited by predatory mites to locate their prey, the herbivorous spider mites (Dicke and Sabelis, 1988; Dicke et al., 1990a,b). Similarly, feeding damage caused by caterpillars induces corn seedlings to release relatively large amounts of terpenes and terpenoids that are attractive to the generalist parasitoid *Cotesia marginiventris* (Turlings et al., 1990, 1991b). Caterpillar-induced emissions of volatiles by plants are not limited to the damaged sites but occur throughout the plant, even in undamaged leaves (Turlings and Tumlinson, 1992). Such a systemic response is also indicated in spider mite-infested plants by the fact that predatory mites are attracted to uninfested leaves of mite-infested plants (Dicke et al., 1990b). Thus, infested plants provide natural enemies of herbivorous arthropods with chemical signals that guide them to the vicinity of potential hosts or prey.

The plant responses induced by mites or caterpillars cannot be mimicked with artificial damage (Dicke et al., 1990a; Turlings et al., 1990). Corn seedlings release only minor amounts of terpenes in response to artificial damage. However, when caterpillar regurgitate is applied to artificially damaged sites, corn seedlings released terpenoids in similar amounts as seedlings that had been damaged by caterpillars (Turlings et al., 1990). The specificity of the response indicates the presence of a factor in caterpillar regurgitate that causes plants to emit chemical signals. In the current study, we developed a bioassay in which terpene release could be induced in corn seedlings severed at the stem and placed in dilutions of caterpillar regurgitate. This easily standardized bioassay, whereby surface damage to the leaves was avoided, was used to test various alternative sources for activity. Additional bioassays with different caterpillar diets, different caterpillar species, and a grasshopper species were used to further characterize the active component(s) in the regurgitate. Treated seedlings also were

tested for their attractiveness to *Cotesia marginiventris*, a parasitoid that attacks a wide variety of lepidopterous caterpillars and *Microplitis croceipes*, which attacks *Heliothis* and *Helicoverpa* species only.

METHODS AND MATERIALS

Insects. All caterpillars were obtained from the USDA rearing facilities in Gainesville, Florida. They were reared according to the procedure described by King and Leppla (1984). *Schistocerca americana* grasshoppers were obtained from a colony maintained by Dr. J.L. Capinera at the Entomology Department at the University of Florida.

Cocoons of *Cotesia marginiventris* and *Microplitis croceipes* were obtained from colonies maintained at the USDA-ARS, Insect Biology and Population Management Research Laboratory, Tifton, Georgia. Both wasps were reared according to the procedure described by Lewis and Burton (1970) and held as described by Turlings et al. (1989). Flight-tunnel tests were conducted with 3- to 5-day-old mated females 4–8 hr into the photophase.

Corn. Corn (*Zea mays* L., var. Ioana sweet corn) was grown in metal trays (9 × 35 × 50 cm) in a greenhouse, approximately 60 seeds per tray. Seeds were planted in a 50:50 mixture of moist vermiculite and potting soil. Natural light was supplemented with 400-W high-pressure sodium lamps placed 1 m above the trays. Experiments were conducted with 8- to 10-day-old seedlings that carried three leaves.

Collection of Regurgitate. One day before their regurgitate was collected, 7- to 9-day-old caterpillars were placed on a specific diet (corn leaves unless stated otherwise). Regurgitation was induced by holding the caterpillars with a pair of light-weight forceps and gently pinching the head region with another pair. The regurgitate was collected by holding the caterpillar over a 100-μl pipet that inserted into a 3-ml vial through a rubber septum, and attached to low vacuum via another pipet. This allowed the regurgitate to slowly drip into the vial through the pipet. Grasshopper regurgitate was collected in a similar fashion from hand-held insects. Depending to some extent on their diet, 10–25 μl regurgitate could be collected from each caterpillar, while the grasshoppers produced 20–40 μl. All regurgitate was centrifuged for 10 min at 11,750*g* and the supernatant was filtered through a 0.22-μm sterile Millipore (Millex-GV) filter to remove bacteria.

Alternative Treatment Materials. To determine the specificity of the active component(s) in the caterpillar regurgitate, several arbitrarily chosen alternative sources were tested. A total of 1 ml of feces from BAW larvae fed on corn was collected, diluted in 5 ml of distilled water, and vigorously shaken for several minutes. The solution was centrifuged at 14,000 rpm for 30 min, the supernatant

filtered through a 0.22-μm Millipore filter, lyophilized, and rediluted with distilled water to a total volume of 1 ml. BAW hemolymph was obtained by piercing the larvae with a needle and gently squeezing the hemolymph out. Hemolymph was centrifuged and the supernatant filtered through a 0.22-μm filter. Corn juice was obtained from corn seedlings by first pulverizing them with a mortar and pestle. The seedlings were then wrapped in aluminum foil and squeezed so that additional fluid would drip from an open end in the foil back into the mortar. The corn juice was also centrifuged and the supernatant filtered.

Treatment of Seedlings. Seedlings were placed in 1-ml vials that contained either 500 μl of distilled water only (control) or 50 μl of treatment material (in most cases caterpillar regurgitate) diluted in 450 μl distilled water. Six treatments (including control) were tested on a given day with three seedlings per treatment. Experiments were replicated six times. The seedlings were cut from the trays in the greenhouse and placed in vials at 9–11 PM. The following day at 9–10 AM, they were removed from the vials, the submerged part of the stem was cut off, and the severed end was wrapped in wet cotton wool.

Collection and Analysis of Volatiles. The volatile collection system used was the same as previously described by Turlings et al. (1991b). Each group of three seedlings that underwent the same treatment was placed in a glass chamber (3 cm ID $\times$ 15 cm long). Using a push–pull technique (compressed air and vacuum), humidified clean air (500 ml/min) was passed through the chamber and through an adsorbent (25 mg Super Q) that trapped the volatiles emitted by the seedlings. Six collection systems were used in parallel and collections lasted 2 hr.

After collection, the traps were rinsed with 150 μl methylene chloride and an internal standard (600 ng of nonyl acetate in 30 μl methylene chloride) was added. Two microliters of each sample was analyzed on a 50-m $\times$ 0.25-mm-ID bonded methyl silicone (007) (0.25-μm-thick film) capillary gas chromatography column combined with a 10-m $\times$ 0.25-mm deactivated retention gap. The Hewlett-Packard model 5890 gas chromatograph (GC) was equipped with an on-column injector system and a flame ionization detector and helium (19 cm/sec) was used as a carrier gas. Following injection, column temperature was maintained at 50°C for 3 min and then programmed at 7°C/min to 190°C. A PE Nelson (Cupertino, California) data collection system was used, and the eight most predominant compounds were quantified using Turbochrome 3.1 software. The total amounts of these eight compounds were used to compare induced releases among different treatments.

Flight-Tunnel Bioassays. Attractiveness of seedlings to the parasitoids *C. marginiventris* and *M. croceipes* was tested in the Plexiglas flight tunnel described by Turlings et al. (1991a). Conditions in the tunnel were: 15 cm/min airflow;

55–70% relative humidity; 27.5–29°C; and approximately 500 lux illumination. For each experiment, groups of three seedlings that had undergone the same treatment were placed in one vial. Two vials with seedlings were placed 20 cm apart on a 40-cm-high stand at the upwind end of the tunnel. Each vial contained seedlings that had undergone different treatments: they were seedlings that had been standing either in distilled water only, diluted BAW regurgitate, or grasshopper regurgitate. Female wasps were released 80 cm downwind from the seedlings. Before release the wasps had been given a 20-sec contact experience with a complex of corn seedlings fed upon by BAW larvae (in the case of *C. marginiventris*) or *Helicoverpa zea* larvae (in the case of *M. croceipes*). Such experiences greatly increase their responses to host-related odors (Drost et al., 1986; Eller et al., 1988; Turlings et al., 1989). On a given day, six wasps of one species were tested to each of the three possible combinations of seedlings; this was repeated on six different days ($N = 36$). We recorded the numbers of females landing on each group of seedlings.

RESULTS

Induced Release of Volatiles. The amounts of volatiles emitted by seedlings that had been standing in a 10-fold dilution of BAW regurgitate differed dramatically compared with the volatiles emitted by seedlings that spent the same amount of time (12 hr) in water only (Figure 1). The identity of the components, mostly terpenes, had been determined in a previous study (Turlings et al., 1991b).

Alternative Sources. The effects of BAW regurgitate on the volatiles released by corn seedlings were compared with the releases caused by BAW feces, BAW hemolymph, corn juice, and regurgitate from another herbivorous insect, the grasshopper *Schistocerca americana*. After having been placed in vials with 10-fold dilutions of the various treatments, all seedlings released volatiles in significantly larger total amounts than did control seedlings (Figure 2). The regurgitate treatments, however, resulted in significantly larger total amounts of terpenoids; the grasshopper regurgitate was particularly active. Of the five treatments, BAW feces and corn juice were the least active.

Different Diets. Regurgitate from BAW larvae fed on a variety of diets was tested to determine if diet influences activity. For this purpose regurgitate was collected from BAW larvae that, for one day, had been fed corn leaves, cotton leaves, soy leaves, artificial diet (a pinto bean-based diet; King and Leppla, 1984), or sucrose water-sprinkled filter paper (Whatman 1). While regurgitate from all diets induced the release of terpenes in corn seedlings, regurgitate from caterpillars fed on soy leaves was most active and regurgitate from cotton- and filter paper-fed larvae was the least active (Figure 3).

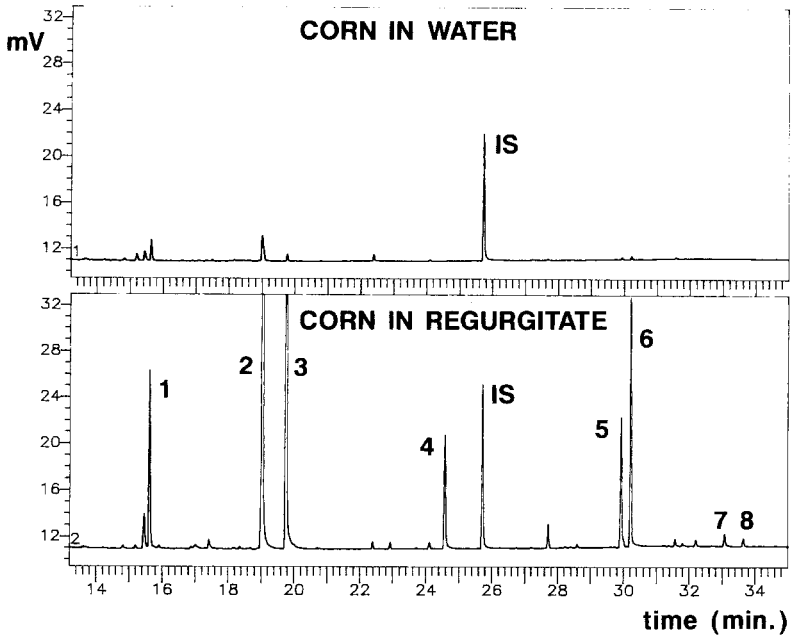

FIG. 1. Comparison of chromatographic profiles of volatiles released by corn seedlings that were placed for 12 hr in vials with distilled water or vials with a 10-fold dilution of BAW regurgitate. The identities of the various compounds have been determined previously (Turlings et al., 1991b); they are: 1, (*Z*)-3-hexen-1-yl acetate; 2, linalool; 3, (3*E*)-4,8-dimethyl-1,3,7-nonatriene; 4, indole; 5, α-*trans*-bergamotene; 6, (*E*)-β-farnesene; 7, (*E*)-nerolidol; and 8, (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. As an internal standard (IS) we used nonyl acetate (600 ng).

Different Caterpillar Species. To learn more about the specificity of regurgitate activity, regurgitate from five species of caterpillars was collected and tested. In addition to regurgitate from BAW, we collected regurgitate from *Spodoptera frugiperda* (fall armyworm = FAW), *H. zea* (corn earworm = CEW), *Trichoplusia ni* (cabbage looper = CL), and *Anticarsia gemmatalis* (velvetbean caterpillar = VBC). All caterpillars had been feeding on artificial diet. The regurgitate of each species caused corn seedlings to initiate the release of terpenoids (Figure 4). Regurgitate from BAW, CEW, and CL was consistently more active than regurgitate from FAW and VBC.

In all cases, treatments induced the release of the same blend of eight compounds. Differences were only consistently observed in the total amounts released, but not in the relative ratios of the compounds.

Parasitoid Responses. In a flight tunnel, female wasps were given a choice between combinations of corn seedlings that had been standing in either distilled

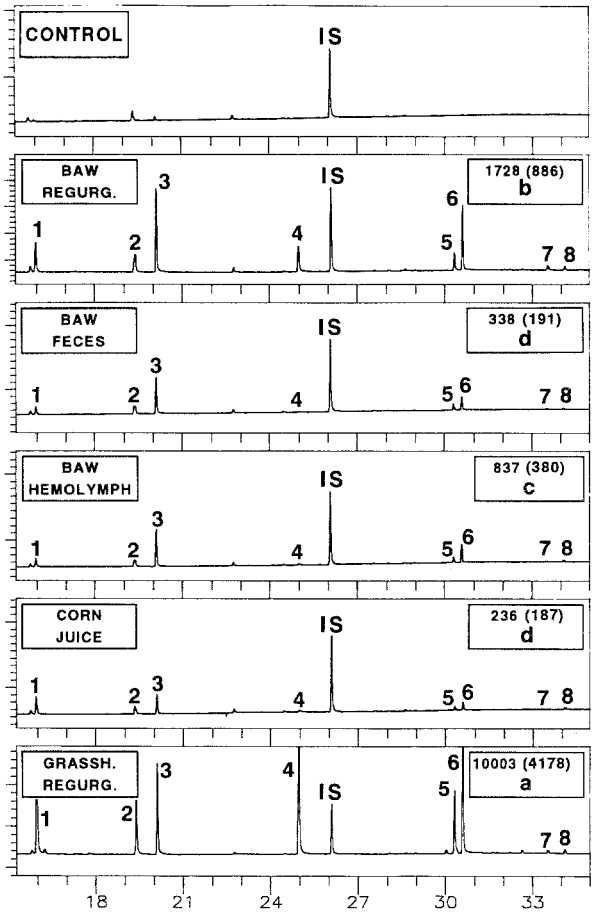

FIG. 2. Comparison of volatiles released by corn seedlings that were placed in distilled water (CONTROL), BAW regurgitate, BAW feces, BAW hemolymph, corn juice, and grasshopper regurgitate. Identities of peaks are given in Figure 1. Values in the right-hand corners are the mean ($N = 6$) total amounts (ng/3 seedlings/2 hr) of the eight compounds released and their standard deviations. Means with different letters are significantly different (paired t test, $P < 0.05$).

water, diluted BAW regurgitate, or diluted grasshopper regurgitate. Both *C. marginiventris* and *M. croceipes* showed a clear preference for seedlings treated with regurgitate (Figure 5). Neither wasp showed a significant preference when given a choice between corn seedlings treated with either caterpillar regurgitate or grasshopper regurgitate, although *C. marginiventris* may have shown a tendency to fly more often to grasshopper regurgitate-treated corn.

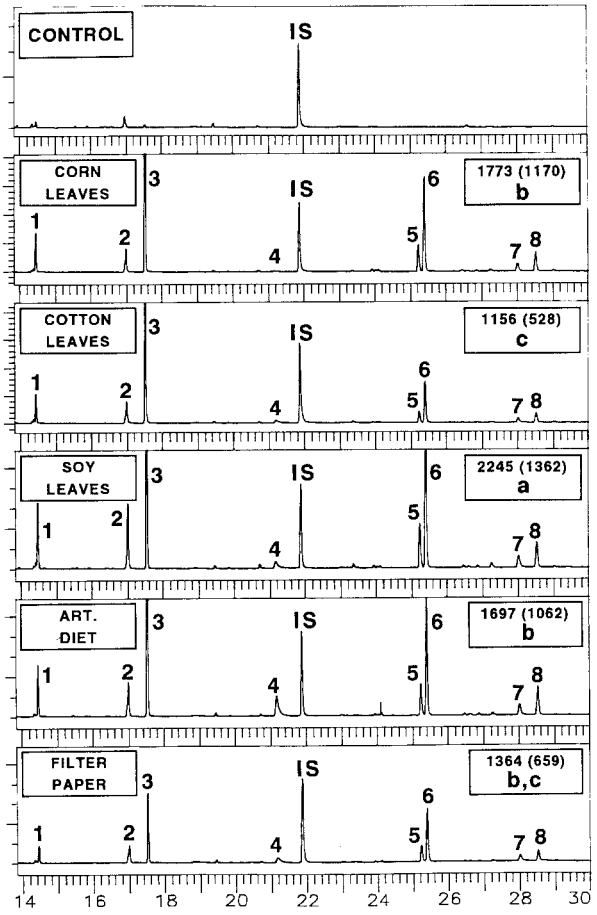

FIG. 3. Comparison of volatiles released by corn seedlings that were placed in regurgitate from BAW caterpillars that had been fed different diets. Identities of peaks are given in Figure 1. Values in the right-hand corners are the mean ($N = 6$) total amounts (ng/3 seedlings/2 hr) of the eight compounds released and their standard deviations. Means with different letters are significantly different (paired t test, $P < 0.05$).

DISCUSSION

Injury inflicted by herbivores can induce the production of specific chemicals in various plants. These chemicals may serve to heal the wounds, as antibiotics to prevent secondary infections by pathogens, or as toxins or repellents against herbivores (Levin, 1976; Kogan and Paxton, 1983; Rhoades, 1979, 1985; Schultz, 1988; Tallamy and Raupp, 1991). More recent work (Dicke and

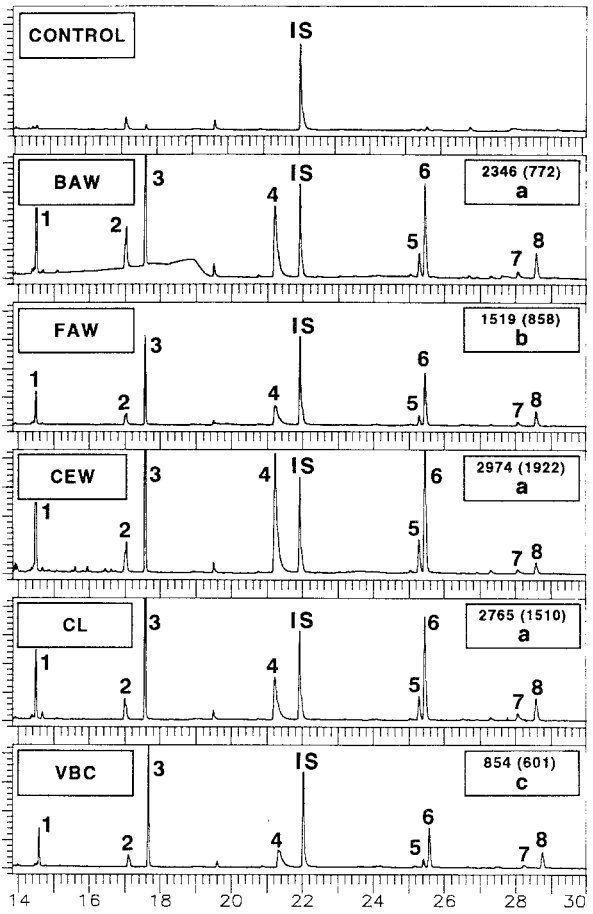

FIG. 4. Comparison of volatiles released by corn seedlings that were placed in regurgitate from different caterpillar species (BAW = beet armyworm; FAW = fall armyworm; CEW = corn earworm; CL = cabbage looper; VBC = velvetbean caterpillar). Identities of peaks are given in Figure 1. Values in the right-hand corners are the mean ($N = 6$) total amounts (ng/3 seedlings/2 hr) of the eight compounds released and their standard deviations. Means with different letters are significantly different (paired t test, $P < 0.05$).

Sabelis, 1988; Dicke et al., 1990a,b; Turlings et al., 1990, 1991b; Turlings and Tumlinson, 1991) also shows that plants, in response to damage caused by herbivorous arthropods, may actively release volatile chemicals that attract natural enemies of the herbivores.

The generalist parasitoid *C. marginiventris* (Turlings et al., 1990, 1991a,b) and the specialized parasitoid *M. croceipes* (Elzen et al., 1987; Drost et al.,

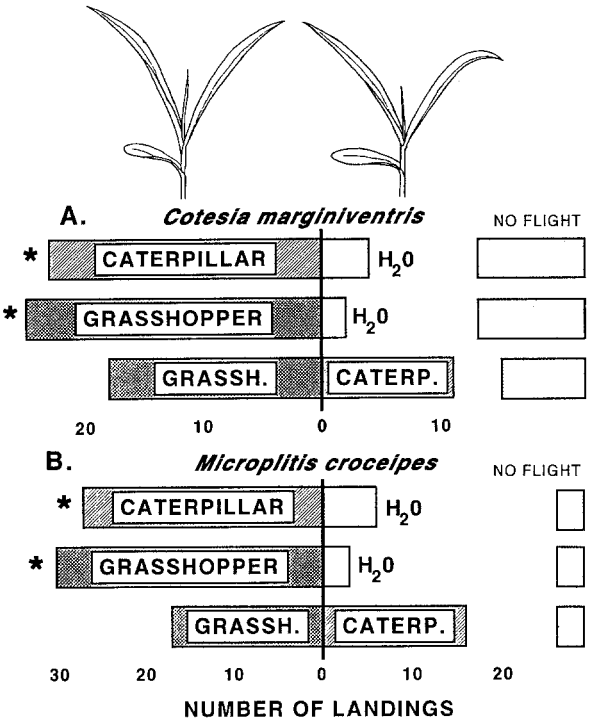

FIG. 5. Number of landings by *Cotesia marginiventris* (A) and *Microplitis croceipes* (B) females on corn seedlings during two-choice tests in a flight tunnel. Seedlings had spent 12 hr in either distilled water, BAW (caterpillar) regurgitate, or grasshopper regurgitate. Asterisks indicate statistically significant preferences for indicated treatments (chi-square, $P < 0.05$). The "No Flight" bars represent those wasps that did not land on a plant.

1988; Eller et al., 1988; McCall et al., 1993) both use plant-released volatiles in host habitat location. Here we show that the release of volatiles that strongly attract these parasitoids can be induced in corn with an elicitor in the regurgitate of herbivorous insects, without actually damaging the surface of the leaves. This confirms previous findings that the plant response is systemic and that the volatile releases are not limited to injured sites, but occur throughout the plant (see also Turlings and Tumlinson, 1992). However, in order for a plant to respond, the elicitor does need to enter the plant (in this case through the severed stem); rubbing regurgitate over intact leaves is not enough to cause a response (Turlings et al., 1990).

The Elicitor and Its Mode of Action. The exact source of the elicitor(s) was not pinpointed in this study, but since activity did not depend on the diet of the caterpillars (even regurgitate of caterpillars fed on filter paper was active), the

compound(s) are likely to be insect produced and are perhaps excreted from glands near the mouth parts. Furthermore, the results show that eliciting factors are common to lepidopterous larvae and may occur in a variety of herbivorous insects. Since they occur not only in regurgitate of caterpillars but also in small quantities in other natural sources and in very large quantities in grasshopper regurgitate, it is likely that they are common natural compounds. We propose that these factors indicate to the plant that it is being attacked by phytophagous organisms, and that, as hypothesized previously (Turlings et al., 1990; Turlings and Tumlinson, 1991), the plant response is a defensive reaction primarily directed at invading herbivores and/or pathogens. Lin et al. (1990) found that such induced resistance in soybean could be greatly enhanced by applying soybean looper regurgitate to damaged plant tissue. Detling and Dyer (1981) showed that grasshopper regurgitate reduces growth in blue grama grass, which also might indicate that energy investments are shifted towards defenses.

The elicitor appears to be taken up by the plant and induces the synthesis and/or release of specific terpenes. Many questions remain concerning the mode of action of the elicitor and the biochemical pathway(s) it may trigger. It may be that the elicitor is transported throughout the plant, or that it simply induces the production and transportation of other products at the point of entry. It is also still unknown whether actual synthesis takes place or whether the terpenoids are mobilized from elsewhere in the plant. Thompson et al. (1974) found only minor amounts of the terpenoids in the essential oil from healthy corn, suggesting that the plant response either involves synthesis of the volatiles or that they are bound in healthy plants and freed when the plant is attacked.

Functions of Plant-Released Volatiles. Terpenes and sesquiterpenes are clearly important for prey and host location by predators and parasitoids of phytophagous arthropods. However, the release of these chemicals is most likely a direct defense against herbivores and against pathogens that may invade the injured plants. Examples of the detrimental effect of terpenes and terpenoids on the development of insect larvae are ample (Mabry and Gill, 1979; Turlings and Tumlinson, 1991), and these chemicals are frequently implicated in an antibiotic role (Loomis and Croteau, 1980; Brooks and Watson, 1985). Yet the defensive roles of induced chemical changes in plants have recently been questioned (e.g., Fowler and Lawton, 1985; Myers and Williams, 1987; Coleman and Jones, 1991; Faeth, 1988, 1991). Many of the studies on plant defenses involve tree species that may largely rely on constitutive defenses rather than induced defenses and are mainly subjected to attacks by well-adapted species. This could explain the limited evidence for the detrimental effect of the induced chemical chances on individuals or communities of the phytophagous insects. Defensive strategies are different in many fast-growing annuals that often possess highly flexible defense expressions that are activated only when the plants are under attack (Coley et al., 1985).

Often studies in this area do not make the distinction between artificial (physical) damage or insect damage (Faeth, 1991). Yet induced chemical plant defenses may be specific to insect attack, as is illustrated here with the corn–caterpillar interaction (see also Turlings et al., 1990).

Obviously this study, conducted under laboratory conditions and with an artificially selected crop plant, provides no answers to any coevolutionary questions. However, it does show that specific insect-induced plant responses do occur and that the observed chemical changes are not necessarily responses to damage alone. These specific chemical changes may benefit the plants not only by directly deterring phytophagous organisms, but also by attracting natural enemies of such organisms.

Parasitoid Responses. Both species of wasps were highly attracted to regurgitate-treated corn independent of the source of the regurgitate (Figure 2). Thus, under these conditions the wasps could not distinguish between caterpillar- and grasshopper-treated plants. This supports the notion that plants provide the wasps with highly detectable cues, but that these cues may not always reliably indicate the presence of suitable hosts (Vet et al., 1991; Vet and Dicke, 1992; Wäckers and Lewis, 1993). Another indication that there are some constraints on the ability of the wasps to recognize host-infested plants is that in this study *M. croceipes*, which attacks only *Heliothis* and *Helicoverpa* species, would readily fly to corn treated with BAW (a nonhost) regurgitate. Similarly, McCall et al. (1993) found that *M. croceipes* is not always able to distinguish corn earworm (= host) odors from BAW or cabbage looper (= nonhost) odors in flight, when these caterpillars are feeding on plants of the same species (McCall et al., 1993). Our chemical data (Figure 4) and those of McCall et al. (unpublished data) indicate that, if occurring at all, differences in emitted odors are very subtle. Possible differences may be learned by the wasps if given enough training. For instance, Eller et al. (1992) found that repeated experiences will improve this wasp's ability to distinguish certain different odor sources. Under more natural conditions, visual learning will add to the effects of odor learning (Wäckers and Lewis, 1992), thereby enhancing the effectiveness of host location by these wasps. Yet discrimination between hosts and nonhosts may commonly occur at close range through innately recognized contact kairomones mainly present in host feces. *Microplitis croceipes* readily distinguishes host feces from nonhost feces (Lewis, 1970; Lewis and Tumlinson, 1988; McCall et al., 1993).

This study confirms the importance of actively released plant volatiles for host-seeking parasitoids. The induction of the release is greatly enhanced by a factor in caterpillar regurgitate. Currently, we focus on isolating and identifying the elicitor, for its identity may answer many of the remaining questions. A synthetic elicitor would allow us not only to further study the mechanisms involved, but also may provide us with a tool to use in the possible manipulation of crops to directly and indirectly enhance control of pests.

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REFERENCES

BROOKS, C.J.W., and WATSON, D.A. 1985. Phytoalexins. *Nat. Prod. Rep.* 2:427–459.

COLEMAN, J.S., and JONES, C.G. 1991. A phytocentric perspective of phytochemical induction by herbivores, pp. 3–45, *in* D.W. Tallamy and M.J. Raupp (eds.). Phytochemical Induction by Herbivores. John Wiley & Sons, New York.

COLEY, P.D., BRYANT, J.P., and CHAPIN, F.S., III. 1985. Resource availability and plant antiherbivore defense. *Science* 230:895–899.

DETLING, J.K., and DYER, M.I. 1981. Evidence for potential plant growth regulators in grasshoppers. *Ecology* 62:485–488.

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, A.E. 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.

DROST, Y.C., LEWIS, W.J., ZANEN, P.O., and KELLER, M.A. 1986. Beneficial arthropod behavior mediated by semiochemicals. I. Flight behavior and influence of pre-flight 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.

ELLER, F.J., TUMLINSON, J.H., and LEWIS, W.J. 1992. Effect of host diet and preflight experience on the flight response of *Microplitis croceipes* (Cresson). *Physiol. Entomol.* 17:235–240.

ELZEN, G.W., WILLIAMS, H.J., VINSON, S.B., and POWELL, J.E. 1987. Comparative flight behavior of parasitoids *Campoletis sonorensis* and *Microplitis croceipes*. *Entomol. Exp. Appl.* 45:175–180.

FAETH, S.H. 1988. Plant-mediated interactions between seasonal herbivores: enough for evolution or coevolution? pp. 391–414, *in* K.C. Spencer (ed.). Chemical Mediation of Coevolution. AIBS Symposium Volume, Academic Press, New York.

FAETH, S.H. 1991. Variable induced responses: Direct and indirect effects on oak folivores, pp. 293–323, *in* D.W. Tallamy and M.J. Raupp (eds.). Phytochemical Induction by Herbivores. John Wiley & Sons, Inc., New York.

FOWLER, S.V., and LAWTON, J.H. 1985. Rapidly induced defenses and talking trees: The devil's advocate position. *Am. Nat.* 126:181–195.

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.

KOGAN, M., and PAXTON, J.D. 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.

LEVIN, D.A. 1976. The chemical defenses of plants to pathogens and herbivores. *Annu. Rev. Ecol. Syst.* 7:121–159.

LEWIS, W.J. 1970. Study of species and instars of larval *Heliothis* parasitized by *Microplitis croceipes*. *J. Econ. Entomol.* 63:363–365.

LEWIS, W.J., and BURTON, R.L. 1970. Rearing *Microplitis* in the laboratory with *Heliothis Zea* as hosts. *J. Econ. Entomol.* 63:656–658.

LEWIS, W.J., and TUMLINSON, J.H. 1988. Host detection by chemically mediated associative learning in a parasitic wasp. *Nature* 331:257–259.

LIN, H., KOGAN, M., and FISCHER, D. 1990. Induced resistance in soybean to Mexican bean beetle (Colleoptera: Coccinellidae): Comparison of inducing factors. *Environ. Entomol.* 19:1852–1857.

LOOMIS, W.D., and CROTEAU, R. 1980. Biochemistry of terpenoids, pp. 363–418, *in* P.K. Stumpf (ed.). The Biochemistry of Plants, Vol. 4. Academic Press, New York.

MABRY, T.J., and GILL, J.E. 1979. Sesquiterpene lactones and other terpenoids, pp. 501–537, *in* A. Rosenthal and D.H. Janzen (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, New York.

MCCALL, P.J., TURLINGS, T.C.J., LEWIS, W.J., and TUMLINSON, J.H. 1993. The role of plant volatiles in host location by the specialist parasitoid *M. croceipes* Cresson (Braconidae: Hymenoptera). *J. Insect Behav.* In press.

MYERS, J.H., and WILLIAMS, K.S. 1987. Lack of short or long term inducible defenses in the red alder–western tent caterpillar system. *Oikos* 48:73–78.

NORDLUND, D.A., LEWIS, W.J., and ALTIERI, M.A. 1988. Influences of plant produced allelochemicals on the host and prey selection behavior of entomophagous insects, pp. 65–90, *in* P. Barbosa and D. K. Letourneau (eds.). Novel Aspects of Insect–Plant Interactions. Wiley, New York.

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 Interactions with Secondary Plant Metabolites. Academic Press, New York.

RHOADES, D.F. 1985. Offensive–defensive interactions between herbivores and plants: Their relevance in herbivore population dynamics and ecological theory. *Am. Nat.* 125:205–238.

SCHULTZ, J.C. 1988. Plant resistances induced by herbivores. *Trends Ecol. Evol.* 3:45–49.

TALLAMY, D.W., and RAUPP, M.J. 1991. Phytochemical Induction by Herbivores. John Wiley & Sons, New York.

THOMPSON, A.C., HEDIN, P.A., and GUELDNER, R.C. 1974. Corn bud essential oil. *Phytochemistry* 13:2029–2032.

TUMLINSON, J.H., TURLINGS, T.C.J., and LEWIS, W.J. 1992. The semiochemical complexes that mediate insect parasitoid foraging. *Agric. Zool. Rev.* 5:221–252.

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., and TUMLINSON, J.H. 1992. Systemic release of chemical signals by herbivore-injured corn. *Proc. Natl. Acad. Sci. U.S.A.* 89:8399–8402.

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., TUMLINSON, J.H., and LEWIS, W.J. 1990. 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. 1991a. Larval-damaged plants: source of volatile synomones that guide the parasitoid *Cotesia marginiventris* to the microhabitat of its host. *Entomol. Exp. Appl.* 58:75–82.

TURLINGS, T.C.J., TUMLINSON, J.H., HEATH, R.R., PROVEAUX, A.T., and DOOLITTLE, R.E. 1991b. Isolation and identification of allelochemicals that attract the larval parasitoid *Cotesia marginiventris* (Cresson) to the microhabitat of one of its hosts. *J. Chem. Ecol.* 17:2235–2251.

VET, L.E.M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

VET, L.E.M., WÄCKERS, F.L., and DICKE, M. 1991. How to hunt for hiding hosts: The reliability-detectability problem in foraging parasitoids. *Neth. J. Zool.* 41:202–213.

VINSON, S.B., ELZEN, G.W., and WILLIAMS, H.J. 1987. The influence of volatile plant allelochemics on the third trophic level (parasitoids) and their herbivorous hosts, pp. 109–114, *in* V. Labeyrie, G. Fabres, and D. Lachaise (eds.). Insects–Plants. Dr. W. Junk Publishers, Dordrecht.

WÄCKERS, F.L., and LEWIS, W.J. 1993. Olfactory and visual learning and their interaction in host site location by *Microplitis croceipes*. *Biocontrol* In press.

WILLIAMS, H.J., ELZEN, G.W., and VINSON, S.B. 1988. Parasitoid host plant interactions, emphasizing cotton (*Gossypium*), pp. 171–200, *in* P. Barbosa and D.K. Letourneau (eds.). Novel Aspects of Insect–Plant Interactions. Wiley, New York.

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AVOIDANCE OF BIRD REPELLENTS BY MICE (*Mus musculus*)

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Abstract—It is believed that mammalian chemosensory irritants are not aversive to birds and vice versa. Nevertheless, few avian repellents have been tested against mammals. For that reason, we evaluated the efficacy of 1.0% w/v methyl anthranilate, orthoaminoacetophenone, 2-amino-4′,5′-methoxyacetophenone, 2-methoxyacetophenone, and veratryl amine as mouse repellents in 3-hr no-choice drinking tests. Relative to ingestion of plain water, all test substances significantly reduced ($P < 0.05$) intake. Orthoaminoacetophenone was the most effective repellent, with intake reduced to levels statistically indistinguishable from zero.

Key Words—Chemical repellents, chemosensory, mice, *Mus musculus*, orthoaminoacetophenone.

INTRODUCTION

Limited data are available that document agricultural losses caused by vertebrates (Salmon, 1988). Nonetheless, it is generally recognized that rats (e.g., *Rattus norvegicus*, *Rattus rattus*), mice (e.g., *Mus musculus*, *Peromyscus maniculatus*), voles (e.g., *Microtus pinetorum*, *Microtus pennsylvanicus*), various ground squirrels (e.g., *Spermophilus* spp.), and other rodents probably cause greater economic harm than any other vertebrate group (Brooks et al., 1990). Agricultural losses are believed to be substantial (Marsh, 1988), and damage is likely to increase in the future as conservation tillage practices become more

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widespread (Castrale, 1987). Besides crop damage, commensal rodents undermine and weaken structures (Marsh, 1988; Timm, 1982) and chew through electrical and telephone cables. They also may serve as primary reservoirs or hosts to vectors for human and livestock diseases, including viral zoonoses (e.g., Venezuelan equine encephalitis), rickettsial diseases (e.g., Rocky mountain spotted fever) and bacterial diseases (e.g., salmonella) (Gratz, 1988).

At present, there are a number of effective lethal strategies that can be employed for rodent control. There are few nonlethal options available, however, even though there are situations where nonlethal control is desirable. For example, rat and mouse infestations at swine confinement facilities often are maintained by the presence of palatable livestock feed. The development of a nontoxic rodent repellent that could safely be added to these feeds would have considerable utility, provided that it did not affect the palatability of the feed to swine. No available rodent repellent has this characteristic. Thus, although repellents like capsaicin or denatonium saccharide and denatonium benzoate are available, these materials are either broadly offensive to all mammals (Meehan, 1988) or show considerable inter- and intraspecific variability in effectiveness. Moreover, all of these materials are curiously inoffensive to birds (e.g., Beauchamp and Mason, 1991).

In the present experiment, we evaluated the repellency of five candidate bird repellents to house mice (*Mus musculus*). Although the available data suggested that at least some of these repellents were inoffensive to humans, livestock (e.g., Glahn et al., 1989; Mason et al., 1991c), and deer mice (*Peromyscus maniculatus*) (Schafer and Bowles, 1985), the overall evidence was sparse.

METHODS AND MATERIALS

Subjects. Seventy-two experimentally naive male CF-1 mice (*Mus musculus*) served as subjects. Animals were individually caged (27 × 21 × 14 cm) under a 12 : 12 light–dark cycle at 23°C and given free access to 8604-00 Wayne Rodent Blox.

Chemicals. We selected five stimuli previously shown to have avian repellent properties for this study: (1) methyl anthranilate (MA; CAS #134-20-3) (Kare, 1961; Kare and Pick, 1960), (2) orthoaminoacetophenone (OAP; CAS #551-93-9) (Mason et al., 1991b; Clark and Shah, 1991), (3) 2-amino-4′,5′-methoxyacetophenone (AMAP; CAS #4101-30-8) (Clark et al., 1991), (4) 2-methoxyacetophenone (MAP; CAS #4079-52-1) (Clark et al., 1991), and (5) veratryl amine (VA; CAS #5763-61-1) (Mason et al., 1991a). All five chemicals were NPLC grade and were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin). Each was added to deionized, distilled water to yield saturated emulsions with concentrations of 1.0% (w/v).

Procedure. Twelve mice (25–35 days old) were randomly assigned to each

of six treatment groups and adapted to an 18-hr water deprivation schedule. Adaptation was followed immediately by a four-day pretreatment period. On each pretreatment day, all animals were given 3-hr access to tapwater in 10-ml syringes fitted with sipper tubes. At the end of the 3-hr period, ingestion was measured, and the mice were permitted an additional 3-hr ad libitum access to water. Water tubes were then removed from cages, and animals were deprived until the following day.

A four-day treatment period immediately followed pretreatment. Treatment procedures were similar to those described for pretreatment, except that five groups were presented with their respective compounds in aqueous solution during the 3-hr measurement period. We continued to give the sixth group plain tapwater as a control.

Analysis. A three-way analysis of variance (ANOVA) with repeated measures over periods (two levels) and days (four levels) was used to assess the results (Winer, 1971). Chemical (six levels including the control group) was the independent factor in this analysis. In addition, for each chemical, a two-way repeated measures ANOVA was used to test for period and day effects. In all cases, Tukey tests (Winer, 1971, p. 201) were used to isolate differences among means ($P < 0.05$).

We also tested whether intake of treated water differed from a theoretical value of zero ingestion. This analysis required a slight modification in calculation of the treatment sums of squares, where the grand mean was replaced by zero and the degrees of freedom reflected the number of treatments considered in the experiment. Estimates of the error term remained the same as in a standard ANOVA.

RESULTS

Mice responded differently for each chemical across time (Figure 1) ($F = 4.5$, df 12, 165, $P < 0.001$). Relative to the pretreatment period, intake of all chemicals declined (AMAP: $F = 194.26$, df 1, 11, $P < 0.001$; MA: $F = 478.30$, df 1, 11, $P < 0.001$; OAP: $F = 736.55$, df 1, 11, $P < 0.001$; MAP: $F = 151.78$, df 1, 11, $P < 0.001$; VA: $F = 442.91$, df 1, 11, $P < 0.001$), with the exception of the control group, for which intake during the pretreatment and treatment periods was equal ($F = 1.79$, df 1, 7, $P = 0.223$). Intake of AMAP decreased across treatment days ($F = 2.88$, df 3, 33, $P = 0.051$), while mice increased their intake of MA, MAP, and VA ($F = 4.23$, df 3, 33, $P = 0.012$; $F = 12.94$, df 3, 33, $P < 0.001$; $F = 2.95$, df 3, 33, $P = 0.047$, respectively). Ingestion of OAP and water ($F = 0.72$, df 3, 33, $P = 0.549$; $F = 0.49$, df 3, 21, $P = 0.69$, respectively) remained constant across days.

OAP was the only chemical that reduced intake to a level not significantly different from zero ($F = 4.34$, df 1, 11, $P = 0.061$).

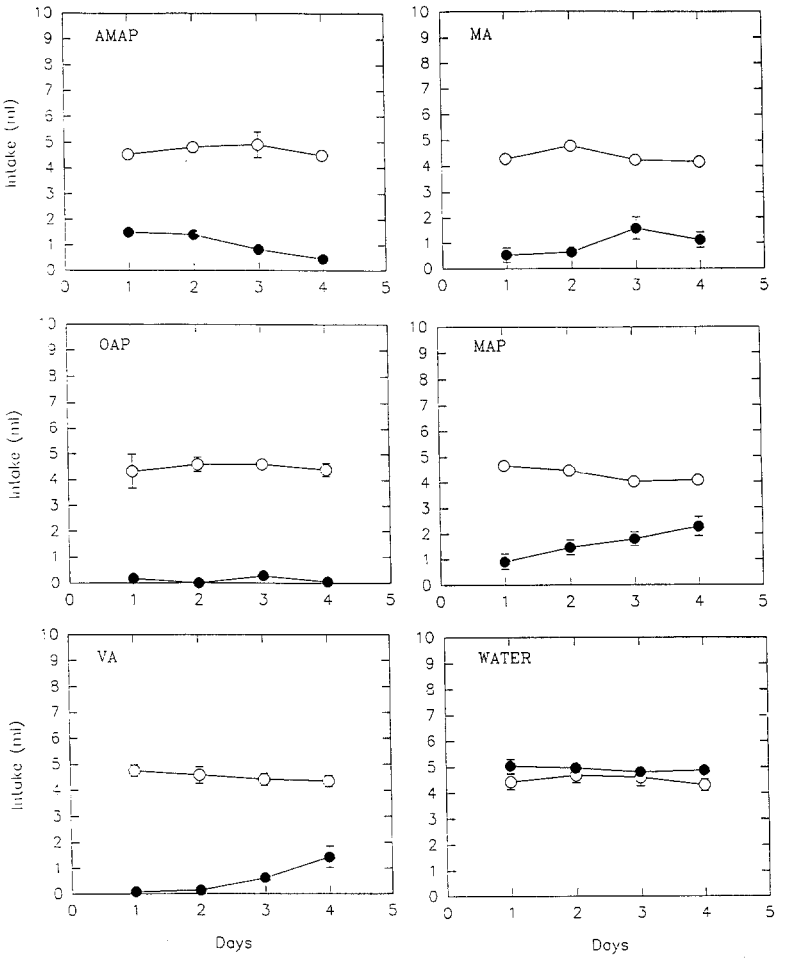

FIG. 1. Intake by mice of water during a four-day pretreatment period (open circles) and subsequent intake of 2-amino-4′,5′-methoxyacetophenone (AMAP), methyl anthranilate (MA), orthoaminoacetophenone (OAP), 2-methoxyacetophenone (MAP), veratryl amine (VA), and water (control) during a four-day treatment period (solid circles).

DISCUSSION

All five chemicals substantially reduced intake relative to pretreatment levels, although MA, MAP, and VA showed signs of habituation (i.e., animals ingested more of these substances on the last day than the first day of treatment). Intake of these chemicals on the last day of the treatment period, however, was still substantially below levels of water drunk during the pretreatment period.

The most repellent material in the present experiment was OAP. This material effectively eliminated ingestion. The fact that the material appears substantially more repellent than MA is consistent with evidence showing that OAP is superior to MA as a bird repellent (Mason et al., 1991b).

Decreased ingestion of AMAP over time suggests that avoidance of this chemical involved learning. In other words, its effectiveness may depend partly on sensory factors and partly on food avoidance conditioning based on post-ingestional malaise.

Overall, the present results were surprising. At least one of the repellents (i.e., MA) is palatable to humans and livestock (Furia and Bellanca, 1975, p. 346; Glahn et al., 1989), and a prior report indicated that it is accepted by deer mice (Schafer and Bowles, 1985). Clearly, the notion that bird repellents are generally palatable to mammals is too broad. At least some bird repellents are offensive to at least some mammals.

Management Implications. While we are cautious about extrapolating from the laboratory to the field, the present results have clear practical implications. For example, one or another of the substances tested in the present experiment might be used as an additive to granular agricultural chemicals to reduce the hazards that these substances present to birds and rodents. In addition, they might be used as rodent and bird repellent seed treatments or livestock feed additives. Both laboratory (Mason et al., 1989) and field (Glahn et al., 1989) data suggest that anthranilate derivatives are effective bird repellent feed additives. The possibility that these materials also repel rodents would make them even more beneficial. Finally, we speculate that the repellents tested here might be incorporated into packaging, fabrics, and plastics to prevent damage by gnawing to electrical cables, containers, and other products where repellency must be instantaneous (i.e., no amount of damage is acceptable). All of the repellents tested in the present experiment appeared to have such immediate effects (i.e., they were offensive at first contact). Pilot tests in our laboratory suggest that OAP, MA, and VA do prevent gnawing of electrical cable, at least in short-term tests.

REFERENCES

BEAUCHAMP, G.K., and MASON, J.R. 1991. Comparative hedonics of taste, pp. 159–183, *in* R.C. Bolles (ed.). The Hedonics of Taste. Lawrence Erlbaum Assoc., Hillsdale, New Jersey.

BROOKS, J.E., AHMAD, E., HUSSIAN, I., MUNIR, S., and KHAN, A.A. 1990. A Training Manual on Vertebrate Pest Management. Pakistan Agricultural Research Council, Islamabad, Pakistan. 206 pp.

CASTRALE, J.S. 1987. Impacts of conservation tillage practices on farmland wildlife in southeastern Indiana. Indiana Statewide Wildlife Research Report No. W-26-R-18. 28 pp.

CLARK, L., and SHAH, P.S. 1991. Nonlethal bird repellents: In search of a general model relating repellency and chemical structure. *J. Wildl. Manage.* 55:538–545.

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. Exp. Zool.* 260:310–322.

FURIA, T.E., and BELLANCA, N. 1975. Handbook of Flavor Ingredients. CRC Press, Cleveland, Ohio. p. 771.

GLAHN, J.F., MASON, J.R., and WOODS, D.R. 1989. Dimethyl anthranilate as a bird repellent in livestock feed. *Wildl. Soc. Bull.* 17:31–320.

GRATZ, N.G. 1988. Rodents and human disease: A global appreciation, pp. 101–170, *in* I. Prakash (ed.). Rodent Pest Management. CRC Press, Boca Raton, Florida.

KARE, M.R. 1961. Bird repellent. U.S. Patent 2,967,128.

KARE, M.R., and PICK, H.L. 1960. The influence of the sense of taste on feed and fluid consumption. *Poult. Sci.* 39:697–706.

MARSH, R.E. 1988. Rodent problems on the North American continent, pp. 1–12, *in* I. Prakash (ed.). Rodent Pest Management. CRC Press, Boca Raton, Florida.

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.

MASON, J.R., BEAN, N.J., SHAH, P.S., and CLARK, L. 1991a. Taxon-specific differences in responsiveness to capsaicin and several analogues: Correlates between chemical structure and behavioral aversiveness. *J. Chem. Ecol.* 17:2539–2550.

MASON, J.R., CLARK, L., and SHAH, P.S. 1991b. Orthoaminoacetophenone repellency to birds: Similarities to methyl anthranilate. *J. Wildl. Manage.* 55:334–340.

MASON, J.R., CLARK, L., and SHAH, P.S. 1991c. Taxonomic differences between birds and mammals in their responses to chemical irritants, pp. 290–296, *in* D. Müller-Schwarze, R. Silverstein, and D. Doty (eds.). Chemical Signals in Vertebrates, VI. Plenum Press, New York.

MEEHAN, A.P. 1988. Chemical repellents, pp. 399–406, *in* I. Prakash (ed.). Rodent Pest Management. CRC Press, Boca Raton, Florida.

SALMON, T.P. 1988. Evaluating rodenticide use impacts on agricultural production, pp. 115–127, *in* S.A. Shumake and R.W. Bullard (eds.). Vertebrate Pest Control and Management Materials: 5th Volume. American Society for Testing and Materials, Philadelphia, Penn.

SCHAFER, E.W., and BOWLES, W.A. 1985. Acute oral toxicity and repellency of 933 chemicals to house and deer mice. *Arch. Environ. Contam. Toxicol.* 14:111–129.

TIMM, R.M. 1982. An IPM approach to rodent control on midwestern farms, pp. 147–150, *in* R.E. Marsh (ed.). Proceedings of the Tenth Vertebrate Conference, University of California, Davis.

WINER, B.V. 1971. Statistical Principles in Experimental Design. McGraw-Hill, New York, 907 pp.

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SUGAR BEET CROWN BORER, *Hulstia undulatella* (CLEMENS)[1]: IDENTIFICATION AND FIELD TESTS OF FEMALE SEX PHEROMONE GLAND COMPONENTS

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Abstract—Electroantennogram profiles of saturated and monounsaturated 12-, 14-, and 16-carbon acetates, and 12- and 14-carbon alcohols implicated (*Z*)-9-tetradecen-1-ol acetate (Z9-14:Ac) as a component of the female sex pheromone of *Hulstia undulatella* (Clemens). Gas chromatography–mass spectrometric analysis of extract of the female sex pheromone glands showed the presence of Z9-14:Ac (8.5 ng/female), (*Z*)-9-tetradecen-1-ol (Z9-14:OH), and (*Z*)-11-hexadecen-1-ol acetate (Z11-16:Ac) in a ratio of 100:4:21, respectively. In tests in sugar beet fields, Z9-14:Ac alone produced some trap catch. Addition of Z9-14:OH did not increase catch while addition of Z11-16:Ac eliminated catch, but addition of both Z9-14:OH and Z11-16:Ac increased catch sevenfold. A combination of Z9-14:OH and Z11-16:Ac without Z9-14:Ac did not produce trap catch. A lure of 200 μg Z9-14:Ac + 16 μg Z9-14:OH + 42 μg Z11-16:Ac is suggested for use in monitoring traps.

Key Words—Lepidoptera, Pyralidae, Phycitinae, *Hulstia undulatella*, sex pheromone, (*Z*)-9-tetradecen-1-ol acetate, (*Z*)-9-tetradecen-1-ol, (*Z*)-11-hexadecen-1-ol acetate.

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[1]Lepidoptera: Pyralidae: Phycitinae.

INTRODUCTION

The sugar beet crown borer, *Hulstia undulatella* (Clemens), occurs throughout North America but is only an economically important pest in the West (Essig, 1926; Grote, 1878) where it attacks sugar beets, spinach, and broccoli (Hawley, 1923). *H. undulatella* was first reported as an important pest of sugar beets in California, Washington, and Oregon in 1904. Since that time it has been a sporadically important pest where commercial sugar beets are produced (Lange, 1950). Most recently it has been a problem in Idaho where infestation levels have reached 30–40% of crop stand (Baird et al., 1989).

The larvae feed on the crown and leaf bases. First-generation larvae may cause severe stand mortality. Second-generation larvae do not harm healthy, vigorous beets directly, but create an opportunity for secondary bacterial infection and fungal growth in harvested beets, which results in decomposition and subsequent heating during storage in beet piles (Baird et al., 1989).

We undertook a study of the sex pheromone of *H. undulatella* in order to develop a lure that could be used to further study this insect and to be a monitor for control programs. This project was part of a terminated program at this laboratory. It was necessary to forego some of the experiments that would logically have been dictated. Nevertheless, the study was complete enough so that we can report the identification of three essential components of the sex pheromone, which provide an effective lure for detecting and monitoring population changes of this pest.

METHODS AND MATERIALS

Insects. H. undulatella hibernaculae containing prepupae from a second (summer) generation of borers were collected from soil samples in an infested sugar beet field near Nyssa, Oregon, during July 1989. Voucher specimens from this study have been placed in the James Entomology Collection, Washington State University at Pullman. Hibernaculae were placed in a clear plastic shoe box (31 × 17 × 9 cm) containing 5–6 cm of soil collected from the field. The box was covered with a clear plastic lid and held in a rearing room at 21°C, 55–60% relative humidity, and at a 15:9 hr light–dark cycle. Emergent adults (ca. 30 total) were collected daily 1 hr before scotophase and placed in individual glass vials (9 × 2 cm).

Collection of Pheromone. Females moths (3–4 days old) in their vials were collected 1 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 were severed and steeped for 15–30 min in dichloromethane. Then the solution of gland extract was removed with a syringe.

Electroantennogram (EAG) Determinations. EAG profiles (Roelofs, 1984) of model compounds were made with a previously described apparatus (McDonough et al., 1980) and 60-μg charges were used. Duplicate determinations were made for each compound. The model compounds used were synthesized by the procedure of Voerman (1988). 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 monounsaturated 12-, 14-, and 16-carbon acetates, and the corresponding 12- and 14-carbon alcohols.

Gas Chromatography–Mass Spectrometry (GC-MS). A Hewlett-Packard (Avondale, Pennsylvania) gas chromatograph (model 5790) with a quadrapole mass spectrometer (model 5970) was equipped with a DB-Wax capillary column, 60 m × 0.25 mm ID (J&W Scientific, Folsom, California). For analysis of sex pheromone gland extract, the column was held at 80°C for 2 min, programmed at 20 min to 200°C, and maintained isothermally thereafter.

Field Tests. Z9–14:Ac, Z9–14:OH, and Z11–16:Ac used for field tests were obtained from commercial sources and were pure by GC analysis and contained less than 1% of the geometrical isomer. Candidate lures in 100 μl of dichloromethane were impregnated into red rubber septa (The West Co., Phoenixville, Pennsylvania); dichloromethane alone was added to control septa. Pherocon 1C sticky traps (Tréce 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 sugar beet foliage, usually 30–60 cm high, where it was believed that most flight activity occurred. In April 1990 male flight activity in response to synthetic lures was observed about 1 hr before dusk and lasted about 2 hr; another flight in response to synthetic lures occurred the following morning at 1100 hr and continued until 1400 hr.

The position of each trap in the experimental plot was drawn randomly and traps were placed at approximately 10-m intervals. Each test lure was replicated four or six times, 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

EAG Profiles. Table 1 summarizes the EAG studies. Z9–14:Ac produced the strongest response of the tested compounds, and Z9–12:Ac and Z7–12:Ac produced responses almost equivalent. Based on these responses, one or more of these three compounds are likely to be pheromone components. Minor pheromone components often do not produce strong EAG responses (Kamm and

TABLE 1. SUMMARY OF ELECTROANTENNOGRAM PROFILES EXPRESSED AS PERCENT OF REFERENCE COMPOUND, Z9-14:Ac[a]

Compound series	Compounds with strongest response (%)	Response range of remaining compounds in series (%)
14:Ac	Z9-14:Ac, 100	29-70
	E9-14 Ac, 75	
12 Ac	Z9-12 Ac, 99	24-78
	E9-12 Ac, 83	
	Z7-12 Ac, 92	
16:Ac	Z9-16:Ac, 61	4-38
14:OH	Z9-14:OH, 58	0-54
	E9-14:OH, 58	
12:OH	Z7-12:OH, 78	12-57

[a]Each "compound series" includes saturated and all monounsaturated derivatives.

McDonough, 1980), and therefore these results do not rule out any of the other tested compounds as possible pheromone components.

GC-MS Analysis. A GC-MS analysis of extract of eight female sex pheromone glands of *H. undulatella* on a polar liquid phase (DB-Wax) produced the total ion monitor chromatogram shown in Figure 1. The analysis covered the volatility range of the 12-, 14-, and 16-carbon acetates and alcohols. The most abundant material (peak B, Figure 1) had the same retention time (18.16 min) and mass spectrum as Z9-14:Ac (M−60 = 194; M−60-28 = 166; $CH_3CO_2H_2$ = 61). The amount of Z9-14:Ac corresponded to 8.5 ng/female. The peak at 20.74 min (peak C, Figure 1) had the same retention time and mass spectrum as Z9-14:OH (M−18 = 194; M−18-28 = 166), and the peak at 26.81 min (peak D, Figure 1) had the same retention time and mass spectrum as Z11-16:Ac (M−60 = 222; M−60-28 = 194; $CH_3CO_2H_2$ = 61). These retention times were repeatable to ±0.02 min and were unique to each isomer of identified configuration and position. Thus these compounds were identified unambiguously. Other peaks in the chromatogram were saturated hydrocarbons or benzenoid compounds except for the peak at 17.00 min (peak A, Figure 1). This latter peak had a retention time identical to 14:Ac and its mass spectrum contained characteristic peaks of 14:Ac (M−60 = 196; M−60-28 = 168; $CH_3CO_2H_2$ = 61), but also contained peaks at 165 and 180 and other smaller, spurious peaks that were not in the standard. The ratio of the four compounds, Z9-14:Ac/Z9-14:OH/Z11-16:Ac/14:Ac was 100:4:21:11.

Field Tests. Field tests of the three identified monoenes were conducted to determine if these compounds could evoke trap catch. In Table 2 (first test),

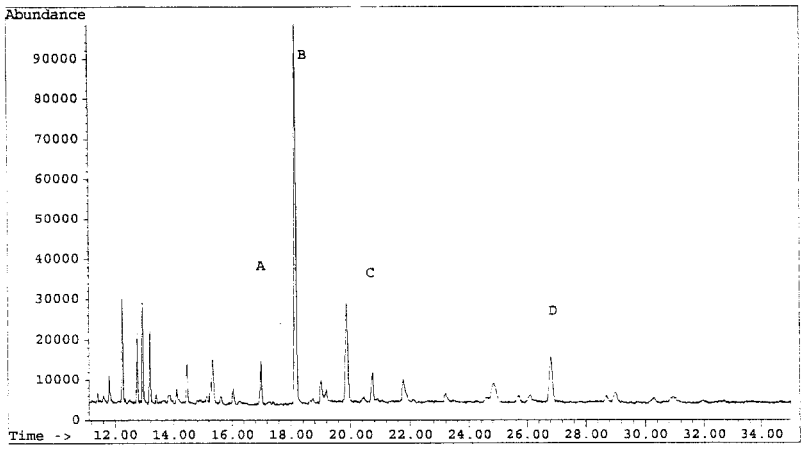

FIG. 1. Gas chromatography–mass spectrometric analysis of extract of eight female sex pheromone glands of *H. undulatella*. Ordinate is total ion abundance; abscissa is in minutes. A, B, C, and D indicate compounds that were candidates as sex pheromone components (see text).

three compositions were compared. Z9–14:Ac and Z9–14:Ac + Z9–14:OH produced significant and equivalent catches, while Z9–14:Ac + Z9–14:OH + Z11–16:Ac produced catches about seven times that produced by the other two lures. In the second test (Table 2), Z9–14:OH + Z11–16:Ac produced no catch and again the three-component lure caught large numbers. In the third and last test (Table 2), Z9–14:Ac + Z11–16:Ac produced catches equivalent to blank traps while the three-component lure again caught large numbers. Clearly, Z9–14:Ac alone is an attractant and Z9–14:OH + Z11–16:Ac together are not. The addition of Z9–14:OH to Z9–14:Ac did not increase trap catch, whereas the addition of Z11–16:Ac to Z9–14:Ac reduced trap catch to that of the controls. Nevertheless, a dramatic sevenfold increase in trap catch occurred when both Z9–14:OH and Z11–16:Ac were added to Z9–14:Ac.

In Table 3 (test 1), various dosages of the three-component lure at the ratios found in the gland extract are compared. All doses produced significant catch and, except for the lowest dose (10 μg Z9–14:Ac), produced catches equivalent to each other. In test 2 (Table 3), the effect of varying proportions of Z9–14:OH were evaluated. At ratios of Z9–14:Ac to Z9–14:OH of 100:4, 100:8, and 100:16 catches were equivalent to one another and superior to catches from ratios of 100:2 and 100:1. Z9–14:OH is the most volatile component (McDonough, 1991) and, because amounts of Z9–14:OH higher than the ratio found in the female sex pheromone gland did not decrease catch, the field life of the lure can be increased by using a ratio higher than the gland ratio. These

TABLE 2. CAPTURE OF MALE *H. undulatella* WITH VARIOUS COMBINATIONS OF THREE FEMALE SEX PHEROMONE GLAND COMPONENTS

Test	Test period	Lure components	Dose (μm)	Cumulative captures and significance
1	Mar. 30–Apr. 2, 1990	Z9-14:Ac	100	72a
	(4 replicates)	Z9-14:Ac + Z9-14:OH + Z11-16:Ac	100 + 4 + 14.5	490b
		Z9-14:Ac + Z9-14:OH	100 + 4	73a
		Blanks		1c
2	Apr. 3–5, 1990	Z9-14:Ac + Z9-14:OH + Z11-16:Ac	100 + 4 + 21	540a
	(4 replicates)	Z9-14:OH + Z11-16:Ac	4 + 21	0b
		Blanks		0b
3	Jul. 24–Aug. 13, 1991	Z9-14:Ac + Z11-16:Ac	300 + 21	4a
	(6 replicates)	Z9-14:Ac + Z11-16:Ac	300 + 63	4a
		Z9-14:Ac + Z11-16 Ac	300 + 210	2a
		Z9-14:Ac + Z11-16 Ac	300 + 630	8a
		Z9-14:Ac + Z9-14:OH + Z11-16:Ac	100 + 4 + 21	512b
		Blanks		3a

TABLE 3. CAPTURE OF MALE *H. undulatella* IN TRAPS BAITED WITH VARIOUS DOSAGES AND RATIOS OF FEMALE SEX PHEROMONE GLAND COMPONENTS

Test	Test period	Lure components	Dose (μg)	Cumulative captures and significance
1	Apr. 3–5, 1990 (4 replicates)	Z9–14:Ac + Z9–14:OH + Z11–16:Ac	10 + 0.4 + 2.1	115a
		Z9–14:Ac + Z9–14:OH + Z11–16:Ac	30 + 1.2 + 6.3	226b
		Z9–14:Ac + Z9–14:OH + Z11–16:Ac	100 + 4.0 + 21	409b
		Z9–14:Ac + Z9–14:OH + Z11–16:Ac	300 + 12 + 63	397b
		Z9–14:Ac + Z9–14:OH + Z11–16:Ac	0 + 0 + 0	1c
2	Aug. 14–21, 1991 (6 replicates)	Z9–14:Ac + Z9–14:OH + Z11–16:Ac	200 + 2 + 42	56a
		Z9–14:Ac + Z9–14:OH + Z11–16:Ac	200 + 4 + 42	71a
		Z9–14:Ac + Z9–14:OH + Z11–16:Ac	200 + 8 + 42	160b
		Z9–14:Ac + Z9–14:OH + Z11–16:Ac	200 + 16 + 42	170b
		Z9–14:Ac + Z9–14:OH + Z11–16:Ac	200 + 32 + 42	234b
		Z9–14:Ac + Z9–14:OH + Z11–16:Ac	0 + 0 + 0	0c

data indicate the most effective lure of these three components for monitoring populations would consist of 200 μg Z9–14:Ac + 16 μg Z9–14:OH + 42 μg Z11–16:Ac. Because of the small number of *H. undulatella* available, the potency of this lure relative to female-baited traps was not tested. Nevertheless, the lure appears to be reasonably potent. For example, one of us (D.D.B.) conducted monitoring tests at sites in Idaho and Oregon in 1991. The tests revealed three flights corresponding to the three known generations. At the peak of the flights, captures of 45 males/trap-day were common. These data are being incorporated into a pest management program under development by Dr. Edward Bechinski at the University of Idaho.

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REFERENCES

BAIRD, C.R., BOWERS, D.D., STOLTZ, R.L., and JOHNSTON, R.L. 1989. The sugarbeet crown borer in Idaho. University of Idaho, Cooperative Extension Service Information Series No. 845. 2 pp.

DUNCAN, O.B. 1955. Multiple range and multiple *F* tests. *Biometrics* 11:1–41.

ESSIG, E.O. 1926. Insects of Western North America. MacMillan, New York, p. 711.

GROTE, A.R. 1878. Preliminary studies on the North American Pyralidae. U.S. Department of Interior Bulletin of the Survey, IV, No. 3. pp. 698–699.

HAWLEY, I.M. 1923. Notes on the insect pests of Utah. *J. Econ. Entomol.* 16(4):379.

KAMM, J.A., and MCDONOUGH, L.M. 1980. Synergism of the sex pheromone of the cranberry girdler. *Environ. Entomol.* 9:795–797.

LANGE, H.W. 1950. New sugarbeet pest. *Calif. Agric.* 4(5):5, 14.

MCDONOUGH, L.M. 1991. Controlled release of insect sex pheromones from a natural rubber substrate, pp. 106–124, *in* P.A. Hedin (ed.). Naturally Occurring Pest Bioregulators. ACS Symposium Series 449. American Chemical Society, Washington, D.C.

MCDONOUGH, L.M., KAMM, J.A., and BIERL-LEONHARDT, B.A. 1980. Sex pheromone of the armyworm, *Pseudaletia unipuncta* (Haworth) (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 6:565–572.

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-Verlaag, New York.

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.

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QUINOLIZIDINE ALKALOIDS IN *Genista acanthoclada* AND ITS HOLOPARASITE, *Cuscuta palaestina*

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Abstract—About 20 quinolizidine alkaloids were identified in *Genista acanthoclada* by capillary GLC and GLC-MS, such as sparteine, 11,12-dehydrosparteine, retamine, *N*-methylcytisine, cytisine, 17-oxosparteine, lupanine, α-isolupanine, 5,6-dehydrolupanine, 10-oxosparteine, *N*-carbomethoxycytisine, 17-oxoretamine, *N*-formylcytisine, *N*-acetylcytisine, and anagyrine. Its phloem-feeding holoparasite *Cuscuta palaestina* contained alkaloids too, such as sparteine, 11,12-dehydrosparteine, retamine, *N*-methylcytisine, cytisine, 17-oxosparteine, lupanine, *N*-carbomethoxycytisine, and anagyrine. Whereas sparteine, retamine, 17-oxosparteine, and cytisine are the main alkaloids of *G. acanthoclada*, lupanine, cytisine, *N*-methylcytisine, and anagyrine are abundant and enriched in *C. palaestina*. Since these alkaloids figure as antiherbivoral chemical defense compounds in *Genista*, it is assumed that the parasite can exploit the acquired allelochemicals for its own protection.

Key Words—*Genista acanthoclada*, holoparasite, *Cuscuta palaestina*, alkaloid sequestration, phloem transport, chemical defense.

INTRODUCTION

Hemi- and holoparasitic plants exploit their host plants in many ways: Both rely on water and inorganic ions that are transported in the vascular tissues. Whereas hemiparasitic plants partly use their own photosynthesis, the nonphotosynthetic

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holoparasites take all essential nutrients (i.e., amino acids and sugars) from their host plants (Steward and Press, 1990).

Plants have to defend themselves against herbivores and microorganisms. The production of chemical defense compounds or allelochemicals is a major means in this context (Harborne, 1988; Rosenthal and Janzen, 1979; Swain, 1977; Wink, 1988, 1992a–c).

Since parasitic and hemiparasitic plants are also endangered by herbivores and microorganisms, the question is whether and how they protect themselves. In general, the following alternatives exist: (1) no defense of their own, but relying on the defenses of the respective host plant; (2) mechanical protection by thorns, spines, or trichomes; (3) utilization of chemical defense by production of indigenous defense compounds and/or by sequestration of the allelochemicals from the host plant.

Genista acanthoclada DC. is a perennial scrub that inhabits arid and semi-arid coastal places of the eastern Mediterranean (Greece and the Aegean region) and often forms large associations. The aerial parts of *G. acanthoclada* consist of prickly spines, which certainly have an antiherbivore function. In addition, this species produces quinolizidine alkaloids, which are known herbivore deterrents and toxic allelochemicals (Wink 1985, 1987, 1988, 1992a,b). At the natural habitat in Crete, we could not observe any adverse effects by herbivory even though goats and sheep are abundant.

In eastern Crete we found many specimens of *G. acanthoclada* that were parasitized by *Cuscuta palaestina* Boiss., which formed thick mats on top of the broom. *Cuscuta* is known to be a predominantly phloem-feeding holoparasite.

In this study we analyzed whether *Cuscuta palaestina* is able to exploit the defense chemicals of its host plant, i.e., whether it takes up and stores them as acquired defense compounds.

METHODS AND MATERIALS

Plants. Aerial parts of *G. acanthoclada* DC. were collected in eastern Crete near Sitia in August 1991. *Cuscuta palaestina* Boiss. were manually picked from the broom host plants and stored separately.

Alkaloid Isolation and Analysis by Capillary GLC and GLC-MS. Plant material was homogenized in 0.5 M HCl and left standing overnight. Then the homogenate was made alkaline with ammonia (to pH 12) and poured onto a standard Chemelut column for solid–liquid extraction with CH_2Cl_2. The alkaloid-containing eluate was concentrated in vacuo and analyzed by high-resolution gas chromatography (HRGC) and GLC-MS. HRGC was performed with a gas chromatograph employing a capillary column (DB-5, 15 m $\times$ 0.258 mm,

0.1-μm film thickness; J&W Scientific, Folsom, California). GC conditions were: injector, 250°C; detector, 300°C; oven, 150°C; 1 min isothermal, 150–300°C with 15°C/min, at 300°C 10 min isothermal. GC-MS was performed with a Carlo-Erba 5160 GC and a Finnigan MAT 4515 quadropole mass spectrometer. EI-MS spectra were recorded at 40 eV and evaluated with the INCOS data system. Sparteine, lupanine, or cytisine were used as external standards for quantification.

RESULTS AND DISCUSSION

Quinolizidine Alkaloids in Genista acanthoclada. Alkaloid extracts of aerial parts of *Genista acanthoclada* were separated by capillary GLC and GC-MS (Figure 1), which allow the separation and identification of complex QA mixtures (reviewed in Wink, 1992c).

Since most of the alkaloids present had been analyzed before in our laboratories (Wink, 1992c; Wink et al., 1981, 1983, 1991; Wink and Witte, 1991; Wink and Römer, 1986), we could identify the following QA unambiguously according to their Kovats retention indices and mass spectra (Table 1). Thus sparteine, retamine, 17-oxosparteine, and cytisine can be considered as major and α-isosparteine, 11,12-dehydrosparteine, 17-oxoretamine, 5,6-dehydrolupanine, lupanine, α-isolupanine, *N*-methylcytisine, *N*-carbomethoxycytisine, *N*-formylcytisine, *N*-acetylcytisine, and anagyrine as minor components of the green parts of *G. acanthoclada*. A few minor alkaloids could not be identified with certainty, such as the dehydrosparteines (compounds 3, 4, and 6) and oxosparteines (10 and 15) (Tables 1 and 2) due to the scarcity of the material. Only cytisine, *N*-methylcytisine, anagyrine, retamine, and lupanine have been described for *G. acanthoclada* (Faugeras and Paris, 1971).

Quinolizidine Alkaloids in Cuscuta palaestina *Boiss.* About 1 g of dried plant material of *Cuscuta palaestina*, consisting of sprigs and flowers, which were definitely free of host-plant tissues, was extracted and analyzed by HRGC and GC-MS. As can be seen from Figure 1B, *Cuscuta palaestina* sequesters some of the alkaloids that are also present in the host plant. We assume that these alkaloids were not made by *Cuscuta palaestina* indigenously but were taken up from its host plant by phloem-feeding, since the overall pattern of alkaloids is rather similar. *Cuscuta reflexa* and *C. platyloba*, which were kept on *Lupinus albus* as a host plant were analyzed recently (Czygan et al., 1988; Bäumel et al., 1991). In this case an uptake of QA from the host plant was also observed. This is in agreement with earlier studies showing that QA are transported in the phloem (Wink, 1992a–c; Wink and Witte, 1991).

Major alkaloids of *C. palaestina* are cytisine-type alkaloids (such as cytisine, *N*-methylcytisine, and anagyrine), which show strong antiherbivore activ-

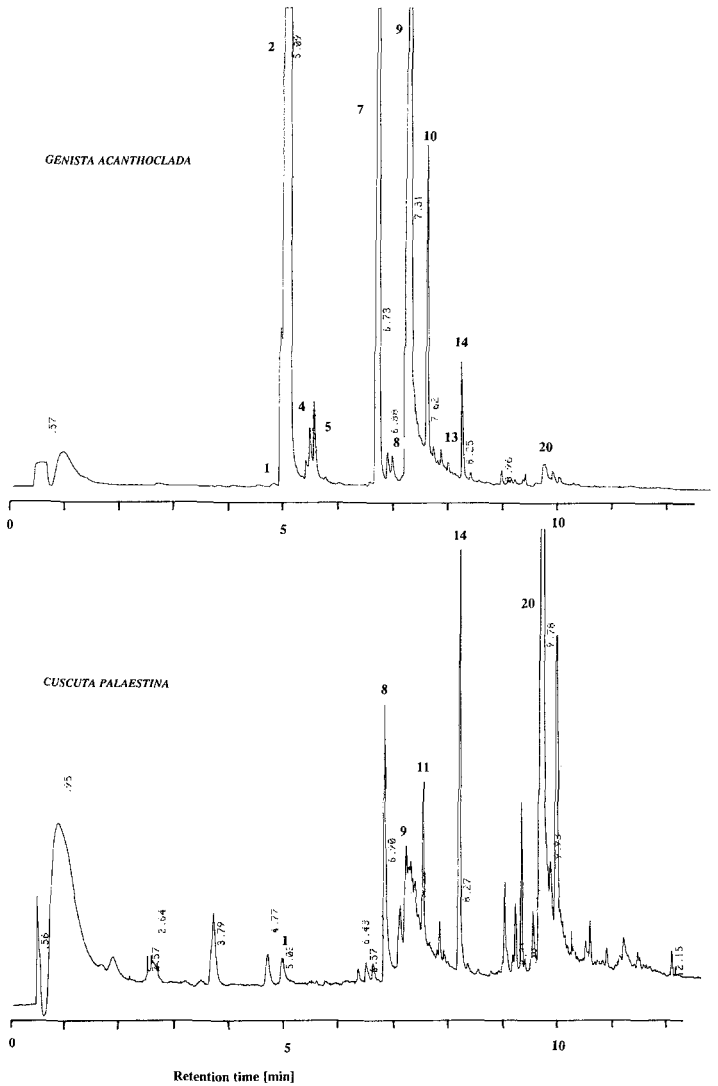

FIG. 1. Separation of alkaloid extracts from *Genista acanthoclada* and its holoparasite *Cuscuta palaestina* by capillary GLC (nitrogen-specific detection) Numbering is according to Table 1.

ities, e.g., cytisine activates acetylcholine receptor-coupled ion channels of neurons (Wink, 1992a–c). The cytisine level found in *Cuscuta* should be high enough to provide significant antiherbivore protection.

Considering the survival strategies mentioned in the introduction, it is likely

TABLE 1. IDENTIFICATION OF QUINOLIZIDINE ALKALOIDS FROM *Genista acanthoclada* AND *Cuscuta palaestina* BY GC-MS

Alkaloid	RI	M+	5 abundant ions (abundance %)
1. α-Isosparteine	1710	234	234(25) 193(15) 137(50) 98(100) 84(20)
2. Sparteine	1785	234	234(20) 193(30) 137(100) 98(100) 84(20)
3. Dehydrosparteine	1810	232	232(50) 191(15) 148(30) 134(100) 98(95)
4. Dehydrosparteine	1825	232	232(65) 189(30) 148(30) 134(90) 98(100)
5. 11,12-Dehydrosparteine	1840	232	232(35) 191(5) 148(20) 134(100) 97(85)
6. Dehydrosparteine	1855	232	232(45) 191(10) 148(20) 134(40) 98(100)
7. Retamine	1940	250	250(5) 232(20) 207(25) 134(35) 98(100)
8. *N*-Methylcytisine	1952	204	204(15) 146(5) 58(100)
9. Cytisine	1990	190	190(65) 160(25) 147(80) 146(100) 134(25)
10. Oxosparteine	2015	248	248(25) 229(10) 150(35) 135(25) 97(100)
11. 17-Oxosparteine	2070	248	248(40) 220(20) 136(40) 110(75) 97(100)
12. α-Isolupanine	2105	248	248(20) 149(30) 136(70) 98(30) 57(100)
13. 5,6-Dehydrolupanine	2128	246	246(12) 134(12) 98(100) 97(35) 84(10)
14. Lupanine	2165	248	248(30) 149(50) 136(100) 110(20) 98(25)
15. 10-Oxosparteine	2180	248	248(45) 220(15) 136(95) 110(60) 97(100)
16. *N*-Carbomethoxycytisine	2240	248	248(25) 160(15) 146(100) 102(90) 58(50)
17. 17-Oxoretamine	2250	264	264(25) 247(80) 136(25) 98(100) 97(75)
18. *N*-Formylcytisine	2310	218	218(50) 190(5) 160(20) 146(100) 134(15)
19. *N*-Acetylcytisine	2320	232	232(15) 218(30) 190(5) 160(20) 146(100)
20. Anagyrine	2382	244	244(20) 136(20) 122(20) 98(100)

TABLE 2. ALKALOID PROFILES OF *Genista acanthoclada* AND ITS PARASITE *Cuscuta palaestina*

	Alkaloid composition (total alkaloids = 100%)		
		C. palaestina	
Alkaloid	*G. acanthoclada*	Sample 1	Sample 2
1. α-Isosparteine	tr		
2. Sparteine	46.1	tr	tr
3. Dehydrosparteine	0.2		tr
4. Dehydrosparteine	0.9		tr
5. 11,12-Dehydrosparteine	1.2		tr
6. Dehydrosparteine	tr		
7. Retamine	16.6		tr
8. *N*-Methylcytisine	0.4	7.5	5.9
9. Cytisine	25.3	33.1	12.1
10. Oxosparteine	tr		
11. 17-Oxosparteine	4.9		4.9
12. α-Isolupanine	tr		
13. 5,6-Dehydrolupanine	tr		
14. Lupanine	1.2	25.4	7.2
15. 10-Oxosparteine	tr		
16. *N*-Carbomethoxycytisine	tr	tr	
17. 17-Oxoretamine	tr		
18. *N*-Formylcytisine	tr		
19. *N*-Acetylcytisine	tr		
20. Anagyrine	0.7	24.9	56.1
Alkaloid content (μg/g dry weight)	3560	ND	680

that *Cuscuta* exploits the allelochemicals of its host plant as acquired defense chemicals. The exploitation of host plant-produced allelochemicals seems to be a general phenomenon in parasitic plants, examples are: uptake of QA by *Cuscuta reflexa* and *C. platyloba* from *Lupinus*, *Cytisus*, or *Spartium* host plants, respectively (Czygan et al., 1988; Bäumel et al., 1991); by *Orobanche rapum-genistae* from *Cytisus scoparius* (Wink et al., 1981); by *Pedicularis semibarbata* from *Lupinus fulcratus* (Stermitz et al., 1989); by *Viscum cruciatum* growing on *Lygos sphaerocarpa* (Cordero et al., 1989); of PA, QA, and iridoid glycosides by *Castilleja* species from *Lupinus*, *Senecio*, or *Penstemon* host plants (Stermitz et al., 1986; Stermitz and Harris, 1987).

A remarkable difference can be seen in the abundance of individual alkaloids in *G. acanthoclada* and *C. palaestina* (Table 2) in that sparteine, 11,12-dehydrosparteine, and retamine are missing in *Cuscuta* or are only present in traces, whereas *N*-methylcytisine, anagyrine, and lupanine are clearly enriched

(Table 2). Since sparteine and retamine are abundant in the same organ of the host plant parasitized by *C. palaestina* and since their phloem transport has been demonstrated in other instances (Wink and Witte, 1991), we assume that QA uptake and storage by *C. palaestina* is, in part, a specific process. In *Viscum cruciatum* from *Lygos sphaerocarpa* lupanine, anagyrine, cytisine, and *N*-methylcytisine were detected as well as retamine, whereas the host plant contained mainly retamine and sparteine (Cordero et al., 1989), showing a somewhat similar qualitative discrimination as found in our *C. palaestina* plants. QA do not pass biomembranes, such as the tonoplast, by simple diffusion but by carrier-mediated transport (Mende and Wink, 1987; Wink and Mende, 1987). In larvae of the pyralid moth, *Uresiphita reversalis*, which sequesters QA from its host plant, we observed a substantial chemical discrimination in that alkaloids of the 10-oxosparteine-type were not resorbed, but eliminated with the feces, whereas the more toxic cytisine and derivatives were stored. Since we can explain this discrimination in terms of a specific QA carrier in midgut epithelia (Wink et al., 1991), we would expect, in analogy, the presence of a selective alkaloid transporter (at least for *N*-methylcytisine and anagyrine) in the haustoria or other biomembranes of *C. palaestina*, which has not been described in biochemical terms so far. In addition, we cannot rule out that sparteine was taken up and metabolized by *Cuscuta* into lupanine and 17-oxosparteine. When comparing the QA profiles from *C. platyloba* and *L. albus*, it was found that 13-hydroxylupanine was enriched in *C. platyloba* whereas 13-*trans*-cinnamoyloxylupanine had decreased. Bäumel et al. (1991) explain this difference by hydrolysis of the ester alkaloids in *Cuscuta*. Thus, biotransformation could be a second factor besides selective transport to influence the alkaloid profiles of the parasite.

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REFERENCES

BÄUMEL, P., LURZ-GRESSER, G., VEEN, G., WITTE, L., PROKSCH, P., and CZYGAN, F.-C. 1991. Uptake of host-plant alkaloids by parasitic *Cuscuta* species. *Planta Med. Suppl.* 2:A95.

CORDERO, C.M., AYUSO, M.A., RICHOMME, P., and BRUNETON, J. 1989. Quinolizidine alkaloids from *Viscum cruciatum*, hemiparasitic shrub of *Lygos shaerocarpa*. *Planta Med.* 55:196.

CZYGAN, F.C., WESSINGER, B., and WARMUTH, K. 1988. *Cuscuta* and its ability to take up and to accumulate alkaloids of the host plant. *Biochem. Physiol. Pflanz.* 183:495–501.

FAUGERAS, G., and PARIS, R. 1971. Nouvelles recherches phytochimiques sur les Papilionacees-Genestees d'Europe. *Boissiera* 19:201–218.

HARBORNE, J.B. 1988. Introduction to Ecological Biochemistry, 3rd ed. Academic Press, New York.

MENDE, P., and WINK, M. 1987. Uptake of the quinolizidine alkaloid lupanine by protoplasts and isolated vacuoles of suspension-cultured *Lupinus polyphyllus* cells. Diffusion of carrier-mediated transport? *J. Plant Physiol.* 129:229–242.

ROSENTHAL, G.A., and JANZEN, D.H. 1979. Herbivores. Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

STERMITZ, F.R., and HARRIS, G.H. 1987. Transfer of pyrrolizidine and quinolizidine alkaloids to *Castilleja* (Scrophulariaceae) hemiparasities from composite and legume host plants. *J. Chem. Ecol.* 13:1917–1925.

STERMITZ, F.R., HARRIS, G.H., and JING, W. 1986. Iridoids and alkaloids from *Castilleja* (Scrophulariaceae) host plants for *Platyptilia pica*. Rhexifoline content of *P. pica*. *Biochem. Syst. Ecol.* 14:499–506.

STERMITZ, F.R., BELOFSKY, G.N., NG D., and SINGER, M.C. 1989. Quinolizidine alkaloids obtained by *Pedicularis semibarbata* (Scrophulariaceae) from *Lupinus fulcratus* (Leguminosae) fail to influence the specialist herbivore *Euphydryas editha* (Lepideoptera). *J. Chem. Ecol.* 15:2521–2530.

STEWARD, G.R., and PRESS, M.C. 1990. The physiology and biochemistry of parasitic angiosperms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 41:127–151.

SWAIN, T. 1977. Secondary compounds as protective agents. *Annu. Rev. Plant Physiol.* 28:479–501.

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. *ACS Symp. Ser.* 330:524–533.

WINK, M. 1988. Plant breeding: Importance of plant secondary metabolites for protection against pathogens and herbivores. *Theor. Appl. Genet.* 75:225–233.

WINK, M. 1992a. Roles of quinolizidine alkaloids in plant–insect interactions, Vol. IV. pp. 133–169, *in* E.A. Bernays (ed.). Focus on Insect–Plant Interactions. CRC Press, Boca Raton.

WINK, M. 1992b. Allelochemical properties or the raison d'etre of Alkaloids. *In* G. Cordell (ed.). The Alkaloids. In press. Academic Press, New York.

WINK, M. 1992c. Quinolizidine alkaloids. *In* Methods of Plant Biochemistry, Vol. 8. Academic Press, New York. 197–239 P. Waterman (ed.).

WINK, M., and MENDE, P. 1987. Uptake of lupanine by alkaloid-storing epidermal cells of *Lupinus polyphyllus*. *Planta Med.* 53:465–469.

WINK, M., and RÖMER, P. 1986. Acquired toxicity-the advantages of specializing on alkaloid-rich lupins to *Macrosiphum albifrons*. *Naturwissenschaften* 73:210–212.

WINK, M., and WITTE, L. 1991. Storage of quinolizidine alkaloids in *Macrosiphon albifrons* and *Aphis genistae* (Homoptera: Aphididae). *Entomol. Gener.* 15:237–254.

WINK, M., WITTE, L., and HARTMANN, T. 1981. Quinolizidine alkaloid composition of plants and of photomixotrophic cell suspension cultures of *Sarothamnus scoparius* and *Orobanche rapum-genistae*. *Planta Med.* 43:342–352.

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., MONTILLOR, C., BERNAYS, E.A., and WITTE, L. 1991. *Uresiphita reversalis* (Lepidoptera: Pyralidae): Carrier-mediated uptake and sequestration of quinolizidine alkaloids obtained from the host plant *Teline monspessulana*. *Z. Naturforsch.* 46c:1080–1088.

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TRAIL FOLLOWING AND RECRUITMENT: RESPONSE OF EASTERN TENT CATERPILLAR *Malacosoma americanum* to 5β-CHOLESTANE-3,24-DIONE AND 5β-CHOLESTAN-3-ONE

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Abstract—Studies were conducted to determine the relative effectiveness of 5β-cholestane-3,24-dione (diketone) and 5β-cholestan-3-one (monoketone) in eliciting trail following from eastern tent caterpillars, *Malacosoma americanum*. In Y maze tests, trails prepared from the monoketone were followed preferentially over diketone trails, even when the diketone trail was several orders of magnitude stronger. Under field conditions, colonies readily abandoned well-developed trail systems in favor of artificial trails that were established with the monoketone. Other tests in which the caterpillars selected trails prepared from the monoketone (but not the diketone) more often than their own recruitment trails indicate that the monoketone constitutes the chemical basis of recruitment communication in this insect. The study also shows that tent caterpillars are highly sensitive to small differences in the amount of monoketone in a trail and can distinguish between new and aged trails prepared from the compound.

Key Words—5β-Cholestane-3,24-dione, 5β-cholestan-3-one, trail following, recruitment, eastern tent caterpillar, *Malacosoma americanum*, Lepidoptera, Lasiocampidae.

INTRODUCTION

The trail system of the eastern tent caterpillar, *Malacosoma americanum*, consists of exploratory and recruitment components. Hungry caterpillars forage in groups and leave the tent in search of food several times a day. As they move over previously untraversed branches, the caterpillars lay down an exploratory trail by pressing the ventral surface of their last abdominal segment against the bark. These exploratory trails serve to hold the foraging column together and

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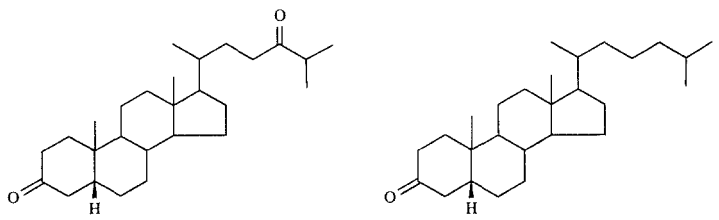

1 2

FIG. 1. (1) 5β-cholestane-3,24-dione, (2) 5β-cholestan-3-one.

enable the caterpillars to find their way back to the tent. Caterpillars that fail to find food, or those that do not feed to repletion, add little or no trail marker to the branches they follow as they return to the tent (Fitzgerald and Peterson, 1983). In contrast, successful foragers overmark their exploratory trails by dragging their sterna against the substrate as they return. Trails so marked by fed caterpillars are followed preferentially and serve to recruit hungry caterpillars to new food finds (Fitzgerald, 1976). Although some species of ants also use trail systems consisting of exploratory and recruitment components (Maschwitz and Mühlenberg, 1975; Chadab and Rettenmeyer, 1975; Topoff et al., 1980), it is currently unknown for any species how exploratory and recruitment trails differ.

Recent studies show that two steroids, 5β-cholestane-3,24-dione (diketone) and 5β-cholestan-3-one (monoketone) (Figure 1), are active components of the sternal secretion of the eastern tent caterpillar (Crump et al., 1987; Webster et al., in preparation). Both compounds elicit trail following from caterpillars when applied to narrow paper strips at rates of 10^{-12} g/mm of trail or greater and are competitive with authentic exploratory trails (Crump et al., 1987). The present paper reports the results of studies to assess the relative effectiveness of these two compounds in eliciting trail following. In addition, tests were conducted to determine how the caterpillars responded to aged trails prepared from the monoketone and to trails that differed only in the amount of the monoketone they contained.

METHODS AND MATERIALS

Commercial 5β-cholestan-3-one was obtained from Sigma Chemical Company; 5β-cholestane-3,24-dione was synthesized in the laboratory (Crump et al., 1987). Eastern tent caterpillars were hatched from eggs collected in the field and were fed the leaves of black cherry, *Prunus serotina*, to maintain them in the laboratory. Field studies were conducted in central New York State.

The relative effectiveness of the monoketone and several different concentrations of diketone in eliciting trail following behavior was assessed in Y maze studies. Mazes were cut from paper as described previously (Peterson and Fitzgerald, 1991). Monoketone samples were prepared by dissolving the chemical in hexanes and serially diluting the solution so that 5 μl applied to the 2-cm-long arm of a maze produced a concentration of 10^{-9} g of pheromone per millimeter of trail. Preliminary studies showed that this concentration readily elicited trail following when absorbed onto paper. Diketone solutions were similarly prepared to produce concentrations of 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} g/mm of trail. To conduct a test, the monoketone was applied to one of the randomly selected arms of the maze and the diketone at a given concentration to the other. The stems of the mazes were treated alternately with either the monoketone or diketone at the same concentration as was used on the arm. A second- or third-instar caterpillar was placed at the base of the stem and observed directly as it moved up the maze to determine the arm it selected. Each test comparing the monoketone to a given concentration of diketone was replicated with 20 caterpillars. Mazes were used once then discarded.

Tests were also conducted to determine the response of caterpillars to different concentrations of the monoketone applied to the alternate arms of a Y maze. Concentration ratios of 1:2, 1:4, 1:10, and 0.1:1 were tested using the same experimental protocol described above. A concentration of 1 corresponded to 10^{-9} g of monoketone per millimeter of trail.

Tests involving whole colonies reared in the open on wooden stands were conducted to assess the ability of the monoketone and diketone to compete with authentic recruitment trails. To obtain authentic recruitment trails, a colony was allowed to establish an exploratory trail on a narrow paper strip during a normal activity period. When the paper strip was well marked, 10 caterpillars were allowed to walk off of it onto a small twig bearing young leaves of black cherry. After feeding to repletion, the caterpillars returned immediately to the tent. They were observed under magnification as they crossed the paper strip to assure that they were laying down recruitment trails. After all of the caterpillars had returned, half of the strip bearing the authentic recruitment trail was arranged to form one arm of a Y maze, with a similar strip of paper marked with the synthetic pheromone at the rate of 10^{-9} g/mm used to form the other arm. The other half of the authentic trail served as the stem of the maze. To conduct a test, unfed caterpillars were allowed to move from the tent onto the stem of the maze, one at a time, and to chose between the alternate arms. Strips treated with the synthetic pheromones were used once then discarded. To prevent a possible positional bias, a random procedure was used to assign a treatment to the right or left arm for each replicate of the test.

Previous studies of trail following by eastern tent caterpillars showed that they can distinguish between aged and newly deposited trails. Although the monoketone is a large molecule, the material is used in small amounts and it is

possible that the loss of a small quantity through volatilization over time allows the caterpillars to distinguish old from new trails. This possibility was assessed by allowing caterpillars to choose between the alternate arms of paper Y mazes treated with 10^{-10} g/mm of the monoketone just prior to testing or up to 24 hr previously, using the same experimental protocol described above.

Artificial trails made from the monoketone were established under field conditions to determine if they were competitive with trails established by colonies of tent caterpillars. Pheromone was applied at the approximate rate of 10^{-9} g/mm along previously untraversed branches originating near to a tent or trunk trail and terminating at a previously unexploited patch of leaves. The pathways ranged in length from 50 to 90 cm and involved at least three choice points (forks in branches). The synthetic trail was established while a colony was assembling on the tent in preparation for a foraging bout. Colonies were then observed during the bout to determine their response to the synthetic trails. A response was considered positive when the caterpillars left their established trail system after encountering the synthetic pathway and followed it directly to its distant end. The study was replicated with 23 colonies of fourth- to fifth-instar caterpillars.

RESULTS

When allowed to choose between the arms of Y mazes marked with synthetic monoketone or diketone, the caterpillars showed a strong preference for the monoketone trails even when the concentrations of the pheromones differed in strength by several orders of magnitude (Table 1). The caterpillars were also

TABLE 1. NUMBER OF EASTERN TENT CATERPILLARS SELECTING TRAILS ON ALTERNATE ARMS OF Y MAZES MARKED WITH 5β-CHOLESTAN-3-ONE (MONOKETONE) OR 5β-CHOLESTANE-3,24-DIONE (DIKETONE)[a]

Trail concentration ratio (monoketone : diketone)	Number selecting trail (N = 20)		P^b
	Monoketone	Diketone	
1 : 1	19	1	**
1 : 10	18	2	**
1 : 100	17	3	**
1 : 1000	14	6	NS
Totals	68	12	**

[a] A concentration of 1 = 10^{-9} g/mm of trail.
[b] Probability for χ^2 test, **<0.01, NS = not significant ($P > 0.05$).

TABLE 2. NUMBER OF EASTERN TENT CATERPILLARS SELECTING TRAILS MARKED ON ALTERNATE ARMS OF Y MAZES WITH DIFFERENT CONCENTRATIONS OF 5β-CHOLESTAN-3-ONE[a]

Relative trail strength	Number selecting trail (N = 20)		P^b
	Weaker	Stronger	
1:2	5	15	*
1:2	3	17	**
1:4	4	16	**
1:10	4	16	**
0.1:1	0	20	**
Totals	16	84	**

[a] A concentration of 1 = 10^{-9} g/mm of trail.
[b] Probability for χ^2 test, *<0.05, **<0.01, NS = not significant ($P > 0.05$).

sensitive to small differences in the amount of monoketone used to mark the arms of mazes. As shown in Table 2, they preferred the stronger trails in all tests, even when the trails differed in strength by a factor of only two.

In all of six separate tests with whole colonies, caterpillars selected authentic recruitment trails over those prepared from the diketone applied at the rate of 10^{-9} g/mm (Table 3). In contrast, artificial trails made with the monoketone applied at this same rate were chosen significantly more often than authentic recruitment trails (Table 3). When the concentration of the diketone was increased by a factor of 10^3, there was no significant difference in the numbers of caterpillars following artificial and authentic trails (Table 3). During the tests with both compounds, most caterpillars were observed to swing their head from trail to trail at the choice point before moving out onto one or the other. In some instances, larvae moved onto one trail without apparently sensing the presence of the alternative pathway. Because the authentic and artificial trails were close enough to allow a caterpillar to swing its head from one to the other, caterpillars were sometimes observed to become aware of the presence of a second trail only while they were moving down the other. If the caterpillar crossed over to the newly detected trail immediately after contacting it, it was considered to have selected that trail. In a few instances involving the monoketone trails, the caterpillars were clearly ambivalent, shifting several times from one trail to another. Although such response indicated that the monoketone was clearly competitive with the authentic trail, the ambiguous result was excluded from the data set.

Field colonies were highly responsive to trails marked with the monoketone. In 19 of the 23 tests, the caterpillars abandoned their heavily used trails

TABLE 3. NUMBER OF EASTERN TENT CATERPILLARS SELECTING AUTHENTIC RECRUITMENT TRAILS (A) OR TRAILS PREPARED FROM 5β-CHOLESTAN-3-ONE (MONOKETONE) OR 5β-CHOLESTANE-3,24-DIONE (DIKETONE) APPLIED TO PAPER STRIPS AT RATE OF 10^{-9} OR 10^{-6} g/mm OF TRAIL

	Number of caterpillars selecting trail (N = 10)								
Test	A	Monoketone (10^{-9})	P^b	A	Diketone (10^{-9})	P	A	Diketone (10^{-6})	P
1	0	10	**	9	1	**	5	5	NS
2	3	7	NS	8	2	*	7	3	NS
3	0	10	**	9	1	**	3	7	NS
4	1	9	**	10	0	**	7	3	NS
5	1	9	**	8	2	*			
6	5	5	NS	10	0	**			
7	3	7	NS						
8	0	10	**						
Totals	13	67	**	54	6	**	22	18	NS

[a]Probability for χ^2 test, *<0.05, **<0.01, NS = not significant ($P > 0.05$).

to move onto the synthetic trail when they encountered it. Typically, large numbers of larvae pushed forward onto the trail simultaneously, converging *en masse* on the designated feeding site. In those instances where caterpillars failed to move onto the trail, the colony moved off in a different direction and did not come in contact with the experimental trail during the observation period.

In tests involving the response of caterpillars to new and aged monoketone trails, the larvae selected new trails over trails aged 6 hr or more (Table 4). The

TABLE 4. NUMBER OF EASTERN TENT CATERPILLARS SELECTING NEW AND AGED TRAILS OF 5β-CHOLESTAN-3-ONE APPLIED TO ALTERNATE ARMS OF Y MAZES AT RATE OF 10^{-9} g/mm

Test	Number of hours aged	Number selecting		P^a
		Aged	New	
1	24	4	16	**
2	18	3	17	**
3	6	5	15	*
4	2	8	12	NS
5	2	6	14	NS

[a]Probability for χ^2 test, *<0.05, **<0.01, NS = not significant ($P > 0.05$).

caterpillars showed no preference for new trails over trails that were aged only 2 hr.

DISCUSSION

This study shows that synthetic trails prepared from the monoketone are followed preferentially over diketone trails. Furthermore, while trails prepared from the diketone are competitive with authentic recruitment trails only at very high application rates, trails prepared from the monoketone are capable of defeating authentic trails at markedly lower concentrations. That caterpillars actually preferred the monoketone trails over their authentic recruitment trails in most tests is likely attributable to my decision to apply the chemical at a rate that gave unambiguous results, apparently exceeding that deposited by the caterpillars in these tests. Unlike ants that sense their trail pheromones with antennae that are held above the trail to intercept the volatilizing compound, tent caterpillars rely on contact chemoreceptors that occur on the maxillary palps (Roessingh et al., 1988). Thus, only a small fraction of the chemical absorbed by the paper strips likely comes into contact with the palps as they sweep the surface of the artificial trail. Although it is not known how much material they incorporate into their trail, the caterpillars are likely to deposit significantly less than that applied by us in the studies reported here, since they brush the material onto the substrate in a narrow band concentrated on the surface.

The ability of synthetic pheromone trails to draw caterpillars off their heavily silked trunk trails under field conditions corroborates a previous study (Fitzgerald and Edgerly, 1982) showing that while the silk that caterpillars deposit as they move over branches may enhance the trail following response, extrasilk factors play the more important role in trail following. It remains likely, however, that tactile or visual properties of the silk trail provide additional orientational cues.

The possibility that chemicals associated with feeding also play some role in recruitment communication cannot be completely precluded. Feeding caterpillars are likely to pick up host juices on their bodies and mouthparts as they move over damaged leaves while feeding. This material might then be passively deposited on the trail as they return to the tent. Odors of crushed leaves wafted onto the tent clearly excited caterpillars preparing to initiate a foraging bout. Yet, despite repeated attempts, it was not possible to demonstrate that extracts of crushed leaves or crop contents added to a pheromone trail had any effect on trail following. If such host chemicals are involved they are likely to play a subordinate role to the steroids, and this study shows that they are not required to defeat authentic recruitment trails.

Tent caterpillar trails become less effective in eliciting trail following as they age (Fitzgerald and Gallagher, 1976), and the caterpillars can readily dis-

tinguish between new recruitment trails and those laid down during the colony's last *en masse* foraging bout (Fitzgerald, 1976). Y maze studies showing that caterpillars prefer new to aged trails prepared form the pure pheromone indicate that loss through volatilization or degradation of the pheromone may account, at least partially, for this phenomenon. Experiments with tritium-labeled monoketone are currently being conducted to determine if simple volatilization can account for this result.

The chemical basis of bilevel trail systems is unknown for any species. It is thought that the army ants *Eciton* (Chadab and Rettenmeyer, 1975) and *Neivamyrmex* (Topoff et al., 1980) use qualitatively different chemicals to mark their exploratory and recruitment trails, but no chemical has yet been identified. The ant *Leptogenys* (Maschwitz and Mühlenberg, 1975) also employs a trail system with features similar to that of the tent caterpillar, but as is the case for the army ants, it is not understood how these ants distinguish long-lived trunk trails from their more ephemeral recruitment trails.

Our study indicates that eastern tent caterpillars are highly sensitive to small differences in the amount of pheromone used to mark trails. Moreover, it was recently shown that the forest tent caterpillar *Malacosoma disstria* secretes the monoketone but not the diketone (Fitzgerald and Webster, in preparation). This indicates that the diketone is not merely a passive precursor or by-product of monoketone synthesis and suggests that both steroids have behavioral significance in the eastern tent caterpillar. Thus, consistent with our current understanding of trail chemistry and larval behavior, it appears likely that the bilevel, trail-based communication system of the eastern tent caterpillar is dependent upon both the qualitative and quantitative chemistry of the trails. A fuller understanding of chemical communication in this insect awaits the development of analytical procedures that are sensitive enough to enable precise quantitative measurements of the amounts of pheromonal components that occur in variously aged authentic exploratory and recruitment trails.

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REFERENCES

CHADAB, R., and RETTENMEYER, C.W. 1975. Mass recruitment by army ants. *Science* 188:1124–1125.

CRUMP, D., SILVERSTEIN, R.M., WILLIAMS, H.J., and FITZGERALD, T.D. 1987. Identification of the trail pheromone of the 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.S. 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.M. 1976. A chemical trail factor from the silk of the eastern tent caterpillar *Malacosoma americanum* (Lepidoptera: Lasiocampidae). *J. Chem. Ecol.* 2:564–574.

FITZGERALD, T.D., and PETERSON, S.C. 1983. Elective recruitment communication by the eastern tent caterpillar (*Malacosoma americanum*). *Anim. Behav.* 31:417–423.

MASCHWITZ, U., and MÜHLENBERG, M. 1975. Zur Jagdstrategie einiger orientalischer *Leptogenys*-Artren (Formicidae: Ponerinae). *Oecologia* 20:65–83.

PETERSON, S.C., and FITZGERALD, T.D. 1991. Chemoorientation of eastern tent caterpillars to trail pheromone 5β-cholestane-3,24-dione. *J. Chem. Ecol.* 17(10): 1963–1972.

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.

TOPOFF, H., MIRENDA, J., DROUAL, R., and HERRICK, S. 1980. Behavioural ecology of mass recruitment in the army ant *Neivamyrmex nigrescens*. *Anim. Behav.* 28:779–789.

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ENANTIOMERS OF METHYL SUBSTITUTED ANALOGS OF (*Z*)-5-DECENYL ACETATE AS PROBES FOR THE CHIRALITY AND COMPLEMENTARITY OF ITS RECEPTOR IN *Agrotis segetum*[1]: SYNTHESIS AND STRUCTURE–ACTIVITY RELATIONSHIPS

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Abstract—The enantiomers of analogs of (*Z*)-5-decenyl acetate, a pheromone component of *Agrotis segetum*, substituted by a methyl group in the 2, 3, 4, 7, and 8 positions and dimethyl substituted in the 4,7 positions, have been synthesized and studied by an electrophysiological single-cell technique and by molecular mechanics calculations. The results demonstrate that the electrophysiological activity as well as the ability of the (*Z*)-5-decenyl acetate receptor to differentiate between enantiomers depends on the position of the methyl substituent. For analogs methyl substituted in the 2, 4, or 8 position, no differences in the activities of the enantiomers could be observed. In contrast, the enantiomers of the 3- and 7-methyl analogs display a significant difference in the activities, the *R*-enantiomers being more active than the *S*-enantiomers. From an analysis of the structure–activity results of the enantiomers of the 4,7-dimethyl-substituted analogs, the chiral sense of the alkyl-chain of the natural pheromone component on binding to its receptor could be deduced.

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[1] Schiff., Lepidoptera: Noctuidae.

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Key Words—Lepidoptera, Noctuidae, *Agrotis segetum*, (*Z*)-5-decenyl acetate, pheromone analog, methyl substitution, structure-activity, single-sensillum recordings, receptor interaction, conformational analysis, molecular mechanics, enantiomers, chirality.

INTRODUCTION

We have, in previous studies on the steric complementarity between the (*Z*)-5-decenyl acetate receptor of *Agrotis segetum* and its natural substrate, systematically introduced substituents into various positions of (*Z*)-5-decenyl acetate (**1**, Figure 1) (Jönsson et al., 1991a,b, 1992). By introducing methyl groups to **1** as space probes for the characterization of the effective dimensions of the receptor and the steric complementarity between the substrate and its receptor,

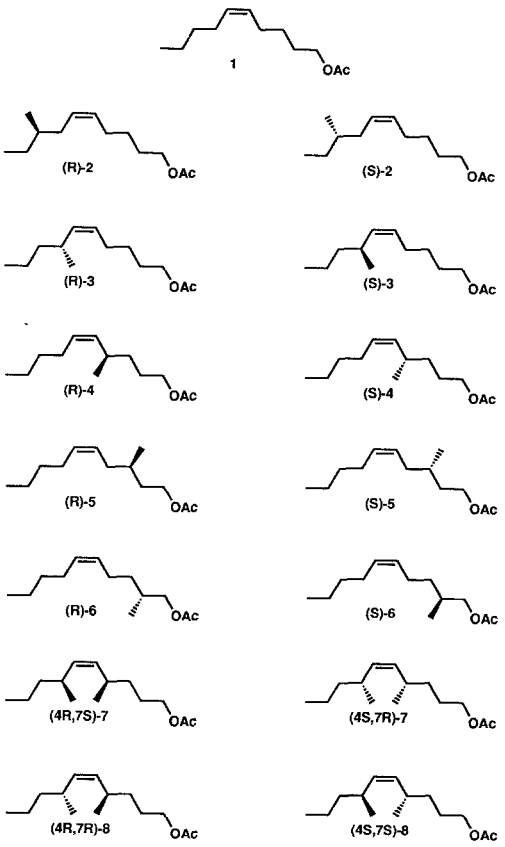

FIG. 1. Compounds studied.

we have demonstrated that the receptor tuned to **1** interacts with its natural substrate in a highly complementary way. This high steric complementarity accounts for the very high affinity of the pheromone component for its receptor and the high selectivity of the substrate–receptor interaction.

Pheromone component **1** is a multiply prochiral compound. Our previous studies on methyl-substituted analogs of **1** (Jönsson et al., 1991a, 1992) were restricted to the racemates of these analogs. On the basis of the results of these studies, in some cases it was not possible to draw final conclusions on the nature of the interaction between an analog and the receptor. An investigation of some of the compounds was needed to clarify some of the ambiguity found in earlier investigations. Such a study also gives an opportunity to elucidate the chiral properties of the receptor cavity and the chiral sense of the receptor-bound pheromone component. For this purpose, we have prepared the enantiomers of analogs of **1** substituted by a methyl group in the 2, 3, 4, 7, and 8 positions and the four stereoisomers of 4,7-dimethyl substituted **1** (Figure 1). For these compounds, structure–activity relationships have been studied by the electrophysiological single-cell technique. As in our previous work, the possible effects on the biological activity due to conformational effects have been studied by molecular mechanics calculations using the MM2(85) method (Burkert and Allinger, 1982).

Very few studies on chiral analogs of an achiral pheromone component of the monoene type have been reported and, to our knowledge, no previous study using the single-cell technique has been published. Chapman et al. (1978a,b) have demonstrated that moth receptors are able to differentiate between enantiomers. In an EAG study on enantiomers of 13-methyl-(*Z*)-11-pentadecenyl acetate, Bestmann et al. (1980) found that the receptors of *Ostrinia nubilalis* and *Argyrotaenia velutinana*, tuned to (*Z*)-11-tetradecenyl acetate, clearly could differentiate between the enantiomers of the chiral analog. The results of a sex stimulation study on the same compounds were consistent with the EAG results of Bestmann et al.; however, these compounds were inactive in a flight tunnel assay (Schwarz et al., 1989).

Very recently, Bestmann et al. (1992), using single-cell recordings, reported that receptor cells for dienic pheromone components in *Bombyx mori*, *Manduca sexta*, *Antheraea pernyi*, and *Antheraea polyphemus* are able to differentiate between enantiomers of methyl substituted analogs.

METHODS AND MATERIALS

Synthesis

N,N'-Dimethyl-*N,N'*-propylene urea (DMPU) was used as substitute for the carcinogenic HMPA as cosolvent and stored under argon over 4 Å molecular sieves. The crude pheromone products were chromatographed over Merck Silica

gel 60H with petroleum ether–ethyl acetate (20:1). All products were then further purified by argentation chromatography (Houx et al., 1974), to remove *E*-isomers and finally by high-performance liquid chromatography (HPLC) on a semipreparative straight-phase column (Kromasil, particle size 10 μm, 250 × 10 mm ID with petroleum ether–ethyl ether, 50:1) and monitored with a differential refractometer. The purity of the isolated final products was >99% and no trace (<0.05%) of the natural pheromone component **1** could be detected by GLC.

^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ solution on a Varian XL-300 spectrometer. The $CHCl_3$ signal was used as an internal reference (7.26 ppm). Chemical shifts (δ) are given downfield relative to TMS.

Analytical GLC was performed on a Varian 1400 using a 2-m column (3% OV-101 on Chrom Q 100/120) or a Varian 3400 GLC equipped with a capillary column (DB-Wax, 30 m × 0.25 mm ID).

High resolution mass spectra were recorded on a Jeol JMS-SX 102 spectrometer.

The synthetic schemes for the preparations of compounds **2–8** are shown in Figures 3–5 below.

(R)- and (S)-*2-Methyl-4-pentenol* [(R)- *and* (S)-***10a***]. 2-Methyl-4-pentenoic acid (**9a,** Figure 2) was prepared by alkylation of ethyl methylmalonate (171 ml, 1.0 mol) with allyl bromide (91 ml, 1.05 mol) followed by hydrolyzation and decarboxylation according to the method described by Ställberg-Stenhagen (1946). The crude acid was treated with excess thionyl chloride (95 ml, 1.3 mol), stirred overnight, and distilled: bp 130–138°C, 82.2 g (62%); NMR (60 MHz): δ 1.2 (d, 3H, CH_3), 2.0–2.8 (m, 3H, CH, CH_2), 4.8–6.2 (m, 3H, CH_2=CH).

(*S*)-α-Methylbenzylamine was obtained by resolution of its α-methylbenzylammonium salt of (2*R*,3*R*)-(+)-tartaric acid (Ault, 1965). After five recrystallizations from methanol, the recovered (*S*)-amine was ≥99.9%

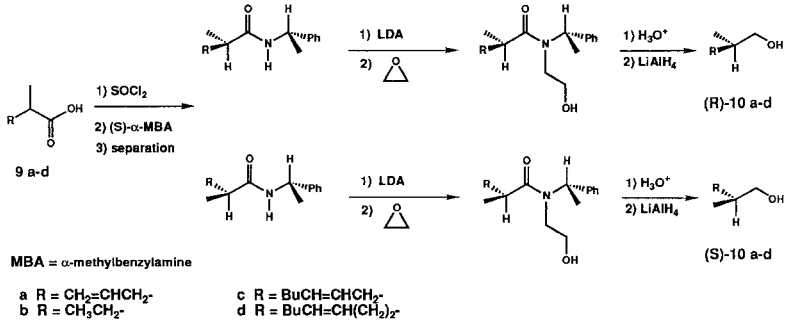

FIG. 2. Scheme for the preparation of the enantiomerically pure α-methylalcohols.

pure as determined by GLC (DB-Wax) as the amide of α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) (Dale and Mosher, 1973). To the pure (*S*)-α-methylbenzylamine (70 ml, 0.55 mol) and triethylamine (76 ml, 0.55 mol) in methylene chloride (600 ml), cooled on an ice-bath, was slowly added the prepared 2-methyl-4-pentenoyl chloride (64 g, 0.5 mol). The mixture was stirred for 2 hr, washed with 2 M HCl, dried, and the solvent removed to give the diastereomeric pair of amides as a semisolid mixture (98.9 g, 91% yield). The amide pair was separated chromatographically on a preparative scale on silica gel (Merck Kieselgel 60) with petroleum ether–ethyl acetate (7:3) as eluent. Enriched fractions were recycled, and the diastereomerically pure amides were obtained in almost quantitative yields. The purifications were monitored by TLC plates with spots visualized by iodine vapor. Determination of the absolute configuration of the diastereomers was done according to the method of Helmchen et al. (1977), based on the elution order of the amide pair. TLC (hexane–ethyl acetate 7:3), R_f for $R_{acid}S_{amine}$ = 0.29 and $S_{acid}S_{amine}$ = 0.24; GLC (DB-Wax 160–215 2°C/min), R_t for $R_{acid}S_{amine}$ = 16.63 and $S_{acid}S_{amine}$ = 15.94; NMR (60 MHz): δ 1.1 (d, 3H, $\mathbf{CH_3}$CH), 1.4 (d, 3H, $\mathbf{CH_3}$CHN), 2.0–2.4 (m, 2H, $CH_2C{=}$), 4.8–6.2 (m, 3H, $CH_2{=}$CH), 7.3 (m, 5H, aryl H).

The labilization and cleavage of the purified amides was accomplished essentially as described by Sonnet et al. (1987). Thus, the pure amide (43 g, 0.2 mol) was added to a THF solution (400 ml) of lithium diisopropylamide (0.3 mol) at 0°C. After 15 min of stirring, the mixture was cooled to −30°C and ethylene oxide (20 ml, 0.4 mol) was injected. External cooling was removed and the solution stirred overnight, then worked up with 2 M HCl and extracted with ether. Removal of the solvent gave a crude oil in quantitative yield. This was dissolved in THF (250 ml), containing 2 equiv. of conc. HCl (33 ml) and heated under reflux. The transacylation of the hydroxyethylated amide to the aminoester was monitored by GLC (OV-101), and after 2 hr all starting material was found to be consumed. The solution was cooled down and immediately concentrated on a rotary evaporator. Then, ether was added, the remaining water separated off, and the solution was dried over $MgSO_4$. The dried solution was then added dropwise to $LiAlH_4$ (12 g) in ether (1000 ml) at 0°C. The suspension was stirred overnight, quenched with water and 15% NaOH, and after 1 hr of stirring was dried over $MgSO_4$. The mixture was filtered, and the filter cake was thoroughly washed with anhydrous ether. The filtrate and washings were combined, concentrated, and the residue distilled to give 10.4–11.6 g (52–58% yield from the amide) of the enantiomerically pure alcohol, bp 143–145°C, lit. (Fanta and Erman, 1972) bp 57–58°C/12 mm Hg; $[\alpha]_D^{25}$ = −2.44 (*c* = 10, $CHCl_3$), lit. (Gramatica et al., 1988) $[\alpha]_D^{25}$ = −2.45, (Rossi and Conti, 1977) $[\alpha]_D^{18}$ = −2.38 for (*S*)-2-methyl-4-pentenol. NMR data were identical to those reported for the racemate by Fanta and Erman (1972).

(R)- *and* (S)-*2-Methylbutanol* [(R)- *and* (S)-***10b***]. These were obtained in the same manner as described above for (*R*)- and (*S*)-2-methyl-4-pentenol, from

commercially available 2-methyl-1-butyric acid (Figure 2). The separation of the diastereomeric amides was more cumbersome than that for the corresponding amides from 2-methyl-4-pentenoic acid, described above. This is due to the lower separation factor of the amide from 2-methyl-1-butyric acid. Determination of the absolute configuration of the diastereomers was done according to the method described for (*R*)- and (*S*)-2-methyl-4-pentenol. TLC (hexane–ethyl acetate 7:3), R_f for $R_{acid}S_{amine}$ = 0.25 and $S_{acid}S_{amine}$ = 0.22; GLC (DB-Wax 160–215 2°C/min), R_t for $R_{acid}S_{amine}$ = 12.93 and $S_{acid}S_{amine}$ = 12.70. The enantiomerically pure alcohols were distilled, bp 127–129°C to give ca. 1.7 g (54–60% yield from the amide). Boiling points, NMR data, and GLC retention times were in agreement with those of a commercial racemic sample of 2-methylbutanol (Aldrich Chemical Co).

(R)- *and* (S)-*2-Methyl-(Z)-4-nonenol* [(R)- *and* (S)-***10c***]. The acid **9c** (Figure 2) was prepared by alkylation of ethyl methylmalonate (31.3 g, 180 mmol) using sodium (4.2 g, 180 mmol), dry ethanol (250 ml), and (*Z*)-2-heptenyl bromide (30.0 g, 170 mmol) (Jönsson et al., 1992), the ester was hydrolyzed, and the resulting diacid decarboxylated at 150–170°C (55% yield). Crude **9c** was then converted by means of thionyl chloride into its acid chloride, which was reacted with (*S*)-α-methylbenzylamine, as described for **9a,** to give the amide pair in essentially quantitative yield. Determination of the absolute configuration of the diastereomeric amides was done according to the chromatographic elution order of the amide pair on GLC and TLC. TLC (hexane–ethyl acetate 7:3), R_f for $R_{acid}S_{amine}$ = 0.39 and $S_{acid}S_{amine}$ = 0.31; GLC (DB-Wax 120–220 4°C/min), R_t for $R_{acid}S_{amine}$ = 36.96 and $S_{acid}S_{amine}$ = 35.67. The diastereomerically pure amides were labilized and cleaved, as described for **10a,** and the resulting alcohols were purified on a SiO_2 column to yield pure (*R*)- and (*S*)-**10c** (49–55% yield from the amide).

(R)-*and* (S)-*2-Methyl-(Z)-5-decenol* [((R)-*and* (S)-***10d***)]. These were prepared in the same manner as described for the preparation of (*R*)- and (*S*)-**10a** from racemic 2-methyl-(*Z*)-5-decenoic acid (1.1 g, 6.0 mmol) (Jönsson et al., 1992). Determination of the absolute configuration of the diastereomeric amides was done according to the method of Helmchen et al. (1977). TLC (hexane–ethyl acetate 7:3), R_f for $R_{acid}S_{amine}$ = 0.39 and $S_{acid}S_{amine}$ = 0.31; GLC (DB-Wax 180–220 3°C/min), R_t for $R_{acid}S_{amine}$ = 27.51 and $S_{acid}S_{amine}$ = 25.89. The diastereomerically pure amides were cleaved and converted to the corresponding alcohols to give 150–200 mg (30–40% yield) of **10d.**

Preparation of Bromides. Bromine (5.1 ml, 0.1 mol) was added dropwise to a stirred and ice-cooled solution of triphenylphosphine (26.2 g, 0.1 mol) in anhydrous methylene chloride (120 ml) (Figure 4). To this milky mixture was added the alcohol (80 mmol), and the resulting clear solution was stirred overnight. The main part of the solvent was evaporated off and the residue treated with pentane to precipitate triphenylphosphine oxide. The mixture was then

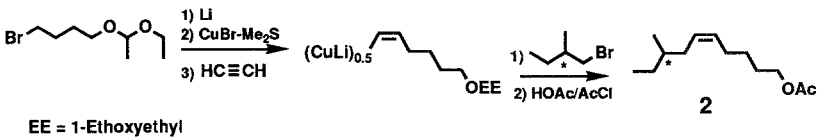

FIG. 3. Scheme for the preparation of compound **2.**

filtered through a column of silica gel, which was thereafter rinsed with pentane. The collected eluents were carefully evaporated to give the pure bromides in >90% yields.

(Z)-5-decenyl acetate **(1)** was prepared as previously described (Olsson et al., 1983). Racemic samples of compounds **2–6** were obtained as previously described (Jönsson et al., 1991a, 1992).

(R)- *and* (S)-*8-Methyl-(Z)-5-decenyl Acetate* [(R)-*2 and* (S)-*2*]. For details regarding the synthetic method, see Jönsson et al. (1991a). 4-(1-Ethyoxyethoxy) butyl lithium was prepared from ethoxyethyl protected 4-bromobutanol and lithium in dry ether (Figure 3). This ethereal solution (25 mmol) was added to CuBr–Me_2S complex (2.88 g, 14 mmol) at −50°C. After 30 min of stirring, acetylene (0.6 l, 25 mmol) was bubbled slowly into the solution. This was allowed to warm to −25°C, and DMPU (6 ml) in THF (40 ml) was added and thereafter (*R*)- or (*S*)-2-methylbutyl bromide (1.8 g, 12 mmol). The mixture was left to stand overnight at room temperature. Then, it was quenched with

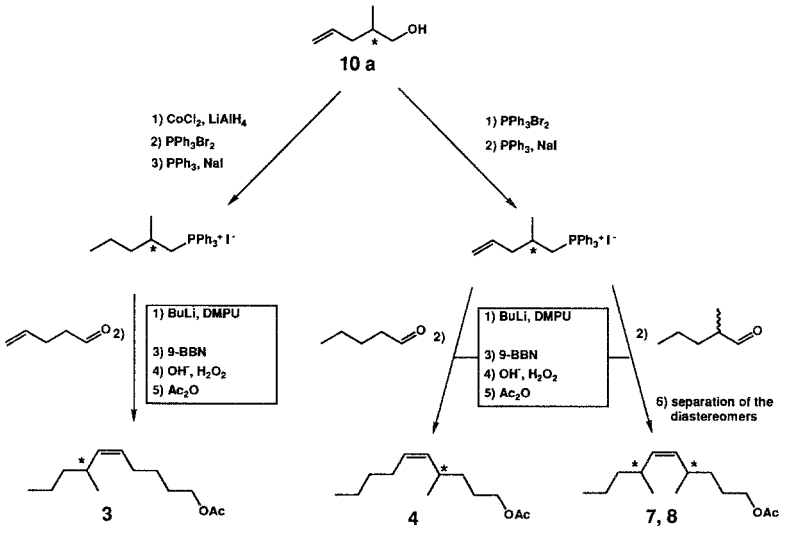

FIG. 4. Scheme for the preparation of compounds **3, 4, 7,** and **8.**

HCl, worked up, and the crude product acetylated with acetic acid (15 ml) and acetyl chloride (1.5 ml) overnight at 40°C. Subsequent work-up gave 0.96–1.06 g (38–42%). The products were fully examined by GLC, ^1H, and ^{13}C NMR, and the data obtained were identical to those of a reference racemic sample (Jönsson et al., 1991a).

(R)- *and* (S)-*7-Methyl-(Z)-5-decenyl Acetate* [(R)-***3*** *and* (S)-***3***]. Enantiomerically pure 2-methyl-4-pentenol, **10a** (1.4 g, 14 mmol), was syringed into a solution of $CoCl_2$ (3.9 g, 30 mmol) in THF (25 ml) (Figure 4). This solution was cooled to −72°C and $LiAlH_4$ (1.14 g, 30 mmol) was carefully added under a stream of nitrogen. After 24 hr of stirring at ambient temperature, the mixture was quenched with water and worked up by extraction with ether. No traces of the starting material could be detected by GLC, yields >92%. The hydrogenated alcohol, 2-methylpentanol, was then converted to the corresponding bromide and the obtained bromide (3.3 g, 20 mmol), triphenylphosphine (5.3 g, 20 mmol), and sodium iodide (7.5 g, 20 mol) in acetonitrile (25 ml) were then refluxed for 72 hr. The reaction mixture was allowed to settle. The supernatant solution was then transferred to a 100-ml one-necked bottle and concentrated on a rotary evaporator. Dry THF (60 ml) was added to the sticky grayish product, and the mixture was cooled on an ice-bath under nitrogen. *n*-Butyllithium (20 mmol) was injected, and the resulting dark-red solution was stirred for 1 hr. The ylid solution was cooled to −72°C and DMPU (9 ml) was added. Freshly prepared 4-pentenal [1.7 g, 20 mmol, prepared by oxidation of 4-pentenol with pyridinium chlorochromate (Corey and Suggs, 1975)] was injected neat. The cooling bath was removed and the yellow mixture stirred overnight. The mixture was then concentrated on a rotary evaporator, the residue filtered through a column of silica gel, and rinsed with pentane to give ca. 1.0 g of 7-methyl-(Z)-1,5-decadiene (33% yield from the bromide). The diene (1.0 g, 6.5 mmol) was dissolved in THF (10 ml) and cooled with an ice bath. 9-Borabicyclo[3.3.1]nonane (9-BBN, 13 ml, 0.5M in THF) was injected, and the solution was stirred at room temperature overnight. A mixture of 3 M NaOH (2.4 ml) and 30% H_2O_2 (2.8 ml) was then added dropwise. After 1 hr of stirring at 40°C, the product was worked up with water and ether. The crude alcohol was acetylated with acetic anhydride (1.5 ml) in pyridine (5 ml) and worked up to give the pure acetate. Analysis by GLC indicated the presence of <8% of the *E*-isomer, which was removed by argentation column chromatography. The NMR and GLC data were indistinguishable from those of the racemic compound. (Jönsson et al., 1991a).

(R)- *and* (S)-*4-Methyl-(Z)-5-decenyl Acetate* [(R)-***4*** *and* (S)-***4***]. Enantiomerically pure 2-methyl-4-pentenol, **10a,** was converted to the bromide and then to the corresponding phosphonium salt, followed by a Wittig reaction as described above for **3** with valeraldehyde in place of 4-pentenol to give the title compounds. The NMR spectra and GLC retention times were identical to those of a racemic sample (Jönsson et al., 1992).

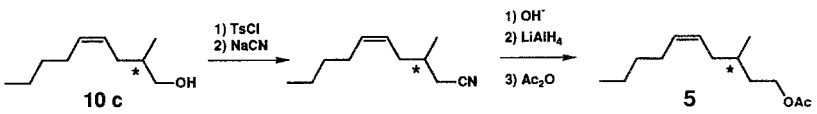

FIG. 5. Scheme for the preparation of compound **5**.

(R)- *and* (S)-*3-Methyl-(Z)-5-decenyl Acetate* [(R)-***5*** *and* (S)-***5***]. The enantiomers of **10c** were converted to the title compounds as described previously (Jönsson et al., 1992) and shown in Figure 5. *p*-Toluene-sulfonyl chloride (1.1 g, 5.6 mmol) was added to an ice-cooled solution of **10c** (0.7 g, 4.5 mmol) in pyridine (6 ml) and stirred for 4–6 hr. It was then worked up with 1 M HCl, dried ($MgSO_4$), and concentrated. The crude tosylate was dissolved in DMSO (8 ml) and NaCN (0.6 g) was added. After stirring at 60°C for 12 hr, the mixture was worked up with water and ether, and thereafter hydrolyzed with NaOH (3 g) in 65% ethanol–water under reflux for 30 hr. The crude acid was reduced with lithium aluminum hydride (0.5 g) and the resulting alcohol acetylated with acetic anhydride (3 ml) in pyridine (5 ml) (yield 50% from the amide). The spectral properties (NMR, MS) and GLC data were identical with those of racemic samples previously reported by us (Jönsson et al., 1992).

(R)- *and* (S)-2-Methyl-(Z)-*5-decenyl Acetate* [(R)-***6*** *and* (S)-***6***]. The enantiomers of **10d** were converted to the title compounds in quantitative yields by acetylation of the alcohols with acetic anhydride in pyridine as described above. The compounds showed the same GLC retention times and the same mass and NMR spectra as a racemic reference sample (Jönsson et al., 1992).

(S,R)-, (R,S)-*4,7-Dimethyl-(Z)-5-decenyl Acetate* [*(4S,7R)-7 and (4R,7S)-7*] *and* (S,S)-, (R,R)-*4,7-Dimethyl-(Z)-5-decenyl Acetate* [*(4S,7S)-8 and (4R,7R)-8*]. These compounds were prepared via the same reaction sequence as described for compound **4** from (*R*)- and (*S*)-**10a** (Figure 4), but using 2-methylvaleraldehyde in place of valeraldehyde (yield 29–35%, from the bromide). From each of the enantiomeric phosphonium salts was obtained a diastereomeric pair of acetates, 4*R*,7*R*/*S* and 4*S*,7*R*/*S*, respectively. Each pair of diastereomers was cleanly separated by GLC (DB-Wax 100–215 4°C/min). The pair of diastereomers derived from the (*R*)-phosphonium salt (4*R*,7*R*/*S*) showed two peaks R_t = 11.10 min (56%) and R_t = 11.48 min (44%), respectively. These diastereomers were cleanly separated by preparative HPLC [Kromasil, petroleum ether–ether (50:1), 5 ml/min] to give the two pure components: a less polar component R_v = 260 ml with R_t = 11.10 min and a more polar component R_v = 270 ml with R_t = 11.48 min. In order to assign the stereoisomers, the reaction sequence was repeated using an enantiomerically pure phosphonium salt, generated from (*R*)-2-methyl-4-pentenyl bromide, and (*R*)-2-methylvaleraldehyde obtained from oxidation of (*R*)-2-methylpentanol (82% ee) (described above under **3**) with pyridinium chlorochromate. The GLC analysis

(under the same conditions as described above) of the product showed two peaks: R_t = 11.10 (19.6%) and R_t = 11.48 (80.4%), respectively. In a coinjection test, the main peak (R_t = 11.48 min) coincided with the peak of the more polar component of the diastereomers described above. Therefore, the peak at R_t = 11.48 was assigned to the 4*R*,7*R*-isomer. To confirm this assignment, analytical samples of 4*S*,7*R*/*S* and 4*S*,7*S* were treated with ozone at low temperature in acetone followed by oxidation with Jones reagent (Gramatica et al., 1988). The mixtures of acids obtained were then converted to corresponding acyl chlorides by treatment with oxalyl chloride and subsequent conversion to amides by treatment with (*S*)-α-methylbenzylamine as described above. The amides derived from the 4*S*,7*R*/*S*-sample showed two peaks of equal magnitude upon GLC analysis, which were identical with peaks from amides derived from (*R*,*S*)-2-methylpentanoic acid, while the assigned 4*S*,7*S*-sample exhibited a major peak that coincided with the peak with the lower R_t value of the two peaks. The low R_t value for amides derived from the (*S*)-α-methylbenzylamine is in accordance with the expected elution order on GLC for the (*S*)-2-methylpentanoic acid derived amide (Helmchen et al., 1977).

The racemates of compounds **7** and **8** were obtained in the same manner as described above from racemic starting materials. Their ^{1}H NMR spectra and chromatographic data were identical with those of the enantiomers.

Compound (R,S)-*7*. (R_t = 11.10 min, R_v = 260 ml); δ_H (300 MHz) 0.87 (t, 3H, CH_3CH_2), 0.90–0.96 (dd, 6H, CH_3CH), 1.14–1.41 (m, 6H, CH_3CH_2, CH_2CC=, =$CCCH_2$), 1.51–1.66 (m, 2H, CH_2CO), 2.04 (s, 3H, $COCH_3$), 2.35–2.48 (m, 2H, =CCH) 4.03 (t, 2H, CH_2O), 5.00–5.12 (m, 2H, J = 10.9 Hz, CH=C); δ_C (75.4 MHz) 14.3, 20.6, 21.0, 21.7, 26.7, 31.7, 31.8, 33.7, 39.9, 64.7, 134.1, 135.6, 171.2; high-resolution CI mass spectrum, for $C_{14}H_{30}O_2N$ ($M+NH_4^+$), calcd 244.2276, found 244.2279.

Compound (R,S)-*8*. (R_t = 11.48 min, R_v = 270 ml); δ_H (300 MHz) 0.87 (t, 3H, CH_3CH_2), 0.90-0.96 (dd, 6H, CH_3CH), 1.14–1.41 (m, 6H, CH_3CH_2, CH_2CC=, =$CCCH_2$), 1.51–1.66 (m, 2H, CH_2CO), 2.04 (s, 3H, $COCH_3$), 2.35–2.48 (m, 2H, =CCH) 4.03 (t, 2H, CH_2O), 5.00–5.12 (m, 2H, J = 10.9 Hz, CH=C); δ_C (75.4 MHz) 14.2, 20.7, 21.0, 21.6, 26.7, 31.7, 31.8, 33.7, 39.9, 64.7, 133.9, 135.5, 171.2; high-resolution CI mass spectrum, for $C_{14}H_{30}O_2N$ ($M+NH_4^+$), calcd 244.2276, found 244.2275.

Determination of Enantiomeric Purity by NMR. The enantiomeric purities of compounds **3–8** were determined by using chiral binuclear lanthanide(III)–silver(I) NMR shift reagent (Wenzel and Sievers, 1982). In a typical test run a stock solution was prepared by carefully weighing tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato], europium(III) derivative, 99% [$Eu(hfc)_3$] and Resolve-Al AgFOD obtained from Aldrich Chemical Co. This moisture- and light-sensitive mixture was then dissolved in $CDCl_3$ and protected from light. For the NMR experiment, 0.8 ml of this stock solution was added to the pheromone analog (3.5–4.0 mg). The fresh solution was then filtered through

a cotton plug in a Pasteur pipet into an amberized standard NMR tube, demagnetized, and the proton spectra recorded at ambient temperature.

Determination of Enantiomeric Purity by GLC. Samples of the prepared (*R*)- and (*S*)-2-methylbutanol, **10b,** along with reference samples of racemic and (*S*)-(−)-2-methylbutanol obtained from a commercial source (Aldrich Chemical Co.) were carefully oxidized to the corresponding acids by Jones reagent (Fieser and Fieser, 1967). These acids were then treated with $SOCl_2$ and thereafter coupled with enantiomerically pure (*S*)-α-methylbenzylamine to give the corresponding amides, analogous to the procedure described above. The enantiomeric purities and absolute configurations of the alcohols were then determined by GLC (DB-Wax).

Electrophysiology

Electrophysiological activities were determined by the single sensillum (single-cell) technique (Kaissling, 1974). Olfactory receptor cells specifically tuned to (Z)-5-decenyl acetate **(1)** are present in antennal sensilla type SW1 of *Agrotis segetum* (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 in detail (Liljefors et al., 1987; Bengtsson et al., 1990). Dose–response curves were constructed using five different stimulus amounts. The amounts used for the pheromone component **1** were 10^{-4}–1 μg and for the analogs 10^{-3}–100 μg, in decadic steps. For each stimulus amount, 10 replicates were recorded. The mean value of the number of action potentials generated during 1 sec from the onset of stimulation was calculated and used to construct dose–response curves. Errors are expressed as standard errors of the mean (SEM). The electrophysiological activity of each compound in relation to that of **1** is expressed as the reciprocal of the relative quantities required to elicit the same response from the receptor cell, as the natural pheromone component **1.**

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 are based on vapor pressure data for saturated compounds (pentane to tridecane) and their methyl substituted analogs (Weast, 1971). The correction factors used are 2.2 for the monomethyl analogs and 4.6 for the dimethyl analogs.

Molecular Mechanics Calculations

Calculations of geometries and conformational energies were done using the molecular mechanics program MM2(85) (Burkert and Allinger, 1982). Starting structures for the molecular mechanics program were obtained from the molecular modeling system MIMIC (Liljefors, 1983; von der Lieth et al., 1984).

RESULTS

Chemicals. The key intermediates for the synthesis of the enantiomers of compounds **3, 4, 7,** and **8** were the enantiomerically pure (*R*)- and (*S*)-2-methyl-4-pentenols [(*R*)- and (*S*)-**10a**]. Coupling of the phosphonium salts derived from these intermediates and the appropriate aldehydes by the Wittig reaction should furnish the eight target molecules with known stereochemistry (Figure 4). The success of this plan was dependent on our ability to prepare the optically pure key intermediates. For this purpose we have used the efficient synthesis of various optically pure α-methylcarbinols from readily available starting materials, reported by Sonnet (1984). The synthetic scheme is shown in Figure 2. 2-Methyl-4-pentenoic acid was obtained by standard malonic ester synthesis. Decarboxylation was effected by heating the acid at low pressure, which allowed the product to distill off and, thus, lactonization was avoided. The acid was then converted to the amide with (*S*)-α-methylbenzylamine. This amine was obtained in >99.5% purity by recrystallization of its monotartrate salt. The diastereomeric pair of amides could be separated by fractional crystallization from aqueous ethanol. After four recrystallizations, one of the diastereomers was obtained in >96% purity, but in modest yield (<30%). An alternative to fractional crystallization for the separation of the pairs of amides is chromatographic separation. The pair of amides shows a high separation factor, $\alpha = 1.21$, which facilitates an efficient separation on a preparative scale. In this way both diastereomers can be obtained simultaneously. Separation of the diastereomeric pair was carried out by chromatography on a silica gel column. Fractions were analyzed by TLC and enriched fractions combined and recycled. The obtained diastereomers exhibited an excellent purity, 94 to >99.5% as determined by GLC. The amides were then hydroxyethylated with ethylene oxide, refluxed with 2 equiv. of conc. HCl in order to rearrange the hydroxyethylated amide to the aminoester, and thereafter immediately reduced to 2-methyl-4-pentenol with $LiAlH_4$. This procedure has been shown to afford alcohols with preserved enantiomeric purity from diastereomerically pure amides (Sonnet et al., 1987). The assignment of the absolute configurations of the alcohols (see Methods and Materials) was done in analogy with the above related study by Sonnet et al., based on the correlation between stereochemistry and elution order on GLC and HPLC for the intermediate amide (Helmchen et al., 1977). The assigned absolute configurations of the amides were confirmed by a comparison of the optical rotations of the prepared alcohols with their literature values (Gramatica et al., 1988). The key intermediate alcohols obtained, (*R*)- and (*S*)-2-methyl-4-pentenol, were transformed to bromides and then to phosphonium salts to be used in subsequent Wittig reactions (Figure 4). In these or the following steps in the synthesis, racemization at the chiral center does not occur (Sonnet, 1984; Bestmann et al., 1990). Compounds (*R*)- and (*S*)-**4** were thus obtained by Wittig

reactions between the prepared phosphonium salts and valeraldehyde in THF with DMPU as cosolvent. The resulting alkadienes were oxidized with 9-BBN and thereafter acetylated. (4*S*,7*R*)-**7** (4*R*,7*S*)-**7,** (4*S*,7*S*)-**8,** and (4*R*,7*R*)-**8** were similarly prepared from the same intermediate phosphonium salts and racemic 2-methylvaleraldehyde. Each of the enantiomerically pure phosphonium salts yielded a diastereomeric pair 4*S*,7*R*/*S* and 4*R*,7*R*/*S*, respectively. The diastereomers were separated by preparative HPLC, which provided all four isomers diastereomerically pure. The assignment of the isomers were established by synthesis between chiral intermediates with known stereochemistry in both the 4 and 7 positions (see Methods and Materials).

Compounds (*R*)- and (*S*)-**3** were obtained by hydrogenating the key intermediate, 2-methyl-4-pentenol under nonepimerizing conditions (Oppolzer et al., 1985) by $LiAlH_4$ and $CoCl_2$ (Ashby and Lin, 1978). The hydrogenated alcohols were then transformed to their phosphonium salts via the bromides and employed in Wittig reactions with 4-pentenal. Subsequent oxidation and acetylation yielded the target molecules (Figure 4).

The enantiomers of the 3- and 2-methyl substituted analogs of **1** [(*R*)-**5,** (*S*)-**5,** (*R*)-**6** and (*S*)-**6**] were prepared from the enantiomers of **10c** and **10d,** respectively, according to procedures previously described for the preparation of the corresponding racemic compounds **5** and **6** (Jönsson et al., 1992).

(*R*)- and (*S*)-**4** were obtained by alkylation of (*Z*)-alkenyl cuprate with chiral α-methylalkyl halides (Figure 4). This synthetic method affords products of very high *Z*-purity (Gardette et al., 1985). The chiral intermediates, (*R*)- and (*S*)-2-methylbutanol, were resolved analogously to (*R*)- and (*S*)-2-methyl-4-pentenol (Figure 2). The assignments of the absolute configurations were made by GLC analysis as described in Methods and Materials and by chiroptic comparison with a sample of (*S*)-(−)-2-methylbutanol (Aldrich Chemical Co).

Enantiomeric Purities of Compounds 2–8. The enantiomeric purities of compounds **3–8** were determined by the use of the chiral binuclear complex formed from $Eu(hfc)_3$ and Ag(fod) (Wenzel and Sievers, 1982).

When 1 equiv. $Eu(hfc)_3$ and 1 equiv. Ag(fod) were added to 0.5 equiv. of racemic compound **3** (see Methods and Material), the ^1H NMR spectrum showed a pair of well resolved CH_2O— triplets at 4.12 ppm and 4.18 ppm along with a pair of CH_3CO— singlets, which were buried in a cluster of signals at 2.15 ppm. Samples of (*S*)- and (*R*)-**3** were investigated and the NMR spectra showed that the low-field triplet belonged to the *R* form and the high-field triplet to the *S* form. When the enantiomeric samples were mixed, the ratio between the triplets changed according to mixed amounts of (*R*)- and (*S*)-**3** (Figure 6).

The olefin/$Eu(hfc)_3$/Ag(fod) complex ratio used above was not effective for resolving the CH_2O— protons of the 4-methyl analogs **(4).** The europium(III)/silver(I) ratio was thus varied to optimize the resolution of the acetate methyl singlets. This was achieved by decreasing the concentration of silver(I),

which resulted in a substantial downfield shift of these signals to 3.25 and 3.31 ppm (Figure 6). The acetate methyl signals could also be used to estimate the enantiomeric purities of (*R*)-**5**, (*S*)-**5**, (*R*)-**6**, and (*S*)-**6**.

The results of the ^{1}H NMR analysis show a high purity, 98% ee for (*R*)-**4**, (*R*)-**3**, and 97% ee for (*S*)-**4**, while (*S*)-**3** shows 88% ee. (*S*)-**3** was run from a different batch of the chiral intermediate, (*S*)-2-methyl-4-pentenol, which

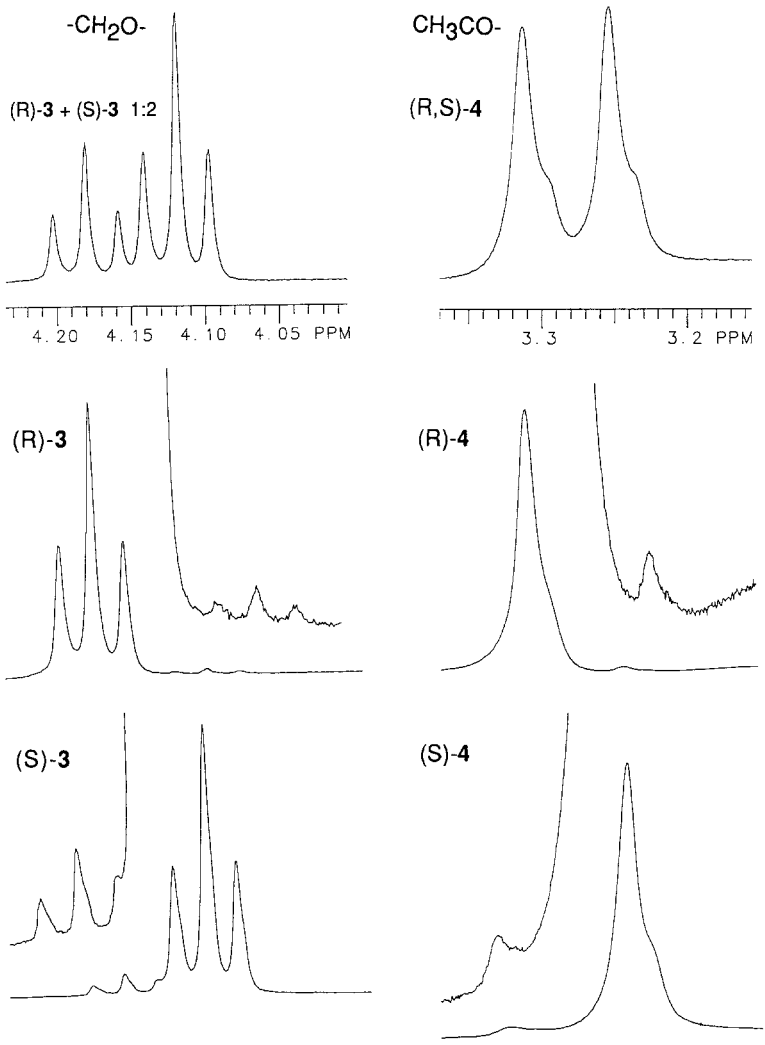

FIG. 6. Left: NMR spectra of compound **3** in $CDCl_3$ [product/Eu(hfc)$_3$/Ag(fod) = 1:0.5:0.6]. Right: compound **4** [product/Eu(hfc)$_3$/Ag(fod) = 1:0.5:0.3].

explains the lower enantiomeric excess of (*S*)-**3** than that of (*S*)-**4.** (*R*)-**5** and (*S*)-**5** both show 90% ee. The enantiomeric purities of (*R*)- and (*S*)-**6** are 94 and 96% ee, respectively. The ee values of compounds **3–8** as determined by NMR are essentially identical to the diastereomeric excess values of the diastereomerically pure amides, described above. This indicates that no racemization occurs during the reaction steps from the amides to the final products.

The enantiomeric purities of the enantiomers of compounds **7** and **8** were determined with the same complex ratio as for **4** (Figure 6). For these compounds, resolved signals from the enantiomers were observed in the spectra between 0.7 and 1.2 ppm. The two doublets for each enantiomer in this region are the resonances for the protons of the methyl groups in the 4 and 7 positions. The results showed 97% ee for (4*S*,7*R*)-**7** and (4*S*,7*S*)-**8,** while (4*R*,7*S*)-**7,** and (4*R*,7*R*)-**8** showed 88% ee.

In contrast to the excellent enantiomeric shifts of compounds **3–7** and **8,** signals from the enantiomers of compound **2** were less well resolved. The enantiomeric purities of (*R*)- and (*S*)-**2** were determined by GLC analysis of the amides obtained from (*R*)- and (*S*)-2-methylbutanol by successive Jones oxidation and amidation with enantiomerically pure (*S*)-α-methylbenzylamine and found to be >96% ee (see Methods and Materials). The subsequent reactions to the target molecules are not expected to endanger the asymmetric center.

Receptor Cell Responses. The single-cell responses of compounds **1–8** are shown in Figures 7 and 8. The biological activities, which include corrections for differences in volatility (see Methods and Materials) are shown relative to the natural pheromone component **1.**

For compound **4,** which is methyl substituted in the 4 position, none of the enantiomers or the racemate display a measurable electrophysiological activity (Figure 7). However, methyl substitution in the 7 position (compound **3**),

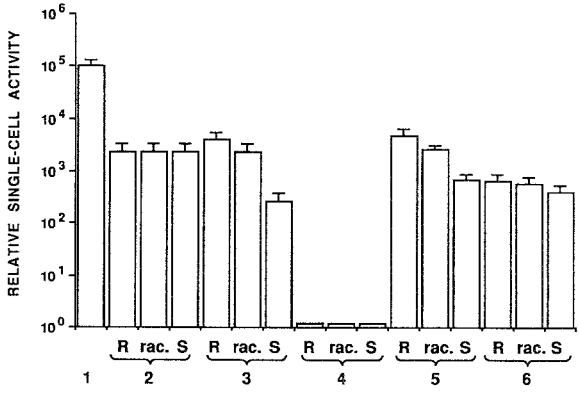

FIG. 7. Relative single-cell activities (+SEM) for compounds **1–6.**

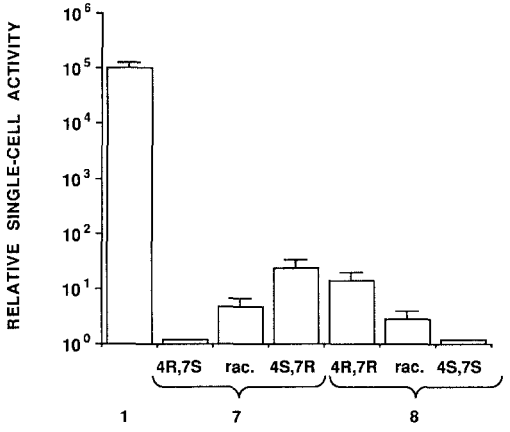

FIG. 8. Relative single-cell activities (+SEM) for compounds **1**, **7**, and **8**.

gives a significant difference in the activities of the two enantiomers. (*R*)-**3** shows an activity decrease compared to **1** by a factor of 26, while (*S*)-**3** displays a decrease by a factor of 370. Thus, the *R* enantiomer is more active than the *S* enantiomer by a factor of 14. It should be noted that (*S*)-**3** contains about 6% of the more active (*R*)-**3.** A similar activity difference between enantiomers was observed for the 3-methyl-substituted compound **5.** (*R*)-**5** is more active than (*S*)-**5** by a factor of 7.

Methyl substitution in the 2 or 8 position of **1** (compounds **6** and **2,** respectively) does not cause any significant differences in the electrophysiological activities of the enantiomers (Figure 7).The observed decrease in the activity for these analogs compared to that of **1** is a factor of 45 for (*R*)- and (*S*)-**2,** a factor of 148 for (*R*)-**6** and a factor of 251 for (*S*)-**6.**

Dimethyl substitution in the 4,7 positions gives two diastereomers, each with a pair of enantiomers. Of these isomers, (4*S*,7*R*)-**7** is the most active one with an observed activity approximately 4200 times lower than that of **1** (Figure 8). This activity is surprising in view of the complete inactivity of (*S*)-**4.** (4*R*,7*R*)-**8** shows an activity decrease of approximately 7300 times compared to the activity of **1,** while (4*R*,7*S*)-**7** and (4*S*,7*S*)-**8** are found to be essentially inactive (Figure 8).

Conformational Energies. As mentioned in the introduction, we have, as in our previous work on alkyl substituted analogs of **1** (Jönsson et al., 1991a, 1992), attempted to separate the activity-decreasing effects due to steric repulsive interactions between the substrate and the receptor and the effects due to unfavorable conformational energies. This has been done as previously described (Bengtsson et al., 1987; Liljefors et al., 1987) by calculating the energy required for each compound to acquire its biologically active conformation. For each

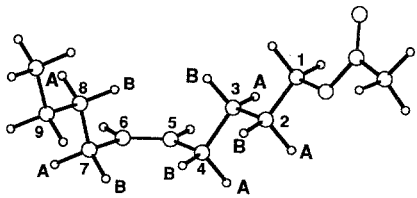

FIG. 9. The proposed biologically active conformation of compound **1** and the possible methyl group directions in 2-, 3-, 4-, 7-, and 8-methyl-substituted analogs of **1.**

compound this energy has been by calculated as the energy difference between the thermodynamically most stable conformation and the conformation mimicking the three-dimensional structure of **1** shown in Figure 9. In our previous studies (Bengtsson et al., 1987; Liljefors et al., 1987) we have concluded that this conformation most probably is the biologically active one for **1.** All calculations have been done by the molecular mechanics program MM2(85) developed by Allinger and coworkers (Burkert and Allinger, 1982). The results of the calculations are shown in Table 1. The calculated conformational energies for the 7- and 8-substituted analogs (compounds **3** and **2**) given in Table 1 differ slightly from the energies reported in our previous analysis (Jönsson et al., 1991a). This is due to a revised conformational analysis of **1,** which includes conformers of the "coiled" type (Jönsson et al., 1992).

TABLE 1. CALCULATED CONFORMATIONAL ENERGY DIFFERENCES (kcal/mol) BETWEEN PROPOSED BIOLOGICALLY ACTIVE CONFORMATION AND LOWEST-ENERGY CONFORMATION OF COMPOUNDS **1–8**[a]

Compound	A	A + B	B
1		0.0[b]	
2	0.1		1.2
3	0.2		1.8
4	1.0		5.0
5	1.7		1.5
6	1.3		1.0
7 (4A, 7A)-		0.9	
(4B, 7B)-		9.7	
8 (4A, 7B)-		0.7	
(4B, 7A)-		3.1	

[a]The energy values are given in relation to that of **1**. A and B denote alkyl group directions according to Figure 9. The data for **4–6** are taken from Jönsson et al. (1992).
[b]The A/B designation is not applicable in this case.

An alkyl group in positions 2, 3, 4, 7, or 8 (compounds **2–6**) may adopt two different directions, denoted A and B in Figure 9. Substitution in different directions corresponds to different enantiomers. The calculated results for the monomethyl substituted compounds **2–6** have been discussed before (Jönsson et al., 1991a, 1992). For each of these compounds, with the exception of compound **4** with a methyl group in the B direction, the rearrangement from the thermodynamically most stable conformation to the biologically active one is calculated to require only a modest amount of energy (less than 2 kcal/mol), irrespective of the substituent direction. A methyl group in the B direction of position 4 suffers from substantial intramolecular repulsions with the hydrogen atom in the B direction of position 7, resulting in an energy penalty of 5.0 kcal/mol (Table 1).

4,7-Dimethyl substitution (compounds **7** and **8**) gives four isomers. The isomer with both methyl groups in B directions [(4B,7B)-**7**, Table 1], is calculated to require a very high conformational energy to acquire the active conformation, 9.7 kcal/mol. This is caused by severe steric repulsions between the methyl groups in the biologically active conformation. Moreover, the isomer with one methyl group in the 4B direction, [(4B,7A)-**8**], is calculated to have a substantial conformational energy penalty, 3.1 kcal/mol, on binding to the receptor. For the remaining two isomers of **7** and **8** the energy penalties are calculated to be small (Table 1).

DISCUSSION

The introduction of methyl substituents to compound **1** is anticipated to affect the interaction between the substrate and the receptor by a possible development of steric repulsive interactions with the receptor "wall" along with the possibility of a reduced interaction energy of the receptor–substrate complex due to unfavorable conformational effects of the modified substrate. It is possible to evaluate the latter contribution with our conformational energy model, for which ca. 2 kcal/mol of conformational energy penalty corresponds to an activity decrease by a factor of 10 (Bengtsson et al., 1987; Liljefors et al., 1987). Activity decreases in excess of those due to unfavorable conformational energies should then be due to repulsive steric interactions between the substrate and the receptor.

Methyl substitution in the 4 position of the pheromone component **1**, compounds (*R*)-**4** and (*S*)-**4**, provokes no measurable electrophysiological activity. This was predicted in our previous study on the racemate of **4** and its 4,4-dimethyl substituted derivative (Jönsson et al., 1992). As the calculated conformational energy penalty for a substituent in the A direction of **4** (Figure 9) is low (1.0 kcal/mol, Table 1), the lack of electrophysiological activity **4** is

likely caused by severe repulsive steric interactions with the receptor. A methyl substituent in the B direction might escape severe repulsion with the receptor, but this compound will instead suffer from a high conformational energy penalty (5.0 kcal/mol, Table 1) to acquire the active conformation. However, the conformational energy alone can not explain the inactivity, indicating involvement of steric repulsive interactions with the receptor site as well.

Methyl substitution in the 8 position of **1** (compound **2**) results in quite high and essentially identical electrophysiological activities for the enantiomers (and the racemate) (Figure 7). The calculated conformational energies for the methyl group in the A and B directions are 0.1 and 1.2 kcal/mol (Table 1), respectively, which, in terms of our conformational energy model, corresponds to an activity decrease, compared to the activity of **1,** by less than a factor of 5. The observed activity decrease is a factor of 45 (Figure 7). The results therefore suggest steric repulsions with the receptor of both enantiomers of **2,** but both with somewhat stronger repulsions for a methyl group in the A direction compared to a methyl group in the B direction. The relationship between the absolute configuration and the A/B nomenclature will be discussed below.

The 2-methyl-substituted compound **6** also displays essentially identical activities for the enantiomers. As the activities compared to that of **1** are 150–250 times lower and as the conformational energies for methyl substitution in the A and B directions are very similar and do not account for the activity decrease (Table 1), the conclusion is that a methyl group in the A as well as in the B direction causes steric repulsive interactions with the receptor.

Methyl substitution in the 7 position of **1** results in a significant difference in measured activities of the enantiomers (*R*)-**3** and (*S*)-**3,** in line with our predictions from a previous study on the racemate of **3** and its 3,3-dimethyl-substituted analog (Jönsson et al., 1991a). (*R*)-**3** displays a high electrophysiological activity, only 26 times less than that of **1,** while (*S*)-**3** was found to be 370 times less active than **1** (Figure 7). The racemate shows an activity decrease by a factor of 45 in accordance with earlier results (Jönsson et al., 1991a). As mentioned above, (*S*)-**3** contains 6% of the *R* isomer. This amount of the more potent *R* isomer can account for a large part of the observed activity for (*S*)-**3.** We therefore expect the activity of pure (*S*)-**3** to be considerably lower than displayed in Figure 7.

In our previous study on the racemic 3-methyl analog of **1** and the corresponding 3,3-dimethyl compound (Jönsson et al., 1992), we found that monomethyl substitution caused an activity decrease by only a factor of 22, while 3,3-dimethyl substitution resulted in a decrease of the activity by a factor of 1100. We also found that the much lower activity of the 3,3-dimethyl analog could not be explained by conformational effects. We then concluded that one of the enantiomers of the 3-methyl-substituted analog should be more active than the other one (Jönsson et al., 1992). The observed activities for (*R*)-**5** and

(*S*)-**5** in the present study (Figure 7) show this to be the case. The *R* enantiomer is significantly more active than the *S* enantiomer. As the less active *S* enantiomer contains 5% of the *R* enantiomer, the enantioselectivity of the receptor is underestimated by the data shown for (*R*)- and (*S*)-**5** in Figure 7.

The results clearly show that the receptor is able to differentiate between enantiomers, which implies that the receptor contains elements of chirality. If we consider the deduced biologically active conformation of **1** (Figure 9), we must also include its mirror conformation as a candidate for the active conformation. Thus two "enantiomeric" conformations are possible, which are readily interconvertable through rotation about the C-4–C-5 and C-6–C-7 bonds of **1.** Only one of these conformations of the substrate should be able to fit into the receptor cavity. Substitution in a prochiral position of **1** removes the enantiomeric relationship of these conformations as shown for (*R*)-**3** in Figure 10. The (*R*)-methyl group is positioned in the A or B direction depending on the chiral sense of the cisoid chain. Thus, in order to be able to relate the A and B directions to the electrophysiological activities for the *R* and *S* enantiomers, the chiral sense of the receptor bound pheromone component **1** must be known. For example, two interpretations are possible to explain the activities of compound **3** in terms of steric repulsive interactions and conformational energies. If the *R* enantiomer on binding to the receptor corresponds to the upper structure in

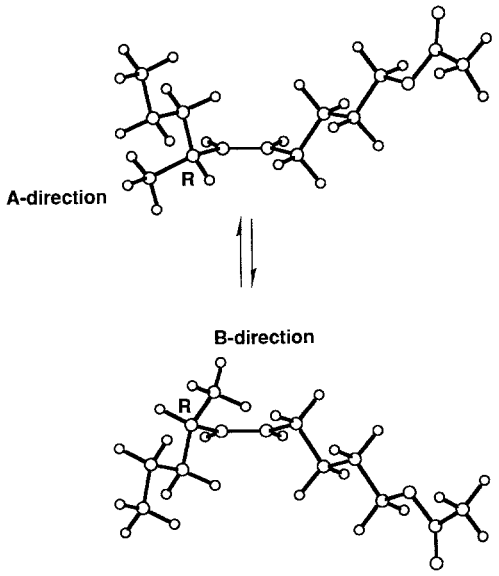

FIG. 10. The two possible active conformations of (*R*)-**3** with a different chiral sense of the alkyl chains.

Figure 10 (with the methyl group in the A direction), the activity must be determined by a moderate steric repulsion with the receptor wall since the conformational energy is small, only 0.2 kcal/mol (Table 1), while the low activity of the *S* enantiomer will be determined partly from a high conformational energy, 1.8 kcal/mol, and inevitably by severe steric repulsions as well. On the other hand, if the *R* enantiomer on binding to the receptor corresponds to the lower structure in Figure 10 (with the methyl group in the B direction), the reduction in activity of this compound can be completely explained by the conformational energy effect, while the (*S*)-methyl enantiomer is suggested to suffer from severe steric repulsions in the A direction. Either of the two interpretations is sustained by previous investigations of 7,7-dimethyl and 7-propyl substituted analogs of **1.** The results of these studies clearly indicate a severe destructive interaction in at least one of the directions at this position (Jönsson et al., 1991a).

We have attempted to answer the question of which of the "enantiomeric" conformations of **1** is the biologically active one by investigating the effect of dimethyl substitution in the 4,7 positions of **1.** This pattern of substitution gives two diastereomers, compounds **7** and **8,** each with a pair of enantiomers (Figure 1). The calculated conformational energy penalty for compound **7** substituted in the 4A,7A directions is only 0.9 kcal/mol, while the other enantiomer, which is substituted in the 4B,7B directions is calculated to need as much as 9.7 kcal/mol to bring this compound into its required conformation for a successful interaction with the receptor (Table 1). According to the relationship between conformational energy and electrophysiological activity discussed above, 9.7 kcal/mol is expected to be a too high energy for a productive receptor interaction. This means that dimethyl substitution in the 4A,7A directions with regard to conformational energies is expected to give some activity, while substitution in 4B,7B directions should give an inactive compound. Consistent with this prediction, only one of the enantiomers of **7** shows measurable activity, albeit quite low. (4*S*,7*R*)-**7** displays a drop of activity by a factor of approximately 4200 compared to the activity of **1,** while (4*R*,7*S*)-**7** is found to be completely inactive (Figure 8). The two cisoid conformations of the active enantiomer, (4*S*,7*R*)-**7,** with different a chiral sense of the chain are shown in Figure 11. As discussed above, the lower structure is calculated to have a prohibitively high conformational energy for binding to the receptor. This indicates that the chiral sense of the chain of (4*S*,7*R*)-**7** on interacting with the receptor should be as shown in the upper structure of Figure 11. This implies that the chiral sense of the chain of the natural pheromone component **1** on binding to the receptor should be as shown in Figure 10. The most active enantiomer of compound **3,** (*R*)-**3,** is thus concluded to have its methyl group in the A direction (Figure 10, upper structure). Furthermore, the most active enantiomer of the 3-methyl-substituted compound **5,** that is (*R*)-**5,** is concluded to have its methyl group in the B direction. Since a 3-methyl group in this direction is "shielded" by the terminal alkyl

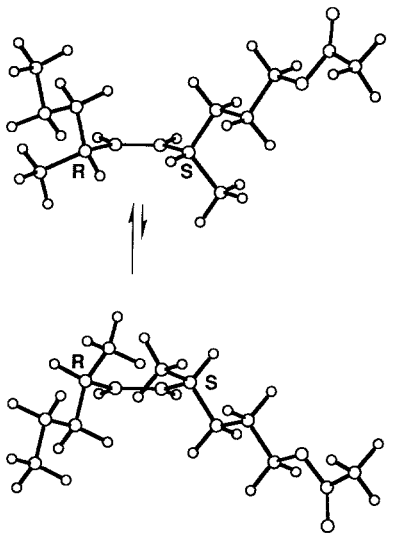

FIG. 11. The two possible active conformations of (4*S*,7*R*)-**7** with a different chiral sense of the alkyl chains.

chain, this is also the most probable methyl group direction for the more active enantiomer (Jönsson et al., 1992).

Of the two enantiomers of compound **8**, only (4*R*,7*R*)-**8** shows measurable activity (Figure 7). Thus, of the four isomers of compounds **7** and **8**, only the isomers with an *R* configuration at position 7, as in the most active enantiomer of **3**, show electrophysiological activities.

Considering the lack of activity of both enantiomers of compound **4** it is somewhat surprising that (4*S*,7*R*)-**7** and (4*R*,7*R*)-**8** show some electrophysiological activity. However, compounds **7** and **8** are much less flexible than compound **4** and should thus lose less conformational entropy on binding to the receptor, thereby increasing the probability of a productive receptor interaction for the dimethyl substituted compounds **7** and **8** compared to that of **4**.

As mentioned in the introduction, Bestmann et al. (1992) have recently studied the ability of some receptors for dienic pheromone components to differentiate between enantiomers of methyl-substituted analogs of the natural pheromone component. The analogs are methyl-substituted in a position corresponding to that of the 7-methyl group in (*R*)- and (*S*)-**3** in the present study. In four of seven cases, the observed activity difference between enantiomers was only a factor 3 or less. In two of these cases, the *S* enantiomer was the slightly more active one, while the *R* enantiomer was the slightly more active one in one case. The "aldehyde receptor" of *Bombyx mori* did not differentiate between the enantiomers of 14-methylbombykal.

In the remaining three cases, including the "aldehyde receptors" of *Manduca sexta*, *Antheraea pernyi*, and *Antheraea polyphemus*, the receptors were shown to be able to differentiate between the enantiomers by factors of 10–100 with the *R* enantiomer as the most active enantiomer in each case. As reported above, the *R* enantiomer is also the most active enantiomer of compound **3.**

CONCLUSIONS

The structure–activity results of the enantiomers of methyl-substituted analogs of **1** studied in this work demonstrate that the electrophysiological activity as well as the ability of the receptor to differentiate between enantiomers depends on the position of the methyl substituent. For analogs methyl substituted in the 2, 4, and 8 positions no differences in the activities of the enantiomers could be observed. The 8-methyl enantiomers display quite high activities, while the 4-methyl-substituted ones were found to be inactive, demonstrating a high sensitivity of the electrophysiological activity on the presence of steric bulk at this position. The enantiomers of the 3- and 7-methyl analogs display a significant difference in the activity, the *R* enantiomer being more active than the *S* enantiomer in both cases.

From an analysis of the electrophysiological responses of the 4,7-dimethyl substituted analogs of **1,** in conjunction with calculated conformational energies, the chiral sense of the alkyl chain of the natural pheromone on binding to the receptor could be deduced.

The studies reported in this work and in previous work on alkyl substituted analogs of **1** (Jönsson et al., 1991a, 1992) demonstrate that the entire hydrocarbon part of **1** interacts with its receptor in a highly complementary way. This is illustrated in Figure 12, which shows a superimposition of the active conformation of **1** and the positions of the hydrogen atoms of the methyl groups of

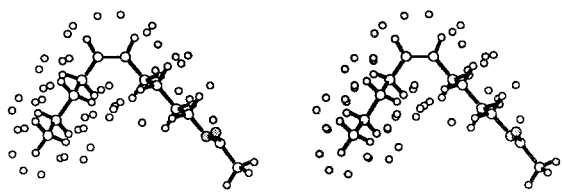

FIG. 12. Superimposition (in stereo) of the deduced active conformation of **1** and the positions of the methyl hydrogen atoms of the mono-methyl-substituted analogs of **1** showing steric repulsive interactions with the receptor. The positions of the hydrogens of methyl groups attached to the double bond are taken from Jönsson et al. (1991a, 1992).

methyl substituted analogs of **1** for which repulsive steric interactions with the receptor have been concluded.

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REFERENCES

ASHBY, E.C., and LIN, J.J. 1978. Selective reduction of alkenes and alkynes by the reagent lithium aluminium hydride-transition-metal halide. *J. Org. Chem.* 43:2567–2572.

AULT, A. 1965. Resolution of D,L-α-phenylethylamine. *J. Chem. Ed.* 42:269.

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.

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.

BESTMANN, H.J., FRIGHETTO, R.T.S., FRIGHETTO, N., and VOSTROWSKY, O. 1990. Synthese der Enantiomeren von 10-Methyldodecyclacetat und 12-Methyltetradecylacetat, chiralen Pheromonkomponenten von *Adoxophyes*-Arten (Lepidoptera: Tortricidae). *Liebigs Ann. Chem.* 1990:829–831.

BESTMANN, H.J., WU, C.H., W., REHEFELD, C., KERN, F., and LEINEMANN, B. 1992. Do pheromone receptors that receive the same or similar signal molecules have the same or similar structure? *Angw. Chem. Int. Ed. Engl.* 31:330–331.

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

CHAPMAN, O.L., KLUN, J.A., MATTES, K.C., SHERIDAN, R.S., and MAINI, S. 1978a. Chemoreceptors in Lepidoptera: Stereochemical differentiation of dual receptors for an achiral pheromone. *Science* 201:926–928.

CHAPMAN, O.L., MATTES, K.C., SHERIDAN, R.S., and KLUN, J.A. 1978b. Stereochemical evidence of dual chemoreceptors for an achiral sex pheromone in Lepidoptera. *J. Am. Chem. Soc.* 100:4878–4884.

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.* 31:2647–2650.

DALE, J.A., and MOSHER, H.S. 1973. Nuclear magnetic resonance enantiomer reagents. Configurational correlations via nuclear magnetic resonance chemical shifts of diastereomeric mandelate, *O*-methylmandelate, and α-methoxy-α-traifluoromethylphenylacetate (MTPA) esters. *J. Am. Chem. Soc.* 95:512–519.

FANTA, W.I., and ERMAN, W.F. 1972. Studies related to the synthesis of (±)-dihydro-β-santalol. *J. Org. Chem.* 37:1624–1630.

FIESER, L.F., and FIESER, M. 1967. Reagents for Organic Synthesis, Vol. 1. Wiley-Interscience, New York, pp. 142–144.

GARDETTE, M., ALEXAKIS, A., and NORMANT, J.F. 1985. Carbocupration of alkynes by organocopper reagents bearing a protected hydroxy or thiol function. *Tetrahedron* 41:5887–5899.

GRAMATICA, P., MANITTO, P., MONTI, D., and SPERANZA, G. 1988. Regio- and stereoselective

hydrogenation of methyl substituted pentadien-1-ols by baker's yeast. *Tetrahedron* 44:1299–1304.

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

HELMCHEN, G., VÖLTER, H., and SCHÜHLE, W. 1977. Directed resolution of enantiomers via liquid chromatography of diastereomeric derivatives III. A convenient method to determine the absolute configuration of carboxylic acids R^1R^2HCOOH. *Tetrahedron Lett.* 16:1417–1420.

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.

JÖNSSON, S., LILJEFORS, T., and HANSSON, B.S. 1991a. Alkyl substitution in terminal chain of (*Z*)-5-decenyl acetate, a pheromone component of turnip moth, *Agrotis segetum*. Synthesis, single-sensillum recordings, and structure–activity relationships. *J. Chem. Ecol.* 17:103–122.

JÖNSSON, S., LILJEFORS, T., and HANSSON, B.S. 1991b. Replacement of the terminal methyl group in a moth sex pheromone component by a halogen atom. Hydrophobicity and size effects on the electrophysiological single-cell activities. *J. Chem. Ecol.* 17:1381–1397.

JÖNSSON, S., LILJEFORS, T., and HANSSON, B.S. 1992. Introduction of methyl gruops to acetate substituted chain of (*Z*)-5-decenyl acetate, a pheromone component of turnip moth, *Agrotis segetum*. Synthesis, single-sensillum recordings, and structure–activity relationships. *J. Chem. Ecol.* 18:637–657.

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

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.

OPPOLZER, W., DUDFIELD, P., STEVENSON, T., and GODEL, T. 1985. Camphorsulfonamide-shielded, asymmetric 1,4-additions and enolate alkylations; synthesis of a southern corn rootworm pheromone. *Helv. Chim. Acta* 68:212–215.

ROSSI, R., and CONTI, C. 1977. Relationship between absolute configuration and sign of the rotatory power of 5-bromo-2-methyl-1-pentanol. *Chim. Ind.* 59:248–249.

SONNET, P.E. 1984. General approach to synthesis of chiral branched hydrocarbons in high configurational purity. *J. Chem. Ecol.* 10:771–781.

SONNET, P.E., PROVEAUX, A.T., ADAMEK, E., SUGIE, H., SATO, R. and TAMAKI, Y. 1987. Stereoisomers and analogs of 14-methyl-1-octadecene, sex pheromone of peach leafminer moth, *Lyonetia clerkella*, L. *J. Chem. Ecol.* 13:547–555.

STÄLLBERG-STENHAGEN, S. 1946. Optically active higher aliphatic compounds. I. The synthesis of (+)-2-methyldodecanoic and (+)-2-methylhexacosanoic acids. *Ark. Kemi. Min. Geol.* 23A(15):1–14.

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.

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.

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

WENZEL, T.J., and SIEVERS, R.E. 1982. Nuclear magnetic resonance studies of terpenes with chiral and achiral lanthanide(III)–silver(I) binuclear shift reagents. *J. Am. Chem. Soc.* 104:382–388.

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(3*R**,5*S**,6*R**)-3,5-DIMETHYL-6-(METHYLETHYL)-3,4,5,6-TETRAHYDROPYRAN-2-ONE, A THIRD SEX PHEROMONE COMPONENT FOR *Macrocentrus grandii* (GOIDANICH) (HYMENOPTERA: BRACONIDAE) AND EVIDENCE FOR ITS UTILITY AT ECLOSION

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Abstract—The compound (3*R**,5*S**,6*R**)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one was identified as a sex pheromone component of *M. grandii*. Laboratory and field bioassays demonstrated that it elicits flight initiation, upwind anemotaxis, and casting in male wasps. The compound acts synergistically with (*Z*)-4-tridecenal, a previously identified sex pheromone component of female *M. grandii*, to increase male response to the aldehyde component. The source of the lactone was determined to be the mandibular glands of male and female wasps. At eclosion a majority of male–female and female-only cocoon masses released the lactone and attracted male wasps. Male-only cocoon masses were not attractive at eclosion and the lactone component was either not released or released at below-threshold concentration. Mating was observed to occur following eclosion in laboratory and field studies.

Key Words—Pheromone, synergist, (3*R**,5*S**,6*R**)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one, (*Z*)-4-tridecenal-4-tridecenal, parasitoid, *Macrocentrus grandii*, *Macrocentrus iridescens*, Hymenoptera, Braconidae.

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INTRODUCTION

Macrocentrus grandii Goidanich is a polyembryonic larval parasitoid of *Ostrinia nubilalis* Hübner, the European corn borer. *M. grandii* was introduced into North America as early as 1926 to control the corn borer. Today this parasitoid accounts for the majority of corn borer larval parasitism throughout the corn belt (Winnie and Chiang, 1984).

In a previous communication, we reported that a hexane extract of female *M. grandii*, when partitioned using open column chromatography with Florisil, yielded three Florisil fractions attractive to male wasps. These were the hexane, 5% ether in hexane (5E), and 50% ether in hexane (50E) fractions (Swedenborg and Jones, 1992a). In addition, a hexane extract of male *M. grandii*, when partitioned using identical column chromatography, yielded a 50E fraction attractive to male wasps. In a subsequent communication (Swedenborg and Jones, 1992b), the female-derived Florisil hexane fraction component was identified as a series of (*Z*,*Z*)-9,13-hydrocarbon dienes of 27–41 carbon atoms. In addition, the female-derived 5E fraction component was identified, in part, as (*Z*)-4-tridecenal, an air oxidation product common to all of the dienes. Oxidation to the aldehyde is apparently responsible for the pheromonal activity of the (*Z*,*Z*)-9,13-dienes. The female-derived diene and aldehyde components, synthetic (*Z*,*Z*)-9,13-heptacosadiene, and synthetic (*Z*)-4-tridecenal were each shown to elicit flight initiation, upwind anemotaxis, casting, landing on the source, walking, wing fanning, and copulatory attempts by male wasps in a wind tunnel. A second attractive element of the 5E fraction elicited similar behaviors in laboratory bioassay as (*Z*)-4-tridecenal; however, its origin and nature were not determined.

The 50E fraction component (male- or female-derived) elicited flight initiation, upwind anemotaxis, and casting by male wasps in a wind tunnel (Swedenborg and Jones, 1992a). However, landing on the source and subsequent courtship behavior following upwind flight were infrequent. When the male- or female-derived 50E fraction was paired with the female-derived Florisil hexane fraction, male responses were increased synergistically in laboratory and field bioassays (Swedenborg and Jones, 1992a). Furthermore, when the female-derived 50E fraction was paired with synthetic (*Z*,*Z*)-9,13-heptacosadiene or (*Z*)-4-tridecenal, male response in field bioassays was increased synergistically (Swedenborg and Jones, 1992b). Chemically the 50E component was determined to have the polarity of a primary alcohol and be quite volatile and/or unstable (Swedenborg and Jones, 1992a). This paper presents the identification, source, and bioassay of the 50E component.

Recently, we observed in the field that male wasps of natural populations of *Macrocentrus iridescens* French were attracted to male–female and female *M. iridescens* cocoon masses during synchronous eclosion near sunset (Swed-

enborg, unpublished data). Males landed on the leaf or leaves enclosing the cocoon mass and actively searched for females. As females emerged, males were observed to elicit wing fanning directed at the female. Subsequently, within seconds of female emergence, mating occurred on the leaf-rolled cocoon surface. *Macrocentrus grandii* eclose synchronously from their cocoon masses in the early photoperiod (Shyu, 1981; Wishart, 1946). *Macrocentrus grandii* and *M. iridescens* are both gregarious and endoparasitic on lepidopterous larvae. In addition, their hosts conceal themselves as leaf rollers or borers. Because of the analogous nature and congeneric relationship of the parasitoids and because of their hosts' similar foraging behaviors, these parasitoids may have related courtship behaviors. We present new observations regarding *M. grandii* courtship behavior and the apparent utility of behavioral chemicals by female *M. grandii* wasps at eclosion.

METHODS AND MATERIALS

Wasps, Cocoons, Extracts, and Florisil Fractions. Approximately 20,000 virgin female and 20,000 virgin male *M. grandii* were reared from parasitized corn borer larvae (Ding et al., 1989; Guthrie et al., 1971). Handling, extraction, and initial purification of the extracts on Florisil were as described by Swedenborg and Jones (1992a). All chemical work started with the 50E fraction, which contained the synergist. Cocoons used for bioassay were from our laboratory-reared colony. Field bioassays with natural populations of *M. grandii* were conducted at the Minnesota Agricultural Experiment Station, University of Minnesota, Rosemont, Minnesota, in late July 1991. Voucher specimens have been placed in the University of Minnesota insect museum.

Chromatography. High-pressure liquid chromatography (HPLC) was performed isocratically with a Waters Associates M6000A pump and a R401 differential refractometer detector. An Altex 300 mm × 8 mm ID μ-Spherogel 50 Å size-exclusion column was used. Samples were eluted with 25% ether in hexane at 1.0 ml/min, collected, analyzed by GC, and bioassayed later.

Gas chromatography (GC) was performed with Hewlett Packard 5830A and 5890A instruments, both equipped with flame ionization detectors. The 5830A was modified to contain an effluent splitter and thermal gradient collector (similar to that described by Brownlee and Silverstein, 1968). Columns used were: a Durabond DB-1 (bonded methylsilicone 15 m × 0.53 mm ID, J & W Scientific, Folsom, California) and a SP-2380 (stabilized phase 30 m × 0.53 mm ID, Supelco, Bellefonte, Pennsylvania) on the 5830A instrument for pheromone collections and analyses; and a Durabond DB-5 (bonded methyl 5% phenyl silicone 30 m × 0.53 mm ID, J & W Scientific) and a SLP-Silar-10C (nonbonded 100% cyanopropyl silicone 50 m × 0.25 mm ID, Quadrex, New

Haven, Connecticut) on the 5890A instrument for pheromone characterization and analyses. Key GC temperature programs are noted with the results.

Spectra. Coupled gas chromatography–mass spectrometry (GC-MS) was carried out with either an LKB 9000 or a Kratos MS25 instrument using a Durabond DB-5 (15 m × 0.53 mm ID) column for sample introduction. An ionization potential of 70 eV was used for electron impact (EI) spectra. Methane was the reagent gas for spectra obtained in the chemical ionization (CI) mode.

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with a Nicolet NT 300-WB FT-NMR spectrometer equipped with a decoupler and 5-mm ^{1}H and ^{13}C probes. ^{1}H and two-dimensional phase-sensitive double quantum-filtered COSY spectra also were obtained with a Varian Unity 500 MHz spectrometer equipped with a Sun workstation. All samples for NMR, after purification by chromatography, were dissolved in $CDCl_3$ (99.8 atom % D, Aldrich Chemical Co.). NMR experiments were conducted with ca. 2 mg of sample. Chemical shifts are reported on the δ scale and coupling constants (J) in Hz.

The Fourier-transformed infrared (FT-IR) spectrum was taken with a Mattson Sirius 100 spectrometer equipped with a Starlab data system. The solvent was $CDCl_3$. The IR spectrum was run with approximately 50 μg of sample in a 200-μl KBr microcavity cell and bands were reported in wave numbers (cm^{-1}).

Chemical Tests. All chemical tests specific for functional groups were run in duplicate on separate male- and female-derived active HPLC fractions. Following the test, the product was run on the GC or GC-MS to look for retention and/or spectral changes indicating chemical changes. Internal standards were run with each test to verify the reaction. In addition, reaction products were bioassayed (as described by Swedenborg and Jones, 1992a) to test for synergistic activity with the Florisil hexane fraction.

For ozonolysis, an extract of 20 female equivalents (FE) and 20 male equivalents (ME) in carbon disulfide was treated with ozone for 20 sec following the procedure of Beroza and Bierl (1967).

For hydrogenation, an extract of 10 FE and 10 ME in 100 μl methanol was hydrogenated over 0.5 mg of 10% palladium on activated carbon for 1 min (Parliment, 1973).

For acetylation, to an extract of 20 FE and 20 ME in hexane 100 μl of acetyl chloride was added. After 15 min at 50°C the reaction was quenched with 100 μl water followed by extraction with 100 μl of pentane.

For reduction with lithium aluminum hydride, an extract of 20 FE and 20 ME in 1 ml of anhydrous ether was added to 50 mg of fresh lithium aluminum hydride. After 24 hr at room temperature, sodium sulfate decahydrate was added to decompose excess lithium aluminum hydride.

Source of Pheromone. Female and male wasps (1–2 days old) were killed by freezing (10 individuals per replicate, three replicates), placed into a Petri

dish with insect physiological saline, and dissected under a microscope. Body parts were placed into 300-μl cone-shaped microreaction vials with 100 μl of hexane and allowed to soak overnight. The following day, extracts of each of the body parts were examined on the GC for evidence of the pheromone. For the initial dissection, the insect body was divided into the head, antennae, thorax, wings, legs, abdomen, and ovipositor with sheath. For the final dissection, wasp heads were progressively subdivided using microsurgery knives until the source of the pheromone was identified.

Laboratory and Field Bioassays. Laboratory and field bioassays were conducted as described by Swedenborg and Jones (1992a). A wind tunnel (31 $\times$ 42 $\times$ 92 cm; airspeed ca. 25 cm/sec) was used for the laboratory bioassays. Chemical treatments were applied to 4-cm watch glass plates with each plate attached to a Boston clip. The plates were hung at the upwind end of the tunnel on prepositioned wire hooks. Three plates or treatments were in the wind tunnel for each bioassay. Each bioassay was replicated three times. About 150–200 males were used for a bioassay of the treatments. Male behavior included upwind flight, casting in front of the source, and subsequent landing on the source and wing fanning. Visits at the source were usually brief and the bioassays were quantified by recording the total number of males landing on each plate during a 1-min period. Purified (via Florisil and HPLC) male- and female-derived synergists (1/3 wasp equivalent) were tested in conjunction with 100 ng of synthetic (*Z*,*Z*)-9,13-heptacosadiene [each female wasp has ca. 9 μg of (*Z*,*Z*)-9,13-diene material] or a Hercon polymer bonded slow-release wafer formulation (0.15 mg/cm^2) of synthetic (*Z*)-4-tridecenal. The target release dosage of the aldehyde was 12.5 ng/hr (release rate not verified). Experimental design was a randomized complete block with each bioassay being a block. Data were transformed ($\sqrt{x + 1}$) to stabilize variance and tested for homogeneity with Levene's test. An analysis of variance (ANOVA) was then applied (degrees of freedom for error = 4) with mean separations by Fisher's (protected) least significant difference (PLSD), $\alpha \leq 0.05$ (Steel and Torrie, 1980).

Wind-tunnel tests with cocoons were with pharate cocoon masses, and they were bioassayed during the early photoperiod at eclosion. Prior to eclosion, each cocoon mass was attached to a Boston clip and paired with an unattractive cocoon mass at the upwind end of the wind tunnel on prepositioned wire hooks. Subsequently, male behavioral response was recorded. Male response to attractive cocoons generally included only upwind flight. An attractive cocoon mass was one in which at least 20 male wasps elicited synchronous upwind anemotaxis and casting within 8 cm downwind of the treatments at eclosion. The test period was 5 min.

For the field bioassays of chemical treatments, ca. 1500 laboratory-reared males were released into a test plot of hybrid field corn. Pherocon 1C traps were attached to corn stalks at ca. 1 m above the ground. Treatments were applied to

4-cm watch-glass plates centered inside the trap bottom. Purified (via Florisil and HPLC) female-derived synergist was applied at 15 FE and a Hercon polymer bonded slow-release wafer formulation of (*Z*)-4-tridecenal (0.15 mg isomer/cm^2) was used at a target release dosage of 50 ng/hr (release rate not verified). Field data were transformed to $\sqrt{x + 1}$ to stabilize variance and tested for homogeneity with Levene's test. The control treatment was not included in the analysis. An ANOVA was applied with mean separations by PLSD, $\alpha \leq 0.05$.

The cocoon field tests were conducted in a corn field with feral second-generation adult *M. grandii* present (first-generation corn borers in this test site were ca. 30% parasitized by *M. grandii*). Pharate cocoon masses were placed either in 4-cm × 20-cm screen cylinders or left uncovered and placed in a corn axil. The cocoons were placed in the field in the early evening. Beginning shortly after dawn the following day, the cocoons were visually monitored for eclosion and for the response of the natural population.

GC Test for Release of Synergist at Eclosion. Cocoon masses were monitored for release of the synergist at eclosion. Male, female, and male–female pharate laboratory-reared cocoon masses were placed into separate 2-dram vials and capped. At the start of the next photoperiod, these cocoons were visually monitored for eclosion. Approximately 15 min (15 min was chosen because nearly all the wasps were emerged from a single cocoon mass by this time) following the emergence of the first wasp from a cocoon mass the vial was opened, and the wasps were counted, sexed, and wasps and cocoon mass removed. The empty vial was then rinsed with 300 μl of pentane for 1 min. The pentane was then removed, and 2 μl of the pentane extract was injected onto the GC to quantify the synergist component. One wasp from each cocoon mass also was extracted with 300 μl of pentane, held for 1 hr, and identically run on the GC.

RESULTS

Chromatography. Separate size-exclusion chromatography of male- and female-derived 50E fractions produced similar broad peaks corresponding with biological activity (Figure 1A, fractions 5–7). The retention time of the active peak corresponded to the retention of methanol under identical chromatographic conditions. Combining the active HPLC fractions from either source and pairing them with (*Z*)-4-tridecenal or (*Z*,*Z*)-9,13-heptacosadiene in the wind tunnel produced a synergistic increase in landing response compared to these components alone (Table 1). The response to the paired treatments included flight initiation, upwind anemotaxis, casting, landing on the source, walking, wing fanning, and mating attempts between males. The paired sources dominated the bioassay arena. Only minor attention was given to the single source treatments by the males. Moreover, in a field study using laboratory-reared males, HPLC-purified female-derived synergist in combination with (*Z*)-4-tridecenal was significantly

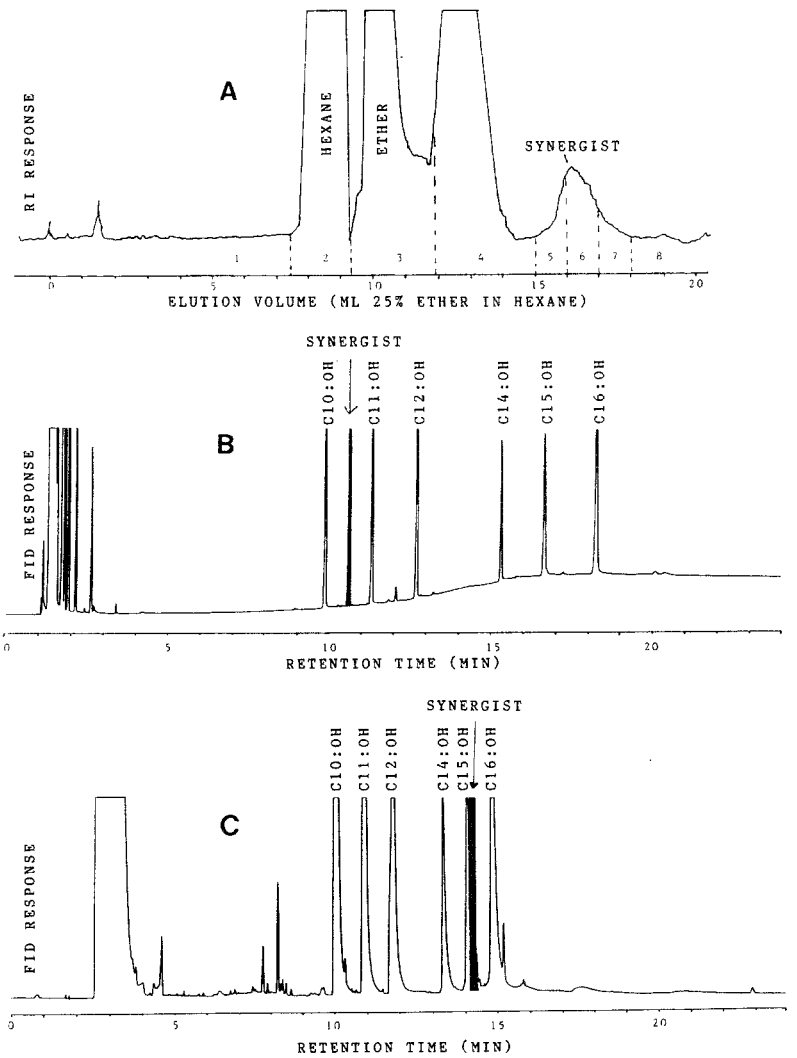

FIG. 1. Chromatograms of *M. grandii* female-derived synergist (3*R**,5*S**,6*R**)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one. (A) HPLC chromatogram using a Spherogel 50 Å size-exclusion column and refractive index (RI) detector. Sample represents 1000 FE from the female-derived 50E fraction. (B) GC chromatogram of the HPLC-purified material using a DB-5 column with internal alcohol standards (C_{10}, C_{11}, C_{12}, C_{14}, C_{15}, C_{16} primary alcohols). (C) GC chromatogram of the HPLC-purified material using a Silar-10C column with same internal alcohol standards. For each GC column samples were injected in the splitless mode at 40°C, held at 40°C for 0.5 min, ramped to 100°C at 20°C/min, then programmed to either 220°C or 205°C, respectively, at 10°C/min and held for 10 min. Helium carrier gas linear velocity at 150°C was 45 cm/sec and 25 cm/sec, respectively.

TABLE 1. LANDING RESPONSES OF MALE *Macrocentrus grandii* TO (Z)-4-TRIDECENAL OR (Z,Z)-9,13-HEPTACOSADIENE AND MALE (M) OR FEMALE (F)-DERIVED SYNERGIST IN A WIND TUNNEL

Treatment[a]	Mean[b] ($N = 3$)
Test 1	
(Z)-4-Tridecenal plus synergist (F)	54.7a
(Z)-4-Tridecenal	6.0b
Synergist (F)	0.3c
Test 2	
(Z)-4-Tridecenal plus synergist (M)	54.3a
(Z)-4-Tridecenal	6.7b
Synergist (M)	0.7c
Test 3	
(Z,Z)-9,13-Heptacosadiene plus synergist (F)	51.3a
(Z,Z)-9,13-Heptacosadiene	7.0b
Synergist (F)	2.3b
Test 4	
(Z,Z)-9,13-Heptacosadiene plus synergist (M)	47.7a
(Z,Z)-9,13-Heptacosadiene	8.6b
Synergist (M)	0.0c

[a] (Z)-4-Tridecenal was released via a Hercon polymer bonded slow-release wafer at a target dosage of 12.5 ng/hr, (Z,Z)-9,13-heptacosadiene was applied at 100 ng, and male- or female-derived synergist was applied at 1/3 wasp equivalent. The source of the synergist was the combined attractive HPLC fractions 5–7.

[b] Each replicate represents a new group of 150–200 untested males 1 to 5 days old. Counts represent the mean number of landings during a 1-min test. In each test, column means followed by different letters are significantly different (PLSD, $\alpha \leq 0.05$).

more attractive than synergist or (Z)-4-tridecenal alone (Table 2). These results supported all previous studies.

On the 5830A GC, using the DB-1 and SP-2380 columns, each of the active HPLC fractions (5–7) eluted one peak for each column. Each peak was collected and produced identical results as the HPLC-purified material in laboratory bioassay. There were no other active peaks or components in the HPLC-purified material.

To characterize the unknown, the active HPLC component was chromatographed on a DB-5 column, and it yielded one major peak at 10.68 min for both the male- and female-derived sources (Figure 1B); a retention similar to a 10- to 11-carbon primary alcohol standard. On a Silar-10C column the same component, from either source, eluted with the retention of a 15-carbon primary alcohol standard at 14.24 min (Figure 1C). Whereas open column chromatography using Florisil had indicated the synergist's polarity was similar to a primary alcohol, the GC retention shift relative to the alcohol standards indicated that the unknown was not behaving as a primary alcohol. Further character-

TABLE 2. RESPONSE OF MALE *Macrocentrus grandii* TO (*Z*)-4-TRIDECENAL AND FEMALE-DERIVED SYNERGIST IN A CORN FIELD

Treatment[a]	Mean[b] ($N = 4$)
Control	0.0
Virgin females (3)	0.5a
Synergist (15 FE)	2.0a
(*Z*)-4-Tridecenal	1.0a
(*Z*)-4-Tridecenal plus synergist (15 FE)	8.5b

[a] (*Z*)-4-Tridecenal was released via a Hercon polymer bonded slow-release wafer at a target dosage of 50 ng/hr. The synergist was female-derived and represented the combined attractive HPLC fractions 5–7.

[b] Approximately 1500 laboratory-reared males were released for this field bioassay. Column means followed by different letters are significantly different (PLSD), $\alpha \leq 0.05$).

ization was performed using two lactone standards (δ-dodecanolactone and γ-decanolactone, Aldrich Chemical Co., Milwaukee, Wisconsin). The lactone standards and the unknown demonstrated very similar chromatographic behavior using open-column and gas chromatography. A quantitative assessment indicated a range of 100–300 ng/male or female of the synergist.

Identification. Based on the chemical and spectral analyses described below, a structure of (3*R**,5*S**,6*R**)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one (Figure 2) is assigned for male- and female-derived synergist. We believe this is the first report of this structure. All analytical work was performed with separate HPLC-purified material from male- and female-derived 50E fractions.

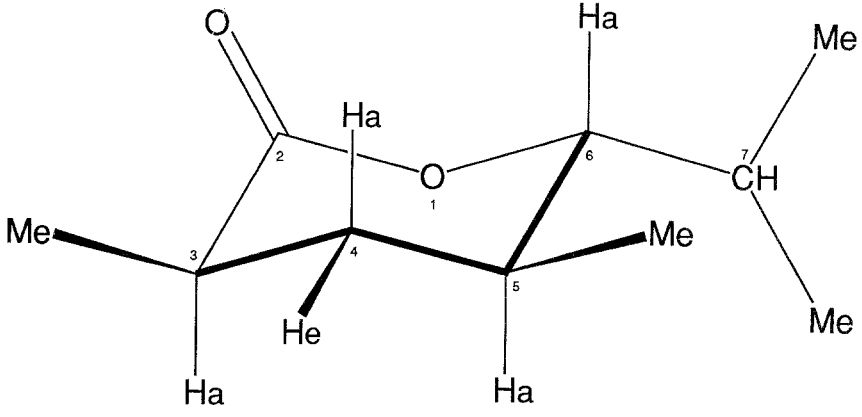

FIG. 2. (3*R**,5*S**,6*R**)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one.

Chemical Tests. The GC retention time of male- and female-derived pheromone was unchanged after hydrogenation or ozonolysis, and it remained active in bioassay. This indicated no unsaturated carbon–carbon bonds. The acetylation test also left the pheromone's GC retention time unchanged and its activity intact, indicating the absence of an alcohol group. Reduction with lithium aluminum hydride destroyed the biological activity of the pheromone. In addition, the reduction gave an apparent diol (an EI mass spectrum recorded significant mass peaks at *m/z* 131, 141, and 156; the CI mass spectrum recorded an M+1 *m/z* 175), indicating that either an ester linkage or two distinct oxygen containing functional groups had been reduced.

Spectra. EI and CI mass spectra of the unknown compound were obtained (Figure 3). The EI spectrum gave a molecular weight of *m/z* 170, which was confirmed by a CI mass spectrum with reagent methane where an M+1 *m/z* 171 was observed. A high-resolution mass spectrum indicated that the parent compound had a mass of 170.1230. This was consistent with the formula $C_{10}H_{18}O_2$ (expected mass 170.1307), which indicated two degrees of unsaturation that could be consistent with two rings or two double-bonds, or one of each in the molecule (if no triple bond). A loss of 43 [$CH(CH_3)_2$] mass units (*m/z* 127) followed by a loss of 28 (CO) mass units (*m/z* 99) was confirmed by the presence of a metastable ion of 77.2.

Peak assignments for undecoupled ^1H NMR were based on chemical shifts and double irradiation experiments (Figure 4). Characteristic signals for the

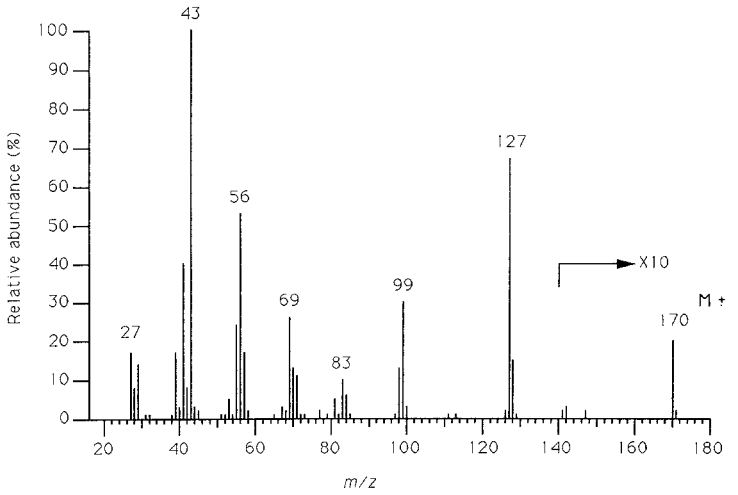

FIG. 3. Electron impact (70 eV) mass spectrum of *M. grandii* female-derived (3*R**,5*S**,6*R**)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one.

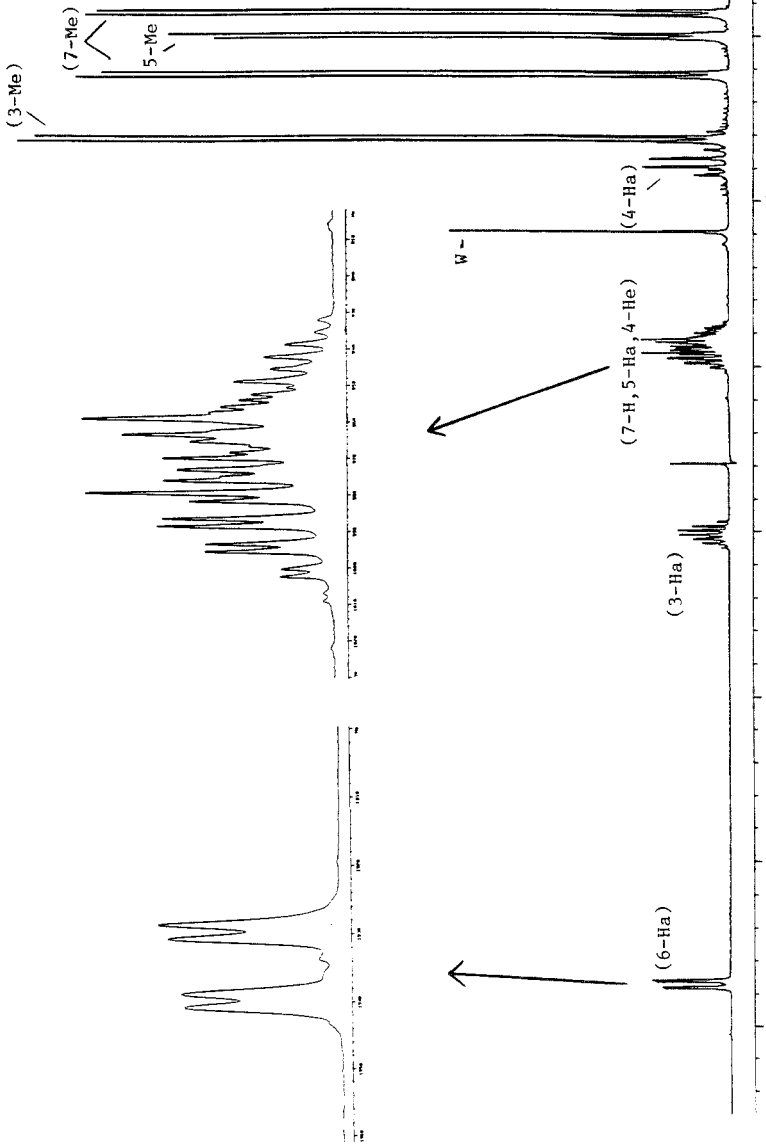

FIG. 4. 500-MHz ^{1}H NMR spectrum of *M. grandii* female-derived (3*R**,5*S**,6*R**)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one. The spectrum represents approximately 2 mg of the purified synergist in $CDCl_3$. The labels 3-Ha, 3-Me, 4-Ha, 4-He, 5-Ha, 5-Me, 6-Ha, 7-H, and 7-Me refer to the carbon protons so labeled on the perspective drawing in Figure 2. (W) is water.

undecoupled ^{1}H NMR spectrum were δ 0.93 (3H; d, J = 7; 7-Me); δ 1.00 (3H; d, J = 6.5; 5-Me); δ 1.11 (3H; d, J = 7; 7-Me); δ 1.31 (3H; d, J = 7; 3-Me); δ 1.38 (1H; q, J = 12.5, 12.5, and 12.5; 4-Ha); δ 1.86–1.96 (2H; m; 4-He and 5-Ha); δ 1.93–2.02 (1H; septet of doublets, J = 7, 7, and 2; 7-H); δ 2.46–2.54 (1H; m, J = 7, 12.5, and 6; 3-Ha); and δ 3.87 (1H; dd, J = 2 and 10; 6-Ha). Decoupling of the three-proton multiplet at δ 1.86–2.02 (7-H, 5-Ha, and 4-He) formed a singlet of each of the three doublets at δ 0.93, δ 1.00, and δ 1.11 (7-Me, 7-Me, and 5-Me); a singlet of the doublet of doublets at δ 3.87 (6-Ha); and a doublet of the quartet at δ 1.38 (4-Ha). Decoupling the proton at δ 2.46–2.54 (3-Ha) changed the doublet at δ 1.31 (3-Me) into a singlet, formed a triplet of the quartet at δ 1.38 (4-Ha), and affected the multiplet at δ 1.86–1.96 (4-He, 5-Ha). Decoupling δ 1.31 (3-Me) changed the multiplet at δ 2.46–2.54 (3-Ha) into an asymmetric triplet. A doublet of doublets was expected (J = 6, 12.5). We believe irradiating δ 1.31 (3-Me) also affected δ 1.38 (4-Ha). Decoupling the doublet of doublets at δ 3.87 (6-Ha) changed the septet of doublets at δ 1.93–2.02 (7-H) into a septet and affected the multiplet at δ 1.86–1.96 (4-He, 5-Ha).

To clarify the resonance positions of the protons, a two-dimensional COSY experiment was conducted on the synergist. The results are summarized in an unsymmetrized contour plot, which graphically displays the connectivities of the adjacent protons (Figure 5). Starting with the diagonal signal with the lowest chemical shift (6-Ha) and using the cross-peaks, the protons were sequentially traced and their positions assigned as shown. The two-dimensional spectrum supported the double irradiation experiments and confirmed location of the methylene on C-4 due to the (3-Ha, 4-Ha) cross-peak and the lack of a (methyl, 4-Ha) cross-peak. In addition, the methyl group giving resonance at δ 1.00 was established as a methyl branch on C-5 due to the (5-Me, 5-Ha) cross-peak correlating with the high-field end of the multiplet δ 1.86–1.97 and the cross-peaks of the isopropyl methyls (δ 1.11, 7-Me and δ 0.93, 7-Me), both correlating with the septet of doublets downfield (δ 1.93–2.02, 7-H). The proton located on C-6 (δ 3.87, 6-Ha) showed cross-peaks with both the high and low end of the multiplet at δ 1.86–2.02 due to coupling with both 5-Ha and 7-H.

Due to the presence of three asymmetric centers, there are eight possible stereoisomers for the lactone. Coupling constants from the one-dimensional proton spectra provided the necessary information for the relative configuration of the synergist as 3*R**,5*S**,6*R**. The quartet at δ 1.38 (4-Ha) showed very large coupling with its two vicinal (5-Ha; $J_{4\text{-Ha},5\text{-Ha}}$ = 12.5 and 3-Ha; $J_{4\text{-Ha},3\text{-Ha}}$ = 12.5) and one geminal (4-He; $J_{4\text{-Ha},4\text{-He}}$ = 12.5) neighbors. This indicated an axial–axial relationship between this proton and both of its vicinal neighbors. The isopropyl and 5-Me moieties were concluded to be equatorial because of the large axial–axial coupling of 5-Ha with 6-Ha ($J_{5\text{-Ha},6\text{-Ha}}$ = 10). The coupling of 6-Ha with 7-H was quite small ($J_{6\text{-Ha},7\text{-H}}$ = 2), suggesting hindered rotation about the bond between C-6 and C-7.

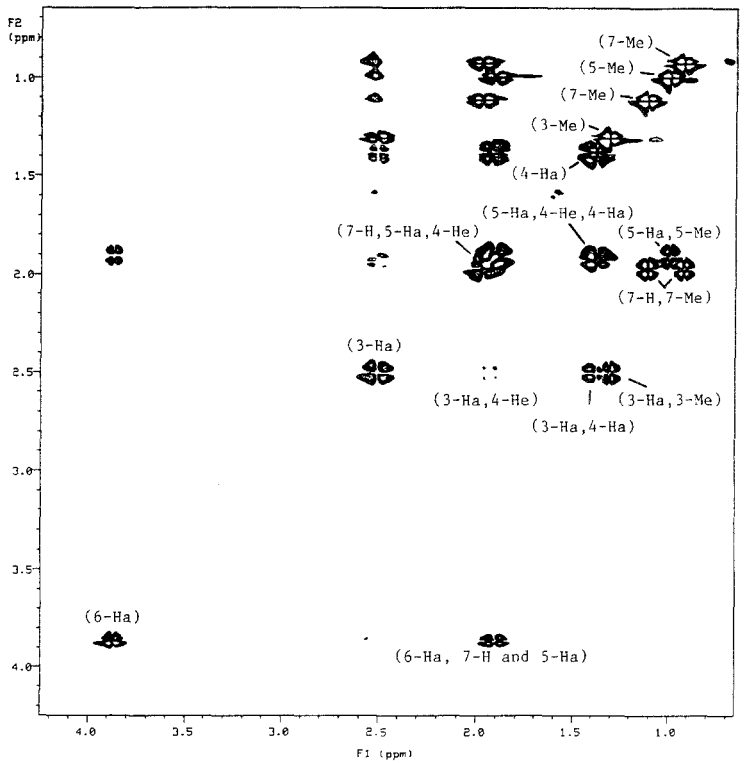

FIG. 5. 500-MHz two-dimensional COSY spectrum of *M. grandii* female-derived (3*R**,5*S**,6*R**)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one. The spectrum represents approximately 2 mg of purified synergist in $CDCl_3$. The labels 3-Ha, 3-Me, 4-Ha, 4-He, 5-Ha, 5-Me, 6-Ha, 7-H, and 7-Me refer to the carbon protons so labeled on the perspective drawing in Figure 2.

Broadband decoupling of the ^{13}C NMR spectra showed nine peaks (Figure 6). However, the peak at δ 17.3 was significantly more intense than the other eight, indicating two carbons with identical shifts and a total of 10 carbons. Peak assignments for the decoupled ^{13}C NMR spectra were based on their correlation with well-known chemical shifts and the proton coupling pattern of each signal. Proton coupling showed the peak at δ 174.9 (C-2) to be fully substituted, and the shift was indicative of a δ-lactone carbonyl. The peak at δ 91.0 was established as a methine and clearly C-6 due to significant deshielding by the adjacent electronegative oxygen and the branching of the isopropyl moiety. The remaining peaks δ 14.0 to δ 37.7 were substantially upfield and apparently not bonded with any heteroatoms. On the basis of the proton coupling patterns, the peak at δ 37.7 was assigned to C-4, the sole methylene carbon of this

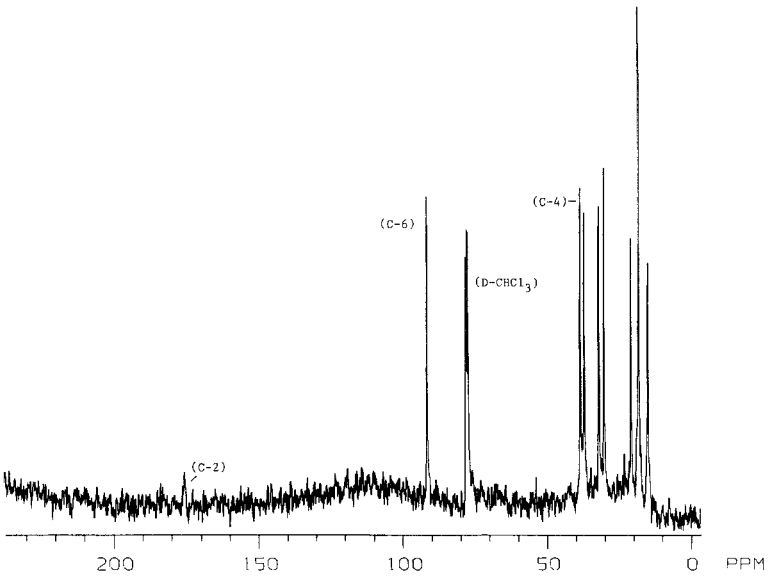

FIG. 6. 300-MHz ^{13}C NMR spectrum of *M. grandii* female-derived (3*R**,5*S**,6*R**)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one. The spectrum represents approximately 2 mg of the synergist. The labels C-2, C-4, and C-6 refer to the carbon atoms so labeled on the perspective drawing in Figure 2.

compound. Peaks δ 36.2, δ 31.1, and δ 29.4 were identified as methine resonances and the remaining upfield signals methyls.

Finally, the IR spectrum showed a strong absorbance at 1711 cm^{-1} (C=O) indicative of the carbonyl of δ-lactone.

Source of Pheromone. GC of the fractions from the dissections showed the source of the synergist to be the head of male and female wasps. There was no chromatographic evidence of the synergist in any other body part. The synergist concentration in the head was 100–300 ng/wasp. Subsequent dissections of the head located the pheromone in the mandibular glands for either sex.

Behavioral and Chemical Studies at Eclosion. Studies at eclosion with laboratory-reared cocoons indicated 1 of 18 male, 9 of 9 female, and 12 of 16 male–female cocoon masses elicited an attractive male response, which included flight initiation, upwind anemotaxis and casting downwind of the cocoon mass. Attractive cocoon masses were very distinctive, for a "cloud" of male wasps would hover and cast in front of the cocoon source, whereas there would be no activity in front of the unattractive cocoon mass. The flight behavior was identical to that observed during male response to the synergist component alone. Males generally did not land on or near the cocoon mass. Only after a female

had emerged and walked away from the cocoon did males then initiate landing, wing fanning, and copulatory attempts. Cocoon attraction coincided with subsequent female emergence. Frequently, it was observed in male–female cocoon masses, when males emerged first, no initial attraction occurred. However, for the same cocoon mass, later in the 5-min assay, suddenly there would be an attractive response by the males in the wind tunnel. This would always be followed by the emergence of a female. Following wasp emergence, all cocoons were no longer attractive. Representative wasps (male or female) were hexane extracted from attractive and unattractive cocoons, and all were found to have the synergist component by GC analysis. The concentration of the synergist ranged from 100 to 300 ng/wasp with no difference between male and female wasps from attractive and unattractive cocoons.

The behavior elicited by the attractive cocoon masses at eclosion clearly was similar to that of the extracted chemical synergist. To verify whether the synergist was released at eclosion, cocoons were placed into vials and early the next morning monitored for eclosion. Chromatographic evidence, using the GC, of synergist released in the vial was found in 6 of 15 male cocoons, 12 of 13 female cocoons, and 11 of 14 male–female cocoons. The mean ($\bar{X} \pm$ SE) concentrations of the synergist per wasp found in the three vial sources were: male cocoons, 0.35 ± 0.19 ng/male; female cocoons, 18.0 ± 6.0 ng/female; and male–female cocoons, 8.1 ± 3.4 ng/wasp. The mean numbers of wasps emerging from the cocoon sources were 15.9, 11.2, and 13.1 for male, female, and male–female cocoon masses, respectively. Female wasps from female-only cocoon masses were releasing 51.4 times more of the synergist than male wasps from male-only cocoon masses. An average female cocoon was releasing 201 ng of the pheromone during this period. An average male cocoon was releasing only 5.6 ng of the pheromone.

In field tests utilizing laboratory-reared *M. grandii* cocoon masses, five of eight predominantly female or female-only cocoon masses attracted feral *M. grandii* males upon adult emergence. Four of four male-only cocoon masses were not attractive to feral males. Feral male behavior included flight attraction to the corn plant containing the attractive cocoon mass and subsequent mate searching. Males used flight primarily to move up and down the plant with intermittent brief landings. The focus of incoming males was not directed at the cocoon mass, as was observed with *M. iridescens*, because no significant landing occurred on the cocoon mass or its immediate vicinity. Newly emerged females generally walked from the cocoon up the corn stalk and/or to the upper side of a leaf. Searching feral males would discover these females or their vicinity, land near the female, walk, and initiate wing fanning and copulatory attempts. Many matings were observed. The attractiveness of a "corn plant" appeared to depend upon how long the females stayed on the plant, suggesting the females were releasing pheromone after emergence. Several "plants" were attracting males

an hour after eclosion, and females could still be found on these plants. However, some cocoon masses were unattractive as adults emerged. These included cocoons that released only males and some male–female cocoons. Newly emerged males generally took flight soon after emergence, and some were observed to take part in the courtship process by searching for females on the corn plant.

Macrocentrus iridescens—*additional notes.* Approximately 50 male and female wasps from 10 cocoon masses (host not determined) were collected from Cook County, Minnesota, on July 15, 1991. The wasps were extracted with pentane and given a cursory chemical review. Open-column chromatography and mass spectral evidence indicated both male and female wasps contained a compound in the 50E fraction identical in structure and concentration to the male- or female-derived *M. grandii* lactone component. When male or female *M. iridescens* 50E fraction sources were substituted for *M. grandii* lactone in laboratory bioassay, an identical upwind male *M. grandii* flight response occurred to the *M. iridescens* 50E sources alone, and a synergistic response occurred when *M. iridescens* 50E sources were paired with the dienes from female *M. grandii*. In addition, argentation chromatography indicated male and female *M. iridescens* contained high concentrations of paraffins and monoene olefins; however, neither male nor female *M. iridescens* sources demonstrated any chromatographic evidence of dienes (dienes make up 40–60% of the total hydrocarbon component in female *M. grandii*). *M. iridescens* paraffins or monoenes were not attractive to male *M. grandii*.

DISCUSSION

The sex pheromone synergist was purified separately from male- and female-derived hexane extractions of *Macrocentrus grandii* and identified via spectral evidence as 3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one. NMR coupling constants suggested a relative configuration of (3*R**, 5*S**, 6*R**). Absolute configuration could not be determined. Chemical work is underway to synthesize the lactone and determine its absolute configuration.

In previous work, the 50E fraction, which contained the lactone, was found to elicit male upwind anemotaxis and to synergize (*Z*,*Z*)-9,13-heptacosadiene and (*Z*)-4-tridecenal (Swedenborg and Jones, 1992b). In this study, a purified form of the synergist increased the number of males responding in bioassays when added to (*Z*,*Z*)-9,13-heptacosadiene or (*Z*)-4-tridecenal, supporting the previous work with unpurified fractions.

The synonymous behavioral response of male *M. grandii* to female-only and male–female cocoon masses and to chemical treatments containing only the synergist suggests the mandibular gland component is released during eclosion. Adult parasitoids cut their exit hole in the cocoon with their mandibles, and this

would be consistent with the release of a mandibular gland secretion. However, male *M. grandii* were found to release significantly less of the lactone than females at eclosion; therefore, its release appears to governed. This may be why virgin females in our field assays and males in our wind tunnel and field studies were not highly attractive (Swedenborg and Jones, 1992a,b). It would be interesting to study the role of mandibular gland secretions in the emergence process. In addition, the fact that males did not land on the cocoons, wing fan, and attempt copulation implies that diene oxidation does not occur in the cocoon, and therefore the aldehyde component is not released until after a female leaves her cocoon. Landing, walking, wing fanning, and mating attempts by males to females are behaviors we have observed to be specific to the aldehyde or diene component. These behaviors were observed after the female left the cocoon and walked up the corn plant in field bioassay or away from the cocoon in laboratory bioassay.

We hypothesize from behavioral tests that the lactone or mandibular gland component is responsible for long-range *M. grandii* male attraction to the mating site, whereas the aldehyde component elicits short-range flight attraction, landing, walking, wing fanning, and copulation. Synchronous female emergence and release of the mandibular gland component was demonstrated by female and male–female gregarious cocoons, and this should enhance male attraction to the mating site and mating efficiency. In male-only cocoon masses or male–female cocoon masses before females emerge, male attraction appears nonexistent, suggesting males do not release the lactone at all or at a high enough concentration at eclosion to elicit attraction. However, male and female wasps may release and utilize the lactone at other times for behavioral purposes. It also is possible that the mandibular gland component may have another function in the parasitoid unrelated to its sex pheromone role, which may have evolved later. Future behavioral studies are necessary to clarify these questions. A combination of (Z)-4-tridecenal and the mandibular gland component can prove useful to monitor *M. grandii* populations for parasitoid activity.

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REFERENCES

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.

BROWNLEE, R.G., and SILVERSTEIN, R.M. 1968. A micro-preparative gas chromatograph and a modified carbon skeleton determinator. *Anal. Chem.* 40:2077–2079.

DING, D., SWEDENBORG, P.D., and JONES, R.L. 1989. Chemical stimuli in host-seeking behavior of *Macrocentrus grandii* (Hymenoptera: Braconidae). *Ann. Entomol. Soc. Am.* 82:232–236.

GUTHRIE, W.D., RUSSELL, W.A., and JENNINGS, C.W. 1971. Resistance of maize to second-brood European corn borer. *Proc. Ann. Corn Sorghum Conf.* 26:165–179.

PARLIMENT, T.H. 1973. Convenient technique for microhydrogenation. *Microchem. J.* 18:613–616.

SHYU, R. 1981. Biological studies of *Macrocentrus grandii* (Hymenoptera: Braconidae) and its response to the kairomones associated with its host, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). MS thesis. University of Minnesota, St. Paul, Minnesota, 50 pp.

STEEL, R.G.D., and TORRIE, J.H. 1980. Principles and Procedures of Statistics. McGraw Hill, New York, 633 pp.

SWEDENBORG, P.D., and JONES, R.L. 1992a. A multicomponent sex pheromone in *Macrocentrus grandii* (Goidanich) (Hymenoptera: Braconidae). *J. Chem. Ecol.* 18:1901–1912.

SWEDENBORG, P.D., and JONES, R.L. 1992b. (*Z*)-4-tridecenal, a pheromonally active air oxidation product from a series of (*Z*,*Z*)-9,13-heptacosadienes in *Macrocentrus grandii* (Goidanich) (Hymenoptera: Braconidae) *J. Chem. Ecol.* 18:1913–1931.

WINNIE, W.V., and CHIANG, H.C. 1984. Seasonal history of *Macrocentrus grandii* (Hym.: Braconidae) and *Eriborus terebrans* (Hym.: Ichneumonidae), two parasitoids of the European corn borer, *Ostrinia nubilalis* (Lep.: Pyralidae). *Entomophaga* 27:183–188.

WISHART, G. 1946. Observations on the emergence of *Macrocentrus gifuensis* Ashm. (Hymenoptera, Braconidae). *Can. Entomol.* 78:162–168.

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PHEROMONE-MEDIATED RESPONSES OF MALE CABBAGE LOOPER MOTHS, *Trichoplusia ni*, FOLLOWING VARIOUS EXPOSURES TO SEX PHEROMONE OR (*Z*)-7-DODECENOL

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Abstract—Prolonged preexposure (three days) of male *Trichoplusia ni* to its six-component sex pheromone blend or its major pheromone component, (*Z*)-7-dodecenyl acetate, reduced subsequent upwind flight responses to a pheromone source. Preexposure to (*Z*)-7-dodecenol increased upwind flight responses to a pheromone source combined with (*Z*)-7-dodecenol. The impact of long-term preexposures was moderate when compared to the more immediate effects of background noise. When (*Z*)-7-dodecenyl acetate was presented as background noise, all male *T. ni* failed to respond to a plume of the full pheromone blend. However, most moths succeeded in locking on to the pheromone plume and contacting the pheromone source in the presence of the five minor pheromone components as background noise. When (*Z*)-7-dodecenol was released as background noise the response rate to a pheromone source containing (*Z*)-7-dodecenol was increased dramatically. This indicates that males became adapted to (*Z*)-7-dodecenol while responding to the pheromone source. The results of this study indicate that both long-term preexposure treatments and immediate exposure to background noise can limit the ability of male *T. ni* to respond to sex pheromone sources.

Key Words—*Trichoplusia ni*, Lepidoptera, Noctuidae, sex pheromone, behavioral inhibitor, (*Z*)-7-dodecenol, wind tunnel, habituation, adaptation.

INTRODUCTION

Mating disruption using synthetic sex pheromones or pheromone components has proven to be effective in many insect species (Justum and Gordon, 1989;

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Minks and Cardé, 1988; Roelofs, 1979). However, the physiological and behavioral mechanisms of mating disruption are not well understood but may include sensory adaptation, central habituation, and false trail following (Bartell, 1982). The nature of exposure of insects to synthetic pheromones used in mating disruption may include, at the extremes, long-term exposure to synthetic pheromone prior to the behavioral response period and short-term exposure that occurs during the response to female-produced pheromone blends. Long-term exposures to sex pheromones have been demonstrated to interfere with subsequent responses to pheromone sources in several species (Bartell and Lawrence, 1973; Kuenen and Baker, 1981; Linn and Roelofs, 1981; Sanders, 1982; Traynier, 1970). Short-term exposures leading to sensory adaptation or habituation and thus decreasing responsiveness to sex pheromones have also been documented (Baker et al., 1985, 1988; Kennedy et al., 1981; Willis and Baker, 1984).

In this study, we examined the effects of long-term preexposure (three days) of male *Trichoplusia ni* (Lepidoptera: Noctuidae) to the full pheromone blend, pheromone components, or (*Z*)-7-dodecenol (*Z*7–12:OH) on subsequent responses to pheromone sources. The three-day exposure period would be approximately equal to the time period from the emergence of moths to the time of maximum behavioral responses to a pheromone source at maturation. We also studied behavioral responses of male *T. ni* to pheromone sources with concurrent introductions of pheromone components or *Z*7–12:OH as background noise to determine the short-term effects of the background noise during responses to pheromone signals.

The pheromone blend of female *T. ni* consists of one major component, (*Z*)-dodecenyl acetate (*Z*7–12:Ac) (Berger, 1966), and five minor components: dodecyl acetate (12:Ac), (*Z*)-5-dodecenyl acetate (*Z*5–12:Ac), 11-dodecenyl acetate (11–12:Ac), (*Z*)-7-tetradecenyl acetate (*Z*7–14:Ac), and (*Z*)-9-tetradecenyl (*Z*9–14:Ac) (Bjostad et al., 1984). *Z*7–12:OH reduces anemotactic flight responses of male *T. ni* to pheromone sources when released together with pheromone blends (Bjostad et al., 1984; Liu and Haynes, 1992; McLaughlin et al., 1974; Tumlinson et al., 1972). It was referred as a (behavioral) inhibitor (Liu and Haynes, 1992; McLaughlin et al., 1974; Tumlinson et al., 1972) or (behavioral) antagonist (Todd et al., 1992).

METHODS AND MATERIALS

Insects. Cabbage loopers were reared on a semisynthetic diet (Shorey and Hale, 1965). Males were separated from females as pupae. Emerged adult males were removed daily and provided with 8% sucrose water. Pupae and adults were kept in 3.8-liter paper cartons at 27 ± 1°C under a 16:8 hr light–dark photoperiod.

Chemicals. All pheromone components and Z7-12:OH were of >99% purity (Pesticide Research Institute, Wageningen, The Netherlands). The relative proportions of compounds in the six-component blend were as follows: 12:Ac (3%), Z5-12:Ac (10%), Z7-12:Ac (100%), 11-12:Ac (3%), Z7-14:Ac (3%), and Z9-14:Ac (2%). When applied to rubber septa, this resulted in a released blend similar to that emitted by calling female *T. ni* (Hunt et al., 1990).

All solutions were prepared in hexane (HPLC grade) and checked by gas chromatography (30-m Carbowax 20 M capillary column in a Hewlett-Packard 5890A gas chromatograph linked to a Hewlett-Packard 5970B mass selective detector) to ensure purity and blend ratios of pheromone components. Rubber septa (Thomas Scientific, Swedesboro, New Jersey; 5 × 9 mm, red) were preextracted with hexane and used as pheromone release substrates. A 50-μl aliquot of a pheromone solution (containing 10 μg Z7-12:Ac) was added to the wide end of a rubber septum, which was then kept in a laboratory exhaust hood for 24 hr before use as a standard pheromone source in wind-tunnel flight experiments.

Test Procedures. Male *T. ni* were confined individually in a 4-cm-diam. × 8-cm-high hardware cloth cages during the last hour of the photophase. Those males that were to be tested immediately after the beginning of the scotophase were kept in the wind-tunnel room. Otherwise, moths were placed in an environmental chamber under the same conditions as above and were tested in the wind tunnel during the fifth through seventh hours of the scotophase. The wind velocity was 50 cm/sec unless specified otherwise. The light intensity was about 0.3 lux. The temperature ranged from 22 to 28°C.

Each moth was released from its cage on a flat metal mesh surface 20-cm above the tunnel floor and 1.5-m downwind from the pheromone source. Each moth was allowed about 1 min to respond after it left the cage. Behaviors of the moth were scored as: locking on (initiation of counter-turning and upwind progress) and source contact.

Experiment 1: Effects of Long-Term Preexposure to Full Blend on Behavioral Responses to Pheromone Source. Newly emerged male *T. ni* (≤24 hr) were separated into two groups and put in separate 3.8-liter paper cartons. A rubber septum loaded with the full pheromone blend (containing 1 mg Z7-12:Ac) was placed in a small hardware cloth cage. The cage was shielded on top with aluminum foil to prevent direct contact of moths with the septum and placed in one of the cartons. Moths in the other carton served as the control. Both cartons were placed in a fume hood to ensure that the two groups of moths experienced the same conditions, while preventing the control group from being exposed to the pheromone. Moths were provided with sugar water daily. The front opening of the fume hood was sealed with black cloth and a 16:8 hr light–dark photoperiod was maintained by switching on or off a light at the ceiling of the fume hood.

In the last hour of the photophase after the three-day exposure, moths from control and treatment groups were confined individually in the small cages. Moths were then tested during the scotophase for their upwind flight responses to a full blend source (containing 10 μg Z7–12:Ac) from 30 min to 4 hr after the end of exposure to the pheromone. A total of 112 moths were tested for each treatment.

Experiment 2: Effects of Long-Term Preexposure to Z7–12:Ac, Five Minor Components, or Full Blend on Behavioral Responses to Pheromone Source. Newly emerged moths were separated into four groups. One group served as the control and the other three were exposed to Z7–12:Ac (1 mg), the five minor components, or the full blend (containing 1 mg Z7–12:Ac). The quantities of the five minor components were the same as in the full blend. Wind-tunnel tests were conducted from 30 min to 3 hr after the exposure treatments. The remaining procedures were the same as in experiment 1. A total of 70 moths were tested for each treatment.

Experiment 3: Effects of Long-Term Preexposure to Z7–12:OH on Behavioral Responses to Pheromone Sources. Newly emerged male *T. ni* were separated into two groups. One group served as the control. The second group was exposed to Z7–12:OH (1 mg) for three days. From 1 to 3 hr after the three-day exposure, moths were evaluated in a wind tunnel for their responses to a full blend (containing 10 μg Z7–12:Ac) and the full blend combined with 10 μg Z7–12:OH in the same septum. The remaining procedures were the same as in experiment 1. A total of 55 moths were tested for each treatment and each pheromone source.

Experiment 4: Effects of Background Noise of Z7–12:Ac or Five Minor Components on Behavioral Responses to Full Blend. A spinning device was constructed to release background noise (Figure 1). It consisted of a bicycle wheel axle fixed at one end to a metal stand. Two 1-mm-diam. metal wires were fixed on the downwind end of the axle sleeve in opposite directions to form two 15-cm-long arms measured from the axle center. The axle was connected with an elastic band to a motor mounted underneath the wind-tunnel floor. Two rubber bands loaded with Z7–12:Ac or the five minor components were stretched along each spinning arm and covered the outer 11 cm of each arm. The rubber bands were preextracted with hexane. About 4 hr before being used in the tests, each rubber band was curled and fitted into a plastic cup and a 100-μl aliquot of Z7–12:Ac solution (containing 100 μg Z7–12:Ac) or a solution of the five minor components (containing the same quantities of each component as the full blend with 100 μg Z7–12:Ac) was applied uniformly on the surface of the rubber band.

The bearing was rotated at about 4 revolutions/sec and Z7–12:Ac or the five minor components solution was released downwind from the rubber bands attached to the spinning arms. At a rotation speed of 4 Hz, a moth flying straight

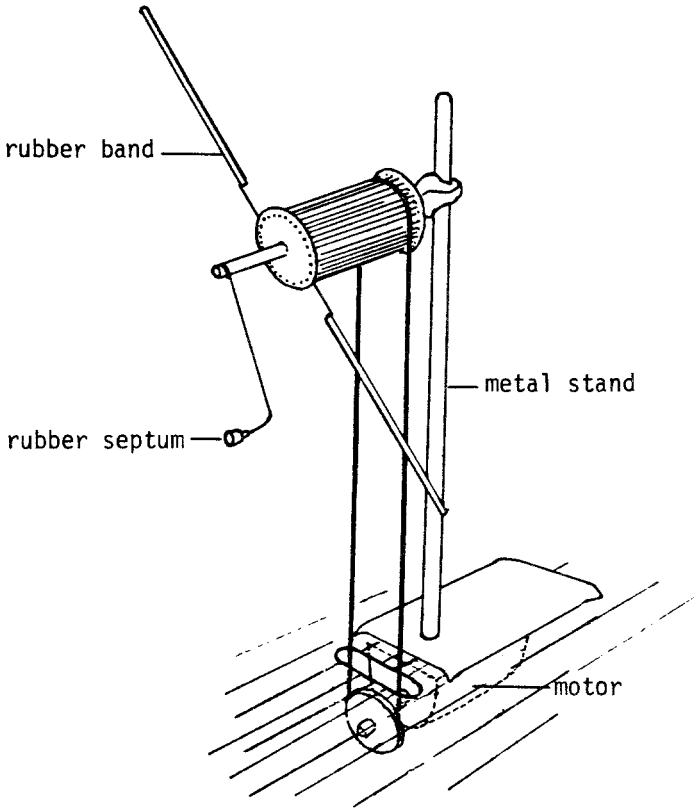

FIG. 1. A diagram of the spinning device used to release background noise. The background noise was released from rubber bands stretched along the spinning arms. The rubber septum was loaded with pheromone and served as a pheromone source. The spinning speed was 4 revolutions/sec.

upwind would encounter background noise released from the rubber bands eight times per second if there was no turbulence. However, as spinning arms cut through air, considerable turbulence was generated as evidenced by turbulent movement of smoke released immediately downwind of spinning arms. This kind of turbulence should help to admix the background noise and clean air and make the background noise generated from the device more cloudlike. A higher rotation speed created even stronger turbulence and greatly distorted the structure of the pheromone plume from the pheromone source positioned downwind of spinning arms. Therefore, it was not used even though it might also help to admix the background noise and clean air more thoroughly. The wind velocity was 40 cm/sec.

Five centimeters downwind from the middle of the spinning arm, a rubber septum loaded with pheromone was positioned to form a pheromone plume within the background noise of Z7–12 : Ac or the five minor components generated by the spinning device. Clean rubber bands were used as a control. After 10 moths were tested, the spinning arms were washed with acetone and the treatment was changed. A total of 60 moths were tested for each treatment.

Experiment 5: Effects of Background Noise of Z7–12 : OH on Behavioral Responses to Mixture of Pheromone and Z7–12 : OH. The procedures were the same as in experiment 4. In this experiment, Z7–12 : OH (100 μg for each rubber band) was used as the background noise. The pheromone source included a full pheromone blend (containing 10 μg Z7–12 : Ac) and 10 μg Z7–12 : OH in the same rubber septum. A total of 80 moths were tested for each treatment.

Statistical Analysis. The *Z* test was used to compare percentage data between control and treatment groups. For experiments with one factor and more than one treatment, each treatment was compared with the control independently as planned. For experiment 3, Ryan's (1960) multiple comparison test was performed.

RESULTS AND DISCUSSION

The three-day exposure of male *T. ni* to the full sex pheromone blend (experiment 1) significantly reduced the subsequent source contact rate in response to a full-blend pheromone source, and the reduction was maintained for the entire 4-hr test period after the exposure (Figure 2). In the second experiment in which male *T. ni* were preexposed to Z7–12 : Ac, the full six-component blend, or the five minor components for three days, the locking-on rates in response to a full blend for the preexposure treatments with Z7–12 : Ac and the full blend were significantly lower than for the control. For the source contact rate, only the preexposure to Z7–12 : Ac yielded a significant reduction at a 95% confidence level (Table 1). After moths were preexposed to Z7–12 : OH for three days (experiment 3), the percentages of locking-on and source contact in response to a full blend were not significantly different from those for untreated moths. However, male moths preexposed to Z7–12 : OH were more likely to lock on to the plume of pheromone plus Z7–12 : OH and were more likely to contact the source than males that had not been preexposed to Z7–12 : OH (Table 2).

When Z7–12 : Ac or the five minor components were released as background noise (experiment 4), the locking-on and source contact rates of male *T. ni* in response to a full-blend pheromone source were reduced dramatically as compared to the control, which lacked the background noise (Table 3). In the treatment with Z7–12 : Ac serving as background noise, no moths contacted

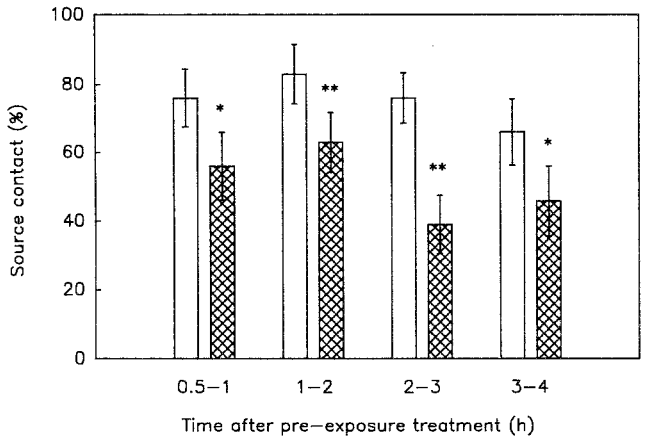

FIG. 2. Behavioral responses of male cabbage looper moths to a full pheromone blend source after being preexposed to full blend for three days. Blank bar: control; cross-hatched bar: pheromone preexposure treatment. Vertical lines represented standard errors of means. Significance of differences between the control and the treatment were denoted by ** ($P \leq 0.05$) or * ($P \leq 0.10$) based on the Z test. The sample sizes for the control or the treatment were: 25, 30, 33, and 24, respectively. The mean percentage of source contact for control (77.7%) was significantly higher than for the treatment (50.9%) (Z test, $P \leq 0.05$).

the source of the full blend. However, 26.7% and 25% of these moths flew upwind along the boundary of Z7–12:Ac background noise and reached the edge of the spinning arms, respectively. When Z7–12:OH was released as background noise (experiment 5), behavioral responses to a rubber septum containing both pheromone and Z7–12:OH improved dramatically (Table 4).

There have been several studies of lepidopteran species, including *T. ni*,

TABLE 1. EFFECTS OF LONG-TERM PREEXPOSURE OF MALE CABBAGE LOOPER MOTHS TO PHEROMONE OR PHEROMONE COMPONENTS ON BEHAVIORAL RESPONSES TO FULL PHEROMONE BLEND

Treatment	*N*	Locking-on (%)	Source contact (%)
Control	70	89.7	80.9
Z7–12:Ac	70	70.0[a]	68.6[a]
Five minors	70	88.2	73.5
Full blend	70	79.7[a]	72.5

[a] Difference between the control and the treatment was significant (Z test, $P \leq 0.05$).

TABLE 2. EFFECTS OF LONG-TERM PREEXPOSURE OF MALE CABBAGE LOOPER MOTHS TO Z7-12:OH ON BEHAVIORAL RESPONSES TO FULL PHEROMONE BLEND AND FULL BLEND COMBINED WITH Z7-12:OH

Blend	Preexposure treatment	*N*	Locking-on (%)	Source contact (%)
Full blend	Control	55	87.3a[a]	83.6a[a]
	Z7-12:OH	55	87.3a	87.3a
Full blend + Z7-12:OH	Control	55	16.4c	10.9c
	Z7-12:OH	55	58.2b	38.2b

[a] Values within a column followed by the same letter were not significantly different based on Ryan's multiple comparison test for proportions ($P > 0.05$) (Ryan, 1960).

TABLE 3. EFFECTS OF BACKGROUND NOISE OF Z7-12:Ac OR FIVE MINOR COMPONENTS ON BEHAVIORAL RESPONSES OF MALE CABBAGE LOOPER MOTHS TO FULL PHEROMONE BLEND SOURCE

Background noise[a]	*N*	Locking-on (%)	Source contact (%)
Control	60	81.7	81.7
Z7-12:Ac[b]	60	0	0
Five minors	60	55.0	55.0

[a] Difference between the control and each treatment was significant for both behavior categories (Z test, $P \leq 0.05$).

[b] 26.7% of moths flew upwind along the edge of Z7-12:Ac background noise and 25% of moths reached the edge of the spinning arms.

TABLE 4. EFFECTS OF BACKGROUND NOISE OF Z7-12:OH ON BEHAVIORAL RESPONSES OF MALE CABBAGE LOOPER MOTHS TO PHEROMONE SOURCE COMBINED WITH Z7-12:OH

Background Z7-12:OH[a]	*N*	Locking-on (%)	Source contact (%)
+	80	61.3	61.3
−	80	21.3	13.8

[a] Difference was significant between the two treatments for both behavior categories (Z test, $P \leq 0.05$).

on the impact of prolonged exposure of moths to pheromone on subsequent behavioral responses to pheromone sources (Bartell and Lawrence, 1973; Bartell and Roelofs, 1973; Farkas et al., 1975; Kuenen and Baker, 1981; Linn and Roelofs, 1981; Sanders, 1982; Traynier, 1970). The exposure time in our experiments was much longer than those used in the previous studies. However, in our study, the reduction levels in source contact rate were moderate (Figure 2, Table 1). The preexposure to Z7–12 : Ac seems to be at least as effective as the preexposure to the full blend in causing subsequent reductions of flight responses to the pheromone source.

Although Kuenen and Baker (1981) reported greater reductions in behavioral responses following exposure of male *T. ni* to Z7–12 : Ac plus 12 : Ac than following exposure to Z7–12 : Ac alone, pheromone sources tested were the same as those used in the preexposure treatments. Thus, their results do not necessarily conflict with our findings. A possible reason for the low reduction rates after the long-term exposure to pheromone sources, in our study, may be that sensory adaptation gives some protection to the central nervous system from habituation.

The effects of preexposure to Z7–12 : Ac could be partly a result of sensory imbalance (Bartell, 1982). When moths became adapted or habituated to the major component, Z7–12 : Ac, they might perceive a full blend as having a distorted blend ratio and therefore would be less likely to respond to it than moths not preexposed to Z7–12 : Ac. In a few species, there is evidence that changes in behavioral responses to pheromone sources may be caused by sensory imbalance (Minks and Cardé, 1988). In *Grapholitha molesta*, preexposure of males to (*E*)-8-dodecenyl acetate enhances their responses to the pheromone blend with higher ratio of (*E*)-8-dodecenyl acetate than in the natural blend (Linn and Roelofs, 1981). In *Pectinophora gossypiella*, when the (Z,Z)-7,11-hexadecadienyl acetate is used to permeate the atmosphere, traps baited with an excess of the Z,Z isomer caught more male moths than traps baited with the gossyplure (1 : 1 ratio of (Z,Z)- and (Z,*E*)-7,11-hexadecadienyl acetates) (Flint and Merkle, 1984).

The improved behavioral responses to pheromone combined with Z7–12 : OH after exposure to Z7–12 : OH suggests that habituation or sensory adaptation has occurred. Because Z7–12 : OH does not stimulate flight activities of male *T. ni*, it should not cause any physical exhaustion of moths, which could otherwise be an alternative explanation for behavioral changes after exposure to the pheromone.

In contrast to moderate reductions in behavioral responses to a pheromone source after the three-day preexposure to the pheromone or pheromone components, the upwind flight responses of male *T. ni* to a full-blend source were greatly reduced when Z7–12 : Ac was introduced as background noise concurrently. It is known that lepidopteran moths fly upwind along the edge of a

pheromone cloud (Baker et al., 1985; Kennedy et al., 1980, 1981; Willis and Baker, 1984). In *T. ni*, male moths appeared to fly upwind along the boundary of cloudlike background noise of Z7–12:Ac. This was evident as *T. ni* males failed to track the plume of a full blend that was positioned in a high concentration of Z7–12:Ac background noise. Those moths that flew upwind made the upwind flight at about the height of the spinning arm and reached the outside of the spinning arm.

However, in *Adoxophyes orana* (Kennedy et al., 1981) and *G. molesta* (Willis and Baker, 1984), male moths were able to trace pheromone plumes superimposed in homogeneous clouds of sex pheromones. The reason for the difference between these results and ours may be that in our study the concentration of background noise was much higher. The rubber bands treated with 100 μg Z7–12:Ac each to release background noise were aged only for 4 hr while the rubber septum containing 10 μg of full blend (measured in Z7–12:Ac) was aged for 24 hr before use as a pheromone source. Furthermore, the area permeated by Z7–12:Ac background noise was largely confined by the length of the two spinning arms. The ratio of strengths of pheromone signal versus background noise may also be critical for moths to trace pheromone plumes in background noise. In *G. molesta*, male moths failed to respond to pheromone sources when the concentration of pheromone background noise was high (Willis and Baker, 1984).

The reason for male *T. ni* flight along the edge of Z7–12:Ac background in the present study probably is that the antennal sensory system of the moth can become adapted to pheromone signals rapidly, and therefore it is only at the edge of the Z7–12:Ac cloudlike plume that their ability to respond is preserved. An electrophysiological study showed that a significant adaptation to Z7–12:Ac by the A neurons in antennal sensilla responding to Z7–12:Ac occurred in 4 sec and the level of adaptation decreased with decreasing concentration of Z7–12:Ac (Borroni and O'Connel, 1992). The rapid sensor adaptation to sex pheromone was also reported in male *Epiphyas postvittana* (Bartell, 1985). In *A. orana*, the anemotactic flight responses to pheromone decrease rapidly in a pheromone cloud and moths need at least 2 sec in clean air to become disadapted (Kennedy et al., 1981). These results and our finding also agree well with results that pulsed pheromone signals induced upwind flight while a relatively uniform pheromone cloud did not (Baker et al., 1985; Kennedy et al., 1980, 1981; Willis and Baker, 1984). Rapid sensory adaptation to sex pheromones seems to be an important cause for the failed upwind flight responses in the presence of background noise or in high-concentration pheromone plumes. Baker et al. (1988) provided evidence that sensory adaptation was a cause of in-flight arrestment of male *Agrotis segetum*. It is likely that rapid sensory adaptation can be an important mechanism in insect communication disruption with synthetic sex pheromones.

The more striking evidence of sensory adaptation was that when Z7-12:OH was introduced as background noise, male *T. ni* showed a remarkable increase in source contact rate (61.3%) to a rubber septum containing a full pheromone blend and equal amount of Z7-12:OH. In the absence of Z7-12:OH as background noise, however, only 13.8% moths succeeded in contacting the pheromone source due to "inhibitory" effects of Z7-12:OH. It has been demonstrated that the high response B neurons of sensilla trichodea on the antenna of male *T. ni* that respond to Z7-12:OH can become adapted to the chemical in 4 sec (Borroni and O'Connel, 1992).

The observation of rapid sensory adaptation while moths were in flight also raises the question of signal discrimination and how insects deal with background noise. Based on the ineffectiveness of behavioral inhibitors released as close as 5 cm crosswind or 10 cm upwind of pheromone sources in disrupting normal anemotactic responses to pheromone sources, it was concluded that a behavioral inhibitor needs to be detected simultaneously with a pheromone signal in the same filament to reduce upwind flight responses to a pheromone source (Liu and Haynes, 1992; Witzgall and Priesner, 1991). Because of the filamentous nature of pheromone plumes, plumes of a behavioral inhibitor and a pheromone source released near each other may overlap, while filaments that carry the inhibitor or pheromone may not mix to a great extent. The integrity of a pheromone signal may therefore be maintained (Liu and Haynes, 1992). The results from the present study seem to suggest that a moth can also cope with background noise by rapid sensory adaptation to the noise, and thus, the moth preserves the ability to respond to a pheromone signal contaminated by background noise.

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REFERENCES

BAKER, T.C., WILLIS, M.A., HAYNES, K.F., and PHELAN, P.L. 1985. A cloud of sex pheromone elicits upwind flight in male moths. *Physiol. Entomol.* 10:257–265.

BAKER, T.C., HANSSON, B.S., LÖFSTEDT, C., and LÖFQVIST, J. 1988. Adaptation of antennal neurons in moths is associated with cessation of pheromone-mediated upwind flight. *Proc. Natl. Acad. Sci. U.S.A.* 85:9826–9830.

BARTELL, R.J. 1982. Mechanisms of communication disruption by pheromone in the control of Lepidoptera: A review. *Physiol. Entomol.* 7:353–364.

BARTELL, R.J. 1985. Pheromone-mediated behaviour of male light-brown apple moth, *Epiphyas postvittana*, correlated with adaptation of pheromone receptors. *Physiol. Entomol.* 10:121–126.

BARTELL, R.J., and LAWRENCE, L.A. 1973. Reduction in responsiveness of males of *Epiphyas postvittana* (Lepidoptera) to sex pheromone following previous brief pheromonal exposure. *J. Insect Physiol.* 19:845–855.

BARTELL, R.J., and ROELOFS, W.L. 1973. Inhibition of sexual response in males of the moth *Argyrotaenia velutinana* by brief exposures to synthetic pheromone or its geometrical isomer. *J. Insect. Physiol.* 19:655–661.

BERGER, R.S. 1966. Isolation, identification and synthesis of the sex attractant of the cabbage looper. *J. Econ. Entomol.* 59:767–771.

BJOSTAD, L.B., LINN, C.E., DU, J.W., and ROELOFS, W.L. 1984. Identification of new sex pheromone components in *Trichoplusia ni*, predicted from biosynthetic precursors. *J. Chem. Ecol.* 10:1309–1323.

BORRONI, P.F., and O'CONNELL, R.J. 1992. Temporal analysis of adaptation in moth (*Trichoplusia ni*) pheromone receptor neurons. *J. Comp. Physiol. A* 170:691–700.

FARKAS, S.R., SHOREY, H.H., and GASTON, L.K. 1975. Sex pheromones of Lepidoptera. The influence of prolonged exposure to pheromone on the behavior of males of *Trichoplusia ni*. *Environ. Entomol.* 4:737–741.

FLINT, H.M., and MERKLE, J.R. 1984. The pink bollworm (Lepidoptera: Gelechiidae): Alteration of male response to gossyplure by release of its component Z,Z-isomer. *J. Econ. Entomol.* 77:1099–1104.

HUNT, R.E., ZHAO, B.-G., and HAYNES, K.F. 1990. Genetic aspects of interpopulational differences in pheromone blend of cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 16:2935–2946.

JUSTUM, A.R., and GORDON, R.F.S. 1989. Pheromones: Importance to insects and role in pest management, pp. 1–13, *in* Insect Pheromones in Plant Protection A.R. Justum and R.F.S. Gordon (eds.). John Wiley & Sons, New York.

KENNEDY, J.S., LUDLOW, A.R., and SANDERS, C.J. 1980. Guidance system used in moth sex attraction. *Nature* 288:475–477.

KENNEDY, J.S., LUDLOW, A.R., and SANDERS, C.J. 1981. Guidance of flying male moths by wind-borne sex pheromone. *Physiol. Entomol.* 6:395–412.

KUENEN, L.P.S., and BAKER, T.C. 1981. Habituation versus sensory adaptation as the cause of reduced attraction following pulsed and constant sex pheromone preexposure in *Trichoplusia ni*. *J. Insect Physiol.* 27:721–726.

LINN, C.E., JR., and ROELOFS, W.L. 1981. Modification of sex pheromone blend discrimination in male Oriental fruit moths by pre-exposure to (*E*)-8-dodecenyl acetate. *Physiol. Entomol.* 6:421–429.

LIU, Y.-B., and HAYNES, K.F. 1992. Filamentous nature of pheromone plumes protects integrity of signal from background chemical noise in cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 18:299–307.

MCLAUGHLIN, J.R., MITCHELL, E.R., CHAMBERS, D.L., and TUMLINSON, J.H. 1974. Perception of *Z*-7-dodecen-1-ol and modification of the sex pheromone response of male loopers. *Environ. Entomol.* 3:677–680.

MINKS, A.K., and CARDÉ, R.T. 1988. Disruption of pheromone communication in moths: Is the natural blend really most efficacious? *Entomol. Exp. Appl.* 49:25–36.

ROELOFS, W.L. 1979. Establishing Efficacy of Sex Attractants and Disruptants for Insect Control. Entomological Society of America, Lanham, Maryland, pp. 1–97.

RYAN, T.A. 1960. Significance tests for multiple comparison of proportions, variances, and other statistics. *Psychol. Bull.* 57:318–328.

SANDERS, C.J. 1982. Disruption of male spruce budworm orientation to calling females in a wind tunnel by synthetic pheromone. *J. Chem. Ecol.* 8:493–506.

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.

TODD, J.L., HAYNES, K.F., and BAKER, T.C. 1992. Antennal neurones specific for redundant pheromone components in normal and mutant *Trichoplusia ni* males. *Physiol. Entomol.* 17:183–192.

TRAYNIER, R.M.M. 1970. Habituation of the response to sex pheromone in two species of Lepidoptera, with reference to a method of control. *Entomol. Exp. Appl.* 13:179–187.

TUMLINSON, J.H., MITCHELL, E.R., BROWNER, S.M., MAYER, M.S., GREEN, N., HINES, R., and LINDQUIST, D.A. 1972. *cis*-7-Dodecen-1-ol, a potent inhibitor of the cabbage looper sex pheromone. *Environ. Entomol.* 1:354–358.

WILLIS, M.A., and BAKER, T.C. 1984. Effects of intermittent and continuous pheromone stimulation on the flight behaviour of the Oriental fruit moth, *Grapholitha molesta*. *Physiol. Entomol.* 9:341–358.

WITZGALL, P., and PRIESNER, E. 1991. Wind-tunnel study on attraction inhibitor in male *Coleophora laricella* Hbn. (Lepidoptera: Coleophoridae). *J. Chem. Ecol.* 17:1355–1362.

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ALLELOCHEMICAL INHIBITION OF RECRUITMENT IN A SEDIMENTARY ASSEMBLAGE

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Abstract—Chemical signals affect recruitment of organisms in many habitats. Most of the described biogenic chemical moieties in marine environments elicit specific positive responses, for example, of predators to prey or of conspecific larvae to suitable habitats. However, organisms also release noxious chemicals that may elicit negative responses from neighboring members of the assemblage. Herein we measured the effect on recruitment of the release of such compounds (halogenated aromatics) into sediments. The common, sediment-dwelling, terebellid polychaete *Thelepus crispus* contains brominated aromatic metabolites and contaminates the sediments surrounding its tube with these compounds. Sediments so contaminated are actively rejected by recruiting *Nereis vexillosa* (Nereidae: Polychaeta). Interestingly, many of these noxious biogenic compounds have low solubility in water and, therefore, potentially long residence times in sedimentary environments. The negative response of larvae to sediment contaminated with them is a novel, potentially common, and very important mechanism in which sediment-dwelling organisms release haloaromatic compounds and thus impose a recruitment filter on their community.

Key Words—Infauna, polychaete, halogenated aromatic, recruitment, allelochemical, negative cue, sediments, *Thelepus*, *Nereis*, allelopathy.

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INTRODUCTION

Chemically mediated processes are important in ecosystem dynamics of many habitats (terrestrial: Feeny, 1976; Rosenthal and Berenbaum, 1991; fresh water: Dawidowicz et al., 1990, Tjossem, 1990; Hanazato, 1991; marine: Faulkner, 1984; Steinberg, 1985; Braekman and Daloze, 1986; Paul, 1987; Davis et al., 1989; Hay and Fenical, 1988). A fundamental structuring and rate limiting process in all communities is the recruitment of new individuals into populations (Underwood and Denley, 1984; Roughgarden et al., 1988). In marine benthic systems, a common theme has been the modulation of recruitment by chemical cues. For a number of species, recruitment rates of larvae are influenced by specific chemical and/or physical cues on or near the ocean bottom (Grosberg, 1981; Hadfield, 1984; Morse and Morse, 1984; Butman, 1987; Johnson and Strathmann, 1989; Pawlik et al., 1990). In marine sedimentary environments, only positive larval responses to biogenic chemical moieties have been described (Highsmith, 1982; Suer and Phillips, 1983). However, a number of common, marine sediment-dwelling organisms produce noxious chemicals such as halogenated aromatic compounds (Sheikh and Djerassi, 1975; King, 1986; Woodin et al., 1987). These compounds are toxic and, if released into the sediment, might act as negative cues. Given the extensive geographic range of the sedimentary habitat, and the abundance of sediment-dwelling organisms (Hemichordata, Polychaeta, and Phoronida) that release noxious compounds [e.g., the polychaete *Lanice conchileca* in the North Sea (Buhr, 1976; Weber and Ernst, 1978) and the hemichordate *Balanoglossus* in the southeastern United States (Peterson and Peterson, 1979)], we suggest that an important control on recruitment may lie in chemical deterrence of recruits through the release of noxious biogenic compounds into the surrounding sediments.

Given the ability of recruiting larvae to differentiate among chemical signals on sediment surfaces, it is probable that recruiting larvae may be sensitive to noxious biogenic substances and differentially reject or accept habitats on that basis. To test this hypothesis, we chose a terebellid polychaete *Thelepus crispus* as a probable source of sediment contamination by halogenated aromatic compounds and a nereid polychaete *Nereis vexillosa* as the response organism. Both are widely distributed, abundant tube-dwelling polychaete species. The terebellid *Thelepus crispus* often is very abundant in the mid-intertidal zone of sheltered areas of the coast of Washington State, U.S.A. It has a strong odor and its congeners are known to produce several brominated aromatic metabolites (Higa and Scheuer, 1975; Higa et al., 1987; Goerke et al., 1991). *Nereis* also is a large, abundant tube dweller. It occurs in the same habitat with *Thelepus*, but rarely within the same square decimeter of sediment. We chose *Nereis* because it is abundant, it overlaps with *Thelepus* in its distribution, its larvae are easy

to raise (Johnson, 1943), and its recruits have a well-defined behavioral response to acceptable versus unacceptable sediments.

If infaunal species contaminate the surrounding sediment with compounds such as halogenated aromatics, and if recruiting larvae of other species respond to those levels of contamination by rejecting the site, then the occurrence of the source organism in an assemblage may affect the entire structure of the assemblage by acting as a recruitment filter through biogenic sediment contamination. Our research focused on the following four aspects: (1) Was the sediment contaminated by such biogenic secretions? (2) Did recruiting individuals of other species respond negatively to the presence of such compounds? (3) Was the response active or passive? (4) Was the response of recruits repeatable in field experiments? As detailed below, *Thelepus* does contaminate surrounding sediments with halogenated aromatic compounds, and this does act as a recruitment filter on the assemblage both in the field and in the laboratory.

METHODS AND MATERIALS

Animal Collection and Maintenance. The large jelly egg masses of *Nereis* were collected in the field and maintained in micro-airlift systems in the laboratory until hatching (Strathmann, 1987). *Nereis* larvae hatch at approximately the three to four setiger stage and are demersal. They do not burrow into sediments, nor do they build tubes until they reach the six setiger stage, approximately one week later (Johnson, 1943; Roe, 1975). At six setigers they will react to offered sediments by either burrowing or by invoking a recognizable rejection behavior (Woodin, 1991). If the sediment is acceptable, the nereids establish burrows. If the sediment is unacceptable, the nereids elevate their bodies off the sediment surface, attached only by a mucous thread from their anal cirri. They stand vertically until water forces break the thread and move them downstream (Woodin and Wethey, unpublished observations). This behavior is typically elicited within 30 sec. Thus, the behavior of *Nereis* at the six setiger stage allows unambiguous characterization of sediments as acceptable or unacceptable.

Sediments for experiments were collected from a site known to lack abundant *Thelepus*, sieved through a 0.5-mm mesh sieve, and conditioned for a minimum of one week in 20-cm-diameter glass dishes with running seawater in the laboratory, either without *Thelepus* (control sediment) or with *Thelepus* added (contaminated sediment). Dishes without *Thelepus* were maintained in tanks without any *Thelepus* to prevent cross-contamination of sediments. Dishes to which *Thelepus* were added were used as sources of contamination sediments only when three or more *Thelepus* had established tubes in the dish. Only the

top 3 mm of conditioned sediment were used; sediment within the feeding areas of *Thelepus* were avoided. Sediments of these two types were used in all laboratory and field experiments.

Sample Extraction and Analysis. The sediment samples for analysis of brominated aromatic compounds were immediately preserved in HPLC-grade methanol and either analyzed immediately or stored at 4°C. The sediment samples were triple extracted in methanol with shaking for 1 hr at 4°C. The combined methanol extracts were diluted 10-fold with pH 2, NaCl-saturated water and mixed with HPLC-grade pentane to partition the brominated compounds into pentane and to remove the water-soluble constituents. The pentane was reduced to a known volume under a stream of nitrogen and directly injected onto a gas chromatograph equipped with an electron capture detector, a digital integrator, and a fused silica bonded-phase capillary column (HP-5). Quantities of 3,5-dibromo-4-hydroxy benzyl alcohol were determined as the methoxy derivative of this compound using the external standard method and a detector response curve from extracted and purified 3,5-dibromo-4-methoxy benzyl alcohol.

Individuals of *Thelepus crispus* were cleaned of attached sediment and tube material, placed in HPLC-grade methanol, and refrigerated at 4°C. The predominant halogenated volatile compound, as its methoxy derivative, was isolated from the methanol extract by fractionation on an HPLC using a C-18 reverse-phase column, a 0.5–95% methanol–water gradient, and a UV detector and monitored by injection of the fractions on a gas chromatograph equipped with an electron capture detector. The compound of interest was eluted in the 95% methanol fraction and was >95% pure by HPLC and capillary GC. GC-MS analyses were conducted on a Finnigan 4521C with an SE-54 capillary column. ^{1}H NMR and ^{13}C NMR spectra were made at −23° on a Bruker AM-500 with a 5-mm broadband probe.

Laboratory Experiments. In the laboratory experiments, a 3-mm layer of experimental sediment was placed in a 7-cm circle onto individual Plexiglas sheets suspended in tanks with running seawater. To each replicate we added 50 six-setiger *Nereis*. The nereids were counted into dishes and added simultaneously with a pipet. In no case were more than two individuals left in the dish after addition. After 24 hr three fourths of the sediment was collected for determination of the number of *Nereis* juvenile recruits remaining. The tank was then drained, and the remaining one fourth of the sediment was collected and used for quantitation of dibromobenzyl alcohol.

To prevent cross-contamination, control and contamination treatments were not run in the same tank. Six tanks were used, two replicates of one treatment type per tank. A nested ANOVA was used for data analysis. In all experiments, the numbers of nereid juveniles remaining were determined by direct counts using a dissecting microscope. The nereids are approximately 500 μm in length. To avoid loss, a 125-μm mesh sieve was used during all transfer steps.

Behavior Observations. To determine the reactions of six-setiger nereids to control and contaminated sediments, we observed the behavior of isolated individuals. Individuals were added with a pipet to one of four dishes containing either control or contaminated sediments. The behavior of each individual was continuously monitored for 5 min with a dissecting microscope. Forty trials of this type were conducted, 20 in contaminated sediments and 20 in control sediments. Only individuals that exited gently from the mouth of the pipet onto the sediment surface were used. These observations were repeated using freshly collected surface sediments from the field. All observations on field sediments were completed within 2-hr of collection. These observations on responses to field sediments were done to determine differences, if any, in the behavior of six-setiger nereids to field contaminated and uncontaminated surface sediments. Field collection sites were surficial sediments (surface to 0.5 cm deep) within beds of *Thelepus* in the field (contaminated) as well as surficial sediments $>$ 1m outside the beds (uncontaminated). Areas disturbed by recent feeding and/or defecation activities were not used.

Field Experiments. A field experiment parallel to the laboratory experiment was conducted in a small intertidal bay containing populations of both *Nereis vexillosa* and *Thelepus crispus*. Our experiment was conducted in an area where *Thelepus* was rare. Cylindrical experimental containers (7.5 cm diameter $\times$ 7.5 cm high) were filled with foundry sand to a height of 5 cm and placed into excavated holes of the same dimensions in the field. Either contaminated or control sediments were added to each until the experimental sediment surface was flush with the surrounding sediments. Eighteen containers, nine of each type, were placed in random order 90 cm apart along a 9-m $\times$ 1-m transect at the 0.0-m tide level. Fifty nereids were added by pipet to the surface sediment of each container. After 24 hr, the experiment was terminated. One quarter of the top 2 cm of sediment was placed into HPLC-grade methanol and used for analysis of brominated aromatic compounds. Three quarters were preserved in buffered formalin for determination of the numbers of *Nereis* recruits remaining.

To determine the distribution and extent of contamination in sediments surrounding populations of *Thelepus*, we sampled a *Thelepus* bed close to our field site. We collected sediments both within the bed and 1 m distant from the edge of the bed. Within each of these locations, two depth intervals were examined. Surficial sediments (surface to 0.5 cm) were collected and placed immediately on ice in HPLC-grade methanol. Additionally, cores of sediment (surface to 8 cm depth) were collected. These samples were handled and extracted as described for those in the laboratory and field experiments.

Data Analysis. All data were analyzed using PC-SAS 6.04 (SAS Institute, 1991, Cary, North Carolina). Data were transformed as indicated in the text. Data were analyzed by analysis of variance (field experiment) or nested analysis of variance (laboratory experiment).

RESULTS

Structure and Production of Principal Volatile Metabolite. Thelepus crispus contains large amounts of brominated aromatic compounds and contaminates the sediments surrounding its tubes with 3,5-dibromo-4-hydroxy benzyl alcohol (Table 1). Closely related species of *Thelepus* produce several brominated metabolites, in addition to this compound (Goerke et al., 1991). To assess the production of such compounds and sediment contamination by *T. crispus*, we determined the structure of the principal halogenated metabolite and assayed the amount of this compound in worms and sediments.

A crude CH_2Cl_2 extract of *T. crispus* showed the presence of a dibromophenol and a large proportion of a dibromobenzyl alcohol by GC-MS analysis. Extracts made with methanol, ethanol, or acetone contained the methoxy, ethoxy, or isopropoxy derivative of the dibromobenzyl alcohol, respectively. A GC-MS analysis of crude methanolic worm extract showed methoxy derivative of the principal volatile secondary metabolite to have M^+ at *m/e* 294 (19), 296 (42), and 298 (18); M − 1 at 293 (5), 295 (12), and 297 (7); M − (OCH_3) at 263 (45), 265 (88), and 267 (36); and M − (BR) at 215 (100) and 217 (93) and other less intense ions consistent with a dibromomethoxy benzyl alcohol. The positions of the substituents on the ring were determined by ^1H NMR and ^{13}C

TABLE 1. AMOUNT OF 3,5-DIBROMO-4-HYDROXY BENZYL ALCOHOL ($\mu g/ml$ OF SEDIMENT) IN EXPERIMENTAL AND FIELD SEDIMENTS[a]

Sediment source	*N*	Mean (SD)	*N*	Mean (SD)
A. Experimental sediments				
		Control Sediments		Contaminated Sediments
Laboratory	6	0.08 (0.06)	6	4.81 (4.13)
Field	6	0.10 (0.07)	7	1.81 (1.28)
Behavior Obs.	2	0.0	2	1.41 (0.13)
B. Field sediments from outside or within *Thelepus* bed				
		1 m outside		Within bed[b]
Surface Sediments[c]	3	0.14 (0.02)	3	4.01 (1.46)
0 to 8 cm cores[d]	3	0.50 (0.37)	3	13.44 (11.72)

[a] For analysis, the data were transformed with a square root transform. For clarity of presentation, the data are not transformed; *N* is number of replicate samples

[b] *Thelepus* densities >100 individuals/m^2.

[c] From top 0.5 cm of sediment.

[d] From integrated vertical core sample 8 cm deep.

NMR using the HPLC-purified compound. The ^{1}H NMR spectrum of the brominated compound was observed to have only three singlets, two aromatic protons at $\delta = 7.43$, two $O-CH_2-AR$ methylenic protons at $\delta = 4.30$, and three methoxy protons at $\delta = 3.31$, indicating no protons on adjacent atoms. The ^{13}C NMR spectrum included signals for eight carbons: 1 methoxy carbon at 58.42, 1 $O-CH_2-AR$ carbon at 73.01, two brominated aromatic carbons at 109.92, two protonated aromatic carbons at 131.76, one aromatic carbon with a $-CH_2-O-$ group at 133.61, and one aromatic carbon with a methoxy group at 149.06. A ^{13}C NMR spectrum using the INEPT technique showed the carbons at 58.42, 73.01, and 131.76 were protonated. A two-dimensional ^{1}H, ^{13}C spectrum using a COLOC technique showed both the signal for the methoxy carbon and for the benzyl alcohol carbon were correlated only to their own protons. On the other hand, the two equivalent protonated aromatic carbons were correlated with their aromatic protons and to the protons of the methylenic group on the benzyl alcohol carbon. In this mixed aromatic–aliphatic system, the 1–3 coupling is expected and shows that the protonated aromatic carbons are one carbon removed from the benzyl alcohol group. In addition, the signal for the brominated carbons was not correlated with any protonated resonances and the quaternary aromatic carbon where the benzyl alcohol group is attached was correlated only to the aliphatic protons of the benzyl group. This suggests that the bromines are 1–3 to the latter carbon. Further, the quaternary aromatic carbon where the methoxy group is attached was correlated only to the aromatic protons. Thus, in the aromatic ring, the protonated carbons are 1–3 to the attachment of the methoxy group. These assignments based on the ^{13}C NMR are consistent with a structure of 3,5-dibromo-4-hydroxy benzyl alcohol as the principal volatile halogenated aromatic occurring in *T. crispus*.

Larval Response to Contaminated Sediment in the Laboratory. Conditioning of sediments with *Thelepus* resulted in contamination of the sediments with halogenated aromatic compounds. Contaminated laboratory sediments had levels of dibromobenzyl alcohol comparable with sediments from the beds of *Thelepus* in the field, while control sediments had undetectable or only trace amounts (Table 1).

In the laboratory experiment, the number of *Nereis* juveniles retained in the contaminated sediments was significantly lower than in the control treatments (Figure 1: nested ANOVA: $df = 1, 5$ $MS = 494.08$ $F = 7.88$ $P < 0.05$). Contamination treatments were significantly contaminated with dibromobenzyl alcohol (Table 1A: nested ANOVA: $df = 1, 5$ $MS = 10.25$ $F = 31.80$ $P < 0.005$). These results suggest that recruiting *Nereis* actively rejected sediments that were contaminated with brominated metabolites from *Thelepus*.

Active rejection of contaminated sediments was confirmed by behavioral observations. Eighty percent of the nereid juveniles burrowed into the control sediments within 30 sec versus less than 10% into the contaminated sediments

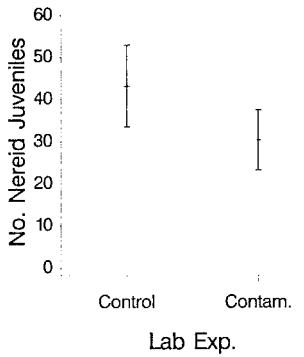

FIG. 1. Laboratory response of recruiting *Nereis vexillosa* to contaminated and control sediments (see Table 1 for sediment concentrations). Means and standard deviations of numbers of nereid juveniles retained in control sediments versus in sediments contaminated by exposure to *Thelepus crispus*.

(Woodin and Marinelli, 1991). The mean time from contact with the sediment surface to initiation of burrowing was 106 sec in the contaminated sediments versus 11 sec in the control sediments. The majority of the individuals in the contaminated sediments engaged in rejection behavior prior to burrowing, while none in the control sediments did. The reaction of the nereid juveniles to freshly collected field sediments was similar. Eighty percent of the nereid juveniles burrowed into the uncontaminated field sediments within 30 sec versus less than 30% into the contaminated field sediments. The mean time from contact with the sediment surface to initiation of burrowing was 71 sec in the contaminated sediments from the field versus 21 sec in the uncontaminated field sediments. In the contaminated field sediments, 25% of the nereid juveniles failed to initiate burrowing within the 5-min period of observation, while in the uncontaminated field sediments all of the individuals burrowed. Thus, as in the laboratory contaminated sediments, surface sediments from within *Thelepus* beds in the field were less acceptable to nereid juveniles than uncontaminated surface sediments from sites >1 m outside the *Thelepus* beds. As with the laboratory contaminated sediments, the nereid juveniles are actively rejecting the field contaminated sediments.

Larval Response to Contaminated Sediment in the Field. To determine whether similar experimental results would be found in the field where numerous other cues and forces are present, we conducted a parallel field experiment. Our laboratory findings were confirmed in our field experiment. Significantly fewer nereids remained in the contamination treatments than in the control treatments (Figure 2: ANOVA: $df = 1, 11$ $MS = 0.294$ $F = 9.94$ $P < 0.01$) (The data were transformed as $\log_{10}(X + 1)$ prior to analysis; for clarity of presentation

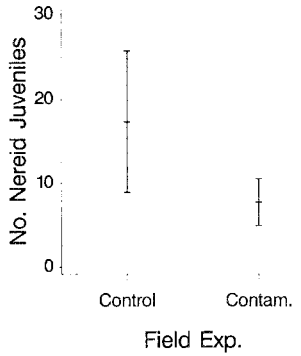

FIG. 2. Field response of recruiting *Nereis vexillosa* to contaminated and control sediments (see Table 1 for sediment concentrations). Means and standard deviations of numbers of nereid juveniles retained in control sediments versus in sediments contaminated by exposure to *Thelepus crispus*. The data for the juvenile nereids were transformed as $\log_{10}(X + 1)$ before analysis. For ease of interpretation, the data presented in Figure 2 are not transformed.

the data in Figure 2 are not transformed). The contamination treatment sediments were significantly contaminated with dibromobenzyl alcohol (Table 1A: ANOVA: $df = 1, 11$ $MS = 3.20$ $F = 24.37$ $P < 0.0005$). The presence of low but detectable concentrations of this compound in the control sediments is presumably due to contamination from the surrounding sediments. Samples of sediments surrounding the experimental area had a mean of 0.05 μg/ml of dibromobenzyl alcohol ($N = 5$, SD = 0.02).

DISCUSSION

The oceans cover more than 60% of the surface of the earth, and the majority of the ocean floor is sedimentary. Thus, marine sediments are one of the most common habitats in the world. The three taxa that typically are the numerical and biomass dominants in marine sediments are the bivalves, polychaetes, and small crustaceans. Of these, the polychaetes often dominate (Knox, 1977). Infauna and particularly polychaetes are well known for their modification of the physical properties of the sediment through burrowing, tube-building, feeding, etc. (see references in Woodin and Marinelli, 1991). This paper documents that they also can alter significantly the chemical properties of the sediment in ways that change the attractiveness of those sediments to new recruits. This biogenic alteration occurs via release of halogenated aromatic compounds (Table 1) and appears to change the perception of the site by the larvae of other organisms (Figures 1 and 2). The larvae of most marine bivalves and polychaetes

spend at least some period of time as released larvae in the plankton (Strathmann, 1987). As such they are subject to water forces that move them and ultimately deposit them into varying locations on the bottom. At the time of deposition or recruitment, specific chemical and/or physical cues on or near the bottom are known to be important (Butman, 1987). After deposition, the larvae react to such cues either by accepting the site and initiating metamorphosis or by rejecting the site. This paper represents the first evidence that biogenic cues can be negative and cause rejection of sites by recruiting individuals (Figures 1 and 2). It is clear from the evidence presented here that the nereid juveniles reject sites as a function of sediment contamination by *Thelepus*, indicating that indirect interactions in benthic sedimentary habitats may be driven by allelopathy. In this way the producer of the chemical acts as a recruitment filter for the rest of the assemblage and can strongly affect the composition of the assemblage.

For biogenic chemical additions to sediments to have a significant impact on the composition of the assemblage, they must be continuously present, which implies either long residence times for the compounds and/or large rates of production and release. In terms of sediment contamination, several lines of evidence suggest that the compounds released by *T. crispus* may have a long residence time in the sedimentary environment. First, our analyses suggest that dibromobenzyl alcohol has only limited water solubility. Second, its concentration drops rapidly outside of the *Thelepus* bed (Table 1B). If the compound were highly mobile, one would expect a more widespread distribution due to diffusive transport through sediment pore waters and biological exchange processes such as irrigation and bioturbation. This was not the case (Table 1B). We expect, therefore, that the effect of such compounds on other fauna is likely to be local to the source organism, with persistence of the compound governed largely by the rate of degradation as well as the rate of release.

Similar characteristics have been described for the majority of halogenated aromatic compounds known to be released by marine worms (Polychaeta, Hemichordata, and Phoronida) (Ashworth and Cormier, 1967; Higa and Scheuer, 1975, 1977; Sheikh and Djerassi, 1975; Weber and Ernst, 1978; Higa et al., 1980; Woodin et al., 1987; Emrich et al., 1990; Goerke and Weber, 1991). These compounds are also released into the sediments (King, 1986; Woodin et al., 1987). These compounds should have limited solubility in water and long residence times. As expected, the data on the sediment distribution of 2,4-dibromophenol released by a hemichordate *Saccoglossus kowalewskii* is similar to that for *Thelepus* (King, 1986). However, an exception to this pattern is found in the capitellid polychaete *Notomastus lobatus*. *Notomastus* releases three bromophenols (Chen et al., 1991). Two of the three are relatively insoluble in water and fit the pattern described above (2,4-dibromophenol and a tribromophenol), but one (4-bromophenol) is moderately soluble. As expected, 4-bromophenol has wider dispersal out from the burrow of the secreting organism than the two

compounds of very limited solubility (Lincoln et al., unpublished observations). However, even in the case of *Notomastus*, two of the compounds released have limited water solubility. Thus, the pattern appears to be that the worms that release halogenated aromatic compounds into the surrounding sediments release forms that in general are of very limited water solubility, enhancing their probable residence time over more water-soluble compounds and increasing the likelihood that they will act as negative recruitment cues.

In conclusion, sedimentary environments are one of, if not the, most common habitats, in the world. The organisms within the sediments that are known to contain halogenated aromatic compounds are members of very common and widely distributed deposit-feeding taxa (Buhr, 1976; Weber and Ernst, 1978; Woodin, 1991). These organisms manipulate, and potentially contaminate, large amounts of sediment and associated pore water in both vertical and horizontal dimensions (Gust and Harrison, 1981; Aller, 1982; Rhoads and Boyer, 1982; Thayer, 1983; Emerson et al., 1984; Aller and Yingst, 1985; Rice, 1986; Meyers et al., 1987; Woodin and Marinelli, 1991). As a result, therefore, there should be ample opportunity for other organisms to encounter contaminated sediments. The extent to which such interactions affect community structure will depend on the physicochemical properties of sediments and the contaminating compound (e.g., the residence time of compounds in sediments and pore water) and the biology of the interacting organisms. Our laboratory and field results show a clear and consistent negative response of recruits to contaminated sediments (Figures 1 and 2). The abundance of these organisms, the resulting sediment contamination, and the consistency of the response of recruits in both laboratory and field experiments suggest strongly that the secretion of such compounds and the consequent contamination of the sedimentary habitat is a novel, and perhaps very important, mechanism by which biological interactions among sediment-dwelling organisms occur.

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REFERENCES

ALLER, R.C., 1982. The effects of macrobenthos on chemical properties of marine sediment and overlying water, pp. 53–102, *in* P.L. McCall and M.J.S. Tevesz (eds.). Animal–Sediment Relations. Plenum Press, New York.

ALLER, R.C., and YINGST, J.Y. 1985. Effects of the marine deposit-feeders *Heteromastus filiformis*

(Polychaeta), *Macoma balthica* (Bivalvia) and *Tellina texana* (Bivalvia) on averaged sedimentary solute transport, reaction rates and microbial distributions. *J. Mar. Res.* 43:615–645.

ASHWORTH, R.B., and CORMIER, M.J. 1967. Isolation of 2,6-dibromophenol from the marine hemichordate *Balanoglossus biminiensis. Science* 155:1558–1559.

BRAEKMAN, J.C., and DALOZE, D. 1986. Chemical defence in sponges. *Pure Appl. Chem.* 58:357–364.

BUHR, K.-J. 1976. Suspension-feeding and assimilation efficiency in *Lanice conchilega* (Polychaeta). *Mar. Biol.* 38:373–383.

BUTMAN, C.A. 1987. Larval settlement of soft-sediment invertebrates: The spatial scales of pattern explained by active habitat selection and the emerging role of hydrodynamical processes. *Oceanogr. Mar. Biol Annu. Rev.* 24:113–165.

CHEN, Y.P., LINCOLN, D.E., WOODIN, S.A., and LOVELL, C.R. 1991. Purification and properties of a unique flavin-containing chloroperoxidase from the capitellid polychaete *Notomastus lobatus. J. Biol. Chem.* 266:23909–23915.

DAVIS, A.R., TARGETT, N.M., MCCONNEL, O.G., 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.

DAWIDOWICZ, P., PIJANOWSKA, J., and CIECHOMSKI, K. 1990. Vertical migration of *Chaoborus* larvae is induced by the presence of fish. *Limnol. Oceanogr.* 35:1631–1636.

EMERSON, S., JAHNKE, R., and HEGGIE, D. 1984. Sediment-water exchange in shallow water estuarine sediments. *J. Mar. Res.* 42:709–730.

EMRICH, R., WEYLAND, H., and WEBER, K. 1990. 2,3,4-Tribromopyrrole from the marine polychaete *Polyphysia crass. J. Nat. Prod.* 53:703–705.

FAULKER, D.J. 1984. Marine natural products: metabolites of marine algae and herbivorous molluscs. *Nat. Prod. Rep.* 1:251–280.

FEENY, P. 1976. Plant apparency and chemical defense. *Recent Adv. Phytochem.* 10:1–40.

GOERKE, H., and WEBER, K. 1991. Bromophenols in *Lanice conchilega* (Polychaeta, Terebellidae): The influence of sex, weight and season. *Bull. Mar. Sci.* 48:517–523.

GOERKE, H., EMRICH, R., WEBER, K., and DUCHENE, J.-C. 1991. Concentrations and localization of brominated metabolites in the genus *Thelepus* (Polychaeta: Terrebellidae). *Comp. Biochem. Physiol.* 99B:302–206.

GROSBERG, R.K. 1981. Competitive ability influences habitat choice in marine invertebrates. *Nature* 290:700–702.

GUST, G., and HARRISON, J.T. 1981. Biological pumps at the sediment-water interface: mechanistic evaluation of the Alpheid shrimp *Alpheus mackayi* and its irrigation pattern. *Mar. Biol.* 64:71–78.

HADFIELD, M.G. 1984. Settlement requirements of molluscan larvae: New data on chemical and genetic roles. *Aquaculture* 39:283–298.

HANAZATO, T. 1991. Effects of a *Chaoborus*-released chemical on *Daphnia ambigua*: Reduction in the tolerance of the *Daphnia* to summer water temperature. *Limnol. Oceanogr.* 36:165–171.

HAY, M.E., and FENICAL, W. 1988. Marine plant-herbivore interactions: The ecology of chemical defense. *Annu. Rev. Ecol. Syst.* 19:111–145.

HIGA, T., and SCHEUER, P.J. 1975. Constituents of the marine annelid *Thelepus setosus. Tetrahedron* 31:2379–2381.

HIGA, T., and SCHEUER, P.J. 1977. Constituents of the hemichordate *Ptychodera flava laysanica*, pp. 35–43, *in* D.J. Faulkner and W.H. Fenical (eds.). Marine Natural Products Chemistry. Plenum Press, New York.

HIGA, T., FUJIYAMA, T., and SCHEUER, P.J. 1980. Halogenated phenol and indole constituents of acorn worms. *Comp. Biochem. Physiol.* 65B:525–530.

HIGA, T., OKUDA, R.K., SEVERNS, R.M., SCHEUER, P.J., HE, C.-H., CHANGFU, X., and CLARDY, J. 1987. Unprecedented constituents of a new species of acorn worm. *Tetrahedron* 43:1063–1070.

HIGHSMITH, R.C. 1982. Induced settlement and metamorphosis of sand dollar (*Dendraster excentricus*) larvae in predator-free sites: Adult sand dollar beds. *Ecology* 63:329–337.

JOHNSON, L.E., and STRATHMANN, R.R. 1989. Settling barnacle larvae avoid substrata previously occupied by a mobile predator. *J. Exp. Mar. Biol. Ecol.* 128:87–103.

JOHNSON, M.W. 1943. Studies on the life history of the marine annelid *Nereis vexillosa*. *Biol. Bull.* 84:106–114.

KING, G.M. 1986. Inhibition of microbial activity in marine sediments by a bromophenol from a hemichordate. *Nature* 323:257–259.

KNOX, G.A. 1977. The role of polychaetes in benthic soft-bottom communities, pp. 547–604, *in* D.J. Reish and K. Fauchald (eds.). Essays on Polychaetous Annelids. Allan Hancock Foundation, University of Southern California, Los Angeles.

MEYERS, M.B., FOSSING, H., and POWELL, E.N. 1987. Microdistribution of interstitial meiofauna, oxygen and sulfide gradients, and the tubes of macro-infauna. *Mar. Ecol. Prog. Ser.* 35:223–241.

MORSE, A.N.C., and MORSE, D. 1984. Recruitment and metamorphosis of *Haliotis* larvae induced by molecules uniquely available at the surfaces of crustose red alga. *J. Exp. Mar. Biol. Ecol.* 65:191–215.

PAUL, V.J. 1987. Feeding deterrent effects of algal natural products. *Bull. Mar. Sci.* 41:514–522.

PAWLIK, J.R., BUTMAN, C.A., and STARCZAK, V.R. 1990. Hydrodynamic facilitation of gregarious settlement of a reef-building tube worm. *Science* 251:421–424.

PETERSON, C.H., and PETERSON, N.M. 1979. The Ecology of Intertidal Flats of North Carolina: A Community Profile. Fish and Wildlife Service, Office of Biological Services. FWS/OBS-79/39, 73 pp.

RHOADS, D.C., and BOYER, L.F. 1982. The effects of marine benthos on physical properties of sediments: a successful perspective, pp. 3–52, *in* P.L. McCall and M.J.S. Tevesz (eds.). Animal–Sediment Relations, The Biogenic Alteration of Sediments. Plenum Press, New York.

RICE, D.L. 1986. Early diagenesis in bioadvective sediments: relationships between the diagenesis of beryllium-7, sediment reworking rates and the abundance of conveyor-belt deposit feeders. *J. Mar. Res.* 44:149–184.

ROE, P. 1975. Aspects of life history and of territorial behavior in young individuals of *Platynereis bicanaliculata* and *Nereis vexillosa* (Annelida, Polychaeta). *Pac. Sci.* 29:341–348.

ROSENTHAL, G.A., and BERENBAUM, M.R. 1991. Herbivores. Their Interactions with Secondary Plant Metabolites, 2nd ed. Academic Press, San Diego.

ROUGHGARDEN, J., GAINES, S.D., and POSSINGHAM, H.P. 1988. Recruitment dynamics in complex life cycles. *Science* 241:1460–1466.

SHEIKH, Y.M., and DJERASSI, C. 1975. 2,6-Dibromophenol and 2,4,6-tribromophenols—antiseptic secondary metabolites of *Phoronopsis veridis*. *Experientia* 31:265–266.

STEINBERG, P.D. 1985. Feeding preferences of *Tegula funebralis* and chemical defenses of marine brown algae. *Ecol. Monogr.* 55:333–349.

STRATHMANN, M.F. 1987. Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast. University of Washington Press, Seattle.

SUER, A.L., and PHILLIPS, D.W. 1983. Rapid, gregarious settlement of the larvae of the marine echiuran *Urechis caupo* Fisher and MacGinitie 1928. *J. Exp. Mar. Biol. Ecol.* 67:243–259.

THAYER, C.W. 1983. Sediment-mediated biological disturbance and the evolution of marine benthos, pp. 480–625, *in* M.J.S. Tevesz and P.L. McCall (eds.). Biotic Interactions in Recent and Fossil Benthic Communities. Plenum Press, New York.

TJOSSEM, S.F. 1990. Effects of fish chemical cues on vertical migration behavior of *Chaoborus*. *Limnol. Oceanogr.* 35:1456–1468.

UNDERWOOD, A.J., and DENLEY, E.J. 1984. Paradigms, explanations, and generalizations in models for the structure of intertidal communities on rocky shores, pp. 151–180, *in* D.R. Strong, Jr., and D. Simberloff, L.G. Abele, and A.B. Thistle (eds.). Ecological Communities: Conceptual Issues and the Evidence. Princeton University Press, Princeton, New Jersey.

WEBER, K., and ERNST, W. 1978. Occurrence of brominated phenols in the marine polychaete *Lanice conchilega. Naturwissenschaften* 65:262.

WOODIN, S.A. 1991. Recruitment of infauna: Positive or negative cues. *Am. Zool.* 31:797–807.

WOODIN, S.A., and MARINELLI, R.L. 1991. Biogenic habitat modification in marine sediments: The importance of species composition and activity, pp. 231–250, *in* P.S. Meadows and A. Tufail (eds.). The Environmental Impact of Burrowing Animals and Animal Burrows. Zoological Society, London, No. 63.

WOODIN, S.A., WALLA, M.D., and LINCOLN, D.E. 1987. Occurrence of brominated compounds in soft-bottom benthic organisms. *J. Exp. Mar. Biol. Ecol.* 107:209–217.

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2-HYDROXY-4,7-DIMETHOXY-1,4-BENZOXAZIN-3-ONE (*N-O*-ME-DIMBOA), A POSSIBLE TOXIC FACTOR IN CORN TO THE SOUTHWESTERN CORN BORER[1]

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Abstract—The southwestern corn borer (SWCB), *Diatraea grandiosella* Dyar, is a major pest of corn, *Zea mays* L., in the southern United States. The damage to corn is caused primarily by larval feeding on leaf, ear, and stem tissues. In this study, 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (*N-O*-Me-DIMBOA) was identified by MS and NMR as present in corn whorl surface waxes. This compound has evidently not been isolated previously, but its glucoside has been reported in corn, wheat, and *Coix lachryma*. It is present in the waxes in a higher concentration than DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) and 6-MBOA (6-methoxybenzoxazolinone). It was toxic to the SWCB in a stress diet, but it was less toxic to this insect than 6-MBOA when incorporated in the standard rearing diet. Nevertheless, it may have some role in the resistance of corn to the SWCB because the total surface wax content is higher in resistant lines than in susceptible lines.

Key Words—Corn, *Zea mays* (L.), southwestern born borer, *Diatraea grandiosella*, Dyar, Lepidoptera, Pyralidae, feeding resistance, 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one, *N-O*-Me-DIMBOA.

INTRODUCTION

The southwestern corn borer (SWCB), *Diatraea grandiosella* (Dyar), is a major pest of corn, *Zea mays* L., in the southern United States. It attacks corn in the vegetative and reproductive stages of plant growth. In whorl stage corn, the

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SWCB larvae feed first on the tender unfurled leaves in the whorl and later within the stem (Davis et al., 1988a,b).

High protein and low fiber content have been correlated with susceptibility of whorl-stage corn to the SWCB (Hedin et al., 1984). Later, high protein and low fiber content along with the distribution of whorl free amino acids have been associated with susceptibility to another important southern corn pest, the fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith) (Hedin et al., 1990). Extensive efforts to associate feeding resistance of corn to both insects with the toxicity of some corn plant allelochemicals have been largely unsuccessful, although 6-methoxybenzoxazolinone (6-MBOA) was found present in both susceptible and resistant lines and manifested some toxicity (ED_{50} = 0.40% in the SWCB, ED_{50} = 0.50% in the FAW) to these insects (Nicollier et al., 1982; Hedin et al., 1984). 6-MBOA is the major degradation product from 4-*O*-glucosyl-2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, which upon injury or crushing, is initially hydrolyzed enzymatically to the corresponding aglycon, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (Klun and Brindley, 1966).

Although DIMBOA is reported to be more toxic than 6-MBOA to the European corn borer (ECB), *Ostrinia nubilalis* (Hübner) (Klun and Brindley, 1966), we have not been able to satisfactorily test DIMBOA in diets for the ECB because it is rapidly converted to 6-MBOA after its incorporation. In any event, repeated GLC analyses for 6-MBOA in whorls of SWCB and FAW leaf-feeding susceptible (S) and resistant (R) corn lines grown in Mississippi were low and not appreciably different (Hedin et al., 1984). Thus, DIMBOA and 6-MBOA may be contributing resistance factors in corn to the SWCB, but they do not appear to be decisive factors.

A long-continuing corn breeding program at this location has led to the release of eight germplasm lines with leaf-feeding resistance to the FAW and the SWCB and several other lepidopterous insects (Davis et al., 1988a,b; Williams et al., 1989). The mechanisms of the resistance have been determined as larval antibiosis and nonpreference for both the SWCB and the FAW (Wiseman et al., 1981; Davis et al., 1989). It is expected that the same or similar chemical and/or physical factors may govern the resistance of corn to these insects.

The initial objective of the present study was to carry out chemical studies on the surface waxes of S and R lines, hoping to find differences that could be associated with insect resistance. Previously, Bianchi and Avato (1984) found that the kernels, husks, and leaves of maize plants were covered by waxes comprised of long-chain alkanes, esters, aldehydes, alcohols, acids, and sterols. In preliminary tests by us, GLC and GLC-MS analyses revealed only minor differences of these expected constituents in our S and R lines, but 6-MBOA, DIMBOA, and an apparently new benzoxazinone were found present. In this study, the structure of the benzoxazinone was deduced, its relative concentration

in several S and R lines was determined, and its toxicity to the SWCB, relative to that of 6-MBOA, was evaluated.

METHODS AND MATERIALS

Plant Material. Five SWCB leaf-feeding susceptible inbred lines (AB24E, GT106, SC229, TX601, and Va35) and five SWCB leaf-feeding resistant inbred lines (Mp496, Mp704, Mp705, Mp707, and Mp708) were used in the study. These inbred lines were grown in 1990 and 1991 in the field in a randomized complete block (RCB) design with four replications by the Corn Host Plant Research Unit (USDA-ARS) at Mississippi State, Mississippi. Corn whorl tissue was collected by replicates from plants grown to the V8–V10 stage of development (Ritchie and Hanway, 1982).

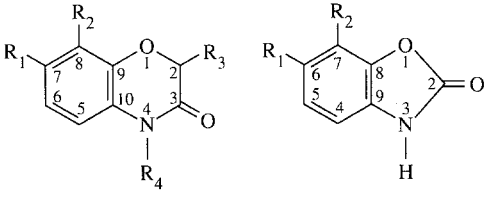
Harvesting of Surface Waxes. Surface waxes of corn whorls (later found to include 6-MBOA, DIMBOA, and related constituents) were collected by dipping and agitating corn whorl leaf sections in CH_2Cl_2 for approximately 1 min. The waxes were stored at −20°C until they were analyzed or evaluated. For GLC-MS analysis, about 200 g of each whorl sample (analyzed in duplicate) was dipped in 2 liters of CH_2Cl_2. Solubles were carefully concentrated and stored at −20°C. For preparative work to isolate adequate quantities for NMR and dietary studies, approximately 4 kg of corn whorls from two resistant inbred lines, Mp707 and Mp708, were solvent-stripped with about 40 liters of CH_2Cl_2 in batches. Yields of waxes averaged 0.047% and 0.069% from fresh tissue for S and R lines, respectively.

Dietary Constituents. The standard casein–wheat germ diet including contents and rearing procedures for the SWCB have been established by Davis (1989). The ingredients of the standard casein–wheat germ diet (used for test comparisons) and the test diet (without the casein and wheat germ) were custom prepared by Bio-Serv, Inc., Frenchtown, New Jersey. The standard diet (530 g) includes 12.6 g casein; 10.6 g wheat germ; 10.6 g agar; 36.8 g of a mixture of sucrose, salts, linseed oil, cholesterol, methyl paraben, sorbic acid, and corn cob grits; 3.7 g of a vitamin mix; 0.3 g of neomycin sulfate; and 456 ml of water. For amino acid diets, 14.17 g of a mixture of essential and nonessential amino acids were used in place of the casein and wheat germ. The contents were based on amino acid analysis of corn whorls (Hedin et al., 1990), and alphacel (alphacellulose; Bio-Serv, Inc., Frenchtown, New Jersey) was used to normalize total weight of the diet. To initiate feeding (and growth) on the amino acid diets, it was found that the addition of 1 g of wheat germ (0.2% of the diet on a wet weight basis, a nonnutritional level) was required (Hedin and Davis, unpublished data). The significance of this requirement is being investigated.

Dietary Studies. Effects of *N-O*-Me-DIMBOA (compound Ib; see Table 1) and 6-MBOA on SWCB larval growth were evaluated using a standard casein-wheat germ diet and the rearing procedure established by Davis (1989). Dietary ingredients and procedures are further listed and described by Hedin et al. (1990). Dietary evaluations were also performed using a nonoptimal synthetic amino acid diet that required the aforementional supplementation with wheat germ (0.2%) to initiate growth (Hedin and Davis, unpublished data). Each treatment was comprised of 7–17 replicates consisting of the growth of one larvae.

To test putative toxins in short supply such as 6-MBOA, 50-ml portions of the prepared diet were further mixed with 1 g of alphacel on which varying amounts of 6-MBOA had been suspended using ethyl ether as a carrier so that

TABLE 1. EI-MS FRAGMENTATIONS (m/z)[a] OF SOME 1,4-BENZOXAZIN-3-ONES AND METHOXYBENZOXAZOLINONES IN CORN WHORLS



I II

(Ia) R_1 = OMe, R_2 = H, R_3 = R_4 = OH (DIMBOA)
(Ib) R_1 = R_4 = OMe, R_2 = H, R_3 = OH (*N-O*-Me-DIMBOA)
(Ic) R_1 = OMe, R_2 = R_4 = H, R_3 = OH
(Id) No Structure Assigned
(IIa) R_1 = OMe, R_2 = H (6-MBOA)
(IIb) R_1 = R_2 = OMe

Ia 211(46), 195(28), 193(10), 180(7), 166(85), 165(100), 150(56), 138(34), 124(18), 122(12), 110(40), 106(28), 95(34)
Ib 225(48), 196(14), 193(17), 166(100), 165(95), 150(64), 138(30), 122(14), 110(42), 106(32), 95(30)
Ic 195(42), 166(100), 165(8), 138(12), 124(20), 110(14), 95(8)
Id 239(15), 225(10), 195(8), 166(34), 165(100), 150(55), 137(60), 122(18), 110(30)
IIa 166(14), 165(100), 150(58), 136(3), 122(12), 106(28), 95(16)
IIb see Ic

[a] M^+ is underlined.

the final diet contained 0.02, 0.05, 0.10, 0.20, and 0.40% of 6-MBOA on a wet weight basis. To test Ib at approximately the same level (0.25%) with only 22 and 24 mg available for two separate tests that were conducted in 1991 and 1992, 10 ml of the diet was mixed with 0.5 g of alphacel on which Ib had been suspended from ethyl ether. The above diets were poured into 45-mm-diameter crystallizing dishes to gel and 7–17 diet plugs (weighing 0.4 g each) were harvested with No. 6 cork borer. The plugs were placed in the center of a paper board cap and infested with one newly hatched SWCB larva. An inverted 30-ml plastic cup containing 2% agar was placed over the cap, which was then snapped into the rim. Similar sized diet plugs were also prepared from the control diets.

In other dietary studies, tests with 0.1 and 0.4% of 6-MBOA added to the modified synthetic amino acid diet were carried out to determine whether initiation of growth might be stimulated at relatively low levels of incorporation of 6-MBOA. Also, S and R waxes were evaluated at 0.12% of the diet for their effect on growth when added to the modified synthetic amino acid diet.

Insects. The SWCB used in these studies were obtained from laboratory colonies maintained by the Corn Host Plant Resistance Research Unit of the Crop Science Research Laboratory located at Mississippi State, using the procedures described by Davis (1989). For the growth tests, the insects were maintained at 27°C, 50–60% relative humidity, and 16:8 hr light–dark photocycle. To maintain vigor, the colony was infused with wild insects every year.

Isolation of N-O-*Me-DIMBOA (Ib).* Surface waxes of S and R lines (0.5-g aliquots) that had been collected as previously described were redissolved in hexane and chromatographed on a 2.5 × 11-cm Biosil A column (silicic acid; Biorad Laboratories, Rockville Center, New York). Fractions were eluted with 200 ml volumes of solvents of increasing polarity: CH_2Cl_2, $CHCl_3$, ethyl acetate, and methanol. The progression of the elution was monitored by silica gel TLC using $CHCl_3$–ethyl acetate 1:1 for development, and I_2 or 1% ethanolic diphenylboric acid 2-aminoethyl ester for visualization. The hydrocarbons, wax esters, and a trace of carotenes were eluted from the column with hexane and CH_2Cl_2. 6-MBOA, DIMBOA, and Ib were eluted with $CHCl_3$–ethyl acetate 1:1. Ib possessed an R_f of 0.50 (Silica gel; $CHCl_3$–ethyl acetate 1:1), while 6-MBOA (IIa) and DIMBOA (Ia) gave R_f values of 0.57 and 0.10, respectively, with the same system. Repeated chromatographic work in 1990 and 1991 yielded 22 and 24 mg, respectively, of Ib. Portions were used for gas chromatographic, spectral, and subsequently, dietary studies.

Synthesis of 6-MBOA. Quantities were available from our previous synthesis work (Nicollier et al., 1982).

^{1}HMR and ^{13}CMR Spectra. Spectra were obtained on 22.0 mg of the isolated compound (Ib) in D_6-acetone with a QE-300 GE NMR spectrometer. The spectral shifts and their assignments are given in Table 2.

TABLE 2. ^{1}H AND ^{13}C NMR SPECTRAL DATA OBTAINED FOR DIMBOA (Ia) AND A 1,4-BENZOXAZIN-3-ONE (Ib) ISOLATED FROM CORN WHORL

	^{1}H NMR (ppm)		^{13}C NMR (ppm)	
Carbon No.	Ia[a]	Ib	Ia[a]	Ib
2	5.72	5.72, s	88.1	92.5
3			208.0	205.5
5	7.25	7.18, d	109.9	107.8
6	6.68	6.73, d	103.6	103.5
7			[b]	157.2
8	6.61	6.63, d	99.2	103.0
9			137.7	137.4
10			117.8	115.9
OCH_3 (C-7)	3.77	3.80, s	51.0	55.1
OH (C-2)	2.85	3.00, s		
OCH_3 (N)		3.93, s		62.2
OH (N-4)				

[a]Campos et al. (1989).
[b]Not assigned.

Mass Spectra. Mass spectra were taken at 70 eV in the positive EI mode with a Hewlett Packard 5985B quadropole mass spectrometer. The sample was introduced into the source of the instrument via a direct insertion probe. The source was maintained at a temperature of 200°C, and the probe was ballistically heated from ambient temperature to 350°C.

Samples were also analyzed by GLC-EI-MS on a methyl silicone fused silica column (25 m × 0.25 mm, film thickness 0.25 μm, programmed from 70° to 250°C at 10°C/min, injection temperature = 200°C, detector temperature = 250°C, carrier gas: helium, 40 cm/sec) that was interfaced to the mass spectrometer. Identifications were assigned by comparison with available standards. An approximation of relative concentration of components was obtained by comparing the MS data system total abundance count of the ion chromatogram with that of appropriate standards. Multiple ion scanning was performed to determine the sequence of fragmentations, concomitantly distinguishing the presence of any isomers.

Statistical Methods. Data on larval weights were subjected to ANOVA. Means were separated using least significant differences $P < 0.05$ test. The statistical design used to compare larval growth on diets was completely random. Each treatment was comprised of 7–17 replicates consisting of the growth of one larvae, as previously stated.

RESULTS AND DISCUSSION

When the isolated surface waxes were chromatographed on a Biosil A column as described in the Methods and Materials section, elution with $CHCl_3$–ethyl acetate 1 : 1 gave a white substance on freeze-drying that could later be crystallized from ethanol or aq. ethanol. In succeeding summers, 22 and 24 mg of a benzoxazinone were collected for structural and dietary studies. The spectral data are summarized in Table 1 and Table 2 and pertinent structures are included.

EI-MS via solid probe of the benzoxazinone (Ib, Table 1) gave an apparent moleculer ion of M^+ 225 with a fragmentation pattern somewhat similar at the lower masses to DIMBOA (Ia) and 6-MBOA (IIa). The mass spectral data listed in Table 1 for 6-MBOA were reported by us previously (Nicollier et al., 1982) and that for DIMBOA was also obtained by us, but has not previously been reported. The MS data for DIMBOA is generally consistent with that reported by Chen and Chen (1976), Venis and Watson (1978), Lyons et al. (1988), and Campos et al. (1989), except that it contained some *m/e* 195 ($M^+ - 16$), which presumably can be attributed to contamination of the DIMBOA with either 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (Ic) or 6,7-dimethoxy-2-benzoxazolinone (IIb). The latter previously was found in corn by Tipton et al. (1967). Finally, evidence was obtained for another component (presumably a benzoxazinone) with an apparent M^+ of 239.

^1H NMR and ^{13}C NMR spectral data for DIMBOA (Ia) and Ib are listed in Table 2. It was possible to reconcile the spectral data reported by Campos et al. (1989) for DIMBOA with those of our isolate Ib for C-2, C-3, C-5, C-6, C-8, C-9, and C-10; the OCH_3 (C-7); and the OH (C-2). Additionally, the purified Ib gave a ^1H shift (three protons) at 3.93 ppm and a ^{13}C NMR shift at 62.2 ppm, both presumptive for an *O*-Me function. The possibility of a C-methyl on the aromatic ring is rejected because the C-5, C-6, and C-8 carbons and their protons have been established, leaving only the C-2 hydroxyl as an alternative methoxylation site to the hydroxy on the ring *N*. Methoxylation of the C-2 OH is unlikely, because of the much lower established ^1H shifts for the methoxy functions of 3.45 ppm in methoxyacetone and of 3.48 ppm in 2-methoxycyclohexanone. Evidence for *N*-OH is lacking by comparison with formaldoxime which gives a singlet at 5.15 ppm. Also, the higher ^1H shift of the *N*-OCH_3 (3.66 ppm) in *N*,*N*-acetylmethoxyaniline (Ito et al., 1980) supports assignment of the *N*-OCH_3. Hence, the structure of Ib is deduced to be 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one.

Further evidence for the *N*-*O*-Me function is provided by NMR data from the work of Nagao et al. (1985), who isolated the 2-*O*-glucoside of Ib and determined its absolute configuration. They list the ^1H NMR and ^{13}C NMR shifts as H 3.89 and 55.4 ppm respectively, both very close to our measurements.

As indicated above, the glycoside of Ib has previously been reported. Hofman and Hofmanova (1970) reported the glucoside of Ib from corn, wheat, and the roots of *Coix lachryma*. In a review article, Gambrow et al. (1986) studied the metabolic fate of Ib glucoside in wheat in which the trivial name HDIBOA-Glc was employed. Nagao et al. (1985) analyzed the stereochemistry of the Ib glucoside in *Coix lachryma* and found it to be 2-*O*-β-Glucopyranosyl-4,7-dimethoxy-1,4-(2H)-benzoxazin-3-one. Neimeyer (1988), in a review article, referred to Ib as HDMBOA-Glc. He further cited the work of Gambrow et al. (1986), who reported that Ib could not be obtained in pure form because of its ease of decomposition. In this present work, multimilligram quantities were isolated with relative ease, probably because the source was the waxy surface layer of the whorls. A review of GLC-MS data collected in this laboratory over a several-year period from whorl extracts, pressed whorl juice, and corn callus failed to reveal any evidence for *N*-*O*-Me-DIMBOA, while 6-MBOA was prominent. Because the wax layer constitutes a relatively small percent of the total whorl tissue, its content in these tissues escaped detection. Evidently, this successful isolation of *N*-*O*-Me-DIMBOA can be attributed to its stability in the wax layer.

The contents of *N*-*O*-Me-DIMBOA, DIMBOA, and 6-MBOA of the waxes from several S and R corn whorl samples as determined by GLC-FID analysis are listed in Table 3. These compounds eluted in an order that was unexpected based on molecular weights; *N*-*O*-Me-DIMBOA; 9.8 min; DIMBOA, 11.0 min; and 6-MBOA, 12.6 min. The unexpected relatively early elution of DIMBOA actually was that of the thermally dehydrated species (*m/e* 193) as later shown by GLC-MS. However, the molecular ion of DIMBOA (M^+ 211) survives solid probe MS, and *m/e* 193 ($M^+ - 18$) is a relatively minor fragment (Table 1).

The total surface wax content, which includes a number of hydrocarbons, esters, and acids in addition to *N*-Me-DIMBOA, DIMBOA, and 6-MBOA, was 46% higher (statistically significant) in resistant lines than in susceptible lines (Table 3), consistent with the visible appearance of resistant whorls, which appear to have a heavier coat of surface waxes. The content (milligrams per gram of whorl) of DIMBOA and *N*-*O*-Me-DIMBOA in resistant waxes appeared to be generally higher than in susceptible lines, while 6-MBOA appeared to be higher in susceptible lines, but the differences were not statistically significant.

Finally, dietary tests were carried out to evaluate the toxicity of *N*-*O*-Me-DIMBOA to SWCB larvae. Table 4 summarizes results from three tests in which larvae were fed on diet plugs for 7 or 13 days. Test A showed that *N*-*O*-Me-DIMBOA was not toxic to insects fed the standard casein–wheat germ diet at the same general level at which 6-MBOA appeared to decrease growth. A semilog extrapolation of data obtained by feeding five levels of 6-MBOA (0.02–0.40%) as incorporated in the standard diet gave an ED_{50} = 0.25% for 6-MBOA, similar to our previous finding of ED_{50} = 0.40 (Nicollier et al., 1982) for the SWCB.

TABLE 3. CONTENT OF *N-O*-Me-DIMBOA, AND 6-MBOA IN SURFACE WAXES OF S AND R CORN WHORLS—GLC ANALYSIS[a]

Line	Waxes (%)	Content (mg/g whorl)		
		N-O-Me-DIMBOA	DIMBOA	6-MBOA
Susceptible				
AB24E	0.048	1.28	0.38	0.16
GT106	0.55	4.82	0.91	0.36
SC229	0.043	1.15	0.74	1.52
TX601	0.046	3.63	1.80	1.16
Va35	0.045	1.61	1.40	1.80
Average	0.047 ± 0.004	2.50 ± 1.47	1.05 ± 0.50	1.00 ± 0.64
Resistant				
Mp496	0.059	8.16	1.71	0.90
Mp704	0.090	3.22	1.17	0.57
Mp705	0.078	11.34	4.10	0.73
Mp707	0.058	3.15	0.52	0.17
Mp708	0.062	[b]	[b]	[b]
Average	0.069 ± 0.013	6.46 ± 3.47	1.88 ± 1.35	0.59 ± 0.27

[a] Mean ± standard deviation, analysis was on a fresh weight basis.
[b] Sample was lost.

Test B evaluated *N-O*-Me-DIMBOA with the dietary nitrogen being supplied by amino acids rather than the casein–wheat germ so as to invoke dietary stress. While SWCB larvae will not grow on a diet in which a synthetic amino acid mixture based on the amino acid analysis of corn whorls is the sole source of protein, FAW larvae grow and emerge to adults on the same diet, although their growth is initially retarded (Hedin et al., 1990) (many other insects can also be reared on amino acid diets). This lag period has been attributed to metabolic adaptation of the young larvae to the free amino acid source. SWCB larvae grew about 30% as well during the first 13 days (32.5 mg) on the described amino acid diet fortified with 0.2% wheat germ, but on the further addition of 0.25% *N-O*-Me-DIMBOA, growth was abolished (1.6 mg). On the other hand, S and R waxes at the 0.12% level did not affect the growth of SWCB larvae on the amino acid diet fortified with 0.2% wheat germ. Finally (test C), addition of 0.1 and 0.4% 6-MBOA to the synthetic amino acid diet in the absence of 0.2% wheat germ had no further negative (or positive) effect on growth.

In summary, the structure of a new benzoxazinone, *N-O*-Me-DIMBOA, found in the surface wax of corn, was deduced, although its glucoside had previously been reported, and some evidence for the presence of two other benzoxazinones was also obtained. This component appears mainly in the surface wax along with DIMBOA and 6-MBOA. *N-O*-Me-DMBOA is present in

TABLE 4. EFFECTS OF 6-MBOA, *N-O*-Me-DIMBOA, AND WHORL SURFACE WAXES ON LARVAL GROWTH OF SOUTHWESTERN CORN BORER

Protein source[a]	Additives	Mean larval weight (mg) days after infestation 7	13
Test A			
C-WG	—	9.6	
C-WG	0.20% 6-MBOA	7.7	
C-WG	0.25% *N-O*-Me-DIMBOA	12.30	
LSD 0.5 value		(3.0)	
Test B			
C-WG	—		104.7
SYN AA	0.2% WG		32.5
SYN AA	0.2% WG + 0.12% S Wax		30.6
SYN AA	0.2% WG + 0.12% R Wax		26.8
SYN AA	0.2% WG + 0.25% *N-O*-Me-DIMBOA		1.6
LSD 0.05 value			(17.2)
Test C			
C-WG			94.4
SYN AA			3.0
SYN AA	0.1% 6-MBOA		6.1
SYN AA	0.4% 6-MBOA		3.7
LSD 0.05 value			(12.6)

[a]C-WG = casein-wheat germ, SYN AA = synthetic amino acids (Hedin et al., 1990)

corn surface waxes in higher concentration than DIMBOA and 6-MBOA. *N-O*-Me-DMBOA was toxic when incorporated in a stress (amino acid) diet, but it was less toxic than 6-MBOA to the SWCB when incorporated in the standard laboratory diet. Methoxylation of the ring *N* may contribute to its relative stability in the wax and to its limited toxicity. Nevertheless, it may have some role in the resistance of corn to the SWCB given that the surface wax content of R lines is higher than in S lines. An explanation for the effect of *N-O*-Me-DIMBOA on larval growth in stressed diets may be that hydroxamic acids inhibit lepidopteran chymotrypsin (Houseman et al., 1992).

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REFERENCES

BIANCHI, G., and AVATO, P. 1984. Surface waxes from grain, leaves, and husks of maize. *Cereal Chem.* 61:45–47.

CAMPOS, F., ATKINSON, J., ARNASON, J.T., PHILOGENE, B.J.R., MORAND, P., WERSTIUK, N.H., and TIMMINS, G. 1989. Toxicokinetics of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) in the European corn borer, *Ostrinia nubilalis* (Hubner). *J. Chem. Ecol.* 15:1989–2001.

CHEN, C.M., and CHEN, M.T. 1976. 6-Methoxybenzoxazolinone and triterpenoids from roots of *Scoparia dulcis*. *Phytochemistry* 15:1997–1999.

DAVIS, F.M. 1989. Rearing southwestern corn borers and fall armyworms at Mississippi State, MS, pp. 27–36, *in* J.A. Mihm (ed.) Toward Insect Resistant Maize for the Third World. Proceedings, International Symposium on Methodologies for Developing Host Plant Resistance to Maize Insects, CIMMYT, El Batan, Mexico.

DAVIS, F.M., NG, S.S., and WILLIAMS, W.P. 1988a. Mechanisms of resistance in corn to leaf feeding by southwestern corn borer and European corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 82:919–922.

DAVIS, F.M., WILLIAMS, W.P., MIHM, J.A., BARRY, B.D., OVERMAN, J.L., WISEMAN, B.R., and RILEY, T.J. 1988b. Resistance to multiple lepidopterous species in tropical derived corn germplasm. Mississippi Agricultural and Forestry Experiment Station Technical Bulletin 157, 6 pp.

DAVIS, F.M., WILLIAMS, W.P., and WISEMAN, B.R. 1989. Methods used to screen maize and to determine mechanisms of resistance to the southwestern corn borer and fall armyworm, pp. 101–108, *in* J.A. Mihm (ed.). Toward Insect Resistant Maize for the Third World. Proceedings, International Symposium on Methodologies for Developing Host Plant Resistance to Maize Insects, CIMMYT, El Batan, Mexico.

GAMBROW, H.J., LUCKGE, J., KLAUSENER, A., and MULLER, E. 1986. Occurrence of 2-(2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazine-3-one)-β-D-glucopyranoside in *Triticum aestivum* leaves and its conversion into 6-methoxy-benzoxazolinone. *Z. Naturforsch.* 41C:684–690.

HEDIN, P.A., DAVIS, F.M., WILLIAMS, W.P., and SALIN, M.L. 1984. Possible factors of leaf-feeding resistance in corn to the southwestern corn borer. *J. Agric. Food Chem.* 32:262–267.

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.

HOFMAN, J., and HOFMANOVA, O. 1970. 1,4-Benzoxazine derivatives in plants. A new type of glycoside from *Zea mays*. *Tetrahedron Lett.* 3213–3214.

HOUSEMAN, J.G., CAMPOS, F., THIE, N.M.R., PHILOGENE, B.J.R., ATKINSON, J., MORAND, P., and ARNASON, J.T. 1992. Effect of the maize-derived compounds DIMBOA and MBOA on growth and digestive processes of European corn borer. *J. Econ. Entomol.* 85:669–674.

ITO, Y., YOKOYA, H., UMEHARA, Y., and MATSUURA, T. 1980. Formation of ketone deperoxides from ozonation of o-methyloximes. *Bull. Chem. Soc. Jpn.* 53:2407–2408.

KLUN, J.A., and BRINDLEY, T.A. 1966. Role of 6-Methoxybenzoxazolinone in inbred resistance of host plant (maize) to first brood larvae of European corn borer. *J. Econ. Entomol.* 59:711–718.

LYONS, P.C., HIPSKIND, J.D., WOOD, K.V., and NICHOLSON, R.L. 1988. Separation and quantification of cyclic hydroxamic acids and related compounds by high-pressure liquid chromatography. *J. Agric. Food Chem.* 36:57–60.

NAGAO, T., OTSUKA, H., KOHDA, H., SATO, T., and YAMASAKI, K. 1985. Benzoxazinones from *Coix lachryma* Jobi Var. Ma-Yuen. *Phytochemistry* 24:2959–2962.

NICOLLIER, G.F., HEDIN, P.A., and DAVIS, F.M. 1982. 5-,6-, and 7-Methoxybenzoxazolinone:

Carbon-13 nuclear magnetic resonance spectra and biological activity. *J. Agric. Food Chem.* 30:1133–1135.

NIEMEYER, H.M. 1988. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defence chemicals in the Graminae. *Phytochemistry* 27:3349–3358.

RITCHIE, S.W., and HANWAY, J.J. 1982. How a corn plant develops. Journal of Iowa State University of Science and Technology Cooperative Extension Service Special Report 48, 21 pp.

TIPTON, C.L., KLUN, J.A., HUSTED, R.D., and PIERSON, M.D. 1967. Cyclic hydroxamic acids and related compounds from maize. Isolation and characterization. *Biochemistry* 6:2866–2870.

VENIS, M.A., and WATSON, P.J. 1978. Naturally occurring modifiers of auxin-receptor interaction in corn: Identification as benzoxazolinones. *Planta* 142:103–107.

WILLIAMS, W.P., BUCKLEY, P.M., and DAVIS, F.M. 1989. Combining ability for resistance in corn to fall armyworm and southwestern corn borer. *Crop Sci.* 29:913–915.

WISEMAN, B.R., WILLIAMS, W.P., and DAVIS, F.M. 1981. Fall armyworm: Resistance mechanisms in selected corn genotypes. *J. Econ. Entomol.* 74:622–624.

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PARTIAL CHARACTERIZATION AND HPLC ISOLATION OF BACTERIA-PRODUCED ATTRACTANTS FOR THE MEXICAN FRUIT FLY, *Anastrepha ludens*[1]

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Abstract—Methods were developed to collect and isolate volatile chemicals produced by a *Staphylococcus* bacterium in tryptic soy culture that are attractive to protein-hungry adult Mexican fruit flies. Centrifugation of bacteria culture yielded a slightly attractive pellet containing most of the bacteria cells and a highly attractive supernatant. Supernatant filtered to remove the remaining bacteria was as attractive as the unfiltered supernatant. Filtrate at pH 7 and above was much more attractive than filtrate at pH 5 and below. Most of the attractiveness was retained on strong cation exchange media under acidic conditions and eluted with base. Attractive principles could not be trapped on adsorbents such as Porapak Q or extracted with organic solvents from aqueous preparations, but they were easily collected by headspace sweeping with steam. The attractive components were efficiently concentrated by rotary evaporation of steam distillate at pH 5, but at higher pH much of the attractiveness distilled. A reverse-phase HPLC method using a negative counter-ion was developed to separate and collect attractive components of concentrated steam distillate. Attractive fractions collected using this method were concentrated and injected onto silica HPLC. Activity eluted from silica in two distinct bands. Results suggest that the most attractive components of the bacterial odor are highly polar, low-molecular-weight amines.

Key Words—Attractants, Mexican fruit fly, Diptera, Tephritidae, *Anastrepha ludens*, bacteria, amines.

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[1] Diptera: Tephritidae.

INTRODUCTION

Attractiveness of bacteria to fruit flies has been investigated for 40 years (Gow, 1954), but few of the chemicals responsible for the attractiveness have been identified. Ammonia is one by-product of bacterial action that has been identified. It has been used in some capacity as a fruit fly attractant since at least the 1930s (Jarvis, 1931; Hodson, 1943), but much disagreement exists concerning its attractiveness. Several authors have stated that attractiveness of bacteria and protein baits is due primarily to components other than ammonia (Gow, 1954; Mazor et al., 1987; Drew and Fay, 1988). Other studies have indicated that ammonia alone or in protein baits is an effective attractant if its release rate is within the optimum range (Bateman and Morton, 1981; Morton and Bateman, 1981; Wakabayashi and Cunningham, 1991).

More recently, 2-butanone and butanol were identified from volatiles produced by *Proteus* species bacteria (Hayward et al., 1977) and shown to be attractive to *Dacus tryoni* (Froggatt) (Drew, 1987). Generally, other volatiles identified from bacteria (Hayward et al., 1977) or protein baits (Morton and Bateman, 1981; Buttery et al., 1983) have not been attractive. The general lack of success in identifying the probable attractive principles of bacteria and protein baits suggests that the problem is difficult and may require unusual approaches.

Robacker et al. (1991a) isolated a bacterium (identified as *Staphylococcus aureus*) from the mouthparts of a female Mexican fruit fly (*Anastrepha ludens* Loew) that was highly attractive to adult Mexican fruit flies in laboratory experiments. That study and a subsequent one (Robacker, 1991) presented evidence that the attraction response of the flies to bacterial odor was motivated by hunger for protein. The attractant chemicals produced by the bacteria were not identified.

This paper reports chemical properties and methods for isolation of the attractive principles of the odor of the *Staphylococcus* species (RGM-1) identified by Robacker et al. (1991a). Evidence is presented that the most important attractants are low-molecule-weight amines that cannot be handled by standard methods for collecting and isolating insect pheromones and other attractants.

METHODS AND MATERIALS

Insects and Test Conditions. Flies were from a culture that had been maintained on laboratory diet for about 80–90 generations with no wild-fly introductions. Recent experiments indicated the culture flies were as vigorous as wild flies in mating competitiveness (Moreno et al., 1991) and had retained much of their natural courtship behavior (Robacker et al., 1991b). Mixed-sex groups of 180–200 flies were kept in 473-ml cardboard cartons with screen tops until used

in tests. Flies were tested when 6–12 days old. Flies were deprived of protein as adults but were fed sucrose and water up until the time of attractiveness testing. All tests were conducted in the laboratory between 0830 and 1430 hr under a combination of fluorescent and natural light. Laboratory conditions were 22 ± 2°C, 50 ± 20% relative humidity, and photophase from 0630 to 1930 hr.

Bacterial Preparations. Bacterial strain RGM-1 (Robacker et al., 1991a) was cultured in tryptic soy broth (Difco Laboratories, Detroit, Michigan) in a shaker for 144 hr at 30°C. Numerous 100-ml samples were prepared as needed for the various experiments. Bacterial culture was centrifuged at 10,000 rpm for 20 min and separated into pellet and supernatant. Supernatant was filtered through 0.45-μm type HA aqueous filters (Millipore Corporation, Bedford, Massachusetts) followed by 0.22-μm type GV aqueous/organic filters (Millipore). The pellet was suspended in water and centrifuged. The new pellet was suspended again in water. The centrifugation and filtration of the supernatant were done to remove bacteria cells. Resuspended pellets (second centrifugation only), supernatants (first centrifugation only), and filtrates were monitored for attractiveness to flies using cage-top bioassays (described below). Test quantities applied to filter papers were 10 μl. Controls were 10-μl quantities of tryptic soy broth. Attractiveness of resuspended pellets was also evaluated against a water control (10 μl). Data were analyzed as paired *t* tests to compare total counts at treatment papers to total counts at control papers.

Cage-Top Bioassay Procedure. Bioassays were conducted by placing four filter paper triangles (3 cm/side), two containing test samples and two containing appropriate controls, near the corners on the top of an inset cage (30 cm/side, aluminum-screened). The numbers of flies beneath each filter paper were counted once each minute for 10 min. The two papers containing test chemicals were positioned diagonally from each other on two corners of the cage top, and the two papers containing controls were positioned diagonally from each other on the other two corners. The filter papers were raised 5 mm above the cage top using plastic rings to ensure that olfaction and not contact chemoreception was solely responsible for the response of the flies. Two cartons of 180–200 flies were used in each bioassay cage.

Effects of pH on Attractiveness of Bacterial Filtrate. Bacterial filtrate had a pH of 7.9. The pH of aqueous bacterial filtrate was raised to 8, 9, 10, 11, and 12 with saturated sodium hydroxide and lowered to 2, 3, 4, 5, 6, and 7 with 85% phosphoric acid. Each pH treatment was bioassayed for attractiveness using cage-top bioassays. Test quantities applied to filter papers were 10 μl. Controls were 10-μl quantities of water. Bioassays were conducted in a randomized complete block experiment. Ten replications of the experiment were conducted. Data used for analysis of variance were differences between total counts at treatment papers and controls.

Retention on Strong Cation Exchange Media. The pH of aqueous bacterial filtrate was lowered to 4.9 with 85% phosphoric acid. The resulting solution (1 ml) was loaded onto a SCX PrepSep extraction column (Fisher Scientific, Fair Lawn, New Jersey) after preparing the column with successive rinses with 5 ml of 0.01 M monobasic sodium phosphate (NaH_2PO_4) (pH 4.9) and 1 ml of water. After loading the 1 ml of bacterial filtrate, the column was eluted with successive rinses with 5 ml of 0.01 M monobasic sodium phosphate (pH 4.9), 5 ml of water, and 1 ml of 0.05 M dibasic sodium phosphate (Na_2HPO_4) (pH 9.0). Since passage through the acidic column always lowered the pH of the 1 ml of 0.05 M dibasic sodium phosphate to about 7–8, one drop of saturated sodium hydroxide was added to the eluate and it was again passed through the column. The attractiveness of all eluates was evaluated with cage-top bioassays after raising their pH to 10 or greater with sodium hydroxide. Bioassay test quantities were 10 μl for the void volume eluting during loading of the bacterial filtrate and for both of the 0.05 M dibasic sodium phosphate eluates, and 50 μl for the water eluate and for the 0.01 M monobasic sodium phosphate eluate. Controls were 10- or 50-μl quantities, as appropriate, of water containing one drop of saturated sodium hydroxide per milliliter. Experimental design was a randomized complete block and data analysis was handled as in the previous experiment. Paired *t* tests also were conducted to compare counts at treatments with counts at controls.

Collection of Volatiles from Bacterial Filtrate. Volatiles were collected using several methods. The first series of methods was to put bacterial filtrate into a glass aeration flask and pull air through the flask and subsequently through a trap. Bacterial filtrate was put into the aeration flask either as 250 ml of liquid or as an incompletely dried residue from 50 ml of the liquid on 2500 cm^2 of filter paper. Both forms of the bacterial filtrate were highly attractive to flies. Four different types of traps were used to collect volatiles. Three employed chemical adsorbents in glass tubes: 250 mg of Tenax-GC (60/80) (Alltech Associates, Inc., Deerfield, Illinois); 250 mg of Porapak Q (50/80) (Alltech Associates); or 20 mg of Super Q (Alltech Associates). Procedures and glassware for the collections using Tenax-GC and Porapak Q were similar to those described in Robacker et al. (1990). Apparatus for collections using Super Q was that described by Heath and Manukian (1992). Aerations were for at least 6 hr. The adsorbent traps were cleaned with 100 ml of 5% acetone in pentane (v/v) before use and extracted after aeration with successive rinses of 1 ml each of 5% acetone in pentane, acetone, and water to obtain bacteria volatiles. The fourth was a cold trap in which the odor-laden air was pulled through a glass flask immersed in a Dry Ice–acetone bath. Volatiles were obtained from the collection flask in the ice that formed during the aeration.

All trap extracts were evaluated for attractiveness using cage-top bioassays. Test quantities applied to filter paper were 100 μl. Controls were 100 μl of

solvents used for extraction (chemical adsorbents collections) or water (cold trap collection).

The final volatile collection method was a headspace sweep with steam. Steam was generated from vigorous boiling of Milli-Q (Millipore) grade water in a two-neck 500-ml flask. The steam was vented into a second two-neck 500-ml flask containing 100 ml of bacterial filtrate heated gently. The steam entered the second flask about 5 cm above the liquid level so as to prevent excessive foaming of the bacterial filtrate. The steam distillate was collected into a 500-ml flask following condensation in a cold-water condenser. The procedure was carried out until 400 ml of steam distillate was collected. Both the steam distillate and the bacterial filtrate residue (the portion of bacterial filtrate that was not swept into the condenser) were evaluated for attractiveness using cage-top bioassays. Test quantities applied to filter papers were 10 μl. Controls were 10 μl of water. Paired t tests were used to compare counts at treatments with counts at controls. A Student's t test was used to compare responses to steam distillate with responses to bacterial filtrate residue. Data for this test were differences between counts at treatment papers and controls.

Solvent–Solvent Extraction. Steam distillate and bacterial filtrate were solvent–solvent extracted in separate experiments with hexane, dichloromethane, and ethyl ether. Quantities of the organic solvents were 10-fold greater than the aqueous distillate or filtrate. Ten-microliter quantities of the aqueous and 100 μl of the organic phases were tested for attractiveness to flies using cage-top bioassays. Controls were 10-μl quantities of water for testing aqueous phases and 100 μl of appropriate organic solvent for testing the organic phases.

Concentration of Bacteria-Produced Attractants. Steam distillate was concentrated using three methods. The first was lyophilization using a Speed Vac concentrator (Savant Instruments, Inc., Farmingdale, New York) connected in series to a refrigerated condensation trap (Savant Instruments, Inc.) and a high vacuum pump (Savant Instruments, Inc.). Steam distillate (100 ml) was concentrated to 1 ml. Dilutions of 1:10 and 1:100 with water were made, and 10-μl samples of the concentrate and the two dilutions were evaluated for attractiveness using cage-top bioassays. Controls were 10 μl of water.

The second method was rotary evaporation using a Rotavapor RE-111 instrument (Buchi-Brinkman Instruments, Inc., Westbury, New York). Rotary evaporation was aided by heating the steam distillate while pulling vacuum on the system. In the first attempt, 250 ml of steam distillate was concentrated to 10 ml. The concentrate, distillate, and 1:10 dilutions of each were evaluated for attractiveness using cage-top bioassays. Test amounts were 10 μl each. As a second attempt, the pH of 250 ml of steam distillate was lowered to 5 with phosphoric acid before rotary evaporation. The concentrate was evaluated when it reached a volume of 10 ml and again after it was evaporated to dryness and redissolved in 10 ml of water. The distillate and dilutions of the concentrates

(1:6 and 1:25) also were evaluated for attractiveness. For these tests, attractiveness was evaluated only after raising the pH to 9 or greater with sodium hydroxide. Test quantities were 10 μl. Controls for all of these tests were 10 μl of water.

Reverse-Phase HPLC. A Waters (Milford, Massachusetts) high-performance liquid chromatograph (HPLC) was used. Detection was at 254 nm (model 490 Programmable Multiwavelength Detector, Waters Associates). Steam distillate was concentrated 200:1 at pH 5 by rotary evaporation. Concentrated steam distillate (pH 5) was fractionated using ion-pairing on a C-18 column (Partisil ODS2, 10-μm particle size, 25 cm × 4.6 mm) (Alltech Associates). Mobile phase was a gradient beginning with 20% methanol and 80% aqueous 0.004 M lauryl sulfate (Sigma Chemical Co., St. Louis, Missouri; purity 99%; pH 3.0) at 1 ml/min. The percentage of methanol was increased to 100% with a linear gradient from 1 to 15 min. The mobile phase remained at 100% methanol for the remainder of the analysis. Injection volume was 100 μl. Consecutive 1-min fractions were collected from 0 to 30 min. One drop of concentrated sodium hydroxide was added to each fraction to raise the pH to 9 or greater. Each fraction was evaluated for attractiveness using cage-top bioassays. Test quantities applied to filter papers were 100 μl. This amount represents a 5:1 concentration over the original bacterial filtrate, assuming no losses during the various preparatory steps. Controls were 100 μl of water containing one drop of saturated sodium hydroxide per 1 ml. Fractions were tested in random order during one day constituting one replication of a randomized complete block experiment. Ten replications of the entire procedure from fraction collection to bioassay were conducted.

Silica HPLC. The HPLC described above was used to further fractionate three fractions collected from the reverse-phase ion-pairing method that were found to be attractive by cage-top bioassays. The fractions were those that eluted at 16–17, 17–18, and 18–19 min. For each of the three fractions, 20 collections of 1 ml each were made using the same HPLC method described above. Collections were combined and concentrated to near dryness by rotary evaporation under vacuum with heat. The dried concentrate was redissolved in 2 ml of methanol, and 0.2 N sodium hydroxide in methanol was added until pH 8 was obtained. Each concentrated fraction was analyzed on a silica column (Lichrosorb SI-60, 5-μm particle size, 25 cm × 4.6 mm) (Alltech Associates). Mobile phase was a gradient beginning with 5% methanol in pentane. The percentage of methanol was increased to 100% with a linear gradient from 1 to 15 min. The mobile phase remained at 100% methanol for the remainder of the analysis. Injection volume was 100 μl. Consecutive 1-min fractions were collected from 0 to 30 min. One drop of 0.2 N sodium hydroxide in methanol was added to each fraction. Each fraction was evaluated for attractiveness using cage-top bioassays. Test quantities applied to filter papers were 100 μl. This amount

represents a 1:2 dilution compared to the original bacterial filtrate. Controls were 100 μl of water containing one drop of 0.2 N sodium hydroxide in methanol per 1 ml. Experimental design was the same as for testing of the fractions from the reverse-phase column.

RESULTS AND DISCUSSION

Attractiveness of Bacterial Preparations. Supernatant obtained by centrifugation of RGM-1 culture and filtrate of the supernatant (containing no bacteria cells) were both significantly more attractive to flies than tryptic soy broth controls (Table 1). The pellet containing the bacterial cells after centrifugation was not as attractive as tryptic soy broth. However, the pellet was significantly more attractive than water.

Previous research (Robacker et al., 1991a) demonstrated that RGM-1 filtrate is at least as attractive as cultures containing living RGM-1 bacterial cells. The results also indicate that the bacteria cells are somewhat attractive in the absence of nutrient broth, as has been reported for several bacteria species attractive to *Bactrocera* (*Dacus*) *dorsalis* Hendel (Jang and Nishijima, 1990) and *Rhagoletis pomonella* Walsh (MacCollom et al., 1992).

Effect of pH on Attractiveness of Bacterial Filtrate. Attractiveness of bacterial filtrate was greatly affected by its pH (Figure 1). Bacterial filtrate was most attractive above pH 6, suggesting that the attractive compounds contain nitrogen capable of accepting protons. According to this interpretation, most of the attractant compounds exist as nonvolatile cations below pH 6 and as volatile neutral molecules above pH 6. The decline in attractiveness above pH 10 is more difficult to interpret but also may relate to ionization. Bacterial filtrate was slightly attractive below pH 6, as demonstrated by paired *t* tests. Except for pH 5, counts of flies at bacterial filtrate were significantly higher than counts at

TABLE 1. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING CENTRIFUGE FRACTIONS AND FILTERED BACTERIAL CULTURES COMPARED TO PAPERS CONTAINING TRYPTIC SOY BROTH (TSB) OR WATER AS CONTROLS[a]

Test	*N*	Test sample	Control
Pellet vs. TSB	6	85.3	143.7
Supernatant vs. TSB	6	211.7	102.0
Filtrate vs. TSB	6	218.3	109.7
Pellet vs. Water	6	95.3	45.3

[a] Means of test samples are significantly different from controls in every test by paired *t* tests ($P < 0.05$).

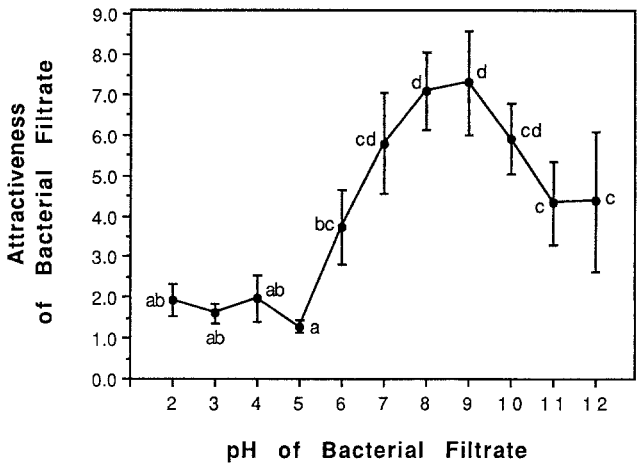

FIG. 1. Means (± SE) of counts of *A. ludens* at filter papers containing bacterial filtrate at various pH levels, divided by counts at filter papers containing water. Means followed by the same letter are not significantly different from each other at the 5% level by LSD.

water (smallest $t = 2.6$, $df = 9$, $P < 0.05$) indicating that some attractant chemicals are neutral under acidic conditions.

Attractiveness of Cationic Exchange Products. All eluates from strong cationic exchange columns (SCX PrepSep) were attractive to flies, indicating that many attractive chemicals are present in the bacterial filtrate (Table 2). The most attractive fraction was the second elution with the Na_2HPO_4 buffer (containing a drop of saturated sodium hydroxide) while the next most attractive

TABLE 2. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING ELUATES FROM STRONG CATION EXCHANGE COLUMNS LOADED WITH BACTERIAL FILTRATE COMPARED TO PAPERS CONTAINING WATER AS CONTROL[a]

Test	*N*	Test sample[b]	Control
Void vs. water	4	32.2a	17.5
NaH_2PO_4 eluate vs. water	4	49.0b	21.5
Water eluate vs. water	4	28.5a	19.0
Na_2HPO_4 eluate 1 vs. water	4	57.0b	22.8
Na_2HPO_4 eluate 2 vs. water	4	92.0c	21.0

[a]Means of test samples are significantly greater than controls in every test by paired *t* tests ($P < 0.05$).

[b]Means of test samples followed by the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$).

fraction was the first elution with the Na_2HPO_4 buffer (pH 9.0). These data show that the most attractive components of bacterial filtrate elute most effectively with alkaline eluants. Again, these data suggest that most of the attractant chemicals contain nitrogen that is cationic under acidic conditions (pH 4.9) and neutral under alkaline conditions (pH $\geq$ 9).

Attractiveness of Bacteria Volatiles Collected by Several Methods. All collections of volatiles using chemical adsorbents including Tenax GC, Porapak Q, and Super Q failed. Table 3 shows data for the Super Q collections. None of the three Super Q extracts were significantly more attractive than solvent controls. Interestingly, the 5% acetone in pentane extracts of Tenax GC, Porapak Q, and Super Q smelled strongly of bacterial culture. Collections using cold traps were slightly attractive but not successful enough to continue using cold-trapping.

Headspace sweeping with steam was very effective. Steam distillate was significantly more attractive than the bacterial filtrate residue (Table 4), even though the steam distillate represented at least a 1:4 dilution compared to the original bacterial filtrate. The pH of steam distillate was 9.5–10. The bacterial

TABLE 3. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING BACTERIA VOLATILES EXTRACTED FROM SUPER Q COMPARED TO PAPERS CONTAINING SOLVENTS USED FOR EXTRACTIONS AS CONTROLS[a]

Test	*N*	Test sample	Control
Acetone–pentane (5:95) extract vs. acetone–pentane (5:95)	4	25.8	27.2
Acetone extract vs. acetone	4	20.0	18.2
Water extract vs. water	4	19.8	17.8

[a]No test samples are significantly different from controls by paired *t* tests ($P < 0.05$).

TABLE 4. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING PRODUCTS OF HEADSPACE SWEEPING WITH STEAM COMPARED TO PAPERS CONTAINING WATER AS CONTROLS[a]

Test	*N*	Test sample[b]	Control
Steam distillate vs. water	5	146.8	50.4
Bacterial filtrate residue vs. water	5	88.6	47.6

[a]Means of test samples are significantly greater than controls in both tests by paired *t* tests ($P < 0.05$).

[b]Means of test samples are significantly different from each by Student's *t* test ($P < 0.05$).

filtrate residue remained more attractive than the water control, indicating that not all of the attractant molecules had been distilled.

We conclude that headspace sweeping with steam is the most effective of the methods attempted in this study to collect volatiles from bacterial filtrate.

Solvent–Solvent Extraction. All organic solvents failed to extract any attractiveness from either steam distillate or bacterial filtrate. The aqueous phases remained attractive in all experiments.

Attractiveness of Concentrated Steam Distillate. Both lyophilization and rotary evaporation at the natural pH of steam distillate produced attractive concentrates. However, dilutions of the concentrates were unattractive relative to the original steam distillate, indicating that much of the attractive material had been lost to the distillate. In fact, the distillate from rotary evaporation was much more attractive (mean count at distillate = 95.0 $\pm$ 6.2 SE) than water (mean count at water = 45.0 $\pm$ 5.7 SE) ($P < 0.05$, $t = 86.6$, $df = 2$).

Rotary evaporation at pH 5 was more effective than lyophilization or rotary evaporation at the natural pH of steam distillate (Table 5). Distillate from rotary evaporation was only slightly more attractive than water, indicating that little of the attractive material had been lost to the distillate. The 1:6 and 1:25 dilutions were prepared to test the combined efficiencies of steam distillation and rotary evaporation at pH 5 with regard to retention of the attractive principles of bacterial filtrate. The 1:6 dilutions were much less attractive than bacterial filtrate (Table 1: mean count at filtrate = 218.3) (no statistical test performed), indicating that much attractive material had been lost from the original bacterial filtrate. The 1:25 dilutions were likewise less attractive than steam distillate (Table 4: mean count at steam distillate = 146.8). Interestingly, the most attractive of the rotary evaporation products was the redissolved dried product (Table 5: concentrate 2), suggesting the possibility that repellent chemicals had been lost during concentration of the final 10 ml to dryness.

We conclude that rotary evaporation at pH 5 is the most effective of the methods attempted in this study to concentrate the attractive principles present in steam distillate.

Reverse-Phase HPLC. Figure 2 shows a representative chromatogram obtained using the ion-pairing technique with injection of concentrated steam distillate. Figure 3 shows that the most attractive region of the chromatogram was between 16 and 19 min retention time. However, paired t tests showed that significant attractiveness also eluted in every 1 min fraction from 2 to 11, 15 to 16, and 19 to 22 min (smallest $t = 3.2$, $df = 9$, $P < 0.05$), suggesting that many attractive components were present in the concentrated steam distillate.

First attempts at reverse-phase HPLC with a water–methanol gradient failed because all attractant activity eluted within 2 min of the void volume. The greatly increased retention of most of the attractiveness when the ion-pairing technique was used is evidence that the major attractants were ionic at pH 5 and were

TABLE 5. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING PRODUCTS[a] OF ROTARY EVAPORATION OF STEAM DISTILLATE AT pH 5 COMPARED TO PAPERS CONTAINING WATER AS CONTROL[b]

Test	*N*	Test sample[c]	Control
Distillate vs. water	4	59.8a	41.5
Concentrate 1 vs. water	4	119.0b	46.0
Concentrate 1 diluted 1:6 vs. water	3	110.7b	48.0
Concentrate 1 diluted 1:25 vs. water	3	97.7b	43.0
Concentrate 2 vs. water	4	169.5c	44.5
Concentrate 2 diluted 1:6 vs. water	3	98.3b	38.3
Concentrate 2 diluted 1:25 vs. water	4	102.8b	38.0

[a]pH of rotary evaporation products was adjusted to 9 or greater before attractiveness bioassays.
[b]Means of test samples are significantly greater than controls in every test by paired *t* tests ($P < 0.05$).
[c]Means of test samples followed by the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$).

retained on the column because of association with lauryl sulfate. These results are consistent with the hypothesis that the most attractive components of bacteria odor contain nitrogen that is positively charged at pH 5.

Silica HPLC. Figure 4 shows a representative chromatogram obtained from injection onto silica of the 17 to 18-min concentrate from the reverse-phase

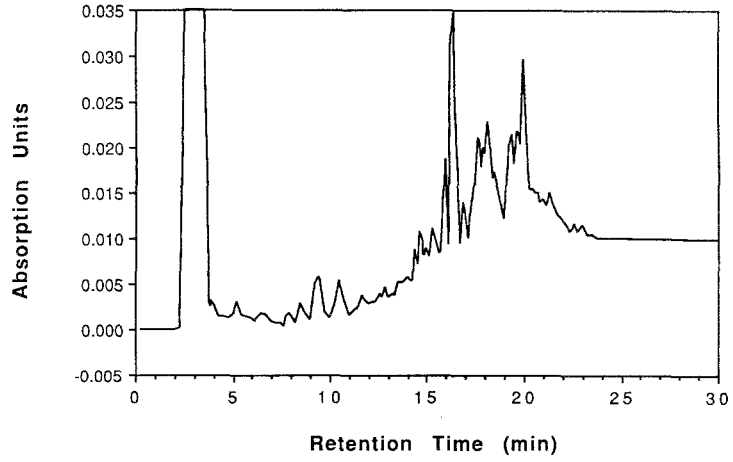

FIG. 2. Reverse-phase ion-pairing HPLC of concentrated steam distillate of RGM-1 bacterial culture.

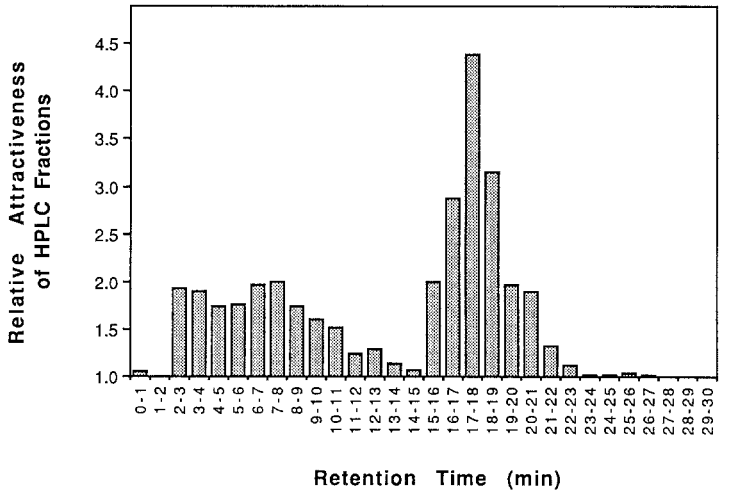

FIG. 3. Means of counts of *A. ludens* at filter papers containing 1-min fractions collected from reverse-phase ion-pairing HPLC, divided by counts at filter papers containing water.

column. A reasonable separation was achieved, although most of the apparent peaks (as detected by 254 nm) eluted close together between 7 and 15 min. A better separation undoubtedly could have been achieved by slowing down the transition to methanol. This was not done because the behavior bioassays indicated the active components were already well separated. As with the reverse-

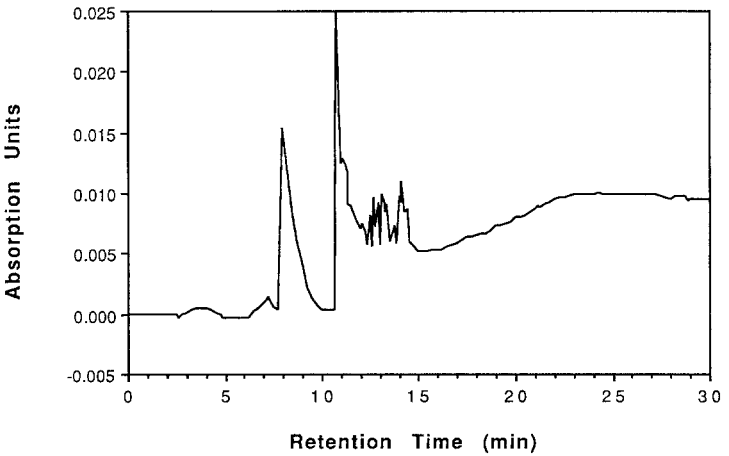

FIG. 4. Silica HPLC of concentrated fraction 17–18 min collected from reverse-phase ion-pairing HPLC.

phase method, all of the components apparently were eluted within the 30-min run.

Figure 5 shows that the attractiveness of the 17 to 18-min concentrate eluted from the silica column in two bands. Only fractions 8–9, 12–13, and 13–14 min were significantly more attractive than controls by paired t tests (smallest t = 4.8, df = 9, $P < 0.001$). These results indicate that at least two attractive components are present in the 17 to 18-min fraction collected from the reverse-phase column.

Chromatograms resulting from injections of the 18 to 19-min concentrate were similar to those from the 17 to 18-min concentrate except the peaks were generally smaller. Again, the most attractive fraction eluted from 12–13 min (mean difference between counts at the test sample and the control = 32.8) ($P < 0.01$, t = 4.1, df = 8). Other significantly attractive fractions by paired t tests were 7–8, 10–11, 11–12, and 13–14 min (smallest t = 2.6, df = 8, $P < 0.05$).

Chromatograms resulting from injections of the 16 to 17-min concentrate indicated that very little separation was occurring as all components apparently eluted between 11 and 17 min (by detection at 254 nm). The reason for this effect is not known. Alternative mobile phases and gradients to achieve better separations were not attempted. As with the 18- to 19-min concentrate injections on silica, the most attractive fraction eluted at 12–13 min (mean difference between counts at the test sample and the control = 56.3) ($P < 0.001$, t = 6.9, df = 9). Other significantly attractive fractions by paired t tests were 2–3,

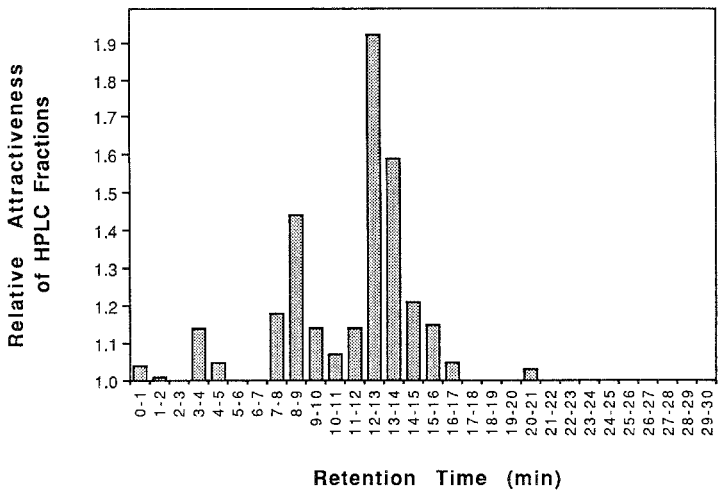

FIG. 5. Means of counts of *A. ludens* at filter papers containing 1-min fractions collected from silica HPLC, divided by counts at filter papers containing water.

11–12, 13–14, 14–15, 15–16, 16–17, and 18–19 min (smallest $t = 2.5$, $df = 9$, $P < 0.05$).

Implications. Data presented in this work show that the most attractive chemicals contained in a bacterial culture that is highly attractive to adult *A. ludens* have properties indicative of highly polar, low-molecular-weight amines. These molecules could not be collected by standard techniques such as adsorption on Porapak Q and similar adsorbents. Further, attractant chemicals apparently could not be extracted from water with organic solvents using our methods. These are important points since most investigations of insect semiochemicals begin with such collections and extractions. A good example is the study of protein baits for fruit flies by Morton and Bateman (1981) that used both adsorption on Chromosorb 105 and liquid–liquid extraction with methylene chloride to identify volatile components. Although over 40 compounds were identified in the volatiles, none was very attractive to adult *D. tryoni*.

The data also suggest that polar, nonionic molecules contribute some attractiveness to the bacterial preparations. Evidence includes significant attractiveness of bacterial filtrate at pH < 6, attractiveness of low pH eluates from strong cation exchange columns, and attractiveness of early-eluting fractions from reverse-phase ion-pairing HPLC. This is not a surprising result since bacterial preparations undoubtedly contain various short-chain alcohols (Hayward et al., 1977) already known to be attractive to *A. ludens* (Robacker et al., 1990). Most noteworthy is that these neutral molecules appear to contribute relatively little to the attractiveness compared to the ionic compounds.

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REFERENCES

BATEMAN, M.A., and MORTON, T.C. 1981. The importance of ammonia in proteinaceous attractants for fruit flies (Family: Tephritidae). *Aust. J. Agric. Res.* 32:883–903.

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.

DREW, R.A.I. 1987. Behavioural strategies of fruit flies of the genus *Dacus* (Diptera: Tephritidae) significant in mating and host-plant relationships. *Bull. Entomol. Res.* 77:73–81.

DREW, R.A.I., and FAY, H.A.C. 1988. Comparison of the roles of ammonia and bacteria in the attraction of *Dacus tryoni* (Froggatt) (Queensland fruit fly) to proteinaceous suspensions. *J. Plant Prot. Tropics.* 5:127–130.

GOW, P.L. 1954. Proteinaceous bait for the Oriental fruit fly. *J. Econ. Entomol.* 47:153–160.

HAYWARD, N.J., JEAVONS, T.H., NICHOLSON, A.J.C., and THORNTON, A.G. 1977. Methyl mercaptan and dimethyl disulfide production from methionine by *Proteus* species detected by headspace gas–liquid chromatography. *J. Clin. Microbiol.* 6:187–194.

HEATH, R.R., and MANUKIAN, A. 1992. Development and evaluation of systems to collect volatile semiochemicals from insects and plants using a charcoal-infused medium for air purification. *J. Chem. Ecol.* 18:1209–1226.

HODSON, A.C. 1943. Lures attractive to the apple maggot. *J. Econ. Entomol.* 36:545–548.

JANG, E.B., and NISHIJIMA, K.A. 1990. Identification and attractancy of bacteria associated with *Dacus dorsalis* (Diptera: Tephritidae). *Environ. Entomol.* 19:1726–1731.

JARVIS, H. 1931. Experiments with a new fruit fly lure. *Queensl. Agric. J.* 36:485–491.

MACCOLLOM, G.B., LAUZON, C.R., WEIRES, R.W., JR., and RUTKOWSKI, A.A. 1992. Attraction of adult apple maggot (Diptera: Tephritidae) to microbial isolates. *J. Econ. Entomol.* 85:83–87.

MAZOR, M., GOTHILF, S., and GALUN, R. 1987. The role of ammonia in the attraction of females of the Mediterranean fruit fly to protein hydrolysate baits. *Entomol. Exp. Appl.* 43:25–29.

MORENO, D.S., SANCHEZ, M., ROBACKER, D.C., and WORLEY, J. 1991. Mating competitiveness of irradiated Mexican fruit fly (Diptera: Tephritidae). *J. Econ. Entomol.* 84:1227–1234.

MORTON, T.C., and BATEMAN, M.A. 1981. Chemical studies on proteinaceous attractants for fruit flies, including the identification of volatile constituents. *Aust. J. Agric. Res.* 32:905–916.

ROBACKER, D.C. 1991. Specific hunger in *Anastrepha ludens* (Diptera: Tephritidae): Effects on attractiveness of proteinaceous and fruit-derived lures. *Environ. Entomol.* 20:1680–1686.

ROBACKER, D.C., TARSHIS MORENO, A.M., GARCIA, J.A., and FLATH, R.A. 1990. A novel attractant for Mexican fruit fly, *Anastrepha ludens*, from fermented host fruit. *J. Chem. Ecol.* 16:2799–2815.

ROBACKER, D.C., GARCIA, J.A., MARTINEZ, A.J., and KAUFMAN, M.G. 1991a. Strain of *Staphylococcus* attractive to laboratory strain *Anastrepha ludens* (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* 84:555–559.

ROBACKER, D.C., MANGAN, R.L., MORENO, D.S., and TARSHIS MORENO, A.M. 1991b. Mating behavior and male mating success in wild *Anastrepha ludens* (Diptera: Tephritidae) on a field-caged host tree. *J. Insect Behav.* 4:471–487.

WAKABAYASHI, N., and CUNNINGHAM, R.T. 1991. Four-component synthetic food bait for attracting both sexes of the melon fly (Diptera: Tephritidae). *J. Econ. Entomol.* 84:1672–1676.

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EFFECTS OF JUGLONE ON GROWTH, PHOTOSYNTHESIS, AND RESPIRATION[1]

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Abstract—The impacts of juglone on plant growth and several other physiological functions were evaluated in this study. Juglone inhibited *Lemna minor* growth, chlorophyll content, and net photosynthesis at treatments between 10 and 40 μM. Soybean leaf disks vacuum infiltrated with as little as 10μM juglone had reduced photosynthesis. Oxygen evolution by chloroplasts isolated from *Pisum sativum* was inhibited by juglone with an I_{50} of 2 μM. Micromolar treatments of juglone stimulated oxygen uptake in mitochondria isolated from *Glycine max*. These data suggest perturbations of chloroplast and mitochondrial functions may contribute to plant growth reductions observed in juglone-mediated allelopathy.

Key Words—Juglone, allelopathy, allelochemical, photosynthesis, chloroplast, mitochondria, *Lemna minor*, *Glycine max*.

INTRODUCTION

The inhibitory effect of black walnut (*Juglans nigra* L.) on associated plant species is one of the oldest stories of allelopathy (Rice, 1984). Davis (1928)

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isolated juglone, 5-hydroxy-1,4-naphthoquinone, as the principle allelochemical causing the detrimental effects from black walnut.

Some of the economic implications of the toxicity of black walnut were reported by Rietveld et al. (1983). They found that a nurse crop of black alder [*Alnus glutinosa* (L.) Gaertn.] trees interplanted with black walnut to improve the nutrient environment eventually died, and they attributed this decline to the toxicity of black walnut. Rietveld (1983) also showed that seedlings of a number of species are inhibited by exposure to micromolar levels of juglone. Ponder and Tadros (1985) isolated sufficient quantities of juglone from the soil in black walnut plantations to conclude that juglone was responsible for the decline of associated black alder species. Weidenhamer et al. (1989) concluded the effect they found on growth of tomatoes in unsterilized black walnut soil was characteristic of toxic substances in the soil.

The physiological action of juglone is not well understood. The aim of this investigation was to determine possible effects of juglone on energy metabolism that may be responsible for its role in growth inhibition.

METHODS AND MATERIALS

Lemna minor *Bioassays. Lemna minor* L. was used as a bioassay plant for initial studies on growth, chlorophyll content, and photosynthesis. Axenic cultures of *L. minor* were grown in 24-well tissue culture cluster dishes following procedures of Einhellig et al. (1985). The inorganic nutrient medium was prepared with 0, 10, 20, and 40 μM of juglone and autoclaved. Preliminary work in our laboratory had shown autoclaving did not alter the effects of juglone on growth. Each well of the tissue culture dish contained 2 ml of growth medium with three *L. minor* fronds added per well; there were six replications for each juglone treatment. Cultures were maintained under constant light (250 μmol/m^2/sec) at 27°C for seven days. At termination of the growth experiment, frond number and tissue dry weight were obtained for each tissue culture well.

Identical growth procedures were followed for determining chlorophyll content of *L. minor*. Chlorophyll was extracted following procedures of Ramirez-Toro et al. (1988). Plants from each culture-dish well were soaked in 6 ml of 95% ethanol for 24 hr in the dark at 4°C. The plants were then soaked for an additional 24-hr period in a second aliquot of ethanol. The two ethanol–chlorophyll extracts were combined and chlorophyll a, b, and total chlorophyll were determined spectrophotometrically (Winterman and DeMots, 1965; Ramirez-Toro et al., 1988).

Tests of the effects of juglone on the rate of photosynthesis in *L. minor* utilized a Gilson Differential Respirometer (model GRP14). The plants were incubated for 48 hr in 125-ml Erlenmeyer flask cultures with 50 ml of medium

containing either 0, 20, or 40 μM juglone prior to measuring oxygen evolution in the respirometer. The manometric tests were conducted in 7-cc flasks containing 2.5 ml of the incubation medium and 15 *L. minor* fronds. There were either four or five replicates for each treatment and the experiment was duplicated. *L. minor* were equilibrated in the respirometer for 1 hr at 25°C, 250 μmol/m^2/sec irradiance before measuring photosynthesis. The photosynthetic rate was measured during the next hour.

The data from each *L. minor* experiment were subjected to analysis of variance (ANOVA) with significant differences among means identified by Duncan's multiple range test, $P < 0.05$.

Leaf Disk Photosynthesis. Soybean [*Glycine max* (L.) Merr., Pioneer 9202] unifoliate leaf tissue from 2- to 3-week-old seedlings was used in these experiments. Seeds were germinated and grown in vermiculite in the greenhouse with periodic watering with one-half strength Hoagland's solution (Hoagland and Arnon, 1950). Plants were placed in low irradiance (10 μmol/m^2/sec) for 1 hr before 3-mm leaf disks were cut for the assay. Following methods of Hensley (1981), leaf disks were vacuum infiltrated, alternating pressure down to -93 KPa, for 1.5 min with the treatment solution. Treatments were 0, 10, 20 and 30 μM juglone in a 10 mM potassium phosphate buffer, pH 6.8, with 0.015% Triton-X. The vacuum infiltrated leaf disks were then incubated in the dark for 30 min before effects on photosynthesis were monitored.

The photosynthesis assay was a floating disk procedure whereby data was obtained on the elapsed time for leaf disks to float after exposure to 100 μmol/m^2/sec irradiance at 25°C. Twenty leaf disks were used per treatment with five replications per treatment, and the experiment was duplicated. The data are reported as a percentage of the number of leaf disks floating in the control at selected time intervals.

Chloroplast Isolation and Experimentation. Chloroplasts were isolated from 10- to 12-day-old little marvel peas (*Pisum sativum* L.). Following procedures modified from Berkowitz and Gibbs (1982) and Burton et al. (1987), peas were grown in vermiculite with a 12:12 hr light–dark cycle, at 20°/16°C, and 250 μmol/m^2/sec. Approximately 1 hr into the light cycle on the day of isolation, 50–80 g of leaf and stem tissue was cut for chloroplast isolation. Using a Waring blender, the tissue was homogenized in 200 ml of a partially frozen (slushlike) grind mix containing 50 mM HEPES–NaOH (pH 7.5), 330 mM sorbitol, 0.1% w/v bovine serum albumin (BSA), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM isoascorbate, and 1.3 mM glutathione. This was accomplished using three, 3- to 5-sec bursts of the blender set at high speed. The homogenate was filtered through six layers of cheesecloth between two layers of miracloth. The filtrate was distributed into four 45-ml glass tubes and centrifuged for 2 min at 750g and 4°C (Sorvall RC-5, HB-4 rotor). The supernatant was discarded and each pellet was gently resuspended in 15 ml of chilled grind mix using a soft

camel's-hair brush. The mixture was carefully layered by pipet onto 15 ml of grind mix containing 33% Percoll and centrifuged at 2500g for 3 min at 4°C. The supernatant was aspirated and each pellet was gently resuspended by brush into 0.3–0.4 ml of grind mix.

The chloroplast suspension was stored in the dark on ice until used. Chloroplasts were viable for at least 2–3 hr after isolation. In order to determine the amount of suspension to use in a test, the chlorophyll concentration was determined immediately according to methods of Arnon (1949). Percent chloroplast intactness was determined using ferricyanide as a Hill oxidant according to a procedure described by Lilley et al. (1975). In this test, a chloroplast suspension equivalent to approximately 80 μg chlorophyll was osmotically lysed in 1 ml of distilled water for 1 min. One milliliter of ferricyanide reaction medium was added. The ferricyanide reaction medium was made double-strength so that the final test medium contained 50 mM HEPES–NaOH (pH 7.5), 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1.5 mM $K_3Fe(CN)_6$ and 10 mM glyceraldehyde. After the solution had equilibrated, NH_4Cl was added as an uncoupler to elicit the accelerated rate of oxygen evolution characteristic of uncoupling. The procedure was repeated without osmotically shocking the chloroplasts. Percent intactness was calculated by comparing the rates of oxygen evolution for the shocked and nonshocked chloroplasts.

Aqueous phase CO_2-dependent oxygen evolution was measured polarographically with a Hansatech Instrument (Kings Lynn, Norfolk, England) with a Clark-type electrode. Oxygen evolution was assayed at 25°C, irradiance of approximately 1300 μmol/m^2/sec, in 2 ml of a reaction medium containing 50 mM HEPES-NaOH (pH 7.5), 330 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 10 mM $NaHCO_3$, 5 mM Na pyrophosphate, 1.5 mM ATP, and chloroplast suspension equivalent to 80–100 μg chlorophyll.

In tests, juglone was dissolved in ethanol and injected into the reaction medium. The amount of ethanol carrier solution was never more than 1% (v/v) of the chloroplast suspension, a concentration that had no effect on chloroplast function. Juglone was injected 1–2 min after optimal oxygen evolution rates were established. Effects of juglone were recorded as a percent of the rate before injection (percent of control) with an I_{50} defined as the concentration required to inhibit oxygen evolution by 50%. Reported data are the means of at least three trials from three different chloroplast isolations. The ferricyanide test for chloroplast intactness was also used to evaluate the possibility that juglone might uncouple the chloroplast electron transport system. In this case juglone was substituted for NH_4Cl in the experimental trials.

Mitochondrial Isolation and Experimentation. Mitochondria were isolated from 4- to 5-day old etiolated soybean [*Glycine max* (L.) Merr., Pioneer 9202] hypocotyls according to procedures described in Rasmussen et al. (1992). Oxygen uptake was measured polarographically at 25°C using a mitochondrial con-

centration equivalent to 0.3–0.5 mg protein in the 2-ml reaction chamber of the Hansatech instrument (Rasmussen et al., 1992). The integrity of mitochondria from each isolation was verified using FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) as an uncoupling agent. State 3 and state 4 designations follow the terminology of Chance and Williams (1955). A respiratory control rate (RCR) and ADP/O ratio were computed for each mitochondrial isolation (Rasmussen et al., 1992).

In tests for effects of juglone, a constant state 3 respiration rate with NADH as the substrate was achieved before introduction of juglone into the reaction chamber. Juglone was dissolved in ethanol and injected into the reaction medium. The addition of ethanol into the reaction chamber never exceeded 20 μl, a quantity that had no effect on oxygen uptake in control tests. Data are reported as a percent of control of state 3 oxygen uptake. Tests were conducted on several different mitochondrial isolations, and the data reported are the means of these trials.

Suspensions of intact mitochondria were analyzed by spectrophotometric methodology (Beckman DU70) to determine if restricted electron flow occurred at specific cytochrome sites following juglone application (Rasmussen et al., 1992).

RESULTS

Growth of *L. minor* as measured by both final frond number and dry weight was significantly inhibited by all levels of juglone tested (Figure 1). In parallel tests, only the 40 μM treatment of juglone altered chlorophyll content of the *Lemna*, and there was no differential effect on the chlorophyll a:b ratio (data not shown). These chlorophyll data are expressed on the basis of final frond number since the ethanol extraction procedures slightly affected the dry weight. Even though chlorophyll content was not affected by 20 μM of juglone, the manometric determination of effects of this allelochemical on photosynthetic rate showed that 20 μM treatments reduced photosynthesis to two thirds the rate of control plants (Figure 1). By inspection, the *Lemna* subjected to 40 μM juglone for two days before the manometric tests did not appear to have a chlorophyll loss, yet their rate of photosynthesis was less than one third that of controls.

Subsequent work with soybean indicated that even 10 μM juglone suppressed photosynthesis in vacuum infiltrated leaf disks (Table 1). At 60 min, when all the control leaf disks had been floating for 15 min, the percent inhibition by 10, 20, and 30 μM of juglone was 23, 52, and 93%, respectively.

Isolated pea chloroplasts also were very sensitive to juglone. In these studies, the control photosynthetic rate averaged 125 μM O_2/mg chl/hr. The isolated

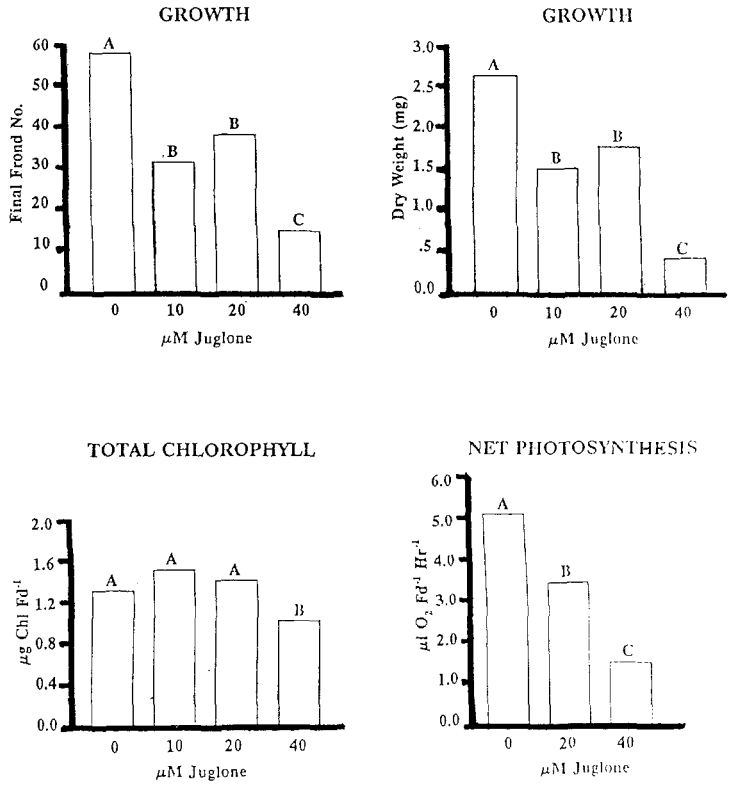

FIG. 1. Effect of juglone on growth, total chlorophyll (Chl) content and photosynthesis of *L. minor*. Bars with different letters within a subfigure are different, $P < 0.05$, ANOVA with Duncan's multiple range test. Fd = frond.

TABLE 1. EFFECTS OF JUGLONE ON FLOTATION OF VACUUM-INFILTRATED LEAF DISKS AFTER EXPOSURE TO LIGHT[a]

Flotation time (min)	Percent of control at μM juglone		
	10	20	30
30	18	9	0
60	77	48	7

[a] 100 μmol/m²/sec at 25°C.

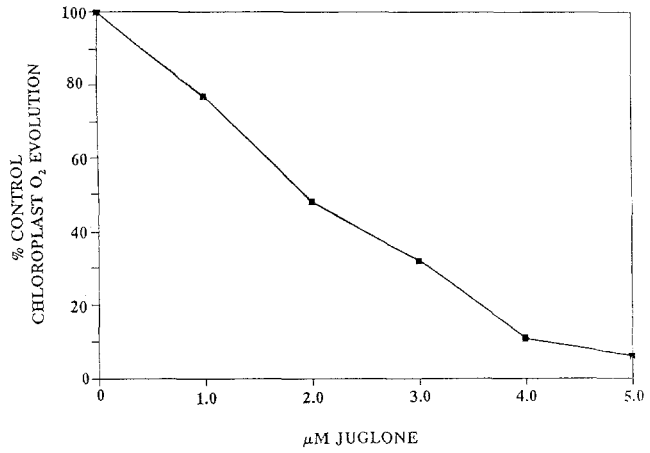

FIG.2. Effect of juglone on CO_2-dependent oxygen evolution of pea chloroplasts, approximately 90 μg chlorophyll.

chloroplasts had an average intactness of 80%. These values are considered very good (Walker, 1980). Amendments of 1 μM or higher concentrations of juglone to the assay containing chloroplast suspension caused an immediate decline in oxygen evolution (Figure 2). The I_{50} was approximately 2.0 μM juglone. When juglone was substituted for NH_4Cl, a known uncoupler used in the ferricyanide intactness test, juglone did not appear to uncouple chloroplast electron transport.

Juglone also disrupted normal functions of isolated mitochondria. The RCR value for untreated soybean mitochondria was between three and four, and the ADP/O ratio for NADH oxidation was typically between one and two. These values are indicative of good structural integrity of the mitochondria (Ikuma and Bonner, 1966; Demos et al., 1975; Nishimura et al., 1982). Injection of FCCP during state 3 respiration increased oxygen utilization two- to threefold until anaerobiosis. Hence, prior to FCCP injection the mitochondria had the electron transport system coupled to ATP synthesis (Douce, 1985). Introduction of juglone into the reaction medium containing mitochondria resulted in an increase in oxygen uptake for all concentrations tested (Figure 3). This enhancement of oxygen uptake was concentration dependent. The average of repeated trials showed that mitochondrial suspension exposed to 25 μM juglone resulted in about 250% of control rates of oxygen uptake. Spectrophotometric analysis of intact mitochondria after juglone addition showed no evidence of spectral peaks or shifts in cytochrome absorption.

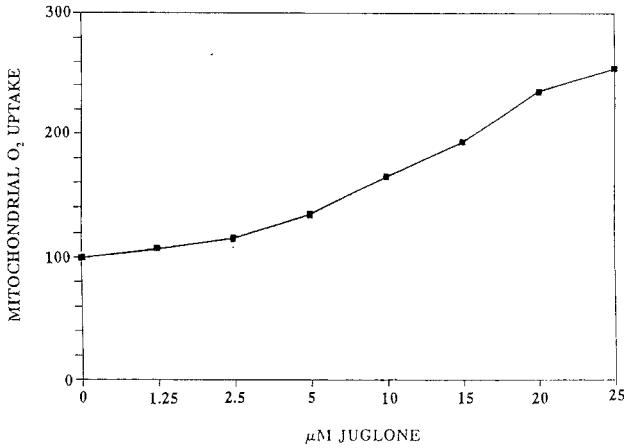

FIG.3. Effect of juglone on NADH state 3 oxygen uptake of isolated soybean mitochondria, approximately 0.3–0.4 mg protein.

DISCUSSION

Juglone inhibited *L. minor* growth in a concentration range comparable to that found by Rietveld (1983) for a number of woody and herbaceous species that might be affected in a field situation. Hence, *L. minor* should be a useful indicator of the possible physiological explanations for growth inhibition. Growth reductions in *Lemna* correlated well with reductions in photosynthetic rate, and this was not caused by loss of chlorophyll. Further evidence of juglone-impairment of the photosynthetic process is evident from its effects on whole leaf disks.

Oxygen evolution in isolated chloroplasts was inhibited by levels of juglone one order of magnitude below the bioassays with whole plant tissue. These chloroplast data suggest that juglone acts directly on the mechanism of photosynthesis, and it is not solely due to some indirect effects, such as stomatal interference or water relationships (Einhellig, 1986).

Juglone also has other direct effects on energy metabolism. Just as juglone affected chloroplast functions, it altered mitochondrial metabolism at the micromolar level. Juglone stimulation of soybean mitochondrial oxygen uptake at this level is comparable to what Koeppe (1972) attributed to a bypass of some or all of the electron transport system (ETS). Although juglone effects on mitochondria mimic the action of an uncoupler of the ETS from ATP production, it did not uncouple chloroplast oxygen evolution. The spectrophotometric data on mitochondria provide evidence that juglone does not seem to block electron flow along the cytochromes, so it appears to provide an alternate pathway for elec-

trons to reduce oxygen. Whatever the mechanism of mitochondrial action, juglone dramatically alters normal oxygen uptake in mitochondria. This effect would work in concert with impairment of photosynthesis to cause the reductions in plant growth that have been observed.

There has been some controversy about the concentration of juglone in the soil and its role in allelopathy (Ponder and Tadros, 1985; Schmidt, 1988, 1990; Weidenhamer et al., 1989; Williamson and Weidenhamer, 1990; De Scisciolo et al., 1990). Certainly, additional studies are required to determine how and at what concentrations juglone from the soil is taken up, metabolized, and transported throughout the plant. However, our data on the effects of juglone on chloroplast photosynthesis and mitochondrial function show that very small quantities of juglone could be detrimental to plant metabolism and account for a reduction in plant growth.

REFERENCES

ARNON, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24:1–14.

BERKOWITZ, G.A., and GIBBS, M. 1982. Effect of osmotic stress on photosynthesis studies with the isolated spinach chloroplast. *Plant Physiol.* 70:1143–1148.

BURTON, J.D., GRONWALD, J.W., SOMERS, D.A., CONNELLY, J.A., GENGENBACH, B.G., and WYSE, D.L. 1987. Inhibition of plant acetyl-coenzyme A carboxylase by the herbicides Sethoxydim and Haloxyfop. *Biochem. Biophys. Res. Commun.* 148:1039–1044.

CHANCE, B., and WILLIAMS, G.R. 1955. Respiratory enzymes in oxidative phosphorylation. III. The steady state. *J. Biol. Chem.* 217:409–428.

DAVIS, E.F. 1928. The toxic principle of *Junglans nigra* as identified with synthetic juglone, and its toxic effects on tomato and alfalfa plants. *Am. J. Bot.* 15:620.

DEMOS, E.K., WOOLWINE, M., WILSON, R.H., and MCMILLAN, C. 1975. The effects of ten phenolic compounds on hypocotyl growth and mitochondrial metabolism of bean. *Am. J. Bot.* 62:97–102.

DESCISCIOLO, B., LEOPOLD, D.J., and WALTON, D.C. 1990. Seasonal patterns of juglone in soil beneath *Juglans nigra* (black walnut) and influence of *J. nigra* on understory vegetation. *J. Chem. Ecol.* 16:1111–1130.

DOUCE, R. 1985. Mitochondria in Higher Plants. Academic Press, Orlando, Florida, 327 pp.

EINHELLIG, F.A. 1986. Mechanisms and modes of action of allelochemicals, pp. 171–188, *in* A.R. Putnam and C.S. Tang (eds). The Science of Allelopathy. John Wiley & Sons, New York.

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.

HENSLEY, J.R. 1981. A method for identification of triazine resistant and susceptible biotypes of several weeds. *Weed Sci.* 29:70–73.

HOAGLAND, D.R., and ARNON, D.I. 1950. The water culture method for growing plants without soil. *California Agriculture Experiment Station Circular* 347.

IKUMA, H., and BONNER, W.D., JR. 1967. Properties of higher plant mitochondria. I. Isolation and some characteristics of tightly coupled mitochondria from dark-grown mung bean hypocotyls. *Plant Physiol.* 42:67–75.

KOEPPE, D.E. 1972. Some reactions of isolated corn mitochondria influenced by juglone. *Physiol. Plant.* 27:89–94.

LILLEY, R.McC., FITZGERALD, M.P., RIENITS, K.G., and WALKER, D.A. 1975. Criteria of intactness and the photosynthetic activity of spinach chloroplast preparations. *New Phytol.* 75:1–10.

NISHIMURA, M., DOUCE, R., and AKAZAWA, T. 1982. Isolation and characterization of metabolically competent mitochondria from spinach leaf protoplasts. *Plant Physiol.* 69:916–920.

PONDER, F., JR., and TADROS, S.H. 1985. Juglone concentration in soil beneath black walnut interplanted with nitrogen-fixing species. *J. Chem. Ecol.* 11:937–942.

RAMIREZ-TORO, G.I., LEATHER, G.R., and EINHELLIG, F.A. 1988. Effects of three phenolic compounds on *Lemna gibba* G3. *J. Chem. Ecol.* 14:845–853.

RASMUSSEN, J.A., HEJL, A.M., EINHELLIG, F.A., and THOMAS, J.A. 1992. Sorgoleone from root exudate inhibits mitochondrial functions. *J. Chem. Ecol.* 18:197–207.

RIETVELD, W.J. 1983. Allelopathic effects of juglone on germination and growth of several herbaceous and woody species. *J. Chem. Ecol.* 9:295–308.

RIETVELD, W.J., SCHLESINGER, R.C., and KESSLER, K.J. 1983. Allelopathic effects of black walnut on European black alder coplanted as a nurse species. *J. Chem. Ecol.* 9:1119–1133.

RICE, E.L. 1984. Allelopathy. Academic Press, Orlando, Florida, 422 pp.

SCHMIDT, S.K. 1988. Degradation of juglone by soil bacteria. *J. Chem. Ecol.* 14:1561–1571.

SCHMIDT, S.K. 1990. Ecological implications of the destruction of juglone (5-hydroxy-1, 4-naphthoquinone) by soil bacteria. *J. Chem. Ecol.* 16:3547–3549.

WALKER, D.A. 1980. Preparation of higher plant chloroplasts. *Methods Enzymol.* 69:94–104.

WEIDENHAMER, J.D., HARTNETT, D.C., and ROMEO, J.T. 1989. Density-dependent phytotoxicity: Distinguishing resource competition and allelopathic interference in plants. *J. Appl. Ecol.* 26:613–624.

WILLIAMSON, G.B., and WEIDENHAMER, J.D. 1990. Bacterial degradation of juglone: Evidence against allelopathy? *J. Chem. Ecol.* 16:1739–1742.

WINTERMAN, J.F.G.M., and DEMOTS, A. 1965. Spectrophotometric characteristics of chlorophylls a and b and their phenophytins in ethanol. *Biochem. Biophys. Acta* 109:448–453.

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CHEMISTRY OF ODORTYPES IN MICE: FRACTIONATION AND BIOASSAY

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Abstract—Mice can discriminate samples of urine obtained from two groups of inbred mice that are genetically identical except in their major histocompatibility complex (MHC) haplotype (congenic mice), whereas they cannot distinguish urine samples from two genetically identical groups of mice. Chemical fractions of urine samples obtained from MHC congenic mice were tested in a Y-maze olfactometer using a method modified to accommodate the bioassay to chemical fractions that might differ in sensory properties from the unfractionated urine. Fractions depleted in protein by several methods were consistently discriminable by mice in the Y maze, providing a direct demonstration that the airborne MHC genotype information can be conveyed by volatile compounds alone.

Key Words—Mouse, urine, social odor, individual identity, mating preference, major histocompatibility complex, *H-2*, class I proteins, odortype.

INTRODUCTION

A mouse can detect the difference in two mice that are genetically identical except in a portion of chromosome 17, known as the major histocompatibility

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complex (MHC) (Yamazaki et al., 1979). The role of the MHC in specifying individuality is well known in its effect on the immune response to transplanted tissues and pathogens. In particular, well-characterized translation products of the MHC, the so-called class I and class II membrane proteins, specify the context in which the immune system can identify proteins as self or nonself in origin. Several class I and class II molecules are encoded by an extraordinary number of alleles in some mammalian species, among which mice and humans have been most extensively investigated (Klein, 1986). In mice it appears that the genetic diversity of these alleles is maintained by mate selection substantially based on MHC genotype (Yamazaki et al., 1976; Potts et al., 1991), but how this identity is specified and why it is regulated by genes of the immune system are unknown.

It is likely that the information that allows mice to distinguish the MHC type of their mate as different from their own is conveyed through chemosensory channels (Yamazaki et al., 1979). Such information can be encoded in airborne chemical signals emitted by a mouse and detected by olfaction or possibly other nasal chemical senses. Chemosensory identity is influenced by environmental factors, such as diet or rearing, so that even two genetically identical mice may be distinguishable (Bowers and Alexander, 1967). However, the existence of genetically determined chemosensory identity, or odortypes, has been rigorously demonstrated in a Y-maze olfactometer experiment using samples of urine from congenic mice that differed only at the MHC (Yamaguchi et al., 1981). Differences at other genetic loci, for example, in the sex chromosomes, are capable of specifying discriminable urinary odors in the Y maze, but training mice to make these distinctions takes significantly more trials, suggesting that odor differences related to MHC differences are, for whatever reason, more salient (Yamazaki et al., 1990). The chemistry of the MHC odortypes of mouse urine is the subject of the investigations described in this report.

Several distinct mechanisms by which the MHC might specify odortype have been recognized: (1) The composition of volatile metabolites in urine may be determined by MHC selected commensal flora (Howard, 1977). (2) Metabolic processes producing a characteristic mixture of volatile compounds may be influenced by MHC genes, directly (Ivanyi, 1978) or indirectly (Schwende et al., 1984). (3) Characteristic volatile compounds may result from catabolism of MHC translation products (Boyse et al., 1991b). (4) Release of odorous compounds from the urine may be regulated by binding to MHC proteins in the urine (Boyse et al., 1987; Singh et al., 1987). (5) The MHC proteins in the urine may become airborne and be detected directly (Singh et al., 1987). In order to answer the question of how the MHC influences the odortype, we need to determine the nature of the chemical signals. We report here on experiments that had the aim of investigating the general chemical properties of these signals;

the results provide direct evidence that odortype in the Y-maze olfactometer is transmitted by volatile molecules and that it does not require proteins.

METHODS AND MATERIALS

Congenic pairs of urine donor panels consisted of 40–60 age-matched, inbred male mice selected so that the members of one panel differed significantly from the members of the second panel only in MHC haplotype. The modified bioassay procedure deemed necessary for testing chemical fractions, which is described in detail below, required that two such congenic pairs of panels be maintained. All panel mice were maintained under uniform conditions in the same animal room. New mice were acclimated for three weeks before using them as donors. Urine was obtained by gentle abdominal pressure and pooled with urine from other members of the panel; it was stored in a −20°C freezer.

Chemical fractionation procedures were performed separately on pooled samples of urine not more than eight weeks old from each of the four panels. The resulting fractions were stored at −20°C and generally were tested within one week of preparation.

Samples of urine were lyophilized for one to three days at a pressure of 0.05 torr. The sublimate was tested directly, and the residue was reconstituted to the original volume in water for bioassay.

Gel permeation chromatography was performed on 5-ml samples of urine on a column of Sephadex G-15 eluted with deionized water. The fractions were analyzed for protein by SDS polyacrylamide gel electrophoresis (Laemmli, 1970).

Proteins in samples of the urine were degraded by treatment with a high concentration (2 mg/ml) of Pronase for 1 hr at ambient temperature. The degradation of protein was confirmed by polyacrylamide gel electrophoresis (Singer et al., 1988). The treated urine samples were tested without further work-up in the bioassay.

Urinary proteins were precipitated and removed by addition of $HClO_4$ to the urine samples to a concentration of 0.24 M, heating at 90°C for 5 min, cooling, and centrifugation at 15,000*g* for 15 min. The supernatants obtained were tested directly.

Dialysis was carried out on 5-ml samples of urine using cellulose tubing with a molecular weight cutoff of 3500 in 500 ml of deionized water for 72 hr at 4°C with two changes of water. The retentate and dialysate were lyophilized and the dried residues were redissolved in 5 ml of water for testing.

Samples of urine from each of the panels were filtered at 5°C in a centrifuge through a membrane with a molecular weight cutoff of 3000. The ultrafiltrate was tested directly.

Extractions were carried out on lyophilized urine by mixing with solvent (ether or ethanol) and filtering. The extracts were prepared from the filtrate for testing by evaporating the solvent to dryness in a rotary evaporator and reconstituting in distilled and deionized water for bioassay. The residues from the extractions were dried in the rotary evaporator to remove residual solvent and reconstituted in water as were the extracts.

The Y-maze olfactometer, the training of mice, and the use of generalization to demonstrate that two samples of urine are of identical odortype have all been described in detail (Yamazaki et al., 1979; Yamaguchi et al., 1981). Air from outside the test room is blown into left and right odor boxes at the end of the two branches of the Y maze. Each odor box has a 3.6-cm Petri dish containing the sample. Left or right placement of a sample is assigned randomly for each trial. An air current conducts the odor from each odor box through a tube into the connected Y-maze branch and then to the foot of the Y, where the subject mouse is initially located. At the beginning of the test, a gate confining the mouse and gates to each of the two branches of the Y are raised simultaneously. Each mouse is trained to choose one of the two odortypes of urine to be used in the experiment. Frequently a trained mouse chooses without pause or after sniffing at the entrance to the branches, or occasionally with brief retracing from one branch to the other. When the mouse has clearly entered one branch, generally 2–3 sec after the start of the test, the gates are lowered. If the mouse makes a choice that is to be rewarded, it receives a drop of water; if the mouse enters the wrong branch, or the trial is designated as unrewarded in the experimental design, the mouse is not rewarded. In any event, it is then returned to the start and within 30 sec, when the samples have been replaced and the drop of water renewed, another trial can begin.

Bioassay of chemical fractions in the Y-maze olfactometer was conducted in two variations (see Discussion). Chemical fractions produced by the dialysis and lyophilization procedures were tested on mice trained to select one of the MHC types of the congenic pair of urine samples used to prepare the fractions. The mice were not rewarded for their choice of chemical fraction even if the choice was concordant with their training. The other chemical fractions were tested by a modified procedure that employed the sequence of sample presentation shown in Table 1. A fresh 0.3- to 0.5-ml sample was used for each testing session on a single mouse, which usually consisted of 48 trials. The chemical fractions were obtained from fractionation procedures performed identically on four samples of pooled urine: one from each of two pairs of panels of congenic mice. In the trials of fractions of urine from congenic mice following the scheme in Table 1, the mice were rewarded for the choice of haplotype source concordant with their training on the unfractionated urine. The trials of the fractions obtained from the second pair of congenic panels were never rewarded. Each of these sets of chemical fractions were usually tested on three or four different

TABLE 1. BIOASSAY SAMPLE SEQUENCE

Trial	Sample type	Choice[a]	Result of correct response
1	urine	b vs. k	reward
2	urine	b vs. k	reward
3	urine	b vs. k	reward
4	urine	b vs. k	no reward
5	chemical fraction	b_1 vs. k_1	reward[b]
6	chemical fraction	b_1 vs. k_1	reward[b]
7	chemical fraction	b_1 vs. k_1	reward[b]
8	chemical fraction	b_2 vs. k_2	no reward[c]
9	urine	b vs. k	reward
10	urine	b vs. k	reward
11	urine	b vs. k	reward
12	urine	b vs. k	no reward
13	chemical fraction	b_1 vs. k_1	reward[b]
14	chemical fraction	b_1 vs. k_1	reward[b]
15	chemical fraction	b_1 vs. k_1	reward[b]
16	chemical fraction	b_2 vs. k_2	no reward[c]

[a] To exemplify the modified scheme used in testing chemical fractions from congenic mice, the following symbols are used in this table: b = pooled urine from two panels of B6 mice, which have *H-2*^{b}; k = pooled urine from two panels of B6 *H-2*^{k} mice; b_1 and b_2 = chemical fractions prepared from urine of first and second panels respectively of B6 mice; k_1 and k_2 = chemical fractions prepared from urine of first and second panels respectively of B6 *H-2*^{k} (congenic to B6) mice.

[b] These are the training trials scored in Table 2.

[c] These are the generalization trials scored in Table 2.

subject mice so that the unrewarded, generalization samples from the second pair of congenic panels had been tested in about 20 trials. The unreinforced chemical fraction samples were coded so that the operator of the bioassay was unaware of the MHC haplotype of the urine donors and thus could not systematically influence the outcome of these trials.

If both the rewarded trials and the unrewarded trials employing chemical fractions gave a significant number of choices concordant with training ($P < 0.05$ as determined from a normal distribution, two-tailed), then they were judged to contain odors capable of indicating MHC haplotype.

RESULTS

The results are contained in Table 2. Applying high vacuum to the urine for three days, under the conditions of lyophilization, did not lead to appreciable

TABLE 2. DISCRIMINATION OF CHEMICAL FRACTIONS OF CONGENIC MOUSE URINE SAMPLES

Fraction	Concordance (%)[a]	
	Training trials	Generalization trials
Lyophilization		
Sublimate	na[b]	52
Residue	na	80***
Gel permeation		
Fraction A	88***	66*
Fraction A1	66***	74*
Fraction A2	64***	54
Fraction A3	64***	73*
Fraction B	53	38
Fraction C	60*	62
Pronase treated urine	84***	83**
Supernatant from $HClO_4$ precipitation	76***	89**
Dialysis		
Retentate	na	55
Dialysate	na	77***
Ultrafiltrate of urine	80***	82**
Extractions		
Ether extract	46	33
residue	73**	86**
Ethanol extract	87***	79**
residue	76***	91**

[a] Concordance values are mean percent of choices concordant with training on unfractionated urine (see Methods and Materials). The following symbols are used in this table: na = not applicable (training was conducted solely on the unfractionated urine); *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

loss of activity when the urine was reconstituted with water; no activity was recovered in the sublimate.

Gel permeation chromatography on a column of Sephadex G-15 with the collection of three fractions (A, B, and C in Table 2) resulted in one active fraction. Only the earliest-eluting fraction, A, was active. This fraction contained the urinary protein as expected from the measured retention of proteins of known molecular weight and as confirmed by SDS polyacrylamide gel electrophoresis. Fractions expected to contain only low-molecular-weight compounds (< 1000 Da), B and C, were not active. In a second fractionation scheme in which the A portion of the former fractionation scheme was collected in three smaller fractions, A1–A3, again the fraction containing the major urinary pro-

teins was active. Activity was also obtained in fraction A3, corresponding in retention volume to the retention of compounds with molecular weight around 10,000.

Urine treated with a high concentration of the protein-degrading enzymes, Pronase, was undiminished in activity in the Y-maze bioassay. The degradation of protein was confirmed by SDS polyacrylamide gel electrophoresis, which showed that less than 0.1% of the urinary protein remained in the enzyme-digested urine.

Perchloric acid precipitation apparently removed urinary protein, judging from the quantity of precipitate obtained. The remaining soluble material (supernatant after centrifugation) was discriminable in the Y-maze bioassay. On dialysis and ultrafiltration, the activity was likewise found in the fraction containing smaller molecules.

Extraction of the residue from lyophilization recovered some active compounds when the extraction solvent was ethanol but none when the solvent was ether. In both cases the extracted residue was active.

DISCUSSION

The data from a large number of Y-maze experiments with a variety of inbred mouse MHC haplotypes indicate that, with the exception of some mutant inbred strains (Yamazaki et al., 1991), there is an odortype corresponding to each MHC genotype in mice (Boyse et al., 1991a). Assuming this is true, there must indeed be a very large number of odortypes corresponding to the exceptionally large number of MHC genotypes (Klein, 1986).

How might a large number of odortypes be specified chemically? In the absence of any definite experimental results bearing on this question, we are considering two possibilities. First, we consider that there may be a specialized class of compounds, the biosynthesis of which is under relatively direct genetic regulation by the MHC. Odortype could be specified by a small number of closely related compounds with numerous possible variations in structure (Voznessenskaya et al., 1992). The possible specification of odortype by soluble class I-derived molecules in the urine (Singh et al., 1987), for example, is a mechanism in which odortype is differentiated by variations in a restricted class of compounds, that is, proteins.

The other possibility to consider is that odortype is encoded by a less restricted mixture of chemically diverse secondary metabolites that varies in composition incidentally to variations in MHC genotype (Yamazaki et al., 1984). Most of the speculations on the mechanism of odortype production listed in the Introduction are based on the idea that odortype is encoded by quantitative variations in the composition of a diverse collection of metabolic by-products.

This possibility is supported chemically by the fact that there are numerous and chemically diverse volatile compounds in mouse urine (Liebich et al., 1977; Schwende et al., 1986).

The possibility that odortype is constituted by a mixture of compounds of diverse chemical functionality prompted us to elaborate on the design of the basic Y-maze experiment with generalization (Yamaguchi et al., 1981). With relatively crude fractionation techniques, such as dialysis or lyophilization, in which the urinary compounds are separated into two fractions of compounds with distinct differences in physical properties, the probability is high that the entire mixture of compounds making up the odortype will be found in one or the other fraction. Mice that had been trained to discriminate urine from congenic mice were indeed able to discriminate samples of one of the two fractions of these urines produced by either dialysis or lyophilization, whereas they were not able to discriminate the other fractions produced by these techniques (Table 2). This result indicates that all or most of the compounds that enable the trained mice to discriminate urine samples from congenic mice were contained in one of the two fractions resulting from each of these techniques that separate compounds on the basis of gross differences in properties.

In testing for the biological activity of fractions produced by techniques such as chromatography that separate on the basis of relatively slight differences in chemical properties, we need to consider the probability that the compounds constituting the odortype, having different functional groups, would very likely be separated into two or more distinct fractions, and thus that the fractions would probably smell different from the unfractionated urine. In this case the mouse trained on whole urine could fail to recognize the fractions and might not respond to fractions actually containing important constituents of the odor. To circumvent this difficulty, the Y maze procedure was modified for testing chemical fractions by the inclusion of training on the fractions, after the mouse had learned to distinguish the odortypes of the unfractionated samples of urine. The unrewarded pairs of chemical fractions for discrimination, which could be coded so that the operator of the Y maze was not aware of their MHC haplotypes, were prepared from a second pair of congenic urine donor panels. This procedure has the added benefit of reducing the possibility of the mice learning an incidental distinction between the two haplotypes introduced accidentally during the fractionation process, because the second pair of chemical fractions is prepared independently.

Use of the modified method for bioassay was validated by the results in Table 2. Subjects in the Y maze bioassay were able to discriminate the odors of a number of chemical fractions produced by diverse methods. The question of whether odortype is conveyed by a restricted class of specialized compounds or diverse metabolic by-products cannot be answered by these results, but the modified bioassay method should make possible the discrimination of odortypes

of fractions containing only a few active components of a complex odorous mixture, if necessary.

A question that was addressed by the data presented here is the question of the involvement of proteins in odortype. Since the signals of individuality detected in the Y maze are necessarily airborne, it might be assumed that only volatile molecules could transmit the odortype information in this apparatus, but it is possible that airborne proteins could be involved. A role for proteins in chemical communication has been established (Singer and Macrides, 1992), and recognizable soluble derivatives of MHC class I membrane proteins have been partially characterized in rat urine (Singh et al., 1988). As evidence that these proteins could become airborne, it is notable that airborne proteins from mouse urine have been demonstrated to cause allergic reactions in a room housing a mouse colony (Schumacher, 1980). The main objection to this argument is that these airborne proteins, which are probably released from dried samples as dust, would be present in extremely low concentration in the Y-maze bioassay, during which they could become airborne from solution in the urine in the Petri dish only by bubbles breaking at the surface forming droplets small enough to remain airborne. The direct involvement of proteins in conveying odortype is inconsistent with the results in Table 2. Whether proteins were eliminated by enzyme degradation, perchloric acid precipitation, dialysis, ultrafiltration, or ethanol extraction, the congenic pairs of fractions containing no protein were consistently discriminable in the Y-maze olfactometer.

Proteins, however, might be less directly involved in odortype specification. One of the mechanisms of genetic regulation of individual odor listed in the Introduction holds that odortype is specified by a mixture of volatile metabolic by-products in which the composition is determined by the selective binding of some of the urinary metabolites to soluble MHC proteins. The plausibility of this mechanism is supported by the recent demonstrations of grooves in human class I membrane proteins (HLA-A and HLA-B proteins) that bind peptide antigens with some selectivity (Garrett et al., 1989; Hunt et al., 1992).

The fact that the early-eluting gel permeation chromatographic fractions, which contain proteins, were discriminated in the Y maze indicates that proteins in the urine may normally bind the compounds constituting odortype, but from the other results it is clear that proteins are not necessary for the discrimination of urine samples from MHC congenic mice in the Y maze and that volatile compounds are capable of conveying the odortype. If urinary proteins are involved in specifying odortype, it is likely that their contribution is to select a distinct mixture of secondary metabolites from the blood serum for secretion by the kidneys (Singh et al., 1987). Another possible function attributed to putative binding proteins in the urine, that they specify the odortype by selectively binding compounds already present in the urine to make the odortype information more persistent (Boyse et al., 1987), is not consistent with the result that destruc-

tion of the protein does not destroy the possibility of discriminating congenic samples of urine.

REFERENCES

BOWERS, J.M., and ALEXANDER, B.K. 1967. Mice: individual recognition by olfactory cues. *Science* 158:1208–1210.

BOYSE, E.A., BEAUCHAMP, G.K., and YAMAZAKI, K. 1987. The genetics of body scent. *Trends Genet.* 3:97–102.

BOYSE, E.A., BEAUCHAMP, G.K., BARD, J., and YAMAZAKI, K. 1991a. Behavior and the major histocompatibility complex of the mouse, pp. 831–846, *in* R. Ader, D.L. Felten, and N. Cohen (eds.). Psychoneuroimmunology, 2nd ed. Academic Press, San Diego.

BOYSE, E.A., BEAUCHAMP, G.K., YAMAZAKI, K., and BARD, J., 1991b. Genetic components of kin recognition in mammals, pp. 148–161, *in* P.G. Hepper (ed.). Kin Recognition. Cambridge University Press, Cambridge, U.K.

GARRETT, T.P.J., SAPER, M.A., BJORKMAN, P.J., STROMINGER, J.L., and WILEY, D.C. 1989. Specificity pockets for the side chains of peptide antigens in HLA-Aw68. *Nature* 342:692–696.

HOWARD, J.C. 1977. H-2 and mating preferences. *Nature* 266:406–408.

HUNT, D.F., HENDERSON, R.A., SHABANOWITZ, J., SAKAGUCHI, K., MICHEL, H., SEVILIR, N., COX, A.L., APPELLA, E., and ENGELHARD, V.H. 1992. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* 255:1261–1263.

IVANYI, P. 1978. Some aspects of the H-2 system, the major histocompatibility system in the mouse. *Proc. R. Soc.* 202B:117–158.

KLEIN J. 1986. Natural History of the Major Histocompatibility Complex. Wiley, New York.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.

LIEBICH, H.M., ZLATKIS, A., BERTSCH, W., VANDAHM, R., and WHITTEN, W.K. 1977. Identification of dihydrothiazoles in urine of male mice. *Biomed. Mass Spectr.* 4:69.

POTTS, W.K., MANNING, C.J., and WAKELAND, E.K. 1991. Mating patterns in seminatural populations of mice influenced by MHC genotype. *Nature* 352:619–621.

SCHUMACHER, M.J. 1980. Characterization of allergens from urine and pelts of laboratory mice. *Mol. Immunol.* 17:1087–1095.

SCHWENDE, F.J., JORGENSON, J.W., and NOVOTNY, M. 1984. A possible chemical basis for histocompatibility-related mating preference in mice. *J. Chem. Ecol.* 10:1603–1615.

SCHWENDE, F.J., WIESLER, D., JORGENSON, J.W., CARMACK, M., and NOVOTNY, M. 1986. Urinary volatle constituents of the house mouse, *Mus musculus*, and their endocrine dependency. *J. Chem. Ecol.* 12:277–296.

SINGER, A.G., and MACRIDES, F. 1992. Lipocalycins associated with mammalian pheromones, *in* R.L. Doty and D. Muller-Schwarze (eds.). Chemical Signals in Vertebrates 6. Plenum, New York. In press.

SINGER, A.G., CLANCY, A.N., MACRIDES, F., AGOSTA, W.C., and BRONSON, F.H. 1988. Chemical properties of a female mouse pheromone that stimulates gonadotropin secretion in males. *Biol. Reprod.* 38:193–199.

SINGH, P.B., BROWN, R.E., and ROSER, B. 1987. MHC antigens in urine as olfactory recognition cues. *Nature* 327:161–164.

SINGH, P.B., BROWN, R.E., and ROSER, B. 1988. Class I transplantation antigens in solution in body fluids and in the urine. *J. Exp. Med.* 168:195–211.

VOZNESSENSKAYA, V.V., PARFYONOVA, V.M., and ZINKEVICH, E.P. 1992. individual odortypes, *in* R.L. Doty and D. Muller-Schwarze (eds.). Chemical Signals in Vertebrates 6. Plenum, New York. In press.

YAMAGUCHI, M., YAMAZAKI, K., BEAUCHAMP, G.K., BARD, J., THOMAS, L., and BOYSE, E.A. 1981. Distinctive urinary odors governed by the major histocompatibility locus of the mouse. *Proc. Natl. Acad. Sci. U.S.A.* 78:5817–5820.

YAMAZAKI, K., BOYSE, E.A., MIKE, V., THALER, H.T., MATHIESON, B.J., ABBOTT, J., BOYSE, J., ZAYAS, Z.A., and THOMAS, L. 1976. Control of mating preferences in mice by genes in the major histocompatibility complex. *J. Exp. Med.* 144:1324–1335.

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.

YAMAZAKI, K., BEAUCHAMP, G.K. THOMAS, L., and BOYSE, E.A. 1984. Chemosensory identity of H-2 heterozygotes. *J. Mol. Cell. Immunol.* 1:79–82.

YAMAZAKI, K., BEAUCHAMP, G.K., BARD, J., and BOYSE, E.A. 1990. Chemosensory identity and the Y chromosome. *Behav. Genet.* 20:157–165.

YAMAZAKI, K., BEAUCHAMP, G.K., BARD, J., BOYSE, E.A., and THOMAS, L. 1991. Chemosensory identity and immune function in mice, pp. 211-225, *in* C.J. Wysocki and M.R. Kare (eds.). Chemical Senses: Genetics of Perception and Communication. Marcel Dekker, New York.

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HERBIVORY INDUCES SYSTEMIC PRODUCTION OF PLANT VOLATILES THAT ATTRACT PREDATORS OF THE HERBIVORE: EXTRACTION OF ENDOGENOUS ELICITOR

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Abstract—It was previously shown that in response to infestation by spider mites (*Tetranychus urticae*), lima bean plants produce a volatile herbivore-induced synomone that attracts phytoseiid mites (*Phytoseiulus persimilis*) that are predators of the spider mites. The production of predator-attracting infochemicals was established to occur systemically throughout the spider mite-infested plant. Here we describe the extraction of a water-soluble endogenous elicitor from spider mite-infested lima bean leaves. This elicitor was shown to be transported out of infested leaves and was collected in water in which the petiole of the infested leaf was placed. When the petioles of uninfested lima bean leaves were placed in water in which infested leaves had been present for the previous seven days, these uninfested lima bean leaves became highly attractive to predatory mites in an olfactometer when an appropriate control of uninfested lima bean leaves was offered as alternative. The strength of this effect was dependent on the number of spider mites infesting the elicitor-producing leaves. Higher numbers of spider mites resulted in an elicitor solution with a stronger effect. In addition, spider mite density was important. The elicitor obtained from one leaf with 50 spider mites had a stronger effect on the attractiveness of uninfested leaves than the elicitor obtained from three leaves with 17 spider mites each. This suggests that the stress intensity imposed on a plant is an important determinant of the elicitor quantity. While the elicitor has a strong effect on the attractiveness of uninfested leaves, spider mite-infested leaves are still much more attractive to predatory mites than elicitor-exposed leaves. The data are discussed in the context of systemic

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effects in plant defense and the biosynthesis of herbivore-induced terpenoids in plants.

Key Words—Plant defense, systemic effect, endogenous elicitor, herbivore-induced synomone, Acari, spider mites, *Tetranychus urticae*, predatory mites, *Phytoseiulus persimilis*, lima bean, *Phaseolus lunatus*, olfactometer, tritrophic interactions

INTRODUCTION

One of the defense options of plants is to promote the effectiveness of natural enemies of herbivores (Price et al., 1980). This may be accomplished by attracting the enemies of the herbivores by volatile infochemicals whose production in the plant is induced by herbivory (Dicke and Sabelis, 1988a; Dicke et al., 1990a; Turlings et al., 1990b; Vet and Dicke 1992); these infochemicals were termed herbivore-induced synomones (Vet and Dicke, 1992). Upon infestation by two-spotted spider mites (*Tetranychus urticae*), lima bean plants and cucumber plants emit large amounts of a blend of volatile chemicals that attract the predatory mite *Phytoseiulus persimilis*, which can exterminate *T. urticae* populations (Dicke and Sabelis, 1988a; Dicke et al., 1990a,b). Similarly, corn plants that are infested by beet armyworm larvae (*Spodoptera exigua*) emit a blend of volatiles that attract the parasitic wasp *Cotesia marginiventris* (Turlings et al., 1990b, 1991). The plant has an active role in this process, and the chemicals that are emitted upon herbivory are either not emitted upon artificial damage or only in minute quantities (Dicke et al., 1990a,b; Turlings et al., 1990b, 1991). Moreover, the herbivore-induced synomones seem to be rather specific: the natural enemies discriminate between different plant-herbivore combinations (Sabelis and Van de Baan, 1983; Dicke, 1988; Dicke and Groeneveld, 1986; Sabelis and Dicke, 1985; Turlings et al., 1990a) and chemical differences between blends emitted by different plant–herbivore combinations have also been recorded (Dicke et al., 1990b; Takabayashi et al., 1991b).

The active role of the plant in the production of these volatile infochemicals has been inferred from two observations: the plant has a more pronounced influence on the composition of the chemical blend than the herbivore (Takabayashi et al., 1991b) and, more importantly, the production of volatile infochemicals that attract natural enemies of herbivores is not restricted to the infested plant parts but occurs systemically throughout the plant [Dicke et al., 1990b; Turlings and Tumlinson, 1992; note that Nadel and Van Alphen (1987) provide a similar conclusion but that their data are not convincing because they did not control for adsorption of infochemicals from the infested leaves onto the uninfested leaves of the same plant].

For several plant–herbivore and plant–pathogen interactions infestation-

induced systemic responses that affect herbivore or pathogen performance directly are known to be mediated by elicitors that are transported from the infested site to the uninfested leaves (Bowles, 1990 a,b; Malamy et al., 1990; Métraux et al., 1990; Ryan and Farmer, 1991). However, an elicitor that mediates a systemic response that affects natural enemy foraging behavior has not been reported to date. Such a systemic effect is known for the tritrophic system consisting of lima bean plants, *T. urticae* spider mites, and *P. persimilis* predatory mites (Dicke et al., 1990b). The existence of an endogenous elicitor mediating this systemic effect has been recently suggested. When spider mite-infested lima bean leaves are placed on wet cotton wool in a tray for seven days and replaced by uninfested lima bean leaves, these latter emit two of the synomone components in much higher amounts than control uninfested leaves that have been placed on wet cotton wool on which *un*infested leaves had been present for seven days (Takabayashi et al., 1991a). These chemical differences also affected the behavior of the predatory mite *P. persimilis*: in an olfactometer they prefer the volatiles from the uninfested treatment bean leaves over the volatiles from the uninfested control bean leaves (Dicke and Dijkman, 1992). Although these data strongly suggest that a water-soluble elicitor was transferred through the wet cotton wool, the data can also be explained by transfer of volatile synomone components to the wet cotton wool or the tray and from there to the uninfested leaves. In the present paper, we provide unambiguous evidence for the existence of a water-soluble endogenous elicitor, and we investigate some aspects of herbivore–plant interactions that affect the strength of the systemic response.

METHODS AND MATERIALS

Plants

Lima bean plants (*Phaseolus lunatus* cv. Carolina or Sieva, W. Atlee Burpee, Pennsylvania) were reared in sterilized soil in a greenhouse. Five plants were grown in a pot 10.5 cm high and 10 cm in diameter. Rearing conditions were 20–30°C and 50–70% relative humidity, and a photoperiod of at least 16 hr. Artificial light (mercury discharge lamps) switched on automatically when the natural daylight intensity during the photoperiod dropped below 500 lux.

Mites

The herbivorous spider mite *Tetranychus urticae* Koch was reared on lima bean plants in a greenhouse under similar conditions as described for rearing of the plants. The predatory mite *Phytoseiulus persimilis* Athias-Henriot was reared in a greenhouse on lima bean leaves infested with *T. urticae*. The leaves were placed on top of clay flower pots that were placed upside down in a water basin.

New leaves were added daily. Environmental conditions were similar to those described for rearing of the plants.

Olfactometer

The response of the predatory mites towards volatiles from lima bean leaves was investigated in a Y-tube olfactometer. The olfactometer consisted of a glass Y-shaped tube in which an iron wire was positioned centrally. With the aid of air pressure and a vacuum system, two air streams of 4 liters/min were generated, each through a different vessel containing a source of volatile chemicals. Each of these air streams entered one arm of the olfactometer. Satiated adult female predatory mites were individually introduced on the iron wire and given a maximum of 5 min to walk towards the end of one of the arms of the olfactometer. When the predator reached the end of one of the olfactometer arms its choice was recorded. After removal of the predator, a new predator was introduced. The stimulus sources were exchanged after testing five predators to neutralize any asymmetric aspect of the setup. The test was performed at 22 $\pm$ 1°C and 70 $\pm$ 10% relative humidity in a cage surrounded by white curtains, with two high-frequency fluorescent tubes (16 W) at the ceiling, to obtain a light distribution as uniform as possible. The choice of the predators was analyzed with a chi-square test ($\alpha = 0.05$). Predators that did not make a choice within 5 min were excluded from the statistical analysis. For more details about the olfactometer, see Takabayashi and Dicke (1992).

Experimental Procedures

The basic experimental procedure (Figure 1A) used two sets of 20 lima bean plants that were ca. 2–3 weeks old with the first trifoliar leaf in the process of expanding. At the start of the experiment, the first set of 20 plants was used. The primary leaves of each plant were detached and placed with the petiole in a glass vial (7 cm high and 1.9 cm in diameter) with ca. 17 ml distilled water. Parafilm was used to close the vials. One leaf of each pair was used in the treatment and the other in the control so as to minimize any interindividual plant variation between treatment and control. The treated leaves were placed in one plastic cage (66 $\times$ 66 $\times$ 100 cm) and the control leaves in another (cages A and B in Figure 1A). Specifics of each treatment and the appropriate controls are given below. Each cage was connected to a house vacuum to remove volatiles emitted from the leaves. The cages were placed in a climate room at 23 $\pm$ 1°C, 60–80% relative humidity. Light was provided by high-frequency fluorescence tubes; the light intensity at the position of the leaves was 1100 lux. After seven days, the treated and control leaves were taken from the vials and used as an odor source in a Y-tube olfactometer experiment to test attractiveness

to predatory mites. The water in the vials was poured into new vials and distilled water was added to replenish the new vial completely. The vials with the transferred solution were placed in new cages (cages C and D in Figure 1A) and leaves from uninfested lima bean leaves (second set of 20 plants) were placed in them, again from each plant one leaf for the treatment and one for the control. Thus, the uninfested leaves of the second set of plants were incubated under conditions where the transferred solution was the only link with the treated or control leaves of the first set of plants. After three days of incubation, the leaves in cages C and D were taken from the vials and used in the Y-tube olfactometer test with predatory mites.

Based on this general experimental procedure, the following experiments were carried out (the cages mentioned refer to Figure 1A).

Isolation of Elicitor Mediating Systemic Production of Herbivore-Induced Synomone. This is the basic experiment whose procedure is depicted in Figure 1A. In cage A, 50 adult female spider mites were introduced per leaf, while the leaves in cage B remained uninfested. Vials in cage C received the water from vials in cage A, and vials in cage D received the water from vials in cage B. To investigate whether a predator attractant was transferred in the water or whether a water-soluble compound was transferred that elicits the production of synomone in uninfested plant tissue, the water from the vials in cages A and B was poured onto filter paper. The two sets of wet filter paper were used as odor sources in the Y-tube olfactometer. This experimental setup is shown graphically in Figure 2A.

Effect of Spider Mite Number. Fifty adult female spider mites were introduced per leaf in cage A and 17 per leaf in cage B. Vials in cage C received the water from vials in cage A and vials in cage D received the water from vials in cage B. The experimental setup is shown graphically in Figure 3A.

Effect of Spider Mite Density. In cage A, 50 adult female spider mites were introduced per leaf, and in cage B the same number of spider mites was divided over three leaves that shared the same vial. Vials in cage C received the water from vials in cage A and vials in cage D received the water from vials in cage B. The experimental setup is shown graphically in Figure 4A.

Relative Attraction of Spider Mite Infested Leaves and Elicitor-Exposed Leaves. This is the only experiment in which cage B remained unoccupied. In cage A, 50 adult female spider mites were introduced per leaf. After seven days, vials in cage C received the water from cage A, and vials in cage D received freshly distilled water. Fifty adult female spider mites were introduced on every leaf in cage D. The experimental setup is shown graphically in Figure 5A. In this experiment only leaves from cages C (elicitor-exposed) and D (spider mite-infested) were compared in the olfactometer.

In every olfactometer experiment, a total of 120 predators was tested over three experimental days.

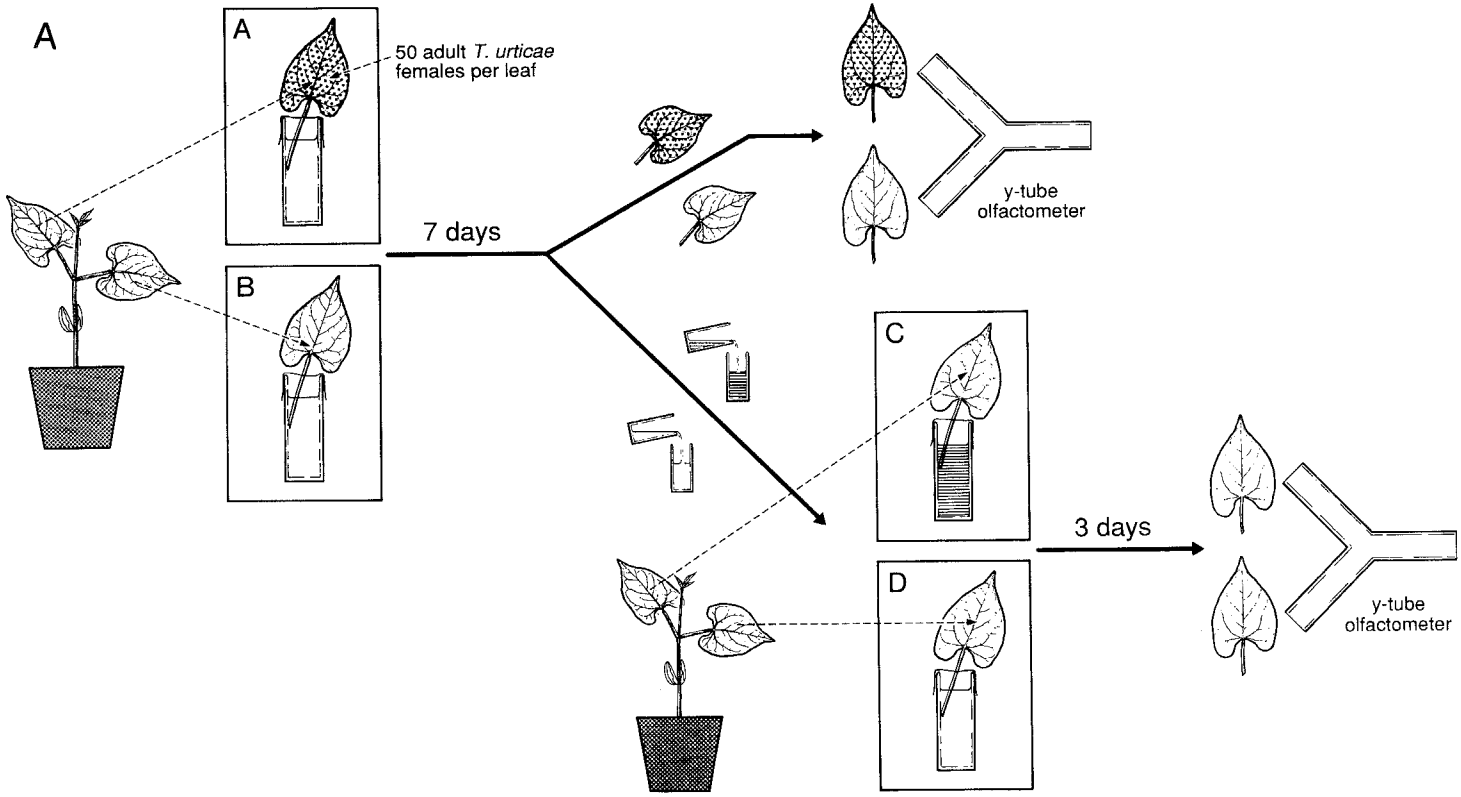

FIG. 1. (A) Basic experimental procedure for extracting an elicitor that mediates induction of systemic production of herbivore-induced synomone. Primary leaves of lima bean plants are detached and incubated in vials with distilled water. One leaf of a plant is assigned to the treatment (cage A) and the other to the control (cage B). After seven days of incubation, the leaves are used in an olfactometer to test the attractiveness to predatory mites. The water from the vials in cage A is transferred to new vials that are placed in cage C and the water from the vials in cage B is transferred to new vials that are placed in cage D. Uninfested primary lima bean leaves are incubated in the vials in cages C and D. Each of the four cages (A through D) is connected to a house vacuum to remove any volatiles produced during incubation from the climate room. For further details see text in Materials and Methods.

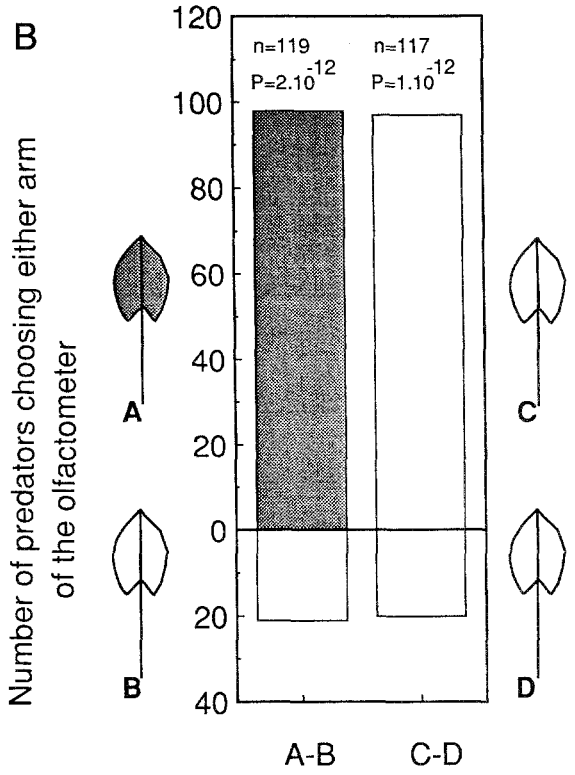

FIG. 1. Continued. (B) Response in Y-tube olfactometer of satiated *Phytoseiulus persimilis* females to spider mite-infested leaves vs. uninfested leaves (leaves from cages A and B, respectively) and to elicitor-exposed uninfested leaves vs. control uninfested leaves (leaves from cages C and D, respectively). The total number of predators tested per experiment is 120; *n* indicates the number of predators that reached the end of either olfactometer arm within 5 min.

RESULTS

Isolation of Elicitor Mediating Systemic Production of Herbivore-Induced Synomone. When leaves infested by spider mites were offered in the olfactometer with uninfested leaves as a control, the majority of the predators (82%) preferred the arm with infested leaves (Figure 1B). This is in agreement with previous data (Sabelis and van de Baan, 1983). A similar percentage (83%) of predators preferred the odor of uninfested leaves that had been incubated in water in which infested leaves had been present versus the odor of uninfested leaves that had

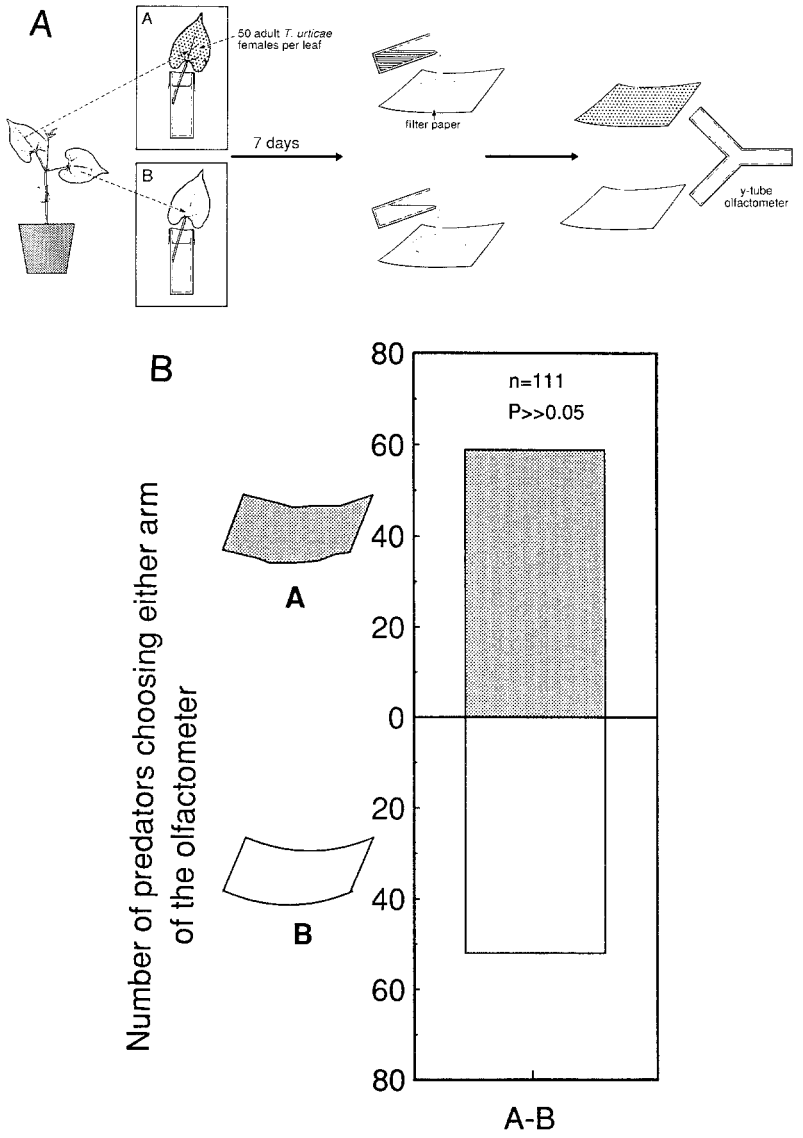

FIG. 2. (A) Experimental procedure used for investigating whether attractants for predatory mites are collected in water in which infested lima bean leaves have been incubated for seven days. (B) Response in Y-tube olfactometer of satiated *Phytoseiulus persimilis* females to filter paper with water in which spider mite-infested leaves or uninfested leaves had been standing for the previous seven days. The total number of predators tested was 120; *n* indicates the number of predators that reached the end of either olfactometer arm within 5 min.

been incubated in water in which uninfested leaves had been present (Figure 1B).

When the predators were offered the water in which spider mite-infested leaves had been present for seven days versus the water in which uninfested leaves had been standing for seven days, the predators did not discriminate between the treatment and control water, which shows that the predator attractants themselves are not in the water (Figure 2B). These data indicate that an elicitor is present in the water that induces the production of synomone in the uninfested leaf.

Effect of Spider Mite Number. The predators significantly preferred the olfactometer arm with leaves infested by 50 spider mites per leaf (73%) over the arm with leaves infested by 17 spider mites per leaf (Figure 3B). Again, it is remarkable to see a similar percentage (77%) of predators preferring uninfested leaves that have been incubated in the water in which the leaves with 50 spider mites had been incubated when the alternative consisted of uninfested leaves that had been incubated in water in which the leaves with 17 spider mites had been incubated (Figure 3B). Thus, the predators not only discriminate between actual numbers of spider mites on the leaves, but also between leaves that have been exposed to elicitor solutions obtained from leaves with different spider mite numbers. This suggests that the amount of elicitor is correlated with the number of spider mites.

Effect of Spider Mite Density. The number of infested leaves per vial was varied while the number of spider mites used per vial to infest the leaves was the same for the treatment and control vials of the first incubation period. In the control, the 50 spider mites were distributed over three leaves and in the treatment they were all on one leaf. The predators preferred the arm with 20 leaves that were each infested by 50 spider mites (66%) over the arm with 60 leaves that were each infested by 17 spider mites (34%) (Figure 4B). This indicates that, rather than the number of herbivores, their density is important in determining herbivore-induced synomone production. In the second part of the experiment, the predators again showed a similar preference towards leaves that had been incubated in water in which the leaves of the first part of the experiment had been incubated during the previous seven days. Sixty-six percent of the predators walked toward the far end of the arm with uninfested leaves that had been incubated in water in which one leaf with 50 spider mites had been incubated and 34% walked to the uninfested leaves that had been incubated in water in which three leaves with 17 spider mites each had been incubated before (Figure 4B).

Relative Attraction of Spider Mite-Infested Leaves and Elicitor-Exposed Leaves. When infested leaves (50 spider mites per leaf) were offered to the predators vs. uninfested leaves that had been exposed for seven days to elicitor solution obtained from leaves with 50 spider mites per leaf, the predators pre-

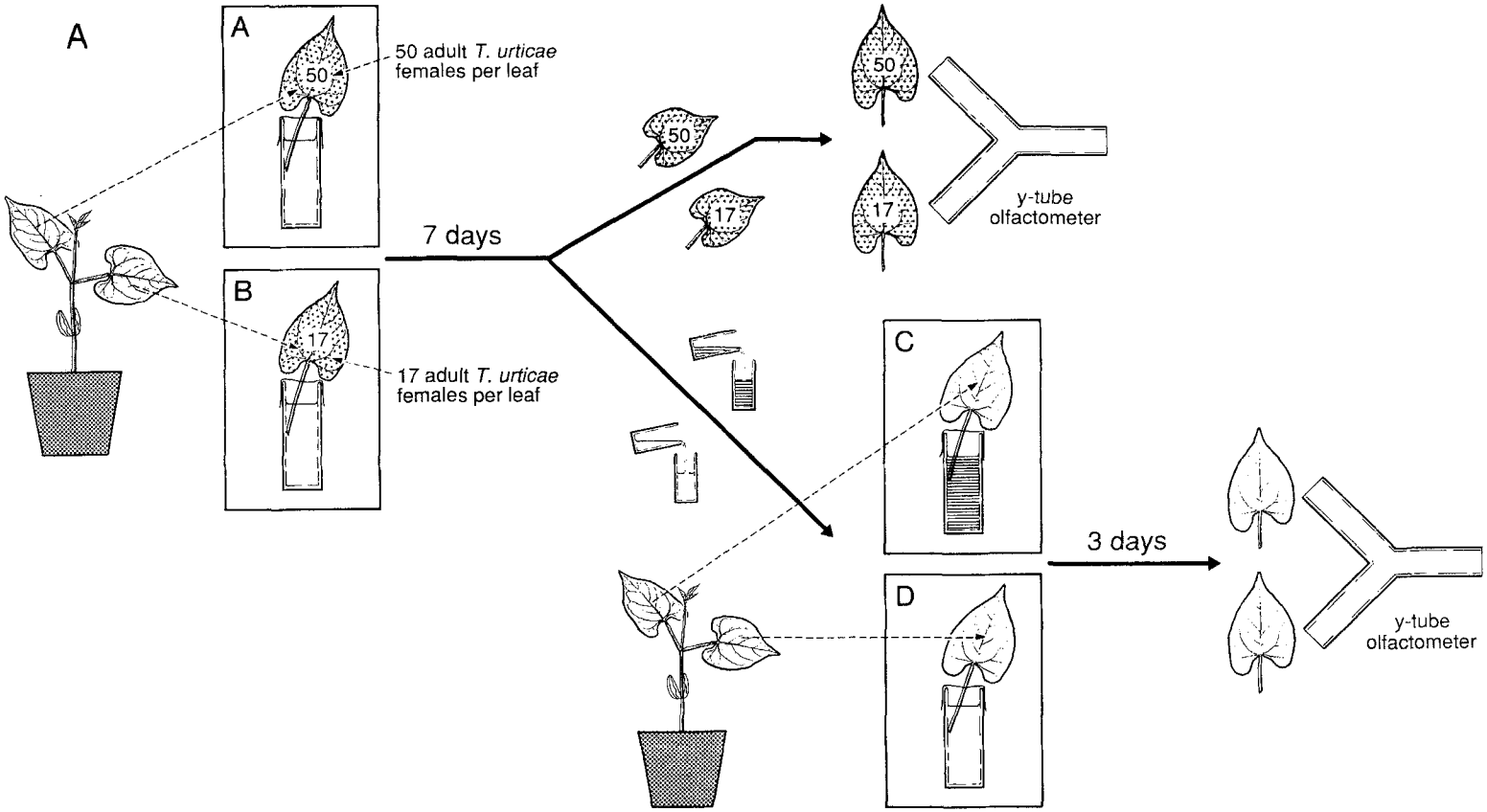

FIG.3. (A) Experimental procedure used for investigating the effect of different spider mite numbers on elicitor production in lima bean leaves.

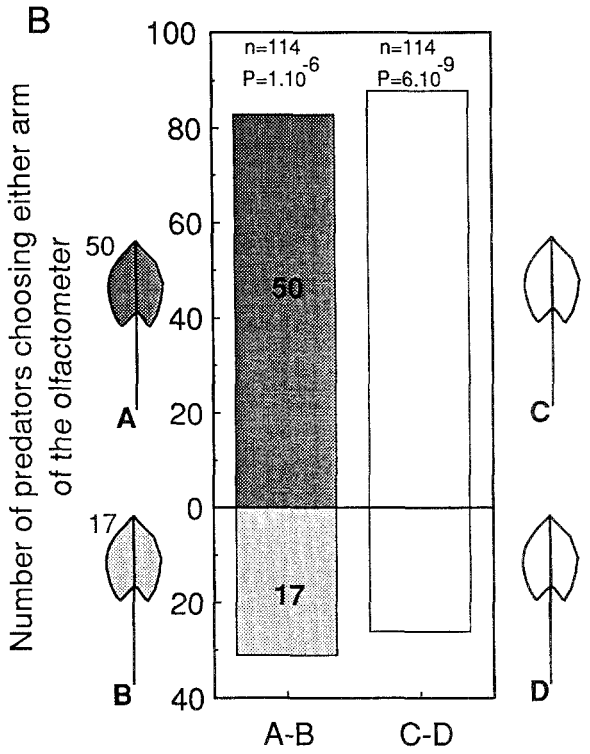

FIG. 3. Continued. (B) Response in Y-tube olfactometer of satiated *Phytoseiulus persimilis* females to spider mite-infested leaves having different level of infestation (50 vs. 17 spider mites per leaf; leaves from cages A and B, respectively) and to uninfested leaves that have been exposed to the corresponding elicitor solutions (leaves from cages C and D). The total number of predators tested per experiment is 120; *n* indicates the number of predators that reached the end of either olfactometer arm within 5 min.

ferred the infested leaves to the elicitor-exposed leaves (Figure 5B). Thus, the predators can discriminate between infested leaves and elicitor-exposed leaves, despite their strong attraction to elicitor-exposed leaves when uninfested control leaves are the alternative (Figure 1B).

DISCUSSION

It was previously shown that uninfested leaves of lima bean plants of which one leaf is infested by spider mites became attractive to predatory mites (Dicke et al., 1990b). In order to extract an elicitor that mediates this systemic response,

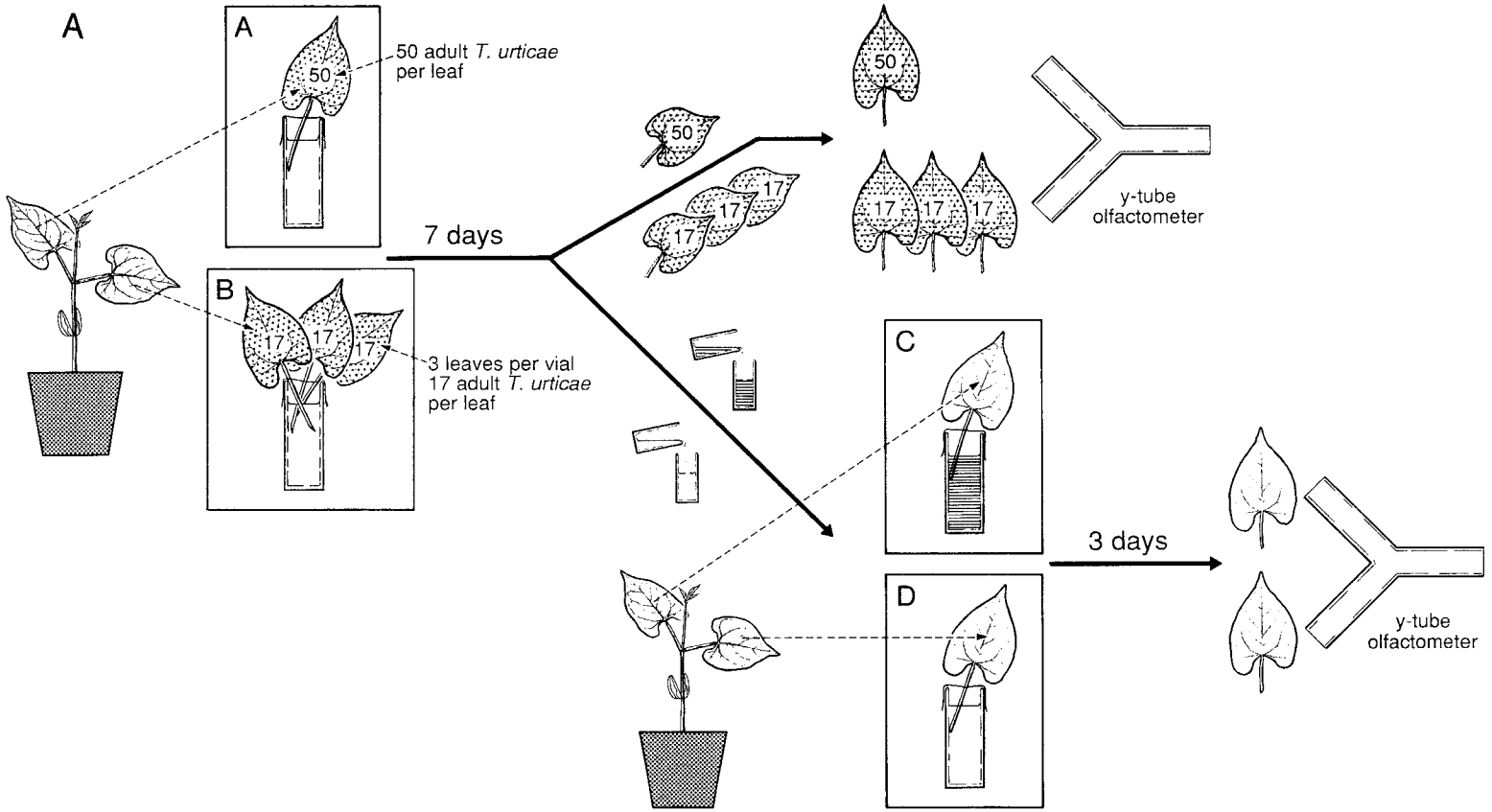

FIG 4. (A) Experimental procedure used to investigate the effect of different spider mite densities on elicitor production in lima bean leaves. Differences in spider mite numbers are corrected for by using more leaves at the lower density.

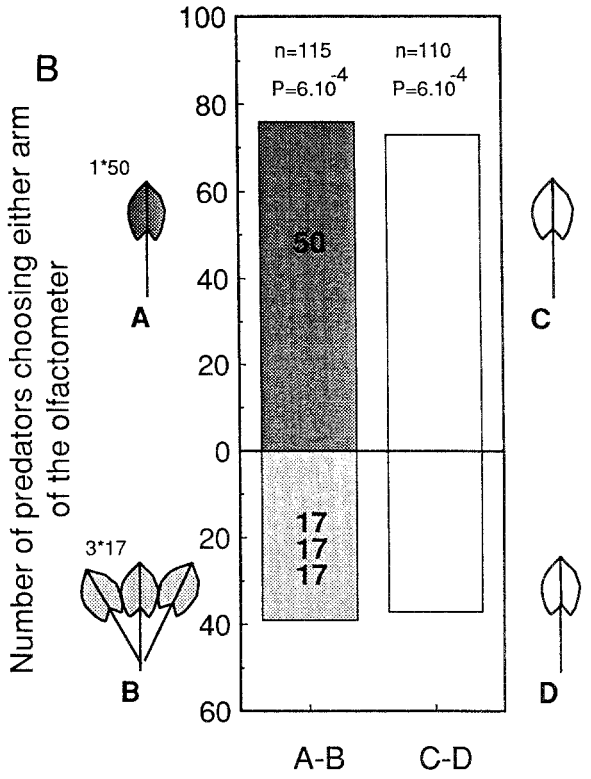

FIG. 4. Continued. (B) Response in Y-tube olfactometer of satiated *Phytoseiulus persimilis* females to spider mite-infested leaves having different spider mite densities, while the total number of spider mites per olfactometer arm is constant (leaves from cages A and B) and to uninfested leaves that have been exposed to the corresponding elicitor solutions (leaves from cages C and D). The total number of predators tested per experiment is 120; *n* indicates the number of predators that reached the end of either olfactometer arm within 5 min.

we detached spider mite-infested leaves to collect substances transported out of them and exposed detached uninfested leaves to these solutions. The resulting data are the first to demonstrate the existence of an endogenous elicitor (or elicitors) that mediates the systemic production by plants of predator attractants upon infestation by herbivores. The elicitor may either induce this production directly or through activation of another step in a reaction chain. The exact mechanism of induction of synomone production remains to be elucidated. To do so, an important contribution would be to identify the chemical structure of the currently demonstrated elicitor. This would also allow quantification of elic-

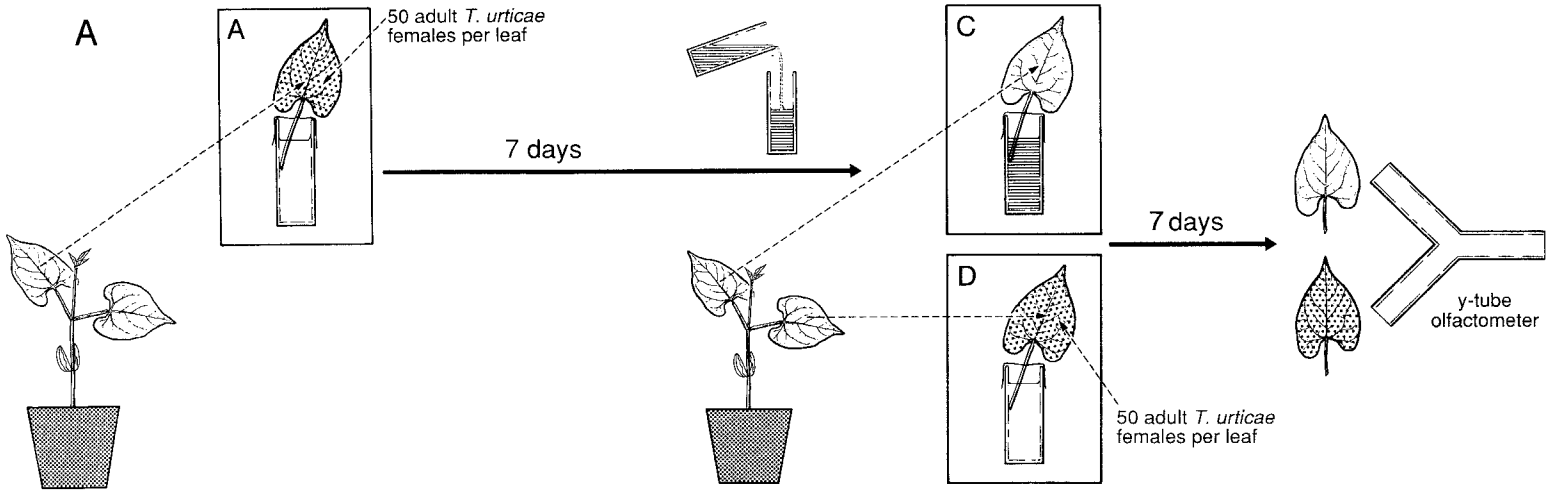

FIG. 5. (A) Experimental procedure used for investigating the effect of spider mite infestation vs. the effect of exposure to endogenous elicitor on the attraction of predatory mites to lima bean leaves.

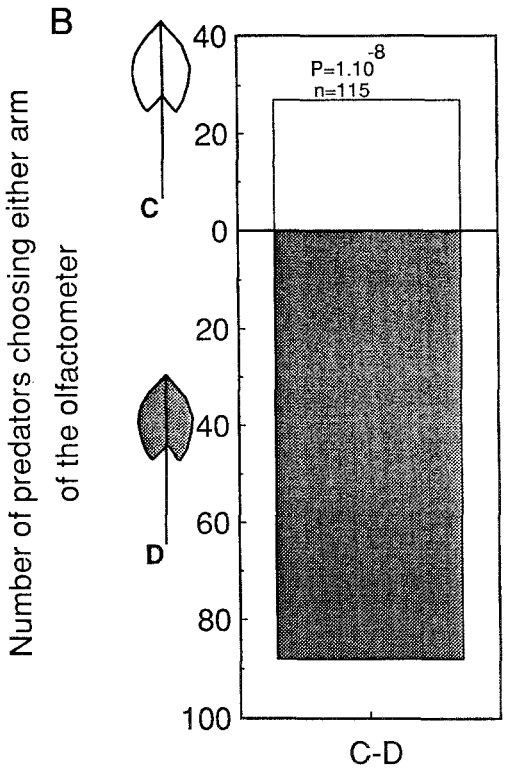

FIG. 5. Continued. (B) Response in Y-tube olfactometer of satiated *Phytoseiulus persimilis* females to spider mite-infested lima bean leaves vs. elicitor-exposed uninfested lima bean leaves (leaves from cages C and D). The total number of predators tested per experiment is 120; *n* indicates the number of predators that reached the end of either olfactometer arm within 5 min.

itor production in differently treated leaves as well as investigation of the transport of the elicitor in vivo. Elicitors that are involved in direct defense that affects herbivore performance through inducing the production of digestibility reducers or phytoalexins have been reported for many systems. Among the identified signaling compounds are many oligosaccharides and other compounds such as a polypeptide, arachidonic acid, abscisic acid, methyl jasmonate, salicylic acid, and inositol phosphates (Malamy et al., 1990; Métraux et al., 1990; Ryan and Farmer, 1991; Pearce et al., 1991). The functioning and recognition of these elicitors and thus their role in activation of plant genes is currently receiving much attention (Bowles, 1990a; Ryan and Farmer, 1991), and recently a synthesis for the knowledge of the systemic induction of proteinase inhibitors in tomato in response to wounding has been presented (Farmer and Ryan, 1992).

With respect to one of the herbivore-induced synomone components that mediate lima bean-predatory mite interactions, the methylene terpene 4,8-dimethyl-1,3(*E*),7-nonatriene (Dicke et al., 1990a), some information is available on its production in herbivore-infested leaves (Gäbler et al., 1991). The compound originates from nerolidol by an oxidative cleavage of its C skeleton by plant enzymes, followed by exclusive loss of H_s-C(5) and formal production of a C_4 compound. Nerolidol probably originates from a phytogenic glycoside, which is presumably cleaved by a spider mite salivary enzyme (Gäbler et al., 1991). Spider mites insert their stylets into parenchymous cells and inject saliva (Tomczyk and Kropczynska, 1985). It seems unlikely that spider mite salivary enzymes are transported through the parenchyma to the phloem and from there to other leaves. Salivary enzymes are assumed to be too large to be able to pass a large number of cell walls. Thus, it seems that the production of 4,8-dimethyl-1,3(*E*),7-nonatriene in uninfested leaves, if affected by an elicitor (cf. evidence presented by Takabayashi et al., 1991a; Turlings and Tumlinson, 1992), cannot be mediated by cleavage of phytogenic glycosides by herbivore enzymes (see also below in next paragraph). It is known for plant responses that directly affect herbivore or pathogen performance through secondary metabolites or phytoalexins that the biosynthetic pathway employed at the site of damage may be distinct from the pathway induced by an elicitor in undamaged parts of the damaged plant (Coleman et al., 1992). The existence of another production mechanism of 4,8-dimethyl-1,3(*E*),7-nonatriene is also suggested by the emission of minute quantities from uninfested leaves of uninfested plants (Dicke et al., 1990b; Turlings and Tumlinson, 1992). Another possibility is that salivary enzymes yield products at the site of infestation that can be transported through the parenchyma to the phloem and thus to other leaves. This is the proposed scenario for elicitor production in systemic responses in other systems (Ryan and Farmer, 1991). In this context, it is interesting that [^{32}P]phosphate, when applied superficially to bean leaves, is transported throughout the plant (Storms, 1971). Similarly, when spider mites that have become radioactive from feeding on a [^{32}P]phosphate-labeled or ^{14}C-labeled plant feed on an unlabeled plant, radioactivity can be found throughout the plant, but especially in the growing parts; i.e., the developing top leaves and the roots (Avery and Briggs, 1968; Storms, 1971). Whether the labeled compounds concerned are salivary enzymes or other spider mite chemicals is not known. further investigations are needed to elucidate whether these translocated chemicals comprise the elicitor; e.g., by comparing the synomone production in leaves in which the radioactive compounds are highly abundant vs. leaves in which the radioactive compounds hardly appear.

There is a good correlation between the response of *P. persimilis* to spider mite infested leaves and the response to uninfested leaves that have been exposed to the elicitor obtained from those spider mite infested leaves. Not only is the

predator response correlated with the number of spider mites that infest the leaves, but the predators also prefer the synomone of the uninfested leaves that have been exposed to elicitor solutions obtained from leaves infested by the higher number of spider mites. However, spider mite numbers themselves are not the most important determinant of predator attraction. It is interesting to note that spider mite density rather than spider mite number determines the attraction of predatory mites to infested leaves as well as to uninfested leaves exposed to corresponding elicitor solutions (Figure 4). This may suggest that the elicitor is not a spider mite-injected chemical (see discussion in previous paragraph) that is transported throughout the plant (cf. Storms, 1971), assuming that a spider mite's saliva injection is not density dependent. In fact, this result adds to the evidence that the plant is more important than the herbivore in determining herbivore-induced synomone characteristics (Takabayashi et al., 1991b; Vet and Dicke, 1992). At the stress levels imposed upon the leaves, the more severe the stress the more attractive both the infested leaves and the elicitor-exposed uninfested leaves are to predatory mites.

In conclusion, it is clear from our data that uninfested lima bean leaves that have been exposed to an elicitor originating from spider mite-infested lima bean leaves are highly attractive to predatory mites. This indicates that the systemic response may add to the attractiveness of a partially infested plant. Thus, predatory mites may perceive volatile infochemicals related to spider mite infestations from three sources: (1) the spider mite-infested leaves (e.g., Sabelis and Van de Baan, 1983), (2) the uninfested leaves of a spider mite-infested plant (Dicke et al., 1990b; this paper), and (3) the downwind neighbors of the spider mite-infested plant (Bruin et al., 1992). Current data indicate that the attractiveness of the uninfested leaves does not affect the ability of the predators to distinguish the infested leaves that harbor the predator's prey. This discrimination may be enabled by quantitative differences and in addition by qualitative differences. These aspects will be the subject of future investigations.

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REFERENCES

AVERY, D.J., and BRIGGS, J.B. 1968. The aetiology and development of damage in young fruit trees infested with tree red spider mite, *Panonychus ulmi* (Koch). *Ann. Appl. Biol.* 61:277–288.

BOWLES, D. 1990a. Defense-related proteins in higher plants. *Annu. Rev. Biochem.* 59:873–907.

BOWLES, D. 1990b. Signals in the wounded plant. *Nature* 343:314–315.

BRUIN, J., DICKE, M., and SABELIS, M.W. 1992. Plants are better protected against spider mites after exposure to volatiles from infested conspecifics. *Experientia* 48:525–529.

COLEMAN, J.S., JONES, C.G., and KRISCHIK, V.A. 1992. Phytocentric and exploiter perspectives of phytopathology. *Adv. Plant Pathol.* 8:149–195.

DICKE, M. 1988. Prey preference of the phytoseiid mite *Typhlodromus pyri*: 1. Response to volatile kairomones. *Exp. Appl. Acarol.* 4:1–13.

DICKE, M., and DIJKMAN, H. 1992. Induced defence in detached uninfested plant leaves: Effects on behavior of herbivores and their predators. *Oecologia* 91:554–560.

DICKE, M., and GROENEVELD, A. 1986. Hierarchical structure in kairomone preference of the predatory mite *Amblyseius potentillae*: Dietary component indispensable for diapause induction affects prey location behavior. *Ecol. Entomol.* 11:131–138.

DICKE, M., and SABELIS, M.W. 1988a. How plants obtain predatory mites as body guards. *Neth. J. Zool.* 38:148–165.

DICKE, M., and SABELIS, M.W. 1988b. Infochemical terminology: should it be based on cost-benefit analysis rather than origin of compounds? *Funct. Ecol* 2:131–139.

DICKE, M., BEEK, T.A. van, POSTHUMUS, M.A., BEN DOM, N., BOKHOVEN, H. van, and GROOT, Æ. de. 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.

FARMER, E.E., and RYAN, C.A. 1992. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* 4:129–134.

GÄBLER, A., BOLAND, W., PREISS, U., and SIMON, H. 1991. Stereochemical studies on homoterpene biosynthesis in higher plants; Mechanistic, phylogenetic and ecological aspects. *Helv. Chim. Acta* 74:1773–1789.

MALAMY, J., CARR, J.P., KLESSIG, D.F., and RASKIN, I. 1990. Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250:1002–1004.

MÉTRAUX, J.P., SIGNER, H., RYALS, J., WARD, E., WYSS-BENZ, M., GAUDIN, J., RASCHDORF, K., SCHMID, E., BLUM, W., and INVERARDI, B. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250:1004–1006.

NADEL, H., and ALPHEN, J.J.M. VAN. 1987. The role of host- and host-plant odors in the attraction of a parasitoid, *Epidinocarsis lopezi*, to the habitat of its host, the cassava mealybug, *Phenacoccus manihoti*. *Entomol. Exp. Appl.* 45:181–186.

PEARCE, G., STRYDOM, D., JOHNSON, S., and RYAN, C.A. 1991. A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* 253:895–898.

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 plant on interactions between insect herbivores and natural enemies. *Annu. Rev. Ecol. Syst.* 11:41–65.

RYAN, C.A., and FARMER, E.E. 1991. Oligosaccharide signals in plants: A current assessment. *Annu. Rev. Plant Physiol. Mol. Biol.* 42:651–674.

SABELIS, M.W., and DICKE, M., 1985. Long-range dispersal and searching behavior, pp. 141–160, *in* W. HELLE and M.W. SABELIS (eds.). Spider Mites. Their Biology, Natural Enemies and Control. World Crop Pests 1B. Elsevier, Amsterdam.

SABELIS, M.W., and VAN DE BAAN, H.E. 1983. Location of distant spider mite colonies by phytoseiid predators: Demonstration of specific kairomones emitted by *Tetranychus urticae* and *Panonychus ulmi*. *Entomol. Exp. Appl.* 33:303–314.

STORMS, J.J.H. 1971. Some physiological effects of spider mite infestation on bean plants. *Neth. J. Plant Pathol.* 77:154–167.

TAKABAYASHI, J., and DICKE, M. 1992. Response of predatory mites with different rearing histories to volatiles of uninfested plants. *Entomol. Exp. Appl.* 64:187–193.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M.A. 1991a. Induction of indirect defence against spider mites in uninfested lima bean leaves. *Phytochemistry* 30:1459–1462.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M.A. 1991b. Variation in composition of predator-attracting allelochemicals emitted by herbivore-infested plants: Relative influence of plant and herbivore. *Chemoecology* 2:1–6.

TOMCZYK A., and KROPCZYNSKA, D. 1985. Effects on the host plant, pp. 317–329, *in* W. HELLE and M.W. SABELIS (eds.). Spider Mites. Their Biology, Natural Enemies and Control. World Crop Pests, Vol. 1A. Elsevier, Amsterdam.

TURLINGS, T.C.J., and TUMLINSON, J.H. 1992. Systemic release of chemical signals by herbivore-injured corn. *Proc. Natl. Acad. Sci. U.S.A.* 89:8399–8402.

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 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 micro-habitat of its hosts. *Entomol. Exp. Appl.* 58:75–82.

VET, L.E.M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

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Book Review

Phytochemical Induction by Herbivores. Douglas W. Tallamy and Michael J. Raupp (eds.). New York: John Wiley & Sons, Inc., 1991. $98.00. 431 pages. ISBN 0-471-63241-4.

Just a few years ago, a young colleague was told that his thesis project on damage-induced plant responses would be inappropriate in a department of neurobiology and behavior, "since plants have neither." Since then, some journals have instituted review series on signal transduction by responding plants, a role for gene expression in induction has been identified, agriculture has begun taking induced immunization seriously, and the literature on such plant behavior has grown rapidly. And here we have an entire book reviewing the status of this research theme; it contains enough thesis projects for scores of young colleagues.

Tallamy and Raupp have collected an interesting sample of the work being done on changes in plants that are apparently induced by herbivory. This sample allows several generalizations, reveals some experimental problems, and clearly identifies a series of critical unanswered questions.

First, the generalizations. Although negative findings are probably not published, the number of plant species exhibiting changes in quality to herbivores (via bioassay) or chemical changes (independent of bioassay) continues to increase. These nearly ubiquitous responses require whole, intact plants, and so investigators must not use excised leaves or other plant parts in bioassays, even if their studies do not focus on induced changes. The quality of plant food for herbivores depends upon the plant's ability to respond systemically.

Induced plant responses are highly variable within species. Almost any factor that could possibly influence plant metabolism, e.g., light, nutrient availability, and water, can increase, decrease, or eliminate the plant's ability to respond to damage. Hence, several authors report changes in the responses of a plant under varying soil, seasonal, or other conditions. These papers confirm a long-held suspicion that younger plants and plant tissues tend to be more responsive than are older tissues, explaining why our ability to elicit induced responses often changes seasonally.

Our ability to detect impacts of damage-induced changes on herbivores depends on the herbivore as well as the plant. Several authors struggle with the observation that induced responses have variable, even positive, effects on her-

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bivores. Yet these same authors have no trouble understanding how adapted, but not unadapted, herbivores can feed successfully in the presence of constitutive chemical defenses. Since many of the induced changes catalogued here merely comprise amplification of constitutive defenses, one would expect all possible herbivore responses. Some authors (e.g., Hartley and Lawton) even question the potential significance of induced changes, evidently failing to appreciate the role of adaptation in coevolution.

Mechanisms by which induced changes influence herbivores remain elusive. Not just a few, but the majority of the chapters in this book conclude that the chemical mechanisms thus far studied fail to account for damage-induced impacts on herbivores. Reviews and new research in such well-studied systems as induced cucurbitacins in cucumber and proteinase inhibitors in Solanaceae indicate that the mechanisms (and even insect responses) originally proposed are woefully off the mark. Indeed, I was shocked to see how few mechanisms have even modest support.

Microbes may be key in eliciting plant responses to damage. Chapters by Raffa and Hartley and Lawton provide strong evidence that plant responses to herbivory may actually be cued by attendant microbes, and none of the other studies described or reviewed here can rule this out. There is plenty of preliminary evidence here that the identity of the attacker influences the nature of the plant response.

These few generalizations, and careful reviews by the authors, clearly point to experimental shortcomings in the study of induction. Chapters by Faeth and Karban bemoan the fact that most investigations have used such disparate methods that their results cannot be compared, even when the study systems are the same. I have already mentioned the use of adapted and unadapted herbivore species, which can produce opposite conclusions about the physiological and ecological impact of plant changes. Only two series of studies (Haukioja's group on birches and the University of Alaska's group on several woody plant species) have been carried out long enough to describe the entire course of an induction response. Both Faeth and Karban suggest useful approaches to resolving these and other problems.

Indeed, few investigations have combined studies of plant changes that impact on the performance of an herbivore and carried this through to ecological consequences for the plant or herbivore. Investigators seem to focus on the mechanistic biochemical or ecological pattern, but rarely combine them. Of course, this is a tall order, but induced changes are a widespread phenomenon with significant ecological (and economic) potential.

Studies are not being constructed to distinguish between herbivore-cued and microbe-cued responses. This is a serious shortcoming. The use of sterile methods and/or the incorporation of microbial contaminants into experimental designs needs to be done on a wide scale.

Perhaps because "a jack-of-all-trades is master of none," the chemistry methods used in induction studies are often inappropriate. Damage-induced changes in phenolics comprise more than half of all the studies cited in this volume. I would judge the analytical methods used to be so flawed in most of these cases as to seriously impair the interpretation of results. With the exception of Clausen *et al.*'s chapter elucidating short-term responses in Salicaceae, none of the studies has examined enzymatic modification (rather than synthesis) of plant metabolites as a primary induction mechanism. Integrating behavior, ecology, and chemistry remains a problem in chemical ecology.

These difficulties and the evident successes reported in this volume leave us with some very important, tantalizing questions that need to be addressed.

First, are induced responses, especially long-term responses, merely an expression of altered growth and physiological (e.g., photosynthetic) activity, or are they specific, potentially adaptive responses to attack? An answer here demands studies of the plant–herbivore interaction at a level of detail similar to that common in plant–pathogen studies. It seems to me that there is no putative ecological phenomenon in which it is more important to have a good understanding of mechanism than this one. In most of these systems we still do not know what produces the impacts on herbivores.

Second, are plants responding to herbivores, damage, or to introduced microbial products? This question also needs an approach commonly seen in plant pathology. This book provides many patterns that resemble responses to pathogens, and good evidence that the identity of the damaging agent influences the plant's response. Yet it contains very few studies involving vertebrates or piercing–sucking insects; the vast majority of examples involve folivory.

Third, are plant responses truly systemic, or merely local (but widespread)? I was very disappointed to find almost no discussion of elicitors or systemic signaling in this book, even though these are among the most rapidly advancing fronts in this area. It is clear from this book that investigators are rarely structuring studies to identify the cues, scale, and internal signaling necessary to define plant changes as responses.

Fourth, we need to know whether induced changes have a heritable basis. Raffa, alone, makes a case for the heritability of terpene induction in conifers. Clearly, if we want to know whether damage-induced changes can be influenced by selection, we need to know their heritable basis, if any.

Fifth, what, if any, is the ecological significance of induced changes? Several authors report induced changes that are less than normal constitutive variation; can these be important? Karban uses some carefully selected references and his own work to suggest that the answer is yes at the herbivore population level. To what degree might this result be influenced by the growing conditions, age, etc., of the plant, and how do these compare with conditions in nature? By what mechanisms might induced changes influence herbivores? I

was frustrated to find only a few paragraphs (by Faeth) about the impact of induced variation on predators, parasites, or pathogens of herbivores, although these are well documented. It is premature to discount the ecological or evolutionary impact of induced responses before we appreciate the importance of plant and animal variation and the mechanisms involved at all three trophic levels.

Overall, the book is well produced, although there are some minor irritations. The page locations of terms in the Index are frequently inaccurate, and there are some mislabeled figures. Less than 2% of the references are more recent than 1988, and this is a fast-moving area. It is clear that the majority of authors did not see other chapters from the same book. Several authors cite published conclusions that are contradicted directly by their book-mate experts in their own chapters.

It is clear that plants respond to herbivory in interesting and potentially important ways. This is a fascinating phenomenon that deserves further (and more careful) study and could have wide-ranging implications for forest and crop management. This book provides a good introduction to and review of much of the work in this area in a series of interesting, well-written chapters by top experts. It is a gold mine of research ideas and directions and can only stimulate more activity in this area. I find it one of the most exciting books on my shelf.

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ANNOUNCEMENT

EIGHTH ANNUAL PENN STATE SYMPOSIUM IN PLANT PHYSIOLOGY

"PLANT SIGNALS IN INTERACTIONS WITH OTHERS"

The 8th PSU Plant Physiology Symposium will be held at State College, PA, on May 20–22, 1993. It will focus on chemical and electrical signals used by plants while interacting with other organisms. Twenty-six talks by international leaders will be presented in five sessions: "General Signalling Mechanisms," "Local Signal Exchange with Enemies," "Systemic Signalling in Response to Pathogens and Pests," "Signals at the Genome Level," and "Signals among Friends." Some specific topics include: sensing touch, signalling with parasitic angiosperms, signalling in interactions with symbiotic bacteria, mycorrhizae and *Rhizobia*, systemic signals in responses to herbivores, molecular genetics of defensive responses, signalling during pollination, self-compatibility signals, and signalling to protective arthropods. All major chemical (e.g., salicylic acid, jasmonate, protein, ethylene, etc.) and electrochemical mechanisms known will be reviewed. Although focusing on mechanism, this symposium should be very interesting and useful to anyone working at any level on plant interactions with microbes, herbivores, pollen vectors, or other plants. We anticipate and wish to promote extensive interaction among basic and applied, academic and industrial scientists of many disciplines. Poster presentations are solicited, and travel awards will be available for student and postdoctoral presenters.

For information: Jack C. Shannon, Intercollege Graduate Program in Plant Physiology, 103 Tyson Building, Penn State University, University Park, PA 16802, Phone: 814-863-2192, FAX: 814-863-6139; or call Jack C. Schultz (814-863-4438) or (Ilya Raskin 908-932-8734) (Co-organizers).

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USE OF PREDATOR ODORS TO PROTECT CHICK-PEAS FROM PREDATION BY LABORATORY AND WILD MICE

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Abstract—Synthetic stoat odor (3-propyl-1,2-dithiolane and 2-propylthietane) and fox fecal odor (2,5-dihydro-2,4,5-trimethylthiazoline) at various concentrations were applied to chick-peas (*Cicer arietinum*) at sowing in an investigation aimed at reducing damage caused by house mice (*Mus musculus*). Stoat odor at 10% concentration exerted a measure of protection, as did 1% fox odor against predation by laboratory mice. Wild mice were less affected by synthetic predator odor and appeared to have a shorter memory for it. Laboratory mice cannot be regarded as surrogate wild mice, when used in experimental situations such as those employed here.

Key Words—Wild mice, laboratory mice, house mice, *Mus musculus*, chick-pea, *Cicer arietinum*, predation, predator odor, 3-propyl-1,2-dithiolane, 2-propylthietane, 2,5-dihydro-2,4,5-trimethylthiazoline.

INTRODUCTION

Growers of chick-peas (*Cicer arietinum*) in the western part of Victoria, Australia, not infrequently experience substantial losses of newly sown peas to mice. The problem is most pronounced in those years when mouse numbers are at plague proportions. It is reported that mice dig up germinating peas from 24 to 72 hr following sowing and eat the emerging shoot (P. Whykes, personal communication). This study was undertaken: (1) to determine whether laboratory mice could be used as "substitute" wild mice when investigating possible solu-

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tions to the problem, and (2) to investigate the effect of treating the peas, prior to planting, with synthetic 3-propyl-1,2-dithiolane and 2-propylthietane [substances naturally found in stoat (*Mustela erminea*) anal gland secretion] (Crump, 1982) and 2,5-dihydro-2,4,5-trimethylthiazoline [naturally found in red fox (*Vulpes vulpes*) feces] (Sullivan et al., 1988a).

The successful use of predator odors as repellents has been examined in a number of studies. Sullivan et al. (1985b) have demonstrated that salal (*Gaultheria shallon*) consumption by black-tailed deer (*Odocoileus hemionus columbianus*) was reduced when the leaves were treated with fox urine and fecal extracts. Dog urine has been shown to minimize browsing damage caused by swamp wallabies (*Wallabia bicolor*) and rabbits (*Oryctolagus cuniculus*) in eucalypt and pine plantations (Montague et al., 1990). Coyote (*Canis latrans*) urine repelled tame mule deer (*O. hemionus*) from feeding on pelleted rations (Andelt et al., 1991) as well as providing consistent suppression of deer browsing on salal (Sullivan et al., 1985b). Allan et al. (1984) demonstrated that foliage of seedlings watered with quadrivalent selenium was browsed less by deer due to the production of a metabolic product with a garliclike odor by the leaves. Sullivan et al. (1985a) demonstrated reduced browsing to lodgepole pine (*Pinus contorta*) by snowshoe hares (*Lepus americanus*) for up to seven days when stoat anal gland secretion was applied to seedlings. Sullivan and Crump (1984) noted that 3-propyl-1,2-dithiolane and 2,2-dimethylthietane induced a negative response of short duration in snowshoe hares when feeding on conifer seedlings in the field and laboratory. The short period of effectiveness was attributed more to evaporative loss rather than habituation by the hares. An enhanced efficiency and durability of repulsion using mixtures of dithiolanes may be due to the ready polymerization of these compounds on contact with the air (Sullivan et al., 1988b). Protection of apple trees against vole attack by *Microtus montanus* and *M. pennsylvanicus* for up to five months has been reported following the application of a 1 : 1 ratio of 2-propylthietane and 3-propyl-1,2-dithiolane to the trees (Sullivan et al., 1988a). Further studies revealed that pocket gophers (*Thomomys talpoides*) clearly avoided 2,5-dihydro-2,4,5-trimethylthiazoline (Sullivan et al., 1988b). Vernet-Maury and Constant (1991) have demonstrated that this compound is effective in repelling Norway rats (*Rattus norvegicus*).

When confronted with a request from Victorian farmers to help them protect chick-peas from mice, we conducted experiments in the laboratory with synthetic extracts from stoat anal glands and fox feces.

METHODS AND MATERIALS

Two types of mice were used in this investigation: wild-caught mice from the university campus and laboratory mice of an outbred strain. All mice used had had the opportunity to eat chick-peas prior to being used in the experiments.

Thirty mice of each type (five groups of six individuals) were established as test subjects. Three groups of six mice, randomly selected from the five established groups, were required for each odor trial. This procedure aimed to minimize bias that may have resulted from repeated exposure of mice to specific odor types. No mouse was used on consecutive nights.

Stoat odor (a 1 : 1 ratio mixture of 3-propyl-1,2-dithiolane and 2-propylthietane) and fox fecal odor (2,5-dihydro-2,4,5-trimethylthiazoline) were prepared at concentrations of 0.1%, 1.0%, and 10% using pure mineral oil as a diluant (Industrial Oil, Shell Australia Ltd.). Urine from a juvenile male New Zealand White rabbit was used as a novel odor.

Chick peas were prepared for testing by applying two strokes of the required odor preparation [stoat, fox, rabbit (i.e., novel odor), mineral oil (control; no odor)] to the pea using an artist's small watercolor brush. Peas were sowed in commercially available seed trays which has 100 individual pots. Ten control and ten odor-treated seeds were planted at random in each tray, and each tray was incubated for 12 hr at 22°C before being presented to the test mice in order to establish germination. Trays were presented in a bank of six test arenas, each of which measured 100 cm × 100 cm × 300 mm, each housing one mouse. A nesting box was provided in each arena as a refuge, and the arena floor was covered in eucalyptus sawdust. Each arena was topped with a tightly fitting Plexiglas lid and supplied with air via a small air pump. An exhaust tube from each arena was plumbed into the laboratory's air conditioning system to prevent odors from one arena contaminating another. After a period of 12 hr the numbers of control and odor peas discovered by each mouse were counted and the arena was thoroughly cleaned.

Following completion of the odor trials, an additional experiment was conducted. One week following exposure of mice to predator odors, during which they had no contact with predator odors, 18 mice were presented with trays of 20 control peas planted randomly. Digging was noted as previously described. This experiment was repeated three weeks later, when there had been a four-week time period between exposure to predator odor and subsequent testing.

RESULTS

Figure 1 shows the effect of the various stoat and fox odor concentrations, rabbit odor, and absence of odor (control) on pea location by wild-caught and laboratory mice. It is apparent that the two types of mice do not respond identically. A two-way ANOVA analysis revealed that odor treatment exerts a significant effect on the pattern of pea discovery ($F_{6,13} = 9.72$, $P < 0.001$); neither the type of mouse nor the interaction of mouse type and odor exerted a significant effect ($F_{1,13} = 1.41$, $P > 0.05$; $F_{6,13} = 1.70$, $P > 0.05$, respectively).

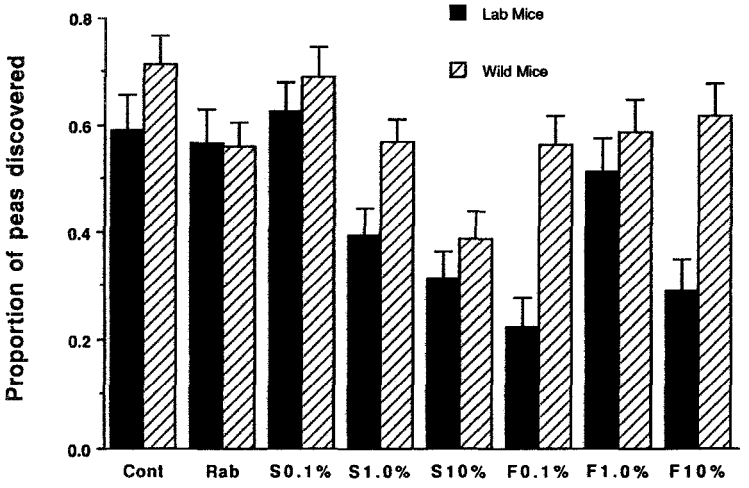

FIG. 1. Proportion of chick-peas discovered by laboratory and wild mice under various odor-repellent treatments. (Error bars ± 1 SE). Cont = control: peas treated only with mineral oil. Rab = rabbit urine: novel odor. S0.1%, S1.0%, and S10% = stoat odor (1 : 1 mixture of 3-propyl-1,2-dithiolane and 2-propylthietane) at various concentrations. F0.1%, F1.0%, and F10% = fox odor (2,5-dihydro-2,4,5-trimethylthiazoline) at various concentrations.

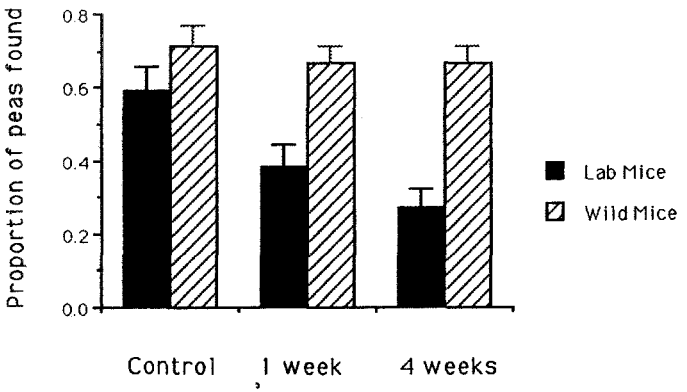

FIG. 2. Proportion of chick-peas discovered by laboratory and wild mice after a specific time interval since last exposure to either stoat or fox odor. (Error bars ± 1 SE). Cont = control: peas treated only with mineral oil; 1 week = time interval of one week, 4 weeks = time interval of four weeks.

Figure 2 examines the effect a prior encounter with predator odors has on subsequent pea detection by mice following the removal of the odor source. The effects of three different odor experiences on the proportion of peas removed were investigated: no history of odor encounter, within one week following an encounter with a predator odor, and contact with predator odor four weeks previously. It is apparent that the two types of mouse removed very different proportions of peas as a result of their past experience with predator odors. A two-way ANOVA of the data revealed that both previous experience ($F_{2,5} = 5.88$, $P < 0.01$, >0.001) and mouse type ($F_{1,5} = 35.1$, $P < 0.001$) exert a significant effect on the data but that the interaction of the two does not. Ryan's test (Day and Quinn, 1989) on the data indicate that the number of peas found in the control phase is significantly different from both odor trials but that there is no difference between these two.

DISCUSSION

The results of this investigation reveal that the treatment of chick-peas with 10% stoat odor and, to a lesser extent, 1% stoat odor, affords the peas a measure of protection against mouse attack. It is apparent from Figure 1 that the level of protection is only partial and that at best only about 70% of peas treated with the most effective odorants are protected. The data further reveal that laboratory mice and wild-caught mice may respond to predator odors in different ways. Laboratory mice appear more readily influenced by predator odor, being more responsive to it than are wild-caught mice (Figure 1). This is the case in all trials. They also appear to have a better memory of predator odor than do wild-caught mice and are able to display that memory over at least a four-week time period (Figure 2). By contrast wild-caught mice show no significant variation in the proportion of peas they find as a result of previous experience (Figure 2). A number of possible genetic and/or behavioral explanations for this difference are possible. Kavaliers (1990) has demonstrated that deer mice taken from an island that lacked weasels reacted to weasel odor in a significantly different manner from those that had been sympatric with weasels. He reported that sympatric mice responded to weasel odor immediately, as evidenced by an increase in latency to a thermal stressor. Insular mice failed to discriminate on this basis between the odor of weasel and rabbit. Discussing aspects of the concept of foraging risk and foraging efficiency in the pika, Holmes (1991) points out that foraging is a risk-sensitive activity. Riskier foraging behavior is associated with the times of year when nutritional need is highest, such as during lactation. Mice presumably face a risk every time they emerge from a refuge to feed; wild-caught mice may thus be better at risk assessment than laboratory mice. Whether they have a less well-developed odor memory than laboratory mice is not known.

The results of this investigation suggest that predator odor is partly effective in curbing the predatory activities of wild-caught and laboratory bred mice, but that the two types of mice respond differently to the odorous repellents used. The study suggests that laboratory mice cannot be used as surrogates for wild mice, at least under the experimental conditions used in this investigation.

REFERENCES

ALLAN, G.G., GUSTAFSON, D.I., MIKELS, R.A., MILLER, J.M., and NEOGI, S. 1984. Reduction of deer browsing of Douglas fir (*Pseudotsuga menziesii*) seedlings by quadrivalent selenium. *For. Ecol. Manage.* 7:161–181.

ANDELT, W.F., BURNHAM, K.P., and MANNING, J.A. 1991. Relative effectiveness of repellents for reducing mule deer damage. *J. Wildl. Manage.* 55:341–347.

CRUMP, D.R. 1982. Synthesis of (2*S*)-2-propylthietane. *Aust. J. Chem.* 35:1945–1948.

DAY, R.W., and QUINN, G.P. 1989. Comparisons of treatments after an analysis of variance in ecology. *Ecol. Monogr.* 59:433–463.

HOLMES, W.G. 1991. Predator risk affects foraging behavior of pikas: Observational and experimental evidence. *Anim. Behav.* 42:111–119.

KAVALIERS, M. 1990. Responsiveness of deer mice to a predator, the short-tailed weasel: Population differences and neuromodulatory mechanisms. *Physiol. Zool.* 63:388–407.

MONTAGUE, T.L., POCOCK, D.C., and WRIGHT, W. 1990. An examination of the animal browsing problem in Australian eucalypt and pine plantations, pp. 203–208, *in* L.R. Davis and R.E. Marsh, (eds.). Proceedings of the 14th Vertebrate Pest Conference, University of California-Davis.

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., NORDSTRUM, 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.

VERNET-MAURY, E., and CONSTANT, B. 1991. Repellent effects of trimethyl thiazoline in the wild rat, *Rattus norvegicus* Berkenhout. Chemical Signals in Vertebrates VI, *Abstract*, 96.

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VANILLIN-HCl METHOD FOR CONDENSED TANNINS: EFFECT OF ORGANIC SOLVENTS USED FOR EXTRACTION OF TANNINS

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Abstract—Tannins are generally extracted using aqueous organic solvents, mainly methanol and acetone. The presence of various concentrations of methanol in the sample containing catechin or tannins did not alter the kinetics of their reaction, but the absorbance depended on the amount of methanol in the sample; the higher the methanol, the higher was the absorbance. Acetone reacted with acidified vanillin to produce a chromogen with λ_{max} at 548 nm, which produce a substantial error in the determination of condensed tannins. In the presence of acetone, the time courses of the reaction for catechin and tannins were different, which depended on the acetone concentration and the reaction temperature. Reaction conditions for catechin and tannins that enable their measurement in the presence of acetone are presented.

Key Words—Condensed tannins, catechin, vanillin-HCl method, organic solvents, methanol, acetone.

INTRODUCTION

The vanillin-HCl method is widely used for the determination of condensed tannins. The chemistry of the reaction involved, its specificity, and various assay procedures have been reviewed by Deshpande et al. (1986). Recently, we observed great variability in the levels of condensed tannins when present in aqueous organic solvents, especially acetone. Therefore, a systematic study was undertaken to examine the effect of various concentrations of methanol and acetone at different assay temperatures in the vanillin-HCl method of Broadhurst

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and Jones (1978), the method in routine use in various laboratories. The study has wide implications as tannins are extracted from plant materials either by using aqueous methanol or aqueous acetone (Hagerman, 1988; Cork and Krockenberger, 1991; Makkar et al., 1990, 1991). Price et al. (1978) have examined several factors that influence the vanillin reaction. To our knowledge, no one has yet drawn attention to the effect of the presence of organic solvents used for the extraction of tannins in the vanillin reaction.

METHODS AND MATERIALS

Catechin and vanillin were purchased from Sigma Chemie GmbH, Deisenhofen, Germany. All other reagents were of analytical grade.

The tannins of *Quercus incana*, *Dichostachys cinerea*, and *Cajanus cajan* leaves were purified by adsorption on Sephadex LH-20 (Strumeyer and Malin, 1975) in 95% ethanol. The tannins were desorbed by 50% acetone after washing the gel with 200 ml of 95% ethanol. Acetone was evaporated at 30°C under vacuum and the rest of the material lyophilized to obtain tannins.

The vanillin-HCl method of Broadhurst and Jones (1978) was used except that the assay volume was reduced by half. In short, to 0.25 ml of the sample was added 1.5 ml of vanillin reagent (4% w/v in methanol) and then 0.75 ml concentrated (37%) HCl. The reaction was carried out in either 1-cm disposable plastic cuvettes in the laboratory environment of 22 ± 1°C (termed 22°C air) or in glass tubes placed in a water bath at 22 ± 1°C (termed as 22°C water) and also at 30°C (termed as 30°C water bath). After addition of the HCl, the contents of the tubes were mixed by shaking them in the water bath for 3–4 sec. The contents of the cuvettes were mixed by covering their mouths with parafilm. Neither cuvettes nor tubes were wrapped in aluminum foil. The reaction was carried out in normal laboratory daylight. Acetone or methanol concentrations were changed in the sample containing catechin or tannins and are expressed as percent in the sample volume of 0.25 ml. Normally, catechin or tannins were dissolved in 50% methanol or 70% acetone. Aliquots (50 μl) of these were taken and made up to 0.25 ml with water and methanol or acetone to get the desired concentration of acetone or methanol in the sample. Acetone concentration in the sample containing tannins was not increased beyond 70%, as this is the maximum level generally used for extraction of tannins from plant materials. Absorbance was recorded at 500 nm at different time intervals using a Hitachi U-2000 Spectrophotometer.

RESULTS AND DISCUSSION

Effect of Acetone Concentration

Figures 1A and B show changes in absorbance with time at 28% and 70% acetone concentration in the sample with and without catechin. The absorbance varied both at 22°C air and 30°C water bath with time and acetone concentra-

tion. The higher the acetone concentration in the sample, the higher was the decrease in absorbance with time. This explains the different values for condensed tannins we observed when following the method of Broadhurst and Jones (1978), according to which the absorbance could be measured between 15 and 60 min with a precision of ± 1 μg tannin. Thus the need to optimize time for measuring absorbance when catechin and/or tannins are present in the acetone containing medium becomes obvious.

Increase of absorbance in the absence of catechin (Figure 1) suggested formation of a chromophore by the reaction of acetone with acidified vanillin, the λ_{max} of which was 548 nm. In the presence of both catechin and acetone (70%) in the sample at 22°C air, the λ_{max} was 498 nm at 2 min after the start of the reaction and shifted to 503 nm after 30 min and to 548 nm after 60 min (Figure 2). The initial temperature appears to be important in determining the reaction between acetone and the acidified vanillin. The addition of concentrated HCl to the assay mixture produced heat. The cuvettes were warmer for a longer period after addition of the HCl compared to when the reaction was carried out in test tubes kept in a water bath at 30°C. This is due to the faster dissipation of heat from the tubes in the water bath because heat is dissipated from the tubes to the water by conduction and from the cuvettes to the atmosphere mainly by

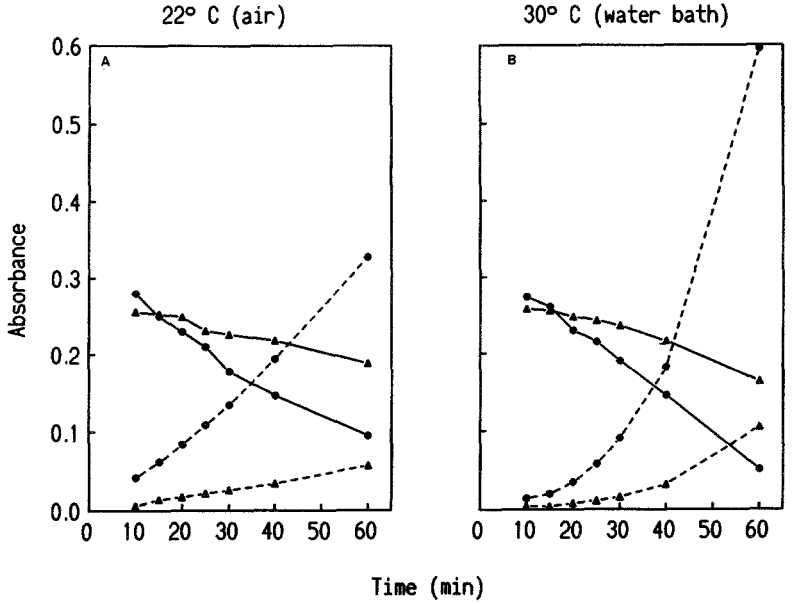

FIG. 1. Absorbances (500 nm) with (—) and without (- -) catechin in the sample containing 28% (▲) and 70% (●) acetone (absorbances without catechin are against acetone-free blanks, and with catechin are against blanks containing same concentration of acetone).

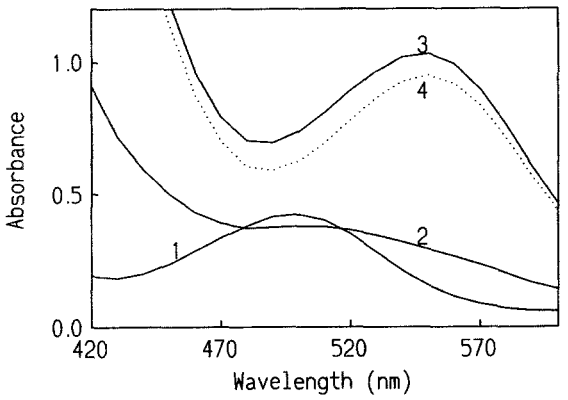

FIG. 2. Spectrum at different start times of the reaction (1, 2, 3 with catechin in 70% acetone at 2, 30, and 60 min, respectively, and 4 without catechin but containing 70% acetone at 60 min).

radiation (and to some extent by conduction from the bottom of the cuvette to the surface on which it is kept). The higher absorbance of the reaction of acetone with acidified vanillin up to 40 min at 22°C air compared to 30°C water bath appears to be due to the initial higher temperature of the assay. Therefore, further studies on optimization of reaction conditions also included the reaction in test tubes placed in a 22°C water bath, as this would further increase the rate of heat dissipation, thereby slowing the unwanted reaction of acetone with acidified vanillin. It was found that at 22°C water bath, the temperature of the assay mixture reached 29°C when concentrated HCl was added, and it came down to 23°C within 3 min, whereas at 22°C air, the temperature reached 39°C and remained above 30°C even 10 min after addition of the HCl.

Absorbance of catechin. Figure 3 shows the absorbance (using blanks containing the same amounts of acetone) at acetone concentrations of 14, 28, 42, and 70% at different time intervals and at different temperatures. At 30°C water bath, the absorbance reached the maximum value in 2 min at all acetone concentrations. At the lower concentration of acetone, the plateau was broad and became narrow with an increase in acetone concentration. At 70% acetone, the absorbance reached its peak at 2 min and then immediately started decreasing (Figure 3A). This could lead to substantial error if absorbance is not recorded exactly after 2 min. At 22°C air, the absorbances were at a plateau at 6–25 min with 14% acetone, 4–10 min with 28% acetone, 4–6 min with 42% acetone, and 1–4 min with 70% acetone (Figure 3B). The trend at 22°C water bath was similar except that the curves were flatter; the plateau was at 10–40 min with 14% acetone, 5–22 min with 28% acetone, 4–8 min with 42% acetone, and 4–7 min with 70% acetone (Figure 3C). For all the acetone concentrations

except 14%, the absorbances were at a plateau at 6 min of incubation at 22°C water bath. Recording of absorbance at 6 min at 14% acetone in the sample would produce an error of 1.5–2%.

Figure 4 shows calibration curves for catechin at different acetone concentrations at 22°C water bath. The absorbances were recorded exactly at 6 min except for the 14% acetone, where the absorbances were read at 10 min. At all

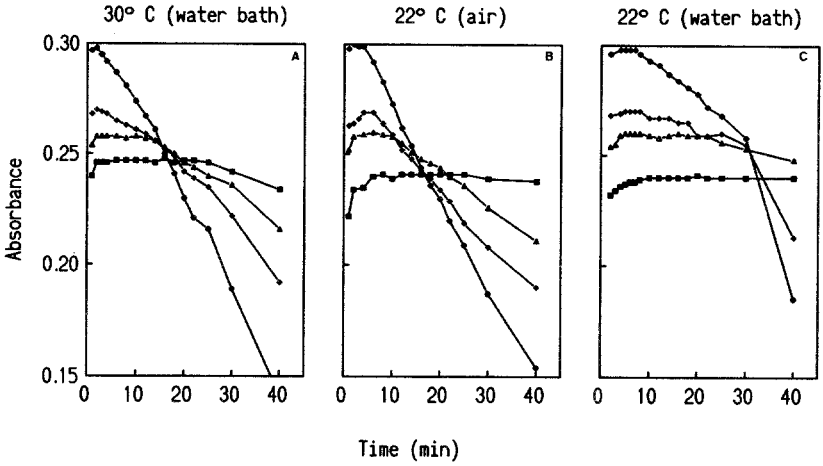

FIG. 3. Change in absorbance (500 nm) of catechin in different acetone concentrations and at different temperatures (acetone concentrations: ■, 14%; ▲, 28%; ♦, 42%; ●, 70%).

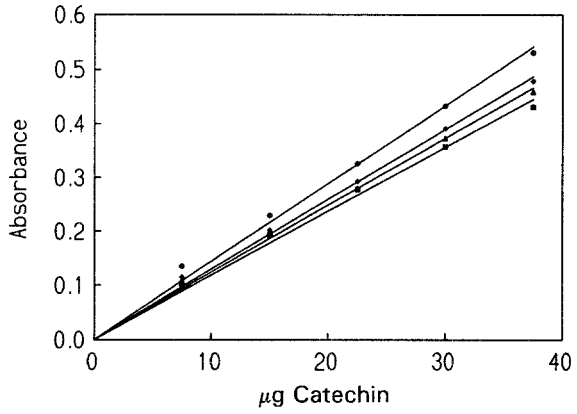

FIG. 4. Calibration curves for catechin at different acetone concentrations (■), 14%; ▲, 28%; ♦, 42%; ●, 70%).

acetone concentrations, there was no statistical difference between these values (Figure 4) and those at 30°C water bath at 2 min of incubation (results not shown). Higher temperature (30°C) decreased the time for obtaining maximum absorbance but not the maximum absorbance. This holds true when the same concentrated HCl was used throughout. We found that for catechin, HCl from a newly opened bottle of concentrated HCl (37%) gave a 15% higher absorbance than that obtained using HCl from an old bottle of concentrated HCl (37%) that had been opened several times. Further experiments with tannins were carried out in test tubes kept in a water bath at 22 ± 1°C as the reaction between acetone and acidified vanillin was minimal and the curves were broader under these conditions.

Absorbance of Tannins. Figure 5 shows changes in absorbance of tannins from *C. cajan*, *D. cinerea*, and *Q. incana* at 22°C water bath. At all acetone concentrations studied (14–70%), the absorbances were at a plateau at 20 min for all three tannins. The pattern for crude extracts prepared from these plant materials in 70% acetone was similar (results not shown). The pattern of the curves vis-à-vis acetone concentration (Figure 5) was similar to catechin (Figure 3), i.e., curves were at a plateau for a greater time at lower acetone concentrations. However, the kinetics of the reaction for catechin and tannins are different. Absorbances for catechin were at maximum at 6 min for 28–70% acetone and at 10 min for 14% acetone, whereas for tannins the absorbances were at a plateau at 20 min.

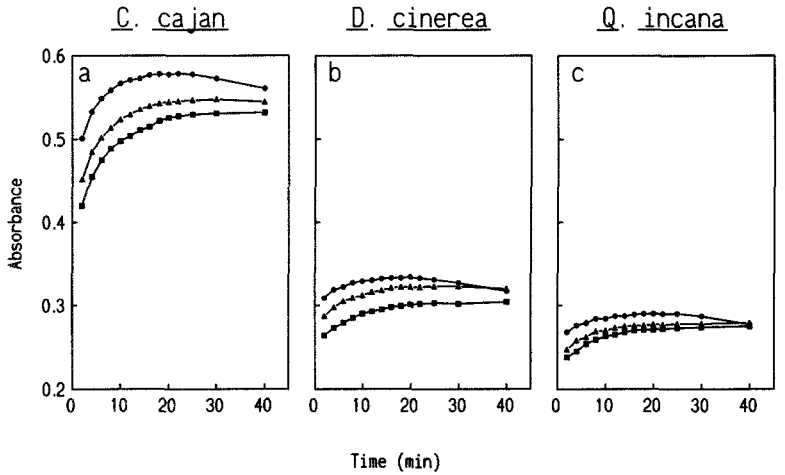

FIG. 5. Change in absorbance (500 nm) of tannins from *Cajanus cajan*, *Dichostachys cinerea*, and *Quercus incana* in different acetone concentrations (■, 14%; ▲, 42%; ●, 70%).

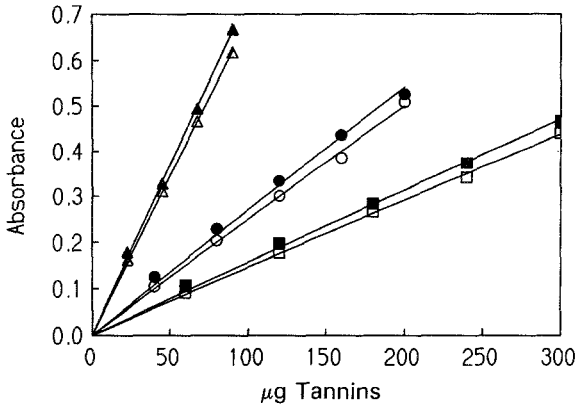

FIG. 6. Calibration curves from tannins from *Quercus incana* (□, ■), *Dichostachys cinerea* (○, ●), and *Cajanus cajan* (△, ▲) in different acetone concentrations (unfilled, 14%; filled, 70%).

The calibration curves using these three tannins are shown in Figure 6. The absorbances were recorded at 20 min. As for catechin (Figure 4), the absorbances were higher at a higher concentration of acetone.

Effect of Methanol

The methanol concentrations in the sample (0.25 ml) containing catechin or tannins were kept at 10%, 50%, and 90%, and the reaction was performed at 22°C air and 30°C water bath. For the catechin and the tannins from *Q. incana*, *D. cinerea*, and *C. cajan*, the absorbances were at a plateau from 20 min to 40 min at all three methanol concentrations studied and at both temperatures studied (results not shown). These results are similar to those obtained by Broadhurst and Jones (1978), where tannins were in water. The absorbances depended on the concentration of methanol and were higher at higher concentrations of methanol (Figure 7), suggesting that the presence of water has an adverse effect on the assay.

Conclusions

A closer look at the calibration curves for catechin (Figures 4 and 7) shows that the curves deviate considerably from linearity. This feature of the reaction has also been noticed by Price et al. (1978). We suggest the use of a regression equation (and not the use of a curve forced to pass through the origin).

The reagents and the recommended procedures are as follows:

Reagents. As described by Broadhurst and Jones (1978).

Procedure. (A) When catechin or tannins are in aqueous acetone:

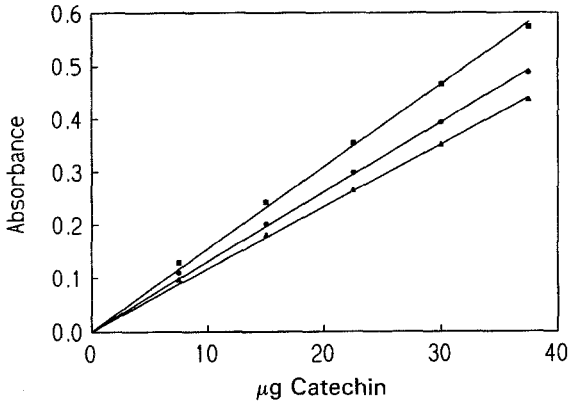

FIG. 7. Calibration curves for catechin at different methanol concentrations (▲, 10%; ●, 50%; ■, 70%).

Carry out the reaction in test tubes placed in a water bath at 22 ± 1°C. Record the absorbance at 6 min for catechin present in 28–70% acetone, and at 10 min when in 14% aqueous acetone in a sample volume of 0.25 ml. For tannins, record the absorbance at 20 min.

(B) When catechin or tannins are in aqueous methanol:

The absorbances should be measured after 20 min for both catechin and tannins. Reaction may be carried out in cuvettes or in test tubes in a laboratory environment of 22–30°C.

The blanks and calibration curves should have the same amount of acetone or methanol as the test/unknown sample.

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REFERENCES

BROADHURST, R.B., and JONES, W.T. 1978. Analysis of condensed tannins using acidified vanillin. *J. Sci. Food Agric.* 29:788–794.

DESHPANDE, S.S., CHERYAN, M., and SALUNKHE, D.K. 1986. Tannin analysis of food products. *CRC Crit. Rev. Food Sci. Nutr.* 24:401–449.

CORK, S.J., and KROCKENBERGER, A.K. 1991. Methods and pitfalls of extracting condensed tannins and other phenolics from plants: Insight from investigations on *Eucalyptus* leaves. *J. Chem. Ecol.* 17:123–134.

HAGERMAN, A.E. 1988. Extraction of tannins from fresh and preserved leaves. *J. Chem. Ecol.* 14:453–461.

MAKKAR, H.P.S., SINGH, B., and NEGI, S.S. 1990. Tannin levels and their degree of polymerisation and specific activity in some agroindustrial byproducts. *Biol. Wastes* 31:137–144.

MAKKAR, H.P.S., SINGH, B., and DAWRA, R.K. 1991. Tannin levels in the leaves of some oak species at different stages of maturity. *J. Sci. Food Agric.* 54:513–517.

PRICE, M.L., SCOYOC, S.V., and BUTLER, L.G. 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *J. Agric. Food Chem.* 26:1214–1218.

STRUMEYER, D.M., and MALIN, M.J. 1975. Condensed tannins in grain sorghum: Isolation, fractionation, and characterization. *J. Agric. Food Chem.* 23:909–914.

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THE ROLE OF OLFACTION IN CHEMOSENSORY-BASED PREDATOR RECOGNITION IN THE FATHEAD MINNOW, *Pimephales promelas*

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Abstract—Solitary fathead minnows (*Pimephales promelas*) were rendered anosmic and exposed to chemical stimuli from a predatory northern pike (*Esox lucius*) to determine the role of olfaction in the minnow's ability to recognize predators on the basis of chemical stimuli. Anosmic fish did not respond to the pike stimuli with a typical fright reaction, while control fish, with intact olfactory receptors, did. These results demonstrate that the olfactory system is necessary for the ability of fathead minnows to recognize northern pike as a predator and that the gustatory and single-celled chemosensory systems are not sufficient for this recognition in the absence of olfactory input. Olfactory impairment was behaviorally confirmed by exposing minnows to alarm substance (Schreckstoff).

Key Words—Olfaction, chemoreception, solitary chemosensory cells, predator recognition, alarm pheromone, Schreckstoff, fathead minnow, *Pimephales promelas*, northern pike, *Esox lucius*.

INTRODUCTION

The recognition of predators through chemoreception occurs in a variety of vertebrates (review: Weldon, 1990). For many prey species, including the fathead minnow (*Pimephales promelas*), the ability to chemically recognize a predator is an important component of an individual's antipredator arsenal, because it may result in a greater number of ecological situations in which predator recognition is possible. For example, prey fishes with chemical predator rec-

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ognition abilities may be able to utilize habitats with poor optic properties. As well, nonvisual detection of cryptic ambush predators, such as the northern pike (*Esox lucius*) (Savino and Stein, 1989), may be critical to the survival of prey fishes (Mathis et al., 1993).

In fishes, predator recognition through chemoreception could result from olfaction, gustation, or single-celled chemoreception (Kotrschal, 1991). Solitary chemosensory cells (SCCs), which are secondary epidermal sensory cells (Whitear, 1971), may be used in predator recognition and avoidance, as they respond to mucus of heterospecific fishes (Baatrup and Doving, 1985; Kotrschal et al., 1989; Peters et al., 1987, 1990, 1991). Little information exists on the role of gustation in predator recognition. However, fishes possess numerous taste receptors (Hara, 1971) that could potentially be used in predator recognition. Olfaction has been demonstrated to be responsible for mediating predator recognition in coho salmon (*Oncorhynchus kisutch*) (Rehnberg et al., 1985), brook trout (*Salvelinus fontinalis*) (Keefe, 1992) and the bitterling (*Rhodeus sericesu amarus*) (Kasumyan and Pashchenko, 1985). Olfaction is based on nerve cell receptors contained within the olfactory pits (Kleerkloper, 1969; Hara, 1971). As these receptors are highly localized in the nares, this sensory system, unlike the gustatory or single-celled chemosensory system, can be blocked.

Mathis et al. (1993) demonstrated that fathead minnows from a population sympatric with northern pike are able to recognize pike as predators through chemoreceptive cues alone. They did not, however, determine the specific chemosensory system used by the minnows to recognize the chemical stimuli from the pike. The goal of this study was to determine the role of olfaction in the chemosensory-based predator recognition ability of fathead minnows. We tested the hypothesis that sham-operated control and olfactorily impaired minnows differed in their response to chemical stimuli from northern pike. If olfactorily blocked minnows exhibit a fright reaction [i.e., defensive behavior that occurs upon recognition of a predator (review: Smith, 1992)] to the chemical stimuli from a pike, then a chemosensory system other than olfaction must be used in predator recognition. This result would not, however, eliminate the possibility that olfaction can be used for predator recognition. In contrast, the lack of a fright reaction by olfactorily impaired minnows would indicate that olfaction is necessary for minnows to identify northern pike as a predator using chemical cues.

To confirm olfactory impairment behaviorally, we also tested control and olfactorily impaired minnows for a fright reaction to minnow alarm substance. The alarm substance, or Schreckstoff, probably hypoxanthine-3-(*N*)-oxide (Pfeiffer et al., 1985), is detected through olfaction (Frisch, 1941; Pfeiffer et al., 1984). Therefore, we tested the hypothesis that control and olfactorily impaired minnows differed in their response to alarm substance.

METHODS AND MATERIALS

Collection and Maintenance. In September 1990, northern pike were collected from Eagle Creek, a tributary of the North Saskatchewan River in south-central Saskatchewan. They were maintained in a 300-liter tank at approximately 15°C on a 14 : 10 light–dark photoperiod and were fed once every five days with fathead minnows.

Fathead minnows were collected in the fall of 1990 from Pike Lake, an oxbow lake of the South Saskatchewan River in south-central Saskatchewan where they occurred in sympatry with northern pike. The minnows were maintained in outdoor pools (approximately 18,000 liters) at temperatures ranging from 15°C (fall months) to 4°C (winter months). In the spring of 1991, the minnows were placed in the laboratory in a 600-liter holding tank at approximately 15–18°C on a 14 : 10 light–dark photoperiod and were fed daily with commercial fish food.

Pike Stimulus Preparation. Prior to the stimulus collection, three pike [mean fork length = 19.2 cm ± 3.55 (1 SD)] were fed approximately equal volumes (range = 3–4 ml, measured by volumetric displacement in water) of swordtails (*Xiphophorous helleri*) for each of three feedings (i.e., once every five days). A swordtail diet was used on the last three pike feedings to eliminate secondary stimuli from the pike's diet of fathead minnows (Mathis and Smith, 1993). Mathis et al. (1993) demonstrated that this stimulus evokes a fright reaction from Pike Lake minnows. Just prior to the last feeding, the pike were moved from their 300-liter holding tank to a separate holding tank (150 liters). Approximately 1 hr later they were removed from the tank and rinsed with dechlorinated tap water to remove any swordtail residue from the pike's skin. The pike were then returned to an identical holding tank (150 liters) that contained clean water. Approximately 16 hr after the last feeding, the pike were placed in separate clear plastic stimulus collection chambers (26 × 8 × 8 cm) that contained 1200 ml of dechlorinated tap water. This 16-hr delay ensured that the pike did not regurgitate their stomach contents (Mathis, personal communication). The stimulus collection chambers were aerated but contained no filtration system. After three days, the pike were removed from the stimulus collection chambers. The stimulus water was pipetted into separate 5-ml polypropylene containers and was frozen at approximately −20°C.

Alarm Substance Stimulus Preparation. The alarm substance stimulus was prepared from 15 donor fathead minnows. The twelve male and three female donors [mean fork length = 5.10 ± .382 (1 SD)] were killed and a skin filet was taken from both sides of each fish. Total area of skin collected from all donors was approximately 29.0 cm^2. Immediately upon removal, the skin samples were placed in 100 ml of chilled glass-distilled water. The skin samples

were then homogenized with a polytron homogenizer and the homogenate was filtered through glass wool to remove any solid particles. The homogenate was diluted with 100 ml of glass-distilled water, resulting in a total volume of 200 ml that was frozen in separate 5-ml polypropylene containers.

Olfaction Impairment. Olfactory impairment was induced in 15 experimental minnows [mean fork length = 6.17 cm ± 0.623 (1 SD)] by injecting hot petroleum jelly (>150°C) into the olfactory pit of minnows anesthetized with 140 mg/liter of MS 222 (Triciane methanesulfonate). The petroleum jelly solidified immediately to block the olfactory pit. This technique was successfully used to induce anosmia in coho salmon (Rehnberg et al., 1985) and brook trout (Keefe, 1992). Upon completion of testing, each minnow was examined under a dissecting microscope to ensure that the plug remained intact.

Fifteen control minnows (mean fork length = 6.23 ± 0.477) were similarly anesthetized and subjected to a sham nasal blocking treatment in which two drops of hot petroleum jelly were deposited just ventral to the nares. Impairment of the olfactory system of the experimental minnows and the sham operation of the control minnows took place three days prior to testing these individuals.

Testing Protocol. Immediately following the olfactory impairment or the sham operation, the minnows were individually placed into separate Plexiglas acclimation tanks (45 × 45 × 20 cm). Clean water was constantly supplied to the acclimation tanks at a rate of approximately 250 ml/min, maintaining a constant water depth of 4–5 cm. The fish were fed daily with commercial fish food and were kept under a 14 : 10 light–dark photoperiod. After two days, the minnows were transferred into separate testing tanks that were identical to the acclimation tanks except that the flow rate was increased to 500 ml/min. The holding and testing tanks were the same as those used by Mathis et al. (1993). Acclimation and testing occurred at a mean water temperature of 16.7°C (range 16–18°C).

The test tanks were surrounded by an Opto-Varimex-Aqua tracking meter (Columbus Instruments), which lays down a grid of light beams across the tanks. A microcomputer that was interfaced with the tracking meter detected changes in the fish's movement by determining the number and location of light beams that were broken between a light source and photocell. Broken light beams were detected at intervals of 0.125 sec. Lemly and Smith (1986, 1987) described this testing apparatus in detail. Our system differed from that of Lemly and Smith (1986, 1987) only in that outflowing water was discarded rather than recirculated. Three measures of activity that typically decrease during a fright reaction in fathead minnows (Lawrence and Smith, 1989) were quantified by the computer: (1) total distance traveled (centimeters), (2) number of stereotypic movements (i.e., activity in which the fish breaks light beams without moving outside one grid square), and (3) total time active (seconds).

Utilizing video equipment, an observer in an adjacent room was able to

view the experimental tanks without disturbing the minnows. The observer was also able to inject the stimulus water into inflowing water lines that passed through the observation room before entering the test tanks. Experimental observations were conducted using the pike stimulus water between 0700 and 1130 hr. These trials lasted a total of 16 min, with 5.0 ml of the pike stimulus being injected at the end of 8 min. Injection of the stimulus water took approximately 5 sec. Subsequent observations of these same minnows were completed later that day between 1200 and 1730 hr using 5.0 ml of the alarm substance stimuli. Injection of the alarm substance stimulus was done in the same fashion as injection of the pike water stimulus. The mean time between tests with the same individuals was 255 min.

The minnows' response was based on three different parameters (total distance traveled, number of stereotypic movements, and total time active), each of which was tested independently. To test for differences in prestimulus activity that may have resulted from the olfactory impairment procedure, prestimulus activities of control and olfactorily impaired minnows were compared using a Wilcoxon-Mann-Whitney test (Siegel and Castellan, 1988) (Wx = Mann-Whitney U: Siegel, 1956) prior to exposure to the first stimulus (i.e., the pike stimulus). The significance of the response of the minnows to both pike stimuli and alarm substance stimuli was determined by the Wilcoxon signed rank test (Siegel and Castellan, 1988) (i.e., prestimulus versus poststimulus activity). For each stimulus, statistical comparisons of control and olfactorily impaired minnows were made using the Wilcoxon-Mann-Whitney test (Siegel and Castellan, 1988). In all cases two-tailed statistical tests were employed with $\alpha = 0.05$ and $N = 15$.

RESULTS

Prestimulus Activity: Control vs. Anosmic Fish. Prior to presentation of the first stimulus (i.e., the pike stimulus), the activity levels of the control and olfactorily impaired minnows were not significantly different, in terms of either distance traveled ($Wx = 221$, $P = 0.646$), number of stereotypic movements ($Wx = 209.5$, $P = 0.352$), or time active ($Wx = 209$, $P = 0.342$). These comparisons demonstrate that the normal activity levels of anosmic minnows were not altered by the olfactory impairment procedure.

Prestimulus vs. Poststimulus Activity. Control minnows significantly decreased their activity following exposure to chemical stimuli from northern pike in terms of distance traveled (mean decrease = 57.3%, Wilcoxon $T = 5.5$, $P < 0.001$), number of stereotypic movements (mean decrease = 40.0%, $T = 9$, $P = 0.002$), and time active (mean decrease = 39.0%, $T = 13$, $P < 0.006$). Control minnows also significantly decreased their activity following

exposure to alarm substance stimuli in terms of distance traveled (mean decrease = 69.8%, $T = 0$, $P < 0.001$), number of stereotypic movements (mean decrease = 49.4%, $T = 1$, $P < 0.001$), and time active (mean decrease = 52.7%, $T = 0$, $P < 0.001$). In contrast, olfactorily impaired minnows exhibited no significant change in distance traveled (mean increase = 0.6%, $T = 51$, $P = 0.639$), number of stereotypic movements (mean increase = 2.5%, $T = 51$, $P = 0.639$), or time active (mean increase = 5.0%, $T = 50.5$, $P = 0.619$) following exposure to chemical stimuli from northern pike. They also did not significantly alter their activity in terms of distance traveled (mean increase = 4.5%, $T = 42$, $P = 0.359$), number of stereotypic movements (mean decrease = 24.0%, $T = 27$, $P = 0.064$), or time active (mean decrease = 14.6%, $T = 29$, $P = 0.083$) following exposure to alarm substance stimuli.

Control vs. Experimental Conditions. In direct comparisons of control and experimental minnows, control minnows exhibited a significantly greater decrease in distance traveled (Wilcoxon-Mann-Whitney test, $Wx = 165.5$, $P = 0.006$) (Figure 1), number of stereotypic movements ($Wx = 167$, $P = 0.007$) (Figure 2), and time active ($Wx = 171$, $P = 0.011$) (Figure 3) than did olfactorily blocked minnows that were exposed to the chemical stimuli from pike. Similarly, control minnows exhibited a significantly greater decrease in distance traveled ($Wx = 155.5$, $P = 0.002$) (Figure 1), number of stereotypic movements ($Wx = 175$, $P = 0.018$) (Figure 2), and time active ($Wx = 175$, $P = 0.018$) (Figure 3) than did olfactorily blocked minnows that were exposed to alarm substance stimuli.

DISCUSSION

The results of this study demonstrate that chemical cues are necessary for fathead minnows to recognize northern pike as a predator. The role of olfaction in predator recognition has been documented in several vertebrates. Kats (1988), for example, demonstrated that larval small-mouthed salamanders (*Ambystoma texanum*) detected fish predators through olfaction. Individual salamanders with plugged nares failed to increase their use of refuges when exposed to chemicals from green sunfish (*Lepomis cyanellus*), while control individuals increased their use of refuges. Webster (1973) reported that kangaroo rats (*Dipodomys merriami*) with olfactory bulbectomies did not avoid sidewinders (*Crotalus cerastes*), while intact individuals did. Olfaction has also been demonstrated to be responsible for the ability of brook trout to recognize chemical stimuli from red fin pickeral (*Esox americanus*) (Keefe, 1992) and for bitterlings to recognize chemical stimuli from northern pike (Kasumyan and Pashchenko, 1984). Furthermore, olfaction mediates the avoidance response of juvenile coho salmon to L-serine (Rehnberg et al., 1985), an active salmon repellent in mammalian skin extract (Idler et al., 1956).

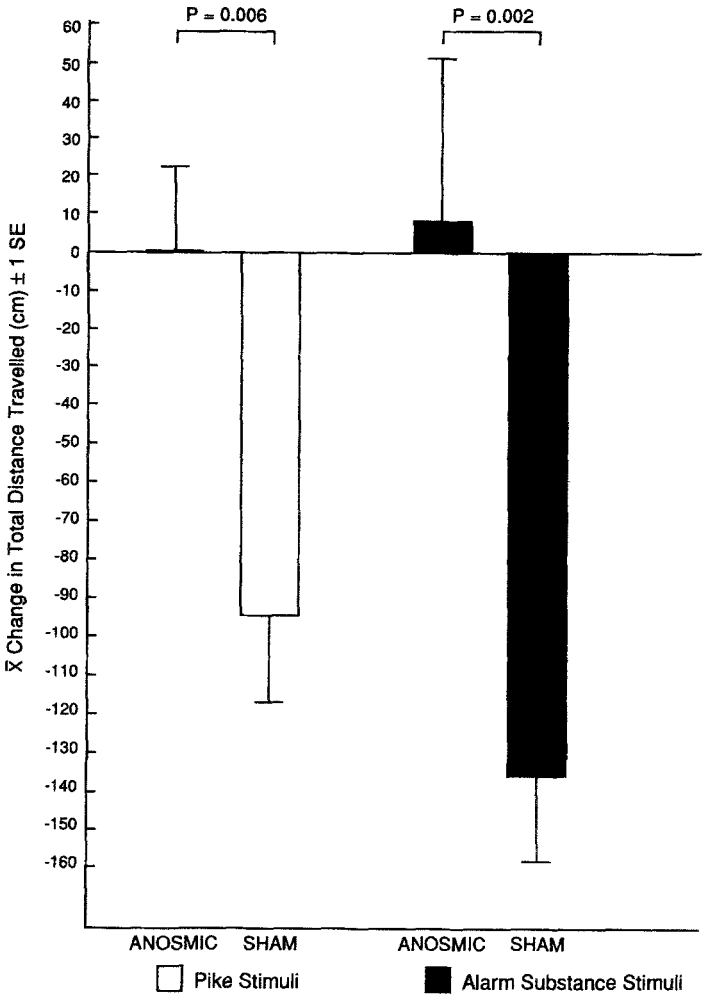

FIG. 1. Mean change in total distance traveled (cm) by sham operated (control) and anosmic fathead minnows following exposure to chemical stimuli from pike and to alarm substance stimuli.

Three lines of evidence confirm that the olfactory system of the experimental fish was impaired. Microscopic analysis upon completion of the tests revealed that the olfactory plugs remained intact in all of the experimental minnows. Control minnows significantly altered their activity following exposure to pike stimuli while olfactorily impaired minnows did not. Furthermore, control minnows significantly altered their activity following exposure to alarm substance stimuli while olfactorily impaired minnows did not.

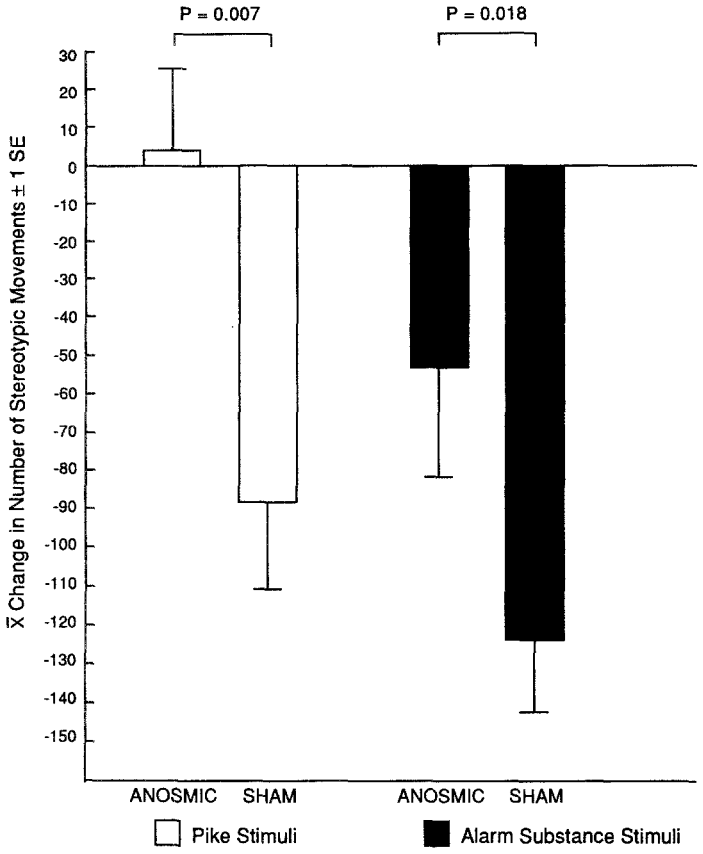

FIG. 2. Mean change in number of stereotypic movements by sham operated (control) and anosmic fathead minnows following exposure to chemical stimuli from pike and to alarm substance stimuli.

There is some controversy as to whether SCCs function in mediating recognition of chemical stimuli from predators or whether their function is related to feeding (Kotrschal et al., 1991). In our study the lack of a defensive response by anosmic minnows suggests that SCCs are not sufficient for recognition of chemical stimuli from pike. Physiological evidence from rocklings (*Ciliata mustela*) suggests that SCCs may be used in predator avoidance, as they appear tuned to the body mucus of heterospecific fishes (Peters et al., 1989, 1991; Kotrschal et al., 1989). Furthermore, oligovillous cells of lampreys, which are structurally comparable to SCCs (Whitear and Lane, 1983), have been shown

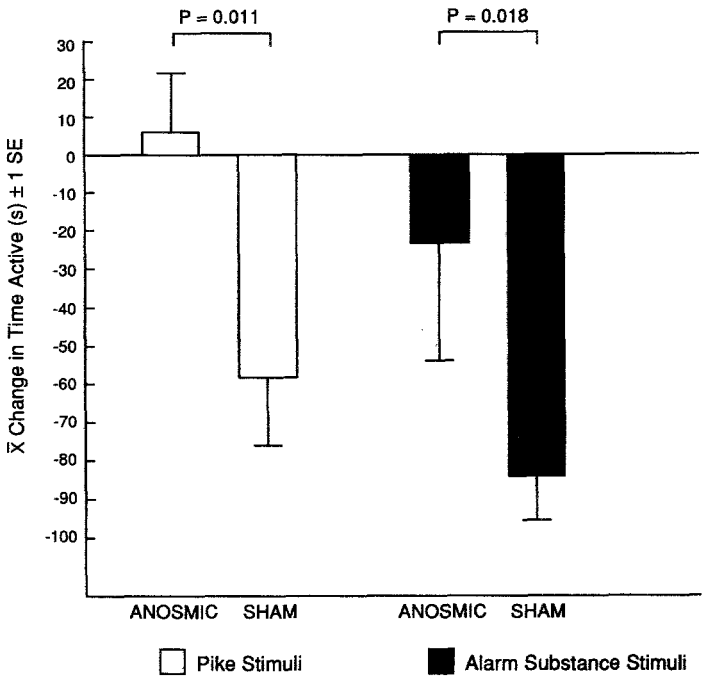

FIG. 3. Mean change in total time active (sec) by sham operated (control) and anosmic fathead minnows following exposure to chemical stimuli from pike and to alarm substance stimuli.

to respond best to mucoid substances (Baatrup and Doving, 1985). In contrast, electrophysiological evidence from the sea robin (*Prionotus carolinus*) suggests that SCCs on the free pectoral fin rays respond best to amino acids, including betaine (trimethylglycine), thereby implicating a function related to feeding and not predator avoidance (Silver and Finger, 1984). The SCCs of fathead minnows, like those of rocklings, may still respond electrophysiologically to chemical stimuli from the pike; however, we have demonstrated that olfaction is necessary for mediating the ability of fathead minnows to recognize chemical stimuli from pike.

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REFERENCES

BAATRUP, E., and DOVING, K.B. 1985. Physiological studies on solitary receptors of the oral disc papillae in the adult brook lamprey, *Lampetra planeri* (Bloch). *Chem. Senses* 10:559–566.

FRISCH, K. von. 1941. Über einen Schreckstoff der Fischhaut and seine biologische Bedeutung. *Z. vergl. Physiol.* 29:46–145.

HARA, T.J. 1971. Chemoreception, pp. 79–114, *in* W.S. Hoar and D.J. Randall (eds.). Fish Physiology, Vol. 5, Sensory Systems and Electric Organs. Academic Press, New York.

IDLER, D.R., FAGERLUND, U.H., and MAYOH, H. 1956. Olfactory perception in migrating salmon. I. L-Serine, a salmon repellent in mammalian skin. *J. Gen. Physiol.* 39:889–892.

KASUMYAN, A.O., and PASHCHENKO, N.I. 1985. Olfactory way of alarm kairomone perception by fishes. *Vest. Mosk. Univ. Biol.* 40:50–54.

KATS, L.B. 1988. The detection of certain predators via olfaction by small-mouthed salamander larvae (*Ambystoma texanum*). *Behav. Neural Biol.* 50:126–131.

KEEFE, M. 1992. Chemically mediated avoidance behaviour in wild brook trout: The response to familiar and unfamiliar predaceous fishes and the influences of fish diet. *Can. J. Zool.* 70:288–292.

KLEEREKOPER, H. 1969. Olfaction in Fishes. Indiana University Press, Bloomington.

KOTRSCHAL, K. 1991. Solitary chemosensory cells—taste, common chemical sense or what? *Rev. Fish Biol. Fish.* 1:3–22.

KOTRSCHAL, K., PETERS, R., and ATEMA, J. 1989. A novel chemosensory system in fish: Do rocklings (*Ciliata mustela* Gadidae) use their solitary chemoreceptor cells as fish detectors? *Biol. Bull.* Woods Hole, Mass. 177:328.

KOTRSCHAL, K., BRANDSTATTER, R., GOMAHR, A., JUNGER, H., PALZENBERGER, M., and ZAUNREITER, M. 1991. Brain and sensory systems, *in* I.J. Winfield and J.S. Nelson (eds.). Cyprinid Fishes: Systematics, Biology and Exploitation. Chapman and Hall, London.

LAWRENCE, B.J., and SMITH, R.J.F. 1989. Behavioral response of solitary fathead minnows, *Pimephales promelas*, to alarm substance. *J. Chem. Ecol.* 15:209–219.

LEMLY, A.D., and SMITH, R.J.F. 1986. A behavioural assay for assessing effects of pollutants on fish chemoreception. *Ecotoxicol. Environ. Safety* 11:210–218.

LEMLY, A.D., and SMITH, R.J.F. 1987. Effects of chronic exposure to acidified water on chemoreception of feeding stimuli in fathead minnows (*Pimephales promelas*): Mechanisms and ecological implications. *Environ. Toxicol. Chem.* 6:225–238.

MATHIS, A., and SMITH, R.J.F. 1993. Fathead minnows (*Pimephales promelas*) learn to recognize pike (*Esox lucius*) as predators on the basis of chemical stimuli from minnows in the pike's diet. *Anim. Behav.* In press.

MATHIS, A., CHIVERS, D.P., and SMITH, R.J.F. 1993. Population differences in responses of fathead minnows (*Pimephales promelas*) to visual and chemical stimuli from predators. *Ethology.* In press.

PETERS, R.C., VAN STEENDEREN, G.W., and KOTRSCHAL, K. 1987. A chemoreceptive function for the anterior dorsal fin in rocklings (*Gaidropsarus* and *Ciliata*: Teleostei: Gadidae): Electrophysiological evidence. *J. Mar Biol. Assoc. U.K.* 67:819–823.

PETERS, R.C., KOTRSCHAL, K., DRAUTGARTNER, W.-D., and ATEMA, J. 1990. A novel chemosensory system in fish: electrophysiological evidence for mucus detection by solitary chemoreceptor cells in rocklings (*Ciliata mustela*, Gadidae). *Biol. Bull.* Woods Hole, Mass. 177:329.

PETERS, R.C., KOTRSCHAL, K., and DRAUTGARTNER, W.-D. 1991. Solitary chemoreceptor cells of *Ciliata mustela* (Gadidae, Teleostei) are tuned to mucoid stimuli. *Chem. Senses* 16:31–42.

PFEIFFER, W., MANGLOLD-WERNADO, U. and NEUSTEUER, P. 1984. Identification of the nerve bundle in the tractus olfactorius of the tench, *Tinca tinca* L., which conducts the nervous excitation elicited by the alarm substance. *Experientia* 40:219–220.

PFEIFFER, W., RIEGELBAUER, G., MEIR, G., and SCHEIBLER, B. 1985. Effects of hypoxanthine-3-(*N*)-oxide and hypoxanthine-1-(*N*)-oxide on central nervous excitation of the black tetra *Gymnocorymbus ternetzi* (Characidae, Ostariophysi, Pisces) indicated by dorsal light response. *J. Chem. Ecol.* 11:507–524.

REHNBERG, B.G., JONASSON, B., and SCHRECK, C.B. 1985. Olfactory sensitivity during parr and smolt development stages of coho salmon. *Am. Fish. Soc. Trans.* 114:732–736.

SAVINO, J.F., and STEIN, R.A. 1989. Behaviour of fish predators and their prey: Habitat choice between open water and dense vegetation. *Environ. Biol. Fish.* 24:287–293.

SIEGEL, S. 1956. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York, 312 pp.

SIEGEL, S., and CASTELLAN, N.J. 1988. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York, 399 pp.

SILVER, W.L., and FINGER, T.E. 1984. Electrophysiological examination of a non-olfactory, non-gustatory chemosense in the searobin, *Prionotus carolinus*. *J. Comp. Physiol. A* 154:167–174.

SMITH, R.J.F. 1992. Alarm signals in fishes. *Rev. Fish Biol. Fish.* 2:33–63.

WEBSTER, D.B. 1973. Audition, vision, and olfaction in kangaroo rat predator avoidance. *Am. Zool.* 13:1346A.

WELDON, P.J. 1990. Responses of vertebrates to chemicals from predators, pp. 500–521, *in* D. W. MacDonald, C. Müller-Schwarze, and R.M. Silverstein (eds.). Chemical Signals in Vertebrates V. Plenum Press, New York.

WHITEAR, M. 1971. Cell specialization and sensory function in fish epidermis. *J. Zool. London* 163:237–264.

WHITEAR, M., and LANE, E.B. 1983. Oligovillous cells of the epidermis: Sensory elements of lamprey skin. *J. Zool. London* 199:359–384.

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SYNERGISM BETWEEN CHEMICAL ATTRACTANTS AND VISUAL CUES INFLUENCING OVIPOSITION OF THE MOSQUITO, *Culex quinquefasciatus* (DIPTERA: CULICIDAE)

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Abstract—Physical factors, such as the color of the oviposition substrate, have seldom been compared with chemical cues in their ability to elicit oviposition behavior in mosquitoes. The role of dyed oviposition waters in attracting ovipositing *Culex quinquefasciatus* was examined in laboratory experiments. Oviposition waters dyed with ink were found to be significantly attractive to ovipositing *Cx. quinquefasciatus* when compared to distilled water controls. Experiments demonstrated that the mosquitoes were responding to the increased optical density of the dyed oviposition water rather than volatile components of the dye. Ink was also considered in combination with chemical oviposition cues. No comparative data exist on the effect of physical and chemical factors presented together on the oviposition behavior of *Cx. quinquefasciatus*. Waters dyed with ink acted synergistically with a five-component chemical attractant mixture (3-methylindole, indole, 4-methylphenol, 4-ethylphenol, and phenol) in inducing oviposition in a 2 × 2 factorial experiment.

Key Words—Insecta, Diptera, Culicidae, *Culex quinquefasciatus*, oviposition behavior, attractants, optical density, visual cues.

INTRODUCTION

There has been great interest in describing and quantifying physicochemical and biotic factors involved in the oviposition site selection of gravid mosquitoes. A thorough knowledge of these factors and the interactions between them is crucial in acquiring an understanding of the oviposition process. The thrust of much of

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the research into mosquito oviposition behavior has been toward the identification of chemical compounds that attract or stimulate gravid female mosquitoes (Bentley and Day, 1989). In natural situations these chemical compounds do not act independently. They interact with other biotic and abiotic factors at the oviposition site, and in sum they present an integrated picture of the quality and attractiveness of the oviposition site.

Organic infusions have been commonly used in mosquito oviposition studies. Kramer and Mulla (1979) found that some organic infusions were attractive to ovipositing *Culex* mosquitoes while others were repellent. An alfalfa hay infusion (Reiter, 1986) is currently utilized in traps for gravid *Culex quinquefasciatus* Say. Millar et al. (1992) replaced alfalfa hay with Bermuda grass (*Cynodon dactylon* L.) and isolated a five-component attractant mixture from an infusion that was strongly attractive to ovipositing *Cx. quinquefasciatus*. A single component of this fraction, 3-methylindole (skatole), was shown to be the most biologically active.

Physical factors such as the color of the oviposition water have also been shown to influence oviposition activity in mosquitoes. In early experiments, Bates (1940) and Lund (1942) found that oviposition pans with darkened bottoms were attractive to ovipositing *Anopheles labranchinae atroparvus* van Thiel and *Anopheles quadramaculatus* Say, respectively. In laboratory experiments on *Aedes triseriatus* (Say), Williams (1962) found that dyed oviposition water was more attractive to ovipositing females than undyed water. In the field, Beehler and DeFoliart (1990) collected nearly four times as many *Ae. triseriatus* eggs in oviposition traps containing dyed tap water as compared to oviposition traps with only tap water.

Interactions between chemical and physical oviposition attractants and cues have been documented primarily in container-bred mosquitoes. O'Gower (1963) found that oviposition activity in *Aedes aegypti* var. *queenslandis* Theobald is influenced by a number of physical and chemical factors including olfactory and visual cues. Wilton (1968) showed effects of olfactory and visual factors in laboratory experiments on *Aedes triseriatus* (Say). Also in laboratory studies with *Ae. triseriatus*, McDaniel et al. (1976) showed that visual factors interacted with factors produced by larval conspecifics.

The objectives of our study were to investigate, in the laboratory, the importance of visual cues on the oviposition behavior of *Culex quinquefasciatus*, a mosquito species whose breeding habitats range from large containers to large polluted temporary ponds, and to determine if visual oviposition cues in the form of dyed waters interact with its chemical oviposition attractants.

METHODS AND MATERIALS

The laboratory colony of *Cx. quinquefasciatus* was established using egg rafts collected from organic infusion-baited oviposition traps placed in Orange County, California in June through October of 1991. The colony was maintained

as described by Kramer and Mulla (1979). Mosquitoes were allowed to mate and blood-feed on chicks seven days before laboratory experiments (University of California animal care protocol #A-9105063). The insectary had a photoperiod of 14 : 10 hr light–dark including a 2-hr dusk/dawn period. The day lighting was provided by two 40-W fluorescent tubes. The 1-hr dusk twilight period and the 1-hr dawn period were provided by a single 15-W incandescent bulb. Insectary temperatures were maintained at 25 ± 3°C.

Six experiments were conducted to determine the effect of water dyed with ink and its interaction with a chemical attractant on the ovipositional response of *Cx. quinquefasciatus*. Experiment 1 was designed to determine the effect of water dyed with ink on oviposition behavior. Twenty gravid laboratory-reared *Cx. quinquefasciatus* were placed at 1700 hr in each of six cages (23 × 23 × 32 cm) in a laboratory insectary. Each of the six cages contained two randomly placed waxed paper oviposition cups (5 × 7.5 cm diam), one cup containing the test solution, which in this case was 25 μl of India ink (Fount India, Pelikan, Hannover, Germany) in 80 ml of distilled water and the other a control, distilled water. Mosquitoes were left in the cages overnight, and the following morning the resulting egg rafts were counted and collected. Egg raft numbers were transformed ($\sqrt{x}$) and a paired t test was used to evaluate the attractancy of the dyed water. The square root transformation with a paired t test is appropriate in situations where the variance is proportional to the mean (Snedecor and Cochran, 1980). This six-cage experiment was repeated over three nights for a total of 18 replicates.

Experiment 2 examined the oviposition response of gravid female *Cx. quinquefasciatus* to dyed oviposition waters under natural twilight conditions. Four screen cages (45 cm on a side) were placed on a window ledge where they were exposed to natural twilight conditions. Twenty gravid *Cx. quinquefasciatus* were placed in each cage with two waxed paper oviposition cups placed randomly in the rear corners of the cage containing either dyed oviposition water or a distilled water control. Mosquitoes were placed in cages at 1600 hr, approximately 2.5 hr before sunset, to be exposed to full crepuscular photoperiodic changes. Temperatures were maintained at 24 ± 2°C. The following morning the resulting egg rafts were removed, the number of egg rafts counted, and the data analyzed as in experiment 1. This experiment was repeated over three nights for a total of 12 replicates.

Experiment 3 determined if the effect of the dyed oviposition water was due to a visual response to the dye or an olfactory response due to volatile compounds contained in the dye. Four cages were set under natural twilight conditions with 20 gravid female *Cx. quinquefasciatus* using the protocol of experiment 2. Two oviposition cups were again placed randomly in the rear corners of each cage, one containing 25 μl of India ink placed in 80 ml of

distilled water and the other containing 25 μl of India ink from which the volatile fraction had been removed in 80 ml of distilled water. The experiment was also repeated over three nights for a total of 12 replicates. Data were analyzed as in experiment 1.

To obtain a profile of the India ink volatiles, purge and trap analysis using a Tekmar LSC 2000 purge and trap device (Tekmar Co., Cincinnati, Ohio) was interfaced to a cryocooled (liquid CO_2) Hewlett-Packard 5890 gas chromatograph (GC) (Hewlett-Packard, Avondale, Pennsylvania) coupled to a Hewlett-Packard 5970 mass selective detector. Ink (5 ml) was loaded into a 25-ml fritted sparge sampler and purged for 12 min at ambient temperature with He (20 ml/min). Purged volatiles were collected on a Tekmar Carbopak B adsorbant cartridge. The adsorbent cartridge was then flushed with dry He for 4 min to drive off water and desorbed for 4 min at 225°C directly into the GC injector. The transfer line temperature was 100°C. The GC was programmed from 0°C for 2 min, 10°/min to 250°C. The injector and detector temperatures were 250°C. Analyses were replicated twice with a blank run between each replicate.

Volatiles were removed from a 20-ml ink sample by concentrating the ink to dryness on a rotary evaporator under reduced pressure at 40°C, followed by pumping the solid residue at 0.02 mm Hg overnight. The resulting black solid was reconstituted with 20 ml of distilled water. The resulting mixture was sonicated until it became homogenous and was then compared with India ink for oviposition attractancy under natural twilight conditions.

In experiments 4 and 5, an oviposition choice test protocol similar to that used in the previous insectary experiment (experiment 1) compared experimental waters treated with a chemical oviposition attractant to those treated with a physical attractant, the India ink. A test oviposition cup containing 80 ml of distilled water was treated with 7.6 μg phenol, 0.65 μg 4-methylphenol, 3.2 μg 4-ethylphenol, 0.4 μg indole, and 2.4 μg 3-methylindole, which was shown by Millar et al. (1992) to be attractive to ovipositing *Cx. quinquefasciatus*. The other cup, also containing 80 ml of distilled water, was treated with 25 μl of India ink. These cups were placed randomly in the rear corners of an oviposition cage containing 20 gravid female *Cx. quinquefasciatus* for one night. This experiment was repeated over three nights for a total of 18 replicates. Experiment 5 was conducted similarly using paired oviposition cups, each containing 80 ml of distilled water treated with 25 μl of India ink. One cup of the pair was randomly chosen and treated with the chemical attractant mixture described above. Data were analyzed as in experiment 1.

In experiment 6, 2 × 2 factorials were performed under natural photoperiodic conditions to determine if there were interactive effects of the physical factor (India ink) and the chemical mixture on the oviposition behavior of *Cx. quinquefasciatus*. These experiments were conducted in cages (45 cm per side) using four oviposition cups (80 ml) placed randomly in each of the four corners.

The cups contained: (1) distilled water (control), (2) water and dye (25 μl), (3) water and chemical attractant mixture (at the above concentrations), and (4) water and chemical attractant mixture and dye. Thirty gravid female *Cx. quinquefasciatus* were placed in each cage and allowed to oviposit overnight. This experiment was repeated six nights with four cages per night for a total of 24 replications. Egg rafts were collected in the morning, data were transformed to square roots, and analyzed using multiple regression methods standard for factorial experiments (Box et al., 1978).

RESULTS AND DISCUSSION

The results of experiments 1 and 2 are summarized in Table 1. In experiments conducted under insectary lighting, cups that contained distilled water dyed with ink collected means of 4.3–6.5 egg rafts/cup, while distilled water controls collected means of 3.0–8.0 egg rafts/cup. The mean number of egg rafts collected in the dyed cups was not significantly higher than the control cups (paired t, $P > 0.05$). In the experiments conducted under natural twilight conditions, the cups that contained distilled water treated with ink collected 14.0–16.5 egg rafts/cup, while the distilled water controls collected means of 2.8–4.8 egg rafts/cup. In all cases the cups treated with ink collected significantly more egg rafts than did the undyed controls (paired t, $P < 0.025$),

TABLE 1. OVIPOSITIONAL RESPONSE OF *Culex quinquefasciatus* TO DYED OVIPOSITION WATER

	Mean N egg rafts/cup	
Night[a]	Dyed water	Undyed water[b]
Insectary lighting		
1	6.5	5.0 NS
2	11.5	8.7 NS
3	4.3	3.0 NS
Natural twilight		
1	16.5	4.8***
2	14.0	4.3**
3	16.0	2.8**

[a] Each experiment consisted of six replicated cages with 20 gravid females per cage repeated over three nights in insectary experiments and four replicated cages also with 20 females per cage in natural twilight conditions.

[b] $**P < 0.025$, paired t; $***P < 0.01$, paired t; NS, not significantly different ($P > 0.05$, paired t).

outnumbering the controls by more than 3:1. Twilight in the insectary experiments was supplied by a single 15-W incandescent bulb, which provided illumination for 1 hr after fluorescent bulbs were switched off. Twilight under natural conditions was significantly longer and the decline in light levels was more gradual. When *Cx. quinquefasciatus* females were exposed to natural twilight conditions, dyed water was clearly attractive to females.

Dyed oviposition water has been shown to be attractive to ovipositing female mosquitoes in a number of field and laboratory studies. The container-breeding mosquito, *Aedes triseriatus*, has been shown to oviposit preferentially in dyed oviposition waters in both field and laboratory studies (Williams, 1962; Wilton, 1968; Beehler and DeFoliart, 1990; Beehler, 1992). Both *Anopheles atroparvus* and *An. quadramaculatus* oviposited preferentially in oviposition waters that appeared darker than controls (Bates, 1940; Lund, 1942). In field studies conducted by the Orange County, (California) Vector Control District, *Cx. quinquefasciatus* oviposited preferentially in traps containing darkened oviposition water (J.P. Webb, personal communication). Our studies provide further evidence that darkened oviposition water attracts ovipositing *Cx. quinquefasciatus* females.

Experiment 3 was designed to study whether the response of gravid *Cx. quinquefasciatus* to dyed water was due to the increased optical density of dyed oviposition water or volatile components of the dye itself. Purge and trap analysis revealed that the India ink used in these experiments contained volatile fractions, including ethanol, isopropanol, hexane, ethyl acetate, dimethoxymethane (methylal), and traces of naphthalene and methyl naphthalene. Headspace chromatography of 29 inks has shown that alcohols are among the most common solvents in ink samples (Rastogi, 1991). Concentration and pumping under vacuum removed all of these volatiles from the India ink sample. The evaporated and reconstituted ink had a purge and trap volatiles profile equivalent to that of the distilled water with which the ink had been reconstituted. Paired tests comparing the reconstituted ink that had the volatiles removed with the unpurged India ink showed no difference in the number of egg rafts collected in the paired cups (paired t, $P > 0.05$). Cups containing distilled water and ink with the volatile components removed collected means of 7.7–13.7 egg rafts/cup and those that contained distilled water and unreconstituted ink collected means of 9.2–12.7 egg rafts/cup (Table 2). These data strongly suggest that the ovipositional responses of the gravid female *Cx. quinquefasciatus* were due to the change in optical density of the experimental waters produced by the ink, not by the volatiles present in the ink itself.

Experiment 4 was conducted under insectary conditions to further determine if the ovipositional response of *Cx. quinquefasciatus* was a response to the optical density of dyed water or to chemical components found in the ink. Experiments 1 and 2 showed that the environment provided by the insectary

TABLE 2. INFLUENCE OF INDIA INK CONTAINING VOLATILE SUBSTANCES AND INDIA INK WITHOUT VOLATILES ON OVIPOSITION BY *Culex quinquefasciatus*

	Mean *N* egg rafts/cup	
Night[a]	Volatiles	No volatiles[b]
1	9.2	13.7 NS
2	12.7	7.7 NS
3	12.5	17.5 NS

[a]The experiment consisted of six replicated cages with 20 gravid females per cage repeated over three nights. Experiments were conducted in natural twilight.
[b]NS, not significantly different (paired t, $P > 0.05$).

cages is only marginally suitable for the use of visual cues in the selection of an oviposition site. Dyed water was not shown to be an oviposition attractant in the insectary, but in cages subjected to natural twilight the ink proved to be attractive to *Cx. quinquefasciatus* females. Therefore, insectary experiments were conducted in conditions in which visual cues were minimized and chemical cues were maximized. In experiment 4, water treated with the oviposition attractant mixture at the concentration used in the earlier experiments collected means of 6.3–10.3 egg rafts compared to 3.0–5.3 egg rafts collected in the dyed water (Table 3). The mean values in the attractant-treated cups were significantly higher (paired t, $P < 0.01$). In experiment 5, waters that contained India ink alone collected significantly fewer (paired t, $P < 0.01$) rafts than did oviposition cups containing ink and attractant mixture (Table 3).

The 2 × 2 factorial experiment is summarized in Figure 1. Control cups containing only distilled water collected a mean of 0.5 egg rafts/cage; cups containing dyed water collected a mean of 3.8 egg rafts/cage; cups containing water plus chemical attractant collected 3.0 egg rafts/cage; and cups containing both attractant and dye collected 19.8 egg rafts/cup. Regression analysis of the 48 replicates over 12 nights shows that both the ink ($F = 4.31$, $P < 0.001$) and the chemical mixture ($F = 4.50$, $P < 0.001$) were significantly more attractive to ovipositing *Cx. quinquefasciatus* than were the distilled water control oviposition cups. Cups containing a combination of both ink and attractant mixture were significantly more attractive ($F = 3.70$, $P < 0.001$) than either of the test substances presented singly or the untreated control. The R^2 for the regression was 0.95.

The ovipositional response to the ink presented in tandem with the chemical attractant mixture appeared to be synergistic. Synergism has been defined as a response that is more than the mean additive effects of the compounds presented

TABLE 3. LABORATORY OVIPOSITION CHOICE EXPERIMENTS WITH *Culex quinquefasciatus* USING DYED OVIPOSITION WATER AND A CHEMICAL ATTRACTANT BLEND.[1]

	Mean N egg rafts/cup	
Night[a]	Attractants	Dye alone[b]
1	10.3	5.3***
2	6.3	3.0***
	Dye + Attractants	Dye alone
1	15.3	2.6***
2	10.3	1.5***
3	13.3	3.0***

[a] Chemical 5-component mixture (Millar et al. 1992)
[b] Each experiment consisted of six replicated cages with 20 gravid females per cage. Experiments were conducted under insectary conditions.
***P < 0.01, paired-t.

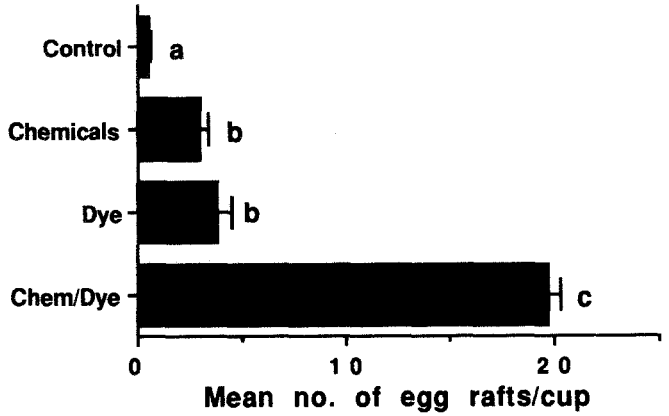

FIG. 1. Synergism between a physical factor (dyed oviposition water) and a chemical factor (five-component oviposition attractant mixture) in a replicated 2 × 2 factorial experiment. Bars followed by different letters are significantly different ($P < 0.05$).

in tandem (Wadley, 1967). The effect of ink and the chemical attractant presented together was tested using the following contrast:

$$(\mu_{ink}) + (\mu_{chemical}) \leq (\mu_{ink + chemical})$$

The mean response to the combination of ink and chemical mixture ($\mu_{ink + chemical}$)

presented together was significantly greater than the sum of the mean effects of the single components [(μ_{ink}) and ($\mu_{chemical}$)] ($P < 0.01$), indicating that the chemical and visual attractants acted synergistically.

In conclusion, these laboratory studies show that gravid female *Cx. quinquefasciatus* mosquitoes use both visual and chemical cues in the selection of an oviposition site. The experiments using India ink show that the mosquitoes readily recognize and oviposit preferentially in natural twilight, but not in the insectary, in waters which have increased optical density. In field conditions, darkened waters are usually the most organically enriched and provide a high-quality nutritional substrate for the larvae. The chemical components of the attractant mixture used are also indicative of a larval habitat that is nutritionally enriched. The chemical and physical cues appear to act synergistically in influencing the ovipositional site selection behavior of gravid female mosquitoes in a way that optimizes the quality of the larval habitat.

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REFERENCES

BATES, M. 1940. Oviposition experiments with anopheline mosquitoes. *Am. J. Trop. Med.* 20:569–583.

BEEHLER, J.W. 1992. The oviposition behavior of *Aedes triseriatus*. *Proc. Calif. Mosq. Vector Control Assoc.* 59:108–113.

BEEHLER, J.W., and DEFOLIART, G.R. 1990. A field evaluation of two suggested *Aedes triseriatus* oviposition attractants. *J. Am. Mosq. Control Assoc.* 6:720–722.

BENTLEY, M.D., and DAY, J.F. 1989. Chemical ecology and behavioral aspects of mosquito oviposition. *Annu. Rev. Entomol.* 34:401–421.

BOX, G.E.P., HUNTER, W.G., and HUNTER, J.S. 1978. Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building. John Wiley & Sons, New York.

KRAMER, W.L., and MULLA, M.S. 1979. Oviposition attractants and repellents of mosquitoes: Oviposition responses of *Culex* mosquitoes to organic infusions. *Environ. Entomol.* 8:1111–1117.

LUND, H.O. 1942. Studies on the choice of a medium for oviposition by *Anopheles quadrimaculatus*. *J. Nat. Malaria Soc.* 1:101–111.

MCDANIEL, I.N., BENTLEY, M.D., LEE, H.P., and YATAGAI, M. 1976. Effects of color and larval-produced oviposition attractants on oviposition of *Aedes triseriatus*. *Environ. Entomol.* 5:553–556.

MILLAR, J.G., CHANEY, J.D., and MULLA, M.S. 1992. Identification of oviposition attractants for *Culex quinquefasciatus* from fermented Bermuda grass infusions. *J. Am. Mosq. Control Assoc.* 8:11–17.

O'GOWER, A.K. 1963 Environmental stimuli and the oviposition behaviour of *Aedes aegypti* var. *queenslandis* Theobald (Diptera, Culicidae). *Anim. Behav.* 11:189–197.

RASTOGI, S.C. 1991. Analysis of organic solvents in printing inks by headspace gas chromatography–mass spectrometry. *J. High Resol. Chromatogr.* 14:587–589.

REITER, P. 1986. A standardized procedure for the quantitative surveillance of certain *Culex* mosquitoes by egg raft collection. *Mosq. News* 2:219–221.

SNEDECOR, G.W., and COCHRAN, W.G. 1980. Statistical Methods. Iowa State University Press, Ames, Iowa.

WADLEY, F.M. 1967. Experimental Statistics in Entomology. Graduate School Press, USDA, Washington, D.C.

WILLIAMS, R.E. 1962. The effect of coloring oviposition media with regard to the mosquito *Aedes triseriatus* (Say) *J. Parasitol.* 48:919–925.

WILTON, D.P. 1968. Oviposition site selection by the tree-hole mosquito, *Aedes triseriatus* (Say). *J. Med. Entomol.* 5:189–194.

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FALL ARMYWORM SENSITIVITY TO FLAVONE: LIMITED ROLE OF CONSTITUTIVE AND INDUCED DETOXIFYING ENZYME ACTIVITY

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Abstract—We used inhibition and induction of detoxifying enzymes to determine whether these enzymes allow a generalist species (*Spodoptera frugiperda;* fall armyworms) to cope with ingestion of the flavonoid, flavone. Flavone induces polysubstrate monooxygenases (PSMO), general esterases (GE), and glutathione *S*-transferases (GST) in *S. frugiperda*, yet this species is affected deleteriously by low dietary concentrations of this allelochemical. First, in a series of experiments, larvae were fed artificial diets containing increasing concentrations of flavone, either alone or with known inhibitors of either PSMO, GE, or GST enzymes. In an additional treatment, flavone and inhibitors of all three enzyme systems were administered in diets simultaneously. PSMO and GE activities were reduced in vivo by their respective inhibitors, whereas that of GST was induced or unchanged. Significant synergism of flavone's growth-reducing activity occurred at the highest concentration tested (0.125% fresh mass, fm) when the PSMO inhibitor, piperonyl butoxide, or the GST inhibitor, diethyl maleate, was added to the diet, and at 0.08% fm flavone, when combined with the GE inhibitor, tri-tolyl phosphate. In many cases, however, the additive effect (i.e., reduction in growth owing to flavone alone + inhibitor alone) was greater than the synergistic effect, and no synergism occurred in the treatment with the three inhibitors combined. In the second approach, caterpillars were preexposed to a concentration of flavone (0.02% fm) that induced these enzymes ca. 1.5- to 2.5-fold, prior to switching larvae to a diet containing a higher (growth-reducing) flavone concentration (0.125% fm). The relative growth rates (RGR) of induced larvae were significantly greater (14%) than those of the uninduced larvae on the 0.125% fm flavone diet. Additionally, in two of the three experiments, relative consumption rate (RCR) was significantly greater (7–

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24%) in induced compared with uninduced larvae. The variable responses to inhibitor treatment and the relatively small benefit of enzyme induction suggest that these enzyme systems have minimal impact on the detoxification of flavone in *S. frugiperda*, even though this allelochemical induces enzyme activity and has been reported to be metabolized in vitro.

Key Words—Detoxification, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, flavone, induction, polysubstrate monooxygenase, glutathione transferase, general esterase, synergism.

INTRODUCTION

Three systems of detoxifying enzymes [i.e., polysubstrate monooxygenases (PSMO), general esterases (GE), and glutathione *S*-transferases (GST)] are commonly regarded as the most important biochemical mechanisms for the metabolism of xenobiotics (Terriere, 1984; Brattsten, 1992) including allelochemicals (Yu, 1986) and pesticides (Hodgson et al., 1991). Xenobiotics may act as inducers by stimulating enzyme synthesis (Yu, 1986). Insects induced by dietary allelochemicals or host plants apparently increase metabolism of several synthetic pesticides, as demonstrated by their increased tolerance to these compounds (Hodgson et al., 1991). Insecticide-resistant strains of insects often have greater detoxifying enzyme activities (Soderlund and Bloomquist, 1990; Yu, 1991), and in at least one example, enzyme inducibility was greater than in a susceptible strain (Ottea and Plapp, 1984). In contrast, little is known about the role of detoxifying enzymes in the tolerance of allelochemicals by insects, although the in vivo metabolism of several allelochemicals has been demonstrated (Proksch et al., 1987; Nitao, 1989, 1990; Dowd, 1990; Harwood et al., 1990; Berge and Rosenthal, 1991; Smirle and Isman, 1992). Additionally, few studies have demonstrated increased allelochemical tolerance in insects attributable to induced enzyme activity (Brattsten et al., 1977; Kennedy, 1984; Gunderson et al., 1985).

Midgut preparations of allelochemical-induced caterpillars metabolize the inducing allelochemical more rapidly in vitro than do those of controls (Yu, 1986; Wadleigh and Yu, 1987, 1988a, b; Nitao, 1989; Harwood et al., 1990). However, to our knowledge only one published study demonstrates increased tolerance of an insect [*Helicoverpa zea* (Boddie)] to an allelochemical (2-tridecanone) following exposure to the same compound (Kennedy, 1984). Although monooxygenase enzyme activity was not measured in that study, induction probably occurred, as cytochrome P-450 content and GST activity were greater in larvae of another noctuid species *Heliothis virescens* (F.) after three days of feeding on diets containing 2-tridecanone compared with controls (Riskallah et al., 1986). For induced detoxifying enzyme activity to be an adaptive response mitigating the negative impact of xenobiotics, we suggest these enzymes should

protect insects from exposure to the same compounds that act as the inducing agents.

In this study we investigated whether there is a benefit of detoxifying enzyme activity to larvae of *Spodoptera frugiperda* (J.E. Smith) in terms of their ability to cope with the ingested allelochemical, flavone (Figure 1). Although this is a generalist species, reported from over 25 plant families (Tietz, 1972), it does not occur on plants known to contain flavone (e.g., *Primula* spp.). Therefore, we would not expect *S. frugiperda* to be specifically adapted to this allelochemical as monophagous species may be to the allelochemicals occurring in their food plants (e.g., Berenbaum et al., 1990). However, flavone represents the basic structure for several thousand flavonoids (Harborne, 1988), many of which occur in plants that comprise this species' host range (Tietz, 1972). Furthermore, this allelochemical is known to induce PSMO, GE, and GST enzyme activities 4.4-, 1.8-, and 3.3-fold, respectively (Yu, 1983, 1984; Yu and Hsu, 1985), in *S. frugiperda* and is metabolized in vitro by midgut preparations from this species (Yu, 1987). Thus, it is likely that *S. frugiperda* larvae have the capacity to detoxify flavone and other flavonoids. We used two approaches to investigate whether there is a benefit to constitutive and induced enzyme activities in terms of tolerating dietary flavone in *S. frugiperda* larvae.

First, caterpillars with inhibited detoxifying enzymes should perform more poorly than those with uninhibited enzymes when fed a performance-reducing dose of an allelochemical if the blocked enzymes normally detoxify the allelochemical. Thus, we used insecticide synergists in an attempt to block the major detoxifying enzyme systems and then measured the change in insect performance when fed a flavone-containing diet. Many reports describe in vitro assays consisting of these inhibitors and insect tissue preparations (e.g., Wadleigh and Yu, 1988a, b; Lee, 1991) but only a few inhibitors have been assayed in vivo after having been fed to caterpillars in diet (Berenbaum and Neal, 1985; Hedin et al., 1988; Lindroth, 1989; Neal, 1989; Lindroth and Bloomer, 1991). However, feeding these enzyme inhibitors directly to insects in their food may present special problems, such as toxicity due to the inhibitor alone, enzyme induction by the inhibitor, or their metabolism by detoxifying enzymes.

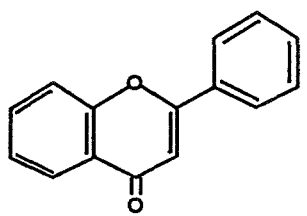

FIG. 1. The structure of flavone.

Second, preexposure of larvae to a subeffective dose of an allelochemical that induces detoxifying enzymes should allow larvae to better tolerate a diet containing a higher concentration of the same allelochemical. Thus, for two days we fed larvae an inducing diet containing flavone at a concentration that would induce detoxifying enzymes but not reduce growth, and then switched the larvae to a diet with a flavone dose that would reduce performance in uninduced larvae. Our hypothesis was that the performance of the preexposed (induced) larvae would be superior to that of the uninduced larvae that were switched directly from a control diet to the performance-reducing diet.

Before starting these experiments, we had to determine: (1) the degree of enzyme inhibition when caterpillars were fed the insecticide synergists; (2) the dose of flavone that induced enzyme activity but caused minimal reduction in caterpillar growth; (3) an effective dose of flavone (i.e., a dose that significantly reduced caterpillar growth); and (4) the extent of enzyme inhibition occurring at these allelochemical concentrations.

METHODS AND MATERIALS

Chemicals. The chemicals used in this study are listed below with the abbreviations and vendors' names in parentheses: aldrin, piperonyl butoxide (PB), and *S,S,S*-tributyl phosphorotrithioate (DEF) (Chem Service Inc., Westchester, Pennsylvania); triphenyltin chloride (TPC) (Aldrich Chemical Company, Milwaukee, Wisconsin); 1-chloro-2,4-dinitrobenzene (CDNB), diethyl maleate (DEM), ellagic acid, flavone (>99% purity by TLC), α-naphthyl acetate, quercetin, and reduced glutathione (Sigma Chemical Company, St. Louis, Missouri), and tri-ortho-tolyl phosphate (TTP) (Eastman Kodak Company, Rochester, New York). All other chemicals were purchased from commercial suppliers.

Insect Growth and Performance. Eggs were obtained from the USDA-ARS Insect Attractants and Basic Biology Laboratory, Gainesville, Florida. Larvae were reared en masse on a modified artificial diet (lacking all preservatives except sorbic acid; Greene et al., 1976) until the beginning of each experiment, at which time they were transferred to individual 30-ml clear plastic cups. All larvae were reared at 27 ± 1°C, 50 ± 15% relative humidity with a 14:10 light–dark photoperiod.

Insect performance was assessed by the calculation of nutritional indices (Waldbauer, 1968; Slansky and Scriber, 1985). Larvae were reared from the late third to early fourth instars [15–25 mg fresh mass (fm); hereafter referred to as mid-instars] through to the pupal stage on the experimental diets. Pupae were frozen and then dried (60°C) for two days in a convection oven before weighing (±0.1 mg). Initial dry biomass was estimated by comparing the mean

mass of a cohort ($N = 10$) of larvae weighed fresh and after drying for two days. Initial dry biomass of the larvae for the preexposure experiment (see "Benefit of Preexposure to Flavone" below) was assessed similarly except that cohorts of mid-instar larvae were reared for two days on each treatment diet to assess biomass at the time larvae were switched. Body mass relative growth rate [RGR; dry biomass gain (mg)/mean dry biomass (mg)/day], body mass relative consumption rate [RCR; dry mass consumed (mg)/mean dry biomass (mg)/day], the efficiency of converting digested food to insect biomass [ECD; biomass gain/(food ingested-feces)], and the approximate digestibility [AD; (food ingested-feces)/food ingested)] were calculated on a dry mass basis from the time the larvae were switched until pupation. For calculation of RGR and RCR, we followed Gordon (1968) in using the exponential mean biomass during the experiment, except that we used dry instead of live biomass.

Treatment Diets. Inhibitors were dissolved in a minimum of acetone, alphacel (ICN Biochemicals Inc., Cleveland, Ohio) was added (1% fm; acetone and alphacel alone for the control), and the mixture was rotoevaporated to dryness at 60°C. This mixture was then added to freshly prepared diet as it cooled. Additionally, enzyme-inducing diets containing flavone, synergism diets containing one or more enzyme inhibitors with flavone, and control diets lacking both synergist and flavone were prepared similarly.

Effects of Enzyme Inhibitors and Flavone on Larval Growth and Survival. The effect of each enzyme inhibitor or flavone on the mortality and RGR of caterpillars fed experimental diets was assessed. Flavone and each synergist were tested at a range of concentrations (Table 1).

Enzyme Activity of Larvae Fed Enzyme Inhibitors and Flavone. Enzyme activity was determined for larvae fed a range of enzyme inhibitor concentrations or flavone. Depending upon the experiment, either mid-instar ($N = 25$) or recently molted sixth-instar larvae ($N = 15$) were fed a test diet for two days, after which their midguts were removed and washed in ice cold (0–4°C) 1.15% KCl. Midguts were homogenized, and enzyme activity was measured in duplicate using standard techniques. Polysubstrate monooxygenase activity was measured by the aldrin epoxidase method (Yu, 1982). Samples comprising the crude homogenate as the enzyme source were incubated for 15 min at 30°C, and the amount of dieldrin produced was extracted with hexane and measured by gas chromatography on a 122 cm × 2 mm ID glass column packed with 3% SE 30 on 100/120 Gas Chrom Q. General esterase activity was measured by the α-naphthyl acetate method, using the crude homogenate as an enzyme source (Van Asperen, 1962; Yu et al., 1984). The amount of hydrolysis product (α-naphthol) was measured spectrophotometrically (600 nm) against a boiled blank. Glutathione transferase activity was measured by detecting CDNB conjugation with glutathione (Habig et al., 1974; Yu, 1984) using the postmitochondrial fraction as the enzyme source. The change in absorbance (340 nm) of the

TABLE 1. MORTALITY, RELATIVE GROWTH RATE (RGR), AND ENZYME ACTIVITY (MEAN ± SE) OF *S. fruqiperda* LARVAE FED INHIBITORS OF DETOXIFYING ENZYMES

Enzyme system/inhibitor	Concentration (% fm)	Mid-instar to prepupa: Mortality (%)	Mid-instar to prepupa: RGR (mg/mg/day)	Mid-instar to prepupa: N	Sixth instar: Enzyme activity (% of control)	Sixth instar: N
Control		0	0.46 ± 0.01	55	100[a]	
PSMO						
Piperonyl butoxide (PB)	0.01	0	0.42 ± 0.02	15	69.2 ± 10.4[b]	6
	0.05	0	0.44 ± 0.02	15	40.5 ± 3.1[b]	7
	0.1	0	0.41 ± 0.02	15	68.9 ± 13.0[b]	7
GE						
Triorthotolyl phosphate	0.01	0	0.43 ± 0.01	15	0.3 ± 0.3[b]	6
(TTP)	0.05	0	0.40 ± 0.02[b]	15	4.2 ± 3.3[b]	4
	0.1	0	0.36 ± 0.02[b]	15	0 ± 0[b]	4
S,S,S-tributyl	0.002	13.3	0.47 ± 0.02	13	4.8 ± 4.8[b]	5
phosphorotrithioate	0.004	0	0.40 ± 0.01	15	0.9 ± 0.7[b]	6
(DEF)	0.006	0	0.42 ± 0.01	15	0 ± 0[b]	2
	0.01	0	0.29 ± 0.02[b]	15	[c]	
	0.05	40.0	0.16 ± 0.02[b]	9		
	0.1	93.3		[d]		
GST						
Diethyl maleate (DEM)	0.01	0	0.46 ± 0.02	15	110.8 ± 8.5	6
	0.05	0	0.41 ± 0.02	15	178.7 ± 10.4	6

	0.1	0	0.47 ± 0.02	15	251.4 ± 30.4	8
	0.15		[c]		265.7 ± 26.6[b]	8
	0.2				362.3 ± 50.0[b]	5
	0.25				522.3 ± 116.3[b]	5
Triphenyltin chloride[f]	0.0004				111.8 ± 18.2	6
(TPC)	0.0005	6.7	0.39 ± 0.01[b]	14		
	0.0006				162.5 ± 12.4	5
	0.0008				139.8 ± 12.5	7
	0.001	13.3	0.15 ± 0.02[b]	5	214.9 ± 29.6	7
	0.0025				179.3 ± 10.3	4
	0.005	100			347.5 ± 144.3[b]	3
Quercetin	0.05	0	0.46 ± 0.01	15		
	0.1	0	0.43 ± 0.01	15	92.6 ± 6.0	5
	0.15	0	0.46 ± 0.01	15		
	0.2	0	0.41 ± 0.02	15	101.8 ± 9.3	5
	0.3				106.1 ± 8.5	5
Ellagic acid[g]	0.1	6.7	0.37 ± 0.02	14	102.0 ± 8.3	5
	0.2	0	0.30 ± 0.01[b]	15	87.0 ± 4.3	4
	0.3	0	0.31 ± 0.01[b]	15	97.8 ± 5.1	4

[a] Mean control activity differed for each enzyme system measured; see Results for values.
[b] Means differ significantly ($P = 0.05$) from the control.
[c] Larvae were used only for mortality and RGR, therefore no enzyme activity data are given.
[d] Insufficient number larvae ($N = 1$) survived to analyze data.
[e] Larvae were used only for enzyme assays, therefore no mortality or RGR data are given.
[f] This compound was also tested at 0.01, 0.05, and 0.1% fm; however, all larvae died at these concentrations.
[g] Ellagic acid was analyzed independently of other compounds. Means compared with control = 0.37 (±0.02) mg/mg/day.

incubation mixture was monitored for 2 min at 25°C. Furthermore, the GST inhibitors were analyzed in vitro by adding each compound directly to the incubation mixture. Ellagic acid is insoluble in the GST buffer (0.1 M, $NaPO_4$, pH 6.5) and therefore was dissolved in 20 μl NaOH (0.1 M), whereas the remaining compounds were dissolved in GST buffer. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Synergism. A series of five experiments was conducted to assess the occurrence of synergism when flavone was combined at several concentrations with each enzyme inhibitor. Each experiment consisted of a single flavone concentration (0.02, 0.04, 0.08, 0.125, or 0.25% fm) combined with each enzyme inhibitor (PB 0.01, TTP 0.01, DEF 0.002, DEM 0.01, or TPC 0.0004% fm). A combined inhibitor treatment also was included (0.04, 0.08, 0.125, and 0.25% fm flavone only) that targeted all three enzyme systems, consisting of three inhibitors (PB 0.01, TTP 0.01, and DEM 0.01% fm). Control diets for each experiment were: (1) flavone-free and inhibitor-free; (2) flavone alone; and (3) each inhibitor alone. Caterpillar mortality and RGR were recorded from the mid-instar through to the pupal stage. Significant synergism occurred if the reduction in RGR due to the synergized treatment (flavone + inhibitor) was significantly greater than the added reduction due to the flavone-alone and the inhibitor-alone controls. Additionally, PSMO, GE, and GST activities were measured (as described above) for each combined flavone (0.02% fm)–synergist treatment.

Benefit of Pre-exposure to Flavone. An experiment was conducted to determine whether pre-exposure to a flavone concentration that induced detoxifying enzymes yet had a negligible negative impact on insect performance would benefit larvae subsequently challenged with an effective flavone concentration that reduced RGR in uninduced individuals (see Results). Following a two-day pre-exposure period, during which larvae (15–23 mg fm) were fed a diet with 0.02% flavone, they were switched to a diet with 0.125% flavone (induced larvae). Other treatments consisted of larvae reared on a control diet for two days and then switched to another control diet (control larvae), and larvae switched to the 0.125% flavone diet directly from the control diet (uninduced larvae). The entire experiment was replicated three times.

Statistical Analyses. Mortality data were analyzed by a G test of independence (Sokal and Rohlf, 1981), whereas all remaining analyses were conducted with PC/SAS (SAS Institute, 1987). To determine the inhibitor levels that did not significantly reduce RGR, the effect of these compounds was analyzed with a one-way analysis of variance (ANOVA) followed by a Dunnett's test ($P = 0.05$) comparing each treatment mean with the appropriate control mean. The effect of flavone concentration on RGR was determined by linear regression. The enzyme activity data were also analyzed by one-way ANOVA

followed by the Dunnett's test. The results from the synergism experiments were analyzed with one-tailed Student's t-test comparisons to test whether the synergist reduction in RGR (i.e., each synergist–flavone combination) was significantly greater than the additive reduction in RGR due to flavone and each synergist alone. The results of the preexposure experiments were analyzed by a two-way ANOVA with interaction, where the main effects were the three experiments and the three treatments. If the interaction was not significant, the data for the experiments were combined. One-tailed orthogonal contrasts were made between: (1) the control and the combined effects of the uninduced and the induced treatments, to determine whether dietary flavone reduced caterpillar performance; and (2) the uninduced versus the induced treatments, to determine if performance of the induced larvae improved relative to that of the uninduced insects.

RESULTS

Effects of Enzyme Inhibitors and Flavone on Larval Growth and Survival. Caterpillar sensitivity to dietary enzyme inhibitors differed greatly among inhibitors. The PSMO inhibitor PB had no effect on RGR or survival at any concentration tested (Table 1). All larvae fed the GE inhibitor TTP survived but their RGR values were reduced at the highest concentrations compared with controls. Larvae fed the other GE inhibitor, DEF, had both greater mortality and reduced RGRs at the highest concentrations. Feeding the GST inhibitor, DEM, resulted in no mortality or reduction in RGR at any concentration tested. However, all larvae died when fed the other GST inhibitor, TPC, at concentrations equal to or greater than 0.005% (0.01, 0.05, 0.1% data not shown). Although mortality was low at 0.0005 and 0.001% TPC, these concentrations significantly reduced RGR. Mortality was low when caterpillars were fed either quercetin or ellagic acid, but there were significant reductions in RGR at ellagic acid concentrations greater than 0.1%.

Flavone significantly reduced RGR with increased concentration (Figure 2). No larvae died when fed diets containing flavone concentrations less than or equal to 0.25%, whereas all died at 0.50 and 0.75% fm.

Enzyme Activity of Larvae Fed Enzyme Inhibitors. Feeding larvae the PSMO inhibitor PB significantly reduced PSMO activity to less than 70% of the control (173.0 $\pm$ 6.0 pmol/min/mg protein) at all concentrations tested (Table 1). General esterase activity was significantly reduced to less than 5% of the control activity (423.7 $\pm$ 81.1 nmol/min/mg protein) by dietary TTP and DEF at all concentrations tested. Inhibition of either of these enzyme systems did not increase with increased concentration of the inhibitors. In contrast, GST activity increased significantly in larvae fed the highest concentrations of DEM (0.15–

0.25%) and 0.005% TPC; maximum activity was 5.2- and 3.5-fold greater, respectively, than the control (365.9 ± 22.6 nmol/min/mg protein). Considerable variation occurred in GST activity at the highest TPC concentration (0.005% fm) tested. No protein was detected in extracts from two of the five TPC experiments conducted, and the data therefore were omitted from the analysis. GST activity was not influenced by quercetin or ellagic acid at any concentration tested. Analysis of in vitro GST incubations that included the inhibitors (10^{-2} M) indicated that under these conditions all synergists tested except DEM significantly reduced enzyme activity (Table 2). Addition of 20 μl of the ellagic acid carrier (0.1 M NaOH) alone did not significantly alter GST activity (85.1 ± 2.3% of control; $P > 0.05$).

Enzyme Activity of Larvae Fed Flavone. All three enzyme systems increased in activity when the mid-instar larvae were fed 0.02% flavone (Table 3). The PSMO, GE, and GST systems had significantly greater activities ($F = 25.6$, *df*

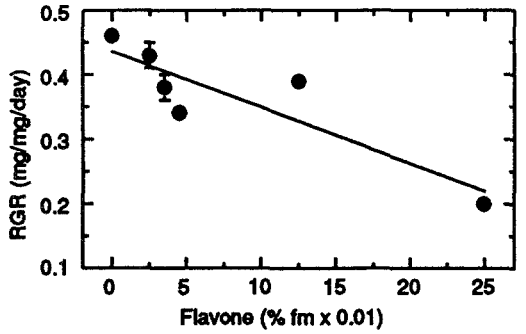

FIG. 2. Relative growth rates (RGR; mean ± SE) of *S. frugiperda* mid-instar larvae fed diets with different flavone concentrations [$R^2 = 0.55$; $P < 0.0001$; RGR = 0.44 − 0.87(flavone concentration)]. Each data point represents the mean of 15 larvae.

TABLE 2. IN VITRO INHIBITION OF GLUTATHIONE TRANSFERASE (GST) ACTIVITY (MEAN ± SE) OF MIDGUT PREPARATIONS FROM SIXTH-INSTAR *S. frugiperda*

Enzyme inhibitor (10^{-2} M)	Enzyme activity (% of control)	N^a
DEM	89.1 ± 9.3	4
TPC	2.3 ± 0[b]	2
Quercetin	2.3 ± 1.6[b]	2
Ellagic acid	5.8 ± 0.8[b]	2

[a] Number of experiments where each assay was conducted in duplicate.
[b] Means differ significantly ($P = 0.05$) from the control.

TABLE 3. ENZYME ACTIVITY (MEAN ± SE) OF MID-INSTAR *S. frugiperda* LARVAE FED FLAVONE (0.02% fm)

Enzyme[a]	Specific activity (nmol/min/mg protein)[b]	
	Control	Flavone
PSMO	93.5 ± 8.8[c]	257.1 ± 31.1[c,d]
GE	383.7 ± 59.9	576.8 ± 108.3[d]
GST	183.4 ± 47.6	319.4 ± 9.8[d]

[a] Polysubstrate monooxygenase (PSMO); general esterase (GE); and glutathione transferase (GST).
[b] Means obtained from six experiments, five for GE/flavone, where each assay was conducted in duplicate.
[c] pmol/min/mg protein.
[d] Significantly different from the control ($P = 0.05$, see text).

$= 1,11$, $P = 0.0005$; $F = 14.31$, $df = 1,10$, $P = 0.016$; and $F = 91.88$, $df = 1,11$, $P < 0.0001$, respectively), that were 2.8-, 1.5- and 1.7-fold greater than the controls, respectively.

These activities also increased in sixth-instar larvae fed flavone over that of the flavone-free control. PSMO activity increased significantly at all flavone concentrations (Figure 3A). GE activity increased significantly only at the highest flavone concentration tested (0.25%) (Figure 3B). GST activity of larvae fed flavone at all concentrations was significantly greater than that of the control (Figure 3C). The PSMO system was the most inducible, with a 3.5-fold increase over the control; the GE and GST systems increased 1.5- and 3-fold, respectively.

Synergism. Larval mortality was very low (0–2.5%) in four of the five experiments; only in the flavone 0.25% experiment was there substantial mortality. Although no larvae died in the flavone- and synergist-free controls in this experiment, mortality for those treatments that included flavone at the 0.25% level ranged from 27% to 60% and was independent of the treatments ($P > 0.1$). Because of the substantial mortality, this experiment (0.25% flavone) was not included in the remaining analyses.

Consistent with the results reported above (Figure 2), RGR decreased with flavone concentrations greater than 0.02% (Figure 4). Additionally, significant reductions in PSMO (PB: $F = 53.27$; $df = 1,26$; $P < 0.0001$) and GE (TTP: $F = 24,701.36$; $df = 1,7$; $P < 0.0001$; and DEF: $F = 37,664.12$; $df = 1,7$; $P < 0.0001$) enzyme activities occurred when larvae were fed combinations of each enzyme inhibitor and flavone (0.02% fm; Table 4), similar to each inhibitor alone (Table 1). Furthermore, GST activity increased significantly when larvae were fed combinations of flavone (0.02% fm) and DEM ($F = 14.75$; $df = 1,6$;

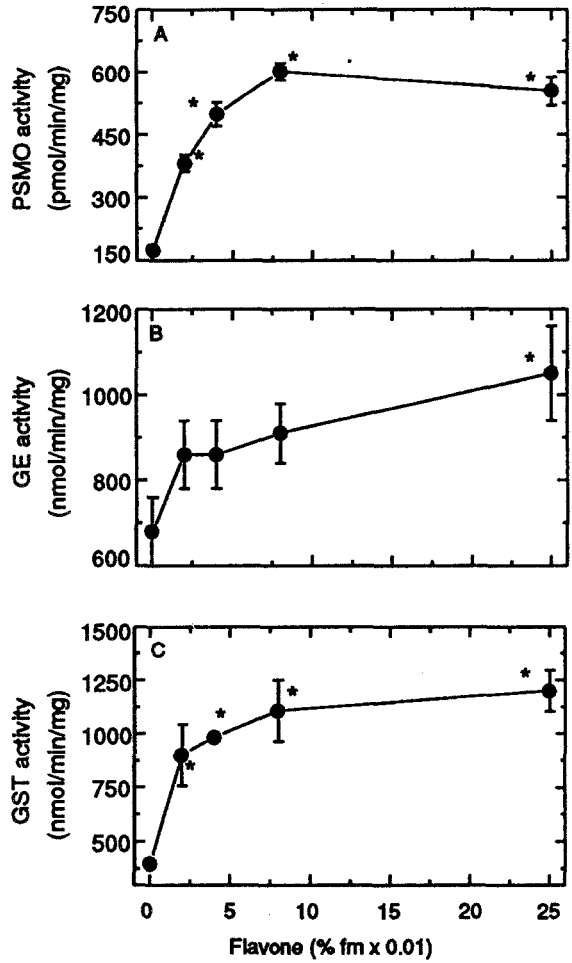

FIG. 3. Polysubstrate monooxygenase (A), general esterase (B), and glutathione *S*-transferase (C) activity (mean ± SE) of *S. frugiperda* sixth-instar larvae fed diets with different flavone concentrations. Each data point represents the mean of four to eight experiments, where each assay was conducted in duplicate. An asterisk next to a data point indicates a significant difference ($P = 0.05$) from the flavone-free control.

$P = 0.009$) or flavone and TPC ($F = 366.75$; $df = 1,7$; $P < 0.0001$), as occurred when flavone alone was fed to larvae (Figure 3). These increases in GST activity were probably due to flavone and not the inhibitors, because at these concentrations neither DEM (0.01) nor TPC (0.0004% fm) significantly altered GST activity (Table 1).

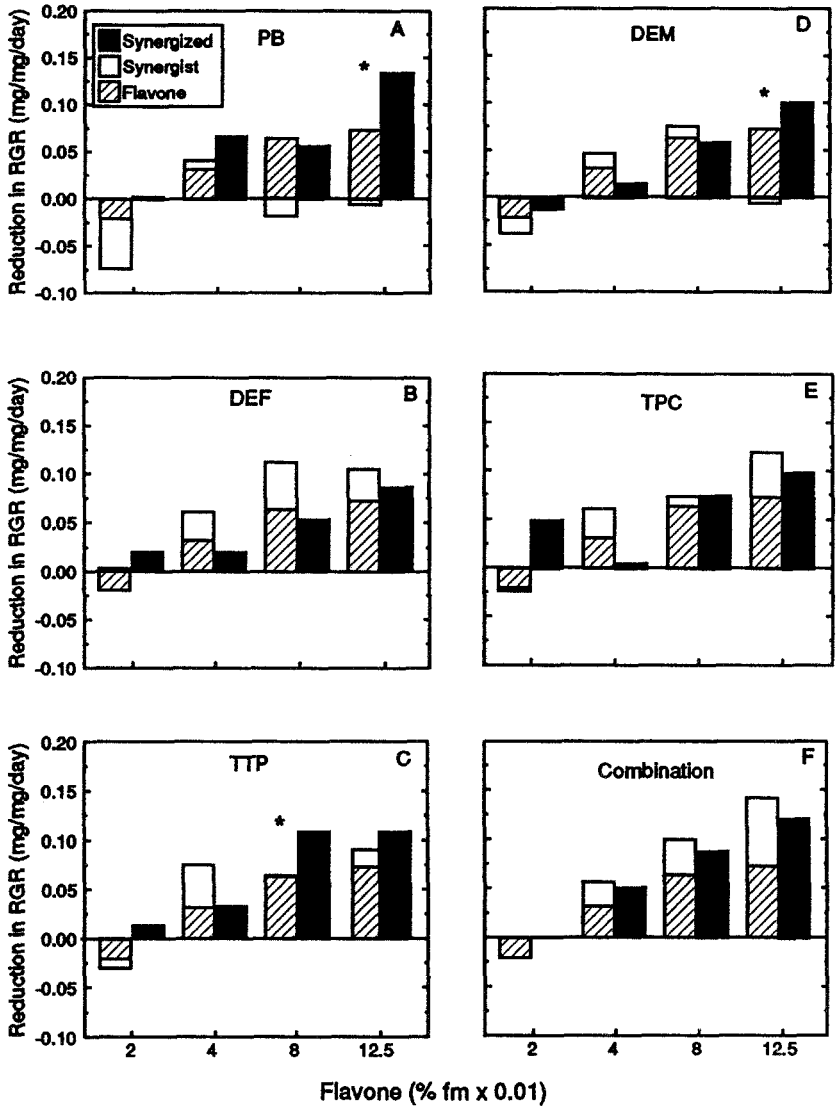

FIG. 4. Reductions in mean RGR for *S. frugiperda* larvae fed diets containing either flavone, an enzyme inhibitor, or flavone plus each enzyme inhibitor. The inhibitors used were piperonyl butoxide (PB); tri-ortho-tolyl phosphate (TTP); *S,S,S*,-tributyl phosphorotrithioate (DEF); diethyl maleate (DEM); and triphenyltin chloride (TPC). The combination treatment consisted of diet containing three inhibitors (PB, TTP, and DEM). Flavone was tested at four concentrations. Significant synergism (indicated by an asterisk) occurred when the reduction in RGR of the synergized treatment was significantly greater than that of the additive effect of flavone and the enzyme inhibitor (indicated by the height of these bars combined). Each bar represents the mean of 15 larvae except for the following treatments: $N = 14$ for flavone and synergized TPC at 0.02%, DEF and synergized TTP at 0.04%, PB, the combination and the synergized PB at 0.08%, DEF at 0.125%, and $N = 11$ for the synergized combination treatment at 0.125% flavone.

TABLE 4. PERCENT OF FLAVONE-FREE CONTROL (MEAN ± SE) ENZYME ACTIVITY OF SIXTH-INSTAR *S. frugiperda* FED FLAVONE (0.02% fm) AND EACH ENZYME INHIBITOR

Enzyme system[a]	Inhibitor (% fm)	Activity (% of control)	N^b
PSMO	PB (0.05)	56.1 ± 4.8[c]	6
GE	TTP (0.05)	5.7 ± 1.4[c]	2
	DEF (0.004)	4.4 ± 1.2[c]	2
GST	DEM (0.01)	261.7 ± 41.8[c]	2
	TPC (0.0004)	256.0 ± 6.4[c]	2

[a] Enzyme systems: Polysubstrate monooxygenase (PSMO); general esterase (GE); and glutathione transferase (GST). Inhibitors: piperonyl butoxide (PB); triorthotolyl phosphate (TTP); *S,S,S,*-tributyl phosphorotrithioate (DEF); diethyl maleate (DEM); triphenyltin chloride (TPC).
[b] Number of experiments, each assay conducted in duplicate.
[c] Significantly different ($P = 0.05$) from the controls (see text).

Significant synergism was found when PB, TTP, and DEM were combined with the higher flavone concentrations. PB and DEM exhibited synergism at 0.125% fm flavone, whereas TTP synergized flavone at 0.08% (Figure 4). However, no significant synergistic effects were found at 0.02 and 0.04% flavone. Furthermore, the inhibitors DEF, TPC, and the combination treatment did not significantly synergize flavone at any concentration tested. The RGR increased slightly, as indicated by the negative bars in Figure 4, when larvae were fed the lowest dose of flavone (0.02%) or several of the synergists alone.

Benefit of Pre-exposure to Flavone. Significant interactions between the main effects (study and treatment) occurred only with RCR ($F = 4.06$; $df = 4,120$; $P = 0.004$); thus, only this performance index was analyzed separately for each study. Two uninduced and four induced larvae died after the dietary switch but before the prepupal stage; however, these differences were independent of the treatments ($P > 0.05$). Biomass of uninduced and induced larvae after the two-day preexposure period was not significantly ($P = 0.17$) different from the control, whereas biomass was significantly reduced in the induced compared with the uninduced treatments ($F = 4.58$; $df = 1,129$; $P = 0.034$; Table 5). Biomass gained following the dietary switch significantly decreased in the uninduced and induced treatments ($F = 34.7$; $df = 1,126$; $P < 0.0001$) compared with the control; however, there was no significant difference between the uninduced and induced larvae. RGR significantly decreased in the uninduced and induced treatments compared with the control ($F = 156.50$; $df = 1,126$; $P < 0.0001$) and, furthermore, RGR of the uninduced larvae was significantly reduced compared with the induced larvae ($F = 6.68$; $df = 1,126$; $P = 0.035$). No significant changes in AD occurred for any of the treatments. ECD decreased

TABLE 5. NUMBER OF *S. frugiperda* LARVAE SURVIVING, THEIR MEAN (±SE) BIOMASS AFTER BEING FED FOR TWO DAYS ON INDUCING DIET, BIOMASS GAIN, RELATIVE GROWTH RATES (RGR), APPROXIMATE DIGESTIBILITY (AD), AND EFFICIENCY OF CONVERSION OF DIGESTED FOOD TO BIOMASS (ECD) AFTER PRE-EXPOSURE

Treatment	N^a	Mean initial biomass (mg fm)	Biomass gain (mg)	RGR (mg/mg/day)	AD (%)	ECD (%)
Control	45/45	73.4 (±2.8)NS[b]	30.1 (±1.1)*	0.23 (±0.01)*	54.2 (±1.1)NS	31.8 (±1.0)*
Uninduced	45/43	73.0 (±3.0)	22.2 (±1.1)	0.14 (±0.01)	51.7 (±1.3)	28.0 (±1.1)
Induced	45/41	64.9 (±2.2)*[c]	23.0 (±0.8)NS	0.16 (±0.01)*	52.3 (±1.4)NS	29.4 (±1.3)NS

[a] Number surviving the pre-exposure treatment/number surviving to prepupal stage.
[b] Control means followed by an asterisk (NS = not significant) are significantly greater than the uninduced and the induced treatments by a one-tailed orthogonal contrast (see Methods and Materials).
[c] Induced means followed by an asterisk (NS = not significant) are significantly different from the uninduced means by a one-tailed orthogonal contrast (see Methods and Materials).

only for the uninduced and induced treatments compared with the control larvae ($F = 10.22$; $df = 1,126$; $P = 0.01$; Table 5). RCR decreased significantly in the uninduced and induced treatments compared with the flavone-free control in experiments 1 ($F = 38.46$; $df = 1,42$; $P < 0.0001$), 2 ($F = 20.66$; $df = 1,42$; $P < 0.0001$), and 3 ($F = 15.94$; $df = 1,38$; $P = 0.0003$). Additionally, the induced larvae had significantly greater RCR values compared with the uninduced larvae only in experiments 1 and 3 (Figure 5).

DISCUSSION

Our experiments were designed to test the importance of detoxifying enzyme activity in the ability of larvae of the polyphagous *S. frugiperda* to tolerate ingested flavone. Little, if any, biologically significant benefit resulted from uninhibited constitutive enzyme activity or from pre-exposure to inducing doses of flavone. If the level of in vitro PSMO activity measured here is representative of the activity of caterpillar midgut tissues in vivo, and if flavone is detoxified by these enzymes, then the inhibition observed with PB (approximately 50%) should effectively double the concentration of flavone in the insect. However, combining PB and flavone in the diet resulted in significant synergism only at the highest flavone concentration tested (0.125%; Figure 4A). Furthermore, a more than two-fold increase in PSMO activity following pre-exposure did not dramatically benefit larvae subsequently exposed to a growth-reducing concentration of flavone (Table 5, Figure 5).

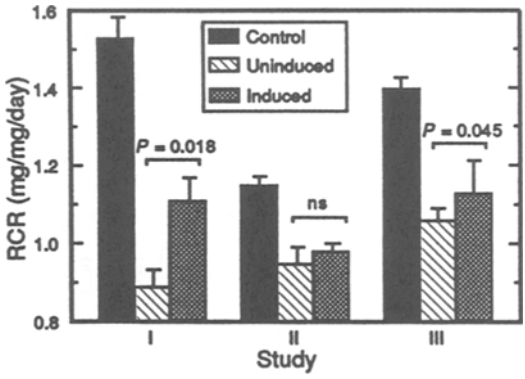

FIG. 5. Relative consumption rates (RCR) (mean ± SE) of *S. frugiperda* larvae fed either control or flavone (0.02%) diets for two days, then switched to a control (control or uninduced) or a flavone (0.125%; induced) diet and reared to pupation. The statistical comparisons indicated are one-tailed orthogonal contrasts between the uninduced and induced treatments.

Synergism. The results of the synergism studies varied considerably and therefore should be interpreted with caution. We predicted that flavone would be metabolized by ring hydroxylation by the PSMO system, a common mode of action for this enzyme system (Hodgson et al., 1991), and that inhibiting this system with PB would have a greater negative impact on caterpillar performance than inhibition of the GE and GST enzyme systems. Although the GE inhibitors were almost 100% effective, we did not expect this inhibition to affect flavone metabolism because this compound lacks the active site (an ester bond; Figure 1) for these enzymes. As none of the GST inhibitors reduced GST activity in vivo, synergism of flavone was not expected, even if these enzymes were involved in flavone detoxification. However, in addition to the effect of PB, significant synergism occurred when flavone diets contained the GE inhibitor TTP or the GST inhibitor DEM (Figure 4C and D, respectively). Synergism by DEM may have occurred because DEM inhibited PSMO activity, as found in vitro in house flies (Welling and DeVries, 1985), although the effect of DEM on PSMO activity was not determined in our experiments. The reason for synergism with TTP and flavone (0.08%; Figure 4C) is unknown. Furthermore, in several of our experiments with various inhibitors (Figure 4B–E), the additive reduction in RGR resulting from flavone and the synergist administered singly was much greater than that of the synergized treatment; the cause of these results is also unknown. Finally, synergism with flavone was not significant in any of the combination treatments, in which an inhibitor of each enzyme system was combined, even though synergism occurred when these same inhibitors were used individually. Possibly the synergists in the combination treatments antagonized one another. Thus, even though we found one example of synergism that fit our prediction (i.e., PB; Figure 4A), many of our results remain unexplained.

Synergism may result when the co-application (usually topical) of an enzyme inhibitor and a toxic pesticide causes a significantly greater reduction in insect performance or an increase in mortality compared with the additive effect of each component in the mixture (Metcalf, 1967). Feeding synergists to caterpillars prolongs exposure to the compounds and thus represents a different situation from that of a single topical dose. Thus, detoxifying enzymes may be induced, possibly resulting in increased metabolism of the synergist (Yu and Terriere, 1974). However, these inhibitors (PB, TTP, DEF, and DEM) have been used successfully to synergize allelochemicals when fed to insect larvae (Hedin et al., 1988; Lindroth, 1989). Furthermore, other workers have reported synergism when larvae of *Heliocoverpa zea* and *Depressaria pastinacella* were fed the allelochemical xanthotoxin with PB or other naturally occurring methylenedioxyphenyl-containing inhibitors (Berenbaum and Neal, 1985; Neal, 1989; Lee and Berenbaum, 1990).

The levels of in vivo PSMO inhibition with PB found in our study were

similar to those of 1-day-old house fly (Yu and Terriere, 1974) and sixth-instar *S. frugiperda* larvae fed PB (Yu and Hsu, unpublished data). Furthermore, they are in the range of previous reports of in vitro inhibition, ranging from 30% to 93% (e.g., Yu, 1987; Nitao, 1989). We found the greatest in vivo inhibition with the GE inhibitors, results that are similar to in vitro analyses reported previously (Yu, 1990). In contrast, although each GST inhibitor except DEM significantly reduced GST activity when added to the in vitro midgut preparations in our study, they did not reduce in vivo GST activity. Instead, the dietary GST inhibitors DEM and TPC induced GST activity, as is often found with dietary xenobiotics (Yu, 1986), including PB in house flies (Yu and Terriere, 1974) and black cutworm larvae (Thongsinthusak and Krieger, 1974). Possibly this biphasic activity occurred in response to DEM and TPC, where GST enzyme inhibition occurred initially, followed by enzyme induction (Yu and Terriere, 1974; Thongsinthusak and Krieger, 1974). However, we are unaware of this biphasic response occurring in insects exposed to any inhibitor other than PB. Induction of detoxifying enzyme activity suggests that the metabolism of these inhibitors may be increased. Neither quercetin nor ellagic acid significantly influenced GST activity, although, as was found in human and rat liver preparations (Das et al., 1986), both compounds (and TPC) reduced in vitro GST activity.

The failure of DEM to inhibit GST enzyme activity in vitro was unexpected, as this is a commonly used insecticide synergist (e.g., Argentine et al., 1989; Pree et al., 1990). Several reasons for this apparent lack of inhibition can be proposed. The use of DEM is based on reduced (by about 50% after 2 hr) GST activity of house fly larvae after topical application (Welling and DeVries, 1985). In mammals, DEM reduces GST activity by depleting the supply of glutathione (see references in Welling and DeVries, 1985). However, in house flies the decrease in glutathione concentration following DEM treatment was not sufficient to cause the observed reduction in enzyme activity (Welling and DeVries, 1985). Possibly, despite depletion of glutathione by DEM (as in mammals), enough glutathione remained in our assays to allow the reaction to proceed normally. Finally, Yu (1989) found six isozymic forms of *S. frugiperda* GST; thus it is possible that the GST isozyme responsible for CDNB conjugation was not sensitive to inhibition by DEM. Furthermore, individual isozymes of the GST system may have been inhibited, as reported by Welling and DeVries (1985), but the substrate we used may have been conjugated by a different GST isozyme. Analysis of individual GST isozymes and inhibition by DEM with additional substrates would be necessary to determine why we failed to find GST inhibition with DEM.

Benefit of Pre-exposure to Flavone. Although PSMO and GST enzyme activities in the flavone-fed (0.02%) larvae were 2.8- and 1.7-fold greater in the mid-instars and 2.2- and 2.3-fold greater in the sixth instars, respectively,

than in the controls, subsequent feeding on the effective-dose diet (0.125% flavone) improved RGR of the induced compared with the uninduced larvae by only 14%. Three possible reasons for this marginal benefit of enzyme induction are discussed here. First, maximum PSMO activity occurs about two to three days following exposure to an inducer (Brattsten and Wilkinson, 1973; Yu, 1982). Detoxifying enzymes in the uninduced larvae may have become induced after the switch to flavone (0.125%), permitting recovery from the toxic effect of ingested flavone. Slansky (unpublished data) found in similar pre-exposure experiments with another allelochemical that the results may depend upon when performance is measured after diet switch. However, induced *S. frugiperda* larvae, allowed to develop for just two days after the switch to the 0.125% flavone diet, did not have greater RGR or RCR values than uninduced larvae (data not shown), in contrast to larvae that were allowed to complete development to the pupal stage. Second, flavonoids (e.g., the pentahydroxy flavone quercetin) inhibit the superoxide dismutase antioxidant enzymes that protect several species of herbivores from superoxide anions (Pritsos et al., 1991). These highly reactive species may react deleteriously with macromolecules such as DNA, RNA, lipids, and proteins (Felton and Duffey, 1991). However, it is not known if flavone influences the antioxidant enzymes in *S. frugiperda*. Third, flavone tolerance or PSMO induction by flavone may have differed among generations of *S. frugiperda*. Both RGR and RCR for all treatments were lower in the second experiment, suggesting that the larvae in this experiment had a reduced ability to respond adaptively to flavone.

Our data showing a benefit of pre-exposure may be the result of habituation to a repellent diet. Both RCR and RGR increased in the induced larvae compared with the uninduced larvae. The improved RGR may simply reflect the greater RCR, rather than an increased tolerance to flavone. An increase in RCR after pre-exposure may be explained by desensitization of deterrent receptors (Szentesi and Jermy, 1990). In a subsequent choice test (Slansky and Wheeler, unpublished), however, flavone at 0.125% deterred *S. frugiperda* larvae, but the effect did not decrease following a two-day exposure to the 0.02% fm diet. Thus, desensitization is considered to be an unlikely explanation for these results.

Induction of detoxifying enzymes may be a generalized response to the presence of xenobiotics (Dowd, 1990). Although flavone induces all of the enzyme systems we investigated, as well as others (Yu, 1986), and is metabolized in vitro by the PSMO system (Yu, 1987), it may not be effectively detoxified in vivo. Similar to our results, Harwood et al. (1990) found that, although all of the peppermint monoterpenes they studied were metabolized in vitro by the PSMO system, apparent inhibition of this system with PB synergized only two of the four allelochemicals tested. Thus, in vitro metabolism may not always reflect in vivo metabolism. Furthermore, the potential flavone metabolites (possibly two to three) recovered from frass produced by uninduced larvae were

each equal in concentration to those produced by the flavone-induced larvae (Wheeler, unpublished data). These results suggest that in vivo metabolism of flavone occurs but the metabolite(s) may be as toxic as the parent compound or more so (Fukami et al., 1967; Isman et al., 1987; Koul et al., 1990). Overall, our findings suggest that, at best, constitutive and induced detoxifying enzymes may marginally enhance flavone tolerance in *S. frugiperda* larvae. However, more studies are needed evaluating the relationship between in vitro and in vivo detoxifying enzyme activity and the benefit of enzyme induction before general conclusions may be drawn regarding the role of detoxifying enzymes in allelochemical tolerance.

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REFERENCES

ARGENTINE, J.A., CLARK, J.M., and FERRO, D.N. 1989. Genetics and synergism of resistance to azinphosmethyl and permethrin in the Colorado potato beetle (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 82:698–705.

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

BERENBAUM, M.R., COHEN, M.B., and SCHULER, M.A. 1990. Cytochrome P450 in plant-insect interactions: Inductions and deductions, pp. 257–262, *in* H.H. Hagedorn, J.G. Hildebrand, M.G. Kidwell, and J.H. Law (eds.). Molecular Insect Science. Plenum Press, New York.

BERGE, M.A., and ROSENTHAL, G.A. 1991. Metabolism of L-canavanine and L-canaline in the tobacco budworm, *Heliothis virescens* (Noctuidae). *Chem. Res. Toxicol.* 4:237–240.

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.

BRATTSTEN, L.B. 1992. Metabolic defense against plant allelochemicals, pp. 175–242, *in* G.A. Rosenthal and M.R. Berenbaum (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

BRATTSTEN, L.B., and WILKINSON, C.F. 1973. Induction of microsomal enzymes in the southern armyworm (*Prodenia eridania*). *Pestic. Biochem. Physiol.* 3:393–407.

BRATTSTEN, L.B., WILKINSON, C.F., and EISNER, T. 1977. Herbivore–plant interactions: Mixed-function oxidases and secondary plant substances. *Science* 196:1349–1352.

DAS, M., SINGH, S.V., MUKHTAR, H., and AWASTHI, Y.C. 1986. Differential inhibition of rat and human glutathione *s*-transferase isoenzymes by plant phenols. *Biochem. Biophys. Res. Commun.* 141:1170–1176.

DOWD, P.F. 1990. Detoxification of plant substances by insects, pp. 181–225, *in* E.D. Morgan and N. B. Mandava (eds.). Handbook of Natural Pesticides, Vol VI: Insect Attractants and Repellents. CRC Press, Boca Raton, Florida.

FELTON, G.W., and DUFFEY, S.S. 1991. Protective action of midgut catalase in lepidopteran larvae against oxidative plant defenses. *J. Chem. Ecol.* 17:1715–1732.

FUKAMI, J., YAMAMOTO, I., and CASIDA, J.E. 1967. Metabolism of rotenone in vitro by tissue homogenates from mammals and insects. *Science* 155:713–716.

GORDON, H.T. 1968. Quantitative aspects of insect nutrition. *Am. Zool.* 8:131–138.

GREENE, G.L., LEPPLA, N.C., and DICKERSON, W.A. 1976. Velvetbean caterpillar: A rearing procedure and artificial medium. *J. Econ. Entomol.* 69:487–488.

GUNDERSON, C.A., SAMUELIAN, J.H., EVANS, C.K., and BRATTSTEN, L.B. 1985. Effects of the mint monoterpene pulegone on *Spodoptera eridania* (Lepidoptera: Noctuidae). *Environ. Entomol.* 14:859–863.

HABIG, W.H., PABST, M.J., and JAKOBY, W.B. 1974. Glutathione *S*-transferases: The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249:7130–7139.

HARBORNE, J.B. 1988. The Flavonoids: Advances in Research Since 1980. Chapman and Hall, New York.

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.

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. *Pestic. Biochem. Physiol.* 32:55–61.

HODGSON, E., SILVER, I.S., BUTLER, L.E., LAWTON, M.P., and LEVI, P.E. 1991. Metabolism, pp. 107–167, *in* W.J. Hays, Jr., and E.R. Laws, Jr. (eds.). Handbook of Pesticide Toxicology. Academic Press, New York.

ISMAN, M.B., PROKSCH, P., and WITTE, L. 1987. Metabolism and excretion of acetylchromenes by the migratory grasshopper. *Arch. Insect Biochem. Physiol.* 6:109–120.

KENNEDY, G.G. 1984. 2-Tridecanone, tomatoes and *Heliothis zea*: Potential incompatibility of plant antibiosis with insecticidal control. *Entomol. Exp. Appl.* 35:305–311.

KOUL, O., SMIRLE, M.J., ISMAN, M.B., and SZETO, Y.S. 1990. Synergism of a natural insect growth inhibitor is mediated by bioactivation. *Experientia* 46:1082–1084.

LEE, K. 1991. Glutathione *S*-transferase activities in phytophagous insects: Induction and inhibition by plant phototoxins and phenols. *Insect Biochem.* 21:353–361.

LEE, K., and BERENBAUM, M.R. 1990. Defense of parsnip webworm against phototoxic furanocoumarins: Role of antioxidant enzymes. *J. Chem. Ecol.* 16:2451–2460.

LINDROTH, R.L. 1989. Biochemical detoxication: Mechanisms of differential tiger swallowtail tolerance to phenolic glycosides. *Oecologia* 81:219–224.

LINDROTH, R.L., and BLOOMER, M.S. 1991. Biochemical ecology of the forest tent caterpillar—responses to dietary protein and phenolic glycosides. *Oecologia* 86:408–413.

METCALF, R.L. 1967. Mode of action of insecticide synergists. *Annu. Rev. Entomol.* 12:229–256.

NEAL, J.J. 1989. Myristicin, safrole, and fagaramide as phytosynergists of xanthotoxin. *J. Chem. Ecol.* 15:309–315.

NITAO, J.K. 1989. Enzymatic adaptation in a specialist herbivore feeding on furanocoumarin-containing plants. *Ecology* 70:629–635.

NITAO, J.K. 1990. Metabolism and excretion of the furanocoumarin xanthotoxin by parsnip webworm, *Depressaria pastinacella*. *J. Chem. Ecol.* 16:417–428.

OTTEA, J.A., and PLAPP, F.W., JR. 1984 Glutatione *S*-transferase in the house fly: Biochemical and genetic changes associated with induction and insecticide resistance. *Pestic. Biochem. Physiol.* 22:203–208.

PREE, D.J., ARCHIBALD, D.E., and COLE, K.J. 1990. Insecticide resistance in spotted tentiform leafminer (Lepidoptera: Gracillariidae): Mechanisms and management. *J. Econ. Entomol.* 83:678–685.

PRITSOS, C.A., PASTORE, J., and PARDINI, R.S. 1991. Role of superoxide dismutase in the protection and tolerance to the prooxidant allelochemical quercetin in *Papilio polyxenes*, *Spodoptera eridania*, and *Trichoplusia ni*. *Arch. Insect Biochem. Physiol.* 16:273–282.

PROKSCH, P., ISMAN, M.B., WITTE, L., and HARTMANN, T. 1987. Metabolites of insecticidal chromenes from the migratory grasshopper *Melanoplus sanguinipes*. *Phytochemistry* 26:2227–2230.

RISKALLAH, M.R., DAUTERMAN, W.C., and HODGSON, E. 1986. Nutritional effects on the induction of cytochrome P-450 and glutathione transferase in larvae of the tobacco budworm, *Heliothis virescens* (F.). *Insect Biochem.* 16:491–499.

SAS INSTITUTE. 1987. SAS Propriety Software Release 6.04. SAS Institute, Cary, North Carolina.

SLANSKY, F., JR., and SCRIBER, J.M. 1985. Food consumption and utilization, pp. 87–163, *in* G.A. Kerkut and L.I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon Press, Oxford.

SMIRLE, M.J., and ISMAN, M.B. 1992. Metabolism and elimination of ingested allelochemicals in a holometabolous and a hemimetabolous insect. *Entomol. Exp. Appl.* 62:183–190.

SODERLUND, D.M., and BLOOMQUIST, J.R. 1990. Molecular mechanisms of insecticide resistance, pp. 58–96, *in* R.T. Roush and B.E. Tabashnik (eds.). Pesticide Resistance in Arthropods. Chapman and Hall, New York.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry, 2nd ed. Freeman, San Francisco.

SZENTESI, A., and JERMY, T. 1990. The role of experience in host plant choice by phytophagous insects, pp. 33–74, *in* E.A. Bernays (ed.). Insect-Plant Interactions. CRC Press, Boca Raton, Florida.

TERRIERE, L.C. 1984. Induction of detoxication enzymes in insects. *Annu. Rev. Entomol.* 29:71–88.

THONGSINTHUSAK, T., and KRIEGER, R.I. 1974. Inhibitory and inductive effects of piperonyl butoxide on dihydroisodrin hydroxylation in vivo and in vitro in black cutworm (*Agrotis ypsilon*) larvae. *Life Sci.* 14:2131–2141.

TIETZ, H.M. 1972. An Index to the Described Life Histories, Early Stages, and Hosts of the Macrolepidoptera of the Continental United States and Canada. A.C. Allyn Museum, Sarasota, Florida.

VAN ASPEREN, K. 1962. A study of housefly esterases by means of a sensitive colorimetric method. *J. Insect Physiol.* 8:401–416.

WADLEIGH, R.W., and YU, S.J. 1987. Glutathione transferase activity of fall armyworm larvae toward α,β-unsaturated carbonyl allelochemicals and its induction by allelochemicals. *Insect Biochem.* 17:759–764.

WADLEIGH, R.W., and YU, S.J. 1988a. Detoxification of isothiocyanate allelochemicals by glutathione transferase in three lepidopterous species. *J. Chem. Ecol.* 14:1279–1288.

WADLEIGH, R.W., and YU, S.J. 1988b. Metabolism of an organothiocyanate allelochemical by glutathione transferase in three lepidopterous insects. *J. Econ. Entomol.* 81:776–780.

WALDBAUER, G.P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol.* 5:229–288.

WELLING, W., and DEVRIES, J.W. 1985. Synergism of organophosphorous insecticides by diethyl maleate and related compounds in house flies. *Pestic. Biochem. Physiol.* 23:358–369.

YU, S.J. 1982. Induction of microsomal oxidases by host plants in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). *Pestic. Biochem. Physiol.* 17:59–67.

YU, S.J. 1983. Induction of detoxifying enzymes by allelochemicals and host plants in the fall armyworm. *Pestic. Biochem. Physiol.* 19:330–336.

YU, S.J. 1984. Interactions of allelochemicals with detoxication enzymes of insecticide-susceptible and resistant fall armyworms. *Pestic. Biochem. Physiol.* 22:60–68.

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.

YU, S.J. 1989. Purification and characterization of glutathione transferases from five phytophagous Lepidoptera. *Pestic. Biochem. Physiol.* 35:97–105.

YU, S.J. 1990. Liquid chromatographic determination of permethrin esterase activity in six phytophagous and entomophagous insects. *Pestic. Biochem. Physiol.* 36:237–241.

YU, S.J. 1991. Insecticide resistance in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). *Pestic. Biochem. Physiol.* 39:84–91.

YU, S.J., and HSU, E.L. 1985. Induction of hydrolases by allelochemicals and host plants in fall armyworm (Lepidoptera: Noctuidae) larvae. *Environ. Entomol.* 14:512–515.

YU, S.J., and TERRIERE, L.C. 1974. A possible role for microsomal oxidases in metamorphosis and reproduction in the housefly. *J. Insect Physiol.* 20:1901–1912.

YU, S.J., ROBINSON, F.A., and NATION, J.L. 1984. Detoxication capacity in the honey bee, *Apis mellifera* L. *Pestic. Biochem. Physiol.* 22:360–368.

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PYRROLIZIDINE ALKALOIDS IN THE ARCTIID MOTH *Hyalurga syma*

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Abstract—The arctiid moth *Hyalurga syma* (subfamily Pericopinae) sequesters pyrrolizidine alkaloids (PAs) from its larval food plant *Heliotropium transalpinum* (Boraginaceae). Colorimetric quantification of total PAs in the larvae, pupae, and adults of *Hyalurga* revealed mean values of about 286–445 μg per individual (1.4–2.6% of dry weight). The PA mixtures found in the moth and its larval food plant were evaluated by GC-MS. Food-plant leaves were found to contain the diastereoisomeric retronecine esters indicine (IIIa), intermedine (IIIb), and lycopsamine (IIIc), and the heliotridine ester rinderine (IIId) only as minor constituents, whereas 3′-acetylrinderine (IVc) (68% of total PAs) and the respective 3′-acetyl esters of indicine (IVa) and intermedine (IVb) (both 17%) were the major alkaloids. Supinine (IIa) is detectable in traces only. The PA mixtures in eggs, larvae, pupae, and imagines of *Hyalurga* were identical: indicine, intermedine, and lycopsamine accompanied by considerable amounts of supinine and amabiline or coromandalinine (IIb/IIc) were the major components. Only larvae were found to store small quantities of a 3′-acetyl derivative. Rinderine and its 3′-acetyl ester were never found in the insects. Low concentrations of the arctiid-specific PA callimorphine (I) were present in larvae, pupae, and imagines. The differences in the PA patterns of the insects and their larval food plant suggest that *Hyalurga* is capable of modifying plant-derived PAs by inversion

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of the 7-OH configuration (conversion of the necine base heliotridine into retronecine), and perhaps the inversion of the 3′-OH [conversion of (+)-trachelanthic acid into (−)-viridifloric acid], although the possibility of a selective sequestration of the respective retronecine esters cannot be excluded. Some trials with the orb-weaving spider *Nephila clavipes*, a common neotropical predator, showed that both freshly emerged and field-caught adults of *Hyalurga syma* are liberated unharmed by the spider. The liberation could be related to the presence of PAs in the moths.

Key Words—Pyrrolizidine alkaloids, alkaloid sequestration, alkaloid transformation, Boraginaceae, *Heliotropium transalpinum*, *Heliotropium peruvianum*, Lepidoptera, Arctiidae, Pericopinae, *Hyalurga syma*, chemical defense, Araneidae, *Nephila clavipes*, orb-weaving spider.

INTRODUCTION

Sequestration of secondary plant substances by phytophagous insects is a well-known phenomenon (Duffey, 1980). Many classes of compounds are involved: cardenolides (Brower, 1984 and references therein), iridoid glycosides (Bowers, 1988), quinolizidine alkaloids (Hartmann, 1991), and pyrrolizidine alkaloids (Boppré, 1990 and references therein). The pyrrolizidine alkaloids (PAs) are a well-known example of plant-derived insect compounds; their functions in insects such as arctiid moths and danaine and ithomiine butterflies are related to chemical defense and pheromone biosynthesis (for reviews see Boppré 1986, 1990).

In this communication we report on the ability of the neotropical arctiid *Hyalurga syma* to sequester food-plant-derived PAs. A comparative analysis of the PAs found in the larval food plant *Heliotropium transalpinum* and the different developmental stages of the moth is given. A bioassay with the spider *Nephila clavipes* suggests that PAs may chemically protect *H. syma* against this predator.

METHODS AND MATERIALS

Organisms. Larvae of *Hyalurga syma* Walker, 1854 (Lepidoptera, Arctiidae, Pericopinae) were collected in the Costa e Silva Wood on *Heliotropium transalpinum* Vell. (Boraginaceae) and an egg cluster was collected in the Monjolinho Arboretum from *Heliotropium peruvianum* L.. Both areas are located in the Fazenda Santa Elisa, Instituto Agronômico, Campinas, São Paulo State, Brazil (22°54′S 47°05′W). The larvae were reared in the laboratory on *H. transalpinum*. The eggs were allowed to hatch on *H. peruvianum*; after five days the larvae were transferred to *H. transalpinum* and were further reared on this species. *H. syma* adults are not very abundant, but they can be found during February–July flying in the forest understory. Sometimes adult moths were observed visiting potential PA sources such as *Eupatorium* species.

Colorimetric Assay. Total PAs (free bases + *N*-oxides) and PA *N*-oxides were quantitatively analyzed using the colorimetric method described by Mattocks (1967a, 1968) and Bingley (1968) and modified by Brown (1985). The insects were placed individually into vials containing 2 ml MeOH; the bodies were punctured with a pair of scissors, and the closed vials were left standing at room temperature for at least one week. Larvae were starved for 6 hr before extraction. For the assay of total alkaloids, one twentieth of the liquid volume was mixed with 0.5 ml of the oxidation reagent ($Na_4P_2O_7$ + H_2O_2 30%, 5 mg/ml, diluted 1:200 with MeOH), heated in a test tube at 100°C for 25 min, cooled to room temperature, and any traces of residual water were removed; isoamyl acetate (1.0 ml) and acetic anhydride (0.1 ml) were added, and the mixture was kept at 100°C for 2 min with the tube tightly stoppered to prevent any water contamination. The sample was again cooled to room temperature and treated with 1.0 ml of freshly prepared modified Ehrlich reagent (8 ml of 20% methanolic BF_3, 72 ml of absolute EtOH, and 1.4 g dimethylaminobenzaldehyde). The tube was stoppered and heated at 60°C for 5 min and cooled to room temperature; the mixture was diluted to 3.8 ml with redistilled acetone and assayed photometrically at 561 nm. For assay of the *N*-oxide fraction, the same procedure was applied, but the oxidation step was omitted, simply drying the methanolic aliquot before addition of isoamyl acetate–acetic anhydride. Heliotrine and heliotrine *N*-oxide were used as standards. All reagents were carefully distilled and purified according to Mattocks (1967a).

Purification of PA Extracts. The MeOH extracts prepared as given above were combined, evaporated under low pressure at low temperature (<40°C), and redissolved in 2 N H_2SO_4. The samples were extracted three times with $CHCl_3$, and the organic phases were set aside. Half of the acid aqueous solution was reduced with Zn dust for 3 hr, alkalinized with NH_4OH, and extracted three times with $CHCl_3$–MeOH (3:1) and once with pure $CHCl_3$. The combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated. The residue represents total PAs as tertiary bases. The remaining half of the acid extract was treated in the same way but the Zn/H^+ reduction was omitted. The final preparation contains the tertiary PAs present in the crude extract. The PA preparations were either directly subjected to TLC analysis according to Mattocks (1967b) or stored at 0°C until GC or GC-MS analysis.

Leaves of *H. transalpinum* and *H. peruvianum* were extracted with MeOH, and the crude PA fraction was purified using the procedure described above.

GC-MS Analysis. Stored and newly prepared PA extracts were further purified by liquid–solid extraction using Extrelut (Merck) columns (Hartmann and Toppel, 1987). The evaporated chloroform Extrelut extracts were redissolved in MeOH and directly used for GC-MS analysis. A Carlo Erba Mega 5160 gas chromatograph equipped with a fused silica column (WCOT, 30 m × 0.32 mm, DB-1, J&W Scientific), was directly coupled to a quadrupole mass spectrometer

(Finnigan MAT 4515). Conditions: injector 250°C; temperature program 150–300°C, 6°C/min; split ratio 1:20; carrier gas He 0.5 bar. RIs were calculated according to Kovats (1958). Reference compounds were prepared from the following sources: intermedine/lycopsamine from *Symphytum officinale*, indicine from *Heliotropium indicum* (Mattocks et al., 1961), rinderine from *H. peruvianum* (Brown, 1984; Trigo, 1988), supinine and amabiline from *Eupatorium laevigatum* (Trigo et al., unpublished), and callimorphine from *Tyria jacobaeae* (Ehmke et al., 1990).

Hydrolysis of 3'-Acetylated PAs. PA extracts of *H. transalpinum* were purified as given above and treated with 1.0 N H_2SO_4 at 50°C for one week. The solution was then adjusted to pH 11 with NH_4OH and extracted four times with $CHCl_3$. The extracts were dried over anhydrous Na_2SO_4, evaporated and redissolved in MeOH, and analyzed by GC-MS.

Spider Bioassay. The *Nephila clavipes* bioassays were carried out in the Costa e Silva Wood, between 0900 and 1600 hr during March to May, when the spiders were abundant. Only healthy female spiders were utilized, that is, the spiders responded immediately when any prey was tossed into their web.

Female spiders behave in a characteristic way when prey items fall into their webs (Vianna, 1972). The spider moves from the radius convergence region to the place where the prey is trapped. The prey is touched with the chelicerae, pedipalps, and the first pair of legs, bitten, and poisoned. The spider then takes the prey to the radius convergence region and afterwards rolls it up with silk from its spinnerets. The prey is sucked for about 15 min after being captured.

Spiders already feeding on any prey were not utilized in the bioassays. Each spider was utilized only once a day.

Laboratory-raised freshly emerged (i.e., age up to one day after eclosion) and field-caught adults of *H. syma* were bioassayed according to Vasconcellos-Neto and Lewinsohn (1984). The pierid *Eurema arbela* (Geyer, 1832), of similar size to *H. syma* and containing no PAs, was utilized as control after the trial with *H. syma*. A response like that described by Vianna (1972) was considered as predation. When the spider cut the web around the prey after touching it and freed it unharmed, the response was described as liberation. A rejection of the prey after it has been killed, as mentioned by Vasconcellos-Neto and Lewinsohn (1984), was not observed in our trials.

RESULTS

Colorimetric Assays. Quantitative PA determination revealed the presence of PAs in larvae, pupae, and imagines of *H. syma* (Table 1); they were also detected but not quantified in eggs and meconium. No significant differences

TABLE 1. CONTENT OF TOTAL PYRROLIZIDINE ALKALOIDS (PAs) IN DIFFERENT DEVELOPMENTAL STAGES OF *Hyalurga syma*[a]

Developmental stages	*N*	Total pyrrolizidine alkaloids		
		Per individuum (μg)	% of dry wt	% as *N*-oxide
Eggs[b]	685	++	NA[c]	NA
Larvae[d]	5	445 ± 159	2.6 ± 0.9	87 ± 10
Pupae	3	353 ± 130	1.4 ± 0.5	91 ± 16
Freshly emerged adults	7	303 ± 251	2.0 ± 1.5	96 ± 35
Field-caught adults	27	286 ± 136	2.1 ± 0.9	103 ± 14

[a]PAs were determined colorimetrically; $\bar{X}$ ± SD.
[b]PAs detectable but not analyzed quantitatively.
[c]NA = not analyzed.
[d]Third-instar larvae.

were found between the total PA level in field-caught and laboratory-raised, freshly emerged adults. In both cases, however, a great variation in the amount per individual was found to exist (20–740 μg in laboratory-raised adults and 27–511 μg in field-caught adults). In both cases PAs were present almost exclusively as *N*-oxides.

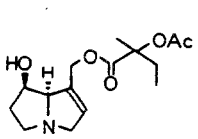
PA Patterns of Hyalurga syma *and its Food Plant.* In the wild areas in the Campinas region, eggs and larvae of *H. syma* were found almost exclusively on leaves of *Heliotropium transalpinum*. The only exception was a population found on *H. peruvianum* in an arboretum. Leaves of *H. peruvianum* contain rinderine as the only PA (Brown, 1985; Trigo, 1988).

The average concentration of total PAs in leaves of the larval food plant *Heliotropium transalpinum* was about 0.045% (dry wt). The PA composition of *H. transalpinum* is shown in Table 2. The major PAs, which together account for 85% of total PAs in the plant, were identified as the 3′-acetyl derivatives of indicine or intermedine, and rinderine (IVa, IVb, and IVc) (Figure 1); RIs 2203 (IVa or IVb) and 2219 (IVc); MS data, *m/z* (%): 341 (M^+ < 1), 326 (<1), 255 (82), 254 (1), 181 (5), 180 (5), 156 (5), 139 (23), 138 (100), 137 (12), 136 (14), 120 (9), 108 (2), 99 (7), 95 (5), 94 (23), 93 (73), 80 (9), 67 (7), 53 (5), 43 (32). The MS data are consistent with data reported for 3′-acetyllycopsamine in *Amsinckia menziesii* (Boraginaceae) (Roitman, 1983). Acid hydrolysis gave two compounds with RI and fragmentation patterns identical to indicine (IIIa, RI 2126), intermedine (IIIb, RI 2126) (not properly resolved with our GC method), and rinderine (IIId, RI 2145). Lycopsamine (IIIc, RI 2139) and rinderine (IIId, RI 2145) were present in low amounts in the total PAs. Other unidentified PAs were detected in trace amounts.

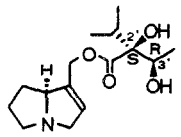
TABLE 2. PATTERNS OF PYRROLIZIDINE ALKALOIDS (PAs) FOUND IN *Hyalurga syma* AND ITS LARVAL HOST PLANT (*Heliotropium transalpinum*) ESTABLISHED BY GC-MS[a]

Alkaloid		RI	M^+	Necine base	Necic acid	Relative abundance (%)						
						Eggs (wild)	Larvae (lab)	Pupae (lab)	Adults (lab)	Meconium	Adults (wild)	Host plant
I.	Callimorphine	1963	297	ret		ND	2	1	2	ND	2	ND
IIa.	Supinine	1974	283	sup	(+)tr	5	4	5	4	12	<1	<1
IIb.	Amabiline or				(−)vd							
IIc.	coromandalinine	1982	283	sup	(+)vd	11	25	9	6	23	4	ND
IIIa.	Indicine and				(−)tr							
IIIb.	Intermedine	2126	299	ret	(+)tr	23	25	24	23	24	51	2
IIIc.	Lycopsamine	2139	299	ret	(−)vd	58	36	57	34	38	37	3
IIId.	Rinderine	2145	299	hel	(+)tr	ND	ND	ND	ND	ND	ND	7
IVa.	3′-Acetylindicine/				(−)tr[b]							
IVb.	3′-Acetylintermedine	2203	341	ret	(+)tr[b]	ND	ND	ND	ND	ND	ND	17
IVc.	3′-Acetylrinderine	2219	341	hel	(+)tr[b]	ND	ND	ND	ND	ND	ND	68
Unknown isomer of IV		2248	341	?	?	ND	12	ND	1	ND	ND	ND
Other unknown PAs						3	5	4	7	3	14	2

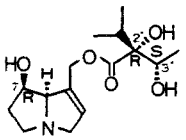
[a]Wild = wild-collected/caught; lab = laboratory raised; ND = not detectable; sup = supinidine; ret = retronecine; hel = heliotridine; tr = trachelanthic acid; vd = viridifloric acid.
[b]3[1]OAc derivative.

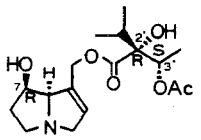


I callimorphine



IIa supinine (2'S, 3'R)
IIb amabiline (2'S, 3'S)
IIc coromandalinine (2'R, 3'R)





IIIa indicine (7R, 2'R, 3'S)
IIIb intermedine (7R, 2'S, 3'R)
IIIc lycopsamine (7R, 2'S, 3'S)
IIId rinderine (7S, 2'S, 3'R)

IVa 3'-acetylindicine (7R, 2'R, 3'S)
IVb 3'-acetylintermedine (7R, 2'S, 3'R)
IVc 3'-acetylrinderine (7S, 2'S, 3'R)

FIG. 1. Pyrrolizidine alkaloids in *Hyalurga syma* and its larval host plant *Heliotropium transalpinum*.

The PA patterns of *H. syma* differ considerably from those of the food plant (Table 2). The major PAs in the insect are indicine (IIIa, RI 2126) and intermedine (IIIb, RI 2126), and lycopsamine (IIIc, RI 2139), both with *m/z* (%): 299 (M^+ < 1), 255 (<1), 254 (<1), 156 (7), 139 (31), 138 (100), 120 (10), 108 (3), 94 (65), 93 (97), 80 (18), 67 (14), 53 (8), 43 (31). A similar fragmentation was found by Pedersen and Larsen (1970) for echinatine (lycopsamine epimer at C-7). Rinderine (IIId, RI 2145) and its 3′-acetylester are completely absent, but one 3′-acetylated PA was found in significant amounts in the larvae only (Table 2), with RI (2248) significantly different from those established for the 3′-acetylesters found in the larval food plant. Two further prominent compounds (IIa, RI 1974) and (IIb, RI 1982) were found in the insect extracts. Both have identical MS fragmentation patterns, given here for IIa: *m/z* (%): 283 (M^+ < 1), 238 (<1), 140 (6), 123 (25), 122 (100), 121 (38), 120 (43), 108 (14), 93 (23), 80 (13), 70 (15), 67 (6), 53 (12), 43 (24). RIs, M^+, and fragmentation patterns, as well as comparison with authentic reference compounds, characterize IIa as supinine and IIb as amabiline or its stereoisomer coromandalinine [first isolated by Mohanraj et al. (1982) from *Heliotropium curassavicum*]. The separation of amabiline and coromandalinine is not possible by our GC method. In the food plant, supinine is only detectable in trace

amounts. Amabiline and other isomers are missing (Table 2). Callimorphine (I, RI 1963) was found in small amounts in all developmental stages, except eggs. Several unidentified PAs were present in trace amounts. In the meconium, IIa, IIb or IIc, IIIa, IIIb, IIIc, and other PAs as traces were found.

Nephila clavipes *Bioassay.* A total of 15 wild-caught adults and eight laboratory-raised freshly emerged adults of *Hyalurga syma* were offered as prey to *Nephila clavipes*. Without exception, all specimens were immediately cut out of the web and liberated by the spider. Control individuals of *Eurema arbela* were preyed by the spider after the bioassay with *Hyalurga syma*. All *Hyalurga* individuals liberated by the spider were found to contain PAs.

DISCUSSION

PAs in Hyalurga syma. *Hyalurga* stores PAs as *N*-oxides. This is in agreement with results from other arctiids such as *Creatonotos transiens* (Nickisch-Rosenegk et al., 1990; Hartmann et al., 1990) and *Tyria jacobaeae* (Ehmke et al., 1990). The great variation in the total amounts of PAs found in the moths needs further study, since all fed on the same leaves.

The differences in the PA patterns between *Hyalurga syma* and its larval food plant could be explained two ways: (1) differential uptake and selective sequestration; and (2) metabolic transformation of the plant-derived PAs by the insect.

Although the first possibility cannot be excluded, the second seems to be more likely. It is known from another arctiid, *Creatonotos transiens*, that in relation to the formation of pheromones such as (7*R*)-hydroxydanaidal, (7*S*)-heliotrine is converted into (7*R*)-heliotrine by larvae and later developmental stages (Wink et al., 1988).

Hyalurga is completely devoid of heliotridine derivatives, which, however, account for 75% of total PAs in the food plant (see Table 2); it seems probable that analogous conversion of 7*S*-configured rinderine into 7*R*-configured intermedine has occurred.

The large proportion of lycopsamine found in the insects could also be explained through an inversion of configuration, comparable to that described above, at the 3′-carbon [conversion of (+)-trachelanthic acid into (−)-viridifloric acid], perhaps accompanying deacetylation. The conversion of rinderine into lycopsamine was also found in experiments with the ithomiine *Mechanitis polymnia* (Trigo et al., unpublished results). The larva's supply of rinderine would come from deacetylation of 3′-acetylrinderine, the major PA of the larval food plant. The acetyl derivative of the macrocyclic PA seneciphylline (12-O-acetylseneciphylline) fed to larvae of the arctiid *Tyria jacobaeae* is deacetylated and only seneciphylline is stored by the insect (Ehmke et al., 1990).

The supinidine derivatives that are found at considerable levels in the insect may be regarded as side products of the 7*S*/7*R* inversion of the hydroxyl, assuming a stereoselective removal and addition of the hydroxyl. In plants, supinidine derivatives are biogenetic precursors of heliotridine or retronecine derivatives (Robins, 1989; Hartmann and Hülsmeyer, unpublished results).

In general, arctiids have been shown to transform plant-derived PAs. Creatonotine in *Creatonotos transiens* (Hartmann et al., 1990) and the related callimorphine (L'Empereur et al., 1989; Ehmke et al., 1990), also present in low amounts in *Hyalurga*, are synthesized in the insects by esterification of retronecine, derived from plant-acquired PAs, with specific necic acids produced by the insect itself from isoleucine.

Feeding experiments with pure or isotopically labeled PAs are needed to test PA transformation in *H. syma*.

Chemical Defense in Hyalurga syma. The PAs identified from alkaloid extracts of *Hyalurga syma* are known to protect Ithomiinae and Danainae against *Nephila clavipes* (Brown, 1984, 1985, 1987; Trigo, 1988). Bioassays by these authors showed that topically applied PAs (around 200 μg) elicited the liberation response of *Nephila clavipes* for palatable butterflies. Thus, it is reasonable to relate the unpalatability of the *Hyalurga* moths to the sequestered PAs. Another arctiid moth, *Utheteisa ornatrix*, is also liberated by this spider due to PAs sequestered from the larval food plant *Crotalaria* (Fabaceae) (Eisner, 1982). In its natural environment, adults of *H. syma* could be well protected against the spider, and perhaps against other predators, although there are no records of natural encounters between the moth and the spider. The chemical defense of *H. syma* against *Nephila clavipes* is a fact, but there is no evidence that the moths sequester PAs in specific response to selective pressure of the spider. The question of the selective pressure responsible for PA acquisition by the *H. syma* in evolutionary time remains to be answered.

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REFERENCES

BINGLEY, J.B. 1968. Solvent and temperature effects in the determination of pyrrolizidine alkaloids with 4-dimethylaminobenzaldehyde. *Anal. Chem.* 40:1166–1167.

BOPPRÉ, M. 1986. Insects pharmocophagously utilizing 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.

BOWERS, M.D. 1988. Chemistry and coevolution: Iridoid glycosides, plants, and herbivorous insects, pp. 133–165, *in* K.C. Spencer (ed.). Chemical Mediation of Coevolution. Academic Press, New York.

BROWER, L.P. 1984. Chemical defense in butterflies. *Symp. R. Entomol. Soc. London* 11:109–134.

BROWN, K.S. 1984. Adult-obtained pyrrolizidine alkaloids defend ithomiine butterflies against a spider predator. *Nature* 309:707–709.

BROWN, K.S. 1985. Chemical ecology of dehydropyrrolizidine alkaloids in adult Ithomiinae (Lepidoptera: Nymphalidae). *Rev. Bras. Biol.* 44:435–460.

BROWN, K.S. 1987. Chemistry at the Solanaceae/Ithomiinae interface. *Ann. Mo. Bot. Gard.* 74:359–397.

DUFFEY, S.S.1980. Sequestration of plant natural products by insects. *Annu. Rev. Entomol.* 25:447–477.

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.

EISNER, T. 1982. For love of nature: exploration and discovery at biological field stations. *BioScience* 32:321–326.

HARTMANN, T. 1991. Alkaloids, pp. 79–121. *in* D.E. Janzen and G. Rosenthal (eds.). Herbivores: Their Interactions with Secondary Plant Metabolism, 2.ed., Academic Press, New York.

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., 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.

L'EMPEREUR, K.M., LI, Y., and STERMITZ, F.R. 1989. Pyrrolizidine alkaloids from *Hackelia californica* and *Gnophaela latipennis*, an *H. californica*-hosted arctiid moth. *J. Nat. Prod.* 52:360–366.

KOVATS, E. 1958. Gas-chromatographische Charakterisierung organischer Verbindungen. Teil 1: Retentionsindices aliphatischer Halogenide, Alkohole, Aldehyde und Ketone. *Helv. Chim. Acta* 41:1915–1932.

MATTOCKS, A.R. 1967a. Spectrophotometric determination of unsaturated pyrrolizidine alkaloids. *Anal. Chem.* 39:443–447.

MATTOCKS, A.R. 1967b. Detection of pyrrolizidine alkaloids on thin-layer chromatograms. *J. Chromatogr.* 27:505–508.

MATTOCKS, A.R. 1968. Spectrophotometric determination of pyrrolizidine alkaloids—some improvements. *Anal. Chem.* 40:1749–1750.

MATTOCKS, A.R., SCHOENTAL, R., CROWLEY, H.C., and CULVENOR, C.C.J. 1961. Indicine: The major alkaloid of *Heliotropium indicum* L.. *J. Chem. Soc.* 1961:5400–5403.

MOHANRAJ, S., SUBRAMANIAN, P.S., and HERZ, W. 1982. Minor alkaloids of *Heliotroium curassavicum*. *Phytochemistry* 21:1775–1779.

NICKISCH-ROSENEGK, E., SCHNEIDER, D., and WINK, M. 1990. Time course of pyrrolizidine alkaloid processing in the alkaloid exploiting arctiid moth, *Creatonotos transiens*. *Z. Naturforsch.* 45c:881–894.

PEDERSEN, E., and LARSEN, E. 1970. Mass spectrometry of some pyrrolizidine alkaloids. *Org. Mass Spectrom.* 4:249–256.

ROITMAN, J.N. 1983. The pyrrolizidine alkaloids of *Amsinckia menziessi*. *Aust. J. Chem.* 36:769–778.

ROBINS, D.J. 1989. Biosynthesis of pyrrolizidine alkaloids. *Chem. Soc. Rev.* 18:375–408.

TRIGO, J.R. 1988. Ecologia química na interação Ithomiinae (Lepidoptera: Nymphalidae)/Echitoideae (Angiospermae: Apocynaceae). MSc thesis. Instituto de Biologia, UNICAMP, Campinas, São Paulo, Brazil, 196 pp.

VASCONCELLOS-NETO, J., and LEWINSOHN, T. 1984. Discrimination and release of unpalatable butterflies by *Nephila clavipes*, a neotropical orb-weaving spider. *Ecol. Entomol.* 9:337–344.

VIANNA, M.J.B. 1972. Contribuição à biologia de *Nephila clavipes* L. (Araneae, Araneidae). PhD thesis. Faculdade de Ciências Médicas e Biológicas de Botucatu, São Paulo, Brazil, 154 pp.

WINK, M., SCHNEIDER, D., and WITTE, L. 1988. Biosynthesis of pyrrolizidine alkaloid-derived pheromones in the arctiid moth, *Creatonotos transiens*: Stereochemical conversion of heliotrine. *Z. Naturforsch.* 43c:737–741.

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IDENTIFICATION AND BIOACTIVITY OF ALARM PHEROMONE IN THE WESTERN FLOWER THRIPS, *Frankliniella occidentalis*

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Abstract—Analysis by gas chromatography (GC) and GC-mass spectroscopy disclosed that droplets of anal fluid produced by second-instar western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), contain a two-component alarm pheromone, comprised of decyl acetate and dodecyl acetate, in a molar ratio of approximately 1.5:1. Both nymphs and adults responded to the pheromone by walking away from the source. The synthetic pheromone was active at a concentration of 1.0 ng, and the proportions of insects responding to the pheromone, but not the distances moved, increased with increasing dose. Each component was active alone, although at low doses, the response to decyl acetate was less than to either dodecyl acetate or the blend. The pheromone also induced some second instars to drop from leaves and reduced oviposition by adult females in both two-choice and no-choice experiments. Because the response of western flower thrips to the alarm pheromone is relatively weak, the potential for its use in pest management is limited, unless it is used in conjunction with other control measures.

Key Words—*Frankliniella occidentalis*, western flower thrips, Thysanoptera, Thripidae, alarm pheromone, decyl acetate, dodecyl acetate, pest management.

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INTRODUCTION

Although alarm pheromones are most commonly found in eusocial insects (Blum, 1969, 1985), they do occur in some presocial or gregarious species. Alarm pheromones have been found in several Homoptera, particularly treehoppers (Membracidae) and aphids (Aphididae). Aggregations of treehoppers disperse in response to alarm pheromones (Nault et al., 1974; Wood, 1976), and aphids also disperse by walking away or dropping from their feeding sites when exposed to alarm pheromone (Bowers et al., 1972; Montgomery and Nault, 1977; Calabrese and Sorensen, 1978; Wholers, 1981b). Nymphs of a pyrrhocorid (Calam and Youdeowei, 1968) and both nymphs and adults of some Pentatomidae (Ishiwatari, 1974; Lockwood and Story, 1987) disperse from aggregations and often drop from feeding sites in response to their alarm pheromones. Alarm pheromones also cause dispersal behavior in species of Cimicidae (Levinson and Bar Ilan, 1971; Levinson et al., 1974), Tingidae (Kearns and Yamamoto, 1981), and Acanthosomidae (Maschwitz and Gutmann, 1979).

The only thysanopteran known to produce an alarm pheromone, perillene, is the gregarious tubuliferan thrips, *Varshneyia pasaniae* Mukaigawa (Suzuki et al., 1988). Western flower thrips, *Frankliniella occidentalis* (Pergande), are not highly gregarious, but do exhibit a tendency toward clumped distributions (Peterson, 1990). Therefore, utilization of an alarm pheromone would be an adaptive trait in this species. Our preliminary observations suggested that this thrips produces an alarm pheromone that is released in droplets of anal fluid.

Herein, we verify the presence of an alarm pheromone in the western flower thrips and report on its isolation and identification and on some behavioral responses of thrips to the pheromone.

METHODS AND MATERIALS

Maintenance of Thrips Colony. Western flower thrips were obtained from colonies at the Agriculture Canada Research Station, Agassiz, British Columbia. A stock colony of thrips was reared in 1-liter glass canning jars. Each jar contained a 2 to 3-cm-deep layer of damp peat moss on the bottom to provide pupation sites. The metal lid of the jar was replaced with filter paper held in place by the screwtop ring. Each jar held a small, water-filled plastic container with a hole in the lid. Thrips were reared on pinto beans, *Phaseolus vulgaris* L., with the severed stems, usually bearing two leaves, extending through the hole in the lid into the water. Leaves were replaced as they wilted or sustained severe feeding damage.

Large quantities of even-aged nymphs were obtained by placing 20–30 adult thrips of both sexes on each of several leaves, as above. Adults were removed from the leaves every one to two days, and the leaves replaced. Adult

thrips were added to the jars as numbers declined. Leaves with eggs were removed from the jars and placed on styrofoam trays floating in water, with the petioles of the leaves extending through holes in the styrofoam into the water. The age of the thrips developing on each leaf was therefore known within a range of one to two days.

Demonstration of Alarm Response. Thirteen adult thrips, 20 second-instar, and 37 first-instar thrips were tested for response to the secretions of second instars. Experiments were conducted in a laboratory at approximately 25°C and with light intensity varying from 600 to 21500 lx. Single thrips of the three stages were isolated on pinto bean leaves (area approximately 25 cm^2) floating adaxial (upper) surface up in 2–3 mm of water in a 10-cm-diameter Petri dish. The thrips were allowed to settle for at least 2 hr.

To collect thrips secretions, second-instar thrips were picked up at the thorax with forceps. A triangular, filter-paper wick (base = 2 mm, sides = 7 mm) was held in a second pair of forceps and the point rubbed gently over the abdomen of the thrips. Any anal droplet produced was absorbed. Secretions of five thrips were collected on one filter-paper wick, after which it was immediately held 2–3 mm above a stationary thrips isolated on a leaf. A new wick was used for each insect tested.

Each thrips was observed for 1 min. Any thrips moving ≥ 2 mm was considered to have responded to the stimulus. The net distance moved from the starting point in 1 min was recorded for each thrips. The control treatment was a clean wick presented to similar numbers of thrips in the same way.

A crude extract was made by crushing second-instar thrips in distilled pentane at −78.5°C. The supernatant was removed and concentrated under a stream of nitrogen to 2 nymph equivalents (NE) per microliter. The extract was stored at −5°C until used. Second-instar and adult thrips were isolated on leaves as above and tested for response to the extract. Five nymph equivalents of extract in 2.5 μl of pentane were placed on a wick and held above a stationary thrips. Numbers responding and distance moved were recorded. The control stimulus was a wick treated with 2.5 μl of pentane.

A chi-square test for comparing two proportions (Zar, 1984) was used to compare the proportion of thrips responding in control and experimental treatments. Mean distances moved were calculated for responding individuals and compared by one-tailed t tests.

Sensitivity of second-instar thrips to the extract of second instars was tested at doses of 0.001, 0.01, 0.025, 0.05, 0.1, 0.5, 1.0, and 5.0 NE. A 2.5-μl aliquot of solvent was used as a control treatment. A linear model for categorical data (Grizzle et al., 1969) with modified Bonferroni t tests (Myers, 1979) was used to compare the proportions of insects responding. Tukey's test (Zar, 1984) was used to compare a logarithmic transformation of distances moved by responding insects. Doses at which only one insect responded were excluded from the analysis.

Pheromone Chemistry. A Hewlett-Packard 5830 gas chromatograph equipped with a capillary inlet system and flame-ionization detector was employed for analyses by gas–liquid partition chromatography (GC). A glass column (30 m × 0.5 mm ID) coated with SP-1000 (Supelco Canada Ltd., Oakville, Ontario) was used. The injection port and detector temperatures were 260 and 270°C, respectively. A Hewlett-Packard 5895B GC/MS/DS was employed for coupled gas chromatography–mass spectroscopy (GC-MS). A fused silica column (0.32 mm ID) coated with DB-1 (30 m) (J & W Scientific, Inc., Folsom, California) was coupled directly into the ion source. The injection port, transfer line, and ion source were 260, 250, and 200°C, respectively. Helium was the carrier gas for GC and GC-MS.

In a cold room, first- or second-instar thrips of mixed sex, or adults of each sex, were transferred directly from the host into a pentane-containing vial set in Dry Ice. The frozen thrips were finely crushed. Any sample not immediately processed and analyzed was stored at −27°C. After removal from Dry Ice, the sample was allowed to warm to room temperature, and the supernatant was transferred to a clean vial. After concentration to approximately 100 μl under a stream of nitrogen at 0°C, the extract was placed on glass wool in a small glass chamber to which was attached an analytical Porapak Q trap (6 mm OD packed with approximately a 30-mm length of absorbent). The volatiles were transferred from the glass wool to the Porapak Q with nitrogen (130 ml/min for 2 hr) and recovered by eluting the Porapak Q trap with 2 ml pentane. The eluent was concentrated under a stream of nitrogen to 300 μl, and then analyzed by GC and GC-MS. Volatiles recovered from the Porapak Q trap were tested against second-instar western flower thrips nymphs. Anal droplets were collected from second instars with very fine micropipets, stored in hexane, and analyzed in the same way.

Decyl acetate (99.2%) was prepared by reaction of decyl alcohol with acetic anhydride in pyridine, isolated by extractive work-up in the usual way, and purified by distillation at reduced pressure. Dodecyl acetate (97%) was purchased from the Aldrich Chemical Co., Milwaukee, Wisconsin.

Bioactivity of Synthetic Pheromone. The bioassy used to test the nymph extract was used to test the bioactivity of the synthetic pheromone. Second-instar thrips were used since they were abundant and easy to handle. Because the response of thrips to the nymph extract had seemed to vary with weather-driven changes in the laboratory, two experiments were conducted to test the hypothesis that low levels of light reduce the response of thrips to their alarm pheromone. These experiments demonstrated that the number of second instars responding to the synthetic pheromone increased significantly (χ^2 test, $P < 0.05$) from 33.3% to 83.3% ($N = 12$) when bioassays were done at 7490 lx and 25°C in a controlled-environment chamber compared to 588 lx and 25°C in the laboratory. Within the chamber, responses increased significantly (χ^2 test,

$P < 0.05$) from 58.3% to 91.7% ($N = 12$) when light intensity was increased from 1712 to 7490 lx. Therefore, all bioassays with synthetic pheromone were conducted in the controlled-environment chamber at 25°C and 7490 lx.

The candidate, two-component alarm pheromone (decyl acetate and dodecyl acetate), in the naturally occurring molar ratio of 1.5:1, and each component alone were tested against second-instar thrips at doses of 0.1, 1.0, 10, 100, 1000, and 10000 ng. Pentane (2 μl) and 1 NE of crude extract served as negative and positive control treatments, respectively. The proportions of thrips responding were analyzed with a linear model for categorical data (Grizzle et al., 1969), with modified Bonferroni t tests (Myers, 1979) used to compare proportional data >0%. The Fisher exact test (Zar, 1984) with modified Bonferroni t tests was used to test for differences between responses of 0% and the next 1–3 higher responses. Tukey's test was used to compare a logarithmic transformation of distances moved. Doses at which only one insect responded were excluded from the analysis.

Effects of Pheromone on Nymphal Dropping. Four experiments were conducted to test the hypothesis that the alarm pheromone causes nymphs to drop from the leaves on which they are feeding. Populations of 50–225 second instars were either knocked onto a bean leaf from an aspirator and allowed to settle for 24 hr, or adults were allowed to oviposit on a bean leaf for 24 hr, and the resulting offspring were used when they became second instars. A cardboard trap coated with Tanglefoot (The Tanglefoot Company, Grand Rapids, Michigan) was placed around the stem of each leaf to trap any thrips dropping from the leaf.

In the first experiment, the synthetic pheromone (decyl acetate–dodecyl acetate, molar ratio 1.5:1) in 10 μl of pentane, was placed on a 1.27-cm-diameter filter-paper disk pierced by a stainless steel pin. When the solvent had evaporated, disks were placed on both the upper and lower surfaces of each leaf by penetrating the leaf with the pins until the paper disks touched the leaf surface. One to four populations of second instars were exposed to each treatment at doses of 0.1, 1.0, and 10 μg of pheromone, divided equally between the upper and lower disk. Pentane was used as a control treatment. The second experiment was similar to the first, but after the disks had been pinned to the leaves, the petiole of each leaf was lightly tapped five times with a pencil.

In the third experiment, the pheromone was sprayed directly on leaves containing thrips. The synthetic pheromone was dissolved in 66.7% ethanol at 10 μg/ml and was dispensed at 5 μg of pheromone per spray from a 15-ml perfume atomizer. A similar atomizer containing 66.7% ethanol was used for control treatments. Each leaf was sprayed with approximately 4–5 μg of pheromone on each surface.

In the fourth experiment, a leaf with thrips was placed in a 140-ml closed chamber. In each chamber, a 1-cm-diameter filter-paper disk treated with 10 μg

of pheromone was placed 1–2 cm from the leaf. The leaves on which the thrips were located were not touched or disturbed during the experiment.

In each experiment, the thrips were observed for 30 min, after which the numbers of nymphs on the sticky traps and the numbers remaining on the leaves were counted. In each experiment, the numbers within treatments were pooled, and the percent dropping calculated. The data were analyzed using a linear model for categorical data (Grizzle et al., 1969).

Effects of Pheromone on Oviposition. In the first of two experiments on oviposition, thrips were allowed to choose between an oviposition substrate treated with the alarm pheromone and one treated with solvent. Bean pod segments, 5 cm long, were used as oviposition substrates. The cut ends of the beans were coated with melted paraffin to prevent thrips from entering the interior of the pods. The synthetic pheromone was dissolved in 66.7% ethanol and applied from a 15-ml perfume atomizer. A similar atomizer containing 66.7% ethanol was used for control treatments. Twelve bean segments were each sprayed with 1–2 μg of pheromone and 12 with 70 μl of solvent. When the solvent had evaporated, a pheromone-treated and a control bean were placed with 20 adult females in a 1-liter canning jar covered with filter paper.

After 24 hr the females were removed and each bean was placed in a separate plastic vial covered with 203 PeCap mesh (B & SH Thompson, Scarborough, Ontario). Six days later, the numbers of nymphs on each bean were recorded. Because western flower thrips eggs are very small and difficult to find, nymphs rather than eggs, were counted. The data were analyzed using a paired sample t test.

In a second, no-choice experiment, single bean segments, treated with either the pheromone or ethanol as above, were confined in mesh-covered plastic vials with 20 adult females for 24 hr (10 replicates) or 48 hr (six replicates). Six days after removal of the females, the numbers of nymphs on each bean were counted. A one-tailed t test was used to compare the mean numbers of nymphs produced per 24 hr.

RESULTS

Demonstration of Alarm Response. The typical response by thrips of all three life stages to both the natural secretions and extract of second instars was to walk away from the stimulus source, usually in a fairly straight line. Turns of $>90°$ were uncommon unless the thrips encountered a large vein or the edge of a leaf. Before moving, a thrips often would twitch its abdomen from side to side. This action was also seen when several thrips were in close proximity to each other. When a second instar was unable to move in response to the alarm stimulus (possibly because its proboscis was stuck in the leaf tissue), a droplet

of anal fluid was often produced. The same response was seen in second instars touched or seized by predators. When a thrips was free to move away from the stimulus, the production of an anal droplet was never observed.

A greater proportion of first- and second-instar thrips responded to second-instar secretions than to control treatments (Table 1). Of the insects that responded, only second instars moved a significantly greater distance in response to the secretions than to the control wick. There was no significant difference between the proportions of adults responding to the two treatments or in the distances moved. Adult thrips are more mobile than nymphs and, during testing, often moved in response to any disturbance or movement in their vicinity.

Second instars and adults responded in greater numbers to the extract of second instar thrips than to the solvent control (Table 2). Thus, the high but not significant response by adults to the anal fluid (Table 1) was apparently real. In no case was the distance moved by thrips responding to the extract significantly greater than for those responding to the control stimulus.

The response of second instars to the nymphal extract increased with increasing dose (Figure 1), the greatest response occurring at 1 NE. The distance moved by responding insects exposed to 0.01 NE was significantly less than the distances moved at doses of 0.05–1.0 NE (Figure 2).

Pheromone Identification. Analysis of second-instar volatiles by GC revealed the presence of only two compounds (Figure 3) in a ratio of approximately 2:1. In another sample the ratio was 1.5:1. The mass spectra of the unknowns were readily obtained by analysis of the volatiles by GC-MS, and each exhibited a base peak at m/z 43, suggesting that the compounds were

TABLE 1. ALARM RESPONSE OF *F. occidentalis* TO SECRETIONS OF FIVE SECOND INSTARS PRESENTED ON FILTER-PAPER WICKS TO STATIONARY THRIPS

Developmental stage of test insect	Treatment	Number tested	Percent response[a]	Distance (mm) moved by responding insects (mean ± SE)[b]
First instar	control	20	25.0a	5.8 ± 2.3a
	secretions of 5 nymphs	20	85.0b	9.5 ± 1.7a
Second instar	control	37	24.3a	5.1 ± 1.7a
	secretions of 5 nymphs	37	67.6b	17.1 ± 2.3b
Adult	control	13	23.1a	10.3 ± 1.9a
	secretions of 5 nymphs	13	46.2a	9.8 ± 3.0a

[a] Paired percents followed by the same letter are not significantly different, χ^2 test, $P < 0.05$.
[b] Paired means followed by the same letter are not significantly different, t test, $P < 0.05$.

TABLE 2. ALARM RESPONSE OF *F. occidentalis* TO CRUDE PENTANE EXTRACT OF SECOND INSTARS PRESENTED ON FILTER-PAPER WICKS TO STATIONARY THRIPS

Developmental stage of test insect	Treatment	Number tested	Percent response[a]	Distance (mm) moved by responding insects (mean ± SE)[b]
Second instar	control	29	20.7a	8.7 ± 2.8a
	extract of 5 nymphs	29	75.9b	12.3 ± 1.6a
Adult	control	20	35.0a	5.7 ± 1.0a
	extract of 5 nymphs	20	65.0b	9.5 ± 1.7a

[a] Paired percents followed by the same letter are not significantly different, χ^2 test, $P < 0.05$.
[b] Paired means followed by the same letter are not significantly different, t test, $P < 0.05$.

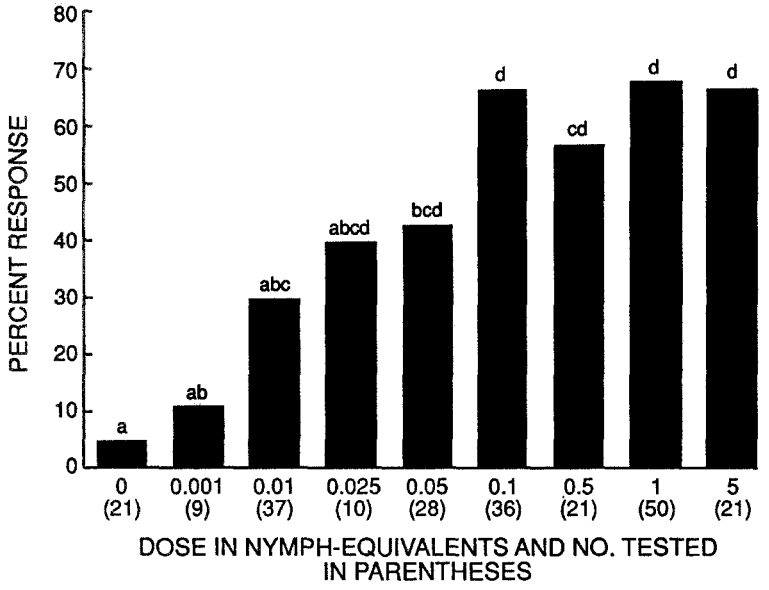

FIG. 1. Percent responses by second-instar *F. occidentalis* exposed to crude pentane extract of second-instar nymphs presented on a filter-paper wick to stationary thrips. Bars topped by the same letter are not significantly different, linear model for categorical data with Bonferroni t tests, $P > 0.05$.

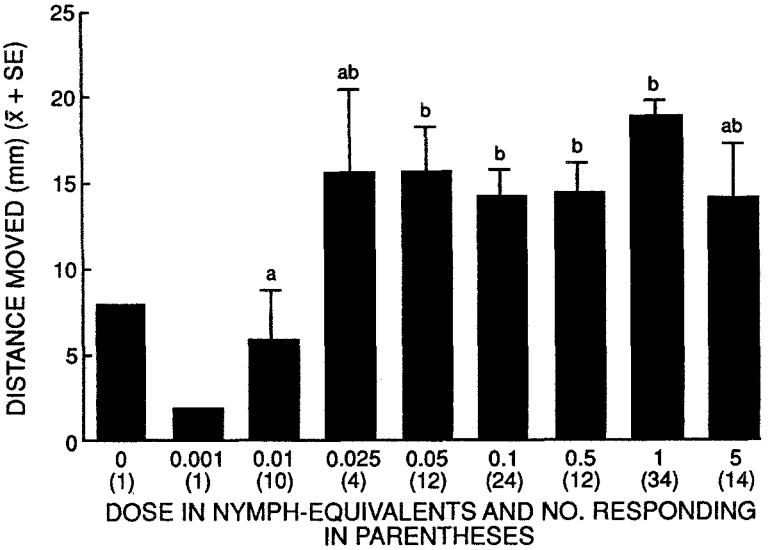

FIG. 2. Distances moved by second-instar *F. occidentalis* exposed to crude pentane extract of second-instar nymphs presented on a filter-paper wick to stationary thrips. Bars topped by the same letter are not significantly different, Tukey's test, $P > 0.05$.

probably acetate esters. The highest observed ions in mass spectra of the unknowns were, in order of elution, *m/z* 140 and 168, respectively, indicating the compounds were homologs differing by two methylene units. Since an acetate ester readily loses acetic acid (mol wt = 60), the fragment ions at *m/z* 140 and 168 were most likely derived from compounds with molecular weights of 200 and 228, respectively. Search of reference spectra revealed that mass spectra of the two unknown compounds matched those of decyl and dodecyl acetate, respectively. The identity of the unknowns was confirmed by comparison of their mass spectra and GC retention times to those of authentic samples. Decyl acetate and dodecyl acetate were also present in first instars and in the anal droplet produced by second instars. Only trace amounts of dodecyl acetate were found in adults of both sexes.

Bioactivity of Synthetic Pheromone. The synthetic pheromone and each component alone were biologically active. The percent responses to each increased significantly with increasing concentration (Figure 4). The response to decyl acetate plus dodecyl acetate (molar ratio 1.5:1) peaked at 10 and 100 ng, and the responses to decyl acetate and to dodecyl acetate peaked at 1000 ng. Responses to the two-component blend were significantly greater than to decyl acetate at doses of 1.0, 10, and 100 ng (linear model for categorical data, $P < 0.05$), but responses to the two-component blend and to dodecyl acetate

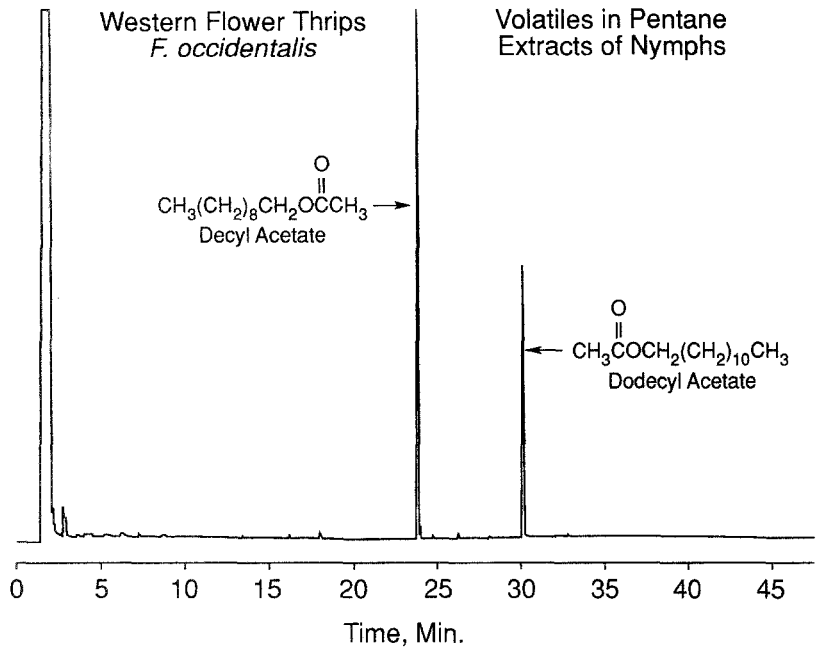

FIG. 3. Gas chromatogram of volatiles from whole crushed second-instar *F. occidentalis*. Column: glass capillary (30 m × 0.5 mm ID) coated with SP-1000. Temperature program: 70°C (2 min), 4°/min to 180°C (20 min).

were never different. The distance moved in response to 0.1 ng of the blend was significantly less than the distances moved at 10 ng and 1.0 NE of extract (Figure 5). The distance moved at 1.0 ng of dodecyl acetate was less than the distances moved at all other doses. There was no significant trend in the distances moved in response to decyl acetate (Figure 5).

Effects on Nymphal Dropping. Treatment with the alarm pheromone caused nymphs to drop from leaves at low rates (Table 3). When the pheromone source was pinned to a leaf, the rate of dropping was significantly greater at 1.0 and 10 μg than in control treatments. A similar result was obtained when the pheromone was diffused through a closed chamber. When the pheromone was sprayed on the leaf, the rate of dropping was not significantly different from that in the control, probably because the thrips became entrapped in the liquid on the leaf and were unable to move. Movement (tapping) of leaves with the pheromone source pinned on them approximately tripled the rate of dropping, compared to when the leaf was not tapped.

Effects on Oviposition. When female thrips were allowed a choice of oviposition sites, fewer than half the number of eggs were laid per 24 hr on phero-

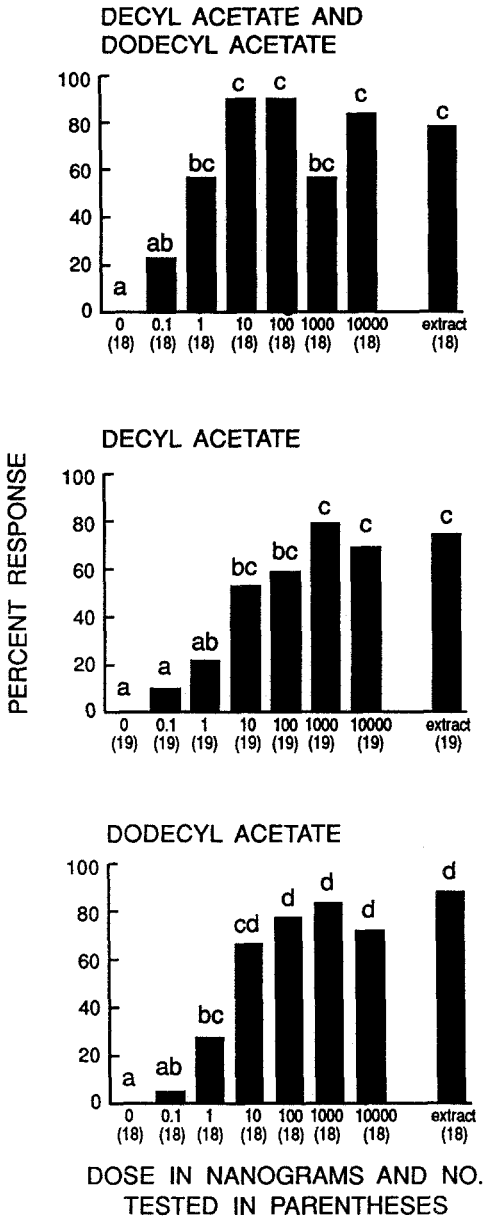

FIG. 4. Percent responses of second-instar *F. occidentalis* to synthetic alarm pheromone components presented on a filter-paper wick to stationary thrips. Bars topped by the same letter are not significantly different, linear model for categorical data and Fisher exact test with Bonferroni t tests, $P > 0.05$.

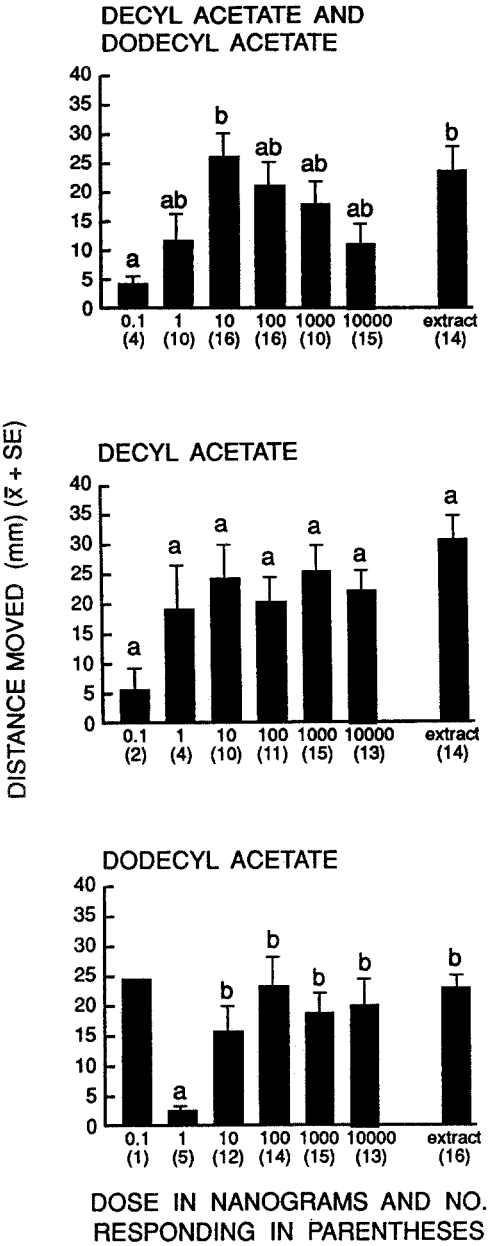

FIG. 5. Distances moved by second-instar *F. occidentalis* exposed to synthetic alarm pheromone components presented on a filter paper wick to stationary thrips. Bars topped by the same letter are not significantly different, Tukey's test, $P > 0.05$.

TABLE 3. PERCENT SECOND INSTAR *F. occidentalis* DROPPING FROM LEAVES IN RESPONSE TO SYNTHETIC ALARM PHEROMONE

		Pheromone source pinned to leaf, leaf not tapped		Pheromone source pinned to leaf, leaf tapped		Pheromone sprayed on leaf		Pheromone diffused in a closed chamber	
Treatment	Dose	Number tested[a]	% drop[b]	Number tested[a]	% drop[b]	Number tested[a]	% drop[b]	Number tested[a]	% drop[b]
Solvent	10 μl	380(4)	0.79a	140(2)	1.43a	185(2)	2.81a	150(3)	0.67a
Pheromone	0.1 μg	80(1)	2.50ab	90(1)	1.11a				
Pheromone	1 μg	320(4)	5.63b	90(2)	14.44b				
Pheromone	10 μg	270(3)	5.56b	60(1)	15.00b	450(2)	6.00a	150(3)	3.33b

[a] Number of populations in parentheses

[b] Percents in a column followed by the same letter are not significantly different, linear model for categorical data, $P < 0.05$.

mone-treated than on control substrates (Figure 6). In the no-choice experiment, significantly fewer eggs were laid per 24 hr on the pheromone-treated beans, but the difference was much less than in the two-choice experiment (Figure 6).

DISCUSSION

The results of these experiments demonstrate the existence of an alarm pheromone in western flower thrips. The only other pheromone identified in a thysanopteran is the monoterpene, perillene, which functions as an alarm pheromone in the gall-inhabiting *V. pasaniae* (Suzuki et al., 1988). Perillene has also been isolated from two other species of gall-inhabiting thrips (Suzuki et al., 1986, 1988). The chemistry of defensive secretions has also been investigated in several species of tubuliferans. γ-Decalactone is the major allomone in *Bagnalliella yuccae* (Hinds) (Howard et al., 1983), and pentadecane and hexadecyl acetate are the primary components of the defensive secretion of *Gynaikothrips ficorum* (Marchal) (Howard et al., 1987). Methylbutyric acid is a very minor, but active component in the defensive allomone of *V. pasaniae* (Suzuki et al., 1988). A number of acetates are found in the secretions of several tubuliferan species, although their functions are not known (Suzuki et al., 1988, 1989). The alarm pheromone of western flower thrips apparently does not have a defensive function. Although the droplet of anal fluid produced by second instars physically deters some predators, decyl acetate and dodecyl acetate act as a kairomone for two thrips predators (Teerling et al., 1993).

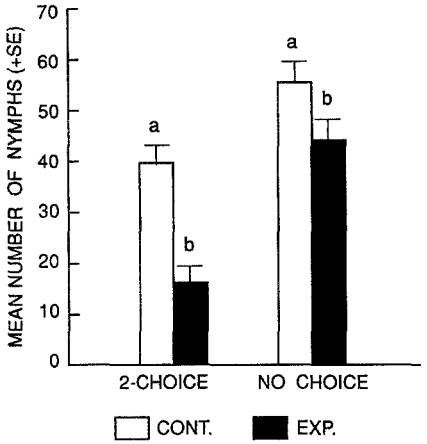

FIG. 6. Numbers of nymphs produced in choice and no-choice experiments by 40 female *F. occidentalis* in 24 hr on alarm pheromone-treated and solvent-treated substrates. Paired bars topped by the same letter are not significantly different, t test, $P > 0.05$.

Although both compounds in the western flower thrips alarm pheromone are independently active (Figure 4), they are not additive or synergistic. As in some attractive pheromone systems (Linn et al., 1984; Borden et al., 1990), such redundancy could serve as a fail-safe method of ensuring perception of and response to a critical stimulus. Alternatively, each component may elicit a slightly different behavior in the several alarm responses (negative taxis, dropping from the host, reduced oviposition) or in aggressive bouts between individual thrips (Crespi, 1986a,b; Terry and Gardner, 1990).

The responses of western flower thrips to their alarm pheromone are quite weak. The distance moved in response to the pheromone is small, but it may be sufficient to remove the thrips from the immediate area where a nymph is under attack. Anthocorids encountering a point source of thrips pheromone seemed to confine their most intensive searches to within 5 mm of the source (Teerling et al., 1993), so thrips moving >5 mm (Tables 1, 2; Figures 2, 5) would reduce their chance of being located by a predator.

The rate of dropping in response to alarm pheromone (Table 3) was much lower in western flower thrips nymphs than in many aphid species (Bowers et al., 1972; Montgomery and Nault, 1977; Calabrese and Sorensen, 1978; Wholers, 1981b). Because thrips are smaller than aphids, the risks of dropping from a feeding site are likely greater. Dropping to the ground involves risk of predation by ground-dwelling predators, and the chance of not regaining a suitable host. For western flower thrips, the benefits of remaining on a leaf, which is a proven source of food, thus seem to outweigh the risks of leaving it. Similarly, adult females may have risked oviposition on a substrate contaminated with alarm pheromone, rather than laying no eggs. Alternatively, females may have adapted or habituated to the pheromone within the 24-hr period, or the pheromone may not have persisted for 24 hr.

Because the responses of thrips to their alarm pheromone are so weak, there is no great potential for direct application of the pheromone in pest management. For example, there is little benefit in inducing dropping from leaves to reduce populations of thrips nymphs already on a crop unless use of the pheromone is integrated with that of some other control agent. Even at the high rates of dropping seen in several aphid species (Bowers et al., 1972; Montgomery and Nault, 1977; Calabrese and Sorensen, 1978; Wholers, 1981b), there is almost no prospect of economically reducing populations already on a plant, since disturbed aphids can return to the host plant within an hour (Montgomery and Nault, 1977; Calabrese and Sorensen, 1978). In addition, aphids rapidly habituate to the pheromone, and climb treated plants (Wholers, 1981a,b).

The alarm pheromone reduced oviposition by female thrips, especially when some oviposition sites were left untreated (Figure 6). Therefore, continuous pheromone release might provide protection for high-value plants, such as floral crops, if some plants or parts of plants were left untreated. Since the alarm

pheromone reduces oviposition, adults may tend to avoid landing and settling on treated plants if given the choice of leaving. Alate aphids avoid landing on plants treated with alarm pheromone, and treatment of plants in a greenhouse reduced colonization (Wholers, 1981a; Dawson et al., 1982). Thus, treatment of a crop with western flower thrips alarm pheromone might reduce adult immigration into the crop from surrounding fields.

Perhaps the most promising use of aphid alarm pheromone in pest management is as a bioirritant. The efficacies of insecticides can be significantly increased by applying a pheromone as a vapor before application of a contact insecticide or by formulating it with an insecticide (Griffiths and Pickett, 1980; Griffiths et al., 1983). Because the synthetic alarm pheromone induces western flower thrips to move, contact with either chemical or microbial insecticide deposits would be increased, possibly to the extent that mortality would be enhanced.

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REFERENCES

BLUM, M.S., 1969. Alarm pheromones. *Annu. Rev. Entomol.* 14:57–80.

BLUM, M.S. 1985. Alarm pheromones, *in* Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 9. G.A. Kerut and L.I. Gilbert (eds). Pergamon Press, Oxford, UK.

BORDEN, J.H., CHONG, L.J., and LINDGREN, B.S. 1990. Redundancy in the semiochemical message required to induce attack on lodgepole pines by the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). *Can. Entomol.* 122:769–777.

BOWERS, W.S., NAULT, L.R., WEBB, R.E. and DUTKY, S.R. 1972. Aphid alarm pheromone: Isolation, identification, synthesis. *Science* 177:1121–1123.

CALABRESE, E.J., and SORENSEN, A.J. 1978. Dispersal and recolonization by *Myzus persicae* following aphid alarm pheromone exposure. *Ann. Entomol. Soc. Am.* 71:181–182.

CALAM, D.H., and YOUDEOWEI, A. 1968. Identification and functions of secretion from the posterior scent gland of fifth instar of the bug *Dysdercus intermedius*. *J. Insect Physiol.* 14:1147–1158.

CRESPI, B.J. 1986a. Size assessment and alternative fighting tactics in *Elaphrothrips tuberculatus* (Insecta: Thysanoptera). *Anim. Behav.* 34:1324–1335.

CRESPI, B.J. 1986b. Territoriality and fighting in a colonial thrips, *Hoplothrips pedicularius*, and sexual dimorphism in Thysanoptera. *Ecol. Entomol.* 11:119–130.

DAWSON, G.W., GIBSON, R.W., GRIFFITHS, D.C., PICKETT, J.A., RICE, A.D., and WOODCOCK, C.M. 1982. Aphid alarm pheromone derivatives affecting settling and transmission of plant viruses. *J. Chem. Ecol.* 8(11):1377–1388.

GRIFFITHS, D.C., and PICKETT, J.A. 1980. A potential application of aphid alarm pheromones. *Entomol. Exp. Appl.* 27:199–201.

GRIFFITHS, D.C., DAWSON, G.W., PICKETT, J.A., and WOODCOCK, C. 1983. Uses of the aphid alarm pheromone and derivatives. *Int. Cong. Plant Protect. Proc.* 1:272.

GRIZZLE, J.E., STARMER, C.F., and KOCH, G.G. 1969. Analysis of categorical data by linear models. *Biometrics* 25:189–195.

HOWARD, D.F., BLUM, M.S., and FALES, M.F. 1983. Defense in thrips: forbidding fruitiness of a lactone. *Science* 220:335–336.

HOWARD, D.F., BLUM, M.S., JONES, T.H., FALES, M.F., and TOMALSKI, M.D. 1987. Defensive function and chemistry of the anal exudate of the cuban laural thrips *Gynaikothrips ficorum* (Marchal). *Phytophaga* 1:163–170.

ISHIWATARI, T. 1974. Studies on the scent of stink bugs (Hemiptera: Pentatomidae) I. Alarm pheromone activity. *Appl. Entomol. Zool.* 9:153–158.

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.

LEVINSON, H.Z., and BAR ILAN, A.R. 1971. Assembling and alerting scents produced by the bedbug *Cimex lectularius* L. *Experientia* 27:102–103.

LEVINSON, H.Z., LEVINSON, A.R., MULLER, B., and STEINBRECHT, R.A. 1974. Structure of sensilla, olfactory perception, and behavior of the bedbug, *Cimex lectularius*, in response to its alarm pheromone. *J. Insect Physiol.* 20:1231–1248.

LINN, C.E., JR., BJOSTAD, L.B., WU, 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.

LOCKWOOD, J.A., and STORY, R.N. 1987. Defensive secretion of the southern green stinkbug (Hemiptera: Pentatomidae) as an alarm pheromone. *Ann. Entomol. Soc. Am.* 80:686–691.

MASCHWITZ, U., and GUTMANN, C. 1979. Trail and alarm pheromones in *Elasmucha grisea* (Heteroptera: Acanthosomidae) *Insectes Soc.* 26:101–111.

MONTGOMERY, M.E., and NAULT, L.R. 1977. Aphid alarm pheromones: Dispersion of *Hyadaphis erysimi* and *Myzus persicae*. *Ann. Entomol. Soc. Am.* 70:669–672.

MYERS, J.L. 1979. Fundamentals of Experimental Design, 3rd ed. University of Massachusetts, Amherst, Massachusetts.

NAULT, L.R., WOOD, T.K., and GOFF, A.M. 1974. Treehopper (Membracidae) alarm pheromones. *Nature* 249:387–388.

PETERSON, B.S. 1990. The effect of host plant on the biological control of western flower thrips by the predatory mite, *Amblyseius cucumeris*. MPM thesis. Simon Fraser University, Burnaby, British Columbia.

SUZUKI, T., HAGA, K., and KUWAHARA, Y., 1986. Anal secretions of thrips: I. Identification of perillene from *Leeeuwenia pasanii* (Thysanoptera: Phlaeothripidae). *Appl. Entomol. Zool.* 21(3):461–466.

SUZUKI, T., HAGA, K., KODAMA, S., WATANABE, K., and KUWAHARA, Y. 1988. Secretion of thrips: II. Secretions of three gall-inhabiting thrips (Thysanoptera: Phlaeothripidae). *Appl. Entomol. Zool.* 23(3):291–297.

SUZUKI, T., HAGA, K., LEAL, W.S., KODAMA, S., and KUWAHARA, Y. 1989. Secretions of thrips. IV. Identification of β-acaridial from three gall-forming thrips (Thysanoptera: Thripidae). *Appl. Entomol. Zool.* 24(2):222–228.

TEERLING, C.R., GILLESPIE, D.R., and BORDEN, J.H. 1993. Utilization of western flower thrips alarm pheromone as a prey-finding kairomone by predators. *Can. Entomol.* In press.

TERRY, L.I., and GARDNER, D. 1990. Male mating swarms in *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae). *J. Insect Behav.* 3(1):133–141.

WHOLERS, P. 1981a. Aphid avoidance of plants contaminated with alarm pheromone (*E*)-β-farnesene. *Z. Angew. Entomol.* 92:329–336.

WHOLERS, P. 1981b. Effects of the alarm pheromone (*E*)-β-farnesene on dispersal behaviour of the pea aphid *Acyrthosiphon pisum*. *Entomol. Exp. Appl.* 29:117–124.

WOOD, T.K. 1976. Alarm behavior of brooding female *Umbonia crassicornis* (Homoptera: Membracidae). *Ann. Entomol. Soc. Am.* 69:340–344.

ZAR, J.H. 1984. Biostatistical Analysis, 2nd ed. Prentice Hall, Englewood Cliffs, New Jersey.

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OLFACTORY RECEPTOR CELL RESPONSES OF *Ips grandicollis* (EICHHOFF) (COLEOPTERA: SCOLYTIDAE) TO INTRA- AND INTERSPECIFIC BEHAVIORAL CHEMICALS

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Abstract—Electrophysiological recordings from antennal olfactory receptor cells were obtained from *Ips grandicollis*. Recordings were made from olfactory receptor cells from nine regions of the antennae in response to stimulation with the semiochemicals α-pinene, frontalin, *endo*-brevicomin, verbenone, *trans*-verbenol, *cis*-verbenol, ipsdienol, and ipsenol. In many cases, up to two cells were recorded concurrently from the same location. When compared to males, females had a greater percentage of cells responsive to the primary pheromones of *Dendroctonus frontalis*, frontalin and *trans*-verbenol, and of *Ips* spp., ipsdienol and ipsenol. Among females, more cells responded to *trans*-verbenol and the *Ips*-produced volatiles than to host or other *D. frontalis*-produced compounds. Olfactory cells of males responded mostly to *cis*-verbenol, followed by α-pinene, verbenone, *trans*-verbenol, and *endo*-brevicomin. Of those cells responsive primarily to one compound, the greatest percentage were responsive to *trans*-verbenol in females and to verbenone in males. The response of the antennal olfactory receptor cells to semiochemicals used by male and female *I. grandicollis* is consistent with the presence of these compounds during the host colonization period for each sex. Our results, which show a lack of specificity in most pheromone and host odor receptor cells, is in contrast with previously published accounts of olfactory receptor cell specificity in other *Ips* species.

Key Words—*Ips*, *Dendroctonus*, Coleoptera, Scolytidae, interspecific attrac-

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tion, behavioral chemicals, single olfactory cells, electrophysiology, pheromones.

INTRODUCTION

Ips grandicollis (Eichhoff) (Coleoptera: Scolytidae) is one of five species of the southern pine bark beetle guild, the most destructive group of softwood forest insects in the southern United States (Thatcher et al., 1980). During epidemic phases of infestation, *I. grandicollis* is found cohabiting pine trees with *Dendroctonus frontalis* (Zimmerman), *D. terebrans* (Oliver), *I. avulsus* (Eichhoff), and *I. calligraphus* (Germar). *Ips grandicollis* is not a pioneering species and relies on the presence of other more aggressive species, typically *D. frontalis*, to weaken defenses of the tree prior to colonization (Vité et al., 1972).

Host tree selection, aggregation, and colonization by these bark beetles is mediated by a complex array of olfactory cues emanating from both hosts and insects (see reviews by Wood, 1982; Lanier, 1983; Birch, 1984; Borden et al., 1986; Byers, 1989). Behavioral investigations have confirmed the existence of intra- and interspecific olfactory communication between conspecific or sympatric beetle species (Vité et al., 1964; Godbee and Franklin, 1976; Payne et al., 1978; Richerson and Payne, 1979; Dixon and Payne, 1980; Birch et al., 1980; Svihra, 1982; Billings, 1985; Phillips et al., 1989; Smith et al., 1990).

Svihra et al. (1980) suggested that the temporal and spatial organization of bark beetles on the host may be determined, in part, by olfactory communication among the species. Of the five primary guild members, *I. grandicollis* has the smallest overlap of its niche, occupying primarily branches in the lower portion of the tree canopy. The other species colonize the lower to upper tree bole (Birch and Svihra, 1979; Dixon and Payne, 1979; Birch et al., 1980; Svihra et al., 1980; Paine et al., 1981). Peak arrival of *I. grandicollis* on the host tree follows arrival of other guild members (Dixon and Payne, 1979; Svihra et al., 1980). Smith et al. (1990) showed that *I. grandicollis* also exhibits the greatest degree of cross-attractancy to semiochemicals produced by the sympatric species, most notably to those produced by *D. frontalis*. They suggested the ability of *I. grandicollis* to locate suitable hosts, yet not compete with sympatric species, may be a result of interspecific olfactory cues.

Electrophysiological experiments support the hypothesis of interspecific olfactory communication, i.e., semiochemicals produced by *Ips* and *Dendroctonus* spp. elicit electroantennograms (EAGs) from all guild members (Smith et al., 1988; Delorme and Payne, 1990). Individual olfactory cell responses to conspecific pheromones and to host volatiles have been recorded from the antennae of *D. frontalis* (Payne and Dickens, 1976; Dickens and Payne, 1977; Payne et al., 1982). However, characterization of the single olfactory cells of other guild members to the chemical stimuli has not been reported. We investigated

single olfactory cell response to interspecific olfactory signals, using *I. grandicollis*, in an effort to elucidate the specificity of the receptor cells and improve our understanding of the chemical ecology of the insect.

METHODS AND MATERIALS

Insects. Ips grandicollis from East Texas were used to infest loblolly pine, *Pinus taeda* L., bolts from southwestern Virginia. The bolts were placed in a ventilated rearing chamber at 20–25°C. Bolts containing mostly pupae and callow adults were placed in an emergence chamber (Browne, 1972). Emerged beetles were placed on moistened filter paper and stored individually at 5°C in 3.7-ml vials until use (one to two days after emergence). Recordings of each antennal location described below were made from five beetles of each sex.

Electrophysiology. Single olfactory cell recording techniques were modified from Dickens (1979). Tungsten electrodes were sharpened to a tip of ca. 1 μm. The reference electrode was inserted into the body of the insect through the oral cavity. The recording electrode was positioned in one of nine regions on the antenna (Figure 1).

Electrophysiological signals were passed through an automatic baseline-restoring preamplifier (G. Johnson) to a Tektronix 122 low-level preamplifier, through a 60-Hz notch filter, and then displayed on a Tektronix R561B oscilloscope. Records were stored on cassette tape with a TEAC R-61 data recorder and displayed on Polaroid film with a Tektronix C-27 oscilloscope camera.

Bioassay. Stimuli used included a terpene of loblolly pine: α-pinene; compounds identified from the frass of *D. frontalis*: frontalin (Kinzer et al., 1969), *endo*-brevicomin (Vité and Renwick, 1971a), *trans*-verbenol, and verbenone (Renwick, 1967); and compounds identified from *Ips* species: *cis*-verbenol, ipsdienol, and the major pheromone component of *I. grandicollis*, ipsenol (Silverstein et al., 1966). Sources and purity of all compounds are listed in Table 1. All compounds were racemic. The compounds were prepared as 10 μg/μl solutions in nanograde pentane. Stimuli were delivered as 5-μl aliquots on a piece of Whatman No. 2 filter paper, inserted into glass cartridges (75 mm long, 5 mm ID), and oriented toward the antennal preparation from ca. 1 cm. Stimuli were presented in random order, following presentation of clean air and a pentane control. Stimulus duration was 800 msec and air flow was 1 liter/min. A 3-min interval separated each stimulation to allow for recovery of the action potential frequency in the cells. To monitor the viability of the preparation, stimulation with the first compound to elicit a response was repeated as a standard once stimulation by all other compounds had been completed.

Data Analysis. Often, more than one amplitude of action potential was observed in a record (e.g., Figure 2). The two largest amplitude spikes were

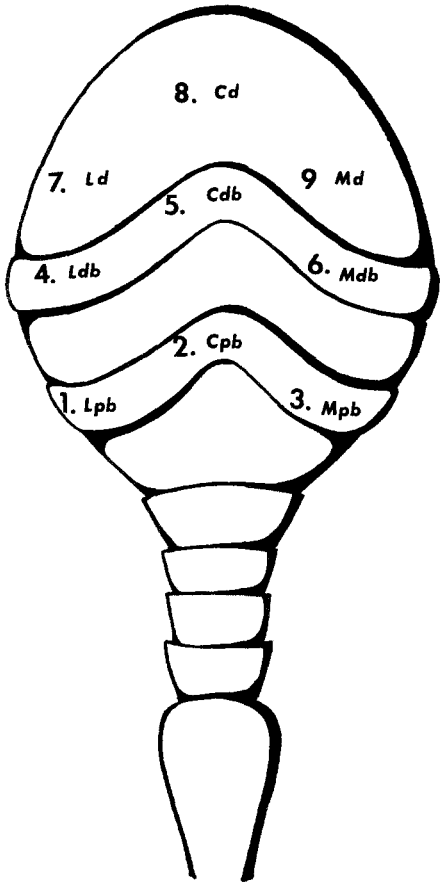

FIG. 1. Diagram of left *Ips grandicollis* antennal flagellum showing regions of the club from which recordings were obtained: position 1. Lpb, lateral proximal band; 2. Cpb, central proximal band; 3. Mpb, medial proximal band; 4. Ldb, lateral distal band; 5. Cdb, central distal band; 6. Mdb, medial distal band; 7. Ld, lateral distal portion; 8. Cd, central distal portion; 9. Md, medial distal portion.

used for separate counts of action potential frequencies. Each of the two spike patterns was considered to be from separate cells. It was impossible to resolve smaller amplitude potentials from background noise. These potentials were ignored, since previous single cell studies with *Ips* spp. have not found more than two cells per record (Mustaparta et al., 1977, 1979). To obtain the change in action potential frequency of each cell due to application of a stimulus, the action potential frequency from the first 500 msec following stimulation was subtracted from two times the number of spontaneously occurring action poten-

TABLE 1. SOURCE AND PURITY OF STIMULUS COMPOUNDS USED IN ELECTROPHYSIOLOGICAL EXPERIMENTS ON *Ips grandicollis*

Compound	Chemical purity (%)[a]	Source
α-Pinene	97	Aldrich Chem. Co.
Frontalin	99	Chem. Samp. Co.
endo-Brevicomin	99	Chem. Samp. Co.
Verbenone	98	Chem. Samp. Co.
trans-Verbenol	95	Borregard Industries
cis-Verbenol	95	Borregard Industries
Ipsdienol	81	Borregard Industries
Ipsenol	89	Borregard Industries

[a] Via GLC.

tials in the 250 msec preceding stimulation. The difference was subjected to analysis. For each cell, mean spontaneous activity prior to stimulation was computed. If a stimulus caused a change in frequency that exceeded the 95% confidence interval of the mean spontaneous activity for that cell, the stimulus was said to have caused a response. Cells for which only one stimulus caused an increased frequency of action potentials that exceeded the upper 95% confidence limit of the mean change in action potential frequency for that cell were said to be primarily responsive to that one stimulus. The percentages of responding cells were ranked and compared using the nonparametric Kruskal-Wallis k-sample test (Quade, 1966).

RESULTS AND DISCUSSION

Sexual Dimorphism. Seventy-four cells were recorded from five females, whereas only 44 cells were recorded from the same number of males. This suggests the possibility of fewer olfactory receptors in males compared to females. A comparison between sex and location of the responding cells showed that in females, the percentage of responding cells in the distal portion of the club and in the two sensory bands did not differ significantly ($F_{2,96} = 0.9$; $P > 0.05$), whereas in males, the distal and proximal sensory bands contained a greater percentage of responding cells than the middle band ($F_{2,96} = 3.5$; $P \leq 0.05$) (Figure 3). Single cell recordings of different antennal regions have been reported by Wadlow (1973) for the carrion beetle, *Necrophorus vespilloides* Herbst and by Mustaparta (1975) for the pine weevil, *Hylobius abietis* L. In both cases, responses were associated with different types of olfactory receptors,

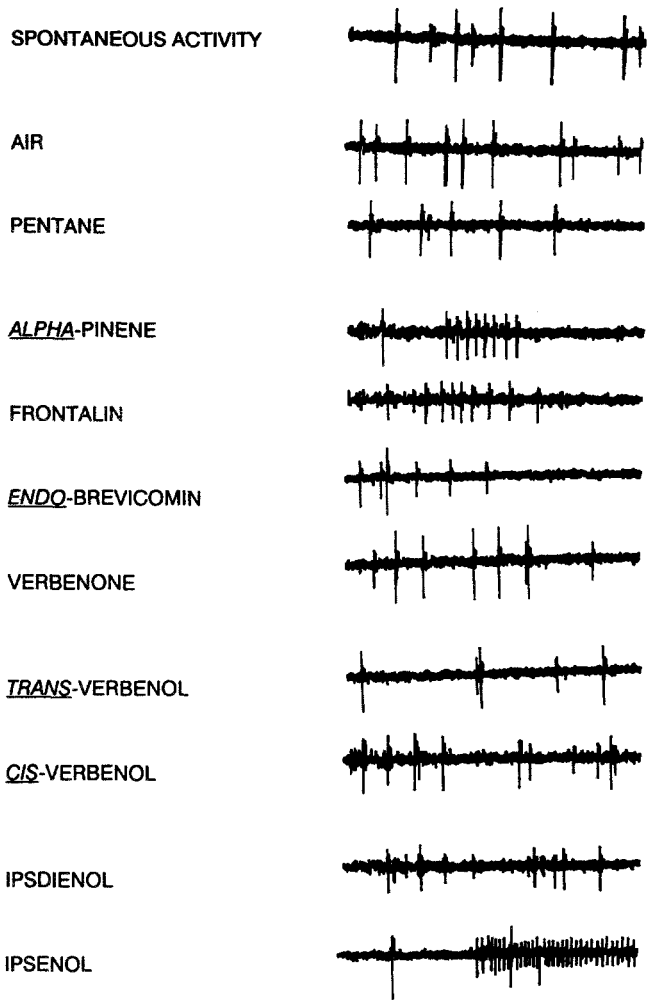

FIG. 2. Representative traces of two cells from the antenna of *Ips grandicollis*. The smaller spike is from a cell that responds primarily to ipsenol, and to a lesser extent to α-pinene, frontalin, and ipsdienol. The firing rate of the larger action potential is not dependent on the application of any of the behavioral chemicals tested.

which were usually localized to specific areas of the antennae. In this study, no attempt was made to differentiate among the different types of receptors sampled.

A significantly greater percentage of cells responded to frontalin, *trans*-verbenol, ipsdienol, and ipsenol in females than in males (Table 2). This finding does not correspond with EAG data of *I. grandicollis*, where differences in

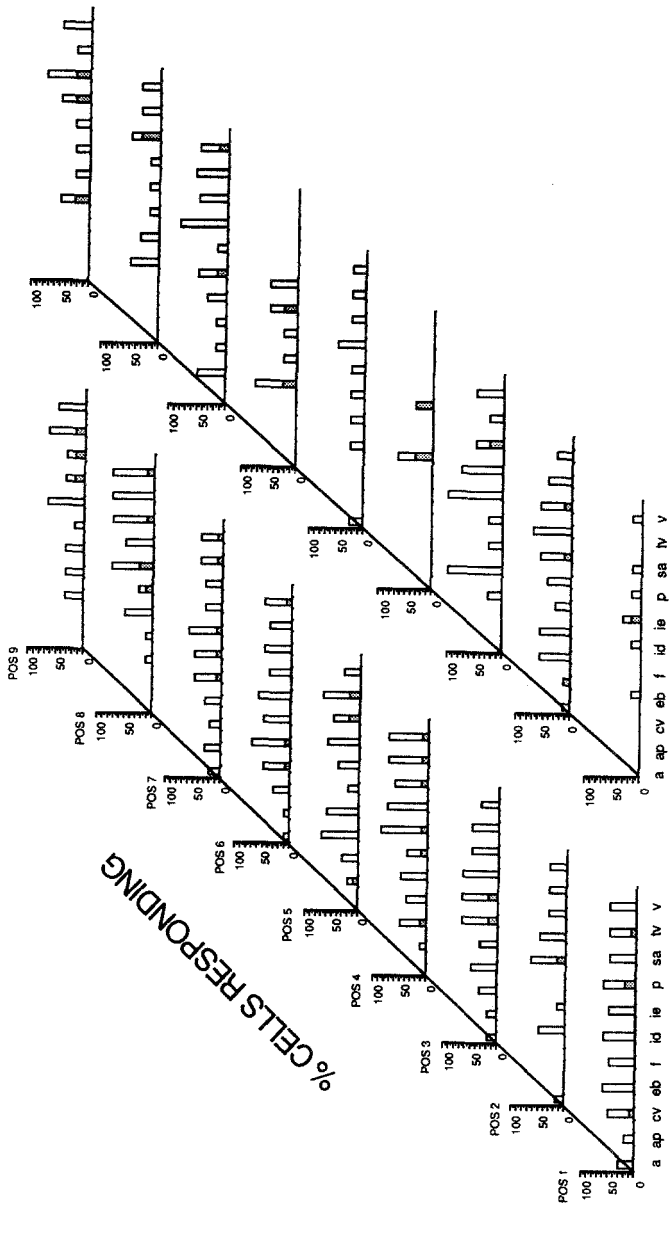

FIG. 3. Percentage of responding cells for each antennal region to the following stimuli: a = air; ap = α-pinene; cv = *cis*-verbenol; eb = *endo*-brevicomin; f = frontalin; id = ipsdienol; ie = ipsenol; p = pentane; sa = spontaneous activity; tv = *trans*-verbenol; v = verbenone. Total bar height represents the total percentage of cells responding, speckled bar height represents the percentage of cells responding only to that stimulus.

TABLE 2. COMPARISON BETWEEN FEMALE AND MALE *Ips grandicollis* OF MEAN PERCENT OF CELLS RESPONDING TO A GIVEN STIMULUS

Stimulus	Female (N = 74 cells)	Male (N = 44 cells)
Spontaneous activity	10.4	4.3
Air	13.2	7.1
Pentane	28.7	11.0
α-Pinene	43.7	43.4
Frontalin	45.9	21.7**[a]
endo-Brevicomin	36.2	32.6
Verbenone	46.7	36.0
trans-Verbenol	63.4	36.0**
cis-Verbenol	52.9	49.6
Ipsdienol	55.4	23.1**
Ipsenol	48.4	28.2*

[a]Significant differences between females and males were determined using Friedman's two-way analysis for block designs (*$P \leq 0.05$; **$P \leq 0.01$).

response were not found between sexes (Smith et al., 1988). The EAG, however, may represent more cells (Boeckh et al., 1965; Schneider, 1969) and thus, could be a more accurate measure of total antennal olfactory response. For *I. paraconfusus* Lanier, equal EAGs were recorded for both sexes in response to ipsenol (Light and Birch, 1979), while in the field, females were more attracted to this pheromone than were males (Byers, 1983). Byers concluded that the behavioral difference between the sexes must be the result of integration within the central nervous system. However, one must not exclude the possibility of the activity of a few cells being responsible for behavioral differences. Such may be the case with *I. grandicollis*, in which more females than males are caught in traps baited with ipsenol and host volatile blends (Smith et al., 1990).

Responses by Females. In females, *trans*-verbenol elicited responses from the greatest percentage of cells, followed by ipsdienol, *cis*-verbenol, and ipsenol (Figure 4), compounds that are all produced by *Ips* species (Vité et al., 1972). Vité and Renwick (1971b) found that ipsenol and *trans*-verbenol both attract a greater number of female than male *I. grandicollis*. The addition of α-pinene, however, increased the ratio of males caught. Smith et al. (1990) showed that female *I. grandicollis* are greatly attracted to traps baited with a blend of racemic ipsdienol, ipsenol, and *cis*-verbenol. They also showed that females were more responsive to a blend of synthetic pheromones of both sexes of *D. frontalis* than to blends produced by either sex alone. In nature, the presence of pheromones of both sexes may be a sign stimulus to *I. grandicollis* of a susceptible host that was successfully attacked by *D. frontalis*. The pheromones of other more aggres-

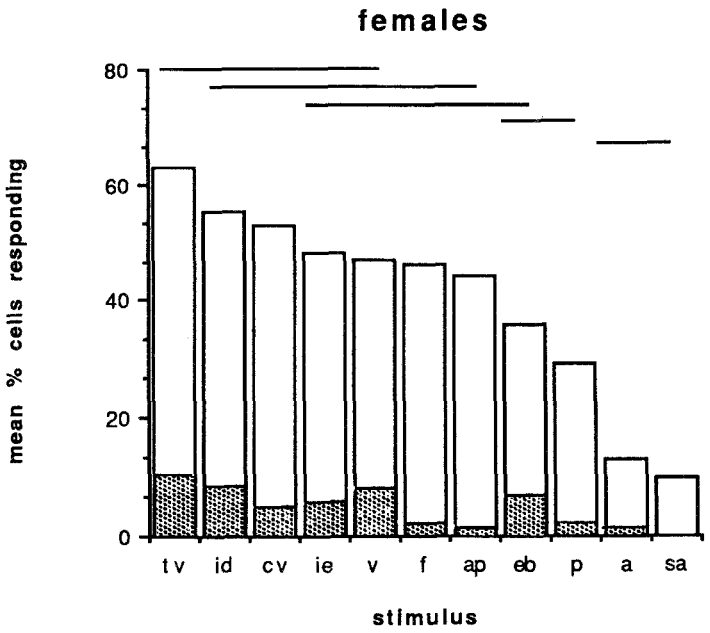

FIG. 4. Mean percent of cells from females responding to a given stimulus. Bar height represents total percentage of cells responding to a compound; speckled bar height represents percentage of cells responding primarily to that stimulus. Means of total percentage of cells responding to a compound grouped under one line are not significantly different ($P \leq 0.05$) as determined by least significant differences (LSD) computed from Friedman's two-way analysis for block designs. For all stimuli, $N = 74$ cells. tv = *trans*-verbenol; id = ipsdienol; cv = *cis*-verbenol; ie = ipsenol; v = verbenone; f = frontalin; ap = α-pinene; eb = *endo*-brevicomin; p = pentane; a = air; sa = spontaneous activity.

sive *Ips* species may also serve as similar sign stimuli for *I. grandicollis* (Smith et al., 1990).

The percentage of cells responding primarily to one stimuli was low (Figure 4). Of all the stimuli tested, only cells responding primarily to *trans*-verbenol and ipsdienol were found more frequently than cells responding primarily to the controls. ($F_{10,88} = 1.99$; $P \leq 0.05$). These results differ from those obtained for *I. pini* (Say), *I. paraconfusus* (Mustaparta et al., 1977, 1979, 1980), and *I. typographus* (Tømmerås et al., 1984). Cells from those species were responsive mostly to a single compound or enantiomer, rather than several compounds.

Responses by Males. cis-Verbenol elicited responses from a significantly greater percentage of cells in males than did ipsenol, ipsdienol, or frontalin (Figure 5). Since *I. grandicollis* produce only trace amounts of *cis*-verbenol

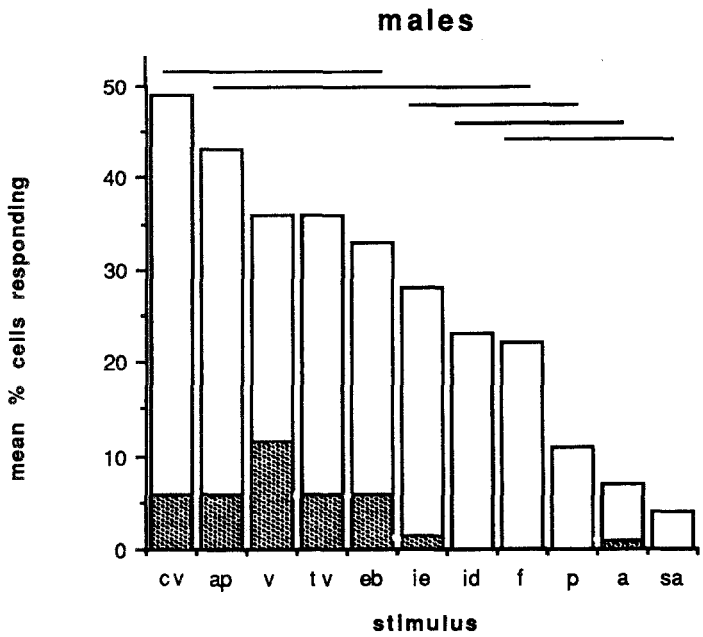

FIG. 5. Mean percent of cells from males responding to a given stimulus. Bar height represents total percentage of cells responding to a compound; speckled bar height represents percentage of cells responding primarily to that stimulus. Means of total percentage of cells responding to a compound grouped under one line are not significantly different ($P \leq 0.05$) as determined by least significant differences (LSD) computed from Friedman's two-way analysis for block designs. For all stimuli, $N = 44$ cells. cv = *cis*-verbenol; ap = α-pinene; v = verbenone; tv = *trans*-verbenol; eb = *endo*-brevicomin; ie = ipsenol; id = ipsdienol; f = frontalin; p = pentane; a = air; sa = spontaneous activity.

(Vité et al., 1972), it appears that males may use *cis*-verbenol produced by *I. calligraphus* (Renwick and Vité, 1972; Hughes, 1974). α-Pinene, verbenone, *trans*-verbenol, and *endo*-brevicomin elicited responses from significantly more cells than did the pentane or air controls, whereas ipsenol, ipsdienol, and frontalin did not. Very few cells must be needed to respond in order to elicit a behavioral response to ipsenol, as males are known to aggregate to their own pheromone, ipsenol (Vité and Renwick, 1971b). It is interesting to note that there was no difference in response to α-pinene between males and females (Table 2). Werner (1972a,b) found that males were more than twice as responsive to α-pinene as females, and in general males were more attracted than females to host terpenes in both laboratory and field tests. Presumably, in addition to interspecific olfactory cues, pioneering males can use host compounds as primary attractants for host tree selection.

In males, the percentage of cells responding primarily to one compound was also low. However, of those that did, verbenone was the most frequent stimulus eliciting such a response (Figure 5). Verbenone, an antiaggregation pheromone of *D. frontalis*, may function as a kairomone, aiding male *I. grandicollis* in the location of weakened trees. In contrast to females, no cells responded primarily to ipsdienol.

Implications to Bark Beetle Ecology. Both sexes of *I. grandicollis* have antennal olfactory cells that are sensitive to pheromones of sympatric species. Some of these kairomones stimulated receptor cells that were also stimulated by host chemicals or by the insect's own pheromone. For some kairomones, however, cells were found that responded to only one of the kairomones tested, i.e., verbenone for males, and *trans*-verbenol and ipsdienol for females.

In a pedestrian laboratory bioassay, *I. grandicollis* oriented toward several host volatiles; however, the attraction was masked by the *D. frontalis* pheromones frontalin or *trans*-verbenol (Werner, 1972b). In contrast, frontalin and *trans*-verbenol, in combination, enhanced attraction of *I. grandicollis* to host compounds in the field. Therefore, it appears that some compounds can elicit both avoidance and attraction of *I. grandicollis* to a source. This being the case, one would expect to find such plastic behavior to be mediated, at least in part, by an across-fiber type of receptor cell patterning, unless combinations of compounds are able to antagonize one another's stimulatory activity on the receptor cell. Smith et al. (1990) demonstrated that ipsenol, specifically the (*S*)-(−) enantiomer, attracted *I. grandicollis*. (*R*)-(+)-Ipsenol masked the effect of its optical enantiomer. Information regarding the optical specificity of ipsenol receptors in this species is unknown.

Both sexes of *I. grandicollis* are attracted to *I. calligraphus*-infested bolts; however, *I. calligraphus* attack and colonization of *I. grandicollis*-infested bolts will reduce the attraction of the bolts to host-searching *I. grandicollis* (Birch et al., 1980; Byers, 1989). Synthetic blends of the pheromones of *I. calligraphus* and *I. avulsus* do attract *I. grandicollis* (Smith et al., 1990). The presence of sympatric species may therefore aid *I. grandicollis* in locating suitable habitats while signaling them to stay away from parts of the host already colonized by *I. avulsus* and *I. calligraphus*.

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REFERENCES

BILLINGS, R.F. 1985. Southern pine bark beetles and associated insects: Effects of rapidly released host volatiles on response to aggregation pheromones. *Z. Angew. Entomol.* 99:483–491.

BIRCH, M.C. 1984. Aggregation in bark beetles, pp. 331–353, *in* W.J. Bell and R.T. Cardé (eds.). Chemical Ecology of Insects. Sinauer Associates, Sunderland, Massachusetts.

BIRCH, M.C., and SVIHRA, P. 1979. Novel approaches to forest insect control: Exploiting olfactory interactions between species of Scolytidae, pp. 135–139, *in* W.E. Walters (ed.). Current Topics in Forest Entomology. John Wiley & Sons, New York.

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.

BOECKH, J., KAISSLING, K.E., and SCHNEIDER, D. 1965. Insect olfactory receptors. *Cold Spring Harbor Symp. Quant. Biol.* 30:263–280.

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, UK.

BROWNE, L.E. 1972. An emergence cage and refrigerator collector for wood-boring insects and their associates. *J. Econ. Entomol.* 65:1499–1501.

BYERS, J.A. 1983. Sex-specific responses to aggregation pheromone: Regulation of colonization density in the bark beetle *Ips paraconfusus*. *J. Chem. Ecol.* 9:129–142.

BYERS, J.A. 1989. Chemical ecology of bark beetles. *Experientia* 45:271–283.

DELORME, J.D., and PAYNE, T.L. 1990. Olfactory responses of the black turpentine beetle, *Dendroctonus terebrans* (Olivier), to beetle pheromones. *J. Chem. Ecol.* 16:1321–1329.

DICKENS, J.C. 1979. Electrophysiological investigations of olfaction in bark beetles. *Mitt. Schweiz. Entomol. Ges.* 52:203–216.

DICKENS, J.C., and PAYNE, T.L. 1977. Bark beetle olfaction: Pheromone receptor system in *Dendroctonus frontalis*. *J. Insect Physiol.* 23:481–489.

DIXON, W.N., and PAYNE, T.L. 1979. Sequence of arrival and spatial distribution of entomophagous and associate insects of southern pine beetle-infested trees. Texas Agriculture Experiment Station Miscellaneous Publication 1432, 28 pp.

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. Ga. Entomol. Soc.* 15:378–389.

GODBEE, J.F., JR., and FRANKLIN, R.T. 1976. Attraction, attack patterns and seasonal activity of the black turpentine beetle. *Ann. Entomol. Soc. Am.* 69:653–655.

HUGHES, P.R. 1974. Myrcene: A precursor of pheromones in *Ips* beetles. *J. Insect Physiol.* 20:1271–1275.

KINZER, G.W., FENTIMAN, A.F., JR., PAGE, T.F., JR., 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.

LANIER, G.N. 1983. Integration of visual stimuli, host odorants, and pheromones by bark beetles and weevils in locating and colonizing host trees, pp. 161–171, *in* S. Ahmad (ed.). Herbivorous Insects: Host Selecting Behavior and Mechanisms. Academic Press, New York.

LIGHT, D.M., and BIRCH, M.C. 1979. Bark beetle enantiomeric chemoreception: Greater sensitivity to allomone than pheromone. *Naturwissenschaften* 69:243–245.

MUSTAPARTA, H. 1975. Behavioral responses of the pine weevil *Hylobius abietis* L. (Col.: Curculionidae) to odours activating different groups of receptor cells. *J. Comp. Physiol.* 102:57–63.

MUSTAPARTA, H., ANGST, M.E., and LANIER, G.N. 1977. Responses of single cells in the pine engraver beetle, *Ips pini* (Say) (Coleoptera: Scolitidae) to its aggregation pheromone, ipsdienol, and the aggregation inhibitor, ipsenol. *J. Comp. Physiol.* 121:343–347.

MUSTAPARTA, H., ANGST, M.E., 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.E., and LANIER, G.N. 1980. Receptor discrimination of enantiomers of the aggregation pheromone ipsdienol, in two species of *Ips*. *J. Chem. Ecol.* 6:689–701.

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.

PAYNE, T.L., and DICKENS, J.C. 1976. Adaptation to determine receptor specificity in insect olfactory communication. *J. Insect Physiol.* 22:1569–1572.

PAYNE, T.L., COSTER, J.E., RICHERSON, J.V., EDSON, L.J., and HART, E.R. 1978. Field response of the southern pine beetle to behavioral chemicals. *Environ. Entomol.* 7:578–582.

PAYNE, T.L., RICHERSON, J.V., DICKENS, J.C., WEST, J.R., MORI, K., BERISFORD, C.W., HEDDEN, R.L., VITÉ, J.P., and BLUM, M.S. 1982. Southern pine beetle: Olfactory receptor and behavior discrimination of enantiomers of the attractant pheromone frontalin. *J. Chem. Ecol.* 8:873–881.

PHILLIPS, T.W., NATION, J.L., WILKINSON, R.C., and FOLTZ, J.L. 1989. Secondary attraction and field activity of beetle-produced volatiles in *Dendroctonus terebrans*. *J. Chem. Ecol.* 15:1513–1533.

QUADE, D. 1966. On analysis of variance for the k-sample problem. *Ann. Math. Stat.* 37:1747–1753.

RENWICK, J.A.A. 1967. Identification of two oxygenated terpenes from the bark beetles *Dendroctonus frontalis* and *Dendroctonus brevicomis*. *Contrib. Boyce Thompson Inst.* 23:355–360.

RENWICK, J.A.A., and VITÉ, J.P. 1972. Pheromones and host volatiles that govern aggregation of the six-spined engraver beetle, *Ips calligraphus*. *J. Insect Physiol.* 18:1215–1219.

RICHERSON, J.V., and PAYNE, T.L. 1979. Effects of bark beetle inhibitors on landing and attack behavior of the southern pine beetle and beetle associates. *Environ. Entomol.* 8:360–364.

SCHNEIDER, D. 1969. Insect olfaction: Deciphering system for chemical messages. *Science* 163:1031–1037.

SILVERSTEIN, R.M., RODIN, J.O., and WOOD, D.L. 1966. Methodology for isolation and identification of insect pheromones with reference to studies on California five-spined *Ips*. *J. Econ. Entomol.* 60:944–949.

SMITH, M.T., BUSCH, G.R., PAYNE, T.L., and DICKENS, J.C. 1988. Antennal olfactory responsiveness of three sympatric *Ips* species [*Ips avulsus* (Eichhoff), *Ips calligraphus* (Germar), *Ips grandicollis* (Eichhoff)], to intra- and interspecific behavioral chemicals. *J. Chem. Ecol.* 14:1289–1304.

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.

SVIHRA, P. 1982. Influence of opposite sex on attraction produced by pioneer sex of four bark beetle species cohabitating pine in the southern United States. *J. Chem. Ecol.* 8:373–378.

SVIHRA, P., PAINE, T.D., and BIRCH, M.C. 1980. Interspecific olfactory communications in southern pine beetles. *Naturwissenschaften* 67:518–519.

THATCHER, R.C., SEARCY, J.L., COSTER, J.E., and HERTEL, G.D. (eds.). 1980. The Southern Pine Beetle. USDA Forest Service Technical Bulletin 1631, 267 pp.

TØMMERÅS, B.Å, MUSTAPARTA, H., and GREGOIRE, J.C. 1984. Receptor cells in *Ips typographus* and *Dendroctonus micans* specific to pheromones of the reciprocal genus. *J. Chem. Ecol.* 10:759–770.

VITÉ, J.P., and RENWICK, J.A.A. 1971a. Inhibition of *Dendroctonus frontalis* response to frontalin by isomers of brevicomin. *Naturwissenschaften* 58:418.

VITÉ, J.P., and RENWICK, J.A.A. 1971b. Population aggregating pheromone in the bark beetle, *Ips grandicollis*. *J. Insect Physiol.* 17:1699–1704.

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 Thomp. Inst.* 22:461–470.

VITÉ, J.P., BAKKE, A., and RENWICK, J.A.A. 1972. Pheromones in *Ips* (Coleoptera: Scolytidae): Occurrence and production. *Can. Entomol.* 104:1967–1975.

WADLOW, U. 1973. Electrophysiology of a new carrion receptor and its relation to the behavior of the carrion beetle (*Necrophorus*). *J. Comp. Physiol.* 83:415–424.

WERNER, R.A. 1972a. Aggregation behaviour of the beetle *Ips grandicollis* in response to host-produced attractants. *J. Insect Physiol.* 18:423–437.

WERNER, R.A. 1972b. Response of the beetle, *Ips grandicollis*, to combinations of host and insect produced attractants. *J. Insect Physiol.* 18:1403–1412.

WOOD, D.L. 1982. The role of pheromones, kairomones, and alomones in the host selection and colonization behavior of bark beetles. *Annu. Rev. Entomol.* 27:411–446.

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ACCUMULATION OF *Dendrobium superbum* (ORCHIDACEAE) FRAGRANCE IN THE RECTAL GLANDS BY MALES OF THE MELON FLY, *Dacus cucurbitae*

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Abstract—4-(4-Hydroxyphenyl)-2-butanone was characterized from flowers of the orchid *Dendrobium superbum* as a specific attractant factor for the male melon fly, *Dacus cucurbitae*. The male flies compulsively licked the flower surface and sequestered the compound in significant quantities in their rectal glands. The compound was detected within 6 hr after ingestion and was retained for more than six days in the rectal gland sacs.

Key Words—*Dacus cucurbitae*, melon fly, Diptera, *Dendrobium superbum*, orchid, 4-(4-hydroxyphenyl)-2-butanone, cue-lure, sequestration, pheromone.

INTRODUCTION

Males of the melon fly, *Dacus (Bactrocera) cucurbitae* Coquillett, show strong affinity to the blossoms of the orchid, *Dendrobium superbum* Rchb. f. (synonym, *D. anosmum* Lindl.) (Flath and Ohinata, 1982; Ichinohe et al., 1983) (Figure 1). Flath and Ohinata (1982) identified several volatile components including benzylacetone (4-phenyl-2-butanone) by a headspace collection of *D. superbum*

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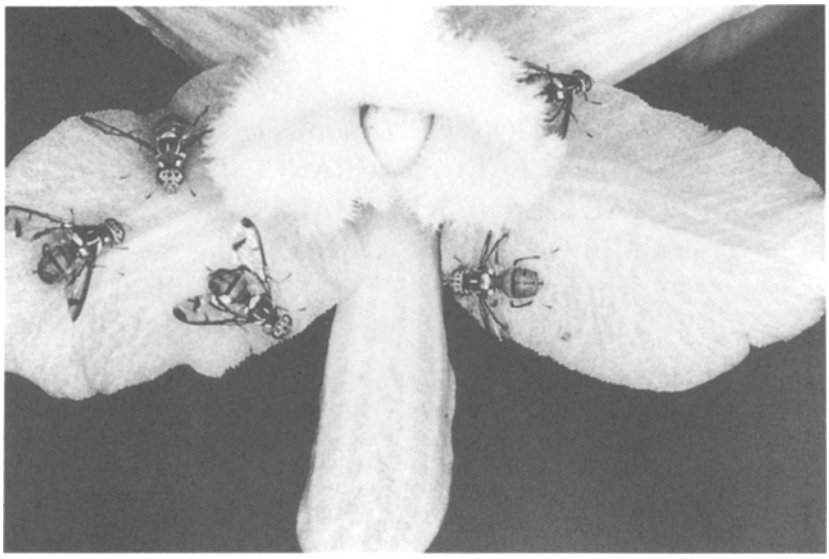

FIG. 1. Males of the melon fly, *Dacus cucurbitae*, congregating and feeding on a *Dendrobium superbum* flower.

flowers. According to our behavioral bioassay, however, the activity of the major attractant chemical in *D. superbum* flowers was more potent than benzylacetone and exhibited a more polar nature on the silica gel chromatography. We have reexamined the attractant chemicals contained in the flowers of *D. superbum*.

Males of *D. cucurbitae* possess a rectal gland complex (Schultz and Boush, 1971) and produce a smokelike substance from the gland during courtship (Kobayashi et al., 1978; Ohinata et al., 1982, Kuba and Sokei, 1988). Alkyl amides, nonan-1,3-diol, 4-hydroxybenzoic esters, and pyrazine derivatives have been identified as the major volatile components of the rectal glands (Baker et al., 1982; Nishida et al., 1990; Perkins et al., 1990). We will demonstrate here the selective accumulation of a *Dendrobium* flower fragrance in the rectal gland, suggesting a possible biological role of the compound in courtship behavior of *D. cucurbitae*.

METHODS AND MATERIALS

Insects. We used two types of melon fly strains, the mass-reared Okinawa strain (Hibino and Iwahashi, 1990) and the Malaysian strain (Nishida et al., 1990). Larvae were reared with an artificial diet (Okinawa strain) (Nakamori

and Kakinohana, 1980) or with cucurbit fruits (Malaysian strain), and adult flies were provided with water and a mixture of protein hydrolysate and sugar. The behavioral bioassay and orchid flower-feeding experiments were conducted with the Okinawa strain. The attractants sequestered in the body tissues were quantified with the Malaysian strain.

Instruments. The gas chromatography-mass spectroscopic (GC-MS) analyses were conducted with a Hitachi M-80 mass spectrometer (20 eV) connected to a GC column (24 m × 0.25 mm fused silica column coated with cross-linked-bonded methyl silicone HP-1, 0.25 μm thick) programmed from 80°C (approximately 2 min holding) to 210°C at a rate of 10°C/min. GC quantifications of volatile chemicals were done on an HP 5790A gas chromatograph with the same capillary column and under the same program conditions by comparing the FID intensities with those of the standard sample of known concentrations and a HP 3390A reporting integrator (Hewlett Packard).

Extraction of Orchid Fragrance. Blossoms of *Dendrobium superbum* were obtained from the cultures grown in Okinawa Island, southeast of Japan, in late April 1990. The flower petals were extracted with absolute ethanol (5 pairs of petals/10 ml). The petal extract, after removal of most of the ethanol in vacuo (20 mm Hg, 32°C), was dissolved in ether (10 ml × 2) and treated with saturated sodium chloride. The ether layer was dried over anhydrous sodium sulfate, and a portion of the concentrated extract was subjected to the GC-MS analysis. To compare the quantities of attractant compound **1** between three parts of the flower—a lip, a petal, and a calyx—each part was extracted with ethanol (10 flower units/50 ml). Portions (100 μl) of the ethanolic extract were concentrated in vacuo (20 mm Hg, 35°C), readjusted to 10 μl with methyl acetate, and a 1-μl portion was injected into the gas chromatograph.

TLC Plate Bioassay. A small portion (50 μl) of the flower petals was subjected to thin-layer chromatography (TLC) on a precoated plate (HPTLC silica gel 60 F_{254}, nano TLC, Merck) and developed with a mixture of benzene and ethyl acetate (4 : 1). The TLC plate was introduced into a small cage containing male flies (approximately 20 males per cage), and the licking behavior was observed for about 10 min.

Detection of Compound 1 from Rectal Gland. Males of *D. cucurbitae* were released in a cage containing a pot of *D. superbum* with flowers. They freely licked the flowers for several hours during the daytime. The feeding was conducted twice, on the 11th and 18th days after adult eclosion (DAE). The male rectal gland sacs were pulled out on the 20th DAE for extraction. Compound **1** in the rectal gland of individual males was quantified by GC analysis.

Quantification of Compound 1 in Body Tissues. Males of *D. cucurbitae* were allowed to feed on pure compound **1** (40 μg/insect offered as a 1-μl solution in 20% ethanol) for 20 min on 14th DAE and then given a normal diet. These males were dissected to remove the rectal gland complex at 6 hr, and one, three,

and six days after treatment. The rectal gland and the body (without rectal gland) of each fly were separately soaked in ethanol (0.25 ml/male). One-microliter portions of the ethanolic extracts after ultrasonication (5-10 min) were used directly for GC analysis. In order to quantify compounds in low concentration, 100-μl portions of the ethanolic solutions were carefully concentrated in vacuo (20 mm Hg, 25°C), readjusted to 10 μl with methyl acetate, and 1 μl was injected into the gas chromatograph.

RESULTS

Observation of Feeding Behavior. The cultivated *Dendrobium superbum* blooms once a year usually around late April to early May in Okinawa. The strong attraction of *D. cucurbitae* males to the blossom can be seen in the open field during this period (Ichinohe et al., 1983). Outdoor observation revealed that the males were attracted to blossoms mostly in the morning (e.g., 10:00-11:00 AM, April 28, 1990, in Okinawa), and they voraciously licked the flower surface. Females paid no attention to the flowers, although Ichinohe et al. (1983) reported that a small number of females were also attracted. The *Dendrobium* flower is composed of a center lip, a pair of petals, and a three-forked calyx. The licking behavior by the males was restricted to the petal area, and the flies seldom visited inside of the lips (Figure 1). Such compulsive feeding did not cause apparent injury to the flowers. The males were also strongly attracted to a piece of filter paper impregnated with an extract of the flowers and licked the filter paper as they did the intact flowers.

Identification of Attractant. The voracious licking behavior of the male flies was observed directly on a developed TLC plate containing the crude extract of the *D. superbum* petals (Figure 2). The males were quickly attracted and licked repeatedly at an R_f value of 0.45. The flies left a clear salivation mark on the plate. The attractant component at $R_f = 0.45$ on the TLC plate corresponded to the peak with a retention time of 11.8 min shown by an arrow in the total ion chromatogram (Figure 3, top). Its mass spectrum exhibited the molecular ion peak at m/z 164 and the base ion peak at m/z 107 (Figure 3, bottom). The compound was identified as 4-(4-hydroxyphenyl)-2-butanone **(1)** by comparison with an authentic sample (Tokyo-Kasei Chemical Industries Co., Ltd.). Petals were found to contain the largest quantity, although the lips and calyxes also contained compound **1** in significant quantities (petal: 60.4 $\pm$ 9.1 μg, lip: 15.4 $\pm$ 0.6 μg, calyx: 20.0 $\pm$ 4.0 μg/flower). Compound **1** was also detected from a variety of *D. superbum* grown in Malaysia (3 μg/whole flower).

Accumulation of Compound 1 in Rectal Glands. D. cucurbitae males incorporated ketone **1** in the rectal glands within a short period after feeding on *D.*

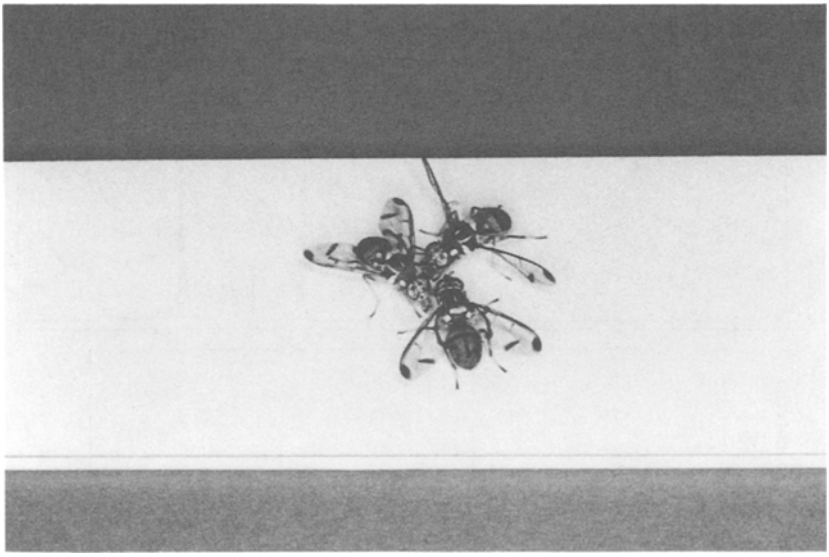

FIG. 2. Males of *Dacus cucurbitae* feeding on an attractive spot ($R_f = 0.45$) in the thin-layer chromatographic bioassay after chromatography of a *Dendrobium* flower extract (HPTLC silica gel 60 F_{254}, nano TLC Merck, developed with benzene–ethyl acetate, 4:1).

superbum flowers. Figure 4 shows the GC-MS trace of rectal gland extracts from *D. cucurbitae* males fed (bottom) and unfed (top) with *D. superbum* flowers. Both were found to contain substantial quantities of *N*-3-methylbutyl acetamide **(2)**, *N*-3-methylbutyl methoxyacetamide **(3)**, 1,3-nonanediol **(4)**, and ethyl 4-hydroxybenzoate **(5)** in common, but ketone **1** was found only in the males fed with *D. superbum* flowers.

The mean content of compound **1** in the rectal sacs in four individual males that had been fed twice on the *Dendrobium* flowers was 0.50 ± 0.26 μg.

Changes in Contents of Ketone 1 in Males. Figure 5 shows the mean contents of ketone **1** in the rectal gland and the rest of the body of *D. cucurbitae* males that had been fed with a pure sample of **1**. The flies appeared to incorporate **1** in the body tissues within 0.25 day after ingestion, and maintained a large portion in the rectal glands for at least six days, with decreasing total contents. The proportion of the content between the gland and body (without gland) at the third day after feeding clearly indicated the selective accumulation of the compound in the gland.

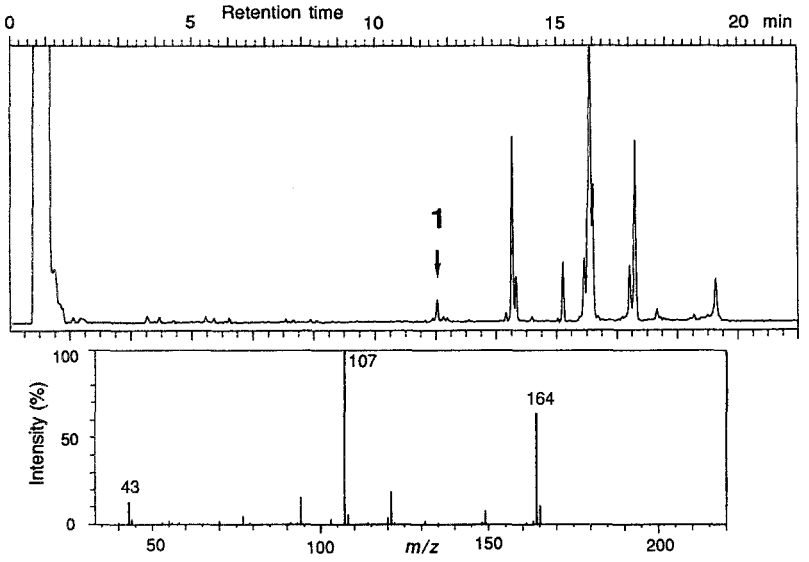

FIG. 3. Top: Mass chromatogram of volatiles in the extract of *Dendrobium superbum* petals. The active peak was found at a retention time of 11.8 min (shown by an arrow). This chromatogram was done on a capillary HP-1 column (24 m × 0.25 mm, programmed from 80 to 210°C, 10°C/min) with a total ion monitor (*m/z* 33–250). Bottom: Mass spectrum of 4-(4-hydroxyphenyl)-2-butanone (**1**), obtained from a scan of the peak with retention time of 11.8 min.

DISCUSSION

4-(4-Hydroxyphenyl)-2-butanone (**1**) has been characterized here as the specific attractant for *Dacus cucurbitae* males from the orchid flower, *Dendrobium superbum*. This compound has already been known as a potent attractant for *D. cucurbitae* males by the name of Willison's lure (Drew, 1974; Drew and Hooper, 1981) and also by the name of raspberry ketone as a characteristic flavor of raspberry (Honkanen et al., 1980; Gallois, 1982). Ketone **1** also has been reported from other plant sources, including Rosaceae, Compositae, and Labiatae (Hirvi et al., 1981; Hirvi and Honkanen, 1984; Lin and Chow, 1984; Marco et al., 1988) and as a fungal metabolite (Ayer and Singer, 1980). We have recently identified the same compound from leaves of the melon fly-attracting coniferous plant, *Juniperus chinensis* (R. Nishida and O. Iwahashi, unpublished). Although our knowledge of the distribution of ketone **1** in the plant kingdom is scanty, it is likely that *D. cucurbitae* males forage ketone **1** from some natural sources wherever available in the field.

Males of *D. cucurbitae* congregate at dusk to form a lek (Kuba et al., 1984;

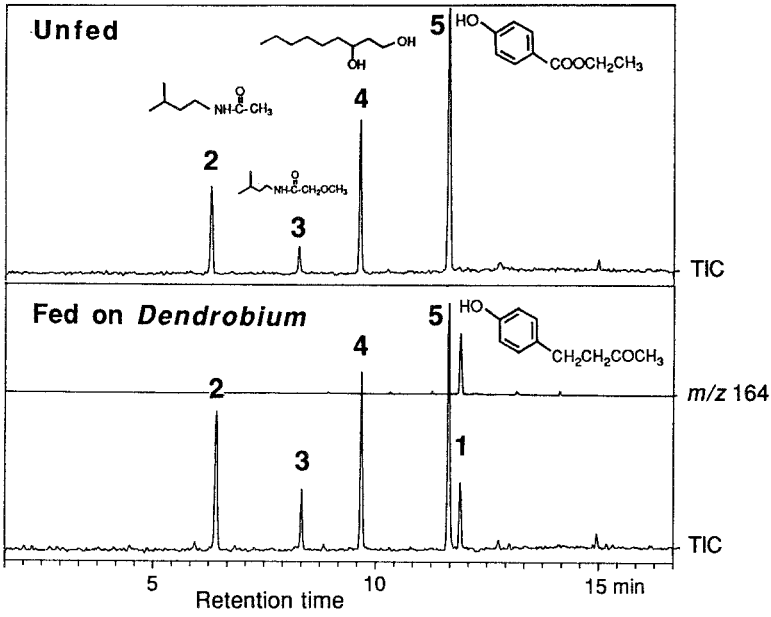

FIG. 4. Gas chromatograms [total ion monitor (TIC *m/z* 33–250); selective ion monitor, *m/z* 164] of volatiles in the rectal gland complex of *Dacus cucurbitae* males fed with *Dendrobium* flowers (bottom) and unfed control (top). Assignments were given by the diagnostic mass spectra: *N*-3-methylbutyl acetamide **(2)** (R_t = 6.4 min), *N*-3-methylbutyl methoxyacetamide **(3)** (R_t = 8.3), 1,3-nonanediol **(4)** (R_t = 9.7), ethyl 4-hydroxybenzoate **(5)** (R_t = 11.7), 4-(4-hydroxyphenyl)-2-butanone **(1)** (R_t = 11.9).

Iwahashi and Majima, 1986) and produce a smokelike substance that originates from the rectal glands during the courtship period (Kobayashi et al., 1978; Ohinata et al., 1982; Kuba and Sokei, 1988). The rectal secretion was attractive to the conspecific females at close range (Kobayashi et al., 1978), but its behavioral role is not fully understood. The volatile portion of the rectal secretion is composed of tetramethylpyrazine, *N*-3-methylbutyl acetamide, *N*-3-methylbutyl methoxyacetamide, 1,3-nonanediol, and methyl, ethyl, and propyl 4-hydroxybenzoates (Baker et al., 1982; Nishida et al., 1990; Perkins et al., 1990). In addition, the rectal sacs incorporated ketone **1** when males were fed either with cue-lure (Nishida et al., 1990) or *Dendrobium superbum* flowers (this study). The selective accumulation of orchid fragrance **1** in the male rectal glands suggests an additional pheromonal function of the compound in nature where the chemical source is available. Neither the rectal volatile mixture nor the intact ketone **1** induced any apparent behavioral response from females (Nishida et al., 1990). We observed that the male flies that had been fed either

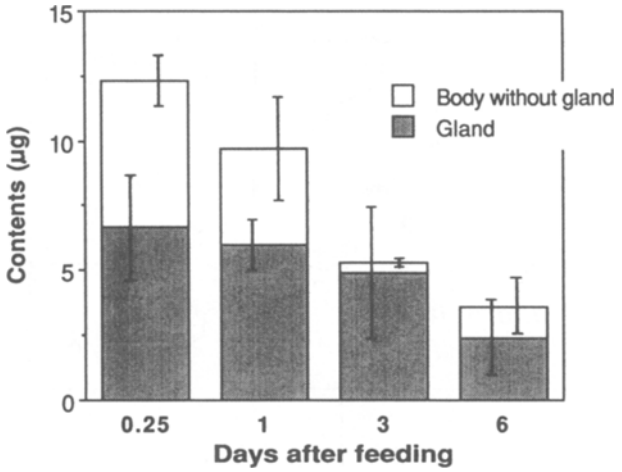

FIG. 5. Mean contents (±SD, μg/male) of 4-(4-hydroxyphenyl)-2-butanone (**1**) in the rectal gland complex and the rest of the body of *Dacus cucurbitae* males fed with compound **1**.

with **1** or cue-lure were persistently chased by unfed males. Many unfed males eagerly licked the surface of the container where the fed males deposited the secretion in spite of the presence of a female in the vicinity. The behavior might be associated with male-to-male competition in the sexual process. Further behavioral bioassay is needed to clarify the significance of ketone **1** together with other rectal volatiles, including male-to-female interactions in terms of direct attraction or aphrodisiac induction, and/or male-to-male interactions in the context of lek formation and mating disruption against competitors.

A parallel investigation has been conducted for the Oriental fruit fly, *Dacus (Bactrocera) dorsalis*. These males selectively accumulated phenylpropanoid metabolites in the rectal glands by foraging from methyl eugenol-containing flowers in the field. They released the rectal components during the courtship period, which suggests a very similar role as pheromone (Nishida et al., 1988; Nishida and Fukami, 1990). It was also suggested that the phenylpropanoids sequestered by *D. dorsalis* males could serve as allomones against predatory animals, since one of the components, 2-allyl-4,5-methoxyphenol, significantly deterred feeding of the sparrow, *Passer montanus* (Nishida et al., 1988; Nishida and Fukami, 1990). In the case of *D. cucurbitae*, however, ketone **1** did not deter feeding of sparrows (R. Nishida, unpublished). Instead, one of the endogenous rectal gland components, 1,3-nonanediol, was found to exhibit some deterrent activity against a lizard, *Hemidactylus frenatus*. Moreover, ketone **1** on the body of fed males was caused by the rectal gland secretion, which was

discharged via reflex action under stress, e.g., during immobilization (K.H. Tan, unpublished).

The flowers also might gain an ecological advantage by attracting a specific pollinator with the fragrant signal (Nishida et al., 1988). Similarly, male euglossine bees pollinate orchids while collecting floral fragrance components that are subsequently utilized to form lek where mating takes place (Dodson, 1975). *D. cucurbitae* males were arrested within the flower petal area and seldom visited inside the lip where pollination occurs, although a significant quantity of ketone **1** also was present in the lips. In contrast, Ichinohe et al. (1983) reported that lips of a variety of *D. superbum* attracted the male flies as well. *D. superbum* is distributed from Laos to the Malay Peninsula, the Philippines, Indonesia, and New Guinea (Karasawa, 1986). Observation of the native *Dendrobium* flowers instead of the cultivated varieties will be necessary to understand the association of the melon fly with the orchid plant in nature.

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REFERENCES

AYER, W.A., and SINGER, P.P. 1980. Phenolic metabolites of the bird's nest fungus *Nidula niveotomentosa*. *Phytochemistry* 19:2717–2721.

BAKER, R., HERBERT, R.H., and LOMER, R.A. 1982. Chemical components of the rectal gland secretions of male *Dacus cucurbitae*, the melon fly. *Experientia* 38:232–233.

DODSON, C.H. 1975. Coevolution of orchid and bees, pp. 91–99, *in* L.E. Gilbert and P.H. Raven (eds.). Coevolution of Animals and Plants. University of Texas Press, Austin.

DREW, R.A.I. 1974. The responses of fruit fly species (Diptera: Tephritidae) in the South Pacific area to male attractants. *J. Aust. 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. Aust. Entomol. Soc.* 20:201–205.

FLATH, R.A., and OHINATA, K. 1982. Volatile components of the orchid *Dendrobium superbum* Rchb. f. *J. Agric Food Chem.* 30:841–842.

GALLOIS, A. 1982. Quantitative evaluation of raspberry ketone using thin-layer chromatography. *Sci. Aliments* 2:99–106 (in French).

HIBINO, Y., and IWAHASHI, O. 1990. Appearance of wild females unreceptive to sterilized males on Okinawa Is. in the eradication program of the melon fly, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae). *Appl. Entomol. Zool.* 26:265–270.

HIRVI, T., and HONKANEN, E. 1984. The aroma of the fruit of sea buckthorn, *Hippophae rhamnoides* L. *Z. Lebensm.-Unters. Forsch.* 179:387–388.

HIRVI, T., HONKANEN, E., and PYYSALO, T. 1981. The aroma of cranberries. *Z. Lebensm.-Unters. Forsch.* 172:365–367.

HONKANEN, E., PYYSALO, T., and HIRVI, T. 1980. The aroma of Finnish wild raspberries, *Rubus idaeus* L. *Z. Lebensm.-Unters. Forsch.* 171:180–182.

ICHINOHE, F., TANAKA, K., ISHIKAWA, K., and MIZOBUCHI, M. 1983. Observation on attraction of the male melon flies, *Dacus cucurbitae*, to the blossoms of a common orchid, *Dendrobium superbum. Res. Bull. Plant Prot. Jpn* 19:1–6 (in Japanese).

IWAHASHI, O., and MAJIMA, T. 1986. Lek formation and male-male competition in the melon fly, *Dacus cucurbitae* Coquillett Diptera: Tephritidae). *Appl. Entomol. Zool.* 21:70–75.

KARASAWA, K. 1986. Orchid Atlas, Vol. 4. Chuo University Press, Tokyo, pp. 210–211.

KOBAYASHI, R.M., OHINATA, K., CHAMBERS, D.L., and FUJIMOTO, M.S. 1978. Sex pheromone of the Oriental fruit fly and melon fly: Mating behavior, bioassay method, and attraction of females by live males and by suspected pheromone glands of males. *Environ. Entomol.* 7:107–112.

KUBA, H., and SOKEI, Y. 1988. The production of pheromone clouds by spraying in the melon fly, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae) *J. Ethol.* 6:105–110.

KUBA, H., KOYAMA, J., and PROKOPY, R.J. 1984. Mating behavior of wild melon flies, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae) in a field cage: Distribution and behavior of flies. *Appl. Entomol. Zool.* 19:367–373.

LIN, Y.L., and CHOW, C.J. 1984. Studies on the constituents of aerial parts of *Scutellaria rivularis* wall. *Kuo Li Chung-Kuo I Yao Yen Chiu So Yen Chiu Pao Kao* 1984:141–165 (in Chinese).

MARCO, J.A., BARBERA, O., RODRIGUEZ, S., DOMINGO, C., and ADELL, J. 1988. Flavonoids and other phenolics from *Artemisia hispanica. Phytochemistry* 27:3155–3159.

NAKAMORI, H., and KAKINOHANA, H. 1980. Mass-production of the melon fly, *Dacus cucurbitae* Coquillett, in Okinawa, Japan. *Rev. Plant Prot. Res.* 13:37–53.

NISHIDA, R., and FUKAMI, H. 1990. Sequestration of distasteful compounds by some pharmacophagous insects. *J. Chem. Ecol.* 16:151–164.

NISHIDA, R., TAN, K.H., SERIT, M., LAJIS, N.H., SUKARI, A.M., TAKAHASHI, S., and FUKAMI, H. 1988. Accumulation of phenylpropanoids in the rectal glands of males of the Oriental fruit fly, *Dacus dorsalis. Experientia* 44:534–536.

NISHIDA, R., TAN, K.H., TAKAHASHI, S., and FUKAMI, H. 1990. Volatile components of male rectal glands of the melon fly, *Dacus cucurbitae* Coquillett (Diptera: Tephritidae). *Appl. Entomol. Zool.* 25:105–112.

OHINATA, K., JACOBSON, M., KOBAYASHI, R.M., CHAMBERS, D.L., FUJIMOTO, M.S., and HIGA, H. 1982. Oriental fruit fly and melon fly: Biology and chemical studies of smoke production by males. *J. Environ Sci. Health* A17:197–216.

PERKINS, M.V., KITCHING, W., DREW, R.A.I., MOORE, C.J., and KÖNIG, W.A. 1990. Chemistry of fruit flies: Composition of the male rectal gland secretions of some species of South-East Asian Dacinae. Reexamination of *Dacus cucurbitae* (melon fly). *J. Chem. Soc. Perkin Trans. I* 1990:1111–1117.

SCHULTZ, G.A., and BOUSH, G.M. 1971. Suspected sex pheromone glands in three economically important species of *Dacus. J. Econ. Entomol.* 64:347–349.

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BEHAVIORAL RESPONSES TO FOOD VOLATILES BY TWO SPECIES OF STORED-PRODUCT COLEOPTERA, *Sitophilus oryzae* (CURCULIONIDAE) and *Tribolium castaneum* (TENEBRIONIDAE)

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Abstract—Laboratory experiments were conducted to study the behavioral activity of grain-derived volatiles as attractants and pheromone synergists for *Sitophilus oryzae*, an internal-feeding pest of sound grain, and *Tribolium castaneum*, an external-feeding pest of damaged grains and flour. Behavioral studies with two-choice pitfall bioassays determined that the fresh grain volatiles valeraldehyde, maltol, and vanillin were attractive to *S. oryzae* at various doses, but *T. castaneum* were not attracted to any dose of any of these three compounds. When oils from pressed grains were bioassayed, sesame oil was significantly repellent and oat and wheat germ oils were attractive to *S. oryzae*. However, rice, soybean, oat, wheat germ, and corn oils were all attractive to *T. castaneum*. A commercial food product composed primarily of soybean oil and wheat germ was highly attractive to *T. castaneum*, but elicited no response from *S. oryzae*. A combination of the three grain volatiles valeraldehyde, maltol, and vanillin with the synthetic pheromone sitophinone was more attractive to *S. oryzae* than either the pheromone alone or the tripartite grain volatile mix. Similarly, a combination of the commercial food product with the pheromone 4,8-dimethyldecanal was more attractive to *T. castaneum* than either food alone or pheromone alone. Behavioral responses to grain volatiles may reflect the ecological niche of the granivore: *S. oryzae* colonizes sound grain and is attracted to volatiles characteristic of fresh grain, while *T. castaneum* utilizes damaged or deteriorated grains and responds best to oils char-

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acteristic of damaged or fungus-infested grain. Synergism of food odors and pheromones suggests that more effective traps can be devised for management of these pest insects.

Key Words—Aggregation pheromone, stored grain, synergism, *Sitophilus oryzae*, rice weevil, *Tribolium castaneum*, red flour beetle, bioassay, sitophinone, 4,8-dimethyldecanal.

INTRODUCTION

Phytophagous insects generally utilize volatile semiochemical cues from host plants during one or more phases of the host selection process. Plant semiochemicals may act as direct attractants for insects or they may synergistically enhance the activity of pheromones produced by insects that have contacted the host plant. These phenomena are known in scolytid bark beetles in which some species respond to terpenes released by stressed or injured host trees and others require that a particular host odor be present to synergize the activity of beetle-produced pheromones (Borden, 1982; Wood, 1982). Various studies on weevils (e.g., Phillips et al., 1984; Dickens, 1989) found that host plant volatiles significantly enhance field responses to male-produced aggregation pheromones. Recent work on certain moth species determined that females and males can orient to host plants in response to odors (Landolt, 1989; Liu et al., 1988) and that plant volatiles can have profound effects on sex pheromone biology of both males and females (Landolt and Heath, 1990; Raina et al., 1992). The use of sex-specific pheromones by phytophagous insects seems so closely tied to the host plant for a variety of functions (e.g., host selection, pheromone biosynthesis, assembly, mating, and oviposition) that the influence of plant odors on orientation and response to pheromones may be a general theme throughout these species (e.g., Dickens et al., 1990).

Stored-product insects present a special situation for research on host plant volatiles because nearly all species are intimately adapted to human-stored grains and grain products, truly nonanthropogenic populations may not exist, and prehistoric host plants and habitats are not well known (Lindsley, 1944). The guilds of insects that infest stored grains are viewed as part of a unique ecosystem (Sinha, 1973). Species can be assigned to ecological niches in which, for example, either fresh, sound grain may be the breeding substrate, or broken, fungus-infected material is used. Volatile attractants for several beetle species that infest broken grain have been identified from cereal grains and their products (e.g., Mikolajczak et al., 1984; Nara et al., 1981; Pierce et al., 1990) and from the fungi that infect stored grains (Pierce et al., 1991). Work on the maize weevil, *Sitophilus zeamais* Motschulsky, a species that attacks sound grain, demonstrated that odors from cracked wheat synergistically enhanced responses to male-produced pheromone (Walgenbach et al., 1987). Trapping technology for

the red flour beetle, *Tribolium castaneum* Herbst, utilizes wheat germ oil as a food attractant in combination with a synthetic male-produced pheromone (Barak and Burkholder, 1985), but synergistic activity was not experimentally addressed. Identification of key host attractants and pheromone synergists in both primary and secondary insect pests could greatly increase efficacy of pheromone-baited traps and aid in pest management.

The objective of this study was to examine the behavioral activity of several grain-derived volatiles as attractants and pheromone synergists for two species of stored-product beetles that occupy different niches in the stored-grain ecosystem. We studied *Sitophilus oryzae* (L), the rice weevil, because it represents internal-feeding insects that infest sound grain. Female *S. oryzae* oviposit in kernels of cereal grain, and the larvae and pupae develop entirely within the kernel. A male-produced aggregation pheromone, (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone, sitophinone, was isolated and identified in studies by Phillips and Burkholder (1981) and Phillips et al. (1985), but specific grain volatiles that may enhance response to pheromone have not been investigated. The second species studied, *T. castaneum*, the red flour beetle, can not penetrate sound grain, but represents species that feed and reproduce on damaged grain, fine material, and milled products. *T. castaneum* also uses a male-produced aggregation pheromone, 4,8-dimethyldecanal (Suzuki, 1980), for which the natural stereochemistry is not known. The grain volatiles we tested fall into two general classes: low-molecular-weight or aromatic flavor constituents common to many cereal grains, particularly when freshly broken (e.g., Maga, 1978), and pressed oils from several species of grains and one legume that contain a complex of compounds (predominantly fatty acids) and represent volatiles typical of older, damaged, and insect- or fungus-infected grains. In addition to these materials, we also tested a commercial food product for activity against the two beetle species.

METHODS AND MATERIALS

Experimental Insects. All experiments were conducted in the laboratory using established colonies of insects. *S. oryzae* were reared on soft spring wheat kernels at 12% moisture content in a growth chamber maintained at 27°C, 60% relative humidity, and 16:8 (light-dark) photoperiod. *T. castaneum* were reared on a mixture of whole wheat flour and brewer's yeast (95:5) under the same environmental conditions as *S. oryzae*. Parent beetles of both species were sifted from cultures one week after inoculation, and new adult progeny were removed for bioassays one week after emergence.

Chemical Compounds. Synthetic grain volatiles and pheromones were 95+% pure and were diluted in hexane at various concentrations prior to bioas-

say. The single compounds we studied were valeraldehyde (1-pentanal), maltol (3-methoxy-2-methyl-4-pyrone), and vanillin (3-methoxy-4-hydroxybenzaldehyde); all were obtained commercially (Aldrich Chemical Company, Milwaukee, Wisconsin). We chose these three grain volatiles because they are common in several species of grain (Maga, 1978) and because preliminary screening revealed some behavioral activity against *S. oryzae* and other beetle species. Cereal oils were 100% pure pressed oils and were used undiluted from newly opened containers obtained from local commercial suppliers. Oils included those from sesame, rice, soy beans, oats, wheat germ, and corn. The commercial food product we used, referred to here as WGN, was studied because of its reputation for becoming infested by various species of secondary beetles while in stored bulk packages. WGN is a prepared, extruded product containing, in descending order of relative amounts, hydrogenated soybean oil, wheat germ, sugar, sodium caseinate (milk protein), soy protein, natural and artificial flavors, and artificial color. The synthetic sitophinone, 5-hydroxy-4-methyl-3-heptanone, was a mix of the *R*S** isomers (Phillips et al., 1985). We used a mixture of 4*R*,8*R* and 4*R*,8*S* diastereomers of 4,8-dimethyldecanal (Zoecon Corporation, Palo Alto, California), which was reported to have optimal activity for *T. castaneum* (Levinson and Mori, 1983).

Bioassays. The majority of experiments employed a two-choice pitfall bioassay in which beetles oriented to one of two holes in the floor of an arena, below which were placed stimulus or control materials in a glass collection dish. Test arenas were open steel cans, 25 cm diam. × 20 cm high; 3-cm-diam. pitfall holes were located directly opposite from each other, 4 cm from the side wall. Glass collection dishes were the bottoms of 15 × 60-mm Petri dishes. The inside top edges of the collection dishes were coated with liquid Teflon to prevent responding beetles from returning to the arena. One experiment utilized a four-choice arena of the same dimensions as the one described above, but in which four pitfall holes were located equidistant from each other in a square arrangement, each 2 cm from the side wall. A single layer of wheat kernels was placed on the floors of all test arenas. We chose wheat to provide a familiar substrate for footing and to approximate conditions in stored grain. Wheat kernels were prevented from falling into the pitfall holes by a 12-mesh screen soldered in place over the holes. The screen allowed easy passage of the insects into the collection dishes. Bioassays were conducted for 2 hr in complete darkness at 27°C and 60 ± 10% relative humidity. Twenty test insects were used as mixed-sex adults in each replicate and were placed under an inverted glass funnel (3 cm diam. at widest point) at the center of the arena for 20 min prior to release to allow for acclimation to the experimental conditions. A response index (RI) for beetles in the two-choice bioassay was calculated as $RI = (T - C/Tot)100$, for which T is the number responding to the treatment, C is the number responding to the control, and Tot is the total number of insects released.

Positive RIs indicate attraction to the treatment and negative RIs indicate repellency; values could theoretically range from −100 for complete repellency, to +100 for complete attraction. Numbers responding to treatment and control were subjected to Student's *t* test for paired comparisons. Mean RIs are reported in all cases for results of two-choice bioassays. In the one four-choice experiment for *T. castaneum*, 50 beetles were released in the arena and the mean number of beetles responding to each treatment was determined. Ten replicates (i.e., 10 separate arena bioassays) were performed for each test material or combination of materials studied.

The first series of bioassays assessed the responses of *S. oryzae* and *T. castaneum* to doses ranging from 1.0 ng to 100 μg of valeraldehyde, maltol, and vanillin. Ten-microliter aliquots of hexane solutions were applied to 1-cm-diam. filter-paper disks, while control disks received 10 μl of hexane only. Treatment and control disks were randomly assigned to collection dishes in the two-choice pitfall arenas. A second series of two-choice bioassays examined the responses of the two beetle species to individual oils. One milliliter of the test oil was placed directly into each treatment collection dish, and 1 ml of mineral oil was added to control dishes; responding beetles were trapped in the oils. A third series of experiments assessed the responses of beetles to 3.0 g of WGN compared to an empty dish as a control. A fourth series of bioassays compared a mixture of grain volatiles only (100 μg valeraldehyde, 100 ng maltol, 100 μg vanillin), synthetic sitophinone only (100 ng), and a combination of the grain volatiles with sitophinone for activity against *S. oryzae*. The last experiment utilized the four-choice bioassay for *T. castaneum* in which 50 beetles were released and could choose among an empty dish (control), 0.5 g of WGN only, 1.0 μg of 4,8-dimethyldecanal only, and a combination of the WGN and 4,8-dimethyldecanal.

RESULTS

In experiments with valeraldehyde (Figure 1), maltol (Figure 2), and vanillin (Figure 3), *S. oryzae* displayed attraction at some doses of all three compounds, but *T. castaneum* were not attracted to any treatments and responded at levels that were not significantly different from responses to controls. *S. oryzae* responded significantly to all doses of valeraldehyde tested (Figure 1), and regression analysis found that there was no significant effect of dose on response ($R^2 = 0.003$, $P = 0.703$). Response of *S. oryzae* to maltol was highest at 0.1 μg, and there was a significant negative effect of dose on response ($R^2 = 0.152$, $P = 0.005$). Responses of *S. oryzae* to vanillin were generally low compared with responses to the other two compounds, resulting in significant attraction only at the 10-μg and 100-μg levels, and there was no significant

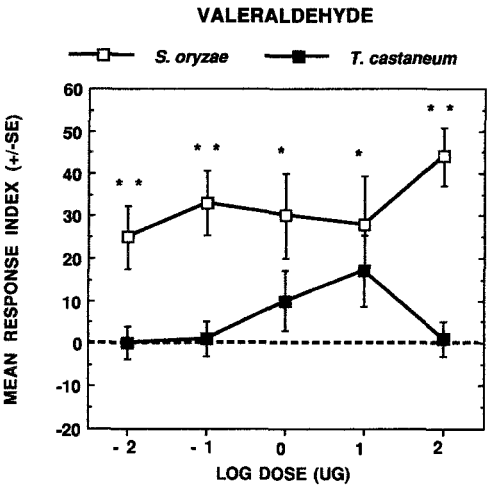

FIG. 1. Response of *S. oryzae* and *T. castaneum* to different doses of valeraldehyde in two-choice bioassays. Response index = $(T - C/Tot) \times 100$, for which T is the number responding to the treatment, C is the number responding to the control, and *Tot* is the total number released. There were no significant differences ($P > 0.05$, t test) between those responding to treatment and those responding to control for *T. castaneum* at any dose. Significant differences between treatment and control responses indicated by * ($P < 0.05$) and ** ($P < 0.01$); $N = 10$ bioassays for each species at each dose.

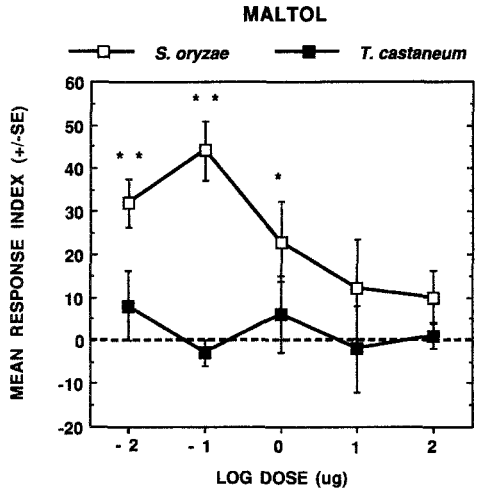

FIG. 2. Response of *S. oryzae* and *T. castaneum* to different doses of maltol in two-choice bioassays. Response index and statistical analyses calculated as in Figure 1.

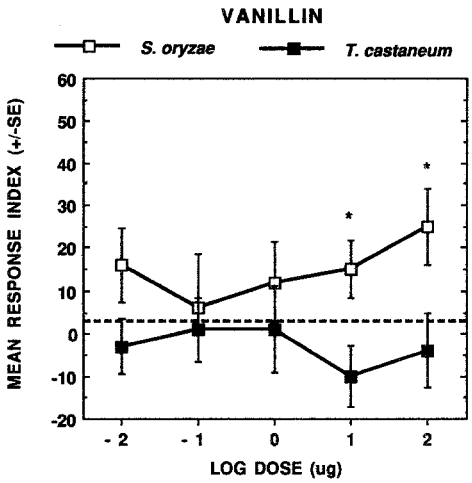

FIG. 3. Response of *S. oryzae* and *T. castaneum* to different doses of vanillin in two choice bioassays. Response index and statistical analyses calculated as in Figure 1.

effect of dose on response ($R^2 = 0.017$, $P = 0.363$). When response indices of *S. oryzae* to the three flavor compounds were compared within dosage levels using analysis of variance, response to maltol was higher than to vanillin at 0.1 μg ($F_{2,18} = 3.819$, $P = 0.041$) and response to valeraldehyde was higher than to maltol at 100.0 μg ($F_{2,18} = 4.614$, $P = 0.024$), but responses did not differ significantly at other doses.

Responses to oils varied among treatments and also differed between the two beetle species (Figure 4). *S. oryzae* was significantly repelled by sesame oil, displayed no significant response to rice, soy bean, or corn oils, but was significantly attracted to oat and wheat germ oils. *T. castaneum* displayed significant attraction to all oils except sesame oil and expressed the highest mean attractive responses to oat, wheat germ, and corn oils. In two sets of bioassays we found that *S. oryzae* did not respond to the WGN food product (mean $RI = -0.5 \pm 0.89$ SE, $P \gg 0.05$), but that *T. castaneum* were highly attracted to WGN (mean $RI = 89.5 \pm 4.62$ SE, $P \ll 0.01$).

Both *S. oryzae* and *T. castaneum* displayed increased responses to their respective pheromones when the pheromones were combined with food odors. A comparison of response indices from three sets of two-choice experiments with *S. oryzae* found that the combination of the grain volatiles valeraldehyde, maltol, and vanillin with the pheromone sitophinone elicited a higher relative response by weevils than did the three grain volatiles alone or sitophinone alone (Figure 5). In a four-choice experiment with *T. castaneum*, more beetles

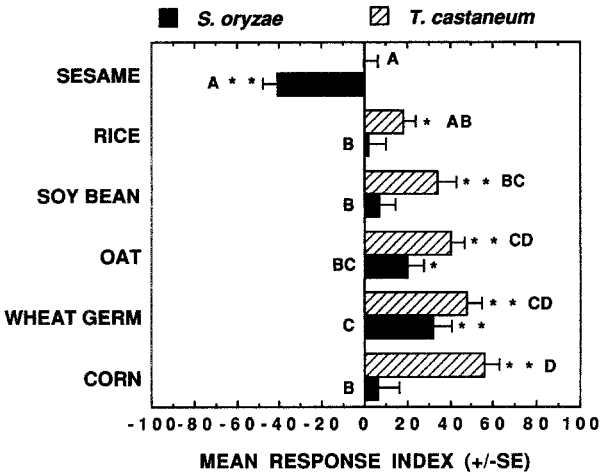

FIG. 4. Response of *S. oryzae* and *T. castaneum* to different oils in two-choice bioassays. Response index was calculated as in Figure 1. Significant differences between treatment and control responses (t test) for each oil within each species indicated by * ($P < 0.05$) and ** ($P < 0.01$); $N = 10$ bioassays for each species with each oil. Mean responses to different oils followed by different letters for *S. oryzae* (left side) and *T. castaneum* (right side) are significantly different (ANOVA, $P < 0.05$, means comparison by Fisher's LSD).

responded to the combination of 4,8-dimethyldecanal and WGN than to either component alone or a blank control (Figure 6).

DISCUSSION

Our results indicate that *S. oryzae* and *T. castaneum* may respond quite differently to the same grain-related volatiles. Orientation of *S. oryzae* to volatile flavor constituents may be correlated to the ecological tendency of this species to breed in fresh grain. Fresh grains contain a number of volatiles, including valeraldehyde, maltol, and vanillin (Maga, 1978). The lack of response by *T. castaneum* to valeraldehyde, maltol, and vanillin suggests that these compounds do not signal a food source for this species. Older grains, particularly if infested by fungi, are known to have a higher fatty acid content than fresh grain (Christensen and Kaufman, 1969). Volatiles from grain oils may signify the quality (i.e., age or level of deterioration) of a food source, or they may facilitate discrimination of suitable species of grain by beetles. The greater response of *T. castaneum* to various oils may reflect the habitat preference for this species of breeding in older and damaged grain substrates. The overall lower responses,

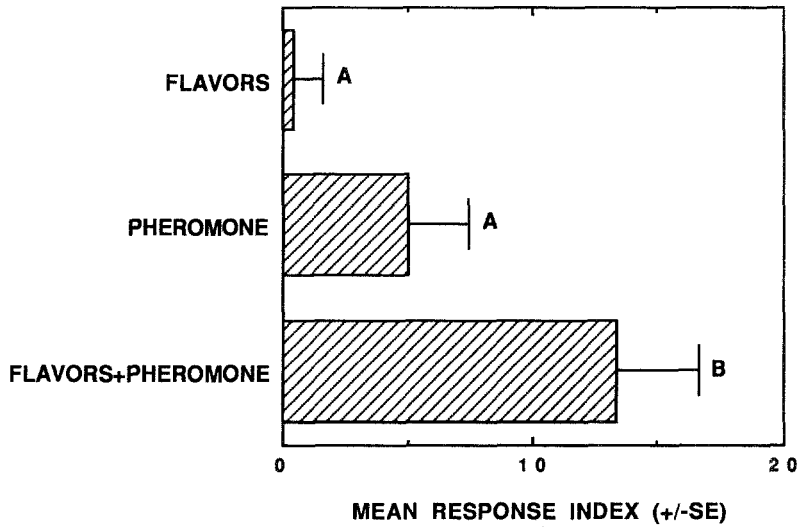

FIG. 5. Response of *S. oryzae* to pheromone and flavor volatiles in three sets of two-choice bioassays in which the controls were solvent only on filter paper. FLAVORS is a combination of 100 μg valeraldehyde, 100 ng maltol, and 100 μg vanillin on filter paper; PHEROMONE is 100 ng of sitophinone on filter paper. Response index = $(T - C/Tot) \times 100$, for which T is the number responding to the treatment, C is the number responding to the control, and Tot is the total number released. Mean response indices followed by different letters are significantly different ($P < 0.05$, ANOVA, with Student-Neuman-Keuls comparison among means; $N = 10$ for each set of bioassays).

and even repellency (e.g., as with sesame oil, Figure 4), of *S. oryzae* to the different oils demonstrates that not all grain oils are attractive for weevils. The very high attractive response of *T. castaneum* to WGN indicates that the processing and combined ingredients of this food product yield a stimulus that may represent an optimal food source for this species. Lack of response by *S. oryzae* to WGN contrasts sharply with attraction of *T. castaneum* to this material and again demonstrates a profound species difference in response to food volatiles.

Both *S. oryzae* and *T. castaneum* exhibited increased responses to their aggregation pheromones when these pheromones were associated with grain odors. More work will need to be done to determine if these combinations can truly act synergistically (e.g., in which the response to the combination is greater than the combined responses to the individual components), but the present data indicate a clear effect of food volatiles. Walgenbach et al. (1987) reported the first record of enhanced response to a combination of natural grain odors and pheromone in *S. oryzae*, and Trematerra and Girgenti (1989) demonstrated a similar phenomenon with *S. oryzae* using intact and broken kernels of rice,

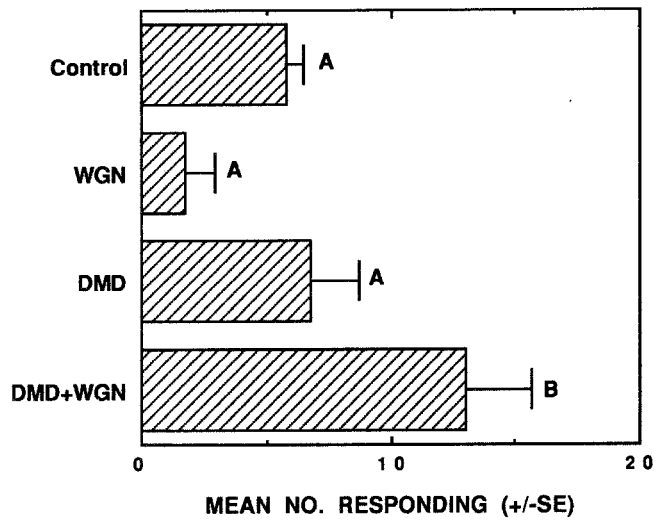

FIG. 6. Response of *T. castaneum* in a four-choice bioassay with food volatiles and pheromone. Control = empty dish, WGN = 0.5 g of a commercial food product (see text for explanation), DMD = 1.0 μg of 4,8-dimethyldecanal. Means followed by different letters are significantly different ($P < 0.05$, ANOVA, with Student-Neuman-Keuls comparison among means; $N = 10$ for each set of bioassays).

corn, and wheat. Our data here support those earlier studies and show that particular synthetic compounds from grain volatiles can increase response of *S. oryzae* to its pheromone. Our result with *T. castaneum* demonstrates enhancement of pheromone activity by addition of food volatiles for this species. Results for both *S. oryzae* and *T. castaneum* suggest that efficacy of pheromone-baited traps used for detecting these species could be increased by the addition of a proper food volatile formulation. Current use of certain food volatiles (e.g., oil mixtures) in commercially available traps may require reevaluation as more information on pheromone and food volatile interactions is collected.

Stored grains can vary in quality as a function of biotic and abiotic factors (Christensen and Kaufman, 1969), and volatiles that come from grain can be indicative of the current grain quality (e.g., Sinha et al., 1988). This study demonstrates that grain volatiles can have significant effects on host selection behavior in granivores and that these effects may differ substantially between species sharing the same resource. Niche partitioning in the stored-grain ecosystem may therefore be facilitated by semiochemicals originating from a heterogeneous food substrate. More research is needed with stored-product insects in which natural volatile extracts from food sources are studied and key semiochemicals isolated that affect various species. Studies of food volatiles will

advance a better understanding of interactions in the stored-product ecosystem and will yield more effective applications of semiochemicals in pest management (Burkholder, 1990)

REFERENCES

BARAK, A.V., and BURKHOLDER, W.E., 1985. A versatile and effective trap for detecting and monitoring stored-product Coleoptera. *Agric. Ecosyst. Environ.* 12:207–218

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.

BURKHOLDER, W.E. 1990. Practical use of pheromones and other attractants for stored-product insects, pp. 497–516, *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.

CHRISTENSEN, C.M., and KAUFMAN, H.H. 1969. Grain Storage, The Role of Fungi in Quality Loss. University of Minnesota Press, Minneapolis, 153 pp.

DICKENS, J.C. 1989. Green leaf volatiles enhance aggregation pheromone of boll weevil, *Anthonomus grandis*. *Entomol. Exp. Appl.* 52:191–203.

DICKENS, J.C., JANG, E.B., LIGHT, D.M., and ALFORD, A.R. 1990. Enhancement of insect pheromone responses by green leaf volatiles. *Naturwissenschaften* 77:29–31.

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.

LEVINSON, H.Z., and MORI, K. 1983. Chirality determines pheromone activity for flour beetles. *Naturwissenschaften* 70:190–192.

LINDSLEY, E.G. 1944. Natural sources, habitats, and reservoirs of insects associated with stored food products. *Hilgardia* 16:187–224.

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.

MAGA, J.A. 1978. Cereal volatiles, a review. *J. Agric. Food Chem.* 26:175–178.

MIKOLAJCZAK, K.L., ZILKOWSKI, B.W., SMITH, C.R. JR., and BURKHOLDER, W.E. 1984. Volatile food attractants for *Oryzaephilus surinamensis* (L.) from oats. *J. Chem. Ecol.* 10:301–309.

NARA, J.M., LINDSAY, R.C., and BURKHOLDER, W.E. 1981. Analysis of volatile compounds in wheat germ oil responsible for an aggregation response in *Trogoderma glabrum* larvae. *Agric. Food Chem.* 29:68–72.

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., SCHMUFF, N.R., and FALES, 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, T.W., WEST, J.R., FOLTZ, J.L., SILVERSTEIN, R.M., and LANIER, G.N. 1984. Aggregation pheromone of the deodar weevil, *Pissodes nemorensis* (Coleoptera: Curculionidae): Isolation and activity of grandisol and grandisal. *J. Chem. Ecol.* 10:1417–1423.

PIERCE, A.M., PIERCE, H.D., OEHLSCHLAGER, A.C., and BORDEN, J.H. 1990. Attraction of *Oryzaephilus surinamensis* (L.) and *Oryzaephilus mercator* (Fauvel) (Coleoptera: Cucujidae) to some common volatiles of food. *J. Chem. Ecol.* 16:465–475.

PIERCE, A.M., PIERCE, H.D., BORDEN, J.H., and OEHLSCHLAGER, A.C. 1991. Fungal volatiles: Semiochemicals for stored-product beetles (Coleoptera: Cucujidae). *J. Chem. Ecol.* 17:581–597.

RAINA, A.K., KINGAN, T.G., and MATTOO, A.K. 1992. Chemical signals from host plant and sexual behavior in a moth. *Science* 255:592–594.

SINHA, R.N. 1973. Ecology of storage. *Ann. Tech. Agric.* 22:351–369.

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.

SUZUKI, T. 1980. 4,8-dimethyldecanal: the aggregation pheromone of the flour beetles *T. castaneum* and *T. confususm* (Coleoptera: Tenebrionidae). *Agric. Biol. Chem.* 44:2519–2520.

TREMATERRA, P., and GIRGENTI, P. 1989. Influence of pheromone and food attractants on trapping of *Sitophilus oryzae* (L.) (Col., Curculionidae): A new trap. *J. Appl. Entomol.* 108:12–20.

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.

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.

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STRUCTURE–ACTIVITY CORRELATIONS AMONG ANALOGS OF THE CURRANT CLEARWING MOTH PHEROMONE

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Abstract—Eleven analogs of (*E*,*Z*)-2,13-octadecadien-1-yl acetate **1**, a main pheromone component of the currant clearwing moth, *Synanthedon tipuliformis* Clerk (Lepidoptera: Sesiidae) were synthesized and tested for their biological activities by electroantennography (EAG). To correct the EAG data for differences in volatility of the analogs, their vapor pressures were estimated by a gas chromatographic method. All structural changes in the parent molecule were found to reduce the biological activity to various degrees. The most active analog tested was the carbamate **12**, whose activity was almost comparable to that of the pheromone component **1**. Structure–activity correlations showed that hydrophobic, steric, and electronic effects of chain terminal groups might be responsible for variations in biological activity of the conformationally unchanged (*E*,*Z*)-2,13-analogs

Key Words—Pheromone analogs, (*E*,*Z*)-2,13-octadecadien-1-yl acetate, EAG, gas chromatography, vapor pressure, currant clearwing moth, *Synanthedon tipuliformis* Clerk, Lepidoptera, Sesiidae.

INTRODUCTION

Pheromone analogs are known to have potential for investigation of the olfactory transduction mechanism and for use in alternative pest control strategies (Prestwich, 1987; Evershed, 1988). It is now generally accepted that the receptor cavity contains highly complementary interaction sites to the three key molecular

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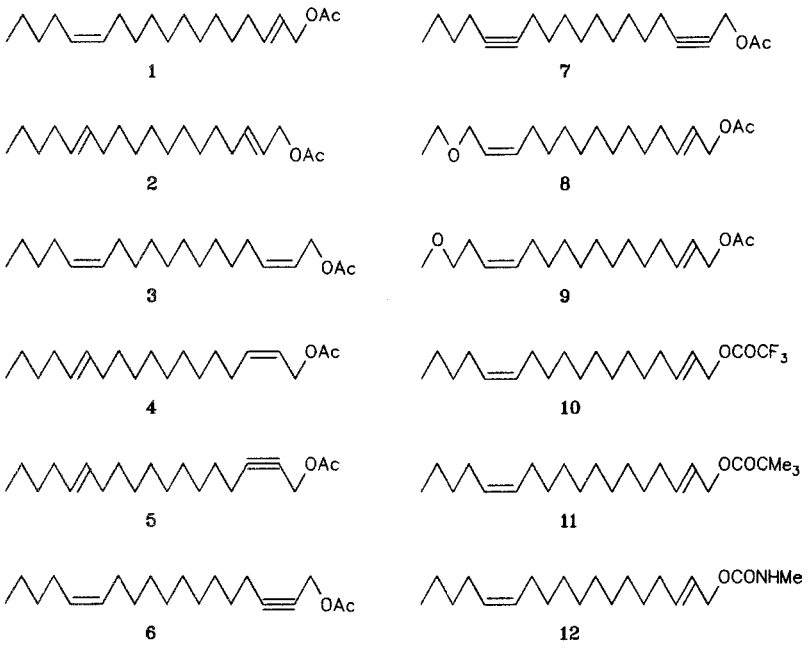
parts of the stimulus molecule, i.e., to the double bond, the terminal alkyl group, and the polar functional group. However, most of the structure–activity studies have been focused on relatively short monounsaturated acetates, e.g., (*Z*)-5-decen-1-yl and (*Z*)-7-dodecen-1-yl acetates (Liljefors et al., 1984, 1985, 1987; Bengtsson et al., 1987, 1990; Jönsson et al., 1991a,b), sex pheromone components of the turnip moth, *Agrotis segetum.* Specialized studies on longer-chain, unconjugated dienic acetates are rather rare and not nearly exhaustive enough (Bestmann et al., 1987) to allow generalizations.

In this study, the currant clearwing moth (CCM), *Synanthedon tipuliformis* Clerk (Lepidoptera: Sesiidae), which is a serious pest of red and black currant and gooseberry in Europe as well as in Asia, North America, and Australia, was selected as a target species. Its female sex pheromone has been identified (Arn et al., 1986) as a two-component mixture consisting of (*E*,*Z*)-2,13-octadecadien-1-yl acetate (**1**, Scheme 1) (94%) and the corresponding C_2-saturated analog, (*Z*)-13-octadecen-1-yl acetate. Several efficient syntheses of **1** have recently been reported (Hoskovec et al., 1990; Sharma et al., 1990; Sorochinskaya and Kovalev, 1991) and its attractant activity confirmed in field tests (Szöcs et al., 1990). Recently, the intraspecific variability in CCM pheromone communication has been observed (Szöcs et al., 1991). Depending on the geo-



SCHEME 1.

graphic region, trace amounts of the (*E*,*Z*)-3,13-isomer were found to cause either synergistic or inhibitory effects.

In an effort to provide clues to the spatial and/or functional group requirements for biological activity of the pheromone component **1,** we conducted an electrophysiological study on the response of CCM males to 11 analogs of **1** (Scheme 1). To corroborate the results of EAG dose–response measurements with respect to differences in volatility between test compounds, vapor pressures of all analogs were estimated by a simple gas chromatographic method and used to correct the original EAG data.

The analogs **2–12** (Scheme 1) proceed from modifications in the key molecular parts that may be directly involved in the interaction process with the receptor. Thus, all the possible double bond configuration changes at positions **2** and **13** are reflected in compounds **2–4.** In the acetylene derivatives **5–7,** one or two double bonds are replaced by a triple bond, whereas in oxa analogs **8** and **9** one of the parafinic carbons is substituted by an oxygen atom. Finally, in esters **10–12,** the acetate moiety is replaced by the trifluoroacetate, pivalate, and *N*-methyl carbamate groups, respectively.

METHODS AND MATERIALS

Insects

Diapausing larvae hidden in currant stems were collected from red and black currant plantages in South Bohemia in March 1991. The larvae were kept outdoors until emergence. Moths hatched in June were collected daily and sexed. The males were held separately in Petri dishes and provided water. Three day-old individuals were used for EAG experiments.

Electrophysiological Recording

Recordings were made from antennae of intact, mechanically immobilized moths. Ag–AgCl electrodes filled with Ringer solution were used to record slow negative EAG potentials generated by olfactory stimulation. The recording electrode was positioned at a cut distal end of the antenna. The EAG potentials were amplified by a high-impedance DC amplifier, monitored on a dual-beam storage oscilloscope, and recorded by a pen recorder. The maximal negative EAG deflections were evaluated.

Stimulation

Serial dilutions of the pheromone component and its analogs were prepared in hexane. From each solution, 5 μl was soaked in a filter-paper disk of 10 mm diameter. After solvent evaporation, the loaded disks were individually inserted

into Pasteur pipets and stored in closed glass vials at −20°C. One hour prior to the experiments, the stimulus cartridges were transferred to attain room temperature. The antennal preparation was continuously blown over by charcoal-filtered and humidified air (1 liter/min). During 0.5-sec stimulation, a constant volume (≈8.3 ml) of air was injected through the stimulus cartridge upon the antenna. The stimuli were delivered from the lowest to the highest concentrations, with 2-min pauses between stimuli at lower concentrations and 5–15 min at concentrations greater than 10^{-1} g/liter. These pauses were adequate for complete recovery of the EAG and for readaptation of receptors after previous olfactory stimulation.

EAG Evaluation

Doses of 10^{-1} g/liter of (*E*,*Z*)-2,13-octadecadien-1-yl acetate **1** were used as standard to (1) normalize responses, and (2) control for viability and constancy of the preparation. Stimulation with the standard both preceded and followed each serial dilution level. Each EAG response to test chemicals was expressed as a percentage of the mean of the two nearest responses to the standard.

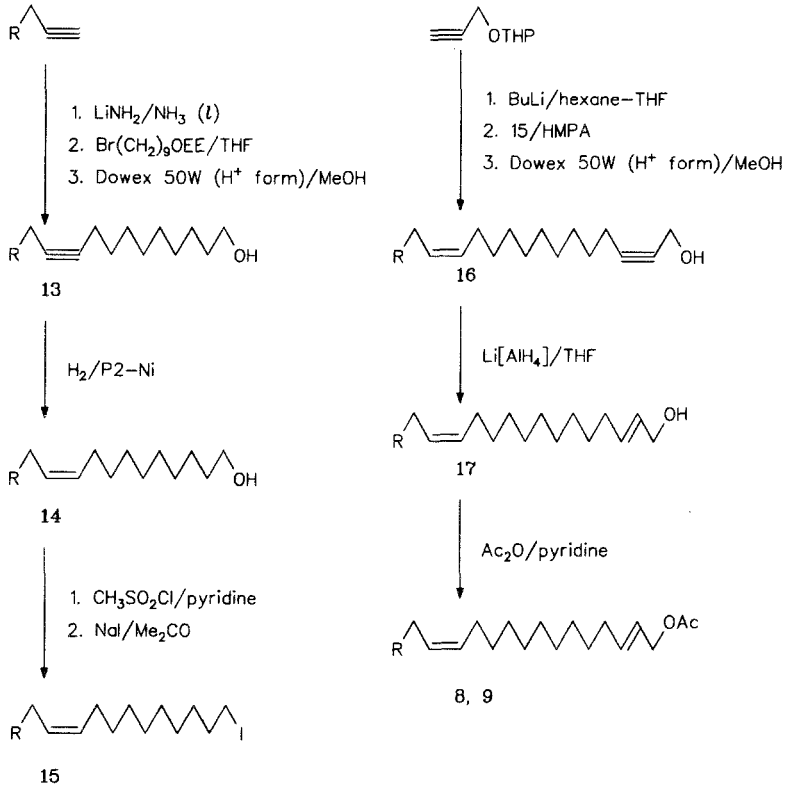
Chemicals

Acetates **1–7** were prepared by previously reported methods (Hoskovec et al., 1990). Trifluoroacetate **10,** pivalate **11,** and *N*-methyl carbamate **12** have been prepared by standard esterifications of the parent (*E*,*Z*)-2,13-octadecadien-1-ol. Their IR, NMR, and mass spectra were fully consistent with the proposed structures. All the doubly unsaturated final alcohols were ultimately purified by argentation chromatography (Voerman and Rothschild, 1978; Voerman et al., 1984) and checked by GC. Their purity was estimated to be 99%. The synthesis of the oxa analogs **8** and **9** is described below (Scheme 2).

^{1}H NMR spectra were determined in $CDCl_3$ solution on Varian UNITY-500 and UNITY-200 spectrometers, operating at 499.5 and 200.1 MHz, respectively, and absorptions are expressed on a δ (ppm) scale relative to TMS. GLC analyses were performed on a Hewlett Packard HP 5880A chromatograph equipped with a FID detector and a 25-m capillary column (internal diameter 0.3 mm, HP5-5% phenyl methylsilicone, cross-linked). Flash chromatography separations were made on Merck 60 silica gel (0.040–0.063 mm) using a Büchi B-680 Prep LC System with a stepwise gradient of ethyl acetate in light petroleum.

13-Oxa-10-pentadecyn-1-ol ***(13a)***

To a suspension of lithium amide (prepared from 1.47 g of lithium and 1000 ml of liquid ammonia) 23.0 g (0.273 mol) of 4-oxa-1-hexyne (Camps et al., 1988) in dry tetrahydrofuran (300 ml) was introduced. After stirring for 1.5



In formulae 8, 13–17a: R= CH_3CH_2O–
9, 13–17b: R= CH_3OCH_2–

SCHEME 2.

hr 1-(1-ethoxyethyloxy)-9-bromononane (51.7 g, 0.175 mol) in dry tetrahydrofuran (300 ml) was added dropwise and stirring was continued for 4 hr. Ammonia was evaporated on standing overnight, and the residue was decomposed with ice-cold water. The mixture was then extracted with ether (4 × 300 ml), and the ethereal extracts were washed with brine and dried over potassium carbonate. Evaporation of the solvent furnished 41.6 g of a red oil, which was dissolved in methanol (1500 ml) and treated with Dowex W50 (H^+ form; 30 g) for 24 hr. The ion exchanger was filtered off and the solvent removed in vacuo. Flash chromatography of the residue yielded 25.9 g (65%) of alkynol **13a.**

Analysis. Calcd. for $C_{14}H_{26}O_2$: C, 74.29; H, 11.58. Found.: C; 74.03, H, 11.41. ^1H NMR: δ 1.23 (t, J = 7.1 Hz, 3H, $C\underline{H}_3CH_2O$—), 1.23–1.43 (m,

12H, $-CH_2-$), 1.53–1.56 (m, 2H, $-C\underline{H}_2CH_2OH$), 2.21 (tt, J = 7.0, 2.2 Hz, 2H, $-C{\equiv}CC\underline{H}_2-$), 3.55 (q, J = 7.1 Hz, 2H, $CH_3C\underline{H}_2O-$), 3.64 (t, J = 6.6 Hz, 2H, $-C\underline{H}_2OH$), 4.12 (t, J = 2.2 Hz, 2H, $-OC\underline{H}_2C{\equiv}C-$).

14-Oxa-10-pentadecyn-1-ol ***(13b)***

Analysis. Calcd. for $C_{14}H_{26}O_2$: C, 74.29; H, 11.58. Found.: C; 74.41, H, 11.73. ^{1}H NMR: δ 1.26–1.68 (m, 14H, $-CH_2-$), 2.18 (tt, J = 2.4, 7.1 Hz, 2H, $-C{\equiv}CC\underline{H}_2-$), 2.47 (tt, J = 2.4, 7.1 Hz, 2H, $-OCH_2C\underline{H}_2C{\equiv}C-$), 3.41 (s, 3H, $C\underline{H}_3O-$), 3.51 (t, J = 7.1 Hz, 2H, $-OC\underline{H}_2CH_2C{\equiv}C-$), 3.68 (t, J = 6.3 Hz, 2H, $-C\underline{H}_2OH$).

(Z)-13-Oxa-10-pentadecen-1-ol ***(14a)***

1,2-Diaminoethane (3.2 g) and **13a** (25.0 g; 0.110 mol) of were added to a suspension of P2-Ni [prepared from 1.98 g of nickel(II) acetate] in ethanol (250 ml) and hydrogenated with stirring at 25°C (Brown and Ahuja, 1973). The hydrogenation was monitored by analyzing aliquots of the solution by GLC. Flash chromatography of the crude product afforded 24.3 g (97%) of (Z)-alkenol **14a.**

Analysis. Calcd. for $C_{14}H_{28}O_2$: C, 73.63; H, 12.36. Found.: C; 73.85, H, 12.29. ^{1}H NMR: δ 1.21 (t, J = 7.1 Hz, 3H, $C\underline{H}_3CH_2O-$), 1.24–1.40 (m, 12H, CH_2), 1.55–1.59 (m, 2H, $-C\underline{H}_2CH_2OH$), 2.06 (bq, J = 6.5 Hz, 2H, $-CH{=}CHC\underline{H}_2-$), 3.49 (q, J = 7.1 Hz, 2H, $CH_3C\underline{H}_2O-$), 3.64 (t, J = 6.6 Hz, 2H, $-C\underline{H}_2OH$), 4.00–4.04 (m, 2H, $-OC\underline{H}_2CH{=}CH-$), 5.51–5.59 (m, 2H, $-C\underline{H}{=}C\underline{H}-$).

(Z)-14-Oxa-10-pentadecen-1-ol ***(14b)***

Analysis. Calcd. for $C_{14}H_{28}O_2$: C, 73.63; H, 12.36. Found.: C; 73.49, H, 12.25. ^{1}H NMR: δ 1.27–1.65 (m, 14H, $-CH_2-$), 2.08 (bq, J = 6.6 Hz, 2H, $-CH{=}CHC\underline{H}_2-$), 2.37 (bq, J = 7.1 Hz, 2H, $-OCH_2C\underline{H}_2CH{=}CH-$), 3.39 (s, 3H, $C\underline{H}_3O-$), 3.42 (t, J = 7.1 Hz, 2H, $-OC\underline{H}_2CH_2CH{=}CH-$), 3.68 (t, J = 6.4 Hz, 2H, $-C\underline{H}_2OH$), 5.40 (dtt, J = 1.3, 6.9, 10.8 Hz, 1H, $-O(CH_2)_2CH{=}C\underline{H}-$), 5.52 (dtt, J = 1.3, 6.9, 10.8 Hz, 1H, $-O(CH_2)_2C\underline{H}{=}CH-$).

(Z)-1-Iodo-13-oxa-10-pentadecene ***(15a)***

This was obtained (Ramiandrasoa and Descoins, 1989) from **14a** mesylate (12.5 g; 0.041 mol) and NaI (12.2 g; 0.082 mol) in dry acetone (100 ml) with 85% (12.6 g) yield.

Analysis. Calcd. for $C_{14}H_{27}IO$: C, 49.71; H, 8.05; I, 37.51. Found.: C, 49.60; H, 8.12; I, 37.63. ^{1}H NMR: δ 1.21 (t, J = 7.1 Hz, 3H, $C\underline{H}_3CH_2O-$),

1.22–1.40 (m, 12H, $-CH_2-$), 1.77–1.83 (m, 2H, $-C\underline{H}_2CH_2I$), 2.04–2.08 (m, J = 6.6 Hz, 2H, $-CH=CHC\underline{H}_2-$), 3.19 (t, J = 6.9 Hz, 2H, $-C\underline{H}_2I$), 3.49 (q, J = 7.1 Hz, 2H, $CH_3C\underline{H}_2O-$), 4.00–4.04 (m, 2H, $-OC\underline{H}_2CH=CH-$), 5.49–5.61 (m, 2H, $-C\underline{H}=C\underline{H}-$).

(Z)-1-Iodo-14-oxa-10-pentadecene ***(15b)***

Analysis. Calcd. for $C_{14}H_{27}IO$: C, 49.71; H, 8.05; I, 37.51. Found.: C, 49.82; H, 8.11; I, 37.39. ^{1}H NMR: δ 1.23–1.42 (m, 12H, $-CH_2-$), 1.79–1.83 (m, 2H, $-C\underline{H}_2CH_2I$), 2.04 (bq, J = 6.4 Hz, 2H, $-CH=CHC\underline{H}_2-$), 2.33 (bq, J = 7.0 Hz, 2H, $-OCH_2C\underline{H}_2CH=CH-$), 3.19 (t, 2H, J = 7.1 Hz, $CH_3OC\underline{H}_2-$), 3.35 (s, 3H, $C\underline{H}_3O-$), 3.38 (t, J = 6.9 Hz, 2H, $-C\underline{H}_2I$), 5.35 [dtt, J = 1.5, 6.9, 10.7 Hz, 1H, $-O(CH_2)_2CH=C\underline{H}-$], 5.50 (dtt, J = 1.5, 6.9, 10.7 Hz, 1H, $-O(CH_2)_2C\underline{H}=CH-$).

(Z)-16-Oxa-13-octadecen-2-yn-1-ol ***(16a)***

Butyllithium (2.5 M solution in hexane; 12.0 ml; 30 mmol) was added to a stirred solution of 1-(2-tetrahydropyranyloxy)-2-propyne (4.21 g, 37.5 mmol) in tetrahydrofuran (100 ml) and HMPA (12 ml) at −20°C under argon (Ramiandrasoa and Descoins, 1989). After 1 hr, iodoalkene **15a** (5.0 g, 14.8 mmol) in HMPA (12 ml) was added dropwise, and the mixture was stirred for an additional 4 hr at room temperature. The solution was then poured into ice-cold water and extracted with light petroleum. After evaporation, the residue was treated as described in the synthesis of **13a.** Yield: 2.79 g (70%) of enynol **16a.**

Analysis. Calcd. for $C_{17}H_{30}O_2$: C, 76.64; H, 11.35. Found.: C, 76.72; H, 11.48. ^{1}H NMR: δ 1.21 (t, J = 7.0 Hz, 3H, $C\underline{H}_3CH_2O-$), 1.24–1.38 (m, 14H, $-CH_2-$), 2.09 (bq, J = 6.6 Hz, 2H, $-OCH_2CH=CHC\underline{H}_2-$), 2.21 (tt, 2H, J = 2.2, 7.0 Hz, $-C\underline{H}_2C\equiv C-$), 3.49 (q, J = 7.0, 2H, $CH_3C\underline{H}_2O-$), 3.98–4.04 (m, 2H, $-OC\underline{H}_2CH=CH-$), 4.25 (t, J = 2.2, 2H, $-C\underline{H}_2OH$), 5.52–5.60 (m, 2H, $-C\underline{H}=C\underline{H}-$).

(Z)-17-Oxa-13-octadecen-2-yn-1-ol ***(16b)***

Analysis. Calcd. for $C_{17}H_{30}O_2$: C, 76.64; H, 11.35. Found.: C, 76.50; H, 11.28. ^{1}H NMR: δ 1.23–1.85 (m, 14H, $-CH_2-$), 2.04 [bq, 2H, $-O(CH_2)_2CH=CHC\underline{H}_2-$], 2.21 (tt, 2H J = 2.2, 7.1 Hz, $-C\underline{H}_2C\equiv CCH_2OH$), 2.33 (bq, J = 6.9 Hz, 2H, $-OCH_2C\underline{H}_2CH=CH-$), 3.35 (s, 3H, $C\underline{H}_3O-$), 3.38 (t, J = 6.9 Hz, 2H, $CH_3OC\underline{H}_2-$), 4.19 (dt, J = 2.2, 15.1, 1H, $-C\equiv CC\underline{H}_2OH$), 4.30 (dt, J = 2.2, 15.1 Hz, 1H, $-C\equiv CC\underline{H}_2OH$), 5.36 [dtt, J = 1.5, 7.1, 10.6 Hz, 1H, $-O(CH_2)_2CH=C\underline{H}-$], 5.47 [dtt, J = 1.5, 7.1, 10.6 Hz, 1H, $-O(CH_2)_2C\underline{H}=CH-$].

(E,Z)-16-Oxa-2,13-octadecadien-1-yl acetate ***(8)***

Lithium alanate (1.21 g, 32 mmol) was added at −20°C under argon to a solution of 1.7 g (6.4 mmol) of alcohol **16a** in dry tetrahydrofuran (75 ml) (Attenburow et al., 1952). After heating for 10 hr at 60°C, the mixture was decomposed with cold water (50 ml) and 10% sulfuric acid (100 ml) and extracted with ether (3 × 100 ml). The ethereal extracts were washed with brine and dried ($MgSO_4$). Removal of the solvent in vacuo and purification of the residue by chromatography afforded 1.5 g (89%) of the (*E,Z*)-dienol **17a.** The isomeric purity of the product was better than 97%. Subsequent argentation chromatography (Voerman and Rothschild, 1978) and acetylation of **17a** gave **8** in 95% yield and 99% isomeric purity.

Analysis. Calcd. for $C_{19}H_{34}O_3$: C, 73.50; H, 11.04. Found.: C, 73.62; H, 11.11. ^{1}H NMR: δ 1.21 (t, J = 7.1 Hz, 3H, $C\underline{H}_3CH_2O-$), 1.23–1.41 (m, 14H, $-CH_2-$), 2.06 (s, 3H, $-OCOC\underline{H}_3$), 2.02–2.08 (m, 2H, $-OCH_2CH=CHC\underline{H}_2-$), 2.02–2.08 (m, 2H, $-C\underline{H}_2CH=CH_2OAc$), 3.49 (q, 2H, $CH_3C\underline{H}_2O-$), 3.99–4.05 (m, 2H, $-OC\underline{H}_2CH=CH-$), 4.51 (dq, J = 1.1, 5.4 Hz, 2H, $-C\underline{H}_2OAc$), 5.51–5.59 (m, 2H, $-OCH_2C\underline{H}=C\underline{H}-$), 5.55 (dtt, J = 1.0, 5.5, 15.4 Hz, 1H, $-CH=C\underline{H}-CH_2OAc$), 5.78 (dtt, J = 1.0, 6.5, 15.4 Hz, 1H, $-C\underline{H}=CH-CH_2OAc$).

(E,Z)-17-Oxa-2,13-octadecadien-1-yl acetate ***(9)***

Analysis. Calcd. for $C_{19}H_{34}O_3$: C, 73.50; H, 11.04. Found.: C, 73.31; H, 10.93. ^{1}H NMR: δ 1.23–1.41 (m, 14H, $-CH_2-$), 2.06 (s, 3H, $-OCOC\underline{H}_3$), 2.02–2.08 [m, 2H, $-O(CH_2)_2CH=CHC\underline{H}_2-$], 2.02–2.08 (m, 2H, $-C\underline{H}_2CH=CHCH_2OAc$), 2.33 (bq, J = 6.5 Hz, 2H, $-OCH_2C\underline{H}_2CH=CH-$), 3.35 (s, 3H, $C\underline{H}_3O-$), 3.38 (t, 2H, $CH_3OC\underline{H}_2-$), 4.50 (dq, J = 5.3, 15.4 Hz, 2H, $-CH=CHC\underline{H}_2OAc$), 5.42–5.50 [m, 2H, $-O(CH_2)_2C\underline{H}=C\underline{H}-$], 5.56 (dtt, J = 1.0, 5.5, 15.4 Hz, 1H, $-CH=C\underline{H}CH_2OAc$), 5.78 (dtt, J = 1.0, 6.5, 15.4 Hz, 1H, $-C\underline{H}=CHCH_2OAc$).

Estimation of Vapor Pressures

Principles. The relevant equations for determining vapor pressures by the GC method (P_{GC}) have been developed by Hamilton (1980). At a constant temperature, the vapor pressures of a test and of a reference compound (subscript t and r, respectively) are related by the ratio of their latent heats of vaporization:

$$\ln P_t = (H_t/H_r) \ln P_r + C \quad (1)$$

where H is the latent heat of vaporization and C is a constant. A similar equation has been developed for the GC retention times t:

$$\ln (t_t/t_r) = (1 - H_t/H_r) \ln P_r - C \quad (2)$$

Therefore, a plot of ln (t_t/t_r) versus ln P_r should have a slope $(1 - H_t/H_r)$ and an intercept $(-C)$. Equation 1 can then be used to determine the vapor pressure of the test compound at any temperature if the vapor pressure of the reference compound at that temperature is known.

Experimental Procedure. Samples were analyzed on a Hewlett Packard HP 5880 chromatograph equipped with a FID detector on a 2-m fused silica capillary column (cross-linked 5% methyl silicone, HP-1), 0.52 μm film thickness; splitless injection. The chromatography was operated isothermally at 10°C intervals from 110°C to 170°C with hydrogen flow 10 ml/min; C_{18} hydrocarbon was used as a reference compound. Vapor pressures of *n*-octadecane at different temperatures were calculated from the equation ln P(torr) $= A + B/T$ with parameters $A = 25.548$, and $B = -10165$ (Macknick and Prausnitz, 1979; Bidleman, 1984). Retention times were determined on an HP 3396A integrator. All runs were made at least in duplicate and average retention times were used for calculations. As recommended (Bidleman, 1984), long retention times of compounds producing unsymmetrical peaks at low temperatures were not taken at the peak maximum, but were estimated at the midpoint between the beginning and the end of the peak.

RESULTS AND DISCUSSION

Vapor Pressures of Compounds ***1–12.*** Since vapor pressures determined by using equations 1 and 2 (P_{GC}) may under- or overestimate the actual vapor pressures (P_L) depending on vapor pressure range and the GC column used (Bidleman, 1984), the P_{GC} estimates have to be generally calibrated to provide reasonable vapor pressure values. Accordingly, the previously developed (Koutek et al., 1992) calibration equation 3 was used to correct the P_{GC} values of all derivatives investigated.

$$\ln P_L(\text{Pa}) = 1.0126 \ln P_{GC} + 0.4440. \quad (3)$$

If the compound is solid (as is the carbamate **12,** with mp of 27°C) another problem arises, since its vapor pressure P_s will be lower than that of the subcooled liquid P_L by the factor of the fugacity ratio P_S/P_L. This has been previously shown (Mackay et al., 1982) to be expressible by

$$\ln (P_S/P_L) = -6.8 (T_M/298 - 1), \quad (4)$$

where T_M is the absolute melting point, and 6.8 is an empirical constant related to the entropy of fusion.

Corrected vapor pressures (P) based on equations 3 and 4 are summarized in Table 1 together with P_{REL} factors that were used to correct the EAG data.

TABLE 1. PARAMETERS OF EQUATION 2 AND VAPOR PRESSURES (25°C) OF COMPOUNDS **1–12**

Compound	H_i/H_r	C	$P^{(25)} \times 10^3$ (Pa) Eq. 1	$P^{(25)} \times 10^3$ (Pa) Eq. 3	P_{REL}[a]
1	1.1600	2.7241	0.930	1.328	1
2	1.1365	2.5654	1.188	1.701	1.281
3	1.1785	2.8208	0.789	1.124	0.846
4	1.1457	2.6409	1.065	1.523	1.147
5	1.2269	3.2673	0.423	0.598	0.450
6	1.2357	3.3202	0.388	0.548	0.413
7	1.2357	3.4286	0.353	0.498	0.375
8	1.2006	2.9845	0.618	0.878	0.661
9	1.1875	2.9191	0.692	0.984	0.741
10	1.1360	1.7715	2.633	3.808	2.867
11	1.2682	4.1164	0.155	0.216	0.163
12	1.3708	5.2620	0.034	0.044[b]	0.033

[a] $P_{REL} = P_{SUBSTANCE}/P_{E2,Z13\text{-}18:Ac}$.
[b] Corrected by using equation 4.

The results impressively demonstrate the importance of vapor pressure corrections in comparing the EAG data for compounds of different volatilities. Note that at 25°C the relative liquid–vapor concentration ratio for the pheromone component **1,** trifluoroacetate **10,** and carbamate **12** follows the order 1:1, 1:2.88, and 1:0.033, respectively. It means that the trifluoroacetate **10** is about 3 times more volatile while carbamate **12** about 30 times less volatile than the pheromone component **1.**

Electrophysiological Properties. Relative EAG activities of compounds **1–12** corresponding to 75% of the relative activity scale are summarized in Figure 1 together with the vapor pressure-corrected data. As expected, the sensitivity of the EAG data to vapor pressure corrections differs significantly with the type of compound. The largest differences are observed in the ester series **1, 10–12,** where the vapor pressure corrections may even cause changes in the activity order.

The following intermolecular forces in substrate–receptor interactions have been generally recognized: electrostatic attraction between charges and partial charges, steric repulsion, hydrophobic bonding, hydrogen bonding, and van der Waals attractions (dispersion, orientation, dipole–dipole, etc.) (Hansch and Leo, 1979). In addition, intramolecular forces in the substrate that affect its conformation may be important, depending on the conformational requirements of the receptor site (Hopfinger, 1980; Liljefors et al., 1985). In this context, the series

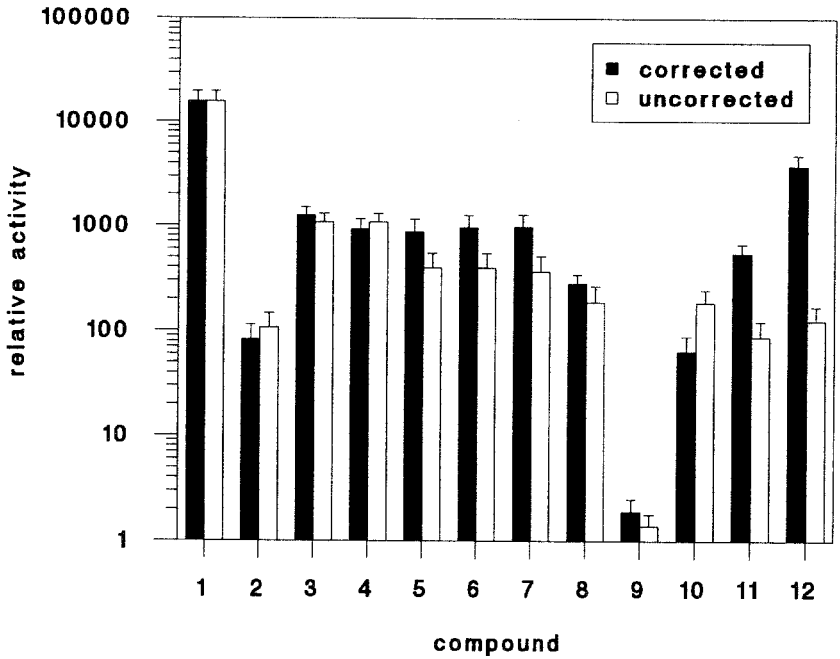


FIG. 1. Experimental EAG activities (+ SEM) for compounds **1–12.** A comparison of uncorrected and vapor pressure-corrected data. The activities are expressed as the reciprocal of the relative number of moles required to elicit the same receptor response.

of analogs **2–12** may be separated into two distinct types of compounds: (1) those where both the acetate and the terminal alkyl functions are constantly present and, as a consequence, conformational requirements may be presumed to be the main factor influencing biological activities (i.e., derivatives **1–7**); and (2) those containing an unchanged 2*E*,13*Z* double bond system, the structural differences taking place on the ester and terminal alkyl functions. Since the conformational properties of these compounds are virtually identical, other factors should be operative to rationalize the observed activity differences.

Inspection of Figure 1 shows that all analogs display a reduced activity compared to the natural pheromone component **1.** The EAG activity of geometrical isomers decreases in the relative ratio of about 188:15:10:1 for the *EZ*(**1**), *ZZ*(**3**), *ZE*(**4**) and *EE*(**2**) isomers, respectively. Thus, replacement of the 13*Z* double bond by its *E* equivalent in the parent acetate **1** seems to cause more drastic decrease in activity than does the *E* ⇒ *Z* change in position 2. Interestingly, some degree of similarity exists between the foregoing results and the EAG data of Tonini et al. (1986) on the leopard moth, *Zeuzera pyrina* L. (Lepidoptera: Cossidae). The *S. tipuliformis* and *Z. pyrina* species not only use

the *E,Z*-acetate **1** as a main pheromone component, but, of the analogs **2–4,** both species also elicit the largest response to the *Z,Z* isomer **3.**

The replacement of a $-CH_2-$ unit by an oxygen atom in the C_{16} and C_{17} positions resulted in a marked decrease of the biological activity. While the activity of the 17-oxa isomer **9** is the lowest of all the analogs investigated, introduction of oxygen into C_{16} position in derivative **8,** is far better tolerated. A linear relationship was observed (Figure 2) between logarithms of the relative activities and Hansch hydrophobic constants π for the terminal alkyl molecular parts $CH_3CH_2CH_2-$(1.55), CH_3CH_2O-(0.38) and CH_3OCH_2- (−0.78). The correlation equation corresponding to the linear least-squares fit is ln (*EAG*) = $3.832\pi + 3.868$ ($n = 3$, $r = 0.998$, SE = 0.413). These results seem to be in line with recent electrosensillography (ESG) findings on the turnip moth analogs (Jönsson et al., 1991b) that substituent size and hydrophobicity of the

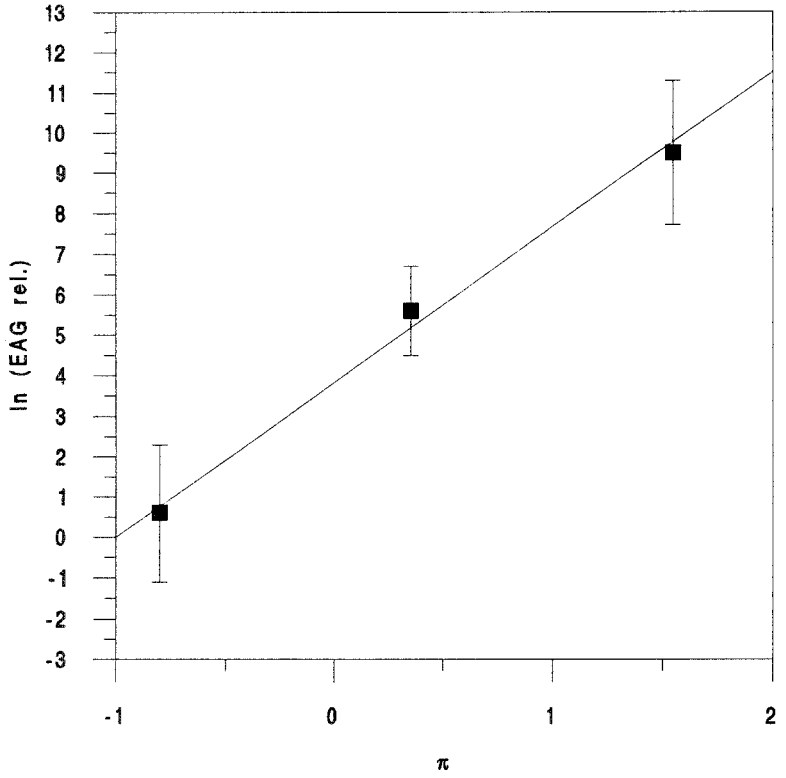


FIG. 2. Relationship between relative EAG activities of the compounds **1, 8,** and **9** and hydrophobic parameters for the terminal substituents $CH_3CH_2CH_2-$, CH_3CH_2O-, and CH_3OCH_2-, respectively. The uncertainty is given as 95% confidence interval.

terminal alkyl group are the most important factors influencing the activity. Because of the suggested bioisostericity of $CH_2 \Rightarrow O$ replacements, the activity of compounds **8** and **9** should be preferentially directed by hydrophobicity alone.

The last group of analogs included the trifluoroacetate **10,** pivalate **11,** and *N*-methyl carbamate **12,** which differ from the acetate **1** only in the R substituent of the ester moiety $-CH_2-O-CO-R$. The EAG activity of these compounds decreases in the order $CH_3 > NHCH_3 > C(CH_3)_3 > CF_3$. Trifluoroacetate **10** showed only less than 1% of the activity elicited by the parent acetate **1.** No clear trend in activity is observed when single parameters describing steric demand (E_s, STERIMOL parameters), liphophilicity, or electronic (F, R) effects are used as substituent descriptors for the ester series. Evidently, one factor is not sufficient to reproduce the EAG data. To rationalize the experimental data, polar effect and size of the substituent R have to be combined. Thus, F (Hansch and Leo, 1979) and L (Verloop et al., 1976) parameters representing the inductive effect and length of the substituents were simultaneously used to express the structural effects numerically. The variation of activity of ester analogs **1, 10–12** is then given by equation $\ln (EAG) = -10.301F - 3.287L + 18.954$ ($N = 4$, $R^2 = 98.22\%$, SE = 0.260). This equation predicts that a substituent with a smaller electron-withdrawing effect and smaller steric demand should have a greater EAG activity. Using this equation, relative EAG activities of 13500, 350, and 65, respectively, are predicted for the acetate **1,** (*E*,*Z*)-2,13-octadecadien-1-yl propanoate and trifluoroacetate **10.** It is interesting to note that the same hierarchical order has been found (Liljefors et al., 1984) for ESG activity of (*Z*)-7-dodecenyl acetate and its propanoate and trifluoroacetate analogs on the turnip moth *Agrotis segetum.* Moreover, in both cases the differences (dE) also follow the same trend, i.e. dE(acetate − propanoate) >> dE(propanoate − trifluoroacetate). Even though a more detailed discussion of these results is precluded owing to the incompatibility of data presentation as well as limited number of points, it seems that an activity order acetate > propanoate > trifluoroacetate could be generally valid for a variety of acetate-responding species (cf. also behavioral studies of Camps et al., 1988 on the processionary moth *Thaumetopoea pityocampa*).

Among the ester analogs investigated, the carbamate **12** deserves a special comment. This derivative belongs to the group of carbamones, as the *N*-methyl carbamate based pheromone analogs have been called. One such carbamone, *Z*9–14:Nmc (*N*-methyl carbamate) was reported as a chemically reactive pheromone analog capable of sensory disruption of the *Heliothis virescens* males (Albans et al., 1984). In contrast, however, no disruption of the perception of *Z*11–16:Ac in *Mamestra brassicae* by the carbamone *Z*11–16:Nmc has been found, in spite of the fact that this carbamate analog elicited an EAG response only slightly less than the natural pheromone component *Z*11–16:Ac (Prestwich, 1987). In view of these findings, the relatively high activity of **12** suggests (but does not prove) a biological activity in the behavioral experiments.

To conclude, we have shown that the CCM males possess receptors capable of responding to analogs of the acetate **1.** The results on analogs only changed in the terminal alkyl group indicate that hydrophobicity plays an important role in the structure–activity relationships. On the other hand, when the analogs are modified on the ester group, the EAG activity order suggests that inductive and steric effects are of primary importance. Single-cell studies will be necessary to provide information whether the analogs interact with the same receptor as **1.** Although the use of a more complete basis set would be needed to supply a more correct representation of the structure–activity relationship, the present data qualitatively agree with the generally accepted view for monoenic acetates as it has been derived from the single-cell measurements.

Significant differences between the vapor pressure-corrected and uncorrected EAG activity data for some of the analogs investigated show that we should be cautious about the validity of conclusions formerly drawn from structure–activity studies without considering different volatilities of the compounds.

REFERENCES

ALBANS, K.R., BAKER, R., JONES, O.T., and TURNBULL, M.D. 1984. Inhibition of response of *Heliothis virescens* to its natural pheromone by antipheromones. *Crop. Prot.* 3:501–506.

ARN, H., TÓTH, M., and PRIESNER, E. 1986. List of Sex Pheromones of Lepidoptera and Related Attractants, OILB-SROP, Paris, 123 pp.

ATTENBURROW, J., CAMERON, A.F.B., CHAPMAN, J.H., EVANS, R.M., HEMS, B.A., JANSEN, A.B.A., and WALKER, T. 1952. A synthesis of vitamin A from cyclohexanone. *J. Chem. Soc.* 1952:1094–1111.

BENGTSSON, M., LILJEFORS, T., and HANSSON, B.S. 1987. Dienic analogs 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.

BESTMANN, H.J., CAI-HONG, W., DÖHLA, B., LI-KEDONG, and KAISSLING, K.E. 1987. Functional group recognition of pheromone molecules by sensory cells of *Antheraea polyphemus* and *Antheraea pernyi* (Lepidoptera: Saturnidae). *Z. Naturforsch.* 42c:435–441.

BIDLEMAN, T.F. 1984. Estimation of vapor pressures for nonpolar organic compounds by capillary gas chromatography. *Anal. Chem.* 56:2490–2496.

BROWN, C.A., and AHUJA, K.K. 1973. "P2-nickel" catalyst with ethylendiamine, a novel system for highly stereospecific reduction of alkynes to *cis*-olefins. *J. Chem. Soc. Chem. Commun.* 1973:553–561.

CAMPS, F., FABRI, S.G., GASOL, V., GUERRERO, A., HERNÁNDEZ, R., and MONTOYA, R. 1988. Analogs of sex pheromone of processionary moth, *Thaumetopoea pityocampa*: Synthesis and biological activity. *J. Chem. Ecol.* 14:1331–1346.

EVERSHED, R.P. 1988. Insect olfaction and molecular structure, pp. 1–33, *in* E.D. Morgan and N.B. Mandava (eds.). CRC Handbook of Natural Pesticides. Vol IV., Pheromones, Part A. CRC Press, Boca Raton, Florida.

HAMILTON, D.J. 1980. Gas chromatographics measurement of volatility of herbicide esters. *J. Chromatogr.* 195:75–83.

HANSCH, C., and LEO, A. 1979. Substituents Constants for Correlation Analysis in Chemistry and Biology. John Wiley & Sons, New York.

HOPFINGER, A.J. 1980. A QSAR investigation of dihydrofolate reductase inhibition by Baker triazines based on molecular shape analysis. *J. Am. Chem. Soc.* 102:7196–7206.

HOSKOVEC, M., ŠAMAN, D., and KOUTEK, B. 1990. A convenient synthesis of 2,13- and 3,13-octadecadienyl acetates, sex pheromone components of the *Synanthedon* species. *Collect. Czech. Chem. Commun.* 55:2270–2281.

JÖNSSON, S., LILJEFORS, T., and HANSSON, B.S. 1991a. Alkyl substitution in terminal chain of (*Z*)-5-decenyl acetate, a pheromone component of turnip moth, *Agrotis segetum*. Synthesis, single-sensillum recordings, and structure-activity relationships. *J. Chem. Ecol.* 17:103–122.

JÖNSSON, S., LILJEFORS, T., and HANSSON, B.S. 1991b. 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. *J. Chem. Ecol.* 17:1381–1397.

KOUTEK, B., HOSKOVEC, M., KONEČNÝ, K., and VRKOČ, J., 1992. Gas chromatographic determination of vapor pressures of pheromone-like acetates. *J. Chromatogr.* 626:215–221.

LILJEFORS, T., THELIN, B., and VAN DER PERS, J.N.C. 1984. Structure-activity relationships between stimulus molecule and response of a pheromone receptor cell in the turnip moth, *Agrotis segetum*. Modifications of the acetate group. *J. Chem. Ecol.* 10:1661–1675.

LILJEFORS, T., THELIN, B., VAN DER PERS, J.N.C., and LÖSSTED, C. 1985. 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.

MACKAY, D., BOBRA, A., CHAN, D.W., and SHIU, W.Y. 1982. Vapor pressure correlations for low volatility environmental chemicals. *Environ. Sci. Technol.* 16:645–649.

MACKNICK, A.B., and PRAUSNITZ, J.M. 1979. Vapor pressures of high-molecular-weight hydrocarbons. *J. Chem. Eng. Data* 24:175–178.

PRESTWICH, G.D. 1987. Chemical studies of pheromone reception and catabolism, pp. 473–528, *in* G.D. Prestwich and G.J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, Orlando, Florida.

RAMIANDRASOA, F., and DESCOINS, C. 1989. A new highly stereospecific synthesis of (*E*,*Z*)-2,13-octadecadienyl acetate, a sex pheromone component of Lepidoptera species. *Synth. Commun.* 19:2703–2712.

SHARMA, M.L., THAPAR, S., and GUPTA, R. 1990. Synthesis of (2*E*,13*Z*)-2,13-octadecadien-1-yl acetate. *Ind. J. Chem.* 29b:657–658.

SOROCHINSKAYA, A.M., and KOVALEV, B.G. 1991. Synthesis of *trans*-2, *cis*-13 and *trans*-3, *cis*-13-octadecadien-1-yl acetates, pheromone component of the *Synanthedon tipuliformis* (Lepidoptera: Sesiidae). (in Russian) *Zh. Org. Khim.* 27:722–727.

SZÖCS, G., MILLER, L.A., THOMAS, W., VICKERS, R.A., ROTSCHILD, G.H.L., SCHWARZ, M., and TÓTH, M. 1990. Compounds modifying male responsiveness to main female sex currant borer, *Synanthedon tipuliformis* Clerk (Lepidoptera: Sesiidae) under field conditions. *J. Chem. Ecol.* 16:1289–1305.

SZÖCS, G., BUDA, V., CHARMILLOT, P., ESBJERG, P., FREIER, B., GOTTWALD, R., KOVALEV, B., MAINI, S., SOLOMON, M.G., SORUM, O., SUBCHEV, M., TÓTH, M., and VAN DER VEIRE, M. 1991. Field tests of (*E*,*Z*)-3,13-octadecadien-1-ol acetate: A sex attractant synergist for male currant borer, *Synanthedon tipuliformis*. *Entomol. Exp. Appl.* 60:283–288.

TONINI, C., CASSANI, G., MASSARDO, P., GUGLIELMETTI, G., and CASTELLARI, P.L. 1986. Study of female sex pheromone of leopard moth, *Zeuzera pyrina* L. Isolation and identification of three component. *J. Chem. Ecol.* 12:1545–1558.

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. Ariens (ed.). Drug Design, Vol. VII. Academic Press, New York.

VOERMAN, S., and ROTHSCHILD, G.H.L. 1978. Synthesis of the two components of the sex pheromone system of the potato tubeworm moth, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) and field experience with them. *J. Chem. Ecol.* 4:531–542.

VOERMAN, S., PERSOONS, C.J., and PRIESNER, E. 1984. Sex attractant for currant clearwing moth *Synanthedon tipuliformis* Clerk (Lepidoptera, Sesiidae). *J. Chem. Ecol.* 10:1371–1375.

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SEX PHEROMONE CATABOLISM IN THE REDBANDED LEAFROLLER MOTH

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Abstract—Tritium-labeled components of the red-banded leaf-roller female sex pheromone, (*Z*)- and (*E*)-[11,12-$^3H_2$]-11-tetradecenyl acetate (57 Ci/mmol), applied to antennae of males and females were degraded causing formation of tritiated 11-tetradecenol, 11-tetradecenoic acid, and water. Results indicate that the catabolic pathway involves acetate hydrolysis, oxidation of alcohol to fatty acid, and degradation of the acid via β-oxidation. Both geometric isomers were degraded equally well by males but degradation proceeded comparatively less rapidly with female antennae. It is surmised that under natural conditions of olfactory sensing, sex pheromone impinging upon the moth's antennae is probably subject to a similar catabolic fate.

Key Words—Lepidoptera, Tortricidae, *Argyrotaenia velutinana*, tritiated pheromone, 11-tetradecenyl acetate, sensory biochemistry, catabolism.

INTRODUCTION

In nature, sex pheromones mediate important behavioral aspects of insect reproduction. However, little is known about biochemical events that underpin olfactory detection of these biologically important compounds. In modern hypotheses of moth olfactory sensory transduction, it has been proposed that the hydrophobic pheromone enters olfactory sensilla on the antennae through pores in the cuticular wall of the sensilla and that the pheromone is solubilized in the hydrophic lumen of the sensilla by becoming bound with pheromone-binding protein (Vogt et al., 1991). The protein-bound pheromone is then presumably

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transported across the sensillar lumen and pheromone is transferred to a membrane-bound pheromone receptor protein (Vogt et al., 1988) on sensory neurons within the sensilla. The binding of the pheromone with the receptor causes generation of an electrophysiological response in the sensory cells, which make labeled-line input into olfactory glomeruli of the moth central nervous system (Hansson et al., 1992). Transduction of pheromone binding to receptors into an action potential may involve second-messenger molecules such as guanine nucleotide binding regulatory proteins according to evidence gathered by Raming and Breer (1990). As a final step in the olfactory chemoreception, receptor-bound pheromone presumably dissociates from the receptor and is catabolized, leaving the receptor ready for fresh incoming pheromone signal.

Pheromone catabolism has been documented in several species of moths (Kasang, 1971; Kasang et al., 1974, 1989; Ferkovich et al., 1972; Ferkovich, 1982; Prestwich 1987; Prestwich et al., 1989, 1990; Tasayco and Prestwich, 1990; Klun et al., 1991, 1992). In many of these studies, catabolic activity was often shown to be highest in or specifically associated with the antenna or sensillar preparations of antennae. Lesser levels of catabolic activity were often associated with nonolfactory tissue such as legs and wings. Activity was often higher in males (the pheromone receivers) than in females (the senders). These observations represent a bank of circumstantial evidence that points to the linkage of catabolism and pheromone perception. Studies of the European corn borer (*Ostrinia nubilalis*) have also provided empirical evidence that catabolic reactions occurring on their antennae and the olfactory chemosensory pathway are linked. Using a combination of chemical and behavioral assays, Klun et al. (1991) showed that when the antennae of European corn borer (ECB) males were treated with microgram doses of pheromone analogs, their ability to respond to female sex pheromone was inhibited, but once the compound was cleared from their antennae by catabolism, response capability to pheromone was concomitantly restored. This is evidence that catabolism plays a postchemoreceptive role by clearing antennal structures of resident pheromone. Exactly where catabolism takes place and how catabolism may be linked to pheromone sensing remains to be determined, but the first question we ask is: what is the overall catabolic fate of pheromone on the insect antennae?

Study of the catabolic fate of pheromone on the moth antennae at doses equivalent to those used naturally in pheromone sensing is technically impossible because of the vanishingly small amount of compound involved. As example, in tests with the ECB it was estimated that a pair of male antennae adsorbed about 80-100 fg(10^{-17} mol) during a 3-min exposure to a physiologically relevant dose of pheromone (Klun et al., 1992). Our approach to studying the fate of pheromone in association with moth antennae has been to use tritiated pheromone of high specific activity, which permits investigation of pheromone processing using 10^{-12} mol/insect. Although this amount exceeds a physiolog-

ically relevant pheromone dose by at least 10^3–10^5 times, it approaches the smallest quantity of compound one can use and remain within tritium radiodetection limits. In earlier work, Klun et al. (1992) investigated catabolism of pheromone on antennae of the ECB using isomers of tritiated 11-tetradecenyl acetate (11–14:OAc). Inasmuch as the female sex pheromone of the redbanded leafroller, *Argyrotaenia velutinana*, was also known to be comprised of the same compounds (Bjostad et al., 1985), we wished to determine, if the redbanded leafroller (RBLR) catabolized pheromone differently from the ECB. We report results that show the RBLR and ECB degrade 11–14:OAc by similar catabolic pathways and the two species differ from one another with regard to the size of the intermediary catabolic pool of one of the catabolites, 11-tetradecenoic acid. In both species, females degraded pheromone less effectively than the males.

METHODS AND MATERIALS

Insects. RBLR pupae were supplied to us by Dr. W.L. Roelofs, Cornell Experiment Station, Geneva, New York. Pupae were caged separately by sex and placed in a reverse photoperiod environmental chamber (80% relative humidity; 16 hr light–8 hr dark, 26°C–20°C) to await adult emergence. All moths were 48-96 hr old when they were taken out of scotophase and used in the experiments.

Radiolabeled Compounds, Chromatography, and Tritium Detection. Materials and methods used here were the same as those reported by Klun et al. (1992). (*Z*)-[11,12-$^3H_2$]-11–14:OAc was prepared by semitritiation of 11-tetradecynyl acetate and the *E* isomer was obtained by isomerization of the *Z* isomer. Geometrically pure isomers were obtained by argentation chromatography, and the isomers each had specific activities of 57 Ci/mmol according to nuclear magnetic resonance studies. Corresponding tritiated alcohols (11–14:OH) and fatty acids (11–14:oic) were prepared from the acetates. The derivatives were used in metabolic studies and/or as chromatographic references. All radioactive compounds used in catabolism studies were repurified at least every four to eight weeks. High-pressure liquid chromatographic (HPLC) analyses or preparative purifications were conducted using a Beckman System Gold (Beckman Instruments, Fullerton, California 92634) chromatographic system equipped with a Beckman model 171 radiodetector fitted with either a solid scintillator or liquid scintillation cell. Tritiated water and toluene standards were purchased from NEN, DuPont Company, Boston, Massachusetts 02118.

Most analyses of the extracts of insect tissues for tritiated pheromone and its catabolites were performed using a 25 cm × 3 mm (ID) column packed with 5 μm Spherisorb ODS (octadecylsilane coated silica), 1 ml/min acetonitrile-water (85:15) pumped as eluant, and using the liquid scintillation detector cell.

A solid scintillator cell was used when compounds were purified by using a 43.5 cm × 3 mm 5 μm Spherisorb silica column eluted with an appropriate mixture of hexane-ethyl acetate. Normal liquid scintillation (LS) counting was done by using a Beckman model LS 6000 counter and 20 ml Ecoscint LS cocktail (National Diagnostics, Manville, New Jersey 08835). Detection efficiency was 23% for tritiated water and 39% for tritiated organic compounds. Counting efficiency was verified by using external-standard tritiated NEN toluene or water calibration compounds.

Gas chromatography was conducted using a Hewlett Packard model 5880A gas chromatograph that was vented to a chemical fume hood. The instrument was fitted with a splitless injector, flame ionization detector (FID), and either a polar 60-m × 0.25-mm DB-WAX column (J & W Scientific, Folsom, California 95630) or a nonpolar 50-m × 0.32-mm Ultra 1 (cross-linked methyl silicone gum, Hewlett-Packard Co., Palo Alto, California 94304). Water from the FID was collected in chilled Pasteur pipets at 1-min intervals throughout chromatographic runs and assayed for tritium by LS. Tests in which picogram amounts of tritiated pheromone components were injected onto the columns showed that ca. 30% of the radioactivity injected was recovered in the FID water coincident with the retention time of the compound on the column. This methodology permitted us to analyze antennal extracts for tritiated pheromone and its metabolites by high-resolution capillary gas chromatography (GC). In advance of such GC analyses, retention-time reference points were established by injecting unlabeled standard mixtures of C_7–C_{14} saturated fatty acid methyl esters, 11-14:OAc, 11-14:OH, 11-tetradecenal (11-14:Ald) and methyl-11-tetradecenoate (Me-11-14:oate) isomers onto the capillary columns.

[11,12-$^3H_2$]-11-14:OAc Catabolism. In the time-course studies of pheromone catabolism in the RBLR, each antenna of a CO_2-anesthetized moth was treated with 0.5 μl heptane containing either tritiated *Z*- or *E*11-14:OAc using a micrometer-driven 10-μl syringe under a dissection microscope. Heptane was used as solvent because, when applied to moth antennae, it does not impair subsequent male behavioral sensitivity to sex pheromone (Klun et al., 1991). By inference, the solvent has no detrimental affect upon chemosensory and associated functions of the antennae. Following the topical treatment, the antennae were either immediately excised (T_0) into 50 μl chloroform-methanol (2:1, v/v) or the moth was enclosed in a plastic jelly cup and permitted to incubate for 3 min (T_3) or 6 min (T_6) before its antennae were excised into solvent. The excised antennae were soaked in the chloroform-methanol for 30 min and the extract was analyzed by ODS HPLC. Using these methods, we monitored degradation of pheromone and followed changes in the proportional distribution of tritium between pheromone component and its metabolites in individual moths as a function of time. Studies were replicated 12 times using each isomer of 11-14:OAc; six males were each treated with 213 × 10^3 dpm (476 pg) *E*

isomer and six with 113 × 10^3 dpm (252 pg) and two sets of six males each were treated with 134 × 10^3 dpm (299 pg) *Z* isomer and 123 × 10^3 dpm (275 pg), respectively. Analyses showed that the different doses of tritiated pheromone had no significant treatment effect; therefore, mean proportions of tritium in 11–14 : OAc and tritiated metabolites were calculated by pooling data obtained with each of the 12 insects treated with each isomer and means were plotted as a function of incubation time. Arbitrarily, best-fit linear regression lines for the data points were computed and drawn by using a personal computer with Cricket Graph software for all catabolic data. (Figures 1–3 below). In time course study of catabolism by RBLR females, 134 × 10^3 dpm (299 pg) *Z* isomer was applied to the antennae of each of six females. Materials and methods were identical to those used with males.

RBLR Hydrolysis of a Microgram Dose of Cold Z11–14 : OAc. The antennae of 10 RBLR males were each treated with 1 μg Z11–14 : OAc in 1 μl heptane, allowed to incubate for 12 min, excised into 50 μl $CHCl_3$ containing 100 ng pentadecane as internal standard, and soaked for 30 min. The extracts were then analyzed quantitatively for 11–14 : OAc and 11–14 : OH by GC using the Ultra 1 capillary column. The purpose of the test was to compare the effect of dose upon the rate of acetate hydrolysis.

[11,12-$^3H_2$]-11–14 : OH Catabolism. The *E*- or Z11–14 : OH was applied to antennae of the RBLR males using the same techniques and HPLC analytical methods as were used with the acetates. Males each were treated with 128.4 × 10^3 dpm *E* isomer (287 pg) or with 126.5 × 10^3 dpm *Z* isomer (258 pg). The tests were replicated six times using each isomer with 0-, 3-, and 6-min incubation periods.

Analyses for Trace Catabolites via GC. The antennae of 30 male RBLR were each treated with 123.5 × 10^3 dpm *Z* isomer. After a 6-min incubation, antennae were excised into 100 μl chloroform. The extract was filtered through a plug of glass wool in Pasteur pipet, 2 μl was analyzed on the ODS HPLC to determine tritium recovery and composition of the sample, and the remainder was reacted with an excess of diazomethane in diethyl ether to convert putative fatty acids in the extracts to methyl esters. A 2-μl aliquot of the methylated sample was reanalyzed on ODS, the remainder was concentrated in a nitrogen gas flow to 10 μl and chromatographed on the DB-WAX and Ultra 1 capillary columns as described earlier to determine the retention times of radiolabeled catabolites in the extracts on the two GC liquid phases. This experiment was replicated twice.

RESULTS AND DISCUSSION

The time-course catabolism studies using males with tritiated *E*- or Z11–14 : OAc each showed formation of three radiolabeled catabolites. In ODS HPLC analyses, the retention volume of one catabolite was coincident with tritiated

water standard and it eluted at void volume. The second had retention volume coincident with 11–14:OH and 11–14:Ald standards. Chromatography of a sample of the extracts on a Spherisorb silica HPLC column showed that the catabolite was 11–14:OH. The third eluted from the ODS column before the alcohol and its retention volume was identical to radiolabeled standard of 11–14:oic. ODS HPLC did not permit verification of the geometry of the catabolites of *E*- and *Z*11–14:OAc, but gas chromatographic analyses on two liquid phases confirmed the identity of the *Z* alcohol and the *Z* acid formed from *Z*11–14:OAc, and it was assumed that the catabolites of *E*11–14:OAc had the corresponding geometry.

In a search for other possible intermediary catabolites of pheromone in the combined extracts of 30 male antennae treated with tritiated *Z*11–14:OAc, ODS HPLC showed that 82% of the tritium in the extracts was in the form of 11–14:oic and the remaining 18% was distributed as: 11–14:OH > 11–14:OAc > water. After the extract was treated with diazomethane, no free 11–14:oic was detected in ODS HPLC and a new chromatographic peak coincident with the retention volume of me-11–14:oate was observed. Replicated GC analyses of the derivatized extracts on the polar and nonpolar capillary columns consistently showed elution of high levels of tritium at the retention time coincident with me-*Z*11–14:oate along with traces of activity associated with the retention times of *Z*11–14:OH, and *Z*11–14:OAc. No other radioactivity above background was detected in GC. The retention time characteristics of the diazomethane-treated radiolabeled catabolites on two liquid phases confirmed the identity of *Z*11–14:OH and *Z*11–14:oic as catabolites of pheromone.

Data in Figure 1A and 1B show that the time-course degradation of both *Z*- and *E*11–14:OAc proceeded via the same pathway. With each isomer, the proportion of tritiated 11–14:OAc and 11–14:OH declined as the proportional amounts of tritiated 11–14:oic and water increased with increasing incubation time. The rate of increase in the tritiated water proportion was similar (slope = 4.7 for *Z* and slope = 4.4 for *E*) from one isomer to another, and this indicated that the rate of catabolism of the isomers was equivalent. The proportional amount of tritium in 11–14:OAc was about 20 for both isomers at T_0 and according to extrapolation no residual 11–14:OAc of either isomer remained after 7 min. In the tests where 1 μg *Z*11–14:OAc was incubated with antennae of each of 10 males, the mean proportion of 11–14:OAc to 11–14:OH was 59:41 after 12 min. The proportionately large amount of 11–14:OAc remaining on the antennae after 12 min of incubation with 1 μg compared to the very rapid hydrolysis of the picogram doses is an indication that the rate of hydrolysis was limited by the availability of esterase. Considering the rapidity with which picogram doses applied to the antennae were hydrolyzed, and realizing that these doses were at least 10^3–10^5 times larger than a male antenna would encounter while following a female sex pheromone plume, it can readily be surmised that

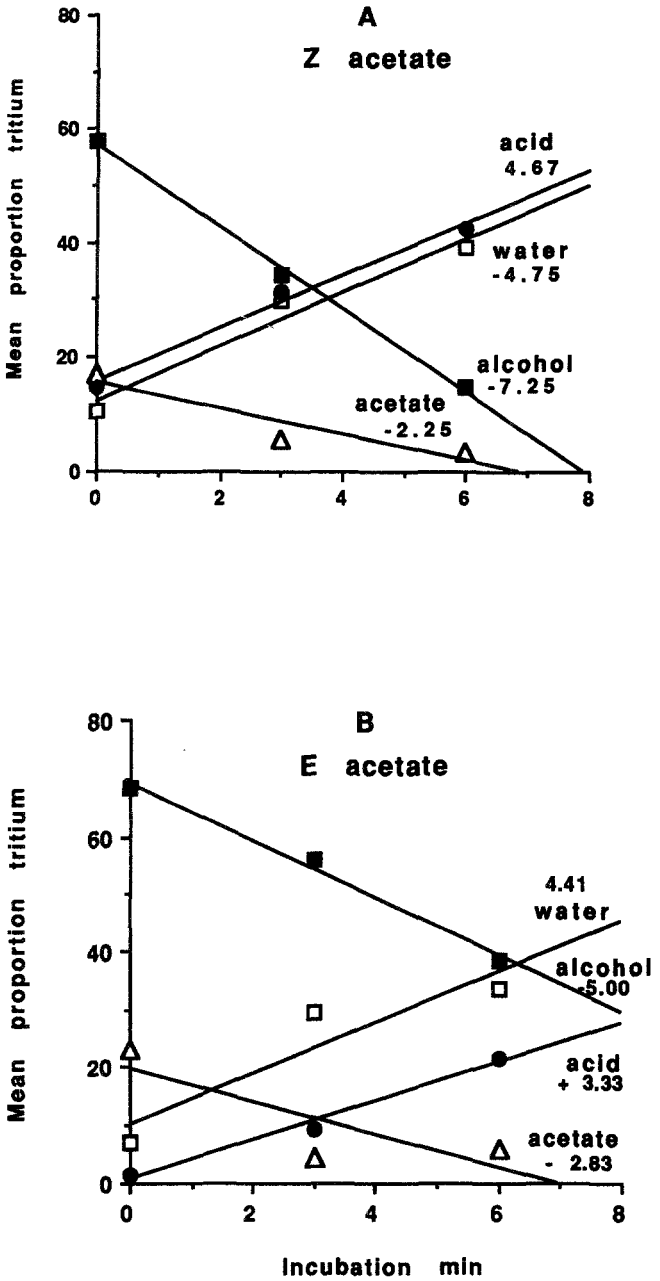

FIG. 1. Mean proportion of tritium in extracts of RBLR antennae 0, 3, and 6 min after tritiated pheromone components were applied to the antennae. (A) *Z* isomer catabolism by males; mean percentage tritium recovery was 14.4, 15.1, and 10.4 for 0-, 3-, and 6-min incubations, respectively. (B) *E* isomer catabolism by males; mean percentage tritium recovery was 16.6, 20.8, and 12.9 for 0-, 3-, and 6-min incubations, respectively. Numbers adjacent to catabolic product names are slopes for respective lines.

the half-life of acetate impinging on the antennae under natural conditions would be extremely brief.

Results obtained with *Z* or *E* alcohol as substrate showed (Figure 2A and 2B) that changes in the proportion of tritium in 11–14:OH and the proportion in 11–14:oic were inversely related over time and that rates of proportional change were virtually identical from one isomer to the other. The results show that the alcohol serves as an immediate substrate for an oxidative process that converts the alcohol to acid.

Figure 3 shows results obtained in the catabolism studies with RBLR females. The data show that *Z*11–14:OAc was hydrolyzed less effectively on the female antennae than on the antennae of males because at T_0 there were nearly equivalent amounts of 11–14:OAc and 11–14:OH present on the female antennae, whereas with males at T_0 there always was a proportionately large amount of 11–14:OH and small amounts of 11–14:OAc present. Additionally, the rate of decrease in the proportional amount of alcohol in females (slope = −1.33) was significantly lower than in males (slope = −5.00 for *E* and slope = −7.25 for *Z*). In males, 11–14:oic was always detectable at T_0 and increased proportionately thereafter, while it was not detected until T_6 in females, indicating that the process of oxidation of alcohol to acid was also less intense in females. Thus, the catabolic pathways are sexually dimorphic.

Moreover, the data indicate that a pheromone-degradation pathway exists on the antennae of RBLR males and females that sequentially involves hydrolysis of acetate, oxidation of alcohol to fatty acid, followed by degradation of the acid. Because no radiolabeled catabolites other than 11–14:oic were detected and because water was the only other radiolabeled catabolite, we surmise that 11–14:oic is most likely degraded by β-oxidation. It is well known that in β-oxidation of long-chain fatty acids the fatty acyl group is transferred to mitochondria, transformed to acyl-CoA, and oxidized entirely in the mitochondrial matrix to acetyl-CoA, which enters the tricarboxylic acid cycle (Lehninger, 1975). The characteristics of the β-oxidation pathway account for why no tritiated catabolites other than 11–14:oic were observed. It also accounts for the rapid appearance of tritiated water because the tritons covalently bonded to carbons 11 and 12 would be incorporated into water by the action of the enzymes enoyl-CoA isomerase and enoyl-CoA hydratase, which are known enzymatic components of β-oxidation.

Comparatively, the pheromone degradative pathway in the RBLR is similar to that of the ECB (Klun et al., 1992). In the RBLR, however, the intermediary catabolic pool of 11–14:oic was comparatively larger, as evidenced by the fact that it was easily detected by HPLC radiodetection in extracts of antennae of individual moths treated with tritiated pheromone. In the ECB, the 11–14:oic pool was so small that it was not detectable in HPLC analysis of individual moths, and it could be detected only through GC radiodetection analysis of the

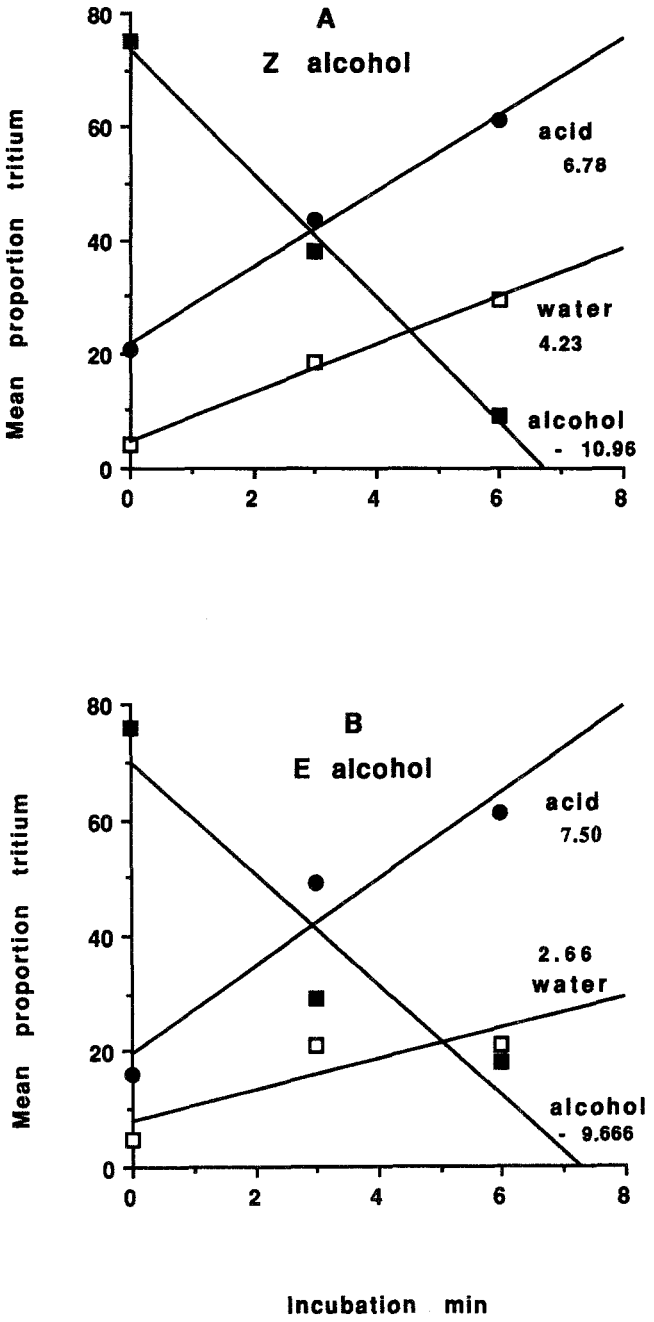

FIG. 2. Mean proportion of tritium in male RBLR antennal extracts as a function of incubation time with 11–14:OH. (A) *Z* isomer; mean percentage of tritium recovery was 15.5, 14.6, and 7.5 for 0-, 3-, and 6-min incubations. (B) *E* isomer; mean percentage tritium recovery was 20.1, 15.7, and 11.0 for 0-, 3-, and 6-min incubations. Numbers adjacent to catabolic product names are slopes of lines.

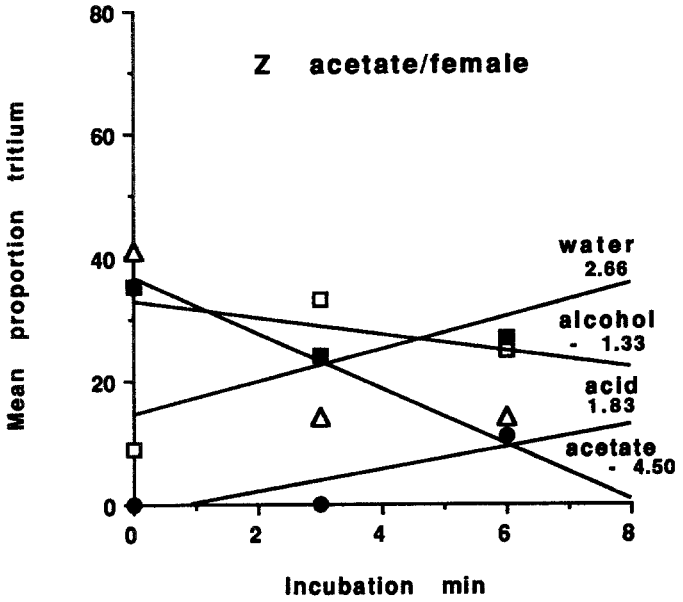

FIG. 3. Catabolism of tritiated Z11-14:OAc by RBLR females. Mean proportion tritium as a function of incubation time. The mean percentage of tritium recovery for 0-, 3-, and 6-min incubations was 13.9, 8.3, and 8.1. Numbers adjacent to catabolic product names are slopes of respective lines.

combined and concentrated extracts of 30 antennal pairs incubated with tritiated pheromone. Furthermore, time course study of 11-14:OH catabolism in the RBLR showed that changes in the proportion of tritium in 11-14:OH and 11-14:oic were inversely related over time. In contrast, time-course studies of ECB catabolism of 11-14:OH showed that changes in the proportion of tritium in 11-14:OH was inversely related to the proportion of tritium in water. This is evidence that the rate of degradation of 11-14:oic is considerably slower in the RBLR than in the ECB and, correspondingly, this accounts for the larger size of the 11-14:oic intermediary catabolic pool in the RBLR.

Comparisons also show that female RBLR and ECB degrade pheromone by a catabolic pathway that is similar to that used by their male counterparts; however, the females degraded pheromone less efficiently than their respective males. This makes sense inasmuch as one would expect the receiver of a chemical signal to process the compound more effectively than the sender because prompt clearing of chemical signal from the receiver's antennae would be essential to maintenance of a high level of chemoreceptive sensitivity

Based upon our research, it is safe to conclude that similar pheromone

catabolic pathways exist on antennae of the RBLB and ECB, but from the results we cannot say exactly where the catabolic events occur on the antenna. It will now be necessary to pinpoint the location and identity of the enzymes that are responsible for each of the catabolic steps in the pathway, to learn how they may be coupled to make a pathway, and to learn if and how the pathway may be linked to olfactory perception itself.

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REFERENCES

BJOSTAD, L., LINN, C., ROELOFS, W., and DU, J.W. 1985. Identification of new sex pheromone components in *Trichoplusia ni* and *Argyrotaenia velutinana*, predicted from biosynthetic precursors, pp. 224–237, *in* Semiochemistry: Flavors and Pheromones. Walter de Gruyter, Berlin.

FERKOVICH, S.M. 1982. Enzymatic alteration 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.

HANSSON, B.S., LJUNGBERG, H., HALLBERG, E., and LÖFSTEDT, C. 1992. Functional specialization of olfactory glomeruli in a moth. *Science* 256:1313–1315.

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., 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., SCHWARZ, M., and UEBEL, E.C. 1991. European corn borer: Pheromonal catabolism and behavioral response to sex pheromone. *J. Chem. Ecol.* 17:317–334.

KLUN, J.A., SCHWARZ, M., and UEBEL, E.C. 1992. Biological activity and in vivo degradation of tritiated female sex pheromone in the male European corn borer. *J. Chem. Ecol.* 18:283–298.

LEHNINGER, A.L. 1975. Biochemistry. Worth Publishers, New York, pp. 544–553.

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., 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.

RAMING, K., and BREER, H. 1990. Comparative studies on G-proteins in the nervous tissue of invertebrates. *Comp. Biochem. Physiol.* 95B:861–864.

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.

VOGT, R.G., PRESTWICH, G.D., and RIDDIFORD, L.M. 1988. Sex pheromone receptor proteins. Visualization using a radiolabeled photoaffinity analog. *J. Biol. Chem*. 263:3952–3957.

VOGT, R.G., PRESTWICH, G.D., and LERNER, M.R. 1991. Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. *J. Neurobiol*. 22:74–84.

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EVIDENCE FOR ALLELOCHEMICAL ATTRACTION OF THE COFFEE BERRY BORER, *Hypothenemus hampei*, BY COFFEE BERRIES

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Abstract—Petri dish choice tests conducted on the coffee berry borer (CBB), *Hypothenemus hampei*, showed that females were able to discriminate between coffee berries at different ripening stages. A Y-shaped glass olfactometer was used to demonstrate that coffee berries emitted volatile chemicals that elicited upwind movement by female CBB. Olfactometer tests with three different solvent extracts of berries showed that at least some of the attractive chemical(s) released by the coffee berries could be extracted with acetone.

Key Words—Coleoptera, Scolytidae, *Hypothenemus hampei*, host selection, kairomones, olfaction, *Coffea* sp.

INTRODUCTION

The coffee berry borer (CBB), *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidae) feeds and develops exclusively in the berries of coffee, especially of the species *Coffea canephora* Pierre. Since the species was first described in 1867 in Uganda, *H. hampei* has spread to most of the world's major coffee-producing areas. The last significant coffee-growing countries not yet infested are Costa Rica, Papua New Guinea, and Hawaii. The CBB is responsible for significant

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crop losses with infestation levels up to 60–80% per crop being reported (Schmitz and Crisinel, 1957; Le Pelley, 1968; Reid and Mansingh, 1985). In New Caledonia, CBB is the major pest of coffee. Control of this pest in New Caledonia can be difficult because it has developed resistance to endosulfan (Brun et al., 1989). In the two major coffee-growing areas of the territory, 94% and 100% of the surveyed CBB populations had resistant individuals (Brun et al., 1990). The spread of the resistant populations is of concern, and new methods to monitor this pest need to be developed.

Surprisingly, little is known about its biology considering the economic significance of this pest. Most of the work on this species has been concerned with its developmental cycle (Bartra et al., 1981; for a review see Borbon-Martinez, 1989). At about 12 days after eclosion, gravid females leave the berry and disperse to colonize and oviposit in new berries. Recently, evidence for parthenogenic reproduction has been reported (Munoz, 1989). Females are able to colonize berries throughout the year, when the harvest is incomplete. Some berries remain on the plant (Baker, 1984). Most of these berries dry on the plant, but some green and red berries can develop.

In contrast with other Scolytidae, little is known about the chemical ecology of CBB, particularly the factors that influence host colonization. Corbett (1933) and Morallo-Rejesus and Baldos (1980) reported that red, ripe berries are preferentially attacked over less ripe berries also present in the field. Morallo-Rejesus and Baldos (1980) dismissed the importance of visual cues in this preferential attack, arguing that insects are usually blind to red. They concluded that other factors, such as odors of ripe berries, may aid the insect in host choice. As a basis for developing a monitoring system for CBB based on host location, we were interested in determining how CBB females recognize a suitable host; i.e., are olfactory or visual cues involved in colonization by CBB females or is colonization of the host plant a result of random search by females. The present study describes laboratory tests determining whether volatile chemicals emitted from different stages of coffee berries are involved in host location by CBB females.

METHODS AND MATERIALS

Insects. Infested berries were collected in the field (Sarramea Valley, New Caledonia) and stored in small black-painted plastic containers in the laboratory, under ambient conditions (26 ± 2°C and relative humidity 80–85%). The positive phototactic response exhibited by female CBB leaving berries was used for collection of CBB females. A transparent plastic tube was connected to the black container so that females that had come out of the berries would walk into the tube. Females were used in experiments within 1 hr after they had left the berry.

Age (after emergence from the pupae) of adult females used for the experiments was approximately 12 days.

Host Plants. Berries were collected at Sarramea 3 hr before each test, from two clones of *C. canephora* var. *robusta* Linden: the primarily produced clone (HB) and a pollinator clone (H 865).

Extracts. Immediately after they were collected, sets of 35 berries were soaked in 70 ml of either hexane, acetone, or ethanol. The berries were left at −18°C for 24 hr in the solvent. Extracts were used without any further purification or condensation.

Choice Bioassay. These bioassays were conducted in plastic Petri dishes (9 cm ID) under ambient laboratory conditions. Natural light was provided through a window and was used to elicit motion. Maximum female flight activity occurs at the beginning of the afternoon (Morallo-Rejesus and Baldos, 1980; Baker, 1984), and therefore the tests were conducted during this period. The Petri dishes were placed at the same distance from the light source. Four different categories of berries were tested: two green, one red, one brown. The two different types of green berries were distinguished by endosperm characteristics: mature green berries have vitrified endosperm, whereas immature berries do not. Red berries are ripe, and brown berries have dried on the trees.

For each test, two different categories of berries were placed on a filter paper, 6 cm apart and perpendicular to the natural light source. The position of each type of berry was reversed after each test and the filter paper renewed. A single female was placed in the middle of a Petri dish, equidistant from the berries. After 3 hr, the test was stopped and berries were checked for infestation. As females do not lay eggs until two days after entry into berries, we used signs of boring activity into berries as a measure of successful colonization rather than the presence of eggs. Females were tested once and each comparison was replicated at least 139 times.

Olfactory Tests. These were conducted in a Y-shaped glass olfactometer (0.5 cm ID for each part of the Y, 40° angle between the arms, length of the main part 5 cm, length of each arm 4 cm) connected to two vertical open containers (2 cm ID) constructed of PVC. The berries, or dental cotton impregnated with 3 ml of extract [i.e., 1.5 berry equivalents (BE)], were placed into one of the PVC tubes and the control (solvent only) placed in the other. The airstream passed through the sources, sucked by a pump providing an airflow of about 1.8 m/sec in the main part of the Y tube. The light conditions were as described previously. Tests were conducted as 26 ± 2°C and 80 ± 10% relative humidity.

The solvent was allowed to evaporate from wicks before the wicks were tested. Tests were run for 20 min following introduction of stimuli and of a single insect. After 20 min, the arm in which the female was located was recorded. About 30% of the tested females did not move into either arm; data

on these females were not included in the statistical analysis. A female released at the extremity of the main arm and reaching the far end of the odorous arm was scored as a positive response. After five replications of each combination of stimuli, the tubes were cleaned in solvents and the positions of the sources reversed. The same tests were then replicated another five times. Chi-squared tests, assuming an equal distribution of females in both arms, were used for statistical analysis of the data.

RESULTS AND DISCUSSION

In the Petri dish experiments, females exhibited distinct preferences for different phenological stages of the coffee berry (Table 1). The proportion of females that did not attack berries was relatively constant for all choice tests and was always less than 10% of the tested insects. These females remained hidden and did not appear to react to the light. Females showed a preference for red berries over mature green berries ($\chi^2 = 31.7$; $df = 1$), for red berries over dry berries ($\chi^2 = 32.8$; $df = 1$), and for dry over immature green berries ($\chi^2 = 18.1$; $df = 1$). In a comparison of the two types of green berries, females showed a preference for mature over immature berries ($\chi^2 = 7.7$; $df = 1$). Field observations (Giordanengo, 1992) indicate that CBB females only oviposit in red berries or in green berries with at least a vitrified endosperm. Nevertheless, they can bore into and inhabit less ripe berries. It has been suggested that feeding upon immature green berries permits adult females to survive until ripe

TABLE 1. CHOICE TESTS IN PETRI DISHES AND LEVEL OF BERRY INFESTATION WHEN SUBMITTED TO CBB FEMALES AT DIFFERENT STAGES OF BERRY MATURATION[a]

Females infesting green immature berries	Females infesting green mature berries	Females infesting fresh red berries (tested within 4 hr of collection)	Females infesting dry berries	Females infesting red berries collected 24 hr ago	Unresponsive females	χ^2 $df = 1$
48	78				13	7.7
	34	101			9	31.7
		101	33		10	32.8
42			92		10	18.1
		88		50	6	10.3

[a]Two different maturation stages of berries were placed at equal distances from a single female. Duration of the test was 3 hr. Green immature berry: aquous endosperm; green mature berry: vitrified endosperm; red berry: mature endosperm; dry berry: dry endosperm.

berries are available for oviposition (Waterhouse and Norris, 1989). However, we found that a gradient of preference in the choice of berries appeared to be related with the degree of maturity of coffee berries. The ability to discriminate between different types of berries could ensure with low expenditure, the choice of a suitable host to establish a new colony. In the final Petri dish experiment (Table 1), red berries tested within 4 hr of collection were preferred ($\chi^2 = 10.3$; $df = 1$) by females than were red berries tested 24 hr after collection. This decrease in attractiveness of coffee berries with time after collection suggested to us that one of the factors involved in the host colonization process may be chemical and that the chemical(s) involved is either volatile (and therefore rapidly evaporates following collection) or labile. This could be advantageous for colonization, since a volatile or labile nature would reduce the likelihood of adsorption of the chemical(s) on the foliage thereby confusing location of the berries.

To test our hypothesis that volatile chemicals and olfaction are involved and perceived by CBB females, an airflow carrying odors of red berries was tested in the Y-shaped olfactometer against a blank stimulus. This experiment showed that volatile chemicals emitted by red coffee berries do influence CBB females. Thus, significantly ($\chi^2 = 25.9$; $df = 1$) more females were found in the end part of arm baited with red berries than in the control arm (Table 2). Most of the tested females walked back and forth through the main part of the olfactometer before entering the arm and walking directly to the source. This movement may be considered as upwind progress, mediated by an anemotactic response. Further results, paralleling those of Petri dish bioassays, showed that the effluvium of red berries in the olfactometer was more attractive ($\chi^2 = 11.5$; $df = 1$) to female CBB than the effluvium of green berries (Table 2).

Due to a shortage of uninfested red berries at the time the experiments involving extracts of berries were conducted, only green berries were used for the olfactometer tests with extracts. Of the three extracts tested, only acetone elicited significant ($\chi^2 = 8.45$; $df = 1$) upwind progress of females in the olfactometer (Table 3).

While our study has shown that volatile chemicals emitted by coffee berries

TABLE 2. RESPONSES OF CBB FEMALES TO EFFLUVIA RELEASED BY DIFFERENT MATURATION STAGES OF COFFEE BERRIES TESTED IN Y-SHAPED OLFACTOMETER

5 red berries	5 green berries	Blank	Unresponsive females	χ^2, $df = 1$
43		7	9	25.9
37	13		7	11.5

TABLE 3. RESPONSES OF CBB FEMALES TO DIFFERENT EXTRACTS OF COFFEE BERRIES TESTED IN Y-SHAPED OLFACTOMETER[a]

2.5 BE, Solvant acetone	2.5 BE, Solvant hexane	2.5 BE, Solvant alcohol	Blank	Unresponsive female	χ^2, $df = 1$
53			27	16	8.45
	45		35	27	1.25
		45	35	23	1.25

[a](BE: berry equivalent).

may be involved in host colonization by female CBB, we have not tested the importance of visual cues such as color and shape in this process. It is possible that female CBB use a combination of these two sensory modalities during host colonization (Prokopy, 1986). Attraction of CBB females to light is related to flight and walking activity but can be switched by the availability of volatiles that act on host choice, as shown in the Petri dish tests. The number of females that did not react during the different tests could be explained by accidental suppression of moving activity, as suggested by Wellington (1980). In CBB, the transition from phototactic orientation to vegetative orientation appeared similar to that previously described for bark beetles (Borden et al., 1986) and for a species of Nitidulidae (Blackmer and Phelan, 1991).

The host colonization phase represents a vulnerable step for *H. hampei*, but orientation of adult females to a suitable host seems to be facilitated by kairomone-like chemicals produced by the berries. The CBB females oriented and walked to odor released by red berries over a short distance. Identification of the volatile compounds involved in host colonization by female CBB is being undertaken. Although our results did not show that a long-range orientation flight mediated by red berry odors occurs, the use of plant odor may eventually allow the development of a field trapping method for monitoring this pest.

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REFERENCES

BAKER, P.S. 1984. Some aspects of the behavior of the coffee berry borer in relation to its control in southern Mexico (Coleoptera, Scolytidae). *Folia Entomol. Mex.* 61:9-24.

BARTRA, C., URRELO, R., and RODRIGUEZ, R. 1981. Biologica de la broca del cafe *Hypothenemus hampei* Ferr. (Coleoptera, Ipide) en Tingo Maria. *Tropicultura* 14:17-31.

BLACKMER, J.L., and PHELAN, P.L. 1991. Behavior of *Carpophilus hemipterus* in a vertical flight

chamber: Transition from phototactic to vegetative orientation. *Entomol. Exp. Appl.* 58:137–148.

BORBON-MARTINEZ, O. 1989. Bioécologie d'un ravageur des baies du caféier *Hypothenemus hampei* Ferr (Coleoptera, Scolytidae) et de ses parasitoides au Togo. PhD thesis. University P. Sabatier de Toulouse, France, 185 pp.

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.). Mechanism in Insect Olfaction. Clarenden Press, Oxford.

BRUN, L.O., MARCILLAUD, C., GAUDICHON, V., and SUCKLING, D.M. 1989. Endosulfan resistance in coffee berry borer, *Hypothenemus hampei* (Coleoptera: Solytidae) in New Caledonia. *J. Econ. Entomol.* 82:1311–1316.

BRUN, L.O., MARCILLAUD, C., GAUDICHON, V., and SUCKLING, D.M. 1990. Monitoring of endosulfan and lindane resistance in the coffee berry borer *Hypothenemus hampei* (Coleoptera: Scolytidae) in New Caledonia. *Bull. Entomol. Res.* 80:129–135.

CORBETT, G. H. 1933. Some preliminary observations on the coffee berry beetle borer, *Stephanoderes (Cryphalus) hampei* Ferr. *Malay. Agric. J.* 21:8–22.

GIORDANENGO, P. 1992. Biologie, écoéthologie et dynamique des populations du scolyte des grains de café *Hypothenemus hampei* (Ferr.) (Coléoptère, Scolytidae) en Nouvelle Calédonie. Thesis. Université de Rennes 1, 110 pp.

LE PELLEY, R.H. 1968. Pests of coffee. Tropical Science Series. Longmans, Green and Co., 590 pp. London.

MORALLO-REJESUS, B., and BALDOS, E. 1980. The biology of the coffee berry borer *Hypothenemus hampei* (Ferr.) (Scolytidae, Coleoptera) and its incidence in the southern tagalog provinces. *Philipp. Entomol.* 4:303–316.

MUNOZ, R. 1989. Ciclo biologico y reproduccion partenogenética de la Broca del fruto del cafeto *Hypothenemus hampei* (Ferr.). *Turrialba* 39:415–421.

PROKOPY, R.J. 1986. Visual and olfactory stimulus interaction in resource finding by insects, pp. 81–89, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanism in Insect Olfaction. Clarenden Press, Oxford.

REID, J.C., and MANSINGH, A. 1985. Economic losses due to *Hypothenemus hampei* Ferr. during processing of coffee berries in Jamaica. *Trop. Pest. Manag.* 31:55–59.

SCHMITZ, G., and CRISINEL, P. 1957. La lutte contre *Stephanoderes hampei* Ferr. Publication I.N.E.A.C. 70, 156 pp.

WELLINGTON, W.G. 1980. Dispersal and population change, pp. 11–24, *in* A.A. Berryman, and L. Safranyik, (eds.). Dispersal of Forest Insects: Evaluation, Theory, and Management Implications. Washington State University, Pullman.

WATERHOUSE, D.F., and NORRIS, K.R. 1989. Biological control: *Hypothenemus hampei*, in Pacific prospects. Supplement 1. Australian Centre for International Agriculture Research, Canberra, 123 pp.

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HOST RECOGNITION AND THE STUDY OF A CHEMICAL BASIS FOR ATTRACTION BY CUCKOO BUMBLE BEES (HYMENOPTERA: APIDAE)

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Abstract—Species of *Psithyrus* (Hymenoptera; Apidae) are obligate bumble bee social parasites. In this study, females of *P. vestalis* and *P. ashtoni* were presented with pentane extracts prepared from different body parts of queens of their respective host species, *Bombus terrestris* and *B. terricola*. Parasites of both species were capable of distinguishing host bees from other bumble bee species using chemical cues contained within extracts. Among extracts of several body parts presented to parasites, the abdomen produced the greatest behavioral response, with Dufour's gland and terminal tergal segments eliciting the greatest response among abdominal regions. Extracts of these two body parts obtained from *B. terrestris* queens shared a number of compounds, identified by GC-MS. Among the identified compounds are a number that have been reported to be of importance in bee sociochemistry.

Key Words—Host recognition, volatiles, social parasitism, cuckoo bumble bees, *Bombus terrestris, Bombus terricola, Psithyrus vestalis, Psithyrus ashtoni,* Hymenoptera, Apidae, Dufour's gland, tergal gland.

INTRODUCTION

Studies of parasites and parasitoids offer considerable opportunities to look at mechanisms of orientation, host specificity, and coevolution (reviewed by Vin-

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son, 1984). In addition, such studies may offer information of applied value in the control of pest species (Nordlund, 1981). Symbioses among the social insects (*sensu* Wilson, 1971) and between social insects and other arthropods represent a further subset of host–parasite relationships. Social insects attract a large number of kleptoparasites, brood parasites, and nest commensals (e.g., Howard et al., 1980; Vander Meer and Wojcik, 1982; Franks et al., 1990). Considering the surprisingly high percentage of bee genera that are exclusively parasitic (20% in North America: Bohart, 1970), the Apoidea would seem to offer a particularly diverse group of parasite–host relationships for examination. Nonetheless, very little is known about bee parasite–host relationships due to problems in designing reliable, quantitative behavioral bioassays and insufficient host records for most bee parasites (Duffield et al., 1984).

These limitations do not appear to be a serious impediment in studies of bumble bee social parasitism. Species of *Bombus* (Hymenoptera: Apidae) are parasitized by bees belonging to the closely related genus *Psithyrus*, all of which are obligate social parasites (inquilines) in nests of their hosts. Host records are well established for most *Psithyrus* species (e.g., Løken, 1984), and a promising basis for chemical ecological studies dealing with bumble bee parasitism has been established. Parasites orient to the odors of host bees and nests and are capable of making distinctions among *Bombus* species on this basis (Fisher, 1983, 1985). Observations by Cederberg (1979, 1983) further suggest that *P. rupestris* Fabricius females are able to detect the odor trails of host *B. lapidarius* L. bees, although the glandular source of the attractive cues and their chemical identity have not been determined. We have attempted to extend these findings by quantifying the attraction of *Psithyrus* females to host-bee cuticular compounds, isolating possible sources of the attractive compounds and identifying a number of compounds emanating from these sources that may be responsible for the observed attraction.

METHODS AND MATERIALS

Insects. Queens of the bumble bee *Bombus terrestris* L. and its cuckoo parasite *Psithyrus vestalis* Geoffroy were caught in the spring of 1987 as they searched for nest sites in the vicinity of Oxford, England. Bees were maintained in the laboratory in individual wire cages with access to sugar water and moist pollen. Queens of *B. terricola* Kirby and its parasite, *P. ashtoni* Cresson, were caught in the springs of 1990 and 1991 in the vicinity of Wolfville, Nova Scotia, Canada, and maintained in the same way as the European bees. Queens of *B. pascuorum* Scopoli also were kept in the laboratory as sources of nonhost extracts for *P. vestalis* studies. Queens of *B. impatiens* Cresson and *B. ternarius* Say were used as sources of nonhost extracts for *P. ashtoni*.

Preparation of Extracts. Queens of *B. terricola* were chilled at 4°C and then narcotized with carbon dioxide. Regions of the body, including exocrine glands, as well as tergites and sternites with their associated glands, were excised under water and transferred to vials containing 1 ml AR grade *n*-pentane (one queen equivalent per vial). Care was taken during excision to avoid contamination of adjacent body regions. For bioassay experiments, extracts were stored at −20°C until use. Extracts of nonhost queens (*B. impatiens* and *B. ternarius*) were prepared similarly.

Extracts of *B. terrestris* (host) and *B. pascuorum* (nonhost) queens were prepared differently. Queens were placed at −36°C for approximately 10 min. Extracts were then prepared by immersing selected body parts for 24 hr at 5°C in small vials containing 1 ml of pentane, i.e., one queen equivalent per vial. Extracts were then decanted into other vials and stored at −36°C until use.

For chemical analyses, bee body parts were steeped in 1 ml dichloromethane (BDH AR grade, redistilled 2× through a Vigreux column) at −20°C for 24 hr. Dodecane (0.5 μg) was added as an internal standard to the decanted solvent, which was evaporated to ~100 μl by a gentle stream of N_2. The resulting extracts were transferred to capillary tubes and concentrated further to ~20 μl. The tubes were sealed and stored at −20°C until analyzed by gas chromatography (GC) and coupled gas chromatography–mass spectrometry (GC-MS).

Parasite Bioassays. The test apparatus consisted of a 15-cm-diameter × 7-cm glass crystallizing dish with a loose-fitting clear plastic lid. The bottom of the dish was lined with a circular filter paper disk. Disks were handled only with only forceps. Pentane extracts were evaporated to 20 μl with nitrogen immediately prior to use. Early observations indicated that parasites responded to an extract concentration of $\frac{1}{2}$ body equivalent. In each experiment, 10-μl aliquots of extract (i.e., $\frac{1}{2}$ body equivalent) were placed on the filter paper in various combinations and presented to *Psithyrus* females.

The body parts selected for making extracts were chosen on the basis of experimental results: those that elicited the strongest behavioral response were examined in more detail in subsequent experiments. Because few parasites were available, the number of extracts in each experiment was varied in order to maximize the amount of information each experiment could provide regarding body part preference, while minimizing the number of times each *Psithyrus* female was tested. The number of different extracts presented to each parasite in a given experiment was varied from two to four.

Depending upon the number of extracts presented in an experiment, the filter paper was divided in half, thirds, or quarters by a light pencil line. In those experiments in which two extracts were presented simultaneously to parasites, a given aliquot was deposited in the center of each half of the disk, and the position of the extract was denoted by lightly circling its outline with a pencil

before the solvent evaporated. Similar procedures were followed in those experiments involving presentation of three or four extracts: aliquots were placed in the center of each pie-shaped filter paper section and their outlines marked by a pencil. The location of extracts was randomized with respect to the section of filter paper on which a given aliquot was placed. After each *Psithyrus* female had been tested, the filter paper was removed and discarded, the dish was rinsed, and another paper disk placed in the dish for the next presentation.

Parasites were carefully removed from their wire cages and lowered into the center of the testing apparatus. The apparatus was illuminated from above with a 25-W red light to minimize flight and escape reactions by the bees. The behavior of each bee was monitored for 3 min. The preference of *Psithyrus* females for different host extracts was measured in different ways corresponding to the behaviors exhibited by parasites when they encounter host odors. Preliminary observations indicated that upon encountering odors that were attractive to them, *Psithyrus* females exhibited pronounced antennal responses (see also Cederberg, 1983), which involved lowering the antennae until they lay flat on the source of the odor. Parasites also would often groom themselves while investigating this substrate.

Thus, the number of females that antennated extracts and the number that groomed themselves while their antennae were within the penciled outline of a given extract were recorded. These numbers were summed, providing a behavioral response score for each *Psithyrus* female; i.e., for an experiment in which 10 *Psithyrus* females were tested, the maximum score for a given extract was 20. In addition, the amount of time spent by parasites investigating each extract spot during the 3-min experimental period was recorded. Responses of parasites to extracts were ranked on this basis. Nonparametric statistical tests were used to compare behavioral responses to extracts. Since only a small number of bees was available for testing, we used a significance value of $P < 0.05$ in order to reject H_0.

In some cases, parasite females appeared to show no interest in the extracts and spent the 3-min period attempting to escape. These responses were considered balks. *Psithyrus* females that balked were removed from the apparatus for 10 min and then retested. If a second balk occurred, the *Psithyrus* female was not retested during that experiment. Balk data were not included in the analysis.

Gas Chromatography. Preliminary analyses of the extracts from body segments and glands were carried out on a Hewlett-Packard 5890 GC equipped with an on-column injector and FID detector. Separations were performed using a 25-m × 0.2-mm-ID WCOT bonded-phase fused silica capillary column coated with 1.0-μm thick DB-1 (J&W Scientific). The column was temperature programmed from 40°C (5 min) to 150°C at 5°C/min, held 1 min, then to 280°C at 10°C/min and maintained at the maximum temperature for 50 min. The on-column injector was held at the GC-oven temperature. Nitrogen carrier gas was 1 ml/min; the detector temperature was 260°C.

Gas Chromatography–Mass Spectrometry. Low-resolution electron-impact GC-MS analyses were performed on a VG70-250S double-focusing magnetic sector mass spectrometer directly coupled via a heated interface to a dedicated HP5890 capillary GC. The same GC column and chromatographic conditions were used as for the capillary GC examination, except that helium replaced nitrogen as carrier gas; capillary column head pressure, 25 kPa; GC-MS interface, 280°C; ion source temperature, 250°C. The scan rate was 0.1 sec/decade with 0.1-sec interscan time. Mass spectral assignments were made with the help of a computer using the NIH/EPA mass spectral data base (Heller and Milne, 1978) available on VG Analytical proprietary search software. Where necessary, MS identifications were confirmed by GC peak enhancement analysis using authentic standards.

RESULTS

Parasite Behavior. The behavior of *P. vestalis* females when presented with various *B. terrestris* body part extracts is summarized in Figure 1. While parasites responded to extracts from nonhost bees, they displayed a clear preference for those of host queens (sign test: $P < 0.05$ for all three tests). In females of *P. ashtoni*, the response to host whole-body extract did not appear to diminish over the course of the experiment, based on similar amounts of time spent investigating extracts of *B. terricola* queens at the beginning and end of the experiment.

A further indication of parasite response was shown by the number of females that antennated extracts and groomed themselves while positioned over extracts. Although these behaviors were elicited by both host and nonhost extracts, the behavioral score of *P. ashtoni* females was greater for extracts of host queens ($X^2 = 9.46$ and 22.69 for the initial and final presentation, respectively, $P < 0.005$ for both).

Parasite Responses to Body Part Extracts. Further tests designed to elucidate which body parts produced the greatest response seemed to pinpoint cues emanating from the abdomen as being more attractive than other body regions (Figure 2). Significant differences were displayed by *P. vestalis* females when they were ranked in terms of the relative time spent investigating extracts of head, thorax plus abdomen, or legs (Friedman two-way ANOVA: $P < 0.05$), and between the thorax and abdomen (sign test: $P < 0.05$). Differences in response to different odor samples were also shown by *P. ashtoni* females (Friedman two-way ANOVA: $P < 0.05$). Similarly, behavioral scores for antennation and grooming differed among extracts. *P. vestalis* scores were higher for abdomen than thorax ($X^2 = 11.43$, $P = 0.005$) and were also higher in *P. ashtoni* for abdomen than other body regions ($X^2 = 14.16$, $P < 0.005$).

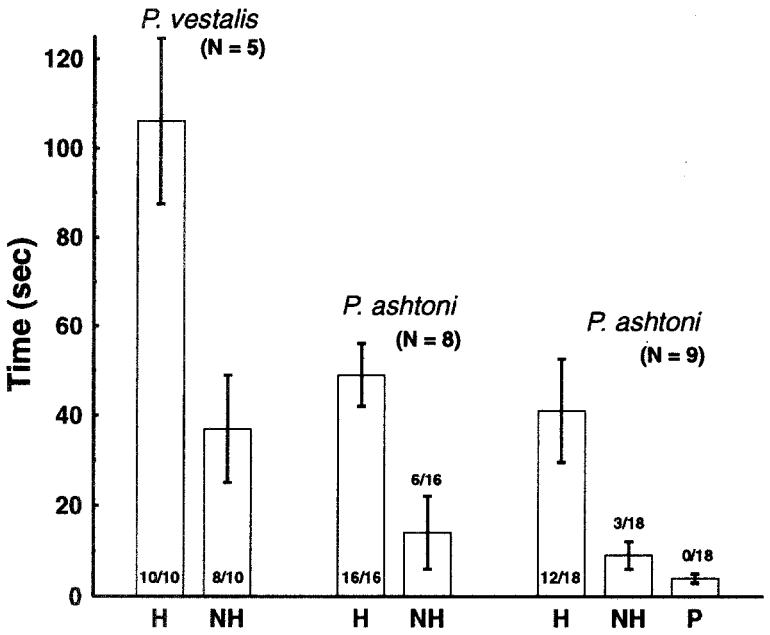

FIG. 1. Relative amounts of time (sec) spent by parasites investigating crude pentane extracts of whole body (H), nonhost whole body (NH), or pentane only (P). Data for *P. ashtoni* represent presentations at the beginning (left) and end (right) of experimentation. Solid bars represent means; vertical lines, SE. Fractions at the base of the bars denote the summed scores of antennation of extracts and grooming behavior by parasites versus the maximum score possible.

Among abdominal portions, Dufour's gland and the terminal tergites elicited greater behavioral responses than other parts (Figure 3). There was a significant difference among behavioral responses of *P. vestalis* females to different abdominal extracts ($X^2 = 17.35$, $P = 0.001$), although there was no significant difference when females were ranked in terms of the amount of time spent investigating extracts (Friedman two-way ANOVA: $P < 0.10$). *P. ashtoni* females exhibited significant differences among both investigation ($P < 0.05$) and behavioral responses ($P < 0.005$).

Chemical Composition of Host Abdominal Extracts. Abdominal cuticular extracts of *B. terrestris* queens were examined by capillary GC and the individual compounds quantified. Duplicate injections from extracts from three to seven individual bees were examined and the means are presented in Table 1. The results depict the first 56 compounds in the order they eluted from the DB-1 column. Compounds eluting after $C_{32}H_{64}$ were ignored. Structural assignments were made by analysis of GC-MS electron-impact (70 eV) data and confirmed

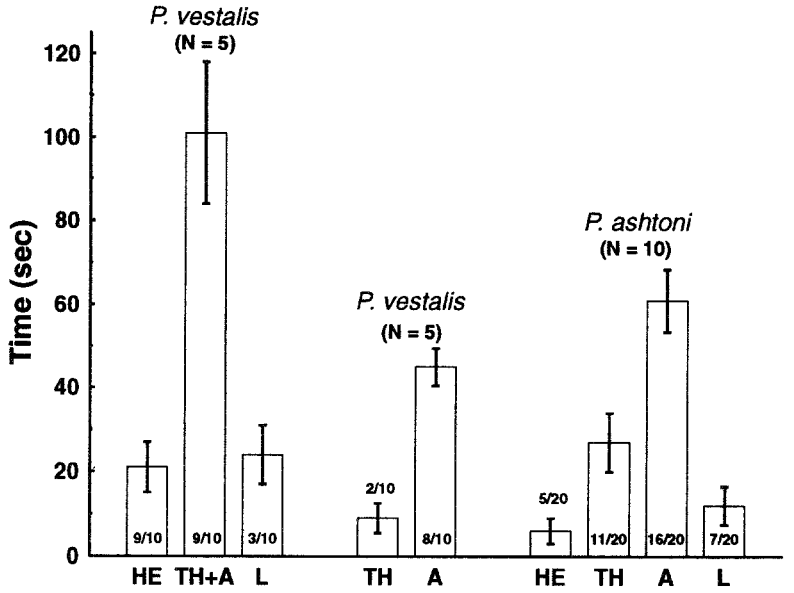

FIG. 2. Relative amounts of time spent by parasites investigating extracts of different host body parts. Fractions as in Figure 1 (A = abdomen, HE = head, L = legs, TH = thorax.)

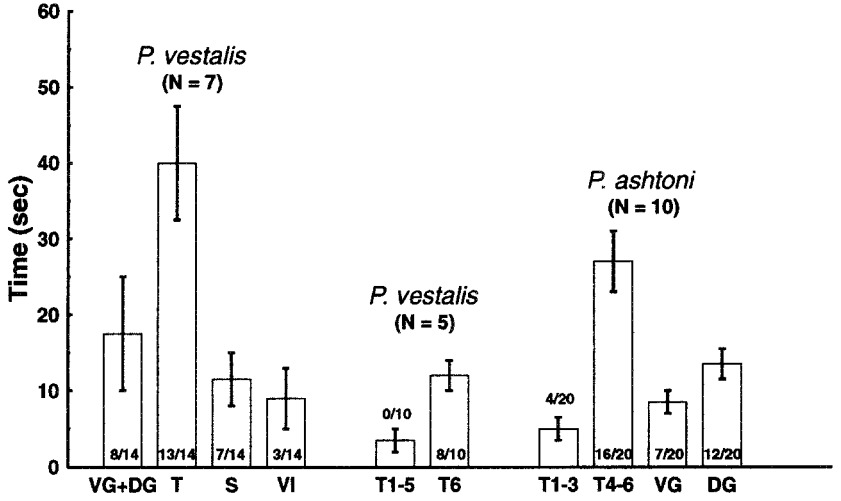

FIG. 3. Relative amounts of time spent by parasites investigating extracts of different host body parts. Fractions as in Figure 1 (DG = Dufour's gland, S = sternites, T = tergites, VG = venom gland, VI = viscera).

TABLE 1. RELATIVE COMPOSITION OF COMPOUNDS PRESENT IN EXTRACTS OF ABDOMINAL REGIONS AND GLANDS OF *B. terrestris* QUEENS

Peak	Compound	Abdominal region or gland source[a]				
		Tergite 5	−T6[b]	Sternites	Dufour's	Tergite 6
1	Int std (dodecane)	1.00	1.00	1.00	1.00	1.00
2	Unidentified	—[c]	—	—	4.62	—
3	Tridecan-2-one	—	—	0.06	0.72	—
4	Pentadecan-2-one	—	—	0.31	18.08	0.04
5	Tridecan-2-ol	—	—	—	1.32	0.01*[d]
6	Unidentified	—	—	—	1.52	0.20*
7	Unidentified	—	—	—	1.84	0.57*
8	Ethyl tetradecanoate	—	—	—	29.66	0.06*
9	Unidentified ester	—	—	0.26	5.79	0.10
10	Heptadecan-2-one	—	0.05	0.31	11.02	0.05
11	Unidentified	0.07	0.19	0.24	0.72	—
12	Unidentified	—	0.30	—	4.02	—
13	Nonadecane	—	0.05	0.12	0.47	0.12
14	Ethyl hexadecanoate	—	—	—	12.62	1.15*
15	Eicosane	—	—	—	2.92	—
16	Unidentified	—	—	—	39.99	—
17	Methyl octadecanoate	—	5.90	—	49.99	0.35
18	Heneicosane	6.08	—	0.31	9.09	2.61
19	Methyl octadecanoate	—	—	—	8.38	7.86*
20	Unidentified	—	—	—	0.63	1.95*
21	Unidentified	—	—	—	2.37	—
22	Ethyl octadecanoate	—	—	—	98.37	—*
23	Docosane	0.73	3.68	3.63	50.99	0.42
24	Unidentified	—	—	—	0.86	1.04*
25	Unidentified	—	—	—	2.01	—
26	Unidentified	0.73	3.60	4.68	2.31	1.07
27	Tricosene	0.60	58.94	138.77	8.51	0.77
28	Tricosane	34.20	—	—	54.98	15.15
29	Branched alkane	—	—	—	3.34	0.36*
30	Tetracosene	0.18	1.11	—	0.68	0.31
31	Tetracosane	0.68	1.09	1.66	1.44	0.32
32	Pentacosene	0.73	2.53	2.91	5.47	0.57
33	Pentacosane	14.79	17.79	37.33	30.08	9.06
34	Hexacosatriene	—	—	—	—	1.12*
35	Hexacosadiene	—	—	—	1.40	—
36	Hexacosane	0.72	1.19	1.65	1.89	0.61
37	Heptacosadiene	—	—	—	2.90	—
38	Heptacosene	3.47	18.17	24.38	21.83	2.97
39	Heptocosane	13.71	28.01	34.57	42.86	15.04
40	Branched alkane	—	—	—	0.86	0.02*
41	Octacosadiene	0.37	1.01	1.63	1.57	0.31
42	Octacosene	0.91	1.11	2.02	9.29	0.47

TABLE 1. CONTINUED

Peak	Compound	Abdominal region or gland source[a]				
		Tergite 5	−T6[b]	Sternites	Dufour's	Tergite 6
43	Octacosane	1.04	0.78	0.73	2.34	0.89
44	Nonacosatriene	—	—	—	—	0.32*
45	Nonacosadiene	2.08	3.19	7.58	21.77	1.69
46	Nonacosene	6.04	20.16	33.84	189.10	9.64
47	Nonacosane	15.81	25.47	49.32	24.27	10.28
48	Branched alkene	9.31	13.78	11.40	0.96	9.51
49	Triacontadiene	0.04	0.04	0.72	9.77	0.03
50	Triacontene	0.07	0.31	0.80	10.39	0.04
51	Hentriacontadiene	3.19	2.62	4.52	91.51	1.57
52	Hentriacontene	1.24	7.50	31.23	76.53	2.45
53	Unidentified	—	—	—	0.25	0.29
54	Octadec-9-eonate ester	—	—	—	3.28	—
55	Octadec-9-enoate ester	—	—	—	— 0.02	7.31
—	56	Unidentified wax ester	—	—	—	1.46
0.32						

[a]Relative to 0.5 μg dodecane, the internal standard, added before extraction.
[b]All tergites minus the sixth tergite and its associated glands.
[c]Absent or below the level of detection.
[d]Compounds of particular interest (see text for details)

by cochromatography with authentic standards where appropriate. All abdominal regions analyzed contained straight-chain saturated hydrocarbons in the series C_{19}–C_{29} with the odd carbon numbers from C_{25}–C_{29} predominating. The presence of unsaturated hydrocarbons was more specific, with tergite 6 being unique in having C_{26}:3 and C_{29}:3 and Dufour's gland alone having C_{26}:2 and C_{27}:2. Branched alkanes were present only in Dufour's gland and tergite 6 extracts.

Simple aliphatic esters and ketones predominated among the oxygenated compounds present. The esters ethyl tetradecanoate, ethyl hexadecanoate, methyl and ethyl octadecanoate, and methyl octadecenoate, as well as high-molecular-weight wax esters were major components of Dufour's gland and to a lesser extent, tergite 6. The ketones tridecan-2-one, pentadecan-2-one, and heptadecan-2-one were present in Dufour's gland with traces in both tergite 6 and the sternites.

The compounds present in tergite 6 and Dufour's gland but absent from tergites 1–5 are potentially the most interesting from a significance point of view to the *Psithyrus* response. These compounds, marked with an asterisk in Table 1, are either branched hydrocarbons, oxygenated, or remain unidentified.

A chromatogram depicting a separation by gas chromatography of volatiles from Dufour's gland of one individual *B. terrestris* queen is shown in Figure 4. The attenuation has been adjusted to depict the minor components. Figure 5 depicts a reconstructed total ion chromatogram from the GC-MS investigation of components in an extract of tergite 6 (T6) from *B. terrestris*. These data underline the relative complexity of the cuticular compounds from T6 and Dufour's gland compared to other abdominal regions and differ considerably with respect to the relative concentration of compounds to that in Table 1, indicating that there was appreciable variation between individual been samples. The extent of this variation is shown in Table 2, where variation in the percentage composition of each of the compounds highlighted in Table 1 is shown as a mean and range of 6 individual bees. In some instances, particular compounds were absent from individual bees.

DISCUSSION

Our results are consistent with other studies (Fisher 1983, 1985) that have shown *Psithyrus* females are capable of distinguishing the odors of host from nonhost *Bombus* species and, further, that this distinction can be made on the basis of chemical cues emanating from crude extracts. Our efforts to pinpoint particular sources of host recognition suggest that they are more prevalent in the abdominal region, although our results indicated that crude whole body extract elicited the greatest response by *Psithyrus* females. This decreased responsiveness may have resulted from smaller quantities of attractive compounds being available for detection as the bodies of host bees were partitioned into smaller portions. However, at present we do not know if the concentration of attractants differs with nest size, or the minimal levels to which parasites are capable of responding.

Alternatively, individual compounds from multiple body sources may act in concert to produce the greatest response. Blum (1986) has suggested that it is unrealistic to expect to find a single volatile compound that is used by social insect species in nestmate recognition. Bumble bees possess a rich variety of exocrine glands (Duffield et al., 1984), the secretions of which, when summed, may produce the greatest attraction. The functions of many of these glands have not been defined, but it is likely that their secretions are used in social behavior. Dufour's gland (Hefetz et al., 1986) and tergal glands (Cruz Landim et al., 1980) have both been implicated in terms of their role in colony integration in studies of bee social behavior. The sixth tergite in bumble bee females is notable in that it contains specialized glands with unknown function (Cruz Landim, 1963). Measurement of the most abundant volatiles contained in Dufour's gland of *B. hypnorum* L. workers shows that there are intraspecific differences in the

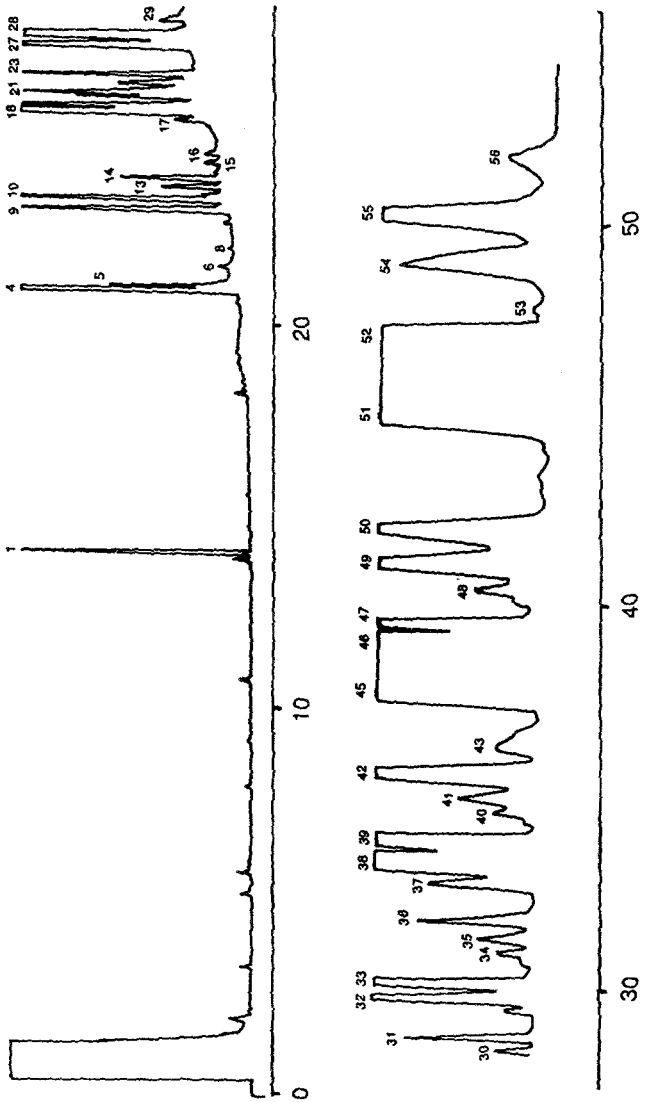

FIG. 4. Capillary gas chromatogram of *B. terrestris* Dufour's gland volatiles. The peak numbers refer to Table 1.

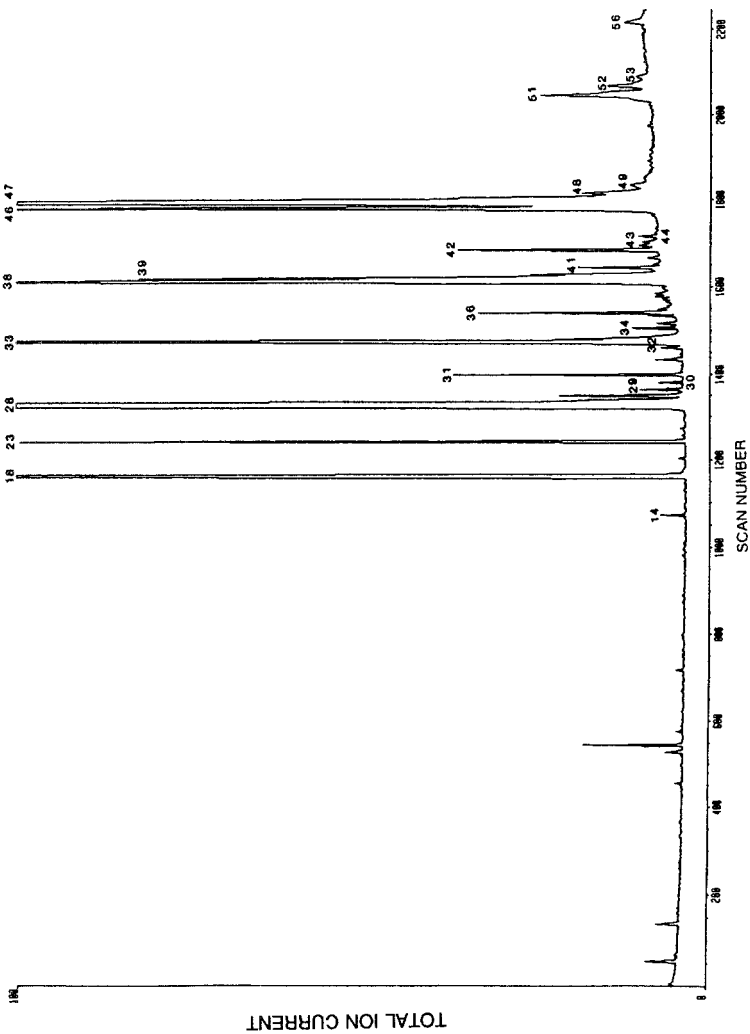

FIG. 5. Reconstructed total ion chromatogram of volatiles from the 6th tergite with its associated glands, from *B. terrestris* queens. The peak numbers refer to Table 1.

TABLE 2. VARIATION IN PERCENT COMPOSITION OF UNIQUE VOLATILE COMPOUNDS IN EXTRACTS OF DUFOUR'S GLAND AND 6TH TERGITE OF *B. terrestris* QUEENS

		Percent composition of fraction			
		Dufour's gland		Sixth tergite	
Peak	Compound	Mean	Range	Mean	Range
5	Tridecan-2-ol	0.10	0.00– 0.13	0.02	0.00– 0.10
6	Unidentified	0.02	0.00– 0.10	0.17	0.00– 0.36
7	Unidentified	0.03	0.00– 0.13	0.27	0.00– 0.99
8	Ethyl tetradecanoate	0.41	0.00– 2.03	0.11	0.00– 0.44
14	Ethyl hexadecanoate	0.71	0.00– 3.30	0.99	0.00– 2.23
16	Unidentified	1.12	0.00– 5.46	0.00	0
19	Methyl octadec-enoate	0.77	0.25– 2.12	8.84	0.00–24.67
20	Unidentified	0.04	0.00– 0.10	1.33	0.00– 3.14
22	Ethyl octadecanoate	7.14	0.07–33.43	0.00	0
24	Unidentified	0.36	0.00– 1.67	0.63	0.00– 1.71
29	Branched alkane	3.47	0.03–16.30	0.30	0.00– 0.64
34	Hexacosatriene	0.00	0	2.83	0.00– 5.00
40	Branched alkane	0.05	0.00– 0.10	0.03	0.00– 0.11
44	Nonacosatriene	0.00	0	0.23	0.00– 0.50

chemical composition of this gland (Tengö et al., 1990), which bumble bees may use to distinguish nestmates. Tengö et al. (1991) have since extended their study to include other *Bombus* species. Similarly, there are differences in the qualitative and quantitative composition of volatiles in the crude whole-body extracts from queens of *B. terrestris*, *B. pascuorum*, and other *Bombus* species using gas chromatography (Fisher, unpublished), which may give *Psithyrus* females the chemical information they require to make distinctions among hosts. Future research in this area will address these questions by coupling bioassays with the fractionation of bioactive extracts, identification of the chemical constituents, and localization of their glandular source.

Volatile compounds from a variety of chemical classes are present in extracts of *B. terrestris* queens. The data collected in the current study are consistent with that of Genin et al. (1984) who investigated caste and sex differences in cuticular and labial gland components from *B. hypnorum*. Many of the compounds that we identified using GC-MS are present as cuticular components of other insect species, where they serve to prevent desiccation as well as regulate cuticular permeability (Hadley, 1980; Howard and Blomquist, 1982). The majority of the compounds present in crude cuticular extracts of *B. terrestris* abdominal segments, for example, are also present in the primitive neotropical stingless bee, *Mourella caerulea* (Wittmann et al., 1989). Tridecanol has been

previously reported as a mandibular gland component from species belonging to the families Andrenidae, Apidae, and Formicidae (see Wheeler and Duffield, 1988). The ketones 2-tridecanone, 2-pentadecanone, and 2-heptadecanone have been found in the mandibular glands of *Trigona* spp., Dufour's gland of formicid workers, and the frontal gland of isopteran soldiers (see Wheeler and Duffield, 1988). 2-Tridecanone has been shown to be the alarm pheromone of the formicid ant, *Acanthomyops claviger* (Regnier and Wilson, 1968). The esters ethyl tetradecanoate and ethyl hexadecanoate are found in the mandibular glands of female bees from the genus *Exoneura* (Anthophoridae) (Cane and Michener, 1983). The latter compound is also found in the mandibular glands of workers of *Melipona interrupta triplaridis* (Apidae) (Smith and Roubik, 1983). Ethyl octadecanoate has been reported as a constituent of Dufour's gland of several colletid bees (see Wheeler and Duffield, 1988) and the methyl and ethyl esters of C_{18}:0 and C_{18}:1 have been recorded in other *Bombus* species (Genin et al., 1984; Tengö et al., 1991). All these compounds may elicit behavioral responses, including sex attraction, host attraction, alarm, and defense (Vander Meer et al., 1989).

Although we found several compounds of potential importance in bee sociochemistry, we urge caution in the interpretation of their relative importance, as our study was separated in time and space, such that we were unable to analyze chemically the makeup of crude extracts used in behavioral experiments. Further, as in other studies of chemical ecology, highly volatile components may diminish by evaporation to concentrations too low to permit chemical analysis or behavioral response. The chemical components of *B. terrestris* queens that attract *P. vestalis* females can be categorized as kairomones, allelochemic signals that are used by host-searching parasites (Nordlund, 1981). Many kairomones are by-products of host physiology that do not appear to have any special significance for the host but are important signals for host parasitoids (Vinson, 1984). Tantalizing evidence that bumble bee volatile compounds act as kairomones comes from observations by Cederberg (1979, 1983) that some bumble bees produce "trail pheromones" that may be detected by searching *Psithyrus* females, although the source of these compounds has not been confirmed. The hypothesis that cuckoo bumble bees identify their hosts using the same chemical signals that hosts use to communicate with one another is an attractive one, awaiting further studies of the proximate chemical bases for bumble bee nestmate recognition.

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REFERENCES

BLUM, M.S. 1986. Specificity of pheromonal signals: a search for its recognitive bases in terms of a unified chemisociality, pp. 401–405, *in* J. Eder and H. Rembold (eds.). Chemistry and Biology of Social Insects. J. Perperny, Munich.

BOHART, G.E. 1970. The Evolution of Parasitism among Bees. Utah State University, 30 pp.

CANE, J.H., and MICHENER, C.D. 1983. Chemistry and function of manidibular gland products of bees of the genus *Exoneura* (Hymenoptera, Anthophoridae). *J. Chem. Ecol.* 9:1525–1531.

CEDERBERG, B. 1979. Odour-guided host selection in *Psithyrus* (Hymenoptera, Apidae). *Entomol. Tidskr.* 100:128–129.

CEDERBERG, B. 1983. The role of trail pheromones in host selection by *Psithyrus rupestris* (Hymenoptera, Apidae). *Ann. Entomol. Fenn.* 49:11–16.

CRUZ LANDIM, C. DA 1963. Evolution of the wax and scent glands in the Apinae (Hymenoptera: Apidae). *J. N.Y. Entomol. Soc.* 81:2–13.

CRUZ LANDIM, C. DA, DOS SANTOS, S.M.F., and HOFLING, M.C.A. 1980. Sex determination in bees. XV. Identification of queens of *Melipona quadrefafasciata anthidioides* (Apidae) with the worker phenotype by a study of the tergal glands. *Rev. Brazil Genet. III* 3:295–302.

DUFFIELD, R.M., WHEELER, J.W., and EICKWORTH, G.C. 1984. Sociochemicals of bees, pp. 387–428, *in* W.J. Bell, and R.T. Cardé (eds). Chemical Ecology of Insects. Chapman and Hall, London.

FISHER, R.M. 1983. Recognition of host nest odor by the bumble bee social parasite *Psithyrus ashtoni* (Hymenoptera: Apidae). *J. N.Y. Entomol. Soc.* 91:503–507.

FISHER, R.M. 1985. Evolution and host specificity: Dichotomous invasion success of a bumble bee social parasite (*Psithyrus citrinus*) in colonies of its two hosts. *Can. J. Zool.* 63:976–981.

FRANKS, N., BLUM, M., SMITH, R.-K., and ALLIES, A.B. 1990. Behavior and chemical disguise of cuckoo ant *Leptothorax kutteri* in relation to its host *Leptothorax acervorum. J. Chem. Ecol.* 16(5):1431–1444.

GENIN, E., JULLIEN, R., PEREZ, R., FONTA, C., and MASSON, C. 1984. Donnees preliminaires sur les mediateurs chimiques du Bourdon *Bombus hypnorum* (Hymenoptere, Apoide, Bombinae). *C.R. Acad. Sci. Paris Ser. III* 299:297–302.

HADLEY, N.F. 1980. Surface waxes and integumententary permeability. *Am. Sci.* 68:546–553.

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(1):197–208.

HELLER, S.R., and MILNE, G.W.A. 1978. EPA/NIH Mass Spectral Data Base, Vols 1–5. National Standards Defence Data Service, National Bureau of Standards, Washington, D.C.

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. 1980. Chemical mimicry as an integrating mechanism: Cuticular hydrocarbons of a termitophile and its host. *Science* 210:431–432.

LØKEN, A. 1984. Scandanavian species of the genus *Psithyrus* Lepeletier (Hymenoptera: Apidae) *Entomol. Scand. Suppl.* 23:1–45.

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. John Wiley & Sons, New York.

REGNIER, F.E., and WILSON, E.O. 1968. The alarm-defence system of the ant *Acanthmyops claviger. J. Insect Physio.* 14:955–970.

SMITH, B.H. and ROUBIK, D.W. 1983. Mandibular glands of stingless bees (Hymenoptera: Apidae): Chemical analysis of their contents and biological function in two species of *Melipona. J. Chem. Ecol.* 9:1465–1472.

TENGÖ, J. HEFETZ, A., and FRANKE, W. 1990. Individual and group specific odours of bumble bee females, pp. 513–514, G.K. Veeresh, B. Mallik, and C.A. Viraktamath (eds.). *in* Social Insects and the Environment. Oxford & IBH Publishing Co., New Dehli.

TENGÖ, J., HEFETZ, A., BERTSCH, A., SCHMITT, U., LUEBKE, G. and FRANKE, W. 1991. Species specificity and complexity of Dufour's gland secretion of bumble bees. *Comp. Biochem. Physiol. B.* 99B:641–646.

VANDER MEER, R.K., and WOJCIK, D.P. 1982. Chemical mimicry in the myrmecopilous beetle *Myrmecaphodius excavaticollis. Science* 218:806–808.

VANDER MEER, R.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(7):2115–2125.

VINSON, S.B. 1984. Parasitoid–host relationships, pp. 205–233, *in* W.J. Bell, and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

WHEELER, J.W., and DUFFIELD, R.M. 1988. Pheromones of Hymenoptera and Isoptera, pp. 59–206, *in* E.D. Morgan and N.B. Mandava (eds.). C.R.C. Handbook of Natural Pesticides. Vol IV: Pheromones, Part B. CRC Press, Boca Raton, Florida.

WILSON, E.O. 1971. The Insect Societies. Harvard University Press, Cambridge, Massachusetts.

WITTMANN, D., LUBKE, G., and FRANKE, W. 1989. Cephalic volatiles identified in workers of *Mourella caerulea*, a rare stingless bee recently rediscovered in southern Brazil. *Z. Naturforsch.* 44C:325–326.

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HYDROCARBONS WITH A HOMOCONJUGATED POLYENE SYSTEM AND THEIR MONOEPOXY DERIVATIVES: SEX ATTRACTANTS OF GEOMETRID AND NOCTUID MOTHS DISTRIBUTED IN JAPAN

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Abstract—Although several sex pheromones of the family Geometridae have been characterized, investigations on Japanese species are limited. In order to obtain more information, screening using known sex pheromones and their analogs was carried out. The (Z,Z,Z)-3,6,9-triunsaturated and (Z,Z)-6,9-diunsaturated hydrocarbons with straight C_{19}–C_{21} chains were synthesized by the Grignard coupling reaction as a key step starting from linolenic and linoleic acids, respectively. Oxidation of the homoconjugated trienes with *m*-chloroperoxybenzoic acid yielded a 1:1:1 mixture of three monoepoxy derivatives that could be separated by silica gel chromatography. The chemical structure of each positional isomer was confirmed using two-dimensional NMR techniques and MS measurements, which enabled characteristic fragment ions from the isomers to be identified. Field tests using lures incorporating only one of the above six hydrocarbons or nine epoxides were carried out in a forest in Tokyo. Consequently, attraction of male moths of 14 geometrid species in addition to four species in another family, the Noctuidae, was observed. It was concluded that hydrocarbons with a homoconjugated polyene system and the monoepoxy derivatives are important components of sex pheromones produced by Japanese lepidopterous insects, particularly the geometrid moths.

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Key Words—Sex pheromone, lepidopterous attractant, field test, unsaturated hydrocarbon, epoxydiene, Geometridae, Noctuidae.

INTRODUCTION

Lepidopterous sex pheromones have been identified from females of more than 300 species since the first investigation on bombykol was carried out. Analyses of their chemical structures (Arn et al., 1986; Ando, personal data base), showed that ca. 85% of them contained linear aliphatic alcohols, aldehydes, or acetates, with a C_{10}–C_{18} straight chain and one or two olefinic linkages. These types of compounds with a terminal functional group, which have been identified from 16 families of Lepidoptera, are among the most ubiquitous pheromone components. Another type of pheromone component lacking in a functional group at the terminal position was identified in the last decade, as shown in Table 1 (Ando, personal data base). To date, hydrocarbons with a homoconjugated triene or diene system and their monoepoxy derivatives have been identified from 38 species mainly in the families of Geometridae and Arctiidae. Pheromone components with the above terminal functional group, however, have not been identified from female moths in these two families. Furthermore, screening tests using these types of synthetic pheromones and the analogs revealed the attraction

TABLE 1. NUMBER OF LEPIDOPTEROUS SPECIES, SEX PHEROMONES OR ATTRACTANTS REPORTED TO BE HYDROCARBONS WITH A POLYENE SYSTEM, AND/OR THEIR MONOEPOXY DERIVATIVES

Superfamily Family	Subfamily	Number of species	
		Sex pheromone	Attractant
Geometroidea			
Geometridae	Ennominae	16	20
	Larentiinae	4	10
	Oenochrominae	1	1
Noctuoidea			
Arctiidae		9	0
Ctenuchidae		2	0
Lymantriidae		1	0
Noctuidae	Catocalinae	4	3
	Herminiinae	0	3
	Hypeninae	0	3
	Ophiderinae	1	1
Total		38	41

of 41 lepidopterous species in Canada and Europe (Millar et al., 1990b; and references therein). Although some of them are distributed in Japan, information about sexual communication utilizing the unsaturated hydrocarbons and epoxides is very limited. Therefore attempts were made to synthesize these types of compounds and to examine their field attractancy against Japanese Lepidoptera.

METHODS AND MATERIALS

Synthesis of 3,6,9-Trienes and 6,9-Dienes. Three homoconjugated trienes (*Z*3,*Z*6,*Z*9–19:H, *Z*3,*Z*6,*Z*9–20:H, and *Z*3,*Z*6,*Z*9–21:H)[4] and three dienes (*Z*6,*Z*9–19:H, *Z*6,*Z*9–20:H, and *Z*6,*Z*9–21:H) were synthesized by a modification of the method described by Conner et al. (1980) and Underhill et al. (1983). A mixture of linolenic and linoleic acids in a ratio of ca. 3:1 (25 g, 90 mmol), purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), was esterified with ethanol and reduced to alcohols with $LiAlH_4$ in dry ether. After silica gel column chromatography, this alcohol mixture was treated with *p*-toluenesulfonyl chloride in pyridine to yield the tosylates (27 g, 72% yield from the acids). A portion of the tosylates (5.0 g, 12 mmol) was supplied for Grignard coupling with methylmagnesium bromide (16.7 ml, 15.9 mmol) [0.95 M tetrahydrofuran (THF) solution, Kanto Chemical Co., Inc., Tokyo, Japan] in dry THF (100 ml) under the mediation of Li_2CuCl_4 (Fouquet et al., 1974) (1 ml) (0.1 M THF solution, Aldrich Chemical Co., Inc., Milwaukee, Wisconsin) to prepare a mixture of *Z*3,*Z*6,*Z*9–19:H, and *Z*6,*Z*9–19:H. The C_{19} triene (1.5 g, 48% yield from the tosylates) and diene (0.6 g, 19% yield) were separated by a silica gel column impregnated with 20% $AgNO_3$ using a *n*-hexane–benzene solvent system. The C_{20} and C_{21} analogs were synthesized in the same manner using ethylmagnesium bromide (0.93 M THF solution, Kanto Chemical Co.) and *n*-propylmagnesium bromide prepared from *n*-propyl bromide, respectively.

Synthesis of Monoepoxy Derivatives. *m*-Chloroperoxybenzoic acid (Tokyo Kasei Kogyo Co., 70% pure, 490 mg, 2.0 mmol) was added to a solution of *Z*3,*Z*6,*Z*9–19:H (470 mg, 1.8 mmol) in dry CH_2Cl_2 (30 ml) and stirred at 0°C for 1 hr. After further stirring at room temperature for 2 hr, the reaction mixture was washed with a saturated aqueous solution of $NaHCO_3$ (20 ml × 2) and dried with Na_2SO_4. Silica gel column chromatography gave a mixed product of the three *cis*-monoepoxy derivatives (240 mg, 48% yield) in a ratio of ca. 1:1:1. Since the epoxydiene mixture showed three separate spots on a silica gel TLC plate (60 F_{254}, Merck, Darmstadt, Germany) [solvent system: *n*-hexane–benzene (1:1), R_f values: epo3,*Z*6,*Z*9–19:H, 0.34; *Z*3,epo6,*Z*9–19:H, 0.43; and

[4]Compounds were abbreviated as follows: *Z*3,*Z*6,*Z*9–19:H is (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene, epo3,*Z*6,*Z*9–19:H is (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene.

Z3,Z6,epo9–19:H, 0.40],[4] each pure sample (a racemic mixture) was obtained by preparative TLC purification. This chromatographic behavior corresponded to that of C_{17} epoxydienes in a normal phase HPLC (Millar et al., 1987). In the same manner, the C_{20} and C_{21} epoxydienes were prepared and purified.

Spectroscopy. NMR spectra of each compound in $CDCl_3$ were analyzed with a JEOL GX 270 Fourier transform spectrometer (270.2 MHz for ^1H and 67.9 MHz for ^{13}C) using TMS as an internal standard. Signal assignments were made by two-dimensional techniques (^1H–^1H and ^1H–^{13}C COSY spectra) using ordinal pulse sequences (Ando et al., 1988). Electron-impact (EI) GC-MS was achieved using a JEOL JNM DX-300 mass spectrometer with a OV-1 capillary column (0.25 mm ID × 25 m, Gasukuro Kogyo Inc., Tokyo, Japan). Ionization voltage of every measurement was 70 eV and ion source temperature was 240°C.

Field Evaluation. Each chemical at 1 mg with purity >95% by GC (i.e., TIC trace from GC-MS) was applied to a white rubber septum (8 mm OD, Aldrich Chemical Co., Ltd.), which was placed in a sticky-type trap (30 × 27 cm bottom plate with a roof, Takeda Chemical Ind., Ltd., Osaka, Japan). A parallel experiment was conducted for each chemical, and the traps were set at a 1.5-m height from the ground. The screening test was carried out from August 1991 to July 1992 in a mixed forest area in the suburbs of Tokyo (Rolling Land Laboratory, Tokyo University of Agriculture and Technology, Hachiohji-shi, Tokyo), and species, sex, and number of moths caught were recorded every two weeks. Each lure was renewed every two months.

RESULTS AND DISCUSSION

Characterization of Synthetic Compounds. The ^1H NMR and MS spectroscopic data of the synthetic hydrocarbons with a homoconjugated triene and diene system corresponded well to those previously reported (Corner et al., 1980; Underhill et al., 1983). The number of C=C double bonds and the carbon length were determined by integration of their olefinic ^1H signals and molecular ions, respectively. The ^{13}C NMR spectra of the unsaturated hydrocarbons were very similar to those of the parent fatty acids. Signals from C-1 to C-15 of the hydrocarbons corresponded to the signals from C-18 to C-4 of the acids. These ^{13}C signals were assigned based on ^1H–^{13}C COSY experiments (see Table 2 for C_{21} diene and triene). Allylic carbon signals (C-5, C-8 and C-11 of dienes, and C-2 of trienes additionally) resonated in a higher field than that calculated for the geometrical isomers (Rossi and Veracini, 1982), indicating all *Z* configurations in the hydrocarbons and the parent fatty acids.

Tables 2 and 3 indicate the ^1H and ^{13}C NMR assignments for three C_{21} *cis*-epoxydienes in addition to C_{21} diene and triene. The NMR spectra of the C_{19} and C_{20} analogs were almost the same as those of the C_{21} compounds except

TABLE 2. ^{1}H NMR ASSIGNMENTS FOR HENEICOSADIENE (Z6,Z9–21:H), HENEICOSATRIENE (Z3,Z6,Z9–21:H), AND EPOXYHENEICOSADIENE (epo3,Z6,Z9–21:H, Z3,epo6,Z9–21:H, and Z3,Z6,epo9–21:H)

	Chemical shift (ppm)												
Compound	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9	H-10	H-11	H-12–H-20	H-21
Z6,Z9–21:H	0.89		1.2–1.4		2.05	~5.35	~5.35	2.78	~5.35	~5.35	2.05	1.2–1.4	0.88
Z3,Z6,Z9–21:H	0.98	2.08	~5.35	~5.35	2.81	~5.35	~5.35	2.81	~5.35	~5.35	2.08	1.2–1.4	0.88
epo3,Z6,Z9–21:H	1.06	~1.6	2.90	2.96	2.22, 2.41	~5.45	~5.5	2.81	~5.3	~5.35	2.04	1.2–1.4	0.88
Z3,epo6,Z9–21:H	0.99	2.07	~5.5	~5.4	2.22, 2.42	2.95	2.95	2.22, 2.42	~5.4	~5.5	2.07	1.2–1.4	0.88
Z3,Z6,epo9–21:H	0.97	2.08	~5.4	~5.35	2.81	~5.5	~5.45	2.22, 2.40	2.95	2.93	~1.5	1.2–1.5	0.88

TABLE 3. ^{13}C NMR ASSIGNMENTS FOR HENEICOSADIENE (Z6,Z9-21:H), HENEICOSATRIENE (Z3,Z6,Z9-21:H), AND EPOXYHENEICOSADIENE (epo3,Z6,Z9-21:H, Z3,epo6,Z9-21:H, AND Z3,Z6,epo9-21:H)

	Chemical shift (ppm)														
Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-11	C-12–C-18	C-19	C-20	C-21
Z6,Z9-21:H	14.08	22.6	31.6	~29.5	27.3	130.20[a]	127.97[b]	25.7	127.99[b]	130.21[a]	27.3	~29.5	32.0	22.7	14.13
Z3,Z6,Z9-21:H	14.3	20.6	132.0	127.2	25.6	128.29[c]	128.34[c]	25.6	127.7	130.4	27.3	~29.5	32.0	22.7	14.1
epo3,Z6,Z9-21:H	10.6	21.1	58.3	56.6	26.2	124.2	130.8	25.8	127.2	130.7	27.3	~29.5	31.9	22.7	14.1
Z3,epo6,Z9-21:H	14.2	20.8	134.4	123.2	26.1	56.5	56.5	26.1	123.7	132.9	27.5	~29.5	31.9	22.7	14.1
Z3,Z6,epo9-21:H	14.3	20.6	132.2	126.7	25.7	130.8	124.2	26.3	56.4	57.2	27.8	~29.5[d]	31.9	22.7	14.1

[a–c] Chemical shift values may be reversed.
[d] C-12 26.6 ppm.

for the intensity of the overlapping signals in the methylene proton region (1.2–1.4 ppm) and methylene carbon region (ca. 29.5 ppm). Chemical structure of each epoxydiene was confirmed based on a ^1H–^1H COSY experiment. Starting from the methyl protons at the 1 position (H-1), correlation peaks of 3,4-epoxides were traced in the following order: protons adjacent to an epoxy ring (H-2), protons at an epoxy ring (H-3 and H-4), allylic protons (H-5), olefinic protons (H-6 and H-7), doubly allylic protons (H-8), olefinic protons (H-9 and H-10) and allylic protons (H-11). The *cis*-configuration of the epoxy ring, predicted based on the reaction property of olefinic epoxidation with a peracid, was indicated by the coupling constant (4.5 Hz) between H-3 and H-4. Homonuclear COSY spectra of 6,7-epoxides and 9,10-epoxides also clearly revealed the position of the epoxy ring. Our ^1H signal assignments corresponded to the published data for C_{17} compounds (Millar et al., 1987). Signal separation of the epoxides was adequate in the ^{13}C NMR spectra, which were very useful to estimate the purity and the structure of contaminating components. A usual ^1H–^{13}C COSY spectrum and a spectrum emphasizing long-range couplings were analyzed in order to assign the ^{13}C signals.

EI-MS data of all nine epoxydienes synthesized for the field screening tests are listed in Table 4. Fragment ions were observed that were characteristic of the position of the epoxy ring. These ions are as follows: 3,4-epoxides: *m/z* M-29, M-72, and 79; 6,7-epoxides: *m/z* M-29, M-69, and 111; 9,10-epoxides: *m/z* M-29, M-69, M-109, 108, and 79. The origin of these ions is indicated in Figure 1. The values of the relative intensity, affixed with *a* in Table 4, were larger than those of the other epoxy ring positional isomers. These fragmentation properties, which were also observed in the mass spectra measured under the other instrumental conditions (Millar et al., 1987; Hansson et al., 1990), are useful in the identification of epoxydienes from a natural pheromone extract.

Detection of Sex Attractants by Field Tests. Field screening tests using the compounds consisting of one major group of sex pheromones, monoenyl (Ando et al., 1977, 1981) and dienyl compounds (Ando et al., 1987) with a terminal functional group, resulted in the specific attraction of more than 200 lepidopterous species in Japan. In the current experiment, a one-year survey was conducted from the summer of 1991 using analogs of another type of pheromonal structure, hydrocarbons with a homoconjugated polyene system and their monoepoxy derivatives, resulted in the attraction of 18 species. Table 5 lists the scientific names of the attracted male moths, abbreviated names of the attractants, time of flight, and total number of moths captured by dual traps. These data suggest that some Japanese lepidopterous insects also utilize hydrocarbons and epoxides as sex pheromones. Chemicals of these types have been identified from the female moths in the following five families: Geometridae, Arctiidae, Ctenuchidae, Lymantriidae, and Noctuidae (see Table 1). Although species in Arctiidae, Ctenuchidae, and Lymantriidae were not captured in these tests, 14

TABLE 4. RELATIVE INTENSITY (%) OF MOLECULAR ION (M^+) AND SOME FRAGMENT IONS IN MASS SPECTRA OF EPOXYDIENES

	Fragment ions								
	m/z 79	*m/z* 108	*m/z* 111	M-109	M-72	M-69	M-29	M-18	M^+
3,4-Epoxide									
epo3,Z6,Z9-19:H	100[a]	9	14	0	23[a]	0	6[a]	11	4[b]
epo3,Z6,Z9-20:H	100[a]	2	19	0	22[a]	0	7[a]	9	7[c]
epo3,Z6,Z9-21:H	100[a]	14	14	0	21[a]	0	5[a]	10	3[d]
6,7-Epoxide									
Z3,epo6,Z9-19:H[e]	36	6	41[a]	0	1	10[a]	3[a]	10	6[b]
Z3,epo6,Z9-20:H[f]	41	6	47[a]	0	1	12[a]	4[a]	10	8[c]
Z3,epo6,Z9-21:H[f]	34	7	39[a]	1	0	6[a]	2[a]	7	4[d]
9,10-Epoxide									
Z3,Z6,epo9-19:H	100[a]	66[a]	3	5[a]	2	2[a]	2[a]	9	4[b]
Z3,Z6,epo9-20:H	100[a]	77[a]	4	3[a]	1	3[a]	2[a]	9	4[c]
Z3,Z6,epo9-21:H	100[a]	81[a]	5	4[a]	1	2[a]	2[a]	8	3[d]

[a] Characteristic ions assumed to be produced by the fragmentation as shown in Figure 1.
[b] *m/z* 278.
[c] *m/z* 292.
[d] *m/z* 306.
[e] Base ion peak was observed at *m/z* 53.
[f] Base ion peak was observed at *m/z* 83.

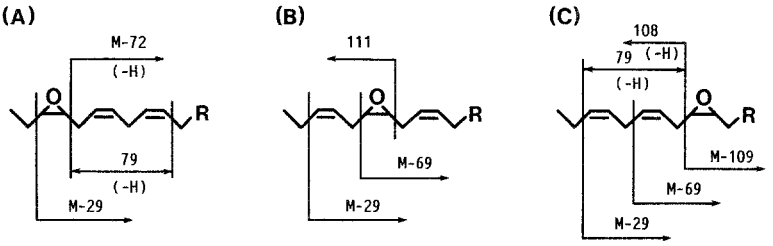

FIG. 1. Characteristic fragment ions of 3,4-epoxides (A), 6,7-epoxides (B), and 9,10-epoxides (C). Molecular ions of epoxynonadecadienes (C_{19} compounds, R = n-C_8H_{17}), epoxyeicosadienes (C_{20} compounds, R = n-C_9H_{19}), and epoxyheneicosadiene (C_{21} compounds, R = n-$C_{10}H_{21}$) were observed at m/z 278, 292, and 306, respectively.

species in Geometridae and four species in Noctuidae were attracted by these synthetic pheromone analogs, indicating that the broad screening test is applicable for research on the pheromones of Japanese geometrid and noctuid moths.

The Geometridae, one of the two families attracted in this experiment, is a large family in the Lepidoptera, which consists of six subfamilies and ca. 800 species in Japan. The pheromones hitherto identified from the geometrid females, which are classified into the subfamilies Ennominae, Larentiinae or Oenochrominae, consisted of hydrocarbons and epoxy derivatives. Furthermore sex attraction by the synthetic hydrocarbons and epoxides has been observed for some other European and Canadian species of the above subfamilies. In addition to some species in these subfamilies, two species of Geometrinae, one of the other three subfamilies, were attracted for the first time by the unsaturated hydrocarbons in this field survey. Males in the subfamilies Sterrhinae and Arcchiearinae had not been attracted by these types of compounds, whereas attraction by the compounds with a terminal functional group had been reported for some species of Sterrhinae (Arn et al., 1986).

The Noctuidae, another large family, is composed of 18 subfamilies and ca. 1200 species in Japan. A large number of pheromone components with a terminal functionality were identified from the species of the following subfamilies: Amphipyrinae, Eustrotiinae, Hadeniinae, Heliothidae, Noctuidae, Plusiinae, and Westermanniinae. Unsaturated hydrocarbons and monoepoxy derivatives have been recently identified as sex pheromones of some species of the subfamilies Catocalinae and Ophiderinae and sex attractants of some species of the subfamilies Herminiinae and Hypeninae. In the current field test, the attraction of males was recorded in Herminiinae and Ophiderinae. It is interesting to note that noctuid moths are classified into two groups according to the chemical structure of their sex pheromones. One group utilizes the compounds

TABLE 5. SEX ATTRACTANTS DETECTED IN FIELD SCREENING TESTS IN A FOREST IN TOKYO FROM 1991 to 1992

Family Subfamily Species	Attractant	Time of flight	Number of attracted moths
Geometridae			
Ennominae			
Aclis angulifera (Butler)	epo3,Z6,Z9–19:H	Sep.–Oct.	18
Ascotis selenaria cretacea (Butler)	epo3,Z6,Z9–19:H	Aug.–Sep.	36
Colotois pennaria ussuriensis Bang-Haas	epo3,Z6,Z9–20:H	Nov.–Dec.	16
	Z3,epo6,Z9–19:H		3
Pachyligia dolosa Butler	Z3,Z6,epo9–21:H	Mar.	9
	Z3,epo6,Z9–21:H		8
Geometrinae			
Agathia carissima Butler	Z3,Z6,Z9–20:H	Aug.	6
Agathia visenda visenda Prout	Z6,Z9–20:H	Apr., Aug.	14
	Z6,Z9–21:H		3
Pachyodes superans Butler	Z3,Z6,Z9–20:H	Aug.–Sep.	17
Larentiinae			
Epirrita viridipurpurescens (Prout)	Z3,Z6,Z9–21:H	Nov.–Dec.	111
Esakiopteryx volitans (Butler)	Z3,Z6,X9–19:H	Mar.–Apr.	384
Operophtera relegata Prout	Z3,epo6,Z9–19:H	Nov.–Dec.	188
	Z3,Z6,epo9–19:H		149
	epo3,Z6,Z9–19:H		41
Sibatania mactata (Felder & Rogenhofer)	Z3,Z6,Z9–21:H	Sep.–Oct.	11
Oenochrominae			
Alsophila japonensis Warren	Z3,Z6,Z9–19:H	Jan.	88
Inurois fumosa Inoue	epo3,Z6,Z9–21:H	Jan.–Feb.	10
Inurois membranaria (Christoph)	epo3,Z6,Z9–21:H	Jan.–Feb.	36
Noctuidae			
Herminiinae			
Paracolax pryeri (Butler)	Z3,epo6,Z9–20:H	May–Aug.	407
Ophiderinae			
Paragabara flavomacula (Oberthür)	Z3,epo6,Z9–21:H	June–Aug.	65
Rivula sericealis (Scopoli)	Z3,epo6,Z9–19:H	Sep.–Oct.	48
Rivula sasaphila Sugi	Z3,epo6,Z9–19:H	Oct.	6

with the most common chemical structure of lepidopterous sex pheromones, and another group utilizes the key components of taxonomically unrelated geometrid moths.

Recently, sex pheromone components of *Ascotis selenaria* (*Boarmia selenaria*: Hampson) and *Colotois pennaria* have been analyzed. From the former species distributed in Israel, the epo3,*Z*6,*Z*9–19:H with 3*S*,4*R* configuration has been identified as a major active component (Becker et al., 1990; Cossé et al., 1992). One of the Japanese subspecies *A. s. cretacea*, a pest insect in a tea garden, was captured by the racemic mixture in this test. *Z*3,epo6,*Z*9–19:H has been identified from *C. pennaria* in Hungary, and a 10:3 mixture with the parent triene exhibited field attractancy (Hansson et al., 1990). Although we have not examined the two-component lure, the Japanese subspecies *C. p. ussuriensis* was more strongly attracted to epo3,*Z*6,*Z*9–20:H than to *Z*3,epo6,*Z*9–19:H. Sex pheromones and attractants of 16 other species captured in this experiment had not been detected so far, although those of some related species had been reported. A C_{19} tetraene (1,*Z*3,*Z*6,*Z*9–19:H) was isolated from *Operophtera accidentalis*, and this compound also attracted *O. occidentalis* (Roelofs et al., 1982). Meanwhile *O. relegata*, one of the Japanese representatives, was attracted by C_{19} epoxydienes in this test. The sex pheromone of *Alsophila pometaria* was found to consist of a mixture of *Z*3,*Z*6,*Z*9–19:H and another C_{19} tetraene (*Z*3,*Z*6,*Z*9,*Z*11–19:H or *Z*3,*Z*6,*Z*9,*E*11–19:H) (Wong et al., 1984), while attraction of *A. quadripunctata* to a mixture of *Z*3,*Z*6,*Z*9–19:H and *Z*6,*Z*9–19:H was reported in Hungary (Szöcs et al., 1984). In the current screening test, *A. japonensis* was attracted to a lure baited with only the C_{19} triene. *Rivula propinqualis* was attracted to a mixture of *Z*3,*Z*6,*Z*9–19:H and *Z*3,epo6,*Z*9–19:H in Canada (Millar et al., 1990a), and *R. sericealis* and *R. sasaphila* were attracted to a single component lure of the epoxydiene in Japan.

Considering the large number of species of Lepidoptera, information about sex pheromones and attractants is still limited. In order to further understand the structure–activity relationship of sex attractants, it is necessary to systematically test other compounds with different chain lengths as well as multicomponent lures.

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REFERENCES

ANDO, T., YOSHIDA, S., TATSUKI, S., and TAKAHASHI, N. 1977. Sex attractants for male Lepidoptera. *Agric. Biol. Chem.* 41:1485–1492.

ANDO, T., KUROKO, H., NAKAGAKI, S., SAITO, O., OKU, T., and TAKAHASHI, N. 1981. Multicomponent sex attractants in systematic field tests of male Lepidoptera. *Agric. Biol. Chem.* 46:487–495.

ANDO, T., KOIKE, M., UCHIYAMA, M., and KUROKO, H. 1987. Lepidopterous sex attractants with a conjugated diene system. *Agric. Biol. Chem.* 51:2691–2694.

ANDO, T., OGURA, Y., KOYAMA, 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., TÓTH, M., and PRIESNER, E. 1986. List of Sex Pheromones of Lepidoptrea and Related Attractants. OILB-SROP/IOBC-WPRS, Secrétariat General, 149 Rue de Bercy, F-75595 Paris cedex 12; ISBN 92-9067-002-9, 123 pp.

BECKER, D., CYJON, R., COSSÉ, A., MOORE, I., KIMMEL, T., and WYSOKI, 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.

CONNER, W.E., EISNER, T., MEER, R.K.V., GUERRERO, A., GHIRINGELLI, D., and MEINWALD, J. 1980. Sex attractant of an arctiid moth (*Utetheisa ornatrix*): A pulsed chemical signal. *Behav. Ecol. Sociobiol.* 7:55–63.

COSSÉ, A.A., CYJON, R., MOORE, I., WYSOKI, M., and BECKER, D. 1992. Sex pheromone components of the giant looper, *Boarmia selenaria* Schiff. (Lepidoptera: Geometridae): Identification, synthesis, electrophysiological evaluation, and behavioral activity. *J. Chem. Ecol.* 18:165–181.

FOUQUET, G., and SCHLOSSER, M. 1974. Improved carbon-carbon linking by controlled copper catalysis. *Angew. Chem. (Int. Ed. Engl.)* 13:82–83.

HANSSON, B.S., SZÖCS, G., SCHMIDT, F., FRANCKE, W., LÖFSTEDT, C., and TÓTH, M. 1990. Electrophysiological and chemical analysis of sex pheromone communication system of the mottled umber, *Erannis defoliaria* (Lepidoptera: Geometridae). *J. Chem. Ecol.* 16:1887–1897.

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-epoxynonadecadiene as sex attractants for two geometrid and one noctuid moth species. *J. Chem. Ecol.* 16:2153–2166.

MILLAR, J.G., GIBLIN, M., BARTON, D., MORRISON, A., and UNDERHILL, E.W., 1990b. 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.

ROELOFS, W.L., HILL, A.S., LINN, C.E., MEINWALD, J., JAIN, S.C., HERBERT, H.J., SMITH, R.F. 1982. Sex pheromone of the winter moth, a geometrid with unusually low temperature precopulatory responses. *Science* 217:657–659.

ROSSI, R., and VERACINI, C.A. 1982. Insect pheromone components. Use of ^{13}C NMR spectroscopy for assigning the configuration of C=C double bonds of monoenic or dienic pheromone components and for quantitative determination of *Z/E* mixtures. *Tetrahedron* 36:639–644.

SZÖCS, G., TÓTH, M., BESTMANN, H.J., and VOSTROWSKY, O. 1984. A two-component sex attractant for males of the geometrid moth *Alsophila quadripunctata*. *Entomol. Exp. Appl.* 38:287–291.

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., PALANISWAMY, P., UNDERHILL, E.W., STECK, W.F., and CHISHOLM, M.D. 1984. Sex pheromone components of fall cankerworm moth, *Alsophila pometaria*. Synthesis and field trapping. *J. Chem. Ecol.* 10:1579–1596.

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C_{18} DIENES AS ATTRACTANTS FOR EIGHTEEN CLEARWING (SESIIDAE), TINEID (TINEIDAE), AND CHOREUTID (CHOREUTIDAE) MOTH SPECIES

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Abstract—By screening singly and binary mixed 2,13- and 3,13-octadecadien-yl acetates and alcohols (2,13- and 3,13-18: Ac/OH)in Lithuania, Armenia, Azerbaijan, Turkmenistan, Ukraine, and the far east of Russia, sex attractants were discovered for 12 Sesiidae, four Tineidae, and one Choreutidae moth species. Males of *Sesia yezoensis* and *Bembecia puella* as well as *Nemapogon flavifrons* were attracted by mixture of *Z*3,*Z*13-18:Ac/OH in a ratio 9:1, *Pyropteron* sp. n. by the same mixture (ratio 1:9), *Bembecia romanovi* and *B. zuwandica* by *Z*3,*Z*13-18:AC and *E*3,*Z*13-18:Ac (9:1), *Synanthedon caucasicum* by the same mixture in the opposite ratio (1:9), *B. scopigera* by 23,213-18:Ac and *E*2,*Z*13-18:OH in a ratio 9:1, *Synasphecia triannuliformis* by *Z*3,*Z*13-18:OH and *E*3,*Z*13-18:OH (9:1), *Similipepsis takizawai* and *Archimeessia sp. n. by E*3,*Z*13-18:OH and *E*2,*Z*13-18:Ac (1:1), *Prochoreutis sechestediana* by a mixture of *E*3,*Z*13-18:Ac plus *E*2,*Z*13-18:OH (1:), *Microsphecia brosiformis by E*3,*Z*13-18:Ac, *Synanthedon conopiformis* by the analogous alcohol, *Synanthedon scoliaeformis* and *Nemaxera betulinella* by *E*2,*Z*13-18:Ac, *Triaxomera fulvimitrella* by *Z*3,*Z*13-18:Ac. An analogous alcohol component is essential for the attraction of *B. ichneumoniformis* males. Inhibitors for *B. romanovi, B. scopigera* and *B. zuwandica* attraction were discovered. Preliminary data on attractants for six other species as well as on the diurnal rhythm of sexual activity of three species are presented. A new method for the stereoselective synthesis of 3,13-18:Ac/OH and *E*2,*Z*13-18:Ac/OH is described.

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Key Words—Lepidoptera, Sesiidae, Tineidae, Choreutidae, (*E*2,*Z*13)-octadecadien-1-ol/acetate, 3,13-octadecadien-1-ol/acetate, isomers, stereoselective synthesis, sex attractants, inhibitors, field trapping.

INTRODUCTION

At present, sex pheromones and attractants are known for about 1500–2000 moth species (Arn et al., 1986). This number is, however, small when compared with the large number of species in the order Lepidoptera and is insufficient to solve chemotaxonomic or evolutionary problems in pheromone communication systems. Hence, the search for attractants aimed at finding new chemical substances as well as the detection of new species with already known compounds remains of great importance.

The identification of sex attractants for clearwing species is equally likely to be of use in taxonomic research. Moths in this family are strong fliers, but are not captured by light traps and, consequently, are more difficult to collect than many other moth species. Many sesiids have limited distributions and appear to be relatively scarce. Thus, their attractants could be useful for both taxonomic and biological studies.

The main components of sex pheromones that have been identified from the females of sesiids are dienes with a chain length of 18 carbon atoms and unsaturation at the 13 as well as the 2 or 3 position (Tumlinson et al., 1974; Schwarz et al., 1983) and, possibly, a corresponding monoene with unsaturation at the same 13 position (Szöcs et al., 1985).

In the present paper we describe a new approach to the synthesis of some C_{18} dienes and field screening trials with a number of these compounds carried out in Lithuania, Armenia, Azerbaijan, Turkmenistan, Ukraine, as well as in the far east of Russia.

METHODS AND MATERIALS

Chemicals. Four isomers of 3,13-octadecadienes with functional alcohol and acetate groups, one isomer of 2,13-octadecadien-1-ol and the corresponding acetate, as well as one monoene with equal carbon chain lengths were used for field screening (Table 1).

Synthesis and Purity. The steps for the synthesis of 3,13-octadecadien-1-yl acetates and alcohols are shown in Figures 1 and 2. The methods for the synthesis of 3,13-octadecadienols are mainly based on the alkylation of metal-acetylides with protected 8-bromo-1-octanol (Uchida et al., 1978; Doolittle et al., 1980) or on the shift of the triple bond in 3-decyn-1-ol to the terminal position under superbasic media (Zhang et al., 1985; Teng and Yuan, 1985).

TABLE 1. C_{18} COMPOUNDS USED FOR SCREENING

Double Bond position	Functional group and isomer	
	Ac	OH
3,13	*ZZ*	*ZZ*
3,13	*EZ*	*EZ*
3,13	*EE*	*EE*
3,13	*ZE*	*ZE*
2,13	*EZ*	*EZ*
13	*Z*	*Z*

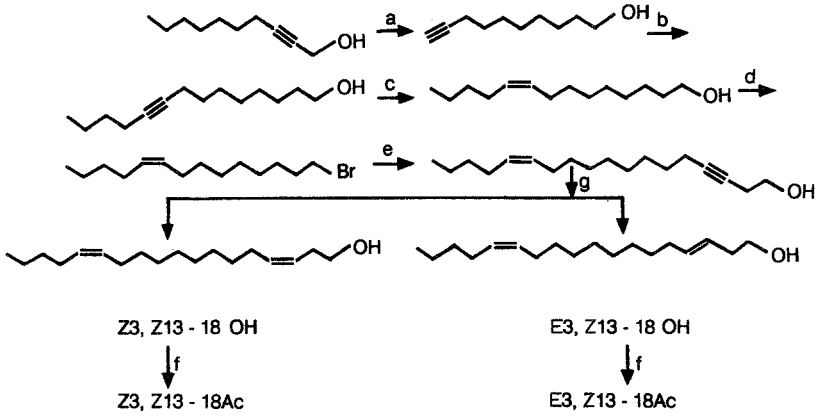

FIG. 1. Stereoselective synthesis of *Z,Z*- and *E,Z*-3,13-18:OH/Ac. For **a g** abbreviations, see Figure 3.

Judging by the yields and complexity of reactions, preference is given to the latter method.

The triple-bond shift in ethylenediamine was undertaken with $NaNH(CH_2)_2NH_2$ at 25°C. The reagent was obtained from $NaNH_2$ and ethylenediamine without specific removal of the ammonia formed. The key stage of the synthesis, namely, the reaction between the 9-tetradecenylhalide and tetrahydropyranyl ether of 3-butyn-1-ol resulted in a relatively low yield of 35%, which agreed with previously published data, but not with the results presented by Hoskovec et al. (1990), where the yield of the product is indicated to be up to 65%. For *E* reduction of the triple bond in 9-tetradecyn-1-ol and 13-octadecen-3-yn-1-ol, Rossi's method appeared to be appropriate (Rossi and Carpita,

1977). Preliminary data on the method of synthesis and the biological activity of (*Z,Z*) and (*E,Z*)-3,13-octadecadienols and their acetates were presented briefly by the present authors at the thirty-second IUPAC Conference (Mäeorg et al., 1989).

(*E*2,*Z*13)-octadecadien-1-ol and the corresponding acetate were synthesized according to the scheme given in Figure 3.

The reaction control and the purity of products were determined by GLC with the chromatograph Chrom-5 using a glass column 2.5 m × 2 mm packed with 10% PDEAS on Chromosorb W-HP 100–120 mesh. The isomer composition and the reduction control of the enynols were checked on a fused silica

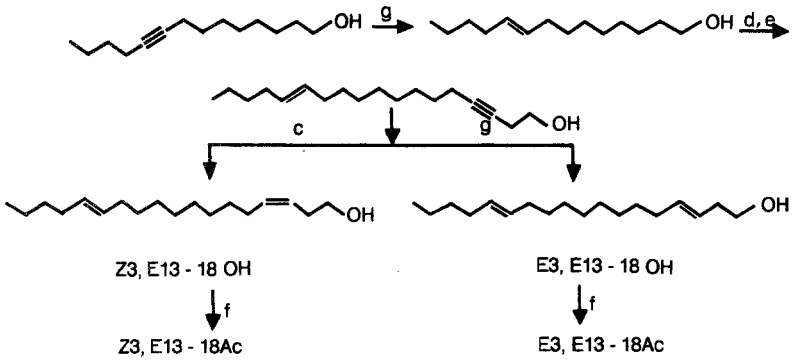

FIG. 2. Stereoselective synthesis of *Z,E* and *E,E* isomers of 3,13-18:OH/Ac. For **c–g** abbreviations, see Figure 3.

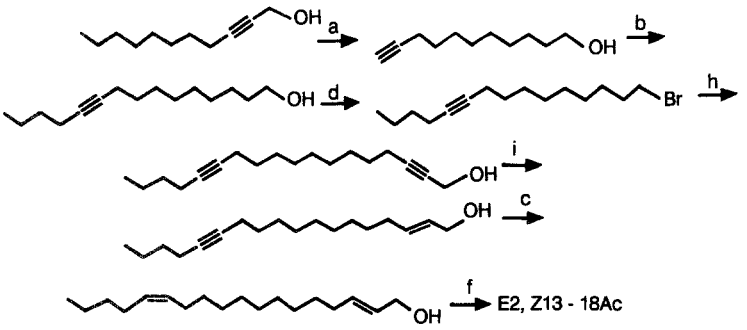

FIG. 3. Stereoselective synthesis of *E*2,*Z*13-18:Ac. **a:** $NaNH(CH_2)_2NH_2$/$H_2N(CH_2)_2NH_2$; **b:** DHP/H^+, $LiNH_2/NH_3$/BuBr, MeOH/H^+; **c:** NiP-2/$H_2N(CH_2)_2NH_2$/C_2H_5OH; **d:** TsCl/Py, LiBr/$(CH_3)_2CO$; **e:** $LiC{\equiv}(CH_2)_2$-$(CH_2)_2$-OTHP/THF/HMPA, MeOH/H^+; **f:** Ac_2O/Py; **g:** $LiAlH_4$/diglyme; **h:** Li-C≡CH_2OH/THF/HMPA, MeOH/H^+; **i:** $LiAlH_4$/THF.

capillary column 25 m × 0.2 mm coated with a liquid crystal *p*-methoxycinnamoyloxy-*p'*-methoxyazobenzol. The structure of products obtained was confirmed by IR and ^{13}C NMR spectroscopy with Spekord 711R and Bruker AC200P instruments, respectively. The isomer purity of *E*2,*Z*13–18:Ac/OH was over 99% and that of *E*3,*E*13–18:Ac/OH was 94.5%, whereas purity of other isomers was lower.

Some of the *E*2,*Z*13–18:Ac was obtained from Dr. S. Voerman (Wageningen); its isomeric purity exceeded 99%. The compound was used at the initial stage of the screening program.

*Z*13–18:Ac/OH was synthesized according to Klun et al. (1975), the isomeric purity exceeding 99%.

Field Experiments. Each compound tested was dissolved in hexane and applied to red rubber tube dispensers (18 × 15 mm; a single cap for compound mixtures), 0.5 mg/cap. Each lure was fixed into an opaque white delta trap measuring 10 × 11 × 10 cm, which had an exchangeable bottom (11 × 18 cm) coated with sticky material (Atracon A trap and Pestifix glue, both from Flora Co., Tartu, Estonia). Traps were fixed to bush or tree branches 0.5–1 m above the ground and were inspected every one or two days or once a week depending on total screening duration. The distance between traps was not less than 15 m.

Screening was carried out from 1986 to 1991 in Lithuania and various areas of European and Asian territories of the former USSR. The trapping period as well as the number of traps in each locality are described below. For brevity, localities are numbered and indicated in the text and Table 2 as follows:

I—Lithuania: **Ia,** vicinity of Vilnius (eastern Lithuania), May 22 to July 24, 1986, two C_{18} compounds were tested separately (see Table 2), six replicates; **Ib,** the same locality, June 23 to July 29, 1987, five replicates of each attractant noted in Table 2; **Ic,** Puvočiai, Varėna District (southern Lithuania), July 22–27, 1990, two replicates.

II—Azerbaijan, vicinity of Lenkoran (southern Azerbaijan), August 4–9, 1987, seven replicates of separate components or mixtures noted in Table 2.

III—Armenia, the Khosrov Reserve (southwestern Armenia), July 10–19, 1989, four replicates.

IV—Turkmenistan: **IVa,** Kara-Kala, Parkhai (southwestern Turkmenistan), September 17–21, 1991, three replicates for each compound and each mixture; **IVb,** Syunt Khoserdag Reserve, Yol-Dere Canyon, May 5–17, 1989, four replicates; **IVc,** the same locality, September 3–15, 1989, five replicates.

V—Far east of Russia: **Va,** Kedrovaya Pad Reserve, Primorye, July 26 to August 12, 1989, four replicates; **Vb,** Partizansky District, Barabash-Levada, July 14–30, 1989, four replicates.

VI—Ukraine, Crimea, Karadag Reserve: **VIa,** August 10–18, 1990, four replicates; **VIb,** July 4–14, 1991, three replicates.

Statistical Analysis. Data from the field tests were transformed $\sqrt{x + 0.5}$

where x is the number of moths captured per trap. Then they were submitted to analysis by Duncan's multiple-range test and significantly different means were separated.

Identification of Moth Species. Captured specimens were identified by analysis of genitalia. Scientific names are given according to Leraut (1980) and Zagulyaev (1964) and descriptions of new species according to Gorbunov (1986, 1987) and Arita and Špatenka (1989). *Bembecia ichneumoniformis* and *B. scopigera* were identified after Špatenka and Laštuvka (1990), *Microsphecia brosiformis* after Laštuvka (1985).

Representative specimens are kept in the insect collection at the Institute of Ecology, Vilnius, Lithuania.

RESULTS AND DISCUSSION

New sex attractants for 13 clearwing (Sesiidae), four tineid (Tineidae), and one choreutid (Choreutidae) moth species as well as inhibitors for three of the sesiids were found during the field screening trials. Detailed data are presented in Table 2. The following species were attracted:

Sesiidae

Microsphecia brosiformis Hubn. (= *Zenodoxus dorsalis* Le Cerf *after* Lastuvka, 1985). A total of 42 males were captured: eight in Armenia **(III)** and 34 in Turkmenistan **(IVa,c).** *E*3,*Z*13–18:Ac and two mixtures both containing 90% of this component showed highest attractivity, which differed statistically significant from all tested compositions (Table 2). The binary mixtures were no more attractive than a single *E*3,*Z*13–18:Ac, even tending to be less attractive; therefore, only *E*3,*Z*13–18:Ac should be considered a sex attractant for this species. Traps baited with this chemical are quite effective in trapping males.

It should be noted that during screening in Portugal the same substance was determined to be a sex attractant for *M. tineiformis* Esp., a very closely related species (Nielsen et al., 1979). This species and *M. brosiformis* were for a time considered synonymous (see Heppner and Duckworth, 1981) and only recently were they classified as two separate species (Laštuvka, 1985). It is not quite clear if the sex attractants for both species are indeed the same. It is highly possible that moths trapped by Nielsen et al. (1979) could be classified as *M. brosiformis* following the latest morphological study (Laštuvka, 1985). For that reason, clearwings trapped to *E*3,*Z*13–18:Ac in Portugal should be reinvestigated and their taxonomy verified. In case the representative specimens are no longer available, *M. tineiformis* sex attractant composition should, in our opinion, be reinvestigated.

Similipepsis takizawai Arita et Spatenka. A total catch of 34 males of the

species was obtained in the Far East **(Va, Vb).** Most moths (15) were attracted to a mixture of *E*3,*Z*13–18:OH and *E*2,*Z*13–18:Ac in a ratio of 1:1. This is the first attractant blend for the genus and the first record of the species outside Japan.

Sesia yezoensis Hampson. Thirty-six males were trapped in the far east of Russia **(Va, Vb).** Most of them were attracted to a mixture of *Z*3,*Z*13–18:Ac and *Z*3,*Z*13–18:OH in a ratio of 9:1 (Table 2). No attractivity was registered, when both components were tested separately or in different ratios.

Synanthedon caucasicum Gorbunov. Captures in Azerbaijan **(II)** totaled 145 males. The most attractive blend for them was that of *Z*3,*Z*13–18:Ac and *E*3,*Z*13–18:Ac in a ratio of 1:9. Separately, the components were not attractive, and only one moth was captured at baits with the main component *E*3,*Z*13–18:Ac. Mixtures in other ratios were ineffective. When alcohol (*E*3,*Z*13–18:OH) is used in the mixture with *E*3,*Z*13–18:Ac, the blend is also attractive, although significantly less than the two acetates. Thus, the *E*3,*Z*13 alcohol might be a syntergist; however, to determine this, tests with three-component mixtures should be made. It is noteworthy that *S. caucasicum* is morphologically very closely related to *S. vespiformis* L. (Gorbunov, 1986), as is the chemical composition of their sex attractants (the present paper and Voerman et al., 1983). Preference of the same attractant indicates that interspecific isolation is unlikely to be determined by differences in pheromone composition.

Our previous indication on *S. caucasicum* single-component attractant (Mäeorg et al., 1989) should be considered as preliminary. A two-component mixture for the attraction of males of this species is necessary.

Synanthedon conopiformis Esp. A total of 25 males were trapped in Lithuania **(Ib).** Five binary mixtures of 13 used were attractive, the common component being *E*3,*Z*13–18:OH. Of the compounds tested singly, only this alcohol was attractive. The total number of moths captured was small, as this species is scarce in the trapping area. Indeed, the species has been recently included in the Red Data Book (of rare species) of Lithuania. The attractant *E*3,*Z*13–18:OH is effective enough for monitoring *S. conopiformis* populations, as was proved by our special program for monitoring this rare species throughout Lithuania, started in 1990.

Synanthedon scoliaeformis Borkh. In Lithuania *(Ia)* 174 moths were captured in traps baited with a single component, *E*2,*Z*13–18:Ac. As it appeared to be an effective attractant, binary mixtures with other C_{18} dienes were not tested.

Pyropteron sp. n. Twenty-eight males were captured in traps baited with a mixture of *Z*3,*Z*13–18:Ac and *Z*3,*Z*13–18:OH in Turkmenistan **(IVb).** The most attractive blend for them was a ratio of 1:9. Three males were attracted to the mixture of the same alcohol and *E*2,*Z*13–18:Ac in a ratio of 1:1.

Synasphecia triannuliformis Freyer. Seventy-three males were trapped in

TABLE 2. TRAP CAPTURES OF MOTHS WITH DIFFERENT C_{18} DIENES AND THEIR BINARY MIXTURES*

Chemical	Ratio	Sesiidae					
		Microsphecia brosiformis	*Similipepsis takizawai*	*Sesia yezoensis*	*Synanthedon caucasicum*	*Synanthedon conopiformis*	*Synanthedon scoliaeformis*
Field testing location		III, IVa, IVc	Va, Vb	Va, Vb	II	Ia, 1b	Ia
Z3,Z13-18:Ac		•	•	4ab	•	•	—
Z3,Z13-18:Ac + Z3,Z13-18:OH	1:1	•	•	•	•	•	•
Z3,Z13-18:Ac + Z3,Z13-18:OH	9:1	•	•	19c	—	—	—
Z3,Z13-18:Ac + Z3,Z13-18:OH	1:9	•	•	•	—	—	—
Z3,Z13-18:Ac + E3,Z13-18:Ac	1:1	1a	•	•	•	•	—
Z3,Z13-18:Ac + E3,Z13-18:Ac	9:1	•	•	2ab	—	—	—
Z3,Z13-18:Ac + E3,Z13-18:Ac	1:9	9b	•	•	97b	•	—
Z3,Z13-18:Ac + E3,Z13-18:OH	1:1	•	•	1a	•	1a	—
Z3,Z13-18:Ac + E3,Z13-18:OH	9:1	•	•	7b	—	—	—
Z3,Z13-18:Ac + E3,Z13-18:OH	1:9	•	5ab	•	•	3ab	—
Z3,Z13-18:Ac + E3,E13-18:Ac	1:1	•	•	1a	—	—	—
Z3,Z13-18:Ac + E3,E13-18:OH	1:1	•	•	•	—	—	—
Z3,Z13-18:Ac + E2,Z13-18:Ac	1:1	•	•	2ab	—	—	—
Z3,Z13-18:Ac + E2,Z13-18:Ac	9:1	•	—	—	—	—	—
Z3,Z13-18:Ac + E2,Z13-18:Ac	1:9	•	—	—	—	—	—
Z3,Z13-18:Ac + E2,Z13-18:OH	1:1	•	—	—	—	—	—
Z3,Z13-18:Ac + E2,Z13-18:OH	9:1	•	—	—	—	—	—
Z3,Z13-18:Ac + E2,Z13-18:OH	1:9	•	—	—	—	—	—
Z3,Z13-18:OH		•	•	•	•	•	—
Z3,Z13-18:OH + E3,Z13-18:Ac	1:1	1a	•	•	—	—	—
Z3,Z13-18:OH + E3,Z13-18:Ac	9:1	•	•	•	—	—	—
Z3,Z13-18:OH + E3,Z,13-18:Ac	1:9	11b	•	•	6a	•	—
Z3,Z13-18:OH + E3,Z13-18:OH	1:1	•	•	•	•	•	—
Z3,Z13-18:OH + E3,Z13-18:OH	9:1	•	•	•	—	—	—
Z3,Z13-18:OH + E3,Z13-18:OH	1:9	•	•	•	•	3ab	—
Z3,Z13-18:OH + E3,E13-18:Ac	1:1	•	•	•	—	—	—
Z3,Z13-18:OH + E3,E13-18:OH	1:1	•	•	•	—	—	—
Z3,Z13-18:OH + E2,Z13-18:Ac	1:1	•	•	•	—	—	—
Z3,Z13-18:OH + E2,Z13-18:OH	1:1	•	—	—	—	—	—
E3,Z13-18:Ac		17b	•	•	1a	•	—
E3,Z13-18:Ac + E3,Z13-18:OH	1:1	•	•	•	21a	•	—
E3,Z13-18:Ac + E3,Z13-18:OH	9:1	1a	•	•	15a	•	—
E3,Z13-18:Ac + E3,Z13-18:OH	1:9	•	5ab	•	•	3ab	—
E3,Z13-18:Ac + E3,E13-18:Ac	1:1	•	•	•	—	—	—
E3,Z13-18:Ac + E3,E13-18:OH	1:1	2a	•	•	—	—	—
E3,Z13-18:Ac + E2,Z13-18:Ac	1:1	1a	•	•	5a	•	—
E3,Z13-18:Ac + E2,Z13-18:OH	1:1	1a	—	—	—	—	—
E3,Z13-18:OH		•	•	•	•	11b	—
E3,Z13-18:OH + E3,E13-18:Ac	1:1	•	7ab	•	—	—	—
E3,Z13-18:OH + E3,E13-18:OH	1:1	•	2a	•	—	—	—
E3,Z13-18:OH + E2,Z13-18:Ac	1:1	•	15b	•	—	4b	—
E3,E13-18:Ac		•	•	•	—	—	—
E3,E13-18:OH		•	•	•	—	—	—
E2,Z13-18:Ac		•	•	•	•	•	174b
E2,Z13-18:OH		•	—	—	—	—	—
Total		42	34	36	145	25	174

Sesiidae							Tineidae				Choreutidae
Pyropteron sp.n.	*Synasphecia triannuliformis*	*Bembecia romanovi*	*Bembecia ichneumoniformis*	*Bembecia scopigera*	*Bembecia puella*	*Bembecia zuwandica*	*Archimeessia* sp.n.	*Triaxomera fulvimitrella*	*Nemaxera betulinella*	*Nemapogon flavifrons*	*Prochoreutis sehestediana*
IVb	III	III	III	VIa, VIb	VIa, VIb	III	Va, Vb	Ia	Ia	IVb	Ic
•	•	13b	•	34b	•	10bc	•	110b	—	12b	•
2a	•	•	•	2a	7b	•	•	—	•	81c	1a
•	•	•	•	•	32c	5b	•	—	—	121d	•
23b	1a	•	4ab	6a	2ab	•	•	—	—	69c	•
•	•	2ab	•	•	•	•	•	—	—	11b	•
•	•	22c	•	9a	1ab	19c	•	—	—	20b	•
•	•	•	•	•	•	•	•	—	—	11b	•
•	•	•	•	2a	2ab	•	•	—	—	12b	•
•	•	1a	•	6a	2ab	•	•	—	—	10b	•
•	•	•	•	•	4ab	•	•	—	—	9b	•
•	•	7b	•	—	—	•	•	—	—	•	•
•	•	•	•	—	—	•	•	—	—	•	•
•	•	3ab	•	5a	•	3ab	•	—	—	15b	•
—	—	—	—	18ab	•	—	—	—	—	—	•
—	—	—	—	2a	•	—	—	—	—	—	—
•	—	—	—	9a	•	—	—	—	—	•	6b
—	—	—	—	71c	•	—	—	—	—	—	•
—	—	—	—	1a	1ab	—	—	—	—	—	—
•	10b	•	2a	•	•	•	—	—	—	•	•
•	1a	•	•	•	•	•	•	—	—	•	•
•	3ab	•	8b	•	•	•	•	—	—	•	•
•	•	•	•	•	•	•	•	—	—	•	•
•	13b	•	2a	•	•	•	•	—	—	•	1a
•	28c	•	2a	•	•	•	•	—	—	•	•
•	3ab	•	•	•	•	•	•	—	—	•	•
•	2a	•	2a	—	—	•	•	—	—	12b	•
•	5ab	•	1a	—	—	•	•	—	—	13b	•
3a	3ab	•	5b	•	1ab	•	•	—	—	•	•
•	•	•	•	•	•	•	—	—	—	•	5b
•	4ab	•	•	•	•	•	•	—	—	•	•
•	•	•	•	•	•	•	•	—	—	•	•
•	•	•	•	•	•	•	•	—	—	•	4ab
•	•	•	•	•	•	•	•	—	—	•	•
•	•	•	•	—	—	•	•	—	—	•	•
•	•	•	•	—	—	•	•	—	—	•	•
•	•	•	•	•	•	•	•	—	—	•	•
•	—	—	—	•	•	—	—	—	—	•	17c
•	•	•	•	•	•	•	•	—	—	•	•
•	•	•	•	—	—	•	•	—	—	•	•
•	•	•	•	—	—	•	•	—	—	•	•
•	•	•	•	•	•	•	260b	—	—	•	•
•	•	•	•	—	—	•	•	—	—	•	•
•	•	•	•	—	—	•	•	—	—	•	•
•	•	•	•	•	•	•	•	•	57b	•	•
—	•	•	•	•	•	•	—	—	—	—	•
28	73	48	26	183	52	37	260	110	57	296	34

*Figures are numbers of moths trapped; numbers in a column followed by different letters differ statistically significantly at $P < 0.05$; dash: not tested; dot: tested, but moths were not trapped and catch corresponds to 0a.

Armenia **(III).** They were attracted by *Z*3,*Z*13–18:OH alone, as was previously shown by Szöcs et al. (1989). However, attractivity increased almost threefold when combined with *E*3,*Z*13–18:OH at a ratio of 9:1 (difference is statistically significant at $P < 0.05$). Almost all binary mixtures tested, which contained *Z*3,*Z*13–18:OH, were attractive for males of this species; therefore, an obvious inhibitor cannot be determined. Sexual activity of *S. triannuliformis* males was observed in the evening before sunset (two moths were attracted to traps between 8:15 and 8:40 PM).

Bembecia romanovi **Bartell.** Forty-eight males were trapped in Armenia **(III).** They were mostly attracted by a mixture of *Z*3,*Z*13–18:Ac and *E*3,*Z*13–18:Ac in a ratio of 9:1. The *ZZ* isomer alone was also a good attractant, although the capture decreased almost by half (Table 2). The *EZ* isomer did not attract any males when applied individually. Captures of *B. romanovi* males with *Z*3,*Z*13–18:Ac as the main attractant component were strongly inhibited by *Z*3,*Z*13–18:OH. Their mixtures did not attract a single moth. A less pronounced inhibitor for males of the species was *E*3,*Z*13–18:OH; only one male was trapped when the compound was added to *Z*3,*Z*13–18:Ac. It should be noted that both inhibitors differ from the attractant components only by the functional group (alcohol).

It is interesting that a melanic form, previously undescribed, was trapped together with the typical golden form of *B. romanovi* males. Melanic individuals comprised 13.3% of the total catch. There were no significant differences between their preferences for attractants.

Males appeared to be attracted to the traps in the morning. Captures were registered between 9:20 and 10:00 AM (based on six males).

Bembecia ichneumoniformis **Den. et Schiff.** ***(sensu*** **Spatenka and Lastuvka,** ***1990).*** Twenty-six months were trapped in Armenia **(III),** of the 26 males trapped with different compositions, most were attracted to three mixtures all containing the same component, namely *Z*3,*Z*13–18:OH (Table 2).

The alcohol is significantly less attractive than binary mixtures with either *E*3,*Z*13–, *E*2,*Z*13– or *Z*3,*Z*13–acetates, which indicates that an acetate admixture increases the attractivity of the alcohol. However, we could not determine which of the three acetates was most effective, because the differences were not statistically significant due to the small number of replicates (four) and short period of trapping (10 days). Therefore, to optimize the attractant composition, attempts should be made to find a coattractant among the acetates.

Bembecia scopigera **(Scopoli),** ***sensu*** **Špatenka and Lastuvka (1990).** One hundred eighty-three males were trapped in the Crimea **(VIa,b).** Males were attracted to traps baited with *Z*3,*Z*13–18:Ac alone, as well as to 11 different binary mixtures containing this chemical. It proved to be an effective attractant; however, the highest attractivity was observed for lure containing *Z*3,*Z*13–18:Ac and *E*2,*Z*13–18:OH at a ratio of 9:1 (Table 2). The effectiveness of

the mixture was significantly higher compared to that of all mixtures tested; therefore, it should be considered the optimal attractant for *B. scopigera* males.

Admixture of *E*3,*Z*13–18:Ac (in a 1:1 ratio) to *Z*3,*Z*13–18:Ac, the main component of the attractive blend, which is also effective when applied singly, is fully inhibiting to *B. scopigera* male capture, so this acetate is potential inhibitor of the most effective attractant blend.

It is interesting to note that although *E*2,*Z*13–18:OH was found in pheromone glands of clearwings (Klun et al., 1990), there have so far been no indications that it could modify males' behavior.

According to the screening data obtained in Hungary, the attractant determined for *B. scopigera* males was *Z*3,*Z*13–18:OH (Szöcs et al., 1989). Authors of the publication mentioned consider *B. scopigera* to be synonymous to *B. ichneumoniformis*, although both taxa have obvious morphological differences and are classified as separate species (Špatenka and Laštuvka, 1990). The data we obtained on differences in the sex attractant composition for both taxa also indicate them to be two separate species.

B. scopigera specimens trapped in Hungary are no longer available (Szöcs, personal communication); therefore, it is impossible to reinvestigate their genitalia. In our opinion, clearwings trapped in Hungary and considered as *B. scopigera* should be attributed to *B. ichneumoniformis* (sensu Špatenka and Laštuvka, 1990).

Bembecia puella Lastuvka. In the Karadag Reserve, Crimea, **(VIa,b)** 52 males were trapped. Most of them (32) were attracted to traps with a mixture of *Z*3,*Z*13–18:Ac and *Z*3,*Z*13–18:OH at a 9:1 ratio. Attractivity to the mixture is highest (Table 2). The same mixture at a 1:1 ratio trapped *B. puella* males, but significantly fewer. Separate components of the mixture were not effective.

Bembecia zuwandica Gorbunov. Thirty-seven males were trapped in Armenia **(III).** Most males were trapped by a mixture of *Z*3,*Z*13–18:Ac with *E*3,*Z*13–18:Ac at a ratio of 9:1 (Table 2). A single *Z*3,*Z*13–18:Ac also appeared to be attractive for males of this clearwing species. The alcohol *E*3,*Z*13–18:OH, which corresponds to the minor attractant component with an acetate functional group, appears to be an inhibitor for this species. It fully inhibited attractivity of *Z*3,*Z*13–18:Ac (Table 2).

Males were trapped in the late morning between 10:05 AM and 12:45 PM (based on 22 males).

It is interesting to note that *B. zuwandica* males are attracted to the same mixture and at the same habitat as *B. romanovi*. Although our observations were based on attractants only, and the actual pheromone blends for the two species could differ slightly, we can assume that the difference in the diurnal rhythm of sexual activity is of great importance for the interspecific isolation of *B. zuwandica* and *B. romanovi*. However, their activity periods overlap slightly; there-

fore, additional isolating mechanisms, e.g., either behavioral, morphological, etc., might be involved.

Trapping data for the remaining sesiid species are not presented in Table 2 as they are either preliminary or only confirm some preliminary data obtained earlier by other authors.

Synanthedon culiciformis L. In Lithuania **(Ia),** 179 males were trapped using *Z*3,*Z*13–18:Ac as bait. It appeared to be a good attractant, and no tests with other potential synergists were undertaken. Our results confirm the data of Fassotte et al. (1986), which are considered as preliminary by Arn et al. (1986).

Screening in Armenia **(III)** and Turkmenistan **(IV)** provided data on sex attractants for another five clearwing species. For a number of reasons, captures of these species were low, so the data can be considered only as preliminary.

Bembecia parthica Led. Five males were captured by traps baited with a mixture of *Z*3,*Z*13–18:Ac and the corresponding alcohol during a three day period. The mixture was more effective at a ratio of 9:1 in **III.**

Bembecia chalciformis Esp. Sixteen males were attracted and trapped in **III** and **IVb** by six different binary mixtures containing *Z*3,*Z*13–18:OH. This compound was unattractive when used singly and effective in a mixture with two acetates. Admixture of *Z*3,*Z*13–18:Ac was effective in Turkmenistan (11 males) and admixture of *E*3,*Z*13–18:Ac attracted moths in Armenia (five males).

Chamaesphecia affinis Staud. Five males were trapped in **III,** four at a mixture of the diene–monoene *E*3,*Z*13–18:Ac and *Z*13–18:OH in a ratio of 1:1. Individual components were not attractive. One male was attracted to a mixture of *E*3,*Z*13–18:Ac and *Z*3,*Z*13–18:OH.

Chamaesphecia sp. n. Eight males were trapped in Turkmenistan **(IVb),** all in two traps with a mixture of *E*3,*Z*13–18:Ac and *E*2,*Z*13–18:Ac in a 1:1 ratio.

Zenodoxus hoplisiformis Mann. Seven males were trapped in **III.** A mixture of *E*3,*Z*13–18:Ac and *E*2,*Z*13–18:Ac appeared to be most attractive at a ratio of 1:1. Two males were captured at a mixture containing the same first component and *E*2,*Z*13–18:OH.

Tineidae

Archimeessia sp. n. In the Far East, 260 males were trapped at the mixture of *E*3,*Z*13–18:OH and *E*2,*Z*13–18:Ac at a ratio of 1:1 (Table 2). All other lures were not attractive. This is the first report of *Archimeessia* tineids captured with synthetic sex attractants.

Tiaxomera fulvimitrella Sod. In Lithuania **(Ia),** 110 males were captured (see Table 2) with *Z*3,*Z*13–18:Ac alone. Mixtures with other components were not tested.

Nemaxera betulinella F. (=corticella *Curt.).* Fifty-seven males of this species were trapped in Lithuania **(Ia),** at *E*2,*Z*13–18:Ac alone (Table 2).

Nemapogon flavifrons Pt. In Turkmenistan **(IVb,c)** 396 moths were trapped. A mixture of *Z*3,*Z*13–18:Ac with a corresponding alcohol in a ratio of 9:1 was the best attractant. When applied singly, the first component was attractive, although considerably less as compared to the binary mixture, whereas the attractivity of the second component was not registered (Table 2).

We obtained data on the sex attractant for another tineid species *Nemapogon clematella* F. (= *arcellus* F.). In Lithuania **(Ia),** 182 males were trapped with *Z*3,*Z*13–18:Ac. This confirms the data of Fassotte et al. (1986), which are considered as preliminary by Arn et al. (1986).

In Russia's far east **(Vb),** 33 males of tineid *Celestica angustipennis* H.-S. were attracted to the blend of *E*3,*Z*13–18:OH and *E*3,*E*13–18:Ac at a ratio 1:1. All moths were caught in only two traps of 4 with the same mixture applied. Therefore, the result should be viewed as preliminary only. It is interesting to note that this species, traditionally considered an inhabitant of Europe and western Asia, was traced in the Far East, which indicates its habitat to be very wide indeed, from the Atlantic in the west to the Pacific in the east.

The presence of dienic alcohols in the attractant blend for *N. personellus* (Szöcs et al., 1989) as well as for *N. flavifrons, T. fulvimitrella,* and apparently for *C. angustipennis* (data of the present paper) suggests that they are as common in the family as the acetates (see Arn et al., 1986).

Choreutidae

Prochoreutis sehestediana F. (= *punctosa* Hw.). Forty-one males were trapped in Lithuania **(Ic).** Most males were trapped by a mixture of *E*3,*Z*13–18:Ac and *E*2,*Z*13–18:OH at a ratio of 1:1 (Table 2). The alcohol in a mixture with either *Z*3,*Z*13–18:Ac or a corresponding alcohol (at a 1:1 ratio) is slightly attractive (Table 2). Captures of the species were low due to very unfavorable weather conditions: during the whole trapping period only two days were without rain.

The present report on sex attractant composition for *P. sehestediana* males is the first recording within the Choreutidae family; another species of the family is attracted by a mixture of (*E*2,*Z*13)-octadecadienol/acetate, as was demonstrated using chromatographically purified chemicals (Būda and Schwarz, in preparation).

It is noteworthy that the similarity in attractant composition within the Sesiidae and Choreutidae families is not accidental. Taxonomically both families are closely related and belong to the same superfamily Sesioidae. A much more complicated question is the relatedness of the families Sesiidae and Choreutidae to Tineidae. Namely, is the similarity in the attractant (pheromone) chemical composition between the latter family and Sesiidae/Choreutidae completely accidental?

The results obtained on the high similarity in the attractant composition for moths of Sesiidae and Choreutidae families suggest that these or very similar compounds could be effective sex attractants for the Brachodidae family as well, because all three families are classified as one superfamily.

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REFERENCES

ARITA, Y., and ŠPATENKA, K. 1989. A new species of *Similipepsis* (Lepidoptera, Sesiidae) from Japan. *Jpn. J. Entomol.* 157:61-66.

ARN, H., TÓTH, M., and PRIESNER, E. 1986. List of sex pheromones of Lepidoptera and related attractants. *Paris Int. Org. Biol. Control.* 123.

DOOLITTLE, R.E., PROVEAUX, A.T., and HEATH, R.R. 1980. Synthesis of sex pheromones of lesser peachtree borer and peachtree borer. *J. Chem. Ecol.* 6:271-284.

FASSOTTE, C., FREROT, B., CHAMBON, J.-P., and DESCOINS, C. 1986. Piégeage sexuel de seize espèces de microlepidoptères nuisibles en vergers de pommiers Belges. *Bull. OILB-SROP* 00:145-152.

GORBUNOV, O.G. 1986. A new species of clearwing moth of the genus *Synanthedon* (Lepidoptera, Sesiidae) from Azerbaijan. *Zool. Zh.* 165:938-940 (in Russian).

GORBUNOV, O.G. 1987. A new species of the genus *Bembecia* (Lepidoptera, Sesiidae) from Talysh. *Vestn. Zool.* 13:12-18 (in Russian).

HEPPNER, J.B., and DUCKWORTH, W.D. 1981. Classification of the superfamily Sesioidea (Lepidoptera: Ditrysia). *Smithson. Contrib. Zool.* 314:1-144.

HOSKOVEC, M., SAMAN, D., and KOUTEK, B. 1990. A convenient synthesis of 2,13- and 3,13-octadecadienyl acetates, sex pheromone components of the *Synanthedon* species. *Collect. Czech. Chem. Commun.* 55:2270-2281.

KLUN, J.A., CHAPMAN, O.L., MATTES, K.C., and BEROZA, M. 1975. European corn borer and redbanded leafroller: Disruption of reproduction behavior. *Environ. Entomol.* 14:871-876.

KLUN, J.A., SCHWARZ, M., LEONHARDT, B.A., and CANTELO, W.W. 1990. Sex pheromone of the female squash vine borer (Lepidoptera: Sesiidae). *J. Entomol. Sci.* 25:64-72.

LAŠTUVKA, Z. 1985. On the taxonomy of *Microsphecia tineiformis* (Esper) and *M. brosiformis* (Hübner) (Lepidoptera). *Acta Univ. Agric. (Brno), Fac. Agron.* 33:183-190.

LAŠTUVKA, Z. 1989. *Bembecia puella* sp. n. aus der Slowakei (Lepidoptera, Sesiidae). *Scripta Fac. Sci. Nat. Univ. Purk. Brun.* 19:85-92.

LERAUT, P. 1980. Liste systematique et synonymique des Lepidoptères de France, Belgique et Corse. Paris. Soc. entomol de France. 333 pp.

MÄEORG, U.J., BŪDA, V.G., KOLONISTOVA, S.F., and KARALIUS, V.A. 1989. Synthesis and biological activity of two isomers of 3,13-octadecadienols and their acetates as attractants for Lepidoptera. Abstracts 32nd IUPAC Congress, August 2-7, Stockholm, p. 71.

NIELSEN, D.G., PURRINGTON, F.F., and SHAMBAUGH, G.F. 1979. EAG and field responses of sesiid males to sex pheromones and related compounds. Pheromones of the Sesiidae (formerly Aegeriidae). *Agric. Res. Results, Northeast. Ser.* 6:11-26.

ROSSI, R., and CARPITA, A. 1977. Insect pheromones. Stereoselective reduction of β- or ω-alkynols to the corresponding (*E*)-alkenols by lithium tetrahydroaluminate. *Synthesis* 8:561–562.

SCHWARZ, M., KLUN, J.A., LEONHARDT, B.A., and JOHNSON, D.T. 1983. (*E,Z*)-2,13-octadecadien-1-ol acetate. A new pheromone structure for sesiid moths. *Tetrahedron Lett.* 124:1007–1010.

ŠPATENKA, K., and LAŠTUVKA, Z. 1990. Zur Taxonomie von *Bembecia scopigera* (Scopoli, 1763), *B. ichneumoniformis* (Denis and Schiffermuller, 1775) und *B. albanensis* (Rebel, 1918) (Lepidoptera, Sesiidae). *Entomofauna, Z. Entomol.* 111:109–121.

SZÖCS, G., SCHWARZ, M., SZIRAKI, G., TÓTH, M., KLUN, J.M., and LEONHARDT, B.A. 1985. Sex pheromone of the female currant borer, *Synanthedon tipuliformis*: Identification and field evaluation. *Entomol. Exp. Appl.* 139:131–133.

SZÖCS, G., TÓTH, M., SZIRAKI, G., and SCHWARZ, M. 1989. 2,13- and 3,13-octadecadienyl compounds composing sex attractants for tineid and sesiid moths (Lepidoptera). *Biochem. Syst. Ecol.* 117:417–422.

TENG, Y.-W., and YUAN, C. 1985. Migration of the triple bond of primary acetylenic alcohol to terminal one with lithium ethylenediamide in ethylendiamide. *Acta Chim. Sinica.* 43:90–91.

TUMLINSON, J.H., YONCE, C.E., DOOLITTLE, R.E., HEATH, R.R., GENTRY, C.R., and MITCHELL, E.R. 1974. Sex pheromones and reproductive isolation of the lesser peachtree borer and the peachtree borer. *Science* 185:614–616.

UCHIDA, M., MORI, K., and MATSUI, M. 1978. Synthesis of (*Z,Z*)-3,13-octadecadienyl acetate and its (*E,Z*)-isomer, the attractant for the cherrytree borer. *Agric. Biol. Chem.* 42:1067–1070.

VOERMAN, S., AUDERMARD, H., and PRIESNER, E. 1983. Sex attractants for clearwing moth: *Synanthedon vespiformis* and *Chamaesphecia tenthrediniformis* (and/or *C. empiformis*). *Entomol. Exp. Appl.* 134:203–205.

ZAGULYAEV, A.K. 1964. Fauna of the USSR. Moths, Vol. 4. Pt. 2. Nauka Press, Moscow (in Russian).

ZHANG XUEHAI, GUO GUANGZHONG, LIN GUOGIANG, WU YUANWEI, WU PEIHENG, LI ZHENYU, and WEI KANGNIAN. 1985. Studies on the identification and synthesis of insect pheromones. XXII. Sex pheromones of poplar twig clearwing moth *Paranthrene tabaniformis* Rott. *Org. Chem.* 2:184–187.

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IRIDOID GLYCOSIDES OF *Chelone glabra* (SCROPHULARIACEAE) AND THEIR SEQUESTRATION BY LARVAE OF A SAWFLY, *Tenthredo grandis* (TENTHREDINIDAE)

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Abstract—Analysis of *Chelone glabra* (Scrophulariaceae) by gas chromatography showed that leaves of this plant contained primarily the iridoid glycoside catalpol, and in a few individuals some aucubin was also detected. There was no difference in the iridoid glycoside content of damaged compared to undamaged plants, nor was there a difference between plants collected from a population in Leverett, Massachusetts, and those from plants in an experimental garden in Cambridge, Massachusetts. Larvae and prepupae of the sawfly, *Tenthredo grandis* (Tenthredinidae) contained catalpol sequestered from the larval host plant. The exuvia also contained catalpol, whereas the frass contained only aucubin. These results indicate that larvae of this sawfly selectively sequester catalpol, eliminating the aucubin in the frass.

Key Words—*Chelone glabra*, iridoid glycoside, plant–insect interactions, sawfly, sequestration, *Tenthredo grandis*, Hymenoptera, Tenthredinidae.

INTRODUCTION

Iridoid glycosides are sequestered from their host plants by a variety of lepidopteran larvae (Bowers, 1988, 1991; Rimpler, 1991) and may render these

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larvae and, in some species, the adults, unpalatable to potential predators (Bowers, 1980, 1981; Bowers and Farley, 1990). Sawfly larvae (Symphyta: Hymenoptera) are also phytophagous, have an appearance very like lepidopteran caterpillars, and show similar behaviors. For example, they can consume substantial amounts of foliage, move from one plant to another to feed, and may be gregarious or solitary (Prop, 1960; Boeve and Pasteels, 1985; Bjorkman and Larsson, 1991). Some sawfly larvae are unpalatable (Prop, 1960; Eisner et al., 1974; Morrow et al., 1976), usually due to regurgitation of the contents of a foregut diverticulum, which contain chemicals derived from the larval host plant (Eisner et al., 1974; Morrow et al., 1976; Bjorkman and Larsson, 1991).

Larvae of the sawfly, *Tenthredo grandis* (Tenthredinidae), feed on turtlehead, *Chelone glabra* (Scrophulariaceae). *Chelone glabra* contains two iridoid glycosides, aucubin and catalpol (Bowers and Fixman, unpublished data). These sawfly larvae are conspicuously colored white with black markings and are quite apparent when resting or feeding on the host plant. Feeding experiments with blue jays showed that these larvae (or the very similarly colored larvae of another species, *Macrophya nigra*, Tenthredinidae; the two species were not distinguished in the feeding trials) were unpalatable and emetic to these birds (Bowers, 1980). Larvae of the checkerspot butterfly, *Euphydryas phaeton* (Nymphalidae) also feed on *C. glabra* and are unpalatable and emetic to blue jays, as are the adults (Bowers, 1980). *Euphydryas phaeton* larvae sequester iridoid glycosides from their host plants, and these compounds are responsible for their unpalatability (Belofsky et al., 1989; Bowers, 1991, 1992). The feeding experiments with blue jays suggested that these sawfly larvae may sequester iridoid glycosides as well.

Sawfly larvae and checkerspot larvae may cooccur in populations of *C. glabra* (Stamp, 1984; Wiernasz, personal observation; Bowers, personal observation). The life history of these two insects is such that the sawfly larvae, which feed in late June and July, may be feeding on plants that have been previously fed upon by checkerspot larvae, which feed in May and early June. Iridoid glycosides in another plant species, *Plantago lanceolata* (Plantaginaceae) may increase in response to larval feeding (Bowers and Stamp, 1993), thus feeding by checkerspot larvae may alter the iridoid glycoside content of turtlehead. The sawfly larvae, therefore, may be feeding on plants that have been induced by previous herbivory.

We collected larvae of *T. grandis* from a single population in Leverett, Massachusetts, to determine if they sequestered iridoid glycosides, and in what amounts. We also collected samples of *C. glabra* from this same location, which were either previously damaged by herbivores, or were undamaged, to determine the iridoid glycoside profile of plants on which sawfly larvae were feeding. For comparison, we collected samples of *C. glabra* from an experimental garden in Cambridge, Massachusetts, that had no herbivore damage.

METHODS AND MATERIALS

Plant Material. *Chelone glabra* samples were collected on May 14, 1988, from a population in Leverett, Massachusetts, and on June 1, 1988, from plants growing in a garden plot at Harvard University, in Cambridge, Massachusetts (14 samples, each from a different individual plant). The *C. glabra* plants growing in Cambridge had been transplanted from the Leverett population three years earlier and had no observable herbivory. Plant samples from Leverett were either damaged by checkerspot caterpillars (*Euphydryas phaeton*) ($N = 10$), or were undamaged ($N = 10$). For each sample, an entire stalk of *C. glabra* was clipped at ground level. All plant material was transported on ice to the laboratory where it was immediately dried at 50°C. For analysis using gas chromatography, all leaves were removed from each stem and ground together to a fine powder; thus each sample was composed of all leaves (of varying ages) from a single stalk.

Insect Material. Sawfly larvae were collected from the *C. glabra* population in Leverett, Massachusetts, in two different years. In July 1986, actively feeding last-instar sawfly larvae were collected, transported to the laboratory, frozen, and dried at 50°C. In August 1988, larvae in the penultimate and ultimate instars were collected, taken back to the laboratory, and reared on *C. glabra* leaves from the Leverett population until those were exhausted, and then on leaves from the Cambridge garden. When these larvae molted to the prepupal stage, indicated by a color change from white and black to tan and black and by a cessation of feeding, they were frozen and dried at 50°C. All specimens were weighed before extraction for chemical analysis.

Frass and Exuvial Material. We collected frass samples on two dates. In 1984, we attempted to rear sawfly larvae collected at the Leverett population to the adult stage on *C. glabra* leaves and collected bulk samples of frass from groups of these larvae in the penultimate and ultimate instars. These samples were dried at 50°C and frozen. In 1988, we reared sawfly larvae individually and collected frass samples from them in the last instar. We separately analyzed frass samples from each of six individual larvae. These samples were collected while the larvae were actively feeding.

Exuvia were collected from larvae in August 1988. All frass and exuvia were dried at 50°C, and weighed before extraction.

Preparation of Samples for Gas Chromatography. Samples were prepared and analyzed using the methods of Gardner and Stermitz (1988). Each sawfly or plant sample was ground to a powder and extracted overnight in screw-cap test tubes with 3–5 ml MeOH. Entire larvae and prepupae were analyzed as were 100-mg samples of frass and plant material. The samples were filtered, and the filtrate was evaporated to dryness. The dry residue was triturated with 2 ml H_2O and 3 ml Et_2O and the triturates were combined in screw-cap test tubes. To each tube was added 1.00 ml of internal standard: phenyl-β-D-glucose

(0.500 mg/ml). The samples were vortexed, centrifuged, and the Et_2O layer was removed. The aqueous layer was washed two more times with 3 ml Et_2O and then evaporated to dryness. To the dry residue was added exactly 1 ml MeOH; this mixture was swirled. A 100-μl aliquot was removed and placed into a small glass sample tube (internal diameter 0.5 mm) and evaporated to dryness. The rest of the sample was placed in a screw-cap vial and stored under refrigeration. To the small sample tube was added 100 μl of silylation reagent (TRI-SIL 'Z', Pierce Chemical Company) and the closed tube was heated at 70–80°C for 20 min. After this mixture was cooled, a 1-μl aliquot was injected into the gas chromatograph.

Gas Chromatography Analysis. All samples were analyzed for iridoid glycosides relative to a standard containing aucubin (0.750 mg/ml), catalpol (0.750 mg/ml), and phenyl-β-D-glucose (0.500 mg/ml), which was silylated as were the samples. GC analyses were performed on the trimethylsilylated derivatives using a gas chromatograph equipped with a split/splitless injector, an FID detector, and a Hewlett Packard 3393 integrator. The column was a DB-1 capillary (J&W Scientific), 30 m long × 0.32 mm ID, with a film thickness of 0.10 μm. The injector was used in the split mode, split ratio of 14 : 1, and at a temperature of 275°C. Carrier gas was high-grade He (99.996%) with a column head pressure of 12 psi and a linear velocity of 30 cm/sec (1.44 ml/min). The detector temperature was 325°C with an H_2 flow of 38 ml/min, air flow of 400 ml/min, and a He (makeup gas) flow of 40 ml/min. Samples were run under the following program: initial column temperature was 200°C for 1 min and was raised to 260°C at a rate of 20°C/min and held for 8 min (aucubin, R_t = 7.50 min; catalpol, R_t = 8.49 min).

RESULTS

Gas chromatography showed that all but four of the *Chelone glabra* samples contained only catalpol, with an average total iridoid content of 0.57% dry weight, although the content varied greatly among individuals (range: 0.06–1.87% dry weight, N = 30) (Table 1). Traces of aucubin were found in three of the damaged (N = 10) and one of the undamaged (N = 10) *C. glabra* samples collected from Leverett, Massachusetts. A comparison of the damaged and undamaged samples from Leverett showed no significant differences in total iridoid glycoside content (one-way ANOVA on arc-sine transformed values, P = 0.18). The *C. glabra* samples from Cambridge contained only catalpol, with a mean of 0.588% dry weight (N = 10) (Table 1). There was no significant difference in the total iridoid glycoside content of undamaged plants from Leverett and Cambridge (one-way ANOVA on arc-sine transformed values, P = 0.478).

GC analysis of sawfly prepupae fed on *C. glabra* showed that they contained only catalpol, with a mean content of 2.15% dry weight, although the content varied over an order of magnitude among individuals (Table 2). The sawfly larvae had about half the catalpol content of the prepupae (Table 2). These larvae also may have contained leaf material in the gut, whereas the prepupae did not. The exuvia also contained only catalpol, with a mean content of 0.344% dry weight (Table 2). Interestingly, GC analysis detected significant amounts of aucubin, but no catalpol, in the frass samples (either bulk or individual) of the sawfly larvae (Table 2).

DISCUSSION

Our chemical analyses showed no significant effect of previous herbivore damage on the iridoid glycoside content of *Chelone glabra*. However, our samples had been collected in the field with no control of timing or amount of

TABLE 1. IRIDOID GLYCOSIDE CONTENT OF *Chelone glabra* LEAVES FROM POPULATIONS IN LEVERETT AND CAMBRIDGE, MASSACHUSETTS

Population	Damage category	*N*	Dry weight (mg) [Mean (SE)]	Aucubin (% dry weight)		Catalpol (% dry weight)	
				Mean (SE)	Range	Mean (SE)	Range
Leverett	Undamaged	10	106.2 (3.6)	0.03 (0.03)	0.00–0.25	0.42 (0.12)	0.06–1.20
	Damaged	10	78.2 (10.7)	0.04 (0.03)	0.00–0.22	0.62 (0.13)	0.16–1.65
Cambridge	Undamaged	14	100.9 (1.2)	0		0.59 (0.14)	0.13–1.87

TABLE 2. IRIDOID GLYCOSIDE CONTENT OF SAWFLY LARVAE, PREPUPAE, FRASS, AND EXUVIA

Life stage	*N*	Dry weight (mg) [Mean (SE)]	Aucubin (% dry weight)		Catalpol (% dry weight)	
			Mean (SE)	Range	Mean (SE)	Range
Larvae	4	22.83 (3.45)	0		1.09 (0.41)	0–1.93
Prepupae	12	32.18 (2.73)	0		2.15 (0.32)	0.11–3.86
Exuvia (5 in bulk)	1[a]	7.84	0		0.34	
Frass (individual)	6[b]	13.07 (1.18)	0.27 (0.17)	0–1.08	0	
Frass (bulk samples)	6[c]	182.65 (85.74)	0.41 (0.12)	0.08–0.66	0	

[a] Five exuvia were combined, weighed, extracted, and analyzed in bulk.
[b] Each frass sample was collected from a single last-instar larva.
[c] Each bulk sample had been collected from a different group of penultimate- and ultimate-instar larvae.

herbivory, both of which factors may affect induction of phytochemicals (Tallamy and Raupp, 1991), including iridoid glycosides (Bowers and Stamp, 1993). Another factor potentially affecting our inability to discern induction of iridoid glycosides in *C. glabra* is that there was substantial variation in the total iridoid glycoside content of individual plants, which may have obscured any induction that occurred. We did find that three of 10 damaged *C. glabra* samples contained aucubin, whereas only one of the undamaged samples contained aucubin. Biosynthetically, aucubin is the precursor of catalpol (Damtoft et al., 1983; Jensen, 1991). Damage to the plant may initiate additional synthesis of iridoid glycosides, resulting in accumulation of detectable amounts of aucubin. This would be in contrast to analyses of the iridoid glycosides of *Plantago lanceolata* (Plantaginaceae), which contains both aucubin and catalpol, that showed that herbivory resulted in increased amounts of catalpol (Bowers and Stamp, 1993).

We did not find any difference between the total iridoid glycoside content of *C. glabra* from Leverett, Massachusetts and Cambridge, Massachusetts. However, four of the 20 samples from Leverett (including both damaged and undamaged plants) contained aucubin, whereas none of the samples from Cambridge (all of which were undamaged) contained aucubin. There was, however, substantial variation in iridoid glycoside concentration of individual plant samples. In both the Leverett and Cambridge populations the iridoid glycoside concentrations varied over an order of magnitude among individual plants. Because each plant sample included all the leaves from an individual stalk of *C. glabra*, this variation reflects variation among stalks or plants, and not variation among leaves.

This variation in iridoid glycoside concentration may be important for insects feeding in these *C. glabra* populations, and especially for insects that sequester iridoid glycosides, such as the sawfly larvae. The individual plant on which a sawfly larva feeds may be critical in determining the iridoid glycoside content of that larva and thus its degree of protection from potential predators. This is the case for another sawfly species, *Neodiprion sertifer* (Diprionidae) that feeds on Scots pine, *Pinus sylvestris*. In this species, feeding on needles of trees that are higher in resin acids (the defensive compounds used by these sawflies) results in lower ant predation rates on the larvae (Bjorkman and Larsson, 1991). Our analyses of *T. grandis* sawfly larvae and prepupae showed that variation in both life stages ranged over an order of magnitude: the larvae varied in their iridoid glycoside concentration from 0 to 1.93% dry weight, whereas the prepupae ranged from 0.11 to 3.86% dry weight. Insects with higher catalpol concentrations may have fed on plants with more catalpol. Other factors may contribute to this variation, such as differences in the iridoid glycoside content of individual leaves fed on by the larvae (as documented in *Plantago lanceolata*, Plantaginaceae; Klockars et al., 1993), or variation among larvae in their sequestrative efficiency (Bowers and Collinge, 1992; Camara, unpublished data). Whatever

the origin of this variation in iridoid glycoside content among individual sawflies, our data indicate that it is substantial. In this sawfly species, therefore, automimicry (Brower et al., 1968, 1970), in which relatively palatable individuals of a population are protected by their resemblance to unpalatable individuals of the same species, is probably an important component of the larval defense.

The difference in the iridoid glycoside content of the larvae and prepupae may have been due to developmental differences or to the different years in which these two developmental stages were collected (larvae were collected in 1986 and prepupae were reared from larvae collected in 1988). We have no way to distinguish between these two possibilities, and both may have been important. For example, there may be year-to-year differences in the chemical content of individual plants due to a variety of factors (Waterman and Mole, 1989). In addition, different life stages of an insect may differ substantially in their chemical content (Nishio, 1980; Bowers and Collinge, 1992).

Although we detected aucubin in some of the *C. glabra* plant samples, none of our insect samples contained any aucubin; they contained only catalpol. In contrast, the frass samples contained only aucubin. One interpretation of these data is that these sawfly larvae are selectively sequestering catalpol and eliminating aucubin in the frass. This is in contrast to what occurs in two species of Lepidoptera, *Junonia coenia* and *Euphydryas phaeton*, that sequester iridoid glycosides. When fed on *Plantago lanceolata*, which contains both aucubin and catalpol (Bowers and Collinge, 1992), neither of these species of larvae eliminate any iridoid glycosides in the frass (Bowers and Collinge, 1992; Bowers and Collinge, unpublished data), and larvae of both species sequester both aucubin and catalpol (Bowers and Collinge, 1992; Bowers et al., 1992). *J. coenia* larvae appear to sequester catalpol more efficiently than aucubin (Bowers and Collinge, 1992). In contrast, sawflies appear to have the ability to physiologically differentiate between aucubin and catalpol and preferentially to sequester catalpol. Chemical analyses of *Euphydryas phaeton* butterflies (Belofsky et al., 1989; Bowers et al., 1992) coupled with feeding experiments with potential predators (Bowers, 1980) suggest that catalpol is the more important of these two iridoid glycosides in determining unpalatability and emeticity of insects sequestering them (Bowers, 1992).

An alternative explanation for the difference in iridoid glycoside content of frass and insects is that catalpol may be converted to aucubin during or after the frass is eliminated. Experiments with *Junonia coenia* larvae fed on artificial diets containing either aucubin or catalpol showed that that species did not appear to convert one of these iridoid glycosides into the other (Bowers and Collinge, 1992). We do not know whether or not conversion occurs in the sawflies.

Thus, the unpalatability of these sawfly larvae (Bowers, 1980) appears to be due to the sequestration of the iridoid glycoside catalpol from the host plant, *Chelone glabra*. In contrast to the lepidopteran larvae of *J. coenia* and *E.*

phaeton, which sequester both aucubin and catalpol, these sawfly larvae appear to be specialized to sequester only catalpol, although both aucubin and catalpol may be found in the host plant. The black and white coloration of the larvae can therefore be considered truly aposematic (conspicuous appearance advertising noxious qualities), reflecting the unpalatability of the larvae, and advertising this feature to potential predators.

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REFERENCES

BELOFSKY, G., BOWERS, M.D., JANZEN, S., and STERMITZ, F.R. 1989. Iridoid glycosides of *Aureolaria flava* and their sequestration by *Euphydryas phaeton* butterflies. *Phytochemistry* 28:1601–1604.

BJORKMAN, C., and LARSSON, S. 1991. Pine sawfly defence and variation in host plant resin acids: A trade-off with growth. *Ecol. Entomol.* 16:283–289.

BOEVE, J., and PASTEELS, J.M. 1985. Modes of defense in nematine sawfly larvae: Efficiency against ants and birds. *J. Chem. Ecol.* 11:1019–1036.

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*, Nymphalidae). *Evolution* 35:367–375.

BOWERS, M.D. 1988. Chemistry and coevolution: Iridoid glycosides, plants and insects, pp. 133–165, *in* K. Spencer (ed.). Chemistry and Coevolution. Academic Press, New York.

BOWERS, M.D. 1991. Iridoid glycosides, pp. 297–325, *in* G. Rosenthal and M. Berenbaum (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites, Vol. 1., 2nd ed. Academic Press, San Diego, California.

BOWERS, M.D. 1992. The evolution of unpalatability and the cost of chemical defense in insects, pp. 216–244, *in* B. Roitberg and M. Isman (eds.). Insect Chemical Ecology: An Evolutionary Approach, Chapman and Hall, New York.

BOWERS, M.D., and COLLINGE, S.K. 1992. Fate of iridoid glycosides in different life stages of the buckeye, *Junonia coenia* (Lepidoptera: Nymphalidae). *J. Chem. Ecol.* 18:817–831.

BOWERS, M.D., and FARLEY, S.D. 1990. The behaviour of gray jays (*Perisoreus canadensis*) toward palatable and unpalatable Lepidoptera. *Anim. Behav.* 39:699–705.

BOWERS, M.D., and STAMP, N.E. 1993. Effects of herbivory by specialist and generalist caterpillars on host plant chemistry, biomass and sexual reproduction. *Ecology*. In press.

BOWERS, M.D., STAMP, N.E., and COLLINGE, S.K. 1992. Early stage of host range expansion by a specialist herbivore, *Euphydryas phaeton* (Nymphalidae). *Ecology* 73:526–536.

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., POUGH, F.H., and MECK, H.R. 1970. Theoretical investigations of automimicry. I. Single trial learning. *Proc. Natl. Acad. Sci. U.S.A.* 66:1059–1066.

DAMTOFT, S., JENSEN, S.R., and NIELSEN, B.J. 1983. The biosynthesis of iridoid glucosides from 8-epi-deoxyloganic acid. *Biochem. Soc. Trans.* 11:594–595.

EISNER, T., JOHNESSEE, J.S., CARREL, J., HENDRY, L.B., and MEINWALD, J. 1974. Defensive use by an insect of a plant resin. *Science* 184:966–969.

GARDNER, D.R., and STERMITZ, F.R. 1988. Hostplant utilization and iridoid glycoside sequestration by *Euphydryas anicia* individuals and populations. *J. Chem. Ecol.* 14:2147–2168.

JENSEN, S.R. 1991. Plant iridoids, their biosynthesis and distribution in angiosperms, pp. 133–158, *in* J.B. Harborne and F.A. Thomas-Barberan (eds.). Ecological Chemistry and Biochemistry of Plant Terpenoids. Oxford University Press, New York.

KLOCKARS, G.K., BOWERS, M.D., and COONEY, B.F. 1993. Leaf variation in iridoid glycoside content of *Plantago lanceolata* (Plantainaceae) and oviposition preference of the buckeye, *Junonia coenia* (Nymphalidae). *Chemoecology*. In press.

LARSSON, S., BJORKMAN, C., and GREF, R. 1986. Responses of *Neodiprion sertifer* (Hym., Diprionidae) larvae to variation in needle resin acid concentration in Scots pine. *Oecologia* 70:77–84.

MORROW, P.A., BELLAS, T.E., and EISNER, T. 1976. Eucalyptus oils in the defensive oral discharge of Australian sawfly larvae (Hymenoptera: Pergidae). *Oecologia* 24:193–206.

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. University Microfilms.

PROP, N. 1960. Protection against birds and parasites in some species of tenthredinid larvae. *Arch. Neerland. de Zool.* 13:380–447.

RIMPLER, 1991. Sequestration of iridoid glycosides by insects, pp. 314–330, *in* J.B. Harborne and F.A. Thomas-Barberan (eds.). Ecological Chemistry and Biochemistry of Plant Terpenoids. Oxford University Press, New York.

STAMP, N.E. 1984. Effect of defoliation by checkerspot caterpillars (*Euphydryas phaeton*) and sawfly larvae (*Macrophya nigra* and *Tenthredo grandis*) on their host plants (*Chelone* spp.). *Oecologia* 63:275–280.

TALLAMY, D., and RAUPP, M.J. 1991. Phytochemical Induction by Herbivores. John Wiley & Sons, New York.

WATERMAN, P.G. and MOLE, S. 1989. Extrinsic factors influencing production of secondary metabolites in plants, pp. 107–134, *in* E.A. Bernays (ed.). Insect–Plant Interactions, Vol. 1. CRC Press, Boca Raton, Florida.

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THE EFFECT OF SHORT-RANGE HOST ODOR STIMULI ON HOST FRUIT FINDING AND FEEDING BEHAVIOR OF PLUM CURCULIO ADULTS (COLEOPTERA: CURCULIONIDAE)

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Abstract—In laboratory assays, we investigated responses of female plum curculios (PCs), *Conotrachelus nenuphar* (Herbst), to host and nonhost fruit or leaf odor when PCs were crawling on experimental tree branchlets or twigs. In choice tests where test specimens were hung from the ends of a wooden crosspiece, PCs made significantly more visits to host plum fruit than to plum leaves, nonhost tomato fruit, wax models of plum fruit, or blanks (wire). In similar tests, PCs made significantly more visits to plum leaves compared to nonhost maple leaves or to blanks. PCs in test chambers that contained host or nonhost odor were significantly more prone to feed on wax plum models in the presence of odor from host fruit or host leaves compared to odor from nonhost fruit or leaves or a water blank. In choice tests offering alternating cluster types on an apple branchlet, PCs visited leaf clusters bearing a host apple fruit more than leaf clusters without a fruit. In tests to assay the distance at which PCs can detect an individual host fruit, PCs crawled from the central stem of an apple branchlet onto a side stem significantly more often when an apple fruit on a side stem was hung 2 cm from the central stem compared to 4 or 8 cm away. Our combined results suggest that PCs use host fruit odor to locate host fruit at close range.

Key Words—*Conotrachelus nenuphar*, Coleoptera, Curculionidae, host odor, host location, feeding.

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INTRODUCTION

The plum curculio (PC), *Conotrachelus nenuphar* (Herbst), is a serious pest of stone and pome fruit east of the Rocky Mountains. In its northern range, PC is an annual threat to commercial apple, pear, plum, and peach orchards, often causing significant economic loss through downgrading of damaged fruit. Despite its importance, PC remains the least understood pest of tree fruit in North America (Whalon and Croft, 1984). PCs overwinter as adults in wooded areas near orchards or unmanaged trees (Lafleur et al., 1987). Coincident with tree bloom or petal fall, PCs leave overwintering sites and arrive on host trees (Lafleur and Hill, 1987), where they feed upon and oviposit into host fruit. PCs are often difficult to control because monitoring procedures to determine need and timing of insecticide application, the only effective management technique, remain primitive. The most widely used method involves examining newly formed fruit for evidence of fresh PC feeding or egg-laying scars (Prokopy et al., 1980). However, extensive PC damage may have already occurred by the time the first feeding or oviposition scar is observed. PCs are particularly active on warm, humid nights and crawl quickly (rarely fly) within a host tree, often causing significant damage to fruit overnight or before the next round of monitoring (Quaintance and Jenne, 1912; Chapman, 1938; Smith and Flessel, 1968; Owens et al., 1982; McGiffen and Meyer, 1986; Butkewich and Prokopy, 1988; Racette, 1988). If new monitoring and control procedures are to be developed for PC, a thorough understanding of the mechanisms involved in host tree and host fruit location is needed.

Previous research hints that PCs may use olfactory cues to guide them to host fruit within a tree. Preliminary laboratory olfactometer tests (Groden, Drummond, and Prokopy, unpublished) showed that PCs crawled upwind more often toward chambers that contained pierced apples than toward chambers containing water-soaked cotton. In laboratory choice tests, PCs visited fruit wounded with four or eight oviposition scars more often than clean fruit (Butkewich et al., 1987). PCs often fed at fruit wound sites and then left without ovipositing. In both of these studies, PCs may have been orienting to volatiles produced by wounded host tissue. The latter study further suggested that wounded host tissue such as punctured fruit may stimulate PCs to feed. PCs also have been reported to feed on petiole abscission scars (Owens et al., 1982).

Here, we conducted tests to determine more definitively the influence of odor on host fruit location and feeding by PC within real and experimental tree branchlets and twigs.

METHODS AND MATERIALS

All tests were conducted using females that were pretested to ensure that they were in a physiological state conducive to oviposition. A single PC was placed in a 35-ml transparent cup that contained a clean plum fruit. The pretest

was complete when a PC oviposited once into each of three clean fruit for a total of three ovipositions. PCs were tested no later than 5 hr after the pretest. Pretests and tests were conducted under low-intensity fluorescent light (110 lux) at 50–60% relative humidity and 24°C. PCs used in these studies were collected from host trees in nature within four weeks after bloom and were tested within one week after collection.

Pretest and test apparatus was designed to seclude PCs from external movement because PCs feign death when disturbed. Where PCs were being pretested or tested simultaneously, opaque dividers were placed between each pretest cup or test cage so that the PCs could not observe other PCs. An opaque white curtain was hung just below eye level to hide the observer's movements from the PCs. A mirror was placed on the wall immediately behind the test arena to aid the observer in visually assessing PC movement from all angles.

All host and nonhost fruit used in the following tests were bagged with mesh cloth to protect against prior insect damage. All natural test fruit or leaves were picked fresh just prior to commencement of an experiment.

Discrimination of Fruit and Leaves. Choice tests were conducted in June 1985, 1986, and 1988 to determine if PCs could discriminate, at close range, among individual host and nonhost fruit or leaves. PCs were offered the following choices: (1) plums vs. blanks, wax plum models vs. blanks, or plums vs. wax plum models; (2) plum leaves vs. blanks, plums vs. blanks, or plums vs. plum leaves; (3) plums vs. blanks, tomatoes vs. blanks, or plums vs. tomatoes; and (4) plum leaves vs. blanks, maple leaves vs. blanks, or plum leaves vs. maple leaves. Data were analyzed according to a G test ($P < 0.05$) (Sokal and Rohlf, 1981). Wax fruit models closely mimicked real plums in color (light green), size (ca. 17 mm diam.), and shape, offering visual stimuli in the absence of other possible plant-emitted cues. A wire that mimicked a fruit petiole was attached to the wax fruit model. Small water-moistened cotton wicks were hung adjacent to the wax plum models to mimic humidity associated with the microenvironment of live plant material. The cotton wicks were also hung adjacent to wire blanks that mimicked the size and shape of a fruit or leaf petiole. All leaf types were rolled vertically and secured to approximately the same surface area as fruit. Immature tomatoes offered a nonhost treatment that mimicked the color, size, and shape of test plums. Hairy pedicels on tomatoes were removed and cut areas were sealed with wax to ensure that physical properties of the tomatoes and plums were similar.

Test cages were transparent cylindrical tubes 11 cm tall × 8.5 cm diam. (Butkewich et al., 1987). Each cage contained a centrally mounted crosspiece made from plum twigs cut at least a year prior to use. Two replicates of each of two treatment specimens were hung by wire from the four ends of the crosspiece in an alternating fashion.

For testing, a PC was released into the test arena in a pretest cup. A test

began when a PC crawled onto the base of the central stem supporting the crosspiece and ended when a treatment specimen was crawled upon. If a PC failed to crawl onto one of the treatment specimens within 30 min after release, the replicate was discounted. Directional location (N, S, E, W) of each treatment specimen was randomized.

Feeding Response to Host and Nonhost Odors. Tests were conducted to determine if host or nonhost odor influenced PC feeding behavior. Test cages were slightly different from those used in the fruit/leaf discrimination tests. The top and bottom of a clear acetate tube (11 cm tall × 8.5 cm diam.) were each closed with a transparent 226-ml Solo plastic cup to form a triple-chamber cage. A wire screen was placed between the open end of each cup and the tube. Host or nonhost parts were hung within each screened cup. The screen served two functions: (1) it allowed odor from plant parts to move into the test cage where wax plum models were hung; and (2) it provided a barrier preventing PCs from contacting the plant material. There was one odor treatment type per cage. Two wax plum models and two wire blanks were hung alternately from the ends of a crosspiece mounted in the central chamber of a test cage. A single PC was confined in a test cage for 1 hr. Wax models were then examined for presence or absence of feeding scars.

In 1986, PCs received odor from five treatments: (1) four immature plums (ca. 17 mm diam.), (2) four unrolled plum leaves (ca. 5 × 7 cm), (3) four unrolled maple leaves (ca. 5 × 7 cm), (4) one tomato (ca. 45 mm diam.), or (5) a vial containing a water-moistened cotton wick as a control. In 1987, tests offering treatments 1, 2, and 5 were repeated along with a new treatment, which consisted of four blackberries (ca. 13 mm diam.). Data from both years were analyzed according to a G test ($P < 0.05$) (Sokal and Rohlf, 1981).

Discrimination of Cluster Types on Host Branchlet. In 1985, studies testing PC response to leaf and fruit clusters on an apple branchlet were conducted to determine: (1) if PCs discriminate between fruit and leaf clusters when searching for a host fruit and, if so, at what plant structural level discrimination occurs (sidestem, cluster, petiole, leaf, fruit), and (2) if PC response to host tissue (leaf clusters) increases when host tissue is wounded.

A freshly cut unsprayed apple branchlet (35 cm tall) with alternating fruit/leaf clusters was placed in the center of a clear acetate cylinder (18 cm diam. × 40 cm tall) that was closed on the top and bottom by sheets of cardboard. To maintain turgidity, the cut end of the branchlet extended through a hole in the cardboard into a beaker of water below. PC behavior was assayed in two sorts of tests: (1) fruit clusters (10 leaves plus a single apple fruit of 20 mm diam.) alternating with leaf clusters (10 leaves without fruit) in ascending order up the central stem, and (2) fruit clusters (10 leaves plus a single apple fruit of 20 mm diam.) alternating with leaf petiole clusters (fruitless clusters where five of 10 leaves were cut off and removed from the proximal end of the petiole just

before testing) in ascending order up the central stem. A single PC was released on the cylinder floor, and visits to the different cluster types during a 15-min interval were recorded. Test time commenced when a PC began ascending the central stem. Data were analyzed according to a test of independence using a 2 × 2 contingency table ($P < 0.05$) (Sokal and Rohlf, 1981).

Distance of Fruit Detection. In 1986, PCs were assayed for ability to detect fruit hung on side stems at varying distances from the central stem of an apple branchlet. Test cylinders were similar to those used in the cluster-type discrimination tests. Two freshly picked unsprayed apples (20 mm diam.) were wired together and hung on a side stem at either 2, 4, or 8 cm from the central stem. A fourth side stem without fruit served as a control. Fruit petioles were sealed with wax to prevent release of volatiles from wounded tissue. A single PC was released onto the floor of the cylinder and allowed to crawl onto and ascend the central stem of the branch. Side stems visited within a 15-min period were recorded. Data were analyzed according to a G test ($P < 0.05$) (Sokal and Rohlf, 1981).

RESULTS

In paired comparison tests where PCs were assayed for propensity to crawl onto assay fruit or leaves hung from a crosspiece (Table 1), PCs made significantly more visits to: plums than to blanks (wire only) (55% vs. 6% of PCs

TABLE 1. NUMBER OF PCS RESPONDING IN CHOICE TESTS OFFERING HOST OR NONHOST FRUIT OR LEAVES HUNG FROM A CROSSPIECE[a]

Series 1		Series 2		Series 3		Series 4	
Plum vs. blank		Plum leaf vs. blank		Plum vs. blank		Plum leaf vs. blank	
17	2*	18	2*	20	1*	18	2*
(31)		(28)		(39)		(28)	
Wax plum model vs. blank		Plum vs. blank		Tomato vs. blank		Maple leaf vs. blank	
2	4	20	1*	16	4*	17	2*
(40)		(39)		(44)		(31)	
Plum vs. wax plum model		Plum vs. plum leaf		Plum vs. tomato		Plum leaf vs. maple leaf	
21	1*	21	5*	16	4*	15	5*
(38)		(36)		(28)		(20)	

[a]Treatments within the same series followed by an asterisk are significantly different from those without according to a G test, $P < 0.05$. Number in parenthesis is total number of PCs tested. Number includes PCs that were discounted because they did not crawl onto a specimen within 30 min.

tested) or wax plum models (55% vs. 3%) (series 1); plums than to blanks (51% vs. 3%) or plum leaves (58% vs. 14%) (series 2); plums than to blanks (51% vs. 3%) or tomatoes (57% vs. 14%) and tomatoes than to blanks (36% vs. 9%) (series 3); and plum leaves than to blank (64% vs. 7%) or maple leaves (75% vs. 25%) and maple leaves than to blank (55% vs. 6%) (series 4). In a test where wax plum models (5%) vs. blanks (10%) were offered, very few visits were made to either treatment relative to other treatment comparisons (series 1).

In tests assessing the influence of host or nonhost odor on the propensity of PCs to "feed" on wax plum models (Table 2), a significantly greater proportion of PCs engaged in feeding when the odor source was plum fruit (series 1 = 41%, series 2 = 25%) or leaves (series 1 = 41%, series 2 = 29%) compared to tomato fruit (series 1 = 9%), blackberry fruit (series 2 = 0%), maple leaves (series 1 = 9%), or a water wick control (series 1 = 14%, series 2 = 0%). Although feeding times were not quantified for all treatments, PCs were observed feeding for 20 min or longer on wax plum models in the presence of plum fruit or leaf odor, comparable to a feeding bout on intact host fruit. Occasionally, PCs made crescent-shaped oviposition scars, usually no more than one scar per model. When the models were dissected, however, no eggs were found within the oviposition-type crevices.

PCs ascending the central stem of an apple branchlet visited a higher proportion (although not significantly so) of clusters on side stems that contained intact fruit plus leaves (62%) than clusters containing intact leaves alone (40%) (Table 3). PCs crawled onto a significantly higher proportion of fruit petioles available (81%) than leaf petioles available (13%) (Table 3). These results suggest that discrimination of host fruit components may occur upon arrival near

TABLE 2. PROPORTION OF PCS FEEDING ON WAX PLUM MODELS IN PRESENCE OF ODOR FROM HOST OR NONHOST FRUIT OR LEAVES[a]

Odor	PCs feeding (*N*)	
	Series 1	Series 2
Plum fruit	9 (22)*	6 (24)*
Plum leaf	9 (21)*	7 (24)*
Maple leaf	2 (22)	
Tomato fruit	2 (22)	
Blackberry fruit		0 (24)
Water blank	3 (21)	0 (24)

[a] Treatments within the same series followed by an asterisk are significantly different from those without according to a G test, $P < 0.05$. Number in parenthesis is total number of PCs tested.

or at the fruit petiole. After crawling onto a petiole, a PC usually continued to crawl onto the surface of the attached fruit (85%) or leaf (68%) (Table 3).

A significantly greater proportion of PCs ascending the stem of an apple branchlet visited leaf clusters where 50% of the leaf blades were removed (leaving exposed cut petioles) (16/24) than intact leaf clusters containing fruit (9/25). Frequently, PCs fed at the cut ends of leaf petioles. Such feeding bouts were brief: 10–15 sec at each wound site.

Tests to assay the ability of PCs to detect apples at various distances from the central stem of a branchlet showed that PCs crawled onto side stems significantly more often when apples were hung 2 cm from the stem–branch juncture (89%) than at distances of 4 (56%) or 8 cm (48%) (Table 4). Although visits to the latter two treatments were proportionately greater than to stems without apples (30%), differences were not significant. These results support

TABLE 3. PROPORTION OF PCs THAT ASCENDED AN APPLE BRANCHLET AND VISITED SIDE BRANCHLETS BEARING CLUSTERS CONTAINING APPLE FRUIT PLUS LEAVES OR LEAVES ONLY[a]

Clusters visited (*N*)/ [clusters visited (*N*) + clusters bypassed (*N*)]		Petioles visited (*N*)/petioles available (*N*)		Fruit or leaf surfaces visited (*N*)/fruit or leaf petioles visited (*N*)	
Fruit + leaves	Leaves only	Fruit petioles	Leaf petioles	Fruit	Leaves
18/29	8/20	13/16*	31/236	11/13	21/31

[a]Treatments with the same column heading followed by an asterisk are significantly different according to a test of independence using a 2 × 2 contingency table, $P < 0.05$. Total number of PCs assayed was 32 per treatment.

TABLE 4. PROPORTION OF PCs THAT ASCENDED THE STEM OF AN APPLE BRANCHLET AND CRAWLED ONTO SIDE BRANCHLETS BEARING DEVELOPING APPLES AT DIFFERENT DISTANCES FROM MAIN STEM[a]

	Distance (cm) of fruit from main stem		
0 fruit	2	4	8
7 (23)	16 (18)*	9 (16)	12 (25)

[a]Treatments followed by an asterisk are significantly different from those without according to a G test, $P < 0.05$. Number in parenthesis is total number of PCs tested. PCs that did not visit any side branch within 15 min were discounted.

the hypothesis that PCs orient to the presence of a fruit on a stem only from close range.

DISCUSSION

Oligophagous insects such as PC that feed on a restricted range of host plants (stone and pome fruit) may have selectively acquired orientation mechanisms that are expressed in response to host cues. Here, the fact that PCs visited host plum fruit more than plum leaves or nonhost tomato fruit, maple leaves, or wax models of plum fruit suggests that PCs use olfactory cues (host odor) to locate host fruit at close range (i.e., within a few centimeters). When near a host fruit, PCs frequently display antennal waving and walk in a stop-and-go manner as if orienting to the fruit. Short-range orientation mechanisms used by insects in response to olfactory stimuli have been classified into two categories: chemotaxis and chemokinesis (Fraenkel and Gunn, 1961; Kennedy, 1977). PC orientation may be either directed, through alternation of lateral deviations (klinotaxis) or by turning to a more or less stimulated side (tropotaxis), or undirected, by moving forward at different rates (orthokinesis) or turning at different rates (klinokinesis). Which of these mechanisms characterizes PC response to individual fruit is unknown.

Under our test conditions, the concentration of host odor most likely was greatest near the source and was dispersed by molecular diffusion outward because air turbulence was minimal within the test cages, as it would be in nature during nights of greatest PC oviposition activity (humid or rainy, calm) (Butkewich and Prokopy, 1988). The fact that host plum or apple fruit received proportionately more visits by PC than plum or apple leaves in assays where test specimens were hung at the ends of a crosspiece or in assays of intact clusters on a branch suggests that unwounded host fruit emit volatiles that are different in quantity or quality from unwounded host leaves.

In cluster-choice tests, PC visitation was reversed in favor of leaf clusters over fruit-bearing clusters when 50% of the leaves in leaf clusters were cut from the petioles. Enhanced volatiles produced by insect-wounded plants can increase insect visitation to such plants (Finch, 1980). PCs may have been responding to volatiles that were in especially high concentration when leaf petioles were wounded. Brief feeding bouts (10–15 sec) were observed frequently on wounded tips of the petioles. Such bouts, which were considerably shorter than typical feeding bouts on host fruit (20 min or longer), may have been stimulated by wounded tissue only to have been interrupted upon detection of an unsuitable host. On the other hand, perhaps PCs ingested all the sap that was available and moved on after the sap supply was exhausted.

The link between PC feeding and oviposition is poorly understood. PCs

may feed without ovipositing, but oviposition does not occur without feeding. Previous studies suggest that PC encounters with wounded host tissue deter oviposition or interrupt the oviposition sequence (Betkewich et al., 1987). Quite probably neural integration of idiothetic (internal) input (e.g., egg load, previous host experience) with allothetic (external) input is critical to the outcome of PC host location and acceptance behavior (Dethier, 1982; Miller and Strickler, 1984).

Our studies suggest that intact host fruit or leaf tissue may stimulate PC feeding. Thus, PCs in test chambers that contained odor from host fruit or leaves held in adjacent chambers tended to feed on inanimate wax plum models that mimicked the visual and external physical properties of real plums. In contrast, the proportion of PCs feeding on models was considerably less in the absence of host odor. Perhaps PCs require a combination of olfactory, visual, and tactile stimuli before feeding is elicited. In the presence of host odor, feeding bouts on wax plums did not appear to be abbreviated, as occurred when PCs fed on injured tissue.

The significantly greater number of PC visits to parts of nonhost plants (tomato fruit and maple leaves) compared with a blank control may have been a response to "general green leaf volatiles" (GLV) (Visser et al., 1979; Visser, 1986). Component blends of six-carbon alcohols and aldehydes are commonly found in plants. Olfactory receptivity to GLVs may aid PCs in the location of a "green oasis," an area where a host plant might be found. PCs may also use GLVs to locate shelter during migration to or from overwintering sites. PC migration is thought to be greatly influenced by abiotic factors such as temperature and relative humidity (McGiffin and Meyer, 1986). If PCs are highly susceptible to desiccation, and adjust their behavior accordingly, then location of a green oasis could provide a refuge of high humidity during migration.

At what level of habitat structure do olfactory cues stimulate orientation mechanisms that guide PCs to a host fruit? After arrival on host trees, PCs are known to engage in extensive crawling behavior among limbs, branches, and twigs (Owens et al., 1982). Our observations in nature reveal that PCs appearing to be in search of host fruit are likely to crawl onto woody structures of progressively decreasing diameter, eventually leading them onto branchlets and petioles that potentially bear fruit. As the environmental hierarchical level narrows from the habitat (tree) to the patch (branch) and to the host source (fruit), once can expect a more finely tuned response pattern to increased concentration of olfactory stimuli at close range (Prokopy, 1986). Upon contact with a fruit or leaf petiole, PCs may discriminate between the two by tactile, visual, or olfactory means, or by some combination of these. Such discrimination may in part explain the comparatively low PC visitation of wax fruit with wire fruit petiole mimics and wire blanks. Presumably the structure of the resource habitat has played a major role in shaping PC foraging behavior.

In sum, our findings indicate that the odor of a host fruit is positively stimulating to a crawling PC. Visual fruit characteristics alone are not stimulating in the absence of host odor. Interaction of host chemical and visual stimuli is known to be important in host-finding behavior of apple maggot fly, *R. pomonella* (Prokopy, 1986); onion maggot fly, *D. antiqua* (Harris and Miller, 1988); and cabbage root fly, *D. radicum* (Prokopy et al., 1983) and may be important in PC short-range host finding as well. According to our results, the odor of an individual host fruit is unlikely to be detected (under calm conditions that often prevail at night when ovipositing PCs are most active) at a distance much beyond ca. 8 cm, if even that far away. The odor of many fruit might be detected by PCs at a greater distance, which suggests that a potential exists for using fruit odor stimuli in traps to monitor PC activity in orchards.

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REFERENCES

BUTKEWICH, S.L., and PROKOPY, R.J. 1988. Singing in the Rain: The effect of weather on plum curculio spring migration. *Fruit Notes. Univ. Mass. Coop. Extens.* 53(2):11-12.

BUTKEWICH, S.L., PROKOPY, R.J., and GREEN, T.A. 1987. Discrimination of occupied host fruit by plum curculio females (Coleoptera: Curculionidae). *J. Chem. Ecol.* 13:1833-1841.

CHAPMAN, P.J. 1938. The plum curculio as an apple pest. *N.Y. State Agric. Exp. Stn. Bull.* 98:467-560.

DETHIER, V.G. 1982. Mechanisms of host-plant recognition. *Entomol. Exp. Appl.* 31:49-56.

FINCH, S. 1980. Chemical attraction of plant-feeding insects to plants, pp. 67-143, *in* T.H. Coaker (ed.). Applied Biology, Vol. 5. Academic Press, New York.

FRAENKEL, G.S., and GUNN, D.L. 1961. The Orientation of Animals, Kinesis, Taxes and Compass Reactions. Dover, New York.

HARRIS, M.O., and MILLER, J.R. 1988. Host acceptance behavior in an herbivorous fly, *Delia atiqua. J. Insect Physiol.* 34:179-190.

KENNEDY, J.S. 1977. Olfactory response to distant plants and other odor sources, pp. 67-91, *in* H.H. Shorey and J.J. McKelvy Jr. (eds.). Chemical Control of Insect Behavior: Theory and Application. John Wiley & Sons, New York.

LAFLEUR, G., and HILL, S.B. 1987. Spring migration, within-orchard dispersal and apple tree preference of plum curculio (Coleoptera: Curculionidae) in southern Quebec. *J. Econ. Entomol.* 80:1173-1187.

LAFLEUR, G., HILL, S.B., and VINCENT, C. 1987. Fall migration, hibernation site selection and associated winter mortality of plum curculio (Coleoptera: Curculionidae) in a Quebec apple orchard. *J. Econ. Entomol.* 80:1152-1172.

MCGIFFIN, M.E., and MEYER, J.R. 1986. Effect of environmental factors on overwintering phenomena and spring migration of the plum curculio, *Conotrachelus nenuphar* (Coleoptera: Curculionidae). *Environ. Entomol.* 15:884-888.

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.

OWENS, E.D., HAUSCHILD, K.I., HUBBELL, G.L., and PROKOPY, R.J. 1982. Diurnal behavior of plum curculio (Coleoptera: Curculionidae) adults within host trees in nature. *Ann. Entomol. Soc. Am.* 75:357–362.

PROKOPY, R.J. 1986. Visual and olfactory stimulus interaction in resource finding by insects, pp. 81–89, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction. Oxford University Press, London.

PROKOPY, R.J., COLI, W.M., HISLOP, R.G., and HAUSCHILD, K.I. 1980. Integrated management of insect and mite pests in commercial orchards in Massachusetts. *J. Econ. Entomol.* 73:529–535.

PROKOPY, R.J., COLLIER, R.H., and FINCH, S. 1983. Visual detection of host plants by cabbage root flies. *Entomol. Exp. Appl.* 34:85–89.

QUAINTANCE, A.L., and JENNE, E.L. 1912. The plum curculio. U.S. Department of Agriculture Bureau of Entomology Bulletin 103, 250 pp.

RACETTE, G. 1988. Daily activity of plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), and implications for control. MSc thesis. McGill University, Montreal, 145 pp.

SMITH, E.H., and FLESSEL, J.K. 1968. Hibernation of the plum curculio and its spring migration to host trees. *J. Econ. Entomol.* 61:193–203.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry. W.H. Freeman, New York.

VISSER, J.H. 1986. Host perception in phytophagous insects. *Annu. Rev. Entomol.* 31:121–144.

VISSER, J.H., VAN STRATEN, S., and MAARSE, H. 1979. General green leaf volatiles: Their isolation and identification in the foliage of potato, *Solanum tuberosum*, a host plant of the Colorado potato beetle, *Leptinotarsa decemlineata*. *J. Chem. Ecol.* 5:13–23.

WHALON, M.E., and CROFT, B.A. 1984. Apple IPM implementation in North America. *Annu. Rev. Entomol.* 29:435–470.

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VOLATILE COMPONENTS IN DEFENSIVE SPRAY OF THE HOG-NOSED SKUNK, *Conepatus mesoleucus*

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Abstract—GC-MS analysis of the anal sac secretion from the hog-nosed skunk, *Conepatus mesoleucus*, showed two major volatile components, (*E*)-2-butene-1-thiol and (*E*)-*S*-2-butenyl thioacetate. Minor volatile components identified from this secretion were phenylmethanethiol, 2-methylquinoline, 2-quinolinemethanethiol, and bis[(*E*)-2-butenyl] disulfide. 3-Methyl-1-butanethiol, a major component in the defensive spray of the striped skunk, *Mephitis mephitis*, and the spotted skunk, *Spilogale putorius*, was absent from this secretion.

Key Words—Mustelidae, *Conepatus mesoleucus*, hog-nosed skunk, anal sac secretion, (*E*)-2-butene-1-thiol, (*E*)-*S*-2-butenyl thioacetate, phenylmethanethiol, 2-methylquinoline, 2-quinolinemethanethiol, bis[(*E*)-2-butenyl] disulfide.

INTRODUCTION

The malodorous anal sac secretion of the striped skunk, *Mephitis mephitis*, has been the subject of many chemical investigations (Swarts, 1862; Löw, 1879; Aldrich, 1896; Aldrich and Jones, 1897; Stevens, 1945; Andersen and Bernstein, 1975; Andersen et al., 1982; Wood, 1990). The volatile components in the spray of the spotted skunk, *Spilogale putorius*, have also been identified (Wood et al., 1991). A single report has been made on the largest genus of skunks, *Conepatus*. Fester and Bertuzzi (1937) reported a thiol with the probable

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molecular formula of C_4H_7SH from the South American skunk, *C. suffocans*. These researchers suggested the odoriferous compound might be 2-butene-1-thiol (crotyl mercaptan) that had oxidized to the disulfide during analysis. In the present study, we report the volatile components of the anal sac from the hog-nosed skunk, *C. mesoleucus*.

METHODS AND MATERIALS

The defensive secretion from a single female hog-nosed skunk from Sierra Vista, Cochise County, Arizona, was examined. A sample of the secretion was obtained by inserting a needle into the anal sac and withdrawing the contents. This was placed in a screw-cap vial, preserved with 0.5 ml of dichloromethane, and shipped at ambient temperature to Arcata, California. A portion of this secretion was placed in diethyl ether and analyzed by 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 that 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.

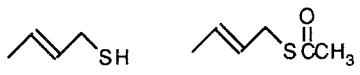
Identification of the components in the hog-nosed skunk secretion was done by comparison of mass spectra and gas chromatographic retention times to reference compounds. The retention time (*RT*), electron impact-mass spectra (EI-MS) and source of these compounds are as follows. (*E*)-2-Buten-1-thiol [*RT* = 2.20 min; m/z = 88(M^+, 37), 73(6), 55(100), 54(42), 53(25), 51(8), 50(8), 47(13), 45(35), and 39(52)] and (*E*)-*S*-2-butenyl thioacetate [*RT* = 6.42 min; m/z = 130(M^+, 10), 88(21), 87(9), 59(5), 55(34), 54(10), 53(9), 50(17), 43(100) and 39(14)] were obtained from striped skunk secretion (Andersen and Bernstein, 1975; Wood, 1990). Phenylmethanethiol [*RT* = 7.17 min; m/z = 124(M^+, 18), 92(9), 91(100), 77(9), 65(31), 63(19), 51(31), 50(16), 45(48) and 39(58)] and 2-methylquinoline [*RT* = 8.58 min; m/z = 144(10), 143(M^+, 100), 142(18), 140(3), 128(18), 117(6), 116(8), 115(18), 101(6) and 76(6)] were purchased from Aldrich Chemical Co. 2-Quinolinemethanethiol [*RT* = 10.18 min; m/z = 176(11), 175(M^+, 100), 174(71), 142(10), 130(37), 129(55), 128(26), 116(11), 115(25) and 102(11)] was prepared as described by Wood (1990). Bis[(*E*)-2-butenyl] disulfide [*RT* = 8.66 min; m/z = 174(M^+, 5), 128(2), 122(2), 120(15), 56(4), 55(100), 53(7), 46(1), 45(10) and 38(12)] was prepared by treatment of the defensive secretion with iodine as described by Wood (1990).

RESULTS AND DISCUSSION

Gas chromatographic–mass spectral (GC-MS) analysis of the anal sac secretion from the hog-nosed skunk showed two major components (Figure 1). These compounds and their amounts (percent total ion current) are (*E*)-2-butene-1-thiol (I, 71%) and (*E*)-*S*-2-butenyl thioacetate (II, 17%). They were identified by comparison of mass spectra and retention times of these compounds from a sample of striped skunk anal sac secretion (Andersen and Bernstein, 1975; Wood, 1990).

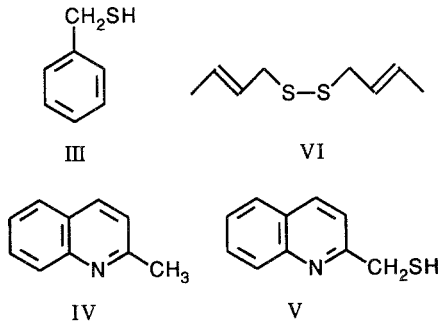
Four minor components were identified from this secretion (Figure 2). They are phenylmethanethiol (III, 1%), 2-methylquinoline (IV, 2%), 2-quinolinemethanethiol (V, 0.5%), and bis[(*E*)-2-butenyl] disulfide (VI, 3%). These compounds were identified by comparison of mass spectra and retention times of authentic samples.

The composition of the anal sac secretion from the hog-nosed skunk is different from previously examined skunks (Table 1). 3-Methyl-1-butanethiol, a major component in this secretion from the striped skunk and the spotted skunk, is absent. 2-Phenylethanethiol, which occurs in moderate amounts in the spotted skunk, could not be detected. Like the striped skunk, (*E*)-*S*-2-butenyl thioacetate, a derivative of (*E*)-2-butene-1-thiol, is found in the hog-nosed skunk.



I II

FIG. 1. Major components from *Conepatus mesoleucus* defensive secretion, (*E*)-2-butene-1-thiol (I) and (*E*)-*S*-2-butenyl thioacetate (II).



III VI

IV V

FIG. 2. Minor components from *Conepatus mesoleucus* defensive secretion, phenylmethanethiol (III), 2-methylquinoline (IV), 2-quinolinemethanethiol (V), and bis[(*E*)-2-butenyl] disulfide (VI).

TABLE 1. COMPOSITION OF MAJOR VOLATILE COMPONENTS OF ANAL SAC SECRETION FROM THREE SPECIES OF NORTH AMERICAN SKUNKS[a]

Compound	Composition (%)		
	Striped skunk	Spotted skunk	Hog-nosed skunk
(*E*)-2-Butene-1-thiol	38–40	30–36	71
3-Methyl-1-butanethiol	18–26	48–66	ND
(*E*)-*S*-2-Butenyl thioacetate	12–18	ND	17
(*S*)-3-Methylbutanyl thioacetate	2–3	ND	ND
2-Phenylethanethiol	trace	2–5	ND
2-Methylquinoline	4–11	trace	2
2-Quinolinemethanethiol	4–12	trace	0.5
S-2-Quinolinemethyl thioacetate	1–4	ND	ND

[a]ND = none detected.

This compound may increase the effectiveness of skunk spray by slowly breaking down to (*E*)-2-butene-1-thiol, a compound with strong mephitic odor. The spray of the spotted skunk does not contain this compound, which has been reported to be less persistent than that of the striped skunk (Wood et al., 1991).

Three compounds were found in all three species (Table 1). Most prominent is (*E*)-2-butene-1-thiol, a highly odoriferous compound, that contributes greatly to the repulsiveness of this secretion. The other two compounds are the alkaloids, 2-methylquinoline and 2-quinolinemethanethiol, minor components of these secretion.

The presence of bis[(*E*)-2-butenyl] disulfide in the secretion from the hog-nosed skunk at 3% may be an artifact generated by air oxidation of (*E*)-2-butene-1-thiol. The sample could not be analyzed immediately after it was collected, and so some oxidation to the disulfide would be expected. Thus, it should not be used in the comparison of these defensive secretions.

Since the hog-nosed skunk's secretion contains a thioacetate derivative of the major thiol and does not contain any 2-phenylethanethiol, it is more like the secretion of the striped than spotted skunk. This may indicate the hog-nosed skunk and striped skunk are more closely related to each other than either is to the spotted skunk. Examination of this secretion from additional species of skunks may give more information about the relationship of different species.

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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.

FESTER, G.A., and BERTUZZI, F.A. 1937. La secrecion del zorrino. *Rev. Fac. Quim. Ind. Agric., Univ. Nac. Litoral* 5:85–87.

LÖW, O. 1879. Notizen über das Stinkthier (*Mephitis texana*). *Ärtzliches Intelligensblatt von München* 252–253 (June).

STEVENS, P.G. 1945. American musk III. The scent of the common skunk. *J. Am. Chem. Soc.* 67:407–408.

SWARTS. 1962. Über das Öl des Stinkthiers. *Justus Liebigs Ann. Chem.* 123:266–270.

WOOD, W.F. 1990. New components in defensive secretion of the striped skunk, *Mephitis mephitis. J. Chem. Ecol.* 16:2057–2065.

WOOD, W.F., MORGAN, C.G., and MILLER, A. 1991. Volatile components in defensive spray of the spotted skunk, *Spilogale putorius. J. Chem. Ecol.* 17:1415–1420.

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(6*Z*,9*Z*-3*R*,4*S*)-EPOXY-HEPTADECADIENE: MAJOR SEX PHEROMONE COMPONENT OF THE LARCH LOOPER, *Semiothisa sexmaculata* (Packard) (LEPIDOPTERA: GEOMETRIDAE)

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Abstract—Gas chromatographic–electroantennographic analysis (GC-EAD) of female larch looper, *Semiothisa sexmaculata* (Packard), gland extracts revealed two EAD-active compounds. Retention index calculations, GC-mass spectroscopy in selected ion monitoring mode, and GC-EAD analysis of authentic standards identified the compounds as (3Z,6Z,9Z)-heptadecatriene (3Z,6Z,9Z-17:H) and (6Z,9Z)-*cis*-3,4-epoxy-heptadecadiene (6Z,9Z-*cis*-3,4-epoxy-17:H). Chirality determination of the monoepoxydiene in gland extracts was impeded by small quantities, but field experiments indicated that male *S. sexmaculata* were most strongly attracted to enantiomerically enriched 6Z,9Z-3*R*,4*S*-epoxy-17:H (69% ee), while male *S. neptaria* (Guenée) responded well to various blends of the *R*,*S*- and *S*,*R*-epoxide enantiomers. Binary combinations of the *R*,*S*-epoxide enantiomer with 3Z,6Z,9Z-17:H significantly inhibited response by male *S. sexmaculata*, but strongly enhanced attraction of sympatric male *S. marmorata* Ferguson. Enantiomerically enriched 6Z,9Z-3*R*,4*S*-epoxy-17:H can be used as a trap bait to monitor populations of the larch-defoliating *S. sexmaculata*. Whether 6Z,9Z-3*R*,4*S*-epoxy-17:H serves as single component sex pheromone in *S. sexmaculata* or

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small amounts of 6*Z*,9*Z*-3*S*,4*R*-epoxy-17:H synergize or suppress optimal attraction, will be tested as chirally pure monoepoxydienes become available.

Key Words—Lepidoptera, Geometridae, *Semiothisa*, *S. sexmaculata*, *S. marmorata*, *S. neptaria*, sex pheromone, sex attractant, enantiomer, (3*Z*,6*Z*,9*Z*)-heptadecatriene, (6*Z*,9*Z*-3*R*,4*S*)-epoxy-heptadecadiene, (6*Z*,9*Z*-3*S*,4*R*)-epoxy-heptadecadiene.

INTRODUCTION

The larch looper, *Semiothisa sexmaculata* Packard, occurs in British Columbia (BC), Idaho, Oregon, and Washington on western larch, *Larix occidentalis* Nutt. and Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco (Furniss and Carolin, 1978). Outbreaks of *S. sexmaculata* in BC were first recorded in 1977 when 4600 ha of western larch northwest of Creston were defoliated. Moderate to severe defoliation of nearly 12000 ha of larch in 1990 in the same general area (Unger and Vallentgoed, 1990) provided the opportunity to study the semiochemical system of this geometrid moth. We report the identification and field testing of sex pheromone components of *S. sexmaculata*.

METHODS AND MATERIALS

Laboratory Analysis. Pupae were collected near Trail, BC, and naturally overwintered in a rain-protected shelter. Pupae were gradually warmed up in the laboratory in February for 20 days at a 14 : 10 light–dark photoperiod.

Pheromone glands of 2 to 3-day-old virgin females were removed 2–3 hr into the scotophase and extracted for 5 min in hexane. Aliquots of one female equivalent (1 FE) of gland extract were subjected to coupled gas chromatographic–electroantennographic analyses (GC-EAD) (Arn et al., 1975) on two fused silica columns (DB-210, DB-1 (J & W Scientific, Folsom, CA). Coupled GC-mass spectroscopy (MS) (Hewlett-Packard 5985B) in selected ion monitoring (SIM) mode on a DB-210 column with isobutane for chemical ionization (CI) was conducted to confirm the identification of EAD-active components in female gland extracts. Full-scan CI mass spectra of synthetic candidate compounds were obtained to select diagnostic ions. In sequence, 200 pg of synthetic compounds, hexane, and a concentrated pheromone gland extract were then analyzed in SIM mode, each time scanning for the diagnostic ions.

Candidate pheromone components, synthesized as described by Millar et al. (1987, 1990c), were generously provided by the National Research Council, Saskatoon.

Field Trapping. Field experiments were conducted in a mixed forest of western larch, lodgepole pine, *Pinus contorta* var. *latifolia* Engelm., and aspen,

Populus tremuloides Michx., 15 km southeast of Castlegar, BC. Unitraps (Phero Tech Inc., Delta, BC, Canada V4G 1E9) were set up in randomized complete blocks with traps and blocks at 15-m intervals. Traps were suspended 2 m above ground from a variety of coniferous and deciduous trees and baited with rubber septa impregnated with lure blends (100 μg each) in HPLC grade hexane. A small diclorvos cube (Green Cross, Division, Ciba-Geigy Canada Ltd., Mississauga, Ontario) placed on the bottom of traps assured retainment of captured moths.

The first 20-replicate experiment comprised the following 10 treatments: (1) (3*Z*,6*Z*,9*Z*)-heptadecatriene (3*Z*,6*Z*,9*Z*-17:H); (2) (6*Z*,9*Z*-3*R*,4*S*)-epoxy-heptadecadiene (6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H) (69% ee); (3–5) binary combinations of treatments 1 and 2 at ratios of 90:10, 50:50, and 10:90; (6) (6*Z*,9*Z*-3*S*,4*R*)-epoxy-heptadecadiene) (6*Z*,9*Z*-3*S*,4*R*-epoxy-17:H) (69% ee); (7–9) binary combinations of treatments 1 and 6 at ratios of 90:10, 50:50, and 10:90; and (10) unbaited control traps (Table 1). The second 11-treatment, 20-replicate

TABLE 1. TOTAL NUMBERS OF MALE *Semiothisa sexmaculata* AND *S. marmorata* CAPTURED IN UNITRAPS (PHERO TECH INC., DELTA, BC, CANADA V4G 1E9) BAITED WITH 3*Z*,6*Z*,9*Z*-17:H ALONE AND IN BINARY COMBINATION WITH ENANTIOMERS OF 6*Z*, 9*Z*-*cis*-3, 4-EPOXY-17:H, TRAIL, BC, 16–30 JUNE 1991, $N = 20$.

Lure composition (μg)	Total numbers of male[a]	
	S. sexmaculata	*S. marmorata*
6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H (100)[b]	182a	7b
6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H (90) + 3*Z*,6*Z*,9*Z*-17:H (10)	67b	74a
6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H (50) + 3*Z*,6*Z*,9*Z*-17:H (50)	5c	82a
6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H (10) + 3*Z*,6*Z*,9*Z*-17:H (90)	0	17b
6*Z*,9*Z*-3*S*,4*R*-epoxy-17:H (100)[b]	52b	13b
6*Z*,9*Z*-3*S*,4*R*-epoxy-17:H (90) + 3*Z*,6*Z*,9*Z*-17:H (10)	7c	13b
6*Z*,9*Z*-3*S*,4*R*-epoxy-17:H (50) + 3*Z*,6*Z*,9*Z*-17:H (50)	0	3c
6*Z*,9*Z*-3*S*,4*R*-epoxy-17:H (10 + 3*Z*,6*Z*,9*Z*-17:H (90)	0	0
3*Z*,6*Z*,9*Z*-17:H (100)	0	1c
Unbaited control	0	0

[a]Numbers within each columns followed by a different letter are significantly different. ANOVA on data transformed by log 10(x + 1), followed by Duncan's multiple range test, $P < 0.05$. Treatments that did not catch any moths were excluded from the analysis.
[b]69% ee.

experiment tested treatments 2 and 6 alone at 10μg each and in binary combinations at nine different ratios (Figure 3).

RESULTS AND DISCUSSION

GC-EAD analysis of pheromone gland extracts on a DB-210 column revealed two EAD-active compounds with respective Kovats retention indices of 1704 and 2108 (Figure 1). As 3Z,6Z,9Z-unsaturated triene hydrocarbons and corresponding monoepoxydiene standards are approximately 400 retention units apart on a DB-210 column, and 3Z,6Z,9Z-triene hydrocarbons (C_{17}–C_{21}) and corresponding monoepoxydienes have been reported as sex pheromone components or attractants in geometrid and noctuid moths (Underhill et al., 1983, 1985; Wong et al., 1985; Millar et al., 1987, 1990a,b; Hansson et al., 1990), we hypothesized that the first EAD-active compound with retention index 1704 was 3Z,6Z,9Z-17:H. Retention time of an authentic standard coincided with

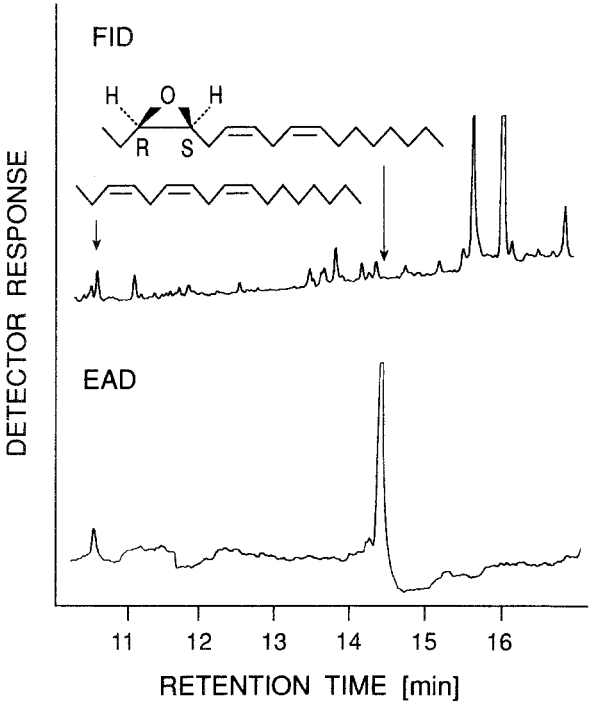

FIG. 1. Detector responses to one female equivalent of *S. sexmaculata* pheromone gland extract chromatographed on a DB-210 column (1 min at 70°C, 10°C/min to 220°C). The antennal recording (EAD) was carried out with an antenna of a male *S. sexmaculata*.

EAD responses to extracts on DB-1 and DB-210 columns, and 200 pg of triene hydrocarbon elicited good antennal responses.

The second EAD-active compound with retention index 2108 was hypothesized to be a corresponding monoepoxy-heptadecadiene. Fifty-picogram standards of racemic 3,4-, 6,7-, and 9,10-monoepoxydienes (C_{16}–C_{21}) were subjected to GC-EAD analysis. 3,4-Monoepoxydienes (C_{17}–C_{19}) elicited the strongest antennal responses (Figure 2), 6,7-monoepoxydienes (C_{17}–C_{19}) were distinctively less EAD-active, and 9,10-monoepoxydienes had no EAD activity. 6*Z*,9*Z*-*cis*-3,4-Epoxy-17:H elicited the greatest electrical potential and coincided with EAD-responses to gland extracts on DB-210 and DB-1 columns.

GC-MS (CI) analyses in SIM mode of a 6 FE gland extract and synthetic 3*Z*,6*Z*,9*Z*-17:H and 6*Z*,9*Z*,-*cis*-3,4-epoxy-17:H resulted in exact retention time and good ion ratio matches of synthetic *versus* female-produced compounds and thereby confirmed the presence of these volatiles in pheromone gland extracts (synthetic 6*Z*,9*Z*,-*cis*-3,4-epoxy-17:H: *m/z* 251 (100, M + 1), 233 (61), gland extract: *m/z* 251 (100), 233 (58); synthetic 3*Z*,6*Z*,9*Z*-17:H: *m/z* 233 (100, M-1), 235 (68, M + 1), gland extract *m/z* 233 (100), 235 (50)

In the mixed larch forest southeast of Castlegar, BC, 3*Z*,6*Z*,9*Z*-17:H,

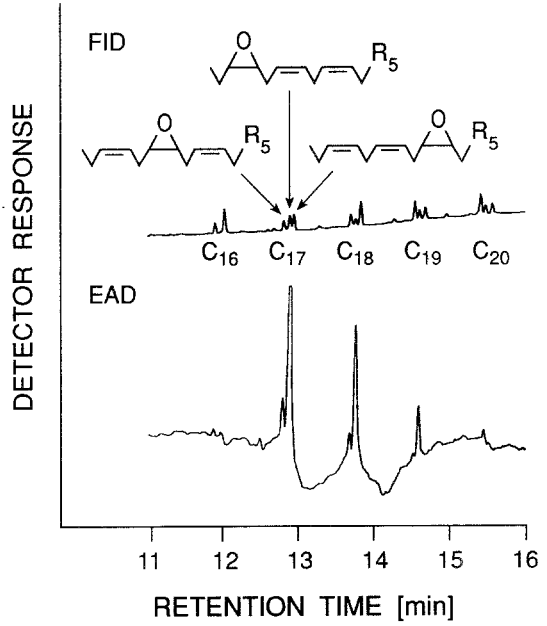

FIG. 2. Detector responses to a 50-pg standards of monoepoxydienes C_{16}–C_{21} chromatographed on a DB-210 column (1 min at 70°C, 10°C/min to 220°C). The EAD was carried out with a single antenna of male *S. sexmaculata*.

6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H, and 6*Z*,9*Z*-3*S*,4*R*-epoxy-17:H tested individually and in binary combinations attracted three species of *Semiothisa* (Table 1, Figure 3): *S. sexmaculata*, *S. marmorata*, and *S. neptaria*. Due to low moth populations, overall trap catches were not very high.

Male *S. sexmaculata* were most strongly attracted to enantiomerically enriched 6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H (Table 1), while male *S. marmorata* were attracted to a 90:10 and 50:50 blend of 6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H and 3*Z*,6*Z*,9*Z*-17:H (Table 1). Although the pheromonal blend of *S. marmorata* has not yet been analyzed in the laboratory, it is conceivable that both compounds are sex pheromone components of *S. marmorata*. As 3*Z*,6*Z*,9*Z*-17:H inhibits response by *S. sexmaculata*, but strongly enhances attraction of *S. marmorata*, it is further conceivable that the presence or absence of the triene hydrocarbon maintains specificity of pheromone communication channels between these two sympatric species.

While pheromone chirality appears to be important in *S. sexmaculata* (Figure 3), male *S. neptaria* responded similarly well to various blends of both the

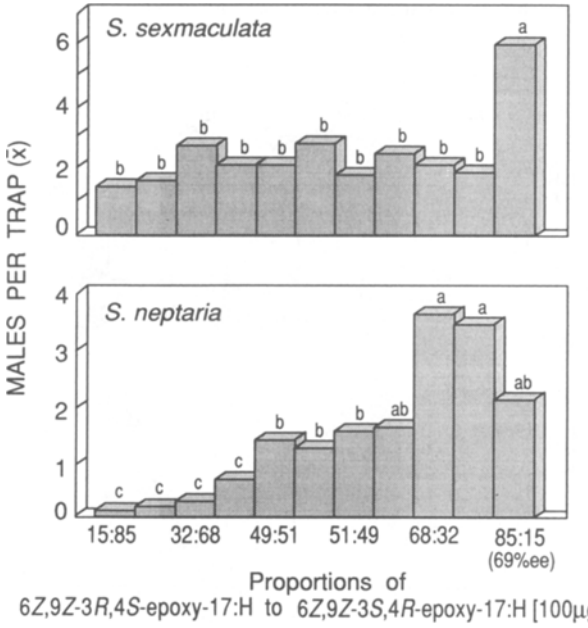

FIG. 3. Mean numbers of male *S. sexmaculata* and *S. neptaria* captured in Unitraps baited with 100 µg of enantiomerically enriched 6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H [69% enantiomeric excess (ee)] and 6*Z*,9*Z*-3*S*,4*R*-epoxy-17:H (69% ee) alone and in binary combinations. ANOVA on data transformed by log10 ($x + 1$) followed by Duncan's multiple-range test, $P < 0.05$. $N = 20$, Trail, BC, 30 June–14 July 1991.

R,S- and *S,R*-epoxide enantiomers (Figure 3). Enantiomeric specificity of behavioral response has also been shown in the geometrid moths *Itame occiduaria* (Packard) and *I. bruneata* (Thunberg). 6*Z*,9*Z*-3*R*,4*S*-Epoxy-17:H (69% ee) and 6*Z*,9*Z*-3*S*,4*R*-epoxy-17:H (69% ee), respectively, combined with 3*Z*,6*Z*,9*Z*-17:H attracted one of the two species while inhibiting response of the other (Millar et al., 1990c).

Fifteen percent chiral impurity of the *S,R* enantiomer in 6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H (69% ee) may have suppressed optimal attraction of *S. sexmaculata*. However, 15% chiral impurity of the *R,S* enantiomer in 6*Z*,9*Z*-3*S*,4*R*-epoxy-17:H (69% ee) still attracted large numbers of male *S. sexmaculata* (Figure 3), suggesting that the *S,R* enantiomer is not inhibitory, but benign. It is also possible that the *S,R* enantiomer in small amounts synergizes attraction to 6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H. Synergism between epoxide enantiomers has already been demonstrated in the noctuid moth, *Bleptina caradrinalis* (Guenée) and in the geometrid moth, *Epelis truncataria* (Walker). Neither enantiomer alone of 3*Z*,6*Z*-*cis*-6,7-epoxy-21:H attracted male *B. caradrinalis*, but a 4:1 mixture of the *R,S* and *S,R* enantiomer was most attractive (Millar et al., 1991). Similarly, racemic 6*Z*,9*Z*-*cis*-3,4-epoxy-17:H was three times more attractive to male *E. truncataria*, than either enantiomer alone (Millar et al., 1990c).

In conclusion, enantiomerically enriched 6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H can be used as a trap bait to monitor populations of the larch-defoliating *S. sexmaculata*. Whether chirally pure 6*Z*,9*Z*-3*R*,4*S*-epoxy-17:H serves as a single sex pheromone component, or whether small amounts of the corresponding *S,R* enantiomer synergize or reduce attraction, will be tested as chirally pure monoepoxydienes become available.

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REFERENCES

ARN, H., STÄDLER, 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.

FURNISS, A.L., and CAROLIN, V.M. 1978. Western forest insects. US Department of Agriculture Forest Service Miscellaneous Publication No. 1339, pp. 654.

HANSSON, B.S., SZÖCS, G., SCHMIDT, F., FRANCKE, W., LÖFSTEDT, C., TÓTH, M. 1990. Electrophysiological and chemical analysis of the sex pheromone communication system of the mottled umber, *Erannis defoliaria* (Lepidoptera: Geometridae). *J. Chem. Ecol.* 16:1887–1897.

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.* 1:2307–2316.

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. Interaction of enantiomers and regioisomers. *J. Chem. Ecol.* 17:2317–2339.

MILLAR, J.G., GIBLIN, M., BARTON, D., and UNDERHILL, E.W. 1991. Chiral lepidopteran sex attractants: Blends of optically active C_{20} and C_{21} diene epoxides as sex attractants for geometrid and noctuid moths (Lepidoptera). *Environ. Entomol.* 20:450–457.

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 erectea*. *J. Chem. Ecol.* 9:1413–1423.

UNDERHILL, E.W., MILLAR, J.G., and WONG, J.W. 1985. Unsaturated hydrocarbons and their oxygenated analogs as a major class of lepidopteran sex attractants. Entomological Society of America National Meeting., December 1985, Hollywood, Florida.

UNGER, L., and VALLENTGOED, J. 1990. Forest insect and disease conditions. Nelson Forest Region, Forestry Canada, FIDS Report 91-3: pp. 1–51.

WONG, J.W., UNDERHILL, E.W., MACKENZIE, S.L., and CHISHOLM, M.D. 1985. Sex attractants for geometrid and noctuid moths. Field trapping and electroantennographic responses to triene hydrocarbons and monoepoxydiene derivatives. *J. Chem. Ecol.* 11:727–756.

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DEFENSIVE SECRETION OF RICE BUG, *Leptocorisa oratorius* FABRICIUS, (HEMIPTERA: COREIDAE): A UNIQUE CHEMICAL COMBINATION AND ITS TOXIC, REPELLENT, AND ALARM PROPERTIES

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Abstract—Defensive secretion produced by adult males and females of *Leptocorisa oratorius*, Fabricius (Hemiptera: Coreidae) living on the host plant, *Oriza sativa*, was analyzed by a combined gas chromatography–mass spectroscopy technique. Both male and female secretions consisted of two major components: (*E*)-2-octenal and *n*-octyl acetate, 76% and 16% (w/w), respectively. The remaining 8% were trace compounds, some of which were identified as hexyl acetate, 3-octenal, 1-octanol, and (*Z*)-3-octenyl acetate. In a survey among 38 coreid defensive secretions, (*E*)-2-octenal and *n*-octyl acetate were found to occur rarely in addition to coreid-specific compounds, while their combination as primary constituents was found to be unique. Toxicity and repellency of this secretion were evaluated using two household pests, *Anoplolepis longipes* and *Sitotroga cerealella*, as test insects, and lethal concentration (LC_{50}) values of 0.24 ppm and 0.14 ppm, respectively, and repellencies of 63% and 58%, respectively, were obtained. Comparing the above values with those of a pentatomid bug, *Coridius janus*, evaluated under the same conditions, it was apparent that this secretion has potential as a repellent to enemies of *L. oratorius* but not as a toxicant to attack them. Bioassay on the alarm activity of this secretion revealed that it elicits alarm responses, alerting and dispersing aggregated male and female *L. oratorius*: this is followed by "self-coating" activities. In addition, some unique behaviors were also noted among alarmed *L. oratorius*.

Key Words—*Leptocorisa oratorius*, Hemiptera, Coreidae, defensive secretion, (*E*)-2-octenal, *n*-octyl acetate, repellent, toxicity, alarm.

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INTRODUCTION

The chemical ecology of insect exocrine secretions has been subjected to thorough investigations in recent years due to their potential in the monitoring and/or control of pest populations. Heteropteran scent substances have received much interest lately. Postulated functions of these secretions are defense against predators and microorganisms and as alarm, aggregation, or sex pheromones (Staddon, 1979). Heteropteran secretions have been thoroughly investigated and as a result there are numerous reports available on the chemical identification (Prestwich, 1976; Taketoshi, 1974; Aldrich, 1988; Weatherston and Percy, 1978). Although the chemicals responsible for defensive, repellent, and deterrent activities are known (Blum, 1964), somewhat less information is available on those for aggregation (Aldrich and Blum, 1978; Biney, 1984; Goodchild, 1977) and alarm (Blum, 1985).

Leptocorisa species (Coreidae) are sucking bugs that occur throughout South and Southeast Asia (Grist and Lever, 1969; Srivastava and Saxena, 1967) and are known to damage the paddy crop by sucking the sap of developing grains, thus affecting grain formation. Damaged grains produce only 40–60% their normal endosperm content, and this has been identified as one of the primary causes of crop loss in tropical countries (Litsinger et al., 1981; Kennard, 1962; Argente and Heinriches, 1983). *Leptocorisa oratorius* is one of the most common and the most important *Leptocorisa* species to damage rice in Sri lanka (Morita and Dhanapala, 1990). *Leptocorisa* species have not been subjected to chemical ecology studies in terms of their secretions. Existence of a host attractant for *L. oratorius*, erroneously identified at the time as *L. acuta* was reported earlier (Gunawardena and Ranatunga, 1989). In this paper we report chemical properties of this secretion and compare them to those of the percentages from 41 other coreid species. The *Leptocorisa* secretion was also bioassayed for fumigant toxicity and repellency against two species of insects, *Anoplolepis longipes* and *Sitotroga cerelella*, which have already been used in evaluating those properties of the defensive secretion of *Coridius janus*, a local pentatomid (Gunawardena and Herath, 1991, 1992). The differences in the efficiency of the above defensive secretions as fumigants and repellents are discussed in terms of their differences in the accompanying compounds.

This secretion was also tested for its alarm activity, and results are discussed in comparison with those of other hemipterans, pentatomids, in particular, and also with those of coleopterans.

METHODS AND MATERIALS

Chemicals (*E*)-2-Octenal and *n*-octyl acetate were purchased from Aldrich Chemicals Co. U.K. and found to be 99.9% by gas chromatography (GC). Diethyl ether and hexane were obtained from Merck Chemical Co. Germany and paraffin oil from BDH, U.K.

Insects and Preparation of Samples. L. oratorius adults were collected from rice fields in the district of Kurunegala, during the end of three seasons; Maha seasons (1989 and 1990) and Yala* season (1991). Insects were brought to the laboratory one day prior to experiments and were caged and fed with rice grains (at the milky stage) and water. Laboratory temperature during all experiments was 30–31°C with relative humidity at 80 ± 4%. The insects were kept on the natural photoperiod, and all bioassays were carried out during the daytime.

Since individual emission of the secretion was in the submilligram range, two methods of sample preparation had to be employed: (1) When neat defensive secretions were needed in more than milligram quantities, adult males and females (5–10) of *L. oratorius* were confined separately in preweighed closed light, glass vials (10 ml) and were shaken thoroughly for a few seconds, holding the vials horizontally until the scent was seen on the walls of the vials. Insects were then quickly allowed to fall to the bottom of the vial, and the vials were then kept at −5°C for 10 min to ensure the retention of all volatiles, before opening them and removing the insects. Required amounts were subsequently weighed from this collection. (2) When the defensive secretion of a single *L. oratorius* was required, one insect was placed in a preweighed glass vial (2 ml) and was made to emit the defensive secretion by pinching a leg with a fine forceps. The insect was removed from the vial immediately after the ejection, and the lid closed, cooled (10–15°C), and weighed. Stock solutions of the concentration 1 mg/ml were made in methylene chloride.

Chemical Analysis. GC of the defensive secretion collected by method 2 was performed on two chromatographs: (1) Shimadzu GC-9A chromatograph fitted with SPB 1 glass capillary column (30 m × 0.25 mm) and flame ionization detector (FID) operated under the following conditions: temperature program 50°C to 225°C at 16°C/min, injector 220°C, detector 260°C, He carrier gas at a rate of 3 ml/min. (2) Hewlett Packard 5890 A gas chromatograph fitted with SE 54 fused silica capillary column (25 m × 0.25 mm), FID detector, injector 220°C, detector 260°C, carrier gas N_2 at a rate of 22 cm/sec, split injection, temperature program, 2 min at 60°C, 60°C to 260°C at 8°C/min, 10 min at 260°C. Gas chromatography–mass spectrometric (GC-MS) analyses were performed on a Shimadzu GC-MS QP 1000A, EI mode at 70 eV, splitless injection, column parameters as in GC 1.

Bioassay for Fumigant Toxicity. Samples were prepared by method 1, described above, with the exceptions that female and male secretions were pooled, and required amounts were weighed into vials as neat liquid. Test insects of *Anoplolepis longipes* and *Sitotroga cerealella* were collected from their natural habitats a few hours prior to bioassay and were fed with sugar solutions and paddy grains. Batches of 25 test insects were taken into fumigating chambers (10 liters), and through the bottom inlet of these chambers, vials containing test substances were inserted halfway, thus allowing volatiles to evaporate into the

*Two cropping seasons which run paralell to two monsoon rainy seasons, for a year

chamber. Complete evaporation of contents into chambers was ensured by slowly heating the bottom of the half-inserted vials, and the test insects were exposed to the above volatiles for 24 hr. Immobile insects were separated and kept under observation for another 24 hr before mortalities were registered. Distilled water was used in the control experiments. Three concentrations were tested, and triplicates were run for each test (Table 1). The LC_{50} values were calculated by probit analysis of data (Busvine, 1957) tested for the calculation of LC_{50} values.

Bioassay for Repellency. A preliminary assay indicated that submilligram quantities of the neat liquid repel insects with 100% efficiency. Using the method of Peschke and Eisner (1987), the defense secretion was diluted in a nonvolatile medium and tested. Thus, 5 μl of a 15% solution of the secretion in paraffin oil was assayed in three replicates. For each assay, 25 test insects were used, and the blank experiments were conducted with paraffin oil and pure water.

A. 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 a direction parallel to a slow stream of air. The outer surface of the stem of a glass funnel was treated with 5 μl of the test solution and was inserted

TABLE 1. FUMIGANT (LETHAL CONCENTRATION: LC_{50} VALUE) VALUES OF DEFENSIVE SECRETION OF *L. oratorius*

Test insect[a]	I	II	III
A. longipes			
Amount of secretion used in assay (mg)[b]	0.90	1.00	6.00
Dose/ppm	0.09	0.10	0.60
Mean % mortality	26.33	46.00	90.33
Range	(24–30)	(40–50)	(85–95)
LC_{50}[c]	0.24 (0.37–0.11)		
S. cerealella			
Amount of secretion used in assay (mg)[b]	0.90	1.40	2.80
Dose/ppm	0.09	0.14	0.28
Mean % mortality	6.66	15.33	89.00
Range	(4–9)	(10–21)	(81–95)
LC_{50}[c]	0.14 (0.18–0.10)		

[a] Batches of 25 insects were exposed to defensive substance in a 10-liter fumigating chamber for a period of 24 hr.
[b] The secretion was used as a net liquid
[c] All values represent mean LC_{50} of three replicates and CI at 95% in the parentheses.

into one end of the tube. Thus, the airflow could carry the repellent throughout the tube with minimum loss of test substance against the direction of the airflow. The number of insects in the original tube was counted 5 min after the introduction of test substance.

For *S. cerealella*, a long glass tube (50 cm long × 3 cm ID) was divided in the middle by marking two parts as A and B. Test insects were introduced into this tube in a dark room and were lured into part A by the use of a light at the end. The test substance (5 μl) spread in a glass funnel as described previously for *A. longipes* was introduced into A. The number of insects in A was counted 10 min after the introduction of test compounds.

Bioassay for Alarm Activity. Fresh rice panicles at the milk stage were each kept horizontally in cylindrical glass jars (20 cm diam. × 10 cm high), and the jars were covered with a piece of net for ventilation. These jars were kept moistened by the use of cotton wool. Adult *L. oratorius* (10) were introduced into the jar, and the jar was kept exposed to diffused natural light. Afterwards one half of the net was covered with a piece of black paper so that all *L. oratorius* aggregated on the uncovered part of the net. Filter paper strips (4 × 0.5 cm) were dosed with three dilutions (100, 500, 1000 μg) of the defensive secretion prepared by method 1. These paper strips were introduced into the jar close to the aggregated insects on the net. The number of *L. oratorius* that were immediately attracted to the filter paper, moved away from it afterwards, and engaged in "self-coating" activities was noted for a period of 40 min. A filter paper dosed with methylene chloride was used as the blank. A total of 120 insects (12 batches, 10 in each) were used at each concentration and *t* test was used to analyze the results.

RESULTS

Chemical Properties. GC analysis showed the presence of only two major components, consisting on average, of 76% and 16% (N = 120), which were identified as (*E*)-2-octenal and *n*-octyl acetate, respectively (peaks 3 and 6, Figure 1) by the MS data as follows; *m/z* peak 3 = 126 (0.38%, $M^{\circ+}$), 125 (1.91%), 108 (6.89%), 98 (15.32%), 97 (17.23%), 83 (70.86%), 70 (100%), 69 (45.9%), 57 (55.54%), 55 (90.02%); peak 6 = 172 (0.37%), 157 (0.37%), 129 (0.75%), 117 (5.29%), 112 (20.41%), 98 (5.29%), 97 (5.29%), 84 (37.8%), 83 (36.28%), 70 (48.38%), 61 (36.28%), 56 (41.58%), 43 (100%).

Retention times (*RT*, GC condition 2) of authentic (*E*)-2-octenal and *n*-octyl acetate were found to be 7.99 and 11.33 min, respectively, and they were identical to those of peaks 3 and 6, respectively. Chemical compositions of male and female secretions were similar, and the major constituents had average ratios of (*E*)-2-octenal to *n*-octyl acetate of 76.70%:15.84% for males and 75.28%:15.41% for females (N = 60 in both cases).

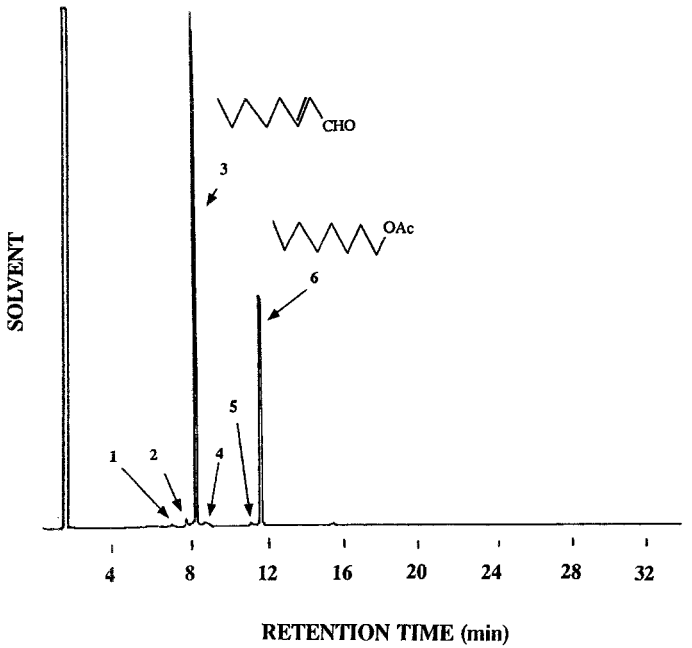

FIG. 1. Gas chromatogram of the defensive secretion of *L. oratorius* (temperature program, 2 min 60°C, 60°C to 260°C at 8°C/min, 10 min at 260°C, SE 54 fused silica capillary column (25 m × 0.25 mm), FID detector, injector 220°C, detector 260°C). Peak 1 = hexyl acetate, peak 2 = 3-octenal, peak 3 = (*E*)-2-octenal, peak 4 = 1-octanol, peak 5 = (*Z*)-3-octenyl acetate.

Minor peaks 1, 2, 4, and 5 (Figure 1) were identified as hexyl acetate, 3-octenal, 1-octanol, and (*Z*)-3-octenol acetate, respectively, by comparing MS data with those available. Confirmation of these compounds with authentic samples were not carried out, however. *m/z* peak 1 = 101(2.0%), 84(22.2%), 69(12.0%), 61(24.6%), 56(58.2%), 55(26%), 43(100%); peak 2 = 126(2.0%), 97(5%), 84(12%), 83(100%), 85(28%), 70(66%), 69(23%), 55(54%), 41(55%); peak 4 = 112(4%), 84.1(52%), 83(32%), 70(55%), 54(48%), 55.9(100%), 43(80%), 41(100%); and peak 5 = 110(32%), 95(11%), 81(48%), 68(38%), 67(38%), 54(68%), 43(100%).

Biological Properties. In response to physical irritation, the scent substance was seen to be emitted as a droplet rather than ejected. This secretion was found to be highly volatile, had an irritating smell, and, on average, 0.7 ± 0.28 mg was emitted by each *L. oratorius*. Our results on 24-hr LC_{50} values obtained by the fumigant method are listed in Table 1. The natural secretion was toxic to both test insects, having mean LC_{50} values of 0.24 ppm (0.11–0.37, confi-

dence intervals at 95%) and 0.14 ppm (0.10–0.18, CI at 95%) for adult *A. longipes* and *S. cerealella*, respectively. Under the same experimental conditions, pure water showed no mortalities for either insect.

In our repellency assay, immediately after the introduction of defensive secretion, the test insects were repelled by it. Repellencies of 63.30% and 58.00% were obtained for *A. longipes* and *S. cerealella*, respectively. Paraffin oil repelled only 8.00% and 6.60% of *A. longipes* and *S. cerealella*, respectively, while pure water showed no repellency at all (Table 2).

In our bioassay for alarm activity, *L. oratorius* raised their antenna and starting scanning in the air, encountering the air currents containing defensive secretion. Significant numbers ($P < 0.01$) compared to that of the blank were attracted (mean of 6.08 and 6.25 for doses 500 μg and 1000 μg, respectively, compare blank 0.41, Table 3) to the scent source within seconds and then dispersed from it to the darkened part of the jar. Means of 6.6 and 9 were obtained for the doses 500 μg and 1000 μg, respectively, and these figures were significantly different ($P < 0.01$) from those of the blank where the mean number dispersed was 1. While being alert, *L. oratorius* were engaged in the characteristic movements of distributing their own secretion over the body, mainly by the use of their legs; this behavior is referred to as "self-coating" (see later). Mean numbers of 6.92 and 6.41 were obtained for the doses of 500 μg and 1000 μg compared to the blank (mean = 0.41, Table 3). From time to time it was seen that *L. oratorius* visited the source of secretion. Doses of 100 μg of the defensive secretion did not evoke alarm responses, while doses of 500 μg and 1000 μg were effective in dispersing *L. oratorius*.

DISCUSSION

Our results show that the defensive substance of *L. oratorius* does not deviate from the usual characteristics of coreids in that it consists of a deterrent accompanied by a nondeterrent. There are, however, appreciable differences noted in this secretion. First, many coreid defensive secretions consist of mixtures of deterrents and accompanying compounds, but those with a single deterrent and accompanying compound as major components together with minor and trace compounds are rather rare. From the 41 coreid taxa investigated (excluding the species under investigation), only 14% had this status (Weatherston and Percy, 1978; Dazzini and Pavan, 1978). Second, C-8 alkenal is somewhat unusual because those that dominate coreid secretions are usually C-6, e.g. hexenal and/or haxanal. Our survey with 41 species of coreids revealed that 80% of the species used any combination of hexanal/(*E*)-2-hexenal and acetic acid, with hexanal being the dominant component. Only 4.8% of the coreids, i.e., *Leptocoris apicalis* and *Leptocorisa varicornis*, use (*E*)-2-octenal

TABLE 2. REPELLENT VALUES OF DEFENSIVE SECRETION OF *L. oratorius*

Test insect[a]	Repellency (%)[b]		
	Defensive secretion[c]	Paraffin oil	Pure waters
A. longipes	63.30 (64.11–62.42)	8.00 (9.34–6.66)	0.00
S. cerealella	58.00 (59.73–56.26)	6.60 (8.43–4.89)	0.00

[a] Batches of 25 insects were used in each assay.
[b] All values represent mean of three replicates; 95% CI is in parentheses.
[c] 5 μl of 15% solution of the defensive substance of *L. oratorius* in paraffin oil.

TABLE 3. BIOASSAY ON ALARM ACTIVITY OF DEFENSIVE SECRETION OF *L. oratorius*

Entry	Dose (μg)	Responses of *L. oratorius* (means)[a]		
		Immediate attraction	Dispersal	Self-coating
1	100	1.66+ (0.30)	3[b] (0.35)	1.41[b] (0.33)
2	500	6.08 (0.39)	6.66 (0.30)	6.92 (0.44)
3	1000	6.25 (0.28)	9 (0.30)	6.41 (0.41)
4	Blank	0.41 (0.19)	1 (0.27)	0.41 (0.20)

[a] Twelve batches of *L. oratorius*, 10/batch, were assayed. Numbers in the parenthesis indicate ±SEM.
[b] Not significant compared to the blank. All other values in entries 2 and 3 are significantly different (t test, $P < 0.01$)

as the only deterrent, while 2% of coreids, i.e., *Acanthocerus galeator*, use a mixture of (*E*)-octenal and (*E*)-hexenal as the deterrent.

Coreid nonaldehydic components are also C-6 acetates as expected from the biosynthetic viewpoint. The usual pattern of distribution observed in our survey was that 51% use hexyl acetate; only 7% of the coreids (*L. apicalis*, *L. varicornis*, and *S. apicalea*) use octyl acetate, while 5% of the coreids (*Amblypelta nitida* and *Anoplocnemis montandorii*) use a mixture of octyl and hexyl acetates. The rest use mainly butyrates and hexanol. A rather high percentage of coreids (34%) do not use accompanying compounds. More interestingly, the combination (*E*)-2-octenal and *n*-octyl acetate as major components was found

to be unique among the 41 coreid defensive secretions surveyed. Two members that use the above combination, although not as major components but with other components, are *Leotocoris apicalis* and *Stenocoris apicales*. The former was found to use the above two compounds with larger amounts (21%) of other compounds, (*E*)-2-decenal being the dominant component, while the latter uses the above combination with two other common deterrents, hexanal and (*E*)-2-decenal (Baggini et al., 1966). *Leptocorisa varicornis*, a member of the same genus, was found to use only (*E*)-2-octenal with traces of 2-butenal, nonenal, and decenal.

The toxic properties of this secretion were found to be much weaker than those of the previously studied local pentatomid (Heteroptera) *Coridius janus*, which uses the (*E*)-2-hexenal–*n*-tridecane (60:40, w/w) combination in 90% of the volatiles. The LC_{50} values obtained for *L. oratorius* were 0.24 ppm and 0.14 ppm for the test insects *A. longipes* and *S. cerealella*, while those of *C. janus*, under the same experimental conditions were, 0.19 ppm and 0.30×10^{-2} ppm, respectively. Assuming that the primary components are largely responsible for the defensive activity of a secretion, the above differences could be attributed to the difference in the nonaldehydic compounds in the two secretions, the former being an acetate while the latter is an alkane. In addition to the previously allocated functions for the accompanying compounds (e.g., modulate evaporation of the deterrent), they also improve penetration of the deterrent into the bodies of enemies (Remold, 1962). The toxic property of the defensive secretion has been shown to derive as much from the alkanes/alkenes as from the deterrents by two new reports on arthropod defensive secretion, namely the stink bug, *Coridius janus* (Pentatomidae, using *n*-tridecane) and the tenebriionid beetle, *Blaps mucronata* (Tenebriionidae, using 1-*n*-tridecene) (Gunawardena and Herath, 1991; Peschke and Eisner 1987).

Although there are no comparable detailed studies in the case of acetate accompanying compounds in coreids, the above functions seem to be applicable to them too, with the exception, however, that acetates may not possess appreciable toxicity similar to alkanes/alkenes. This probably reflects the lower toxicity of this secretion to test insects. In contrast, the repellent property of a defensive secretion has been shown (Gunawardena and Herath, 1991, 1992) to depend largely on the alkenal; hence the repellent values, 63% and 58%, of the defensive secretion of *L. oratorius* for *A. longipes* and *S. cerealella*, respectively, compared well with those of *C. janus* (65% and 63%, respectively) obtained under the same experimental conditions (Gunawardena and Herath, 1991). Based on the above comparisons, it is probable that this secretion is used by *L. oratorius* only as a repellent rather than a direct means of attacking predators. Two more factors support this idea; its highly odoriferous nature and the absence of any ejection mechanism.

Another function of this secretion, made clear by this study, is the high

alarm responses evoked among *L. oratorius*. Doses in the range of an individual equivalent (500–1000 μg) caused three responses—aggregation dispersal, alertness, and self-coating behavior—among significant numbers of *L. oratorius* (Table 3). Alarm activity among hemipterans, Pentatomidae in particular, have been well recognized (Ishiwatari, 1974; Lockwood and Story, 1987; Kou et al., 1989), although the information on coreids is rather scarce. Several hemipterans, such as *Cimex lectularius* (Cimicidae), the bed bug, *Dysdercus* sp. (Pyrrhocoridae), and the cotton stainers, *Erthesina fullo* (Pentatomidae), are known to utilize their defensive secretion parsimoniously as an alarm pheromone to alert and disperse threatened aggregations (Levinson et al., 1974; Calam and Youdeowei, 1968; Kou et al., 1989). Similarly, we believe a bare minimum of defensive secretion has been discharged by a significant number of *L. oratorius* due to their extensive self-coating activities while remaining alert. This behavior has to be viewed as a part of alarm behavior because self-coating reduces the chances of a small insect such as *L. oratorius* being picked up by larger animals such as birds. Self-coating is not a characteristic alarm behavior of pentatomids, however. Due to lack of such data on coreids, we are unable to say anything conclusive at this point. This behavior, however, is well known for coleopteran beetles (Peschke and Eisner, 1987), tenebrionid beetles in particular.

In addition to the above alarm behaviors, the more uncommon observation noted was the immediate attraction of *L. oratorius* to the source of the secretion before dispersal. This kind of behavior has not been noted in connection with alarm activity, and perhaps this may be characteristic of coreids. Some insects also revisited the source of the secretion from time to time. The reasons for these behaviors demand a further study.

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REFERENCES

ALDRICH, J.R., and BLUM, M.S. 1978. Aposematic aggregation of a bug (Hemiptera: Coreidae). *Biotropica* 10:58–61.

ALDRICH, J.R. 1988. Chemical ecology of Heteroptera. *Annu. Rev. Entomol.* 33:211–238.

ARGENTE, A.M., and HEINRICHES, E.A. 1983. Residues of carbaryl, lindane and monocrotophos on rice treated for control of rice bug, *Leptocorisa oratorius* (Fab.). *Crop Prot.* 2:361–369.

BAGGINI, A., BERNARD, I.R., CASNATI, G., PAVAN, M., and RICCA, A. 1966. Richerche sulle secretiozioni difensive di insetti, *Emitteri eterotteri*. *Rev. Esp. Entomol.* 42:7–26.

BINEY, S.H. 1984. A study of the nymphal aggregation of *Dieuches femoralis* Eyles (Heteroptera: Lygaeidae). *Insect Sci. Appl.* 5:131–136.

BLUM, M.S. 1964. Insect defensive secretion; Hex-2-enal in *Pelmatosilpha coriacea* and its repellent values under natural conditions. *Ann. Entomol. Soc. Am.* 57:600–602.

BLUM, M.S. 1985. Alarm pheromones, pp. 193–224, *in* G.A. Kerkut and L.I. Gilbert, (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 9. Pergamon, Oxford.

BUSVINE, J.R. 1957. A Critical Review of the Techniques for Testing Insecticides, The Eastern Press Ltd., London, pp. 167–185.

CALAM, D.H., and YOUDEOWEI, A. 1968. Identification functions of secretions of from posterior scent gland of fifth instar larva of the bug, *Dysdercus intermedius*,. *J. Insect Physiol.* 14:1147–1158.

DAZZINI, V.M., and PAVAN, M. 1978. Scent glands and defensive secretion of Rhyncota. *Pubbl. Inst. Entom.. Univ. Pavia* 5:1–46.

GOODCHILD, A.J.P. 1977. Bionomics, aggregated feeding behaviour and colour variation in the sap sucking bug, *Mygdonia tuberculosa* Sigh (Hemiptera: Coreidae). *Rev. Zool. Afr.* 91:1032–1041.

GRIST, D.H., and LEVER, R.J.A.W. 1969. Pests of Rice, Tropical Science Series, Longmans Green, London.

GUNAWARDENA, N.E., and HERATH, H.M.W.K.B. 1991. Significance of medium chain *n*-alkanes as accompanying compounds in Hemipteran defensive secretions: An investigation based on the defensive secretion of *Coridius janus*. *J. Chem. Ecol.* 17:2449–2459.

GUNAWARDENA, N.E., and HERATH, H.M.W.K.B. 1992. *t*-2-hexenal/*n*-tridecane combination: A natural toxicant of the defensive secretion of *Coridius janus* (Hemiptera: Pentatomidae), *J. Natl. Sci. Counc. Sri Lanka.* 20:209-220.

GUNAWARDENA, N.E., and RANATUNGA, P.R. 1989. Laboratory and field studies of a natural attractant of the rice pest, *Leptocorisa acuta* (Hemiptera: Coreidae). *Trop. Pest Manage.* 35:212–213.

ISHIWATARI, T. 1974. Studies on the scent of stink bugs (Hemiptera: Pentatomidae), I. Alarm pheromone activity. *Appl. Entomol. Zool.* 9:153–158.

KENNARD, C.P. 1962. The paddy bug. *Rice Rev.* 4:34–35.

KOU, R., TANG, D.S., and CHOW, Y.S. 1989. Alarm pheromone of Pentatomid bug, *Erthesino fullo* Thunberg (Hemiptera: Pentatomidae). *J. Chem. Ecol.* 15:2695–2702.

LEVINSON, H.Z., LEVINSON, A.R., and MASCHWITZ, U. 1974. Action and composition of the alarm pheromone of the bed bug, *Cimex lectularis* L. *Naturwissenschaften.* 61:684–685.

LITSINGER, J.A., APOSTOL, R.F., SERANO, O.T., and BARRION, A.T. 1981. Acid fuchsin technique to stain stylet sheaths of the rice bug *Leptocorisa* spp. feeding on rice grains. *Int. Rice Res. Newsl.* 6:19–20.

LOCKWOOD, J.A., and STORY, R.N. 1987. Defensive secretion of the southern green stink bug *Nazara viridula* (L.) (Hemptera: Pentatomidae), as an alarm pheromone. *Ann. Entomol. Soc. Am.* 80:686–691.

MORITA, H., and DHANAPALA, M.P. 1990. "So-called" grain sterility of rice in Sri Lanka in 1985 Yala and 1985/86 Maha cropping seasons. *Jpn. J. Trop. Agric.* 34:20–26.

PESCHKE, K., and EISNER, T. 1987. Defensive secretion of the tenebrionid beetle, *Blaps mucronata*: Physical and chemical determinants of effectiveness. *J. Comp. Physiol. A.* 161:377–378.

PRESTWICH, G.D. 1976. Composition of the scents of eight East African hemipterans, Nymph–adult chemical polymorphism in coreids. *Ann. Entomol. Soc. Am.* 69:812–814.

REMOULD, H. 1962. Scent glands of land bugs, their physiology and biological functions. *Nature* 198:764–768.

SRIVASTAVA, A.S., and SAXENA, H.P. 1967. Rice bug, *Leptocorisa varicornis* and allied species, pp. 525–548, *in* Major Insect Pests of the Rice Plant. Johns Hopkins Press, Baltimore.

STADDON, B.W. 1979. The scent glands of Heteroptera, *Adv. Insect Physiol.* 14:351–418.

TAKETOSHI, I. 1974. Scent of stink bugs (Hemiptera: Pentatomidae). *Appl. Entomol. Zool.* 9:153–158.

WEATHERSTON, J., and PERCY, J.E. 1978. Venoms of Ryncota, pp. 489–509, *in* S. Bettini, (ed.). Arthropod Venoms. Springer Verlag, Berlin.

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INTERACTIONS BETWEEN COMPONENTS OF THE AGGREGATION PHEROMONE DURING CHEMOTAXIS BY THE BARK BEETLE *Ips paraconfusus*

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Abstract—The male-produced aggregation pheromone in *Ips paraconfusus* is composed of three compounds. Female bark beetles were exposed to combinations of these compounds, presented as point sources in an enclosed, circular arena. By itself, *cis*-verbenol (cV) had no effect on the number of beetles that reached the source. Either ipsenol (Ip) alone or ipsdienol (Id) alone strongly increased the number that reached the source, with Id producing a dose–response curve with a much steeper slope. cV moved the onset of the response to Id to higher doses of Id, but the response rose more rapidly after onset than when cV was absent. Overall, cV inhibited the effect of Id except at the highest dose. cV affected the onset of the response to Ip little or none, but strongly increased the slope of the response, synergizing the effect of Ip. The responses to combinations of Id and Ip were related to the log of a linear combination of their doses. The results are consistent with a model where Id and Ip act at a single site of action, but with different potencies, while cV appears to modify the effects of Id and Ip, rather than affecting the site of action directly.

Key Words—Olfaction, ipsenol, ipsdienol, *cis*-verbenol, chemotaxis, perception, discrimination, neural models, logit models, stimulus–response models, *Ips paraconfusus*, Coleoptera, Scolytidae.

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INTRODUCTION

In most sensory modalities, the characteristics of a stimulus have been assigned to one of four dimensions, typically described as quality, intensity, space, and duration (Somjen, 1972). These dimensions are generally treated as though they were separate. In olfaction, perhaps the most studied dimension has been quality discrimination, i.e., the subjective nature of a stimulus (Cain, 1978). As in other modalities, olfactory quality has usually been considered as separate from the intensity of a stimulus.

The term "discrimination" implies that a choice reaction has been made in response to immediately preceding stimulation (Bartley, 1958; Boeckh, 1977; Cain, 1978). Both intensity and quality discrimination may be demonstrated using behavioral responses (Dethier, 1963). Since discrimination along both dimensions is defined on the basis of behavioral choice responses, assuring that the responses are due to the one and not the other dimension can be a problem. If sensory quality is determined by some continuous physical parameter of the stimulus, such as wavelength, then stimuli differing in the value of the putative quality parameter, but of the same energies, may be tested for discrimination among them. Secondly, if quality is independent of intensity, the quality of a stimulus should not change as its intensity is changed. For example, over a wide range of intensities, brightness does not affect the color of light.

Such demonstrations are not always straightforward in olfaction, because the quality aspects of the stimuli often do not relate in any continuous fashion to their chemical, physical, or structural characteristics (Cain, 1978). As a result, the dimensions of quality and intensity may be easily confused. For example, the 10-*trans*-12-*cis*- isomer of bombykol evokes a behavioral response from male *Bombyx mori* at concentrations three to four orders of magnitude lower than any of its other isomers (Schneider et al., 1967). In addition, bombykol by itself evokes a sequence of different behavioral responses as its concentration changes (Schwink, 1958). If two isomers of bombykol were each tested at a single identical concentration, and thereby at nominally "identical" intensities, one isomer might evoke a behavior from early in the sequence and the other might evoke a behavior from later in the sequence. They would appear to have been discriminated. However, from the sensory physiology of the animal, we know the different isomers all stimulate the same set of receptor neurons, but with different efficiencies (Schneider et al., 1967; Kaissling and Priesner, 1970). These two compounds are discriminated on the basis of intensity of stimulation, not quality. Without such knowledge of the animal's sensory physiology, a concentration series of both compounds is required to distinguish whether they are of differing qualities or of different intensities of the same quality. If the compounds cannot be made to elicit distinctly different behaviors over at least some part of their concentration range, the suspicion must be raised that they represent differing intensities of the same quality.

Three compounds together constitute the aggregation pheromone of male *Ips paraconfusus*: ipsenol (Ip), ipsdienol (Id), and *cis*-verbenol (cV) (Silverstein et al., 1966; Wood et al., 1967, 1968). The primary goal of our study was to determine whether the female beetle discriminates between these compounds, i.e., demonstrates different behavioral responses to them. Work with other pheromone systems such as the red-banded leaf roller (*Argyrotaenia velutinana*) (Klun et al., 1973; Baker et al., 1976) and the cabbage looper (*Trichoplusia ni*) (Linn et al., 1984) and indeed earlier work on *I. paraconfusus* itself (Wood et al., 1967, 1968) indicates that multicomponent pheromones often exert their behavioral effects as blends. That is, the compounds may interact in a complex manner and may act in concert in controlling the full sequence of component behavioral steps that lead up to a consummatory act, rather than, for instance, each compound separately controlling different components of the overall response. For instance, our experiments will show that a single compound can have either a synergistic or an inhibitory effect, depending on the other compounds present.

Although it may be relatively small, even the insect nervous system is too complex and contains too many neurons for direct analysis, which is accomplished only by simultaneous (preferably intracellular) recording from connected neurons. As we are limited to more or less inferential methods, behavior provides a minimal benchmark against which hypotheses may be tested. Behavioral responses to systematically manipulated stimuli provide sets of input–output relationships, created by the same neurophysiological mechanisms that we ultimately wish to understand. Any proposal concerning these neural mechanisms must be able to account for these relationships. The far-ranging goal of this study, then, was to provide such sets of relationships and explore whether any simple neural model might explain them.

METHODS AND MATERIALS

The compounds used as stimuli, the experimental arena, the observation chamber, the recording procedures, and the handling of the experimental animal have all been extensively described (Akers and Wood, 1989a; Akers, 1989). Briefly, each treatment had its own arena (Figure 1). The top and bottom of an arena were composed of panes of glass, which were separated by a circular ring 40 cm in diameter, 1 cm high, and with walls approx. 0.5 cm thick. The parts were held together by rubber bands along the edges of the glass panes. The dose for a single treatment, dissolved in 0.1 ml pentane, was applied to a 2 × 2-cm cover slip, which served as the pheromone source, and the solvent was allowed to evaporate. The cover slip was then slipped under the wall of the arena in a standard position next to the ring wall. A dosed arena sat for 1 hr

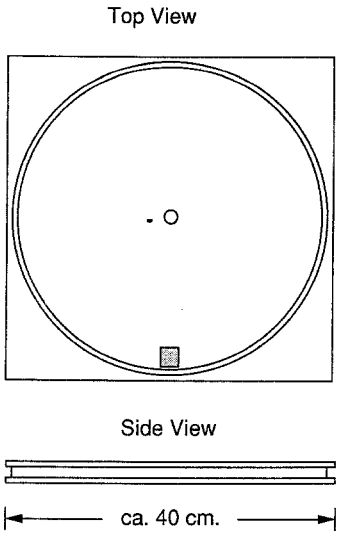

FIG. 1. A schematic of the bioassay arena, approximately to scale. The small circle in the center of the large square represents the access hole in the top pane of glass, through which beetles were introduced into the arena. The small black oblong near the access hole represents a beetle, approximately to scale. The small stippled square at the bottom of the arena is the pheromone source, lying in its typical position next to the inner edge of the ring wall.

before beetles were introduced through a hole in the center of the upper pane of glass. Five beetles were introduced successively within a period of approximately 15 min at the center of the arena, each with an initial orientation roughly perpendicular to the direction of the pheromone source. Each beetle was followed until it contacted the pheromone source, the wall of the arena, or 3 min elapsed. Using a flexible wire passed through the central hole in the arena, each beetle was then turned over near the ring wall to prevent its wandering about, before the next beetle was introduced. All observations were made in an observation chamber that ensured that the beetles wandered at random within the arena when no odor cues were present (Akers and Wood, 1989a). Preliminary experiments demonstrated the beetles did not interact within the arena and that the concentration gradient remained stable over the time the beetles were tested (Akers and Wood, 1989a). An individual beetle was used only once. The parts of an arena were soaked in running hot water for an hour and allowed to air for at least seven days before reuse.

Our study employed a factorial experimental design, with the three pheromone compounds as factors and the number of beetles reaching the pheromone source as the response variable. The purities of the compounds were: ipsenol

95%, ipsdienol 98%, and *cis*-verbenol > 99%. The compounds available were racemic mixtures of the optical isomers. Work since these experiments has demonstrated that the optical isomer composition produced by the beetles is > 95.5% (−)-ipsenol and 1.5–9% (−)-ipsdienol. The ratio of the two compounds in the air around male-infested bolts is 8 : 1 to 26 : 1 ipsenol–ipsdienol, depending on the population and host (Seybold, 1992). Based on preliminary dosage series work with fixed 1 : 1 : 1 ratios of the three pheromonal compounds (Akers and Wood, 1989a), a dosage range of 10^{-3}–1 mg seemed desirable, since the slopes of the response curves were steep and were nearly linear in this range. This would maximize the sensitivity of the assay to changes in the efficacy of the stimulus. Stimuli were also expected to range from very weak (e.g., single compounds) to very potent (e.g., mixtures of all three compounds, also referred to as triplets) (Silverstein et al., 1966; Wood et al., 1967, 1968). On the one hand, then, a wide range of dosages was desirable. On the other hand, the number of treatments goes up as the cube of the number of dosage levels in a factorial experiment with three factors. There were no *a priori* reasons to exclude portions of the response surface, as would be done, for instance, by considering only certain ratios of compounds (Baker et al., 1976). The resulting design, illustrated in the layout of Table 1, was a compromise between obtaining a wider dosage range and limiting the number of treatments. There were 20 replicates per treatment.

The statistical method used to test for significance of effects was based on threshold of tolerance techniques, such as are often used in toxicological studies. Although initial graphical explorations of the data made comparisons using linear regression tempting (e.g., Figure 2F below), a linear model is not an appropriate assumption for these data. Dosage–response curves, such as those generated in this study, are inherently S-shaped because they are bounded below by zero (no beetles reach the source) and above by one (all beetles reach the source). Further, they violate the assumption required by most common linear regression routines that the responses at various treatment levels have equal variances. In threshold-tolerance models, each subject in a population is assumed to have a particular tolerance or threshold dose. A subject responds to the stimulus when the dose exceeds the threshold level. In the present experiments, the number of beetles that reach the cover slip (the pheromone source) is a function of the number of beetles that enter into orientation behavior, which itself seems to be an all-or-nothing phenomenon (Akers and Wood, 1989a). Therefore, we can reasonably use a threshold-tolerance model by assuming that once the dose, D, of a stimulus exceeds the threshold of tolerance for a given beetle, that beetle responds by entering in an all-or-none fashion into orientation. We assumed that the distribution of thresholds across the entire beetle population followed a logistic distribution, and we modeled the probability of response using logit techniques. The Gaussian distribution could likewise be employed, which leads to probit

TABLE 1. NUMBER OF BEETLES REACHING THE SOURCE IN EACH TREATMENT[a]

		Id (mg)				
cV (mg)	Ip (mg)	0	0.001	0.01	0.1	1
0	0	1	—	17	—	17
	0.001	—	2	—	12	—
	0.01	6	—	14	—	18
	0.1	—	12	—	15	—
	1	11	—	15	—	19
0.001	0	—	2	—	17	—
	0.001	6	5	14	—	18
	0.01	—	14	—	17	—
	0.1	15	—	10	—	17
	1	—	18	—	19	—
0.01	0	0	—	7	—	18
	0.001	—	17	—	16	—
	0.01	11	—	18	—	19
	0.1	—	14	—	12	—
	1	20	—	17	—	20
0.1	0	—	4	—	16	—
	0.001	5	—	16	—	19
	0.01	—	11	—	18	—
	0.1	15	—	11	15	16
	1	—	20	—	20	—
1	0	1	—	2	—	19
	0.001	—	6	—	12	—
	0.01	10	—	14	—	17
	0.1	—	18	—	13	—
	1	18	—	20	—	17

[a](*N* = 20 per treatment). (— indicates combination was not used as a treatment.)

models. Logit and probit models give almost identical fits to data in the middle dosage levels (Robertson and Preisler, 1991). In the logit model, the probability of response, p (= the expected proportion of the population responding) is given by

$$P = \frac{e^{\theta}}{1 + e^{\theta}} \tag{1}$$

with the logit line $\theta = \log(p/1 - p) = \alpha + \beta D$, where α and β are regression parameters that are estimated to best fit the curve to the data.

Once the model for the population response has been chosen, the problem

in the present instance becomes choosing an appropriate function for the dose, D. Often, in a toxicological study with chemicals given individually, the logarithm of the dose is a popular choice. In the present circumstances, the effective dose is considered to be a function of the doses of the three compounds. Based on the preliminary graphical analysis (see Results), we tested an additive (similar action) model where the overall effective dose, D, is given by

$$D_{ijk} = \log (Ip_i + \rho_k Id_j) \tag{2}$$

where Ip_i is the ith ipsenol dose level, Id_j is the jth ipsdienol dose level and ρ_k for ($k = 1, \cdots, 5$) is a parameter that reflects the effect of the kth cV dose level on the relative potency of the other two compounds. In this model, Ip and Id have an additive stimulus effect on the same "site of action" in the beetle.

Two other models were used to fit the data and compared with the similar action model: (1) a logit model with the logit line, $\theta = \alpha + \beta_1 \log (Ip) + \beta_2 \log (Id)$, and (2) an independent action model with the probability of response, p, given by $p = p_1 + p_2 - p_1 p_2$, where p_1 is the probability of reponse to Id in the absence of Ip and p_2 is the probability of response to Ip in the absence of Id. The independent action model estimates the expected response if the two compounds, Id and Ip, are acting independently and a response occurs if the dose of at least one of the compounds exceeds the tolerance of the beetle. This kind of response is expected if the action sites of the two compounds are different, i.e., if each compound has a different mode or site of action and these are totally independent.

Strictly speaking, the logit analysis assumes an underlying binomial distribution for the data. However, variance beyond that predicted by the binomial can be taken into account by multiplying estimates of variances by a "heterogeneity factor" (Finney, 1971), which we employed. All estimations were done using the GLIM package (Payne, 1986). The parameter ρ in equation 2 was estimated using a grid search to arrive at an estimate of ρ that maximized the likelihood function.

RESULTS

In exploratory plots of the number of beetles reaching the source versus various combinations of the three compounds, cV alone clearly had no effect on the number of responses (Figure 2A), while Ip and Id both increased the number of beetles reaching the source. The curve for Id alone appeared to be steeper than that for Ip alone (respectively, Figure 2E and 2C). The interaction between cV and Id (Figure 2F) appeared to be the opposite of that between cV and Ip (Figure 2D). cV increased the slope of the response to Ip (Figure 2D) as compared to treatments where cV was absent (Figure 2C). Conversely, cV

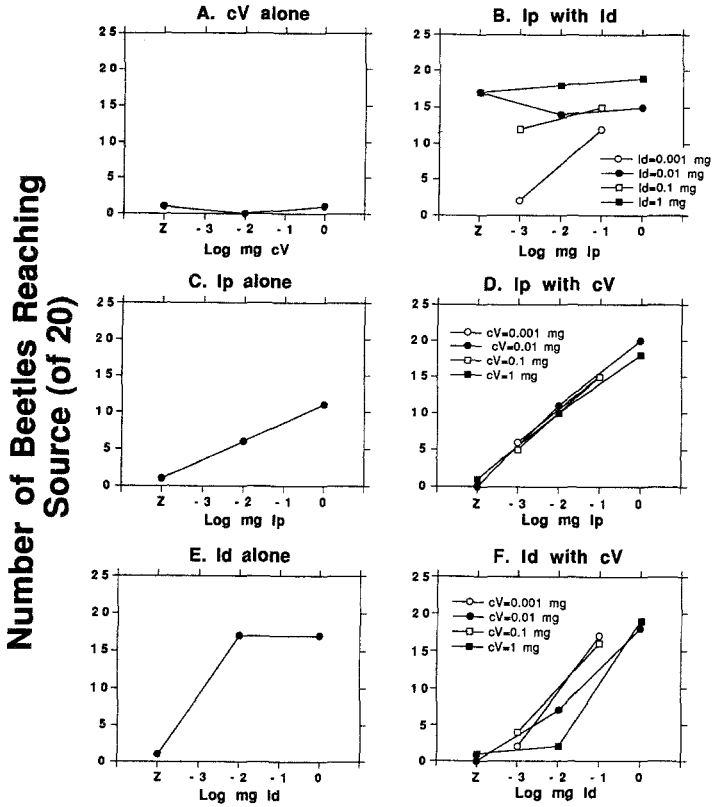

FIG. 2. The number of beetles (of 20 in each treatment) that reached a small source (ca. 2% of the perimeter of the arena) when dosed with different combinations of cV, Id, or Ip. Z indicates the zero-mg dose. (A) The effect of different doses of cV placed by itself on the source. (B) The effects of Ip in combinations with Id. (C) The effect of Ip alone. (D) The effect of Ip in combinations with cV. The different curves in D are not significantly different from each other, but they are all significantly different from the curve in C. (E) The effect of Id alone. (F) The effect of Id in combinations with cV. The different curves in F are not significantly different from each other, but they are all significantly different from the curve in E.

decreased the response to Id by moving the curves to the right (Figures 2E and 2F). Thus, cV appeared to synergize Ip and inhibit Id.

The most apparent pattern seen when all three compounds were present simultaneously was that increasing doses of Ip increased responses at all dose levels of Id but the highest (Figures 3A–D). At the highest Id dose (1.0 mg), Id by itself seemed to produce 100% response (Figures 2E, 2F; 3A–D). When-

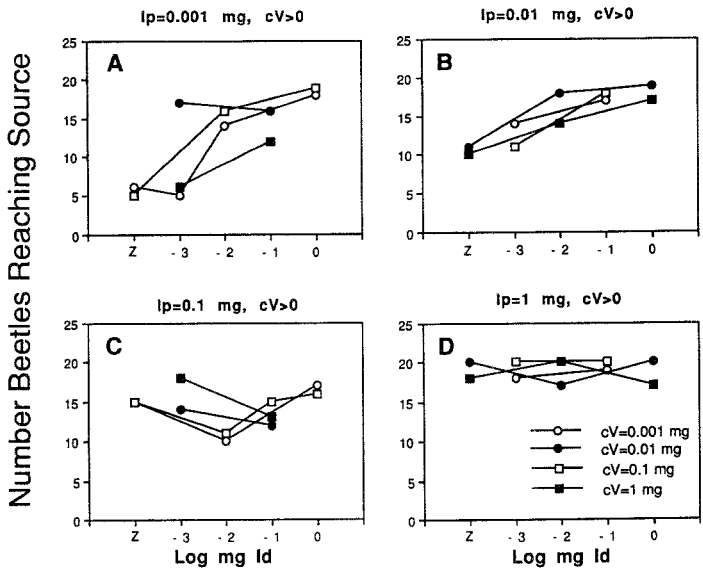

FIG. 3. The number of beetles (of 20 in each treatment) that reached a small source (ca. 2% of the perimeter of the arena) when dosed with different combinations of cV, Id, and Ip, in triplet mixtures. The dose of Id changes along the x axis of each panel, the dose of cV changes as indicated among the different curves within each panel, and the dose of Ip remains constant within a panel, but changes from one panel to the next. Z = 0-mg dose.

ever Id is present at this concentration, any inhibitory effect of cV seems to be obscured by the overwhelming response to Id. Finally, although the presence of any cV (above 0.001 mg) had an effect on response, increasing doses of cV did not seem to substantially increase the effect.

The statistical significance of the effects apparent in the graphical analyses was tested using logit regression (see Methods and Materials). The similar action (log of sum of doses) model gave a much better fit to the data than either the independent action or the sum of the logs of doses model (Table 2), as is indicated by the lower chi-square value, and by noting that the chi-square and the degrees of freedom are nearly equal in value. Further, the hypothesis that the observed responses are not different from the predicted responses is clearly rejected for the independent action and the sum of the logs of doses models, but not for the similar action model. Since the model is predicting the values of 65 treatments using six parameters, this is a good performance. A sense for the agreement between the model and the experimental results can be gained by comparing the predicted (expected) and observed responses in Tables 3 and 4.

TABLE 2. COMPARING GOODNESS-OF-FIT OF THREE MODELS TO DATA

Model	Chi-square goodness-of-fit[a]	*df*	*P* value[b]
1. Similar action $\text{logit}(p) = \alpha + \beta \log(Ip + \rho Id)$	76.1	59	0.066
2. Additive in log dose $\text{logit}(p) = \alpha + \beta_1 \log(Ip) + \beta_2 \log(Id)$	106.8	59	0.0001
3. Independent action $p = p_{Id} + (1 - p_{Id})p_{Ip}$	208.4	57	$\ll 0.0001$

[a] A separate fit was done for the data with cV = 0 and with cV > 0. The chi-square goodness-of-fit from the two fits were added to give the overall chi-square values in the table. Models that better fit the data have lower chi-square values.
[b] The probability that the model and the observed data are the same. Only the similar action model is not rejected.

TABLE 3. ESTIMATES (± STANDARD ERRORS) OF EXPECTED THRESHOLD OF TOLERANCE (ED_{50}) AND PROPORTION OF BEETLES REACHING SOURCE OF Id IN ABSENCE AND PRESENCE OF cV

Dose of cV (mg)	ED_{50} (95% CL)	Percent reaching source: Id = 0.01 mg Exp.	Id = 0.01 mg Obs.	Id = 1 mg Exp.	Id = 1 mg Obs.
0[a]	0.0016 mg (0.0001–0.018)	68.1 (±8.8)	85.0	93.1 (±2.2)	85.0
>0[b]	0.02 mg (0.0094–0.049)	37.4 (±7.2)	22.5	93.1 (±2.2)	92.5

[a] Fitted model was $\text{logit}(p) = 2.6 + 0.92\log(Id)$.
[b] Fitted model was $\text{logit}(p) = 2.6 + 1.56\log(Id)$.

We developed the similar action model because in the graphical analyses the responses to combinations of Id and Ip clearly were not the sums of the responses to the compounds presented individually (Figure 2B vs. 2C and 2E). As noted, the similar action model could be mechanistically interpreted as both compounds having a qualitatively similar effect on a single site of action.

Using the similar action model as a basis for testing for differences, we began by estimating a separate logit curve (= set of parameters) for each of the five levels of cV (Table 5). When we tested for differences among the parameters, the curve when cV was absent was significantly different from the curves

TABLE 4. ESTIMATES (± STANDARD ERRORS) OF EXPECTED THRESHOLD OF TOLERANCE (ED_{50}) AND PROPORTION OF BEETLES REACHING SOURCE OF Ip IN ABSENCE AND PRESENCE OF cV

Dose of cV (mg)	ED_{50} (95% CL)	Percent reaching source: Ip = 0.01 mg Exp.	Ip = 0.01 mg Obs.	Ip = 1 mg Exp.	Ip = 1 mg Obs.
0[a]	0.38 mg (0.04–3.32)	29.8 (±7.1)	30.0	56.3 (±10.0)	55.0
>0[b]	0.008 mg (0.005–0.01)	52.8 (±7.2)	52.5	92.6 (±2.6)	95.0

[a] Fitted model was logit(p) = 0.25 + 0.60log(Ip).
[b] Fitted model was logit(p) = 2.52 + 1.20log(Ip).

TABLE 5. ESTIMATES OF PARAMETERS ± STANDARD ERRORS IN SIMILAR ACTION MODEL, LOGIT(P) = $\theta = \alpha + \beta$Log($Ip + \rho Id$)

Dose of cV (mg)	$\hat{\alpha} \pm$ SE	$\hat{\beta} \pm$ SE	ρ	Chi-square goodness-of-fit (df = 10)
0	0.76 ± 0.22	0.80 ± 0.19	32.0	21.64
0.001	2.17 ± 0.35	1.11 ± 0.20	1.5	18.97
0.01	2.43 ± 0.54	0.86 ± 0.26	0.32	37.88
0.1	2.23 ± 0.38	1.13 ± 0.24	2.0	21.94
1	2.61 ± 0.44	1.20 ± 0.23	0.12	19.63

when cV was present (P = 0.000016) (likelihood ratio test; McCullagh and Nelder, 1989), but there were no significant differences among the curves from among the various nonzero levels of cV (P = 0.79). The same result was observed in the most of the graphs in Figures 2 and 3. This result also indicates that the curves in Figure 2F are not significantly different from one another and should be pooled, even though they may appear to differ from one another on visual inspection. When cV is absent, the situation is qualitatively different from the situation when cV is present, but once cV is present (at or above 10^{-3}mg), its relative level makes little difference. In particular, note that ρ dropped from 32 when cV was absent to values of 1–2 whenever it was present, indicating that the efficacy of Ip increased greatly relative to that of Id whenever cV was present. This change in ρ could be due to either Ip becoming more effective in

the presence of cV or Id becoming less effective in the presence of cV. The following analysis demonstrates that both phenomena are in effect.

Since there was no difference among the nonzero dosage levels of cV, the logit model was simplified by pooling together the curves where cV was greater than zero, which reduces the maximum number of parameters to be estimated from 15 to six. Two situations of interest were then studied: the combinations of cV with Ip versus Ip alone (Figure 2D vs. 2C; Figure 4), and the combinations of cV with Id versus Id alone (Figure 2F vs. 2E; Figure 4). The parameters to be included in the logit models were determined by testing the deviation between observed and expected responses of more complex logit models (more parameters to be estimated) against that of simpler models (fewer parameters to estimate). Because of their greater number of parameters, more complex models will have lower deviations than simpler models. However, this decrease in deviation must be weighed against the loss in degrees of freedom that results from estimating more parameters. A more complex model was accepted over a simpler model when the decrease in deviation more than outweighed the loss in degrees of freedom, as tested by a likelihood ratio test (McCullagh and Nelder, 1989). This procedure led to slightly different optimal models for the two situations, Ip alone vs. Ip plus cV (Ip–cV interactions), and Id alone vs. Id plus cV (Id–cV interactions). For the Id–cV interactions, the intercepts of the logit lines were the same (not significantly different) in the presence and absence of

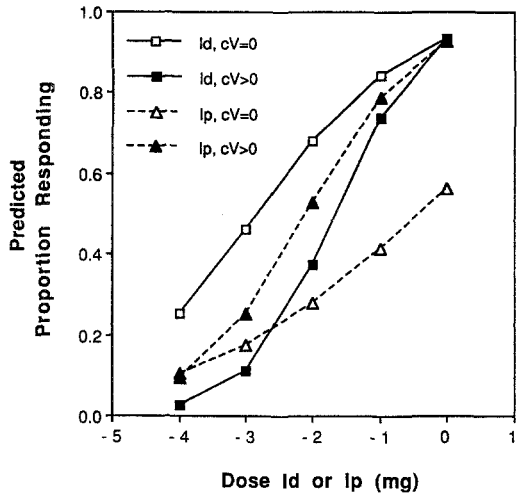

FIG. 4. A plot of the percent response predicted by the logit equations given in the footnotes to Tables 3 and 4, for decade steps in dose. Curves involving Id are indicated by squares; curves for Ip are indicated by triangles. When cV is absent the symbols are open; when cV is present the symbols are filled.

cV (footnote, Table 3), whereas for the Ip–cV interactions the estimates of the intercepts were significantly different (Table 4). Note that, because of the log scale used for dosage, the curve intercepts the y axis when the dose is 1 mg. We then used the optimal models to predict the level of response at a low (0.01 mg) and a high (1 mg) dose of Id or Ip (Tables 3 and 4). We also calculated an estimate of the median threshold of tolerance (ED_{50}, analogous to the LD_{50} of toxicological studies). This exercise reinforced many of the trends observed in the graphical analyses. The response to Id alone is high, and adding cV to it inhibits its effects, especially at low or middle dose levels of Id (Table 3, Figure 4). However, at high doses of Id, the inhibitory effect of cV seems to be overwhelmed by the effect of Id. The opposite occurs for Ip. The effect of Ip alone is low, but cV synergizes it (Table 4, Figure 4).

DISCUSSION

Pheromone Perception System. The results of this study clearly demonstrate a variety of effects and interactions of the pheromone components on the behavior of *I. paraconfusus* due to processing by the nervous system, and rather simple interactions between neural elements can explain them. Electrophysiological evidence from this and a congeneric species (*I. pini*) indicates that the olfactory receptor neurons are largely specific for single compounds (Mustaparta et al., 1977, 1979; Mustaparta, 1979). Clearly, the sensory input generated by Id or Ip alone excites the initiation of approach orientation. The excitation caused by Id appears to be somewhat stronger than that caused by Ip. This could be brought about by sensory neurons that differ in number, threshold, sensitivity, or synaptic coupling coefficients. Recordings from whole antennae indicated that female *I. paraconfusus* are somewhat more sensitive to ipsdienol than ipsenol (Light, 1983a,b). On the other hand, recordings from single sensilla on *I. pini* indicate that roughly equal proportions of sensory neurons are specialized for the two compounds (Mustaparta, 1979). Further, the examples illustrated by Mustaparta (1979) seem to indicate that the two classes of receptor neurons are also similar in threshold and sensitivity. Further clarification is obviously needed, but Mustaparta's work would indicate that the differing levels of reponse to the two compounds are mostly due to a central phenomenon.

cV appears to modify the effects of Id and Ip rather than affecting orientation directly. The increase in slope that cV causes in the response to Ip could be easily accomplished if the cV sensory neurons (or interneurons excited by them) presynaptically facilitated synapses from the Ip sensory neurons (or interneurons excited by them) onto whatever network controls the initiation of orientation. Since such facilitation increases the size of each postsynaptic potential caused by Ip, it is equivalent to multiplying each point on the Ip curve by a

constant, thereby increasing the slope. The value of the constant is directly related to the frequency of impulses arriving along the cV channel. This assumes that interneurons behave approximately as linear operators over a relevant range (Somjen, 1972; MacGregor and Lewis, 1977). The interaction between Id and cV is more complex. cV raises the threshold at which orientation occurs but does not lower the maximal response or the response to the highest levels of Id, relative to Id alone (Figure 4). At least two levels of interneurons would be required to achieve both the change in slope as well as the change in threshold. Activity in the second, higher level of interneurons (I2) would excite the network that controls the initiation of orientation. The increase in threshold could be achieved if input from the Id sensory neurons directly excited I2, while the cV sensory neurons indirectly inhibited I2 through a presynaptic interneuron (I1). In turn, I1 would need to be excited by input from the cV sensory neurons and inhibited by the Id sensory neurons, the latter effect providing the change of slope. If, instead, no interneuron I1 existed and the cV sensory neurons directly inhibited I2, a constant, determined by the frequency of impulses in the cV sensory neurons, would be subtracted from every point along its response to Id. This would move the curve to the right in a parallel fashion. However, with I1 interposed, the inhibitory effect of cV is itself inhibited. Since Id inhibits I1, it prevents much of the activity in I1 when it is at high concentrations, so that the effect of cV is opposed. The net effect is such that cV can exert maximal inhibition only when Id is at the lowest concentrations.

When further information becomes available, the model will no doubt need modification. For instance, we have measured the headings of the beetles with respect to the source as they moved in the arena (Akers and Wood, 1989a). Such measurements hint that cV by itself may not be entirely without effect, but may in fact actually cause movement away from the source. This is consistent with another response surface experiment utilizing an anemotactic response (Akers and Wood, 1989b). That experiment has not been fully analyzed but also suggests that cV by itself may be repellent. If this were true, perhaps there are no interneurons balancing the inputs of cV and Id. Instead, cV may drive a network for orientation away from the source and Id may drive a network for orientation towards the source (and perhaps inhibit the network for orientation away from the source), and the overall outcome is the result of competition between these two networks.

Comparisons with Earlier Work. The present results differ somewhat from earlier behavioral work with *I. paraconfusus*. In lab assays using beetles walking upwind to a point source of pheromone, none of the compounds was attractive by itself (Wood et al., 1966; Silverstein et al., 1966). Field assays monitoring the number of beetles caught at a trap (Wood et al., 1967, 1968) are difficult to interpret because of the low number of replicates, but there was some slight evidence for attraction to Ip or Id delivered alone, at least when synthetic race-

mates were employed. Bark beetles can be very sensitive to the enantiomeric composition of certain compounds that are otherwise chemically pure (Birch et al., 1980; Lanier et al., 1980; Seybold, 1992). Although the sensitivity of *I. paraconfusus* to enantiomeric blends has never been explicitly studied, it would appear to be relatively insensitive, since the beetles responded readily to the racemates used in the present and earlier studies. Putting aside possible enantiomeric effects, in previous studies only doublet mixtures that contained Ip were attractive in lab assays, while only Ip + Id showed any activity in the field. In the present study, both Id and Ip were quite active either singly or together, while the cV + Ip doublet was more active than Ip alone. The differences in the studies may be partly contextual and partly a difference in the sensitivity of the assays. Earlier work had always been based on situations where air movement was present as an orienting cue (anemotaxis). In the anemotaxis response surface alluded to earlier, Id by itself did not appear to produce upwind movement above that of the control, while Ip alone did, although not strongly so. This impression may change with more extensive analysis of the experiment, but it is similar to observations in earlier work, where air movement was always a factor. The relative concentrations of the compounds probably were not drastically different between the taxis and anemotaxis response surface experiments, since gas chromatography indicates that the different pheromonal compounds behave very similarly in terms of their evaporation and diffusion characteristics (Akers and Wood, 1989a).

The dual activity of cV was never hinted at in earlier work on *I. paraconfusus* and indeed is a rather striking and surprising result. However, a similar phenomenon has been noted in another bark beetle, *Dendroctonus pseudotsugae*. In that species, the compound 3-methyl-2-cyclohexen-1-one acts as a synergist when in low concentrations relative to attractants in the aggregation blend, but it acts as an inhibitor at high relative concentrations (Rudinsky, 1973; Rudinsky and Ryker, 1980). It has been termed a "multifunctional pheromone," and cV may prove to be another example. Considering the overlap of compounds used by different species of bark beetles, and the extensive communication both between and within species, perhaps such nuances in the use of pheromonal compounds are to be expected.

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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 beetles. *J. Chem. Ecol.* 15:183–208.

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.* 15:1147–1159.

BAKER, T.C., CARDÉ, and ROELOFS, W.L. 1976. Behavioral responses of male *Argyrotaenia velutinana* (Lepidoptera: Tortricidae) to components of its sex pheromone. *J. Chem. Ecol.* 2:333–352.

BARTLEY, S.H. 1958. Principles of Perception. Harper and Row, New York, 482 pp.

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.* 6:703–718.

BOECKH, J. 1977. Aspects of nervous coding of sensory quality in the olfactory pathway of insects, pp. 308–322, *in* D. White (ed.). Proceedings of the 15th International Congress of Entomology. Entomology Society of America.

CAIN, W.S. 1978. History of research on smell, pp. 197–229, *in* M.P. Friedman (ed.). Handbook of Perception, Vol. VIA: Tasting and Smelling.

DETHIER, V.G. 1963. The Physiology of Insect Senses. Wiley & Sons, New York, 266 pp.

FINNEY, D.J. 1971. Probit Analysis, 3rd ed. Griffin, London.

KAISSLING, K.E., and PRIESNER, E. 1970. Die Riechschwelle de Seidenspinners. *Naturwissenschaften* 57:23–28.

KLUN, J.A., CHAPMAN, O.L., MATTES, K.C., WOJKOWSKI, P.W., BEROZA, M., and SONNET, P.E. 1973. Insect sex pheromones: Minor amount of opposite geometrical isomer critical to attraction. *Science* 181:661–663.

LANIER, G.N., CLASSON, 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.

LIGHT, D.M. 1983a. Sensitivity of antennae of male and female *Ips paraconfusus* (Coleoptera: Scolytidae) to their natural aggregation pheromone and its enantiomeric components. *J. Chem. Ecol.* 9:561–584.

LIGHT, D.M. 1983b. Sensitivity of antennae of male and female *Ips paraconfusus* (Coleoptera: Scolytidae) to its pheromone and other behavior-modifying chemicals. *J. Chem. Ecol.* 9:585–606.

LINN, C.E., BJOSTADT, 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.* 10:1309–1324.

MACGREGOR, R.J., and LEWIS, E.R. 1977. Neural Modelling: Electrical Signal Processing in the Nervous System. Plenum Press, New York, 414 pp.

MCCULLAGH, P., and NELDER, J.A. 1989. Generalized Linear Models, 2nd ed. Chapman and Hall, New York.

MUSTAPARTA, H. 1979. Chemoreception in the bark beetles of the genus *Ips*: Synergism, inhibition, and discrimination of enantiomers, pp. 147–158, *in* F.J. Ritter (ed.). Chemical Ecology: Odor Communication in Animals. Elsevier, New York.

MUSTAPARTA, H., ANGST, M.E., and LANIER, G.N. 1977. Responses of single receptor cells in the pine engraver beetle, *Ips pini* (Say) (Coleoptera: Scolytidae) to its aggregation pheromone, ipsdienol, and the aggregation inhibitor, ipsenol. *J. Comp. Physiol.* 121:343–347.

MUSTAPARTA, H., ANGST, M.E., 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.

PAYNE, C.D. (ed.). 1986. The GLIM system release 3.77 manual. Numerical Algorithms Group, Oxford, U.K.

ROBERTSON, J.L., and PREISLER, H.K. 1991. Pesticide Bioassay with Arthropods. CRC Press, Boca Raton, Florida.

RUDINSKY, J.A. 1973. Multiple functions of the Douglas fir beetle pheromone 3-methyl-2-cyclohexen-1-one. *Environ. Entomol.* 2:579–585.

RUDINSKY, J.A., and RYKER, L.C. 1980. Multifunctionality of Douglas-fir beetle pheromone 3,2-MCH confirmed with solvent dibutyl phthalate. *J. Chem. Ecol.* 6:193–201.

SCHNIEDER, D., BLOCK, B.C., BOECKH, J., and PRIESNER, E. 1967. Die Reaktion der mannlichen Seidenspinner auf Bombykol und seine Isomeren: Elektroantennogramm und Verhalten. *Z. Vergl. Physiol.* 54:192–209.

SCHWINK, I. 1958. A study of olfactory stimuli in the orientation of moths. *Proc. 10th. Int. Cong. Entomol.* 2:577–582.

SEYBOLD, S.J. 1992. The role of chirality in the olfactory-directed behavior of pine engraver beetles in the genus *Ips* (Coleoptera: Scolytidae). PhD dissertation. University of California, Berkeley.

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.

SOMJEN, G. 1972. Sensory Coding in the Mammalian Nervous System. Meredith Corp., New York, 386 pp.

WOOD, D.L., BROWNE, L.E., SILVERSTEIN, R.M., and RODIN, J.O. 1966. Sex pheromones of bark beetles: I. Mass production, bioassay, source, and isolation of the sex pheromone of *Ips confusus* (LeC.) *J. Insect Physiol.* 12:523–536.

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, 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.

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Olea europaea VOLATILES ATTRACTIVE AND REPELLENT TO THE OLIVE FRUIT FLY (*Dacus oleae*, Gmelin)

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Abstract—Comparative study of the headspace (HS) composition from olive leaves, olives of varying degrees of ripeness, and crushed olives revealed significant quantitative and qualitative differences. Three different methods were employed to test the effect of the substances identified on the behavior of *Dacus oleae* gravid females. Attractive/repellent features were tested by presenting the insects with olfactory choices of a water solution of the test chemicals examined, water (blank), and a highly attractive (control) solution of olive maceration water (MW). Toluene and ethylbenzene, present in leaf and half-ripe olive HIS proved attractive, while (*E*)-2-hexenal emitted by the crushed olives was found to be decidedly repellent. An oviposition stimulation/deterrence test was carried out on olives placed in proximity to test chemicals under examination in water solution, with olives from the same crop serving as control. In the oviposition test α-pinene, mostly emitted by the leaves and half-ripe olives, emerged clearly as an activant, *p*-xylene, myrcenone, ethylbenzene, *n*-octane and *o*-xylene as weak activants. (*E*)-2-hexenal and hexanal, both emitted by the crushed olives, displayed an oviposition deterrent effect. The highly repellent property of (*E*)-2-hexenal was again confirmed in this test.

Key Words—*Olea europaea*, allelochemicals, *Dacus oleae*, Diptera, Tephritidae, host-plant volatiles, attractants, repellents, oviposition stimulant, oviposition deterrent.

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INTRODUCTION

The olive fruit fly, *Dacus oleae*, is a very common insect infesting olive plants throughout the Mediterranean basin. Like other species of the Tephritidae family (fruit flies), the pest can cause serious damage to the fruit, boring through the pulp and eating away the potential gains of oil producers. In the last few years, pest controllers have made many attempts to eliminate or reduce chemical sprays by introducing new approaches (integrated control) and products (e.g., sex pheromones) (Gariboldi et al., 1983). Unfortunately current control methods are still based on synthetic insecticides. Fuller understanding of the complex system of host-plant allelochemicals could pave the way for a more efficient approach to fighting the olive fly.

Various authors have investigated the olive–*D. oleae* relationship, and interesting results have been obtained (Cirio et al., 1977). However, despite much work (Bateman, 1972; Fiesta Ros de Ursinos et al., 1972; Prokopy, 1977; Cavalloro, 1982; Levinson and Levinson, 1984; Fletcher, 1987; Metcalf, 1990), the only clear points experimentally demonstrated so far are: (1) the powerfully repellent chemotactile effect of the olive-mill waste waters attributed to β-3,4-dihydroxyphenylethanol (Vita and Cirio, 1977, 1980); (2) the repellent olfactory effect of damaged olives, rejected by *D. oleae* for oviposition (Girolami et al., 1981); and (3) the attractive effect of olive maceration water (see Methods and Materials) (Vita and Barbara, 1976). Moreover, oviposition stimulant effects have been attributed to the oleuropein (Panizzi and Scarpati, 1960) and its aglycone (Girolami et al., 1974). Other authors have noted the return of natural *D. oleae* populations to the olive groves after the first abundant summer rains (Girolami et al., 1982). This phenomenon has yet to be accounted for, although it must certainly be due to volatile compounds emitted by the plants and active on the olive fly.

A number of authors (Fedeli et al., 1973a,b; Montedoro et al., 1978; Nabeta et al., 1988) have identified a great many volatile compounds deriving from various parts of the plant or dissolved in the oil, but no comparative research has been carried out in this area nor, as far as we know, has the activity of these compounds on the insect been investigated.

Thus a common feature of previous findings is the great but confused variety of roles attributed to the plant compounds. The aim of our research is to reinvestigate the entire subject, beginning with preliminary study of all the volatile and nonvolatile components of *O. europaea* in order to ascertain their exact roles and, in particular, to identify the substances active on the behavior of the *D. oleae*.

We agree with Cirio (1971) and Vita and Barbera (1976) in identifying four types of interaction affecting olive fruit fly host acceptance, i.e.: (1) olfactory attraction; (2) chemotactile attraction; (3) olfactory repulsion; and (4)

chemotactile repulsion. We are concerned with all four types of interaction; our findings in sectors 1 and 3 are presented in this paper.

METHODS AND MATERIALS

Chemical Investigation. The volatile organic compounds emitted by the various parts of *O. europaea* were collected with the following method. The substratum was placed in a glass container with openings for the entrance and exit of the carrier gas (purified nitrogen or air). For cryogenic collection of head space (HS) volatiles the carrier gas was passed into a trap at −80°C. For identification of the HS chemical compounds, the nitrogen or air carrier gas at a flow rate of 50 ml/min was purified with an alumina column (70/230 mesh, activity I, Merck). Absorption of the volatiles at the exit of the container was performed with a Supelco Carbotrap 300, 115 mm, ID 4 mm. All connections were made with glass–glass or steel–glass contact secured with Teflon pipes.

The substratum was fluxed for a number of hours depending on the quantity of volatile components emitted (40 min–12 hr). The absorbed components were desorbed by heating the Carbotrap with a Supelco Thermal Desorption Unit (TDU, model 850) with a helium flow and analyzed with a GC-MS (HP 5890/ HP 5971A) system, which was in turn linked with a data processor (HP 59970 MS CHEMSTATION).

Blank or control air collections were made each time as described for samples. Prior to reutilization, the Carbotraps were conditioned by heating with a helium flow in the TDU for 4 min at 100°C and for 14 min at 400°C.

The GC column used was a capillary SUPELCO SPB-1 (1% methyl phenylsilicone), 30 m, ID 0.2 mm, film thickness 0.2 μm.

Laboratory Bioassays. The experiments were carried out under laboratory conditions at 21–25°C and 50–60% relative humidity. Adult olive fruit flies from groves in the Lazio region were transferred to Plexiglas cages 20 × 20 × 20 cm and fed a diet consisting of a mixture of sugar (33%), soy flour (33%), and yeast hydrolysate (33%).

A rearing procedure was adopted using fruits of the Itrana variety, available until April. The insects were transferred to cages 10 days after eclosion and subjected to the tests, during which water was constantly available but food was removed in order to avoid interference.

Three different tests were done in order to ascertain the effect of the olive volatiles identified on the behavior of the gravid females of *D. oleae*, as follows:

Method I. Olfactory Attractancy. Maceration water (MW) were used as control in the attractancy tests. This solution was prepared by totally immersing olives in distilled water and keeping them at room temperature. Variations in attractancy of MW were monitored during that time.

The following procedure was adopted in order to ascertain the attractancy of the olive volatiles. In a Plexiglas cage (20 × 20 × 60 cm) containing mostly gravid females (75 individuals), we placed three 5-cm-diameter Petri dishes as far apart as possible (about 28 cm). They contained 15 ml of: (1) test solution of a pure chemical, (2) control (MW), and (3) blank (water). Every 30 min the dishes were shifted around to eliminate position effects. An insect visiting a container was trapped in the water solution. Testing was carried out before sunset for a duration of 150 min, and the trapped individuals were counted.

Test chemicals (Aldrich, GC purity) were dissolved or suspended in distilled water at a concentration of 10 ppm. Myrcenone (2-methyl-6-methylene-1,7-octadien-3-one), which is not commercially available, was synthesized starting from myrcene (Schulze et al., 1989). The HS volatiles obtained with cryogenic collection were dissolved in a volume of water sufficient for three assays (45 ml).

Method 2. Olfactory Repellency. A test similar to method 1 was adopted to evaluate the repellent effect of the olive volatiles. In this case, the test sample was a solution of the compound in MW at a concentration of 10 ppm, the control was the same MW, and the blank water.

Method 3. Oviposition. The oviposition test was based upon the ability of three gravid females to oviposit on six natural fruits sited at the top of three test tubes containing water (controls) and three filled to 2.5 cm from the rim with test solution. The set of test tubes containing the water solution of the test sample was placed as far as possible from the set of blanks, in a Plexiglas cage (20 × 20 × 20 cm). The olive was placed on the rim of the test tube in such a way that the volatiles evaporating from the solution came into contact with the fly (Figure 1). Six hours later, before sunset, the ovipositions were counted in order to evaluate the stimulant or repressive effect of the natural compounds tested. A response index was defined as the mean ratio of ovipositions on the three olives exposed to the test sample vs. the three exposed only to water.

RESULTS AND DISCUSSION

The HS volatiles from olive leaves, undamaged olives of varying degrees of ripeness (Itrana var.), and MWs were condensed in a cold trap. Thus we were able to ascertain for the first time the attractancy of all the samples (method 1), demonstrating that the olive tree does in fact contain volatile substances acting on the behavior of *Dacus* females (Figure 2A). The HS volatiles from crushed olives showed no attractancy.

GC-MS analyses of the trapped volatiles from small unripe olives yielded limonene, *n*-octane, β-pinene, and myrcenone (Figure 3A), while the analyses of half-ripe olives yielded α-pinene, toluene, limonene, and *p*-cymene (Figure

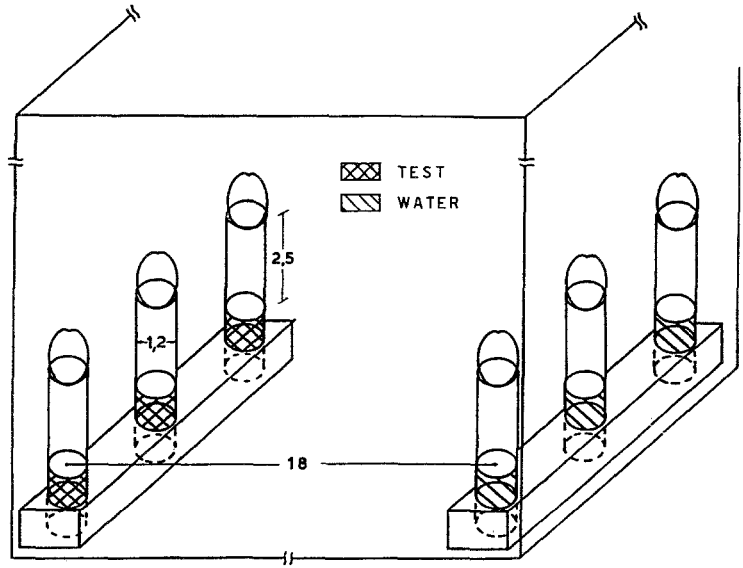

FIG. 1. Method 3. Set-up used in oviposition tests with three gravid females and six olives, test tubes contain chemicals (10 ppm in water) or water (blank). All sizes are expressed in centimeters.

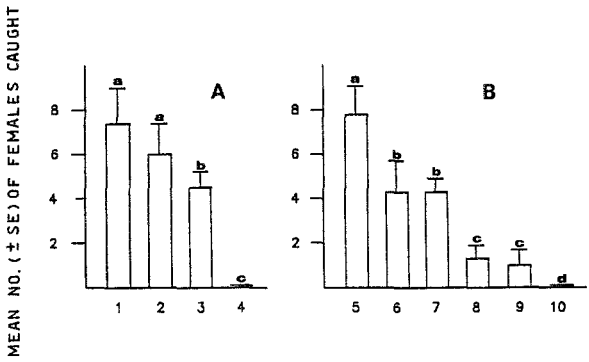

FIG. 2. Olfactory attractancy tests. Attractiveness to *D. oleae* females of HS volatiles (A) and of individual chemicals identified in olive volatiles at 10 ppm concentration in water (B). Replicate numbers (n) are reported in parentheses below. Error bars represent standard error. Significant differences ($P < 0.05$) within each graph are indicated by different lowercase letters, as indicated by Duncan's (1955) multiple-range test. (A) 1, HS of olives (8); 2, HS of MW (6); 3, HS of leaves (6); 4, HS of crushed olives (6). (B) 5, control MW (40); 6, toluene (12); 7, ethylbenzene (12); 8, n-octane (8); 9, α-pinene (8); 10, blank H_2O (40).

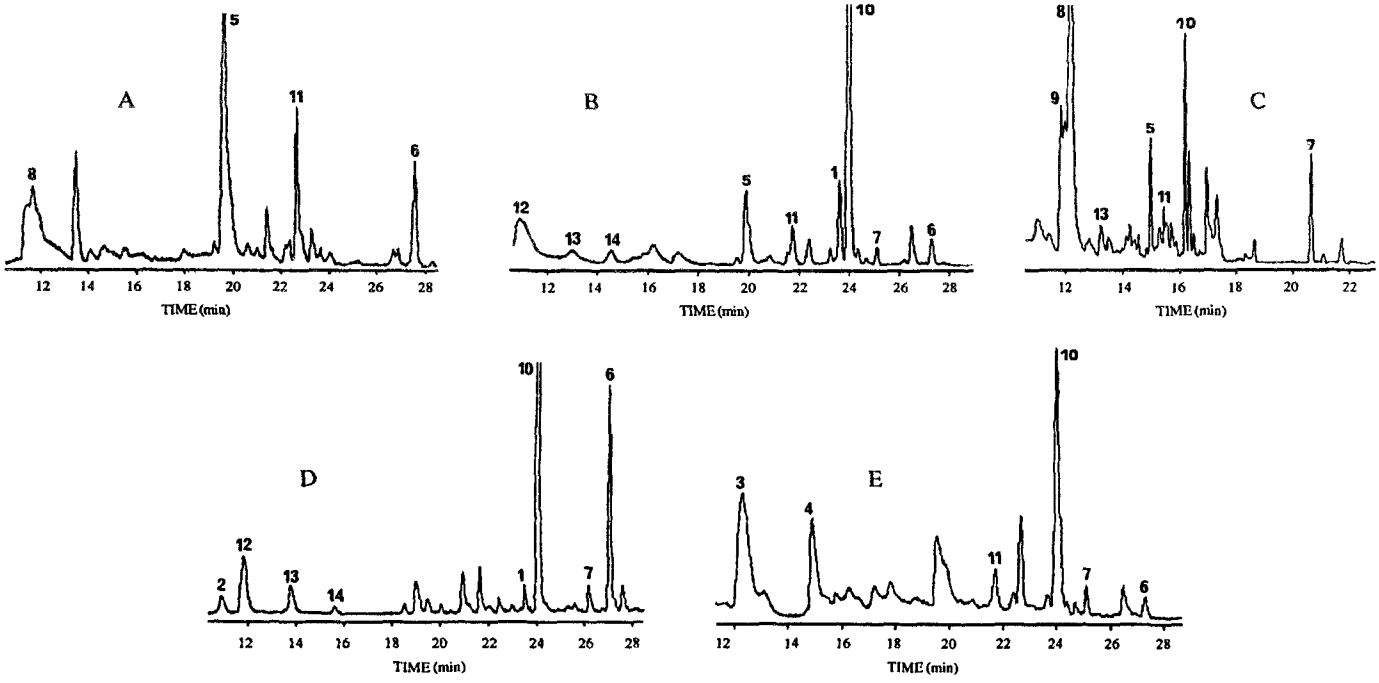

FIG. 3. GC-MS chromatograms of the volatiles from unripe (A), half-ripe (B), ripe olives (C), leaves (D), and crushed olives (E). Conditions: see Methods and Materials. For A, B., D, and E, oven temp.: 50°C, 10 min; 50–180°C at 5°C/min; 180°C, 10 min; for C, oven temp.: 40°C, 5 min; 40–120°C at 3°C/min; 120°C, 1 min; 120–200°C at 20°C/min; 200°C, 10 min. Peak identities: 1, *p*-cymene; 2, ethylbenzene; 3, hexanal; 4, (*E*)-2-hexenal; 5, limonene; 6, myrcenone; 7, nonanal; 8, *n*-octane; 9, 1-octene; 10, α-pinene; 11, β-pinene; 12, toluene; 13, *o*-xylene; 14, *p*-xylene.

3B). Ripe olives yielded *n*-octane, α-pinene, 1-octene, nonanal, limonene, and *o*-xylene (Figure 3C).

The leaves released mainly α-pinene, myrcenone and toluene. Myrcenone, present in other plants (Von Schantz et al., 1973), was identified for the first time in the olive tree (Figure 3D). The crushed olives yielded α-pinene, hexanal, and (*E*)-2-hexenal (Figure 3E). It is known that (*E*)-2-hexenal is produced by the action of lipoxidases on linolenic acid (Hatanaka et al., 1976).

Olive Maceration Water. Preliminary tests were first carried out on the olive MW, whose attractancy was already known, in order to determine variations in attractancy with prolonged soaking of the olives. A peak of activity was found with a solution in which olives had been soaked for eight days, after which a partial decline was noted in the action.

In view of the fact that the water solution of the cold-collected HS volatiles from MW proved attractive (Figure 2A), we can rule out any possibility of the attractancy of the MW being solely nutritional, i.e., attributable to the sugars, mannitol, amino acids, etc., transferred from the olives to the water through prolonged contact.

Olfactory Attractancy Test. The samples to be tested, i.e., the compounds identified in the various HS volatiles (Figure 3), were put in water at a concentration of 10 ppm (method 1), which was found to be optimal for effect on the behavior of *D. oleae*. We synthesized the myrcenone (2-methyl-6-methylene-1,7-octadien-3-one), since it is not commercially available. As control we used the most active MW (eight days). Water was used as blank, in which a very low number (mean value 0.1) of females were caught (70 replicates). With this procedure we compelled the *Dacus oleae* to choose between the test chemical in water, MW, and water alone and kept a continuous check on the attractancy exerted on the insects.

Of all the compounds we identified (Figure 3), the most attractive were found to be toluene and ethylbenzene. *n*-Octane and α-pinene, were weakly active (Figure 2B). The other compounds identified, including limonene, myrcenone, and *p*-cymene, (Figure 3) showed no activity; the last compound was reported to be attractive to *D. oleae* by Gariboldi et al. (1982).

Olfactory Repellency Test. We attempted a great number of preliminary tests, obtaining results of scant significance. The adopted assay consisted of a 10 ppm addition of each sample to the most active MW (method 2). The results are set out in Figure 4, where (*E*)-2-hexenal emerges as the only repellent substance, since it totally eliminates the attractancy of the MW in which it is dissolved. In confirmation, the cold-collected HS of the crushed olives dissolved in water showed no attractancy (Figure 2A). Both hexanal and (*E*)-2-heptenal did not repress MW attractancy; therefore the *D. oleae* olfactory repellency to (*E*)-2-hexenal appears to be highly specific in this assay.

Oviposition Test. We devised a new test to examine oviposition on olives

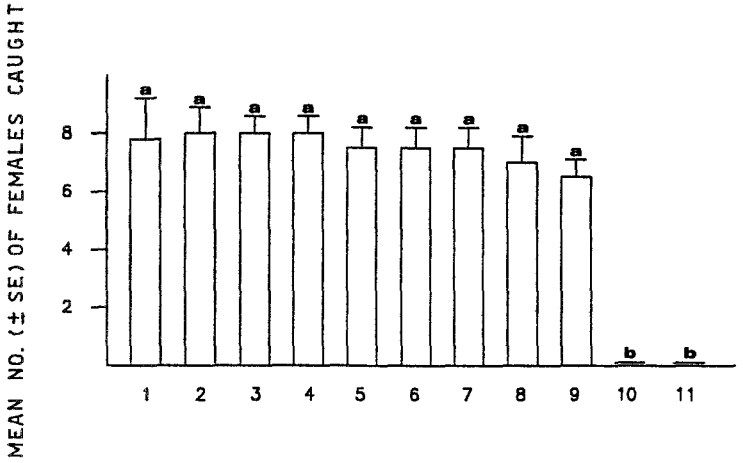

FIG. 4. Olfactory repellency test. Assay of repellency to *D. oleae* females of individual chemicals identified from olive volatiles, at 10 ppm concentration in olive maceration water (MW). Replicate numbers (*n*) are reported in parentheses below. Error bars represent standard error. Significant differences ($P < 0.05$) within each graph are indicated by different lowercase letters, as indicated by Duncan's (1955) multiple-range test. 1, control MW (49); 2, nonanal (6); 3, toluene (6); 4, (*E*)-2-heptenal (3); 5, hexanal (6); 6, *n*-octane (6); 7, α-pinene (4); 8, β-pinene (4); 9, ethylbenzene (4); 10, (*E*)-2-hexenal (10); 11, blank H_2O (49).

while avoiding treatment of the actual surfaces of the fruits with the substances being tested, in contrast to the general practice of brushing (Cirio, 1971; Girolami et al., 1974) or spraying (Vita and Cirio, 1977). These methods cannot be applied with the highly volatile substances we were examining since their effect would wear off in a few minutes while, at the same time, rapid evaporation would lead to saturation of the signal in the cage.

The water solutions or suspensions of the samples were placed close to the olives (method 3) to ensure constant vapor emission throughout the test, with olives unaffected by the vapors as blank (Figure 1). The number of ovipositions to α-pinene and MW (Figure 5) clearly exceeded results obtained with the blank. The substances found to exert weak activity were, in order, *p*-xylene, myrcenone, ethylbenzene, *n*-octane, and *o*-xylene. No significant activity was shown by 1-octene, β-pinene, and *p*-cymene. The substance found to reduce the number of ovipositions drastically was (*E*)-2-hexenal, thus confirming its role as a deterrent, functioning, moreover, in direct relation to oviposition. In addition, hexanal, which is also emitted by crushed olives, showed deterrent oviposition activity. The latter results explain the observation of Girolami et al. (1981) that the number of ovipositions is drastically reduced in artificial fruits (ceresin domes)

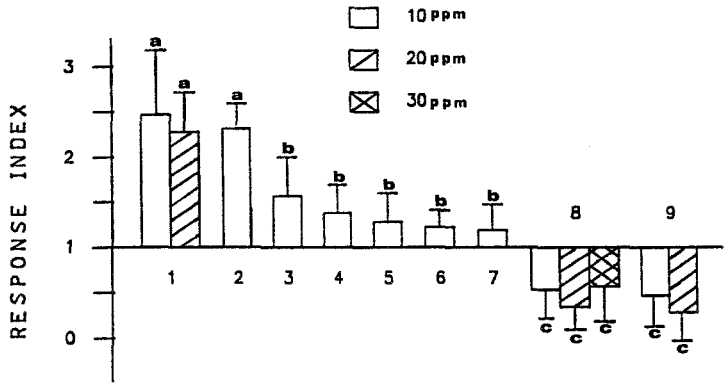

FIG. 5. Oviposition test. The response index was defined as the mean ratio of ovipositions of *D. oleae* on natural fruits exposed to individual samples of *O. europaea* volatiles vs. the ovipositions on the three olives not exposed (see Figure 1). Replicate numbers (*n*) are reported in parentheses below. Error bars represent standard error. Significant differences ($P < 0.05$) within each graph are indicated by different lowercase letters, as indicated by Duncan's (1955) multiple-range test. 1, α-pinene (4); 2, MW (4); 3, *p*-xylene (4); 4, myrcenone (4); 5, ethylbenzene (4); 6, *n*-octane (4); 7, *o*-xylene (4); 8, (*E*)-2-hexenal (10); 9, hexanal (10).

if there is olive pulp near by. Furthermore, in all likelihood, volatiles containing hexanal and (*E*)-2-hexenal are emitted from the oviposition hole; these deterring compounds, together with the marking substances spread by the female around the oviposition site (Cirio, 1971), could discourage other females from ovipositing in the same drupe under field conditions (Cirio and Vita, 1977).

It is very noteworthy that α-pinene is the most active compound in our oviposition test, and it is present in significant amounts in olive leaf volatiles (Figure 3D), strongly suggesting that host-plant odors are oviposition stimulants.

We are now completing research on chemotactile attraction and chemotactile repulsion and the identification of active volatiles of MW. The results reached will be adapted to field research during the natural season of oviposition, with the aim of employing the volatile allelochemicals of the olive against the *Dacus oleae* pest.

REFERENCES

BATEMAN, M.A. 1972. The ecology of fruit flies. *Annu. Rev. Entomol.* 17:493–518.

CAVALLORO, R. (ed.). 1982. Fruit flies of economic importance. Proceedings, CEC/IOBC Symposium, Athens. Balkema, Rotterdam, 642 pp.

CIRIO, U. 1971. Reperti sul meccanismo stimolo-risposta nella ovideposizione del *Dacus oleae* Gmel. *Redia* 52:577–600.

CIRIO, U., VITA, G., FEDELI, E., and JACINI, G. 1977. L'uso di sostanze naturali presenti nell'oliva come prospettiva di lotta contro il *Dacus oleae* (Gmel.). *Boll. Lab. Entomol. Agric. Portici* 34:55–61.

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

FEDELI, E., JACINI, G., BARONE, D., and FAVINI, G. 1973a. Sui componenti odorosi dell'olio d'oliva. Nota II. *Chim. Ind.* 55:681–686.

FEDELI, E., BARONE, D., and JACINI, G. 1973b. Sui componenti odorosi dell'olio d'oliva. Nota III. *Riv. Ital. Sost. Grasse* 50:38–44.

FIESTA ROS DE URSINOS, J.A., GRACIANI CONSTANTE, E., MAESTRO DURAN, N., and VASQUEZ RONCERO, A. 1972. Etude d'un attractif naturel pour *Dacus oleae*. *Ann. S.E.F.(N.S.)* 8:179–188.

FLETCHER, B.S. 1987. The biology of dacine fruit flies *Ann. Rev. Entomol.* 32:115–144.

GARIBOLDI, P., JOMMI, G., ROSSI, R., and VITA, G. 1982. Studies on the chemical constitution and the sex pheromone activity of volatile substances emitted by *Dacus oleae*. *Experientia* 38:441–444.

GARIBOLDI, P., VEROTTA, L., and FANELLI, R. 1983. Studies on the sex pheromone of *Dacus oleae*. Analysis of the substances contained in the rectal glands. *Experientia* 39:502–505.

GIROLAMI, V., PELLIZZARI, G., RAGAZZI, E., and VERONESE, G. 1974. Prospects of increased egg production in the rearing of *Dacus oleae* Gmelin by the use of chemical stimuli. Proceedings of the Symposium: Sterility Principle of Insect Control, IAEA, Vienna, 1975, pp. 209–217.

GIROLAMI, V., VIANELLO, A., STRAPAZZON, A., RAGAZZI, E. and VERONESE, G. 1981. Ovipositional deterrents in *Dacus oleae* (Gmelin). *Entomol. Exp. Appl.* 29:177–188.

GIROLAMI, V., STRAPAZZON, A., and DE GERLONI, P.F. 1982. Insect–plant relationship in olive flies: General aspects and new findings, pp. 258–267, *in* R. Cavalloro (ed.). Fruit Flies of Economic Importance, Proceedings of the CEC/IOBC International Symposium, Athens. Balkema, Rotterdam.

HATANAKA, A., KAJIWARA, T., and SEKIYA, J. 1976. Biosynthesis of *trans*-2-hexenal in chloroplasts from *Thea sinensis*. *Phytochemistry* 15:1125–1126.

LEVINSON, H.Z., and LEVINSON, A.R. 1984. Botanical and chemical aspects of the olive tree with regards to fruit acceptance by *Dacus oleae* (Gmelin) and other frugivorous animals. *Z. Angew. Entomol.* 96:136–149.

METCALF, R.L. 1990. Chemical ecology of Dacinae fruit flies (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* 83:1017–1030.

MONTEDORO, G., BERTUCCIOLI, M., and ANICHINI, F. 1978. Aroma analysis of virgin olive oil by head space (volatiles) and extraction (polyphenols) techniques, pp. 247–280, *in* G. Chaharalamboulos (ed.). Flavour of Foods and Beverages, Chemistry and Technology. Academic Press, New York.

NABETA, K., MATSUO, T., and SUGISAWA, H. 1988. Analysis of volatiles from olive (*Olea europaea* L.). *Res. Bull. Obihiro Univ.* 15:293–299.

PANIZZI, L., and SCARPATI, M.L. 1960. Costituzione della oleuropeina, glucoside amaro e ad azione ipotensiva dell'olivo. Nota II. *Gazz. Chim. Ital.* 90:1449–1485.

PROKOPY, R.J. 1977. Stimuli influencing trophic relations in Tephritidae. *Colloq. Int. CNRS* 265:305–336.

SCHULZE, K., HAUFE, G., and KOHLER, G. 1989. Formale *En*-Chlorierung von Myrcen mittels *N*-Chlor-succinimids. *Z. Chem.* 5:167–168.

VITA, G., and BARBERA, F. 1976. Aspetti biochimici del rapporto piantainsetto nel *Dacus oleae* (Gmel.). *Atti XI Congr. Ital. Entomol.* 151–161.

VITA, G., and CIRIO, U. 1977. Testing the response of *Dacus oleae* to oviposition deterring sub-

stances. Quality control, an idea book for fruit fly workers; E.F. Boller and D.L. Chambers (eds.). Bulletin of SROP WPRS.

VITA, G., and CIRIO, U. 1980. Fruit fly control by chemical attractant and repellents. *Boll. Lab. Entomol. Agric.* 37:127–139.

VON SCHANTZ, M., WIDEN, K.G., and HILTUNEN, R. 1973. Structures of some aliphatic monoterpenoids isolated from the essential oil of *Ledum palustre* L. *Acta Chem. Scand.* 27:551–555.

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ALLELOCHEMIC CONTROL OF BIOMASS ALLOCATION IN INTERACTING SHRUB SPECIES

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Abstract—Aqueous leachates derived from canopy phyllodes of invasive *Acacia cyclops* affected growth of a range of shrub species independently of nutrient input effects. All plants showed a sublethal phytotoxic response. Root mass was generally less adversely affected than shoot mass and, while decreasing significantly in response to the 10% concentration, showed no such response to the 1% solution. Root–shoot biomass ratios increased, except in *Euphorbia burmannii*, which may recognize intrinsic root architecture limitations on extensive exploitation of toxin-free soil. Application of surface plant litter from under *A. cyclops* canopies stimulated the production of basal stems in *Protasparagus capensis* and *Eriocephalus racemosus* but was insufficient to significantly reduce root–shoot ratios. Plant growth inhibition was maximized by canopy leachate compounded by surface litter effects in *Anthospermum spathulatum*. The net effect of leachate at high concentration on biomass allocation in certain shrub species may help explain their patterns of association and disassociation with *A. cyclops*.

Key Words—*Acacia cyclops*, allelopathy, competition, inhibition, growth, leachate, nutrients, phytotoxicity, stimulation.

INTRODUCTION

This paper reports effects of soluble compounds of the Australian *Acacia cyclops* A. Cunn. ex G. Don (Fabaceae) on plants typical of its invasion sites in Africa. Patterns of invasion by the phyllodinous *A. cyclops* have been documented by Macdonald et al. (1986). The degree of association or disassociation

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of indigenous plant species with this invasive and overtopping shrub has been established for certain field sites but, in several cases, not fully explained by competition for limited water, nutrient, and radiation resources (Rutherford and Bösenberg, 1988; Rutherford, unpublished data). Noncompetitive interaction may be implicated.

This study evaluates potential allelopathic susceptibility in five negatively associated species and in two apparently neutral species (Table 1). Consideration was limited to growth and survival of long-lived shrubs rather than species likely to have been recruited on a recently invaded site. Bioassays of growth may be allelochemically more sensitive than germination bioassays (Leather and Einhellig, 1988). Consideration of the movement of the potential allelopathic agents into the environment was limited to that from attached phyllodes and predominantly phyllodinous litter on the ground. Root exudates and other soil phenomena were not considered in the present study. Autotoxicity, an intraspecific form of allelopathy, was not tested.

In this paper, allelopathy describes both the inhibitory and stimulatory biochemical effects of one organism on another (Rice, 1984). Allelochemical usually describes nonnutritional chemicals (Lovett et al., 1989).

The demonstration of allelopathy has long been controversial (Harper, 1975; Williamson, 1990). One of the main problems is the potentially confounding effect of inorganic nutrients in allelochemical tests. Arbitrary nutrient supplements (Qasem and Hill, 1989b) or the inevitable and naturally leached nutrients can effectively mask negative, neutral, or positive effects of allelochemicals.

TABLE 1. RECEPTOR SPECIES AND THEIR DEGREE OF ASSOCIATION WITH ALLELOCHEMIC DONOR *Acacia cyclops* ON STUDY SITE AND THEIR RELATIVE SENSITIVITY TO LONG-TERM SHADING AT ABOUT 90% REDUCTION OF FULL SUNLIGHT (GREATEST SENSITIVITY = 1)[a]

Receptor species	Association with overtopping *Acacia cyclops*	Shade intolerance ranking
Anthosperum spathulatum	negative	1
Eriocephalus racemosus	negative	3
Euclea racemosa	neutral, tending negative	not ranked
Euphorbia burmannii	neutral, tending positive	not ranked
Euphorbia caput-medusae	negative	5
Protasparagus capensis	negative	4
Ruschia macowanii	negative	2

[a]M.C. Rutherford, unpublished data.

Only when stimulatory effects are found are nutrients sometimes considered (Heisey, 1990), but even these effects are often grossly ignored (Rice, 1986).

Apart from the fundamental difference between the role of nutrients and allelochemicals in resource-limited systems, temporal and spatial availability as well as rates of leaching of nutrients and allelochemicals are likely to differ. Most primary metabolites are hydrophilic compounds, while most of the allelopathic secondary metabolites are less soluble (Tang, 1986). Discrimination between the effects of the two main chemical constituents of leachate appears, therefore, desirable and is addressed in this study.

METHODS AND MATERIALS

Study Site. The study site from which plant material was collected was within the West Coast National Park (33°11'S; 18°08'E) a part of the neutral sands coastal platform of southwestern Africa. Mean annual rainfall is 270 mm and occurs predominantly in winter. Fog is fairly frequent.

Leachate and Control. Phyllodes of medium-size evergreen individuals of *A. cyclops* were harvested at intervals throughout summer for leachate production. The phyllodes were immersed in water at a ratio of 1 : 10 (w/v) in the dark for 24 hr at 8°C. The supernatant was removed by straining through a 200 μm mesh and storing it at 2°C up to seven days at the 10% treatment solution. The leachate was diluted 1 : 10 by volume to also provide a 1% treatment. Refrigeration of phyllode material from the field site was necessary to sustain the daily supply of large amounts of leachate. The 1 : 10 ratio for aqueous extraction is commonly used (Richardson and Williamson, 1988; Williamson et al., 1989; Hegazy et al., 1990; Martin et al., 1990; May and Ash, 1990) usually with 24-hr immersion. This can provide a more conservative test of activity than recycling rinses or evaporated rinses (Richardson and Williamson, 1988) or other more drastic forms of plant material preparation such as milling, use of organic solvents, or elevated temperature. No attempt was made to eliminate the microbes associated with the leachate other than storage at low temperature. Osmotic potential of the leachate was determined using a Knauer osmometer.

Our control takes into account the possibility that leachates contain mineral nutrients (Qasem and Hill, 1989a). Most of the secondary compounds implicated so far in plant allelopathy are either terpenoids (mono- or sesquiterpenes) or phenolics (phenols, phenolic acids, cinnamic acids, hydroxyquinones) (Harborne, 1988) and do not generally contain the essential elements associated with inorganic plant nutrients. Therefore, assuming that the potential allelochemic compounds did not contribute significantly to the concentrations of essential elements in the leachate, the aqueous control and the 1% leachate solution were amended to contain levels of essential macro- and microelements similar to those

in the 10% leachate solution. Water was not considered as an appropriate control. Balance of compounds followed relative proportions of the Long Ashton growth solution (Hewitt and Smith, 1975) as far as possible. Successive analyses of nutrient levels in the leachate varied independently of time, and nutrient analysis delays made it difficult to track variations in some elemental concentrations for the controls. In retrospect, the levels of most nutrients used in the control and 1% treatment were conservative relative to those in the 10% leachate. Only nitrate/nitrite and boron may have exceeded that in the leachate, but levels remained below 15% and 50%, respectively, of those in Long Ashton solution. The pH of leachate and control was approximately neutral at between 5.8 and 7.8.

Surface Litter. Surface litter (comprising the dry mass fractions: <200 μm 16%; 200–500 μm 62%; 500–2000 μm 10%; >2 mm 12%) was collected under several *A. cyclops* canopies under dry conditions in early summer, less than a week before treatments commenced, and were stored air dry at room temperature. The litter was well mixed, separated into size fractions, and recombined to ensure a similar range of fragment distribution in each treatment and replicate.

Receptor Species. Potential receptor species from the same locality as the *A. cyclops* were used and not convenient "bioassay species." The evergreen microphyllous shrubs, *Anthospermum spathulatum* Spreng. (Rubiaceae) and *Eriocephalus racemosus* L. (Asteraceae), the evergreen broad-leaved shrub, *Euclea racemosa* Murray (Ebenaceae), and the deciduous microphyllous shrub, *Protasparagus capensis* (L.) Oberm. (Asparagaceae) were propagated from seed, while the stem succulents, *Euphorbia burmannii E. Mey. ex Bioss.*, and *E. caput-medusae* L. (Euphorbiaceae), and the leaf succulent, *Ruschia macowanii (L. Bol.) Schwant.* (Mesembryanthemaceae) were propagated from shoot cuttings to form the test response populations. Material was collected on the West Coast National Park study site, with the exception of *E. racemosa*,[1] for which seed was available from a similar site in the region. The logistic constraints of authentic ecological receptor species selection was illustrated by the failure to propagate adequate numbers of more than half the species originally desired for testing from the field site. Plants were grown in 3-liter pots of sandy soil from the study site and were one and a half years old at start of treatment.

Experimental Design. Treatments were arranged as a 2×3 factorial for each receptor species in a randomized design with five replicates, except for one species (*E. caput-medusae*) with two replicates. This design tested the effect of three levels of phyllode-derived leachate, the effect of two levels of surface

[1] Note that the convention of abbreviating the genus name subsequently in this paper could lead to inadvertent confusion between the unrelated *E. racemosa* and *E. racemosus*.

litter, and possible interaction that could reflect a realistic combination of both water flow through the live canopy and litter layer.

A. cyclops litter was applied to half the set of plants to cover the soil surface to a depth of at least 10 mm. Further additions of leaf litter during the experiment were not needed to match field conditions since no significant phyllode fall occurred during this period. The 10% and 1% leachate solutions and control solution were applied at a rate of 250 ml daily under summer greenhouse conditions. This generally allowed free through-flow and no accumulation of leachate. Treatments were maintained for 10 weeks.

Measurements and Analysis. At the end of treatment, each plant was harvested and aboveground parts sorted, where appropriate, into components of green leaves, dead leaves, stems/twigs, flowers/fruits, and new shoots and old shoots (only in *P. capensis*). Roots were obtained by carefully washing with water. Tissue was dried at 85°C and weighed. Shoot extensions and leaf and flower numbers were also recorded. Owing to the smaller sample size, measures in *E. caput-medusae* were limited to changes in shoot length and number of flowers and leaves from start of treatment. Few significant differences were found with respect to the individual aboveground mass subdivisions, counts, or dimensions, and discussion of results for the aboveground compartment are, therefore, largely restricted to the allocation to the total aboveground shoot mass (shoot is taken as total aboveground material). Data were subjected to two-way analysis of variance and, after confirming the absence of interaction, multiple range test using LSD intervals at the $P < 0.05$ level (Saville, 1990).

Magnitudes of changes are expressed relative to the plant set with 0% leachate and no litter. Relative changes and root–shoot ratios are not necessarily directly comparable between species propagated vegetatively and from seed.

RESULTS

Osmotic Potentials. The leaching process resulted in low osmotic potentials [mean 18.0 milliosmols/kg (SD = 4.2), or mean −0.043 MPa (SD = 0.010) for the undiluted solution]. This, together with the relatively neutral pH values, confirmed that there was no need for pH-adjusted isosmotic solutions in the study.

Plant Mortality. All plants showed a sublethal phytotoxic response or, more rarely, part stimulation by treatment (Figures 1–3).

Shoot Growth. The analysis indicated (Figure 3) that shoot growth of all species [with the possible exception of *E. caput-medusae* (Figure 2)] was significantly inhibited by the 10% leachate solution. *A. spathulatum*, *E. burmannii*, *P. capensis*, and *R. macowanii* displayed greater sensitivity through their susceptibility to the dilute (1%) leachate solution. *A. spathulatum* shoot growth declined uniquely in the litter treatment, which contributed to a distinctive dou-

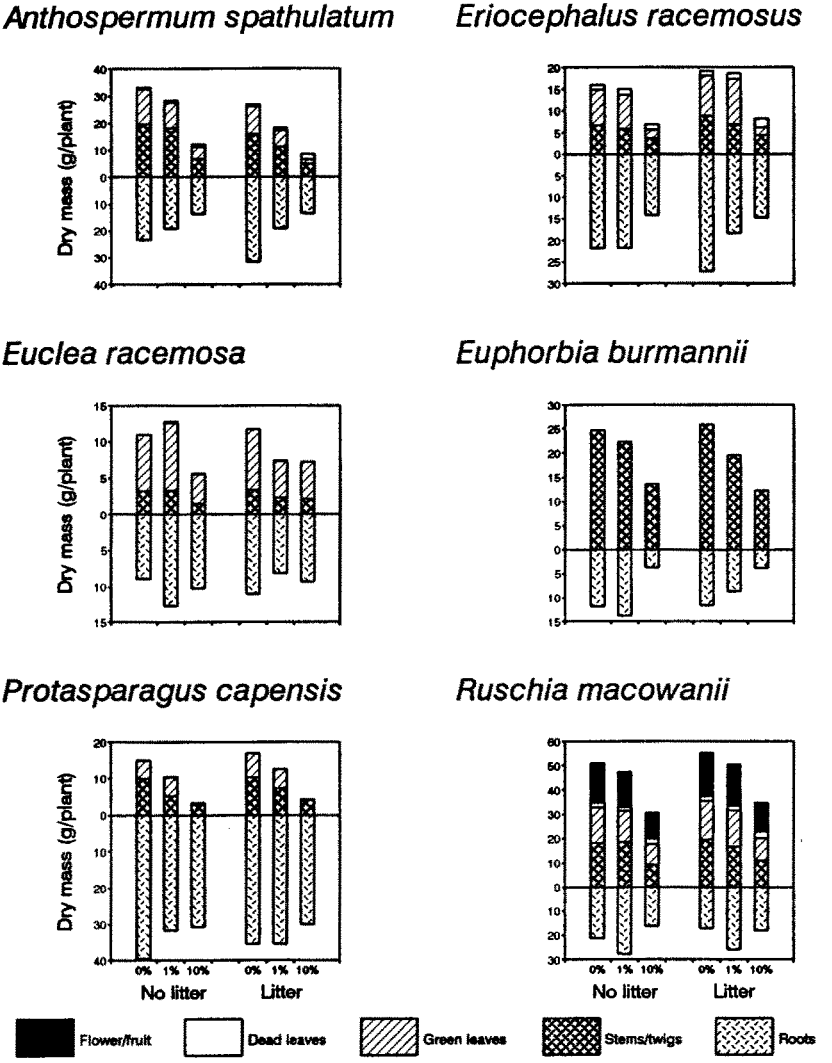

FIG. 1. Responses in allocation of biomass of shrub species to concentrations of aqueous extracts of phyllodes of *Acacia cyclops* and associated ground litter. 0% is the control and the zero horizontal line depicts ground reference plane.

bling of the root–shoot ratio. The net effect of both undiluted leachate and litter application compounded the reduction of this species' shoot mass to 26% of the control (Figure 1). The only other species to exhibit a similar chronic decline in response to the 10% solution was *P. capensis*, where the effect persisted

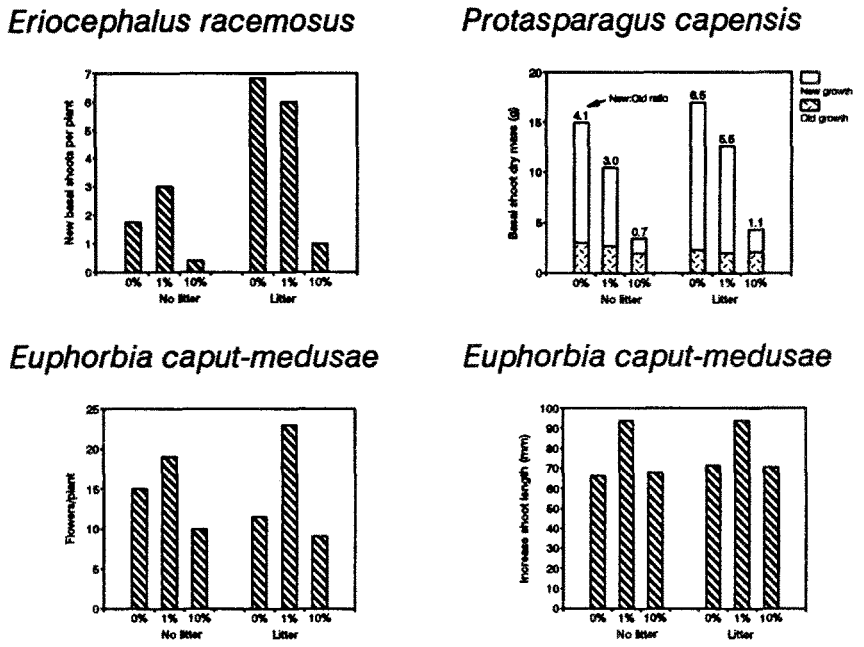

FIG. 2. Aboveground growth response of specific plant organ types to concentrations of aqueous extracts of phyllodes of *Acacia cyclops* and associated ground litter. 0% is the control.

despite a stimulatory trend associated with the litter treatment. Shoot mass of *R. macowanii* was most mildly affected through undiluted leachate and was effectively maintained above 60% of the control (Figure 1). Putatively significant stimulation of shoot growth by litter was evident in this species (Figure 3) as well as in terms of increase in number of basal shoots in *E. racemosus* and in the mass ratio of new basal shoots to old in *P. capensis* (Figures 2 and 3).

Root Growth. No species showed significant inhibition of root growth with the dilute leachate or with litter application (Figure 3). The 10% solution was, however, sufficient for phytotoxic inhibition in all species except in *E. racemosa* and *P. capensis*. While *E. burmannii* roots were more inhibited than shoot growth with the 10% solution, other species showed the reverse (Figure 1). At dilute leachate levels in *R. macowanii*, a polarizing effect becomes evident, with shoot growth declining while root growth is stimulated (Figures 1 and 3), thus elevating the root-shoot ratio. The limited inhibition of root growth in most species, relative to the marked toxic sensitivity in shoots, tends to increase root-shoot mass ratios, for example, to greater than 7.0 in *P. capensis* with 10% solution (Figure 1). *E. burmannii* alone lacked an elevated root-shoot response

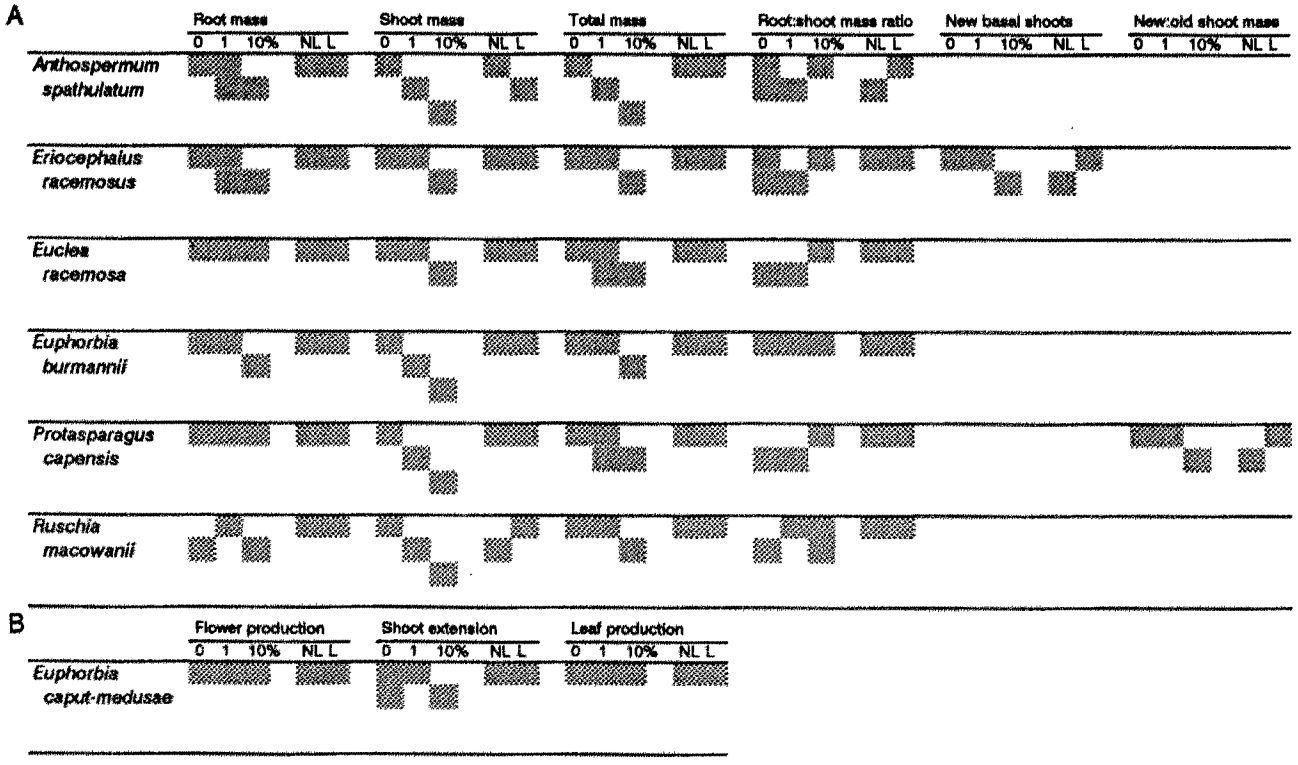

FIG. 3. Statistical comparison of species responses to concentrations of aqueous extracts of phyllodes of *Acacia cyclops* and associated ground litter. Vertical and horizontal separation of shaded squares indicates significant differences at $P < 0.05$ within each contiguous data set. (A) Species with five factorial replicates, (B) species with two factorial replicates.

to leachate application and declined to less than 0.30 with undiluted solution (Figure 1).

Whole Plant. Differential responses of components also affect resulting patterns of total plant mass. All species show a significant decline with the 10% solution (Figure 3). *A. spathulatum* had the most acute inhibition in total mass (Figure 1), to 39% of control, and was also critically sensitive to the 1% solution (Figure 3). *E. racemosa* was most tolerant and with undiluted leachate remained above 80% of the biomass of the control (Figure 1). Litter application had no significant effect on the total mass for any species (Figure 3).

Reproduction. Reproductive material (mostly remains of previous fruiting heads) in *R. macowanii* and flower numbers in *E. caput-medusae* were not significantly affected at the end of the study (Figures 2 and 3). However, in the latter species, a stimulatory effect did occur after seven weeks of treatment but only with the dilute leachate concentration (data not shown).

DISCUSSION

The results are discussed within the context of the specific experiment and relative to the independently assessed degree of association between *A. cyclops* and the various receptor species in the field (Table 1).

Threshold Concentrations. In general, results conformed to the expectation of proportionally inhibited growth at higher concentrations (Kil and Yun, 1992). The arbitrarily selected 10% leachate concentration was associated with phytotoxic inhibition in all study species, while the diluted 1% solution affected plant components in most species. Phytotoxicity levels of concentration are thus relatively closely delimited, and in no species do effective phytotoxic inhibition levels lie above the 10% level. The concentrations selected are not necessarily unrealistic relative to the arid study site. The light rain and even fog that occurs on the site may be more effective in leaching allelochemicals than large volumes of rainwater (Tukey, 1970). Aerial leachates may not be a significant source of allelochemic agents unless fairly infrequent conditions, such as long rainless periods occur (Molina et al., 1991). Concentration of allelochemicals through evaporation from soils has been demonstrated and should be maximized under conditions of low rainfall events (May and Ash, 1990). The extent of allelochemical accumulation at physiologically active sites, however, remains a crucial unknown.

Carbon Allocation. Apart from *E. burmannii*, changes in mass through either leachate or litter application led to elevated root–shoot ratios. This was effected primarily by more intense suppression of the growth of the shoot component. Stimulation of shoot or shoot-related growth of some species by litter was insufficient to significantly reduce the root–shoot ratio, and it is the leachate

that dominates control of the ratio. Shifts in relative allocation of biomass to roots and shoots under conditions of environmental stress can increase root–shoot ratio through, for example, diminished water availability or action of fire or frost. Alternatively, the ratio can be decreased, for example, through light attenuation or greater availability of nutrients (Mooney et al., 1983). Allelopathic effects on the root–shoot ratio may be less predictable and dependent upon the specific site of physiological perturbation. Although greater allelochemical inhibition of root growth than shoot growth has been reported, this may be largely limited to conditions shortly after germination (Achhireddy et al., 1985; Singh et al., 1989). The generally opposite effect in the well-established plants of the present study may relate to toxin-mediated reduced availability of soil resources, possibly coupled to direct metabolic perturbation *en route* to, or within, the stem or leaf organs. This form of adaptive response might carry doubtful survival advantage to plants that have no guarantee of escaping beyond the allelochemic zone of soil before the longer-term demands of the shoot can no longer be met by an excessively extended and possibly weakened root system. The reduction in shoot growth in *R. macowanii* at dilute leachate levels could be ascribed to the stimulation of the root growth rather than to direct negative allelochemic effects at this concentration. Plants with broad-leaved canopies may counter some deleterious allelochemic effects by increasing the probability of expelling the toxins through leaching from the canopy. This could enhance the apparent relative tolerance of *E. racemosa* to the leachate. Alternatively, a relatively greater investment shift to shoot than root (*E. burmannii*), may either limit exposure to toxin or recognize intrinsic root architecture limitations on extensive exploitation of toxin-free soil and rather rely on maximizing plant aerial surface for diluting and expelling toxins by leaching. Injurious effects by allelopathic compounds may be expected to relate to the frequency of contact with roots (Lyu and Blum, 1990). Species with this type of response mechanism would require a sustained allelochemic tolerance for long-term survival. The implications of ecosystem recycling of weakly labile allelochemicals appear to have received little attention and are crucial to the above issues as well as to questions of autotoxicity and links with, for example, coevolutionary telechory or antitelechory.

Growth Stimulation. Shoot or shoot-related growth was stimulated in three species by litter treatments. Although stimulatory effects ascribed to high nutrient levels present in leachate (Keeley and Keeley, 1989) should not apply to the fresh phyllode leachate treatments in the current nutrient-compensated study, the litter treatment could constitute a source of growth-stimulating nutrients. However, this effect may have been negligible since the regular washing of litter without any additions or replacement may soon have leached the soluble nutrients out of the system. Furthermore, any small amounts of nutrient from the litter would be added to the existing levels of nutrients in the washing medium. Two

of the stimulatory effects with litter concerned production of new shoots at the base of the plant. The *A. cyclops* litter layer may directly or indirectly provide a specific environmental stimulus to activate bud primordia at the plant base and thus promote the observed multistemmed condition in shrub forms such as *P. capensis* and *E. racemosus*. Significant shoot growth stimulation by litter in *R. macowanii* supports a stimulatory trend evident in some other species, which may reflect allelochemic action of, for example, growth regulators (Tukey, 1970) or be tied to marginally improved water relations in the litter-covered soil under warm summer conditions. The intensive watering regime and the negative effects of litter on *A. spathulatum* shoot growth would suggest that any transient soil water differences may not have been significant and that an allelochemic stimulation hypothesis should not be discarded prematurely.

The stimulation of root growth of *R. macowanii* at only dilute leachate concentrations accords with previous findings of growth stimulation at low allelochemical concentrations. Low concentrations (0.15 and 1.5%) of similarly prepared *Festuca arundinacea* leachate (but soaked at 1.5 : 10) often stimulated growth of several grass species, while 7.5 and 15% concentrations usually inhibited growth (Buta and Spaulding, 1989). Reasons for the selective species-specific stimulation of growth are unclear.

Litter versus Canopy Effects. The impact of extracts of the canopy phyllodes was far greater than that of the ground litter layer as applied in this study. This merely modificatory role of litter contrasts with the apparent dominant effect of decaying litter over aerial leachate in allelochemical inhibition by *Eucalyptus globulus* (Molina et al., 1991). In *A. spathulatum*, litter components and processes may exacerbate the apparent negative canopy effects, whereas in some other species, the stimulatory trend associated with litter helps counter the magnitude of such effects. In *R. macowanii*, the switch from shoot growth inhibition by live canopy phyllodes to stimulation by dead phyllodes and associated matter in the litter layer suggests detoxification or other chemical conversion of allelochemicals in the litter layer. Possibly, very slow release of bonded allelochemicals is compounded by a slow litter decay rate. Comparisons of canopy and litter effects are potentially complex. The detachment of phyllodes from trees may change the rate of production or release of toxins, since production of secondary compounds sometimes increases in response to wounding or pathogens (Heisey, 1990). Addition of labile organic matter typically stimulates soil microbial populations, causing rapid immobilization of available nitrogen and possibly other plant nutrients. Plants in such treatments would thus suffer from nutrient deficiency and reduced growth (Heisey, 1990). However, addition of nutrients through leaching would tend to counteract nutrient immobilization or deficiency effects. Furthermore, allelochemicals may be leached from plant tissue as water-soluble nontoxic derivatives that are altered chemically once they reach the soil matrix.

Ecological Relationships. Many of the imputed allelopathic effects of *A. cyclops* on the shrub species studied are likely to be indirect. Substances leached from plant material, through the often brief action of aqueous solutions, represent a great diversity of compounds (Tukey, 1970). For example, the volatile extraction of roots of another Australian member of *Acacia* (*A. pulchella*) was found to contain 270 organic components, of which only half could be identified or tentatively identified (Whitfield et al., 1981). Different allelopathic compounds may act synergistically, be subject to photosensitization, microbial conversion and other litter-related processes (see above section); differ in hydrodynamic dispersion, adsorption and decay; and have their activity depend on plant physiological condition and concentration thresholds. Allelopathic inhibition of plants can also result in enhanced competitiveness for resources and, as long as this is not outweighed by the metabolic costs of allelochemic production, autoinhibition, or a counterallelopathic effect, can lead to a net interactive advantage.

The paradoxical apparent tolerance of *E. burmannii* to *A. cyclops* under field conditions (Table 1) in conjunction with the former's susceptibility to the phyllode leachate indicates that it may have developed its own microniche system in the natural ecosystem, which could not be achieved within the confines of the experimental conditions. Although root reduction at the cost of shoot development may represent a means to survive allelopathic conditions (see carbon allocation section above), it may make the plant more susceptible to other environmental stress so that the observed association between these species on this field site may be transient.

That *E. racemosa* was found to be the most tolerant (in terms of total plant mass and its insensitivity to dilute leachate) of the test species to the leachate accords with its tendency toward neutral association with *A. cyclops* (Table 1). The species most severely inhibited by the combination of *A. cyclops* leachate and litter was *A. spathulatum*, which, together with its high sensitivity to shading (Table 1), suggests a compelling explanation for the strong disassociation of this species with the alien invasive plant. *R. macowanii* is virtually as sensitive to shading as the last-mentioned species and the stimulatory effect of *A. cyclops* litter, therefore, does not prevent the strong, negative association with the alien plants. In some of the other negatively associated species (Table 1) with reduced shade sensitivity, the disassociation may link independently or interactively with the putative allelopathic inhibition. If, however, only the 1% concentration of leachate is represented in the field, the negative association of species such as *E. racemosus* may not be satisfactorily explained by allelopathy mechanisms. Although nutrient compensation for the dilute solution is required experimentally, implications for field application do not preclude interaction between allelochemicals and nutrients. However, positive nutrient compensation in the dilute solution clearly had no general stimulatory effect.

The experimental allelopathic reactions obtained appear species specific and not clearly related to plant growth form nor are they clearly generalizable. At a hierarchical level above the chemically specific, a coherent theoretical framework for predicting allelopathic responses remains elusive.

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REFERENCES

ACHHIREDDY, N.R., SINGH, M., ACHHIREDDY, L.L., NIGG, H.N., and NAGY, S. 1985. Isolation and partial characterization of phytotoxic compounds from Lantana (*Lantana camara* L.). *J. Chem. Ecol.* 11:979–988.

BUTA, J.G., and SPAULDING, D.W. 1989. Allelochemicals in tall fescue—abscisic and phenolic acids. *J. Chem. Ecol.* 15:1629–1636.

HARBORNE, J.B. 1988. Introduction to Ecological Biochemistry. Academic Press, London.

HARPER, J.L. 1975. Allelopathy (a review). *Q. Rev. Biol.* 50:493–495.

HEGAZY, A.K., MANSOUR, K.S., and ABDEL-HADY, N.F. 1990. Allelopathic and autotoxic effects of *Anastatica hierochuntica* L. *J. Chem. Ecol.* 16:2183–2193.

HEISEY, R.M. 1990. Evidence for allelopathy by tree-of-heaven (*Ailanthus altissima*). *J. Chem. Ecol.* 16:2039–2055.

HEWITT, E.J., and SMITH, T.A. 1975. Plant Mineral Nutrition. English Universities Press Ltd., London.

KEELEY, J.E., and KEELEY, S.C. 1989. Allelopathy and the fire-induced herb cycle, pp. 65–72, *in* S.C. Keeley (ed.). The California Chaparral. Paradigms Reexamined. No. 34 Science Series, Natural History Museum of Los Angeles County, Los Angeles.

KIL, B.-S., and YUN, K.W. 1992. Allelopathic effects of water extracts of *Artemisia princeps* var. *orientalis* on selected plant species. *J. Chem. Ecol.* 18:39–51.

LEATHER, G.R., and EINHELLIG, F.A. 1988. Bioassay of naturally occurring allelochemicals for phytotoxicity. *J. Chem. Ecol.* 14:1821–1828.

LOVETT, J.V., RYUNTYU, M.Y., and LIU, D.L. 1989. Allelopathy, chemical communication, and plant defence. *J. Chem. Ecol.* 15:1193–1202.

LYU, S.-W., and BLUM, U. 1990. Effects of ferulic acid, an allelopathic compound, on net P, K, and water uptake by cucumber seedlings in a split-root system. *J. Chem. Ecol.* 16:2429–2439.

MACDONALD, I.A.W., KRUGER, F.J., and FERRAR, A.A. (eds.). 1986. The Ecology and Management of Biological Invasions in Southern Africa. Oxford University Press, Cape Town.

MARTIN, V.L., MCCOY, E.L., and DICK, W.A. 1990. Allelopathy of crop residues influences corn seed germination and early growth. *Argon. J.* 82:555–560.

MAY, F.E., and ASH, J.E. 1990. An assessment of the allelopathic potential of *Eucalyptus*. *Aust. J. Bot.* 38:245–254.

MOLINA, A., REIGOSA, M.J., and CARBALLEIRA, A. 1991. Release of allelochemical agents from litter, throughfall, and topsoil in plantations of *Eucalyptus globulus* Labill in Spain. *J. Chem. Ecol.* 17:147–160.

MOONEY, H.A., KUMMEROW, J., MOLL, E.J., ORSHAN, G., RUTHERFORD, M.C., and SOMMERVILLE,

J.E.M. 1983. Plant form and function in relation to nutrient gradients, pp. 55–76, *in* J.A. Day (ed.). Mineral nutrients in mediterranean ecosystems. South African National Science Programmes Rep. 71. Council for Scientific and Industrial Research, Pretoria.

QASEM, J.R., and HILL, T.A. 1989a. On difficulties with allelopathy methodology. *Weed Res.* 29:345–347.

QASEM, J.R., and HILL, T.A. 1989b. Possible rôle of allelopathy in the competition between tomato, *Senecio vulgaris* L. and *Chenopodium album* L. *Weed Res.* 29:349–356.

RICE, E.L. 1984. Allelopathy, 2nd ed. Academic Press, Orlando, Florida.

RICE, E.L. 1986. Allelopathic growth stimulation, pp. 23–42, *in* A.R. Putnam and C.-S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

RICHARDSON, D.R., and WILLIAMSON, G.B. 1988. Allelopathic effects of shrubs of the sand pine scrub on pines and grasses of the sandhills. *For. Sci.* 34:592–605.

RUTHERFORD, M.C., and BÖSENBERG, J. DE W. 1988. Some responses of indigenous western Cape vegetation to the Australian invasive, *Acacia cyclops*, pp. 631–636, *in* F. di Castri, Ch. Floret, S. Rambal, and J. Roy (eds.). Time Scales and Water Stress. International Union of Biological Sciences, Paris.

SAVILLE, D.J. 1990. Multiple comparison procedures: The practical solution. *Am. Stat.* 44:174–180.

SINGH, M., TAMMA, R.V., and NIGG, H.N. 1989. HPLC identification of allelopathic compounds from *Lantana camara*. *J. Chem. Ecol.* 15:81–89.

TANG, C.-S. 1986. Continuous trapping techniques for the study of allelochemicals from higher plants, pp. 113–131, *in* A.R. Putnam and C.-S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

TUKEY, H.B., JR. 1970. The leaching of substances from plants. *Annu. Rev. Plant Physiol.* 21:305–324.

WHITFIELD, F.B., SHEA, S.R., GILLEN, K.J., and SHAW, K.J. 1981. Volatile components from the roots of *Acacia pulchella* R.Br. and their effect on *Phytophthora cinnamomi* Rands. *Aust. J. Bot.* 29:195–208.

WILLIAMSON, G.B. 1990. Allelopathy, Koch's postulates, and the neck riddle, pp. 143–162, *in* J.B. Grace and D. Tilman (eds.). Perspectives on Plant Competition. Academic Press, San Diego.

WILLIAMSON, G.B., FISCHER, N.H., RICHARDSON, D.R., and DE LA PEÑA, A. 1989. Chemical inhibition of fire-prone grasses by fire-sensitive shrub, *Conradina canescens*. *J. Chem. Ecol.* 15:1567–1577.

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VARIATION WITH CASTE OF THE MANDIBULAR GLAND SECRETION IN THE LEAF-CUTTING ANT *Atta sexdens rubropilosa*

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Abstract—In *Atta sexdens rubropilosa*, a strongly polyethic and polymorphic species of myrmicine ant, the contents of the mandibular gland vary with caste. Small workers of head width 0.5–1.8 mm, those generally engaged in duties inside the nest, contain chiefly 4-methyl-3-heptanone. Larger workers, those chiefly engaged in foraging, and the soldier caste contain a mixture dominated by neral and geranial, with very little of the ketone of the smaller workers. The soldiers have massive glands containing milligram amounts of neral and geranial. Virgin and mated females contain essentially only 4-methyl-3-heptanone, the amount increasing after mating, while virgin males have 4-methyl-3-heptanone and 4-methyl-3-heptanol in approximately equal proportions. Mated males have less secretion and lose the 4-methyl-3-heptanol.

Key Words—4-Methyl-3-heptanone, 4-methyl-3-heptanol, citral, neral, geranial, Hymenoptera, Formicidae, caste, *Atta sexdens rubropilosa*.

INTRODUCTION

The mandibular glands of many, perhaps most species of ant contain mixtures of volatile organic compounds. A number of these secretions have been

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analyzed, and the results of these analyses have been reviewed (Blum and Hermann, 1978; Attygalle and Morgan, 1984; Hölldobler and Wilson, 1990, p. 260). In many fewer cases have these compounds been carefully tested for behavioral effects. In general, they have been described as alarm pheromones, attracting or repelling congeners from the source of emission (cf. Cammaerts-Tricot, 1973; Morgan et al., 1978; Bradshaw et al., 1975; Hölldobler and Wilson, 1990, p. 260).

The leaf-cutting or fungus-cultivating ants of the tribe Attini provide particularly interesting problems of pheromone behavior. These ants, spread throughout the neotropics (Cherrett, 1986) are of special interest because of their unique dependence upon their fungus garden for larval food, because of their complex communication systems and well-regulated behavior, and because some of their species cause so much damage to agriculture and arboriculture. Their colonies are territorial and aggressive (Salzemann and Jaffe, 1990). The workers of *Atta* vary enormously in size (cf. Hölldobler and Wilson, 1990, p. 325). Wilson (1980) selected *A. sexdens* for particularly close study since he regards it as having one of the most complex systems of caste and polyethism found in all ants. He divided the workers into four castes according to tasks: gardener–nurses, within-nest generalists, forager–excavators, and defenders (Wilson, 1980). The divisions of labor raise further questions, such as whether they all produce the same pheromone substances and whether they all respond to pheromones in the same way. For all these reasons, the interest in their pheromonal secretions is particularly strong.

In a very early study, Butenandt et al. (1959) identified citral (an equilibrium mixture of geranial, the *trans* form, and neral, the *cis* form) in the mandibular glands of *A. sexdens rubropilosa* and described it as a warning and *schreckstoff* (perhaps best translated as a "frightening compound"). Butenandt et al. (1959) noted the large size of the mandibular glands in the soldier caste and estimated they occupied about one fifth of the volume of the head capsule. The next investigation was by Blum et al. (1968), who examined the mandibular glands of six species of *Atta*, including *Atta sexdens*. They do not state whether this is *A. sexdens nubropilosa* or *A. sexdens sexdens*, but as they were collected in the state of São Paulo, Brazil, near to where those used in our present work were collected, they were most probably *A. s. rubropilosa*. They found 4-methyl-3-heptanone and 2-heptanone in a ratio of 1 : 4 in *A sexdens*. They also identified citral and geraniol. In a simple bioassay of placing a ball of cotton wool impregnated with these substances near a foraging trail, they observed a confused and frantic reaction to 4-methyl-3-heptanone; 2-heptanone was mildly repellent, and neither of the monoterpenes caused any reaction. Moser et al. (1968) and Riley et al. (1974) identified 4-methyl-3-heptanone, 4-methyl-3-heptanol, 3-octanone, and traces of 3-octanol in *A. texana* and *A. cephalotes*, and in addition, 2-heptanone, 2-heptanol, and 3-heptanol in *A. texana*. In both

species they found that (*S*)-(+)-4-methyl-3-heptanone was the enantiomer present, that only this enantiomer, unaffected by the inactive (*R*)-(−)-form, was active in bioassays. The other compounds mentioned were not active.

Schildknecht (1976), by extraction of 20,000 freshly prepared worker heads, identified 16 further constituents in the mandibular glands and gave quantitative figures for 10 of these in micrograms per head. By far the major compounds were geranial and neral (together making 40 μg/head).

All these studies were made on mass extracts and did not distinguish between the different castes. As a preliminary to behavioral tests we have therefore reexamined the mandibular gland contents of all the castes of *A. sexdens rubropilosa* using the technique of analyzing individual glands or insect parts by combined gas chromatography–mass spectrometry, to discover whether there are any differences with caste and in order to be able to provide the appropriate substances and in the correct amounts for behavioral studies. We have indeed found that the mandibular pheromone varies with caste and sex.

METHODS AND MATERIALS

Mandibular glands from a complete range of worker sizes of *Atta sexdens rubropilosa* Forel, with head width varying from 0.6 to 4.8 mm were taken from a single nest in the laboratory at Leuven. In some cases the individual mandibular glands were dissected under water using a binocular microscope, lightly dried, and sealed in soft glass capillaries (Morgan, 1990). In other cases the whole head capsule (if it was small enough) was sealed in the capillary. Large heads were sealed in larger glass capillaries and stored until analyzed. The capillary and head was then crushed in a small tissue grinder with 100 μl of hexane and 1 μl of the solution taken for analysis.

Samples of mandibular glands of virgin and mated males and females of *A. s. rubropilosa* were collected in the mating season near Vicosa (Minas Gerais, Brazil) dissected, sealed, and sent to Keele. All these materials were chemically analyzed as described below.

Gas chromatography–mass spectrometry (GC-MS) was performed on a Hewlett-Packard 5890 gas chromatography coupled to a 5970B Mass Selective Detector as described elsewhere (cf. Bagnères et al., 1991) and using the same immobilized polydimethylsiloxane stationary phase in a 12-m fused silica capillary column (Bagnères et al., 1991). For this work, the sealed capillary was heated in the injection port, which was maintained at 150°C, for 4 min before crushing the capillary (Morgan and Wadhams, 1972). The oven was programmed from 30°C at 8°C/min to 150°C. The split vent was closed before crushing the sample and reopened 30 sec later. The chemical substances separated were identified from their mass spectra and confirmed by comparison of

retention time and mass spectrum with synthetic standards, either purchased or synthesized. In a few cases confirmation was by published mass spectrum. In particular, 4-methyl-3-heptanol and citral were purchased (Aldrich, Gillingham, Dorset, UK), and 4-methyl-3-heptanone was prepared from the corresponding alcohol by chromic acid oxidation. These compounds were used as external standards for quantification of the components.

RESULTS

Some of the mandibular glands were carefully dissected out of the head capsules (for number, see Tables 1 and 2), and the chromatograms obtained from these samples were compared with chromatograms obtained from whole head capsules of approximately the same size of worker. There were no additional peaks in the same range of volatilities from the head capsules, so that results from them could be safely combined with those of the isolated glands. A total of 64 glands or heads of individual workers were analyzed by GC-MS, and the identified compounds and the percentage composition for each worker were entered on a large table. It was evident that for this sample, there was a clear division of type of secretion at a head width of 1.8–2.0 mm. The similar results for workers of head width 0.5–1.8 mm were therefore combined to give the mean values in Table 1. The glands were relatively small (mean of 60 ng secretion) and contained a small number of compounds, heavily dominated by 4-methyl-3-heptanone; there were a few other alcohols, aldehydes, and ketones

TABLE 1. PERCENTAGE COMPOSITION OF MANDIBULAR GLAND SECRETION OF SMALL WORKERS OF *Atta sexdens rubropilosa* (HEAD WIDTH 0.5–1.8 mm) WITH SAMPLE STANDARD DEVIATION, INCLUDING WORKERS FROM WITHIN THE NEST AND FORAGING AREA[a]

Compound	Number in Figure 1A	Composition (% ± SD)
Pentanal		0.02 ± 0.10
4-Methyl-3-heptanone	1	89.5 ± 14.5
4-Methyl-3-heptanol		5.7 ± 9.6
6-Methyl-3-heptanone		0.2 ± 1.1
3-Octanone		0.7 ± 1.6
Geranial		1.6 ± 7.7
2-Decanone		1.6 ± 4.2
Decanal		0.4 ± 2.1

[a]Mean values from 22 dissected glands and 15 whole head capsules. Mean total amount: 0.6 μg ± 2.5.

TABLE 2. PERCENTAGE COMPOSITION OF MANDIBULAR GLAND SECRETION OF LARGER WORKERS AND SOLDIERS OF *A. sexdens rubropilosa* (HEAD WIDTH 2.0–4.8 mm, TOGETHER WITH SAMPLE STANDARD DEVIATION) [a]

Compound	Number in Figure 1B	Composition (% ± SD)
4-Methyl-3-heptanone	1	6.8 ± 8.2
4-Methyl-3-heptanol		0.8 + 2.9
6-Methyl-5-hepten-2-one	2	3.3 ± 3.7
6-Methyl-5-hepten-2-ol		0.1 ± 0.5
Octanal		2.6 ± 5.0
6-Methyl-3-heptanone		0.02 ± 0.1
1-Nonanol		0.3 ± 1.8
Citral-like isomer-1		1.0 ± 1.0
Citral-like isomer-2	3	2.4 ± 2.3
Citral-like isomer-3		2.9 ± 3.2
Decanal	4	3.1 ± 3.0
Neral	5	28.6 ± 9.6
Isogeraniol	6	3.9 ± 9.8
Geranial	7	40.6 ± 11.5
2-Decanone		0.3 ± 1.3
Methyl decanoate		0.1 ± 0.3
Dodecanal	8	0.7 ± 0.9
γ-Decalactone	9	1.0 ± 1.6
Neric acid		0.4 ± 2.0
Geranic acid		0.5 ± 2.4
Geranylacetone	10	1.8 ± 1.7
Unknown ketone		0.4 ± 1.3
Verbenone		0.2 ± 0.9
Farnesol		0.4 ± 1.9

[a] Mean values from 16 dissected glands and 11 whole head capsules.
Mean total amount: head width 2.0–2.8 mm, 34.8 μg; head width 3.0–3.9 mm, 170 μg; and head width 4.0–4.8 mm, 1.43 mg.

in trace amounts, but no citral (Figure 1A). The variability in amount of these minor components is indicated by the large sample standard deviations in Table 1.

The first group of 26 samples of these small workers were taken from outside the fungus compartment of the nest. Recognizing that the duties of the small workers are essentially within the nest (Wilson, 1980), we took a further 11 samples from inside the fungus garden and compared the two sets of results, to be sure there was nothing anomolous about those taken outside. No difference was evident in the two groups, so they were combined in Table 1.

The larger workers and soldiers (head width 2.0–4.8 mm), although varying

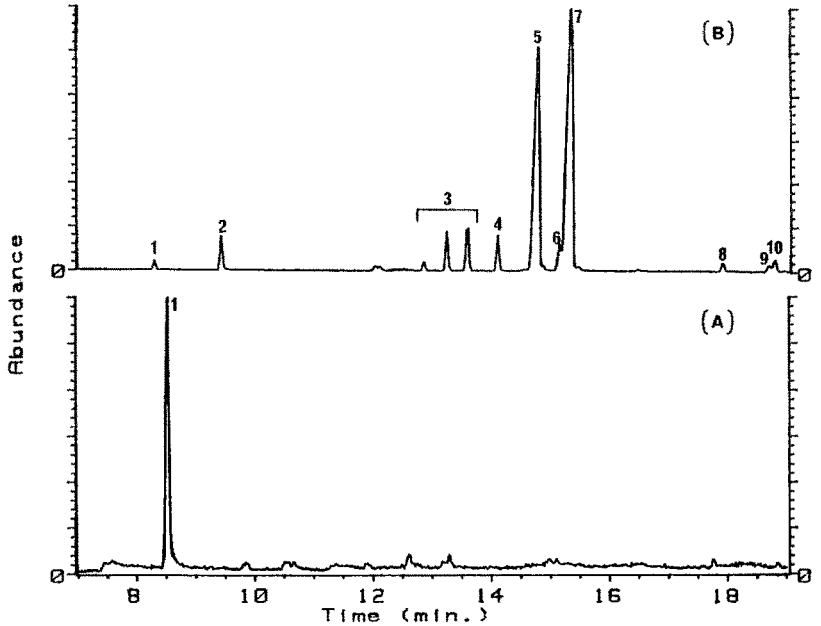

FIG. 1. Gas chromatograms illustrating (A) a single worker mandibular gland from *Atta sexdens rubropilosa*, with head width less than 1.8 mm, and (B) a single worker with head width greater than 2.0 mm. For identification of numbered peaks, see Table 2.

greatly in the size of their mandibular glands, showed a constancy of composition in the secretion, with very little 4-methyl-3-heptanone and having citral (the isomers neral and geranial in their natural ratio) as the major compound pair (Figure 1B). There were a larger number of minor components, many of them terpene-derived (Table 2). Not all the minor components matched those identified by Schildknecht (1976), e.g., β-pinene and 2-phenylethanol were not found here, but γ-decanolactone and farnesol, identified by Schildkneckt were also found in our samples. The 2-heptanone recorded by Blum et al. (1968) was not found. Three minor compounds, eluting before neral and having mass spectra similar to neral and geranial, were found in both the gland and commercial citral. They are isomeric monoterpenes, probably close in structure to citral, but they were not fully identified. The total amount of secretion in the glands varied widely. The mean amount for three groups are given in Table 2.

Mated females collected in Brazil and dissected and sealed there showed a constancy of composition similar to the smaller workers (Table 3) and 4-methyl-3-heptanone comprising about 90% of the secretion (Figure 2A). Unmated females also contained 4-methyl-3-heptanone (Fig. 2B), but there was less secretion (Table 3).

TABLE 3. PERCENTAGE COMPOSITION OF MANDIBULAR GLAND SECRETION OF FOUR SAMPLES OF UNMATED AND 12 SAMPLES OF MATED FEMALE *Atta sexdens rubropilosa*

Compound	Number in Figure 2	Composition (% ± SD)	
		Unmated	Mated
4-Methyl-3-hexanone	1	1.7 ± 1.5	
4-Methyl-3-hexanol	2	t[a]	
3-Heptanone	3	0.2 ± 0.3	0.2 ± 0.6
2-Heptanone	4	t	
4-Methyl-3-heptanone	5	94.8 ± 3.4	91.7 ± 6.7
4-Methyl-3-heptanol	6	0.8 ± 1.7	6.0 ± 3.4
3-Octanone	7	1.1 ± 1.2	0.5 ± 1.5
3-Octanol			t
4,5-Dimethyl-4-hexen-2-one	8	0.2 ± 0.3	t
6-Methyl-3-octanone	9	0.2 ± 0.2	t
4-Methyl-3-octanone		0.5 ± 0.9	
3-Nonanol			t
5-Ethyl-4-methyl-3-heptanone	10	0.4 ± 0.5	t
2-Decanone			0.4 ± 1.4
Decanal		0.3 ± 0.6	
p-Cymen-α-ol		0.1 ± 0.1	t
α-Terpineol			t
Neral			t
Geranial			t
Geraniol			t
Geranylacetone			0.1 ± 0.2
2-Tridecanone			0.2 ± 0.7
3-Undecene			0.1 ± 0.4
Mean total amount (μg)		4.9	8.8 ± 5.6

[a]t = trace, less than 0.1%.

The mated males had a glandular composition similar to that of the mated females, but rather less of the secretion (Figure 3A, Table 4). The unmated males contained a much higher proportion of 4-methyl-3-heptanol than was found in any other group, and more secretion in the gland than in those analyzed after mating.

DISCUSSION

Wilson (1980), by observing the tasks they performed, divided the workers of *A. s. rubropilosa* into four groups. The minims, described as gardener–nurses with mean head width 1.0 mm, and the medians or within-nest generalists of

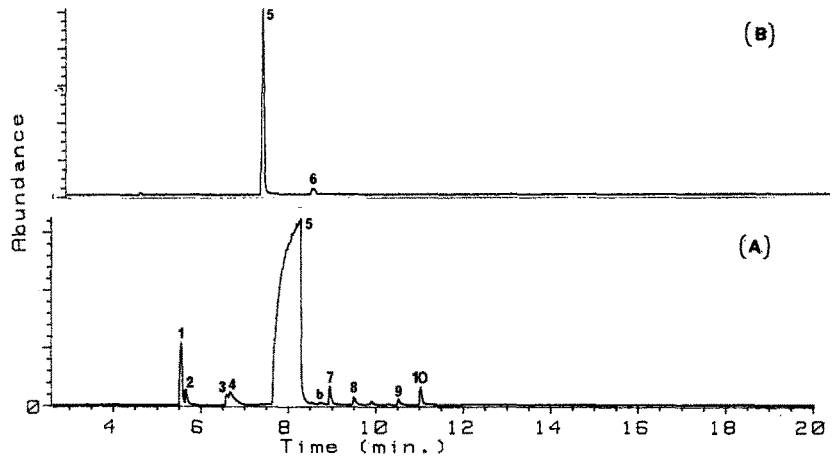

FIG. 2. Gas chromatograms of the mandibular secretion of (A) unmated and (B) mated females of *A. sexdens rubropilosa*. Numbered peaks are identified in Table 3. Retention times are not exactly comparable because of a change of column between two sets of data. One whole head of a female was used for (A); for (B) a solution in hexane containing 1% of the total secretion from one female was used.

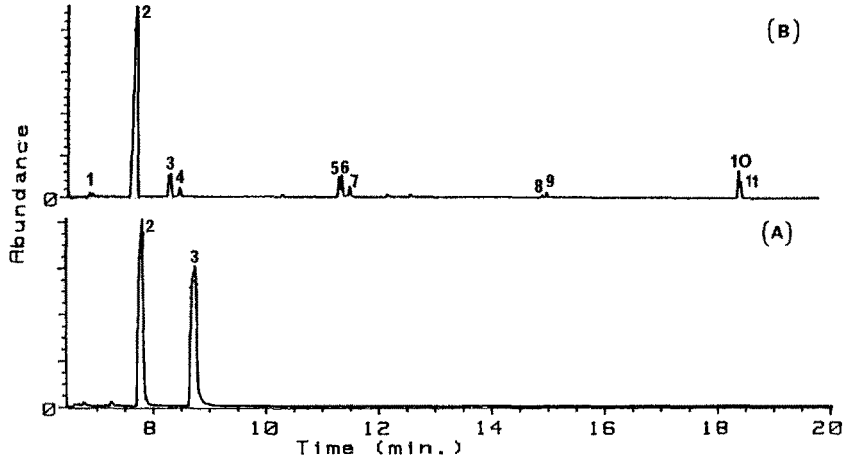

FIG. 3. Gas chromatograms of the mandibular section of (A) unmated and (B) mated males of *A. sexdens rubropilosa*. Numbered peaks are identified in Table 4. Retention times were altered by a change of column between determinations.

TABLE 4. PERCENTAGE COMPOSITION OF MANDIBULAR GLAND SECRETION OF SIX SAMPLES OF UNMATED AND 17 SAMPLES OF MATED MALE *A. sexdens rubropilosa*

Compound	Number in Fig. 3	Composition (% ± SD)	
		Unmated	Mated
4-Methyl-3-hexanone	1	1.0 ± 0.7	2.5 ± 10.2
4-Methyl-3-hexanol		0.2 ± 0.2	
3-Heptanone		0.4 ± 0.9	
3-Heptanol		0.1 ± 0.2	0.2 ± 0.6
2-Heptanone		0.5 ± 0.6	
2-Heptanol		0.4 ± 0.5	
4-Methyl-3-heptanone	2	49.1 ± 7.4	81.8 ± 27.0
4-Methyl-3-heptanol	3	48.0 ± 7.7	8.1 ± 12.2
3-Octanone	4		t
3-Octanol			0.1 ± 0.4
Octanal			0.1 ± 0.2
Decanal			t
Terpinen-4-ol	5	t[a]	0.3 ± 0.6
α-Terpineol	6	0.1 ± 0.1	0.3 ± 0.9
p-Cymen-α-ol	7	t	0.3 ± 0.6
Neral			t
Isogeraniol			t
Geranial			t
Methyl decanoate			t
γ-Decalactone	8		t
Geranylacetone	9		t
9-Heptadecanone	10	0.1 ± 0.1	5.9 ± 22.8
9-Heptadecanol	11	t	t
Mean total amount (μg)		11	2.3 ± 5.1

[a] t = trace, less than 0.1%.

mean head width 1.4 mm both worked within the nest. Those working outside the nest were the major workers or foragers and excavators, centered on head width 2.2 mm, and the defenders, those workers over 3.0 mm in head width.

Rather than use his behavioral divisions of workers, we inspected the complete range of chemical analyses of worker mandibular glands and came to the conclusion that between 1.8 and 2.0 mm there is a sharp chemical division between smaller and larger workers. There is, therefore, chemical support for the divisions proposed by Wilson (1980). Those workers with duties inside the nest have a mandibular gland containing very largely 4-methyl-3-heptanone. Those workers engaged outside the nest, whether foragers or defenders, have a mandibular pheromone based on citral. The clear division in composition between

these two mandibular secretions means that different messages in different contexts are conveyed by these substances. It is difficult to reconcile our findings with the behavioral tests described by Blum et al. (1968). The behavioral responses to these substances of the different castes of workers inside and outside the nest need to be reexamined. The lack of distinction in place and caste in earlier behavioral experiments makes them now of reduced value. The value of the massive quantities of citral stored in the heads of the soldier caste is puzzling if this isomeric pair of compounds causes no reaction in the species as stated by Blum et al. (1968).

In some unpublished work by Morgan and Inwood, we drew air continually over a quietly organized colony of *Myrmica scabrinodis* in an artificial glass nest. We were surprised to find the air contained readily detectable amounts of 3-octanol and 3-octanone, the mandibular gland secretion of this species, yet the ants did not appear disturbed. These observations allow the possibility that the volatile mandibular substances provide short-range nondirectional communication between the workers.

There have been few studies on the mandibular glands of sexuals in ants. Pasteels et al. (1980) have described the mandibular pheromones of *Tetramorium impurum* [wrongly described there as *T. caespitum*, see Pasteels et al. (1981)]. 4-Methyl-3-hexanol and 4-methyl-3-hexanone were found in quantity in males, and in lesser amount in females, whereas only the alcohol was found in workers. Nevertheless, finding such similarity between males and females here was surprising. In view of the short life of about two days of the males once they leave the nest, one wonders what purpose is served. The presence of larger amounts of 4-methyl-3-heptanol in the males would give them a different odor, and this suggests behavioral tests as a sexual attractant. It is noteworthy that among the sexuals, the unmated males have the largest amount of secretion (Table 4). After mating, it drops considerably, and the 4-methyl-3-heptanol is lost. In females, the amount of secretion increases after mating. To complete this study, the chirality of the 4-methyl-3-heptanone must be determined. It is noteworthy that in a marine worm, *Platynereis dumerilli*, 5-methyl-3-heptanone is used as a sexual pheromone, males responding to the *R*-(−) enantiomer and females to the *S*-(+) enantiomer (Zeeck et al., 1992).

Wilson (1980) introduced the hypothesis of a primitive caste and concluded for *A. s. rubropilosa* that the small workers represent the primitive caste. It is perhaps worth noting that it is the mandibular secretion of the small workers that is close to that of the females, and the large workers have developed different substances.

The present work illustrates the value of analyzing insects individually. The mandibular pheromones of *A. sexdens rubropilosa* are more complex than previously recognized. We now propose to produce the blends of substances appropriate to the various castes for behavioral testing.

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REFERENCES

ATTYGALLE, A.B., and MORGAN, E.D. 1984. Chemicals from the glands of ants. *Chem. Soc. Rev.* 13:145–278.

BAGNÈRES, A.G., BILLEN, J., and MORGAN, E.D. 1991. Volatile secretion of Dufour gland of workers of an army ant, *Dorylus (Anomma) molestus*. *J. Chem. Ecol.* 17:1633–1639.

BLUM, M.S., and HERMANN, H.R. 1978. Venom and the venom apparatuses of the Formicidae: Myrmeciinae, Ponerinae, Dorylinae, Pseudomyrmecinae, Myrmicinae and Formicinae, pp. 801–894, *in* S. Bettini, (ed.). Arthropod Venoms, Handbuch der Experimentellen Pharmakologie, 48, New Series. Springer, Berlin.

BLUM, M.S., PADOVANI, F., and AMANTE, E. 1968. Alkanones and terpenes in the mandibular glands of *Atta* species (Hymenoptera: Formicidae). *Comp. Biochem. Physiol.* 16:291–299.

BRADSHAW, J.W.S., BAKER, R. and HOWSE, P.E. 1975. Multicomponent alarm pheromones of the weaver ant. *Nature* 258:230–231.

BUTENANDT, A., LINZEN, B., and LINDAUER, M. 1959. Über einen Duftstoff aus der Mandibeldrüse der Blattschneiderameisen *Atta sexdens rubropilosa* Forel. *Arch. Anat. Microsc. Morphol. Exp.* 48:13–19.

CAMMAERTS-TRICOT, M.-C. 1973. Phéromones agrégeant les ouvrières de *Myrmica rubra*. *J. Insect Physiol.* 19:1299–1315.

CHERRETT, J.M. 1986. History of the leaf-cutting ant problem, pp. 10–17, *in* C.S. Lofgren and R.K. Vander Meer (eds.). Fire Ants and Leaf-Cutting Ants: Biology and Management. Westview Press, Boulder, Colorado.

HÖLLDOBLER, B., and WILSON, E.O. 1990. The Ants. Springer-Verlag, Berlin.

MORGAN, E.D. 1990. 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 material. *J. Chromatogr. Sci.* 10:528–529.

MORGAN, E.D., INWOOD, M.R. and CAMMAERTS, M.-C. 1978. The mandibular gland secretion of the ant *Myrmica scabrinodis*. *Physiol. Entomol.* 3:107–114.

MOSER, J.C., BROWNLEE, R.C., and SILVERSTEIN, R.M. 1968. Alarm pheromones of the ant *Atta texana*. *J. Insect Physiol.* 14:529–535.

PASTEELS, J.M., VERHAEGHE, J.C., BRAEKMAN, J.C., DALOZE, D., and TURSCH, B. 1980. Caste-dependent pheromones in the head of the ant *Tetramorium caespitum*. *J. Chem. Ecol.* 6:467–472.

PASTEELS, J.M., VERHAEGHE, J.C., OTTINGER, R., BRAEKMAN, J.C., and DALOZE, D. 1981. Absolute configuration of (3*R*,4*S*)-4-methyl-3-hexanol—a pheromone from the head of the ant *Tetramorium impurum* Foerster. *Insect Biochem.* 11:675–678.

RILEY, R.G., SILVERSTEIN, R.M., and MOSER, J.C. 1974. Isolation, identification, synthesis and biological activity of volatile compounds from the heads of *Atta* ants. *J. Insect Physiol.* 20:1629–1637.

SALZEMANN, A., and JAFFE, K. 1990. Territorial ecology of the leaf-cutting ant *Atta laevigata*, pp. 345–354, *in* R.K. Vander Meer, K. Jaffe and A. Cedeno (eds.). Applied Myrmecology. Westview Press, Boulder, Colorado.

SCHILDKNECHT, H. 1976. Chemical-ecology—a chapter of modern natural products chemistry. *Angew. Chem. Int. Ed. Engl.* 15:214–222.

WILSON, E.O. 1980. Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*). *Behav. Ecol. Sociobiol.* 7:143–156.

ZEECK, E., HARDEGE, J.D., WILLIG, A., KREBBER, R., and KÖNIG, W.A. 1992. Preparative separation of enantiomeric polychaete sex pheromones. *Naturwissenschaften* 79:182–183.

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OVIPOSITION STIMULANTS FOR THE BLACK SWALLOWTAIL BUTTERFLY: IDENTIFICATION OF ELECTROPHYSIOLOGICALLY ACTIVE COMPOUNDS IN CARROT VOLATILES

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Abstract—Headspace volatiles were collected from undamaged foliage of carrot, *Daucus carota*, a host-plant species of the black swallowtail butterfly, *Papilio polyxenes*. The volatiles were fractionated over silica on an open column, and the fractions were tested in behavioral assays with *P. polyxenes* females in laboratory experiments. The polar fractions, as well as the total mixture of volatiles, increased the landing frequency and the number of eggs laid on model plants with leaves bearing contact-oviposition stimulants. The nonpolar fraction, containing the most abundant compounds in carrot odor, was not stimulatory. Gas chromatographic (GC) separation of the fractions was coupled with electroantennogram (EAG) recordings to identify the compounds perceived by *P. polyxenes* females. The EAG activity corresponded to the behavioral activity of the fractions. None of the nonpolar compounds, identified as various monoterpenes, evoked a major EAG response, but several constituents of the polar fractions elicited high EAG responses. Sabinene hydrate (both stereoisomers), 4-terpineol, bornyl acetate, and (Z)-3-hexenyl acetate were identified by GC-MS as active compounds.

Key Words—*Papilio polyxenes*, Papilionidae, Lepidoptera, *Daucus carota*, Apiaceae, host-plant selection, oviposition behavior, electroantennogram, combined GC-EAG, plant volatiles, sabinene hydrate, 4-terpineol, bornyl acetate, (Z)-3-hexenyl acetate.

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INTRODUCTION

Many phytophagous insects depend on volatile plant compounds as important cues during host-plant selection (Visser, 1986). Among Lepidoptera, olfactory responses to host-plant odor have been shown to influence oviposition behavior in several species of moths (Douwes, 1968; Städler, 1974; Palaniswamy et al., 1986; Leather, 1987; Clark and Dennehy, 1988; Reed et al., 1988; Landolt, 1989; Phelan et al., 1991; reviewed by Ramaswamy, 1988). A number of studies have demonstrated that butterflies make use of visual cues (Vaidya, 1969; Rausher, 1978; Wiklund, 1984; Papaj, 1986) and/or chemicals perceived upon tarsal contact with the leaf surface (Ma and Schoonhoven, 1973; Nishida et al., 1987; Renwick and Radke, 1987; Nishida and Fukami, 1989) when selecting a suitable host plant for oviposition. Few studies, by contrast, have investigated the role of host-plant volatiles in butterfly oviposition behavior (Vaidya, 1969; Saxena and Goyal, 1978).

Feeny et al. (1989) described the influence of host-plant volatiles on oviposition behavior by the black swallowtail *Papilio polyxenes*, whose host range in North America includes various species of the family Apiaceae (Umbelliferae). Although contact chemicals are important in the final decision by *P. polyxenes* females for acceptance or rejection of a plant for oviposition (Feeny et al., 1983, 1988), black swallowtail females landed more often and laid more eggs on plant models treated with contact stimulants when volatiles from the host-plant carrot, *Daucus carota* L., were added. In contrast, volatiles from cabbage, a nonhost plant, inhibited landing on artificial leaves. Electroantennogram (EAG) recordings demonstrated a higher response by *P. polyxenes* females to carrot volatiles than to cabbage volatiles (Feeny et al., 1989), but the chemical compounds contributing to the electrophysiological activity were not identified.

In this paper we describe behavioral assays conducted to investigate the influence of the total mixture of volatile compounds present in the headspace vapor over host-plant foliage and of fractions of this mixture on the oviposition behavior of black swallowtail females. Gas chromatographic (GC) separation of fractions tested in behavioral assays was coupled with EAG recordings to identify volatile host-plant compounds perceived by black swallowtail females.

METHODS AND MATERIALS

Plants and Insects. Six collections (1 kg each) of mature, undamaged foliage from wild carrot, *Daucus carota* L., were made in June 1990 between 0800 and 1000 hr in the vicinity of Ithaca, New York. Females of *Papilio polyxenes asterius* Stoll were obtained from diapausing pupae reared in southern Ontario (Canada).

Collection of Carrot Volatiles. Immediately after collection, the carrot leaves were enclosed in a glass container (30 × 30 × 30 cm) at 25°C. A continuous flow of N_2 (50 ml/min) was passed for 8–10 hr through the container and subsequently through a cold trap cooled with liquid N_2. Every 2 hr, the cold trap was rinsed with 10–15 ml of *n*-hexane (HPLC grade) and vigorously shaken to extract the trapped volatiles. The resulting mixture of hexane and condensed water was decanted from the trap and the hexane and water layers separated. Most of the electrophysiological activity was found in the hexane layer, and a subsequent extraction of the water layer with diethyl ether did not result in extraction of much additional electrophysiological activity. The hexane extract was cooled below 0°C to freeze out remaining water and then concentrated at room pressure and temperature under a stream of N_2. Final concentrations of the extract for electroantennogram recordings typically consisted of the volatiles collected from 1 kg foliage in an 8-hr period, dissolved in 1 ml solvent.

Chromatography of Crude Volatile Mixture. Aliquots (0.5 ml) of the crude volatile mixture obtained from headspace collection were fractionated on silica gel (32–64 μm, 2.5 g) in an open column. Two bed volumes (5 ml each) of hexane, hexane–chloroform (1:1), chloroform, chloroform–acetone (98:2), chloroform–acetone (95:5), and acetone were passed through the column and the eluate collected as two fractions per solvent. To reduce the number of necessary behavioral assays and GC-EAG runs, fractions with similar compositions, as judged by FID gas chromatograms, were combined as follows: nonpolar fraction = hexane fraction (10 ml); intermediate fraction = hexane–chloroform fraction (10 ml); polar fraction A = chloroform fraction (10 ml) + first 5 ml of chloroform–acetone (98:2) fraction; and polar fraction B = remaining fractions. Prior to GC-EAG analysis, the fractions were reduced under N_2 to concentrations corresponding to those in the initial extract.

Assays of Oviposition Behavior. Bioassays were conducted in two walk-in controlled-environment chambers—one each for experiments with volatiles and for control (solvent) experiments—at 16:8 hr light–dark and temperatures of 29°C (light) and 16°C (dark). Each replicate consisted of five female butterflies in a cage (44 × 44 × 60 cm) that contained a model plant bearing four artificial leaves. Cages, model plants, and preparation of artificial leaves are described in detail by Feeny et al. (1989). On each model plant, one leaf was designated as a treatment leaf, to the upper surface of which was applied an aqueous extract containing nonvolatile contact-oviposition stimulants from wild carrot (for preparation see Feeny et al., 1989) at a dose of 1 gram leaf equivalent (gle). The remaining three leaves were blanks, treated only with an equal volume of H_2O.

Bioassays were conducted with the total mixture of headspace volatiles, the combined nonpolar and intermediate fractions, and the combined polar fractions A and B. For every experiment, cages were designated as either experimentals or controls (10–12 replicates each). In the experimentals, the treatment

leaf received volatiles in an amount equivalent to the volatiles collected from 30 g leaves, diluted in 20 μl of hexane (total mixture, combined nonpolar and intermediate fractions), or chloroform (combined polar fractions A and B). In the control cages, equal amounts of pure solvent were applied to the treatment leaf. Volatile solutions or solvents were applied 5 min before the beginning of the bioassays. This allowed for complete evaporation of solvents before exposure of the plant models to butterflies.

Female butterflies were hand-paired one day after eclosion and stored at 5–7°C for up to six days before use in bioassays. They were then caged together with potted carrot plants and hand-fed daily (10% honey water) for three days prior to bioassays, at which stage they had reached their maximum daily oviposition rate (cf. Blau, 1981). The plants were removed 3 hr before the beginning of bioassays, and the females were fed and then assigned randomly to the experimental and control cages. Beginning 5 min after addition of the model plants to the cages, the number of females landing on each leaf was recorded for 50 min. During this observation period, plant models were rotated by 90° every 10 min to minimize potential learning by the butterflies of the position of the treatment leaf. Eggs were counted on all leaves of the model plants after 2 hr.

Each female was used only once for each mixture tested. The total number of replicates for each mixture tested represented the combined results from two batches of butterflies used at different times. These batches differed slightly in their overall oviposition activity. Therefore, for statistical analysis of the two measured parameters (landings and eggs), the result from each cage was standardized by dividing it by the respective total obtained from all cages containing butterflies of the same batch.

GC-EAG Set-Up. Carrot volatiles were separated by gas chromatography on a Hewlett Packard HP5890 gas chromatograph equipped with a FID and a 25-m × 0.2-mm-(ID) HP1 capillary column (0.3 μm cross-linked methyl silicone). GC conditions were: helium as carrier gas (28.7 cm/min linear velocity), splitless injection at 40°C, and temperature programs rising to 200°C (at rates depending on the sample). The column eluent was divided with a glass outlet splitter with pressfit connectors (Perkin Elmer) at a fixed ratio such that 25% of the eluent entered the FID. The remaining 75% was led to the antennal preparation in the manner described by Feeny et al. (1989).

Antennal Preparation for Electrophysiological Recordings. Two- or three-day-old virgin females were used for EAG recordings. Since the EAG preparation techniques were derived from those described by Guerin et al. (1983) and Feeny et al. (1989), only modifications are described here in some detail. Female butterflies were mounted on Plexiglas blocks and the glass capillary of one electrode, filled with saline solution, was inserted into a proximal segment of one antenna. Since inserting the distal electrode into the tip of the antenna (as

described by Feeny et al., 1989) results in partial short-circuiting of the preparation and therefore in a loss of sensitivity, the capillary of the distal electrode was only brought into contact with olfactory sensilla on the antennal surface. For this purpose, the tip of the capillary was polished by pulling it over sandpaper (grade 500) until the diameter of the capillary opening was about one fourth of the diameter of the third last antennal segment. The capillary opening was then pressed against the ventral surface of this segment and kept in place with a 0.4- to 0.7-mm-broad strip of Parafilm (American National Can, Greenwich, Connecticut). About 30% of antennae prepared in this manner gave satisfactory recordings for up to 5 hr without detectable loss of sensitivity. The signals were amplified (differential amplifier LF 252D, input imp. = 2×10^{12} Ω, input bias current = 3 pA, gain = 100) and filtered with an active high-pass filter with variable corner frequency, usually set at 0.02 Hz for a stable baseline and a quick return of the deflection after a peak.

Identification of Compounds. The identity of unknown compounds was determined by GC-mass spectrometry (GC-MS) with the same type of GC and column as for GC-EAG work, connected to a HP 5970B mass selective detector and a HP 59970B workstation (both Hewlett Packard). EI spectra were recorded at 70 eV and compared with published spectra (McLafferty and Stauffer, 1989) and, for confirmation of identifications, with simultaneously recorded spectra of appropriate reference compounds. Where reference material for confirmation was unavailable, Kováts indices of retention times were determined for the unknowns and compared to published indices (Davies, 1990).

Concentration–Response Recordings. Although concentration–response recordings for EAGs with reference material are often carried out with the so-called puff method (e.g., Guerin and Visser, 1980; Feeny et al., 1989), we preferred the application of the above described GC-EAG technique for two reasons: First, the sometimes considerable contribution of impurities in reference compounds to the EAG response could be excluded by separating impurities from compounds by GC prior to EAG analysis. Second, the complete evaporation and transfer of the injected sample to the antenna allows a direct quantitative comparison of compounds that differed in vapor pressure. In the puff method, by contrast, the concentration of the sample in the vapor phase (the only part transferred to the antenna) depends on its boiling point.

GC temperature programs for each compound were adjusted so that retention times were between 9 and 10 min. This resulted in a similar FID peak shape (height-to-width ratio) for every compound, reflecting comparable changes of eluting concentration over time. EAG activity for each compound (diluted in hexane) was recorded at two concentrations (10 ppm and 100 ppm in 2 μl injected), and the height of each EAG deflection was compared to the deflection in response to a standard [100 ppm (*E*)-2-hexenal]. Recordings were performed with pure reference compounds when available. For each of the others, fractions

containing the compound were adjusted to provide concentrations of 10 ppm or 100 ppm.

RESULTS

Oviposition Behavior. The influence of carrot headspace volatiles on overall landing and oviposition activity was determined for *P. polyxenes* females in response to artificial plants emitting carrot volatiles or solvent only (control cages). In the presence of the combined polar fractions A and B of headspace volatiles, *P. polyxenes* females landed significantly more often on artificial leaves than did females in the control cages (Figure 1a). The same trend ($P = 0.13$) was found for the total mixture of headspace volatiles but not for the combined nonpolar and intermediate fractions (Figure 1a). There was considerable variation in numbers of landings within treatments, caused by a considerable, but unpredictable, portion of females within each cage that remained completely inactive during the whole observation period.

The number of eggs laid within 2 hr generally reflected the pattern observed for landings. The presence of the total mixture of headspace volatiles or the polar fractions A and B significantly increased the number of eggs compared with those laid in control cages (Figure 1b). No difference between cages with volatiles and control cages was found for the combined nonpolar and intermediate fractions (Figure 1b).

In addition to comparing the total number of landings in cages with or without volatiles, we analyzed the distribution of the observed landings among the leaves of the model plant within each cage. The proportion of all landings on the treatment leaf, containing contact-oviposition stimulants and volatiles, reflected the ability of swallowtail females to discriminate between the leaf stimulating the whole oviposition sequence and the leaves lacking contact-oviposition stimulants and thus not suitable for oviposition. Both the total headspace volatiles and the combined polar fractions A and B significantly enhanced the proportion of landings on the treatment leaf compared to control cages, in which the treatment leaf contained solvent only (Figure 2). No increased discrimination was found for the combined nonpolar plus intermediate fractions (Figure 2). Most likely, the females learned to associate the presence of volatiles with suitable oviposition substrates. Thus, volatiles permitted females to discriminate more efficiently between artificial leaves with contact stimulants and blank leaves.

In the absence of any cues facilitating discrimination between the leaf treated with contact-oviposition stimulants and the blank leaves, 25% of randomly occurring landings would be expected on the treatment leaf. The observed frequencies on the treatment leaves in the control cages, ranging from 35% to 47% (Figure 2), were tested for differences from the expected values using a

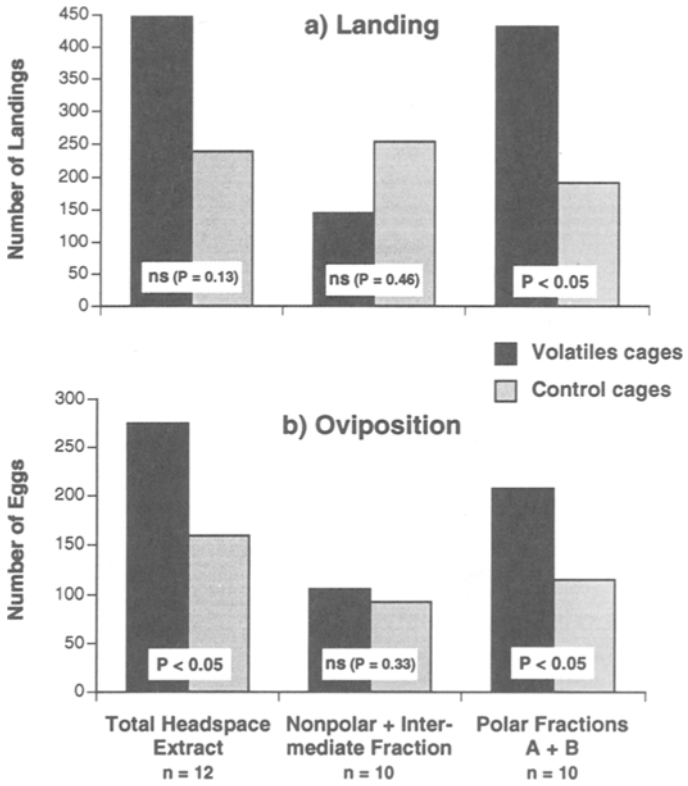
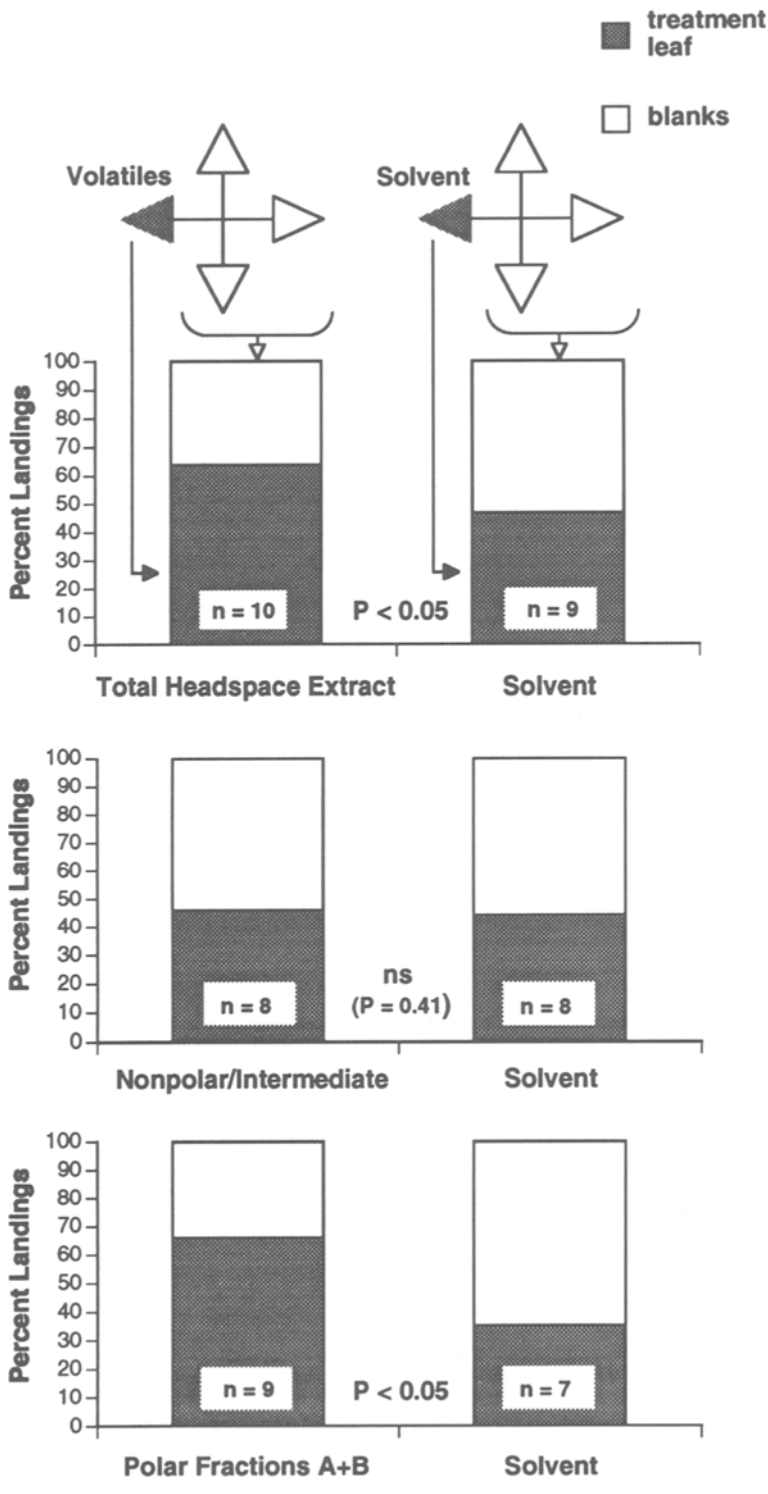


FIG. 1. Effect of carrot headspace volatiles and fractions thereof on landing and oviposition by *Papilio polyxenes* females on all leaves of model plants. Dark grey bars indicate the total number of landings or eggs, respectively, in all cages with volatiles; light grey bars the respective numbers for control cages. n = number of cages per treatment. Five females per cage; plant models were rotated 90° every 10 min during observation period of 50 min for landings; eggs counted after 2 hr. Test for significant differences between volatiles and controls with Wilcoxon signed-ranks test.

Wilcoxon signed-ranks test with observed and expected values for each cage as paired samples. Landing rates on treatment leaves were significantly higher than expected if landings occurred randomly in control cages for experiments with the nonpolar fraction ($P < 0.05$) and for total headspace volatiles ($P < 0.1$). The same trend was also found in the control cages of the experiment with the combined polar fractions ($P = 0.15$). This surprisingly high landing frequency on leaves with contact-oviposition stimulants but no volatiles suggests that the females were, nevertheless, able to discriminate between leaves that were designed to be equal. Besides small visual differences in leaf shape and color,


treatment leaf
blanks
Volatiles
Solvent
Percent Landings
100
90
80
70
60
50
40
30
20
10
0
n = 10
P < 0.05
n = 9
Total Headspace Extract
Solvent
Percent Landings
n = 8
ns
(P = 0.41)
n = 8
Nonpolar/Intermediate
Solvent
Percent Landings
n = 9
P < 0.05
n = 7
Polar Fractions A+B
Solvent

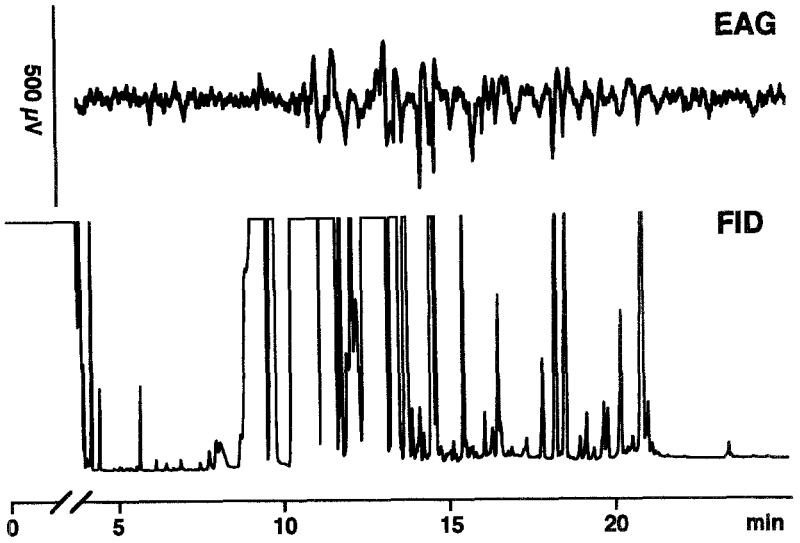


FIG. 3. Gas chromatogram (FID) and simultaneously recorded EAG responses of a *Papilio polyxenes* female. Splitless injection of cold trap-collected headspace vapor dissolved in hexane. Column effluent split at a ratio of 3:1 (EAG:FID). Temperature program: 40°C for 2 min, 10°C/min to 70°C, 3°C/min to 94°C, 15°C/min to 200°C, 200°C for 5 min.

traces of unknown volatiles originating from the contact-oviposition stimulants could have been used by the females as cues and associated with the contact-oviposition stimulants. Furthermore, although models were rotated every 10 min, this may not have been sufficiently frequent to prevent completely short-term association of leaf position with the presence of contact stimulants.

Identification of Perceived Host-Plant Compounds. Gas chromatographic analysis revealed that a large number of compounds in a wide range of concentrations contribute to the volatile blend emanating from carrot foliage. Some of these compounds evoked a distinct EAG response in *P. polyxenes* females (Figure 3). However, the large number of compounds in high concentrations eluting between 80°C and 90°C prevented reliable correlation between EAG and FID peaks. The application of a slower temperature program improved the resolution

FIG. 2. Discrimination by *P. polyxenes* females between treatment leaves and blank leaves within the cages: treatment leaves received contact-oviposition stimulants and either volatiles or solvent only. Only cages with >10 landings included (n = number of cages per treatment, five females per cage). Plant models were rotated 90° every 10 min during observation period of 50 min. Wilcoxon-Mann-Whitney test for significant differences between cages with volatiles and cages with solvent only.

of GC separation but also reduced the sharpness of eluting peaks and hence the quality of the EAG response. The chosen temperature program represented a compromise that permitted good peak sharpness for EAG responses while still providing adequate peak separation (Figure 3).

For further separation, the mixture of carrot volatiles was fractionated on an open silica gel column. The nonpolar fraction contained most of the volatiles (Figure 4a). The most abundant compounds were identified, and all were found to be monoterpenes (Figure 4a, Table 1a). However, no compounds in the nonpolar fraction were found to be electrophysiologically very active (Figure 4a, Table 1a). Terpinolene (peak 9, Figure 4a), although 40 times less concentrated in the nonpolar fraction than the predominant sabinene, consistently elicited a distinct EAG response, while the response to β-myrcene (peak 4, Figure 4a) was very variable among the females tested (Table 1a). The intermediate fraction contained only a few compounds in low concentrations, and none of these showed major EAG activity. Therefore this fraction was not further analyzed.

Most of the EAG activity in terms of number and amplitude of EAG peaks was found in the polar fraction A, as shown for a typical example in Figure 4b. The compounds accounting for three of the largest EAG peaks (peaks 11–13, Figure 4b), were identified as (*E*)-sabinene hydrate, (*Z*)-sabinene hydrate, and 4-terpineol (for structures see Figure 5; for major ions in mass spectra see Table 2). Peak 14 was identified as either (L)-bornyl acetate or isobornyl acetate (structures: Figure 5). These two isomers could not be distinguished by their mass spectra or retention times on our GC system. EAG recordings for both isomers with *P. polyxenes* females did not reveal a significant difference in the magnitude of response ($P = 0.68$, $N = 6$, t test). The mass spectrum of peak 15 showed that this unidentified compound is also an oxygenated monoterpene, consistent with a high sensitivity of the antennal receptors of *P. polyxenes* for this class of compounds.

For all (>30) females tested, (*Z*)-sabinene hydrate (peak 12) elicited the highest response of all compounds in the whole carrot volatile mixture. Even at low concentrations (Table 1, 10 ppm concentration), (*Z*)-sabinene hydrate remained very active. The stereoisomer (*E*)-sabinene hydrate (peak 11), slightly more abundant in the tested volatiles, was less active than the *Z* isomer. The compound 4-terpineol (peak 13) had a specific EAG activity similar to that of (*Z*)-sabinene hydrate (Table 1), but was less abundant in carrot volatiles and therefore accounted for a lower EAG response (Figure 4b).

(*Z*)-3-Hexenyl acetate (peak 10) and (*E*)-2-hexenal, two compounds of the green leaf volatiles complex, showed high EAG activity (Table 1), even at low concentrations. (*Z*)-3-Hexenyl acetate at a concentration of 0.1 ppm still evoked a response of 28 $\pm$ 14% of the response to the (*E*)-2-hexenal standard (mean $\pm$ standard deviation, $N = 3$). However, no green leaf volatiles in major concentrations could be detected in the investigated headspace over carrot foliage.

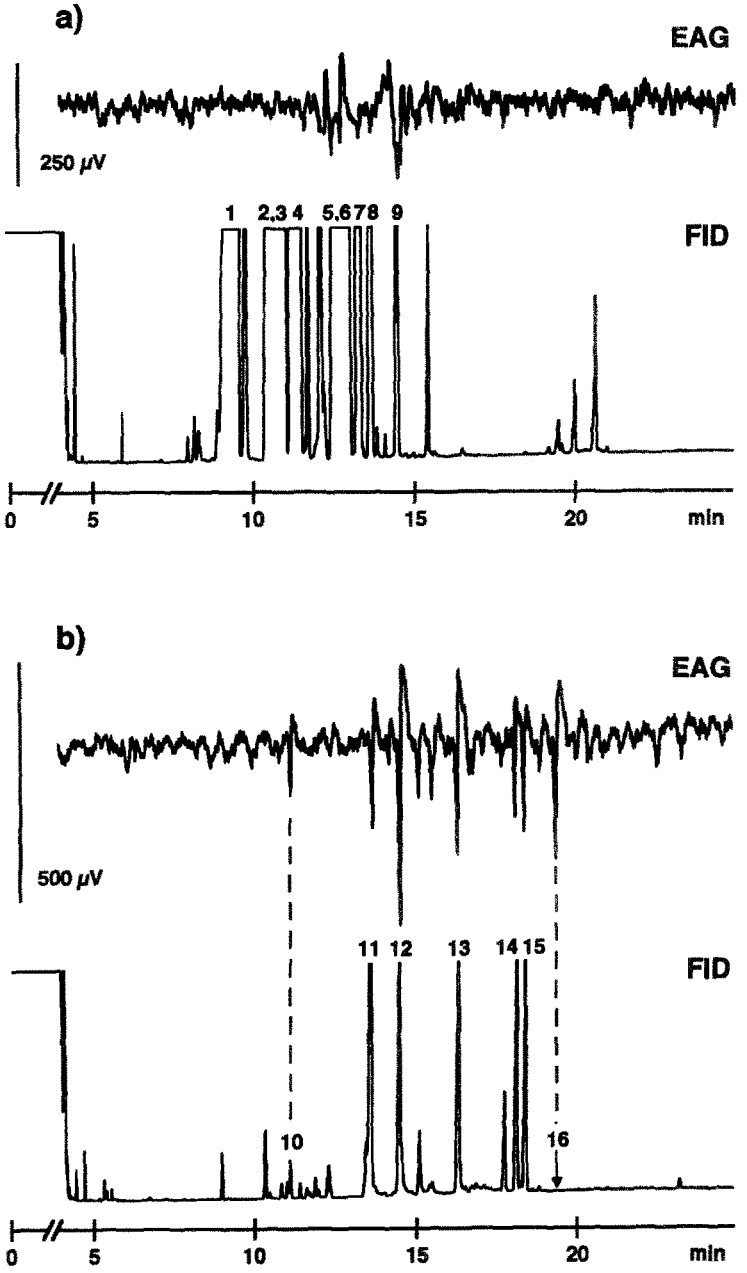


FIG. 4. Gas chromatograms of silica gel fractions of carrot volatiles (for explanation see text) and corresponding EAG responses of a *P. polyxenes* female: (a) nonpolar fraction, (b) polar fraction A. Identified peaks: 1 = α-pinene, 2 = sabinene, 3 = β-pinene, 4 = β-myrcene, 5 = limonene, 6 = (*E*)-β-ocimene, 7 = (*Z*)-β-ocimene, 8 = γ-terpinene, 9 = terpinolene, 10 = (*Z*)-3-hexenyl acetate, 11 = (*E*)-sabinene hydrate, 12 = (*Z*)-sabinene hydrate, 13 = 4-terpineol, 14 = L-bornyl acetate or isobornyl acetate, 15 = unknown monoterpenoid with polar functional group, 16 = trace of unknown compound with high electrophysiological activity. Temperature program as indicated in Figure 3.

TABLE 1. EAG RESPONSE OF *Papilio polyxenes* FEMALES TO IDENTIFIED VOLATILE COMPOUNDS FROM CARROT FOLIAGE

Compound (peak # in Figure 4)	Relative conc. in headspace[a]	EAG activity relative to standard[b]		Identification method[c]
		10 ppm	100 ppm	
a. Major compounds of headspace volatiles (all nonpolar monoterpenes)				
α-Pinene (1)	46	(−)	(−)	R, MSr
Sabinene (2)	100	(−)	14 ± 3 (3)	R, MSr
β-Pinene (3)	11	(−)	(−)	R, MSr
β-Myrcene (4)	52	20 ± 8 (3)	40 ± 18 (7)	R, MSr
Limonene (5)	31	(−)	39 ± 5 (3)	R, MSr
(*E*)-β-Ocimene (6)	9	(−)	25 ± 1 (3)	K, MSp
(*Z*)-β-Ocimene (7)	9	(−)	29 ± 1 (3)	K, MSp
γ-Terpinene (8)	4.4	(−)	25 ± 2 (3)	R, MSr
Terpinolene (9)	2.4	17 ± 3 (3)	40 ± 7 (3)	R, MSr
b. Identified polar compounds with high EAG activity				
(Z)-3-Hexenyl acetate (10)	0.03	56 ± 12 (6)	122 ± 17 (6)	R, MSr
(*E*)-Sabinene hydrate (11)	1.9	29 ± 9 (6)	103 ± 15 (6)	R, MSr
(Z)-Sabinene hydrate (12)	1.6	65 ± 18 (6)	174 ± 19 (6)	R, MSr
4-Terpineol (13)	0.51	42 ± 9 (6)	176 ± 19 (6)	R, MSr
Bornyl acetate[d] (14)	0.26	18 ± 3 (3)	67 ± 17 (12)	R, MSr
c. Standard				
(*E*)-2-Hexenal	ND	61 ± 5 (6)	100 (reference value)	

[a] Concentration in headspace relative to most concentrated compound, expressed in % (calculated from FID peak areas); ND = not detected.
[b] Standard: 100 ppm (*E*)-2-Hexenal; mean ± SD; number of females tested given in parentheses; (−) = response below detection limit (n = 3).
[c] R = comparison of GC-retention time with retention time of reference compound, MSr = comparison with mass-spectra of reference compound, MSp = comparison with published mass-spectra (McLafferty and Stauffer, 1989), K = comparison with published Kovats-indices (Davies, 1990).
[d] (L)-Bornyl acetate or isobornyl acetate (combined data).

Only (Z)-3-hexenyl acetate (major ions in mass spectrum: Table 2) was found and accounted for a minor EAG peak (peak 10, Figure 4b).

Although we have identified the compounds evoking the highest EAG responses of all compounds in the collected carrot headspace vapor, there is evidence that compounds present only in trace amounts contribute significantly to the overall profile of perceived compounds. The response to one of these compounds is seen in Figure 4b (peak 16), and another, eluting at a retention

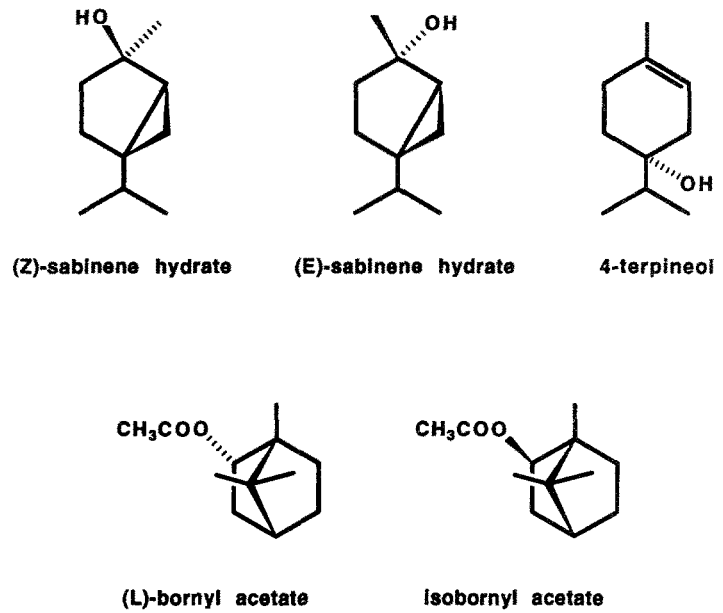


FIG. 5. Electrophysiologically active monoterpenoids identified in the headspace volatiles of carrot.

TABLE 2. MAJOR IONS IN MASS SPECTRA OF IDENTIFIED COMPOUNDS IN POLAR FRACTION OF HEADSPACE VOLATILES FROM *D. Carota* EVOKING HIGH ELECTROPHYSIOLOGICAL RESPONSES IN *P. polyxenes* FEMALES

Compound (peak # in Figure 2)	Highest *m/z*	Main *m/z* relative abundance in %							
(Z)-3-Hexenyl acetate (10)	82	43	67	82	41	39	28	27	55
	(M^+-60)	100	58	28	18	14	13	12	10
(*E*)-Sabinene hydrate (11)	154	43	71	41	27	39	93	55	81
	(M^+)	100	54	40	29	25	24	24	21
(Z)-Sabinene hydrate (12)	154	43	71	41	93	27	81	55	39
	(M^+)	100	48	39	32	29	27	26	24
4-Terpineol (13)	154	71	43	93	41	111	55	27	39
	(M^+)	100	69	46	43	41	34	30	26
L-Bornyl acetate (14)	196	43	95	41	93	121	136	55	39
	(M^+)	100	76	45	38	27	26	20	17

time between the two isomers of sabinene hydrate, was detected in polar fraction B.

The two propenylbenzenes, (*E*)-methylisoeugenol and (*E*)-asarone, described as major attractants in carrot odor for the carrot fly (Guerin et al., 1983) were tested in three preparations of *P. polyxenes* females. Neither of these compounds showed any detectable EAG activity at a concentration of 100 ppm. In addition, no significant EAG activity was detected at the retention times (determined from standards) of 20.30 min for (*E*)-methylisoeugenol or 22.45 min for (*E*)-asarone (Figure 3). Therefore these compounds do not appear to contribute to the odor profile perceived by *P. polyxenes* females.

DISCUSSION

Headspace Volatiles Represent Plant Odor. Feeny et al. (1989) found that volatile compounds in leaf surface extracts from *D. carota* increase the landing frequency and oviposition rate of *P. polyxenes* females on model plants. Given that the volatiles occur in the surface wax layer of leaves, the authors assumed that they would also be present in the air surrounding the plant. We have now shown that volatile compounds indeed emanate from undamaged host-plant leaves and that they have the same effect on the oviposition behavior of *P. polyxenes* as do the constituents extracted from the leaf surface. Comparing the chromatograms from carrot foliage odor with the results of Feeny et al. (1989), the relative lack of "sesquiterpenoid" compounds detected here shows the strong influence of the collection technique on the blend of volatiles obtained. In addition to the distillation of volatiles from leaf surface extracts, other methods have been applied in recent investigations of influences of plant volatiles on insect behavior. These include collection of headspace vapor over shredded or macerated plants on adsorbants (e.g., Hamilton-Kemp et al., 1988) or in cold traps (e.g., Guerin et al., 1983). The method used in the present investigation, cold-trapping of headspace vapors, can be considered to yield relative concentrations of volatile constituents closest to the naturally emitted blend of volatiles (Bergström et al., 1980; Dobson, 1991). Thus, the carrot headspace volatiles analyzed in our study probably represent accurately the carrot odor perceived by the butterflies in nature.

Nonpolar Odor Constituents. Judging from the number of peaks in FID chromatograms from headspace volatiles, carrot odor comprises more than 150 compounds in a wide range of concentrations. Fractionation of the complex mixture of odor constituents revealed that the compounds in the nonpolar fraction, despite their relatively high concentrations, do not contribute significantly to the behavioral activity of the total mixture. Chemical analysis of this fraction revealed that the major volatile compounds released by foliage of wild carrot

are the monoterpenes already known as constituents of odor from roots (Buttery et al., 1968; Cronin and Stanton, 1976; Simon et al., 1980) or stems (Buttery et al., 1968) of cultivated carrot. β-Ocimene, although apparently not reported previously from volatiles of carrot, has been found in essential oils of other umbellifer genera, including *Angelica*, *Apium*, and *Foeniculum* (Formacek and Kubeczka, 1982). The relative concentrations of the major monoterpenes in headspace vapor over foliage differed greatly from the values reported previously from roots and stems. However, none of these compounds elicited a major response by *P. polyxenes* in EAG recordings. Therefore, nonpolar compounds are unlikely to contribute significantly to the perceived profile of host-plant volatiles in this species. This is in accord with the findings of Feeny et al. (1989), who found low sensitivity of *P. polyxenes* to monoterpenoids that lack a polar functional group.

Polar Odor Constituents. All the behavioral activity, as well as the major electrophysiological activity, can be attributed to the polar fraction containing only compounds at comparatively low concentrations relative to the major nonpolar odor constituents. The five compounds of the extract found to elicit the highest EAG responses are all monoterpenoids with oxygenated functional groups. Like their nonpolar analogs, these compounds are not unique to the Umbelliferae, but can be found in odor from a wide range of plants. Sabinene hydrate, probably the least widely distributed of these compounds, has been reported from several families and genera, including *Cuminum* (Umbelliferae; Varo and Heinz, 1970), *Melaleuca* (Myrtaceae; Southwell and Stiff, 1989), *Elettaria* (Zingiberaceae; Masada, 1976) and *Mentha* (Labiatae; Masada, 1976; Formacek and Kubeczka, 1982). The compound 4-terpineol, reported from leaves of plants in the families Labiatae, Verbenaceae, Myrtaceae, and Lauraceae and the fruit peel of several rutaceous species, and bornyl acetate, found in essential oils from leaves of several species of Labiatae and from needles of several coniferous species, are common constituents of plant odor (see Masada, 1976; Formacek and Kubeczka, 1982). They also occur as constituents of carrot-root volatiles (Buttery et al., 1968; Simon et al., 1980).

Green Leaf Volatiles. The high EAG response to (*E*)-2-hexenal (also noted by Feeny et al., 1989) and to (Z)-3-hexenyl acetate indicates a high sensitivity of the olfactory sensilla of *P. polyxenes* to compounds of the green leaf volatiles complex. This group of volatiles is well known for being perceived by many species of herbivorous insects (Visser, 1986). However, in our experiments, they contributed only little to the overall EAG profile of *P. polyxenes* in response to carrot odor because they were not found in appreciable concentrations in headspace vapor from undamaged foliage. Since tissue damage increases the amount of green leaf volatiles released (Wallbank and Wheatley, 1976; Tollsten and Bergström, 1988; Whitman and Eller, 1990), these compounds could permit *P. polyxenes* females to detect damaged host-plant foliage before landing.

Complexity of Perceived Profile of Volatiles. Our results suggest that *P. polyxenes* females perceive a complex profile of volatile compounds from carrot foliage. We therefore expect that several compounds, perhaps with a particular ratio of concentrations, represent the cue that influences oviposition behavior in this species. In addition to the most important compounds identified, several other constituents with minor EAG activity probably contribute to the complexity of the profile used by the black swallowtail. The often widespread occurrence of volatile compounds among plant species may prevent most oligophagous herbivores from relying on single types of compounds as cues for host-plant selection. By contrast, the complete profile of volatile plant compounds, released in species-specific relative concentrations, must be unique for a given plant species in a particular habitat. It is likely, therefore, that phytophagous insects often respond to profiles of several components rather than to single compounds during host-plant selection (Visser, 1986). Several species have nevertheless been found to rely on one or a few characteristic plant compounds for host selection. Prominent examples are herbivores attacking Liliaceae, which respond to certain organic disulfides, and many crucifer-associated insects that are stimulated by isothiocyanates (references in Städler, 1992).

The profile of carrot volatiles perceived by *P. polyxenes* females differs greatly from that perceived by the carrot fly, *Psila rosae*, another herbivorous species associated with carrot. Guerin et al. (1983) found the two propenylbenzenes (*E*)-asarone and (*E*)-methylisoeugenol to elicit the strongest EAG responses of all volatile compounds from carrot foliage and to be effective attractants in field traps, particularly if combined with the green leaf volatile hexanal. However, to induce an oviposition response similar to that from a raw extract of carrot plants, further compounds (e.g., the polyacetylene falcarindiol and furanocoumarins) had to be combined with the propenylbenzenes (Städler and Buser, 1984). Although we cannot exclude the possibility that one or more of the unidentified compounds of high EAG activity for *P. polyxenes* belong to these classes, (*E*)-asarone and (*E*)-methylisoeugenol were not perceived by *P. polyxenes* females in the concentration tested. The different chemical profiles used by *P. polyxenes* and *P. rosae* (Städler, 1992) clearly indicate that herbivorous species associated with a particular plant species may not rely on the same chemical cues to find their host plant. This observation has implications for the potential application of knowledge about plant-emitted insect attractants in agriculture. Efforts to avoid attractiveness for one pest species may have different effects on other pest species of the same plant.

Correlation of EAG Responses with Oviposition Behavior. In this paper we have demonstrated for *P. polyxenes* a correlation between behavioral and electrophysiological activity of fractions of carrot volatiles. The electrophysiological approach greatly reduced the number of compounds that could be responsible for the behavioral activity. However, it would be premature to assume that all

compounds eliciting EAG responses contribute to the behavioral activity of the total headspace mixture. It is possible that some of the perceived host-plant compounds have neutral or even repellent behavioral effects, as has been shown for several insects in the case of nonvolatile (deterrent) compounds perceived upon tarsal contact (reviewed in Städler, 1984, 1992). It is also conceivable that compounds producing a sensory response in only a limited number of receptors, thus producing a weak EAG, may nevertheless have some influence on the behavior, as shown for some pheromone components (Hansson et al., 1989). These restrictions on the interpretation of our EAG results underline the importance of behavioral studies paralleling chemosensory investigations.

CONCLUSION

Our results confirm the finding of Feeny et al. (1989) that host-plant odor is one of the cues involved in the selection of landing sites for oviposition by *P. polyxenes*. The identification of compounds eliciting high EAG responses in black swallowtail females provides a basis for further experiments on the influence of individual chemical compounds on oviposition behavior. In addition, it remains to be determined which characteristics of the perceived profile of host-plant odor actually contribute to the behavioral responses, if and how compounds synergize or antagonize the activity of one another, and to what extent relative concentrations are important. Further experiments are also necessary to improve our knowledge about the influence and the importance of host-plant volatiles on oviposition behavior of the black swallowtail under field conditions.

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REFERENCES

BERGSTRÖM, G., APPELGREN, M., BORG-KARLSON, A.-K., GROTH, I., STRÖMBERG, S. and STRÖMBERG, S. 1980. Studies on natural odoriferous compounds (XXII): Techniques for the isolation/enrichment of plant volatiles in the analysis of *Ophris* orchids (Orchidaceae). *Chem. Scr.* 16:173–180.

BLAU, W.S. 1981. Life history variation in the black swallowtail butterfly. *Oecologia* 48:116–122.

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.

CLARK, L.G., and DENNEHY, T.J. 1988. Host-finding and oviposition behavior of grape berry moth. *Entomol. Exp. Appl.* 47:223–230.

CRONIN, D.A., and STANTON, P. 1976. 2-Methoxy-3-*sec*-butylpyrazine—an important contributor to carrot aroma. *Sci. Food Agric.* 27:145–151.

DAVIES, N.W. 1990. Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicone and Carbowax 20M phases. *J. Chromatogr.* 503:1–24.

DOBSON, H.E.M. 1991. Analysis of flower and pollen volatiles, pp. 231–251, *in* H.F. Linskens and J.F. Jackson (eds.). Modern Methods of Plant Analysis. New Series, Volume 12, Essential Oils and Waxes. Springer-Verlag, Berlin.

DOUWES, P. 1968. Host selection and host finding in the egg-laying female of *Cidaria albulata* L. (Lep. Geometridae). *Opusc. Entomol.* 33:233–279.

FEENY, P., ROSENBERRY, L., and CARTER, M. 1983. Chemical aspects of oviposition behavior in butterflies, pp. 27–76, *in* S. Ahmad (ed.) Herbivorous Insects. Academic Press, New York.

FEENY, P., SACHDEV, K., ROSENBERRY, L., and CARTER, M. 1988. Luteolin 7-*O*-(6″-*O*-malonyl)-β-D-glucoside and *trans*-chlorogenic acid: oviposition stimulants for the black swallowtail butterfly. *Phytochemistry* 27:3439–3448.

FEENY, P., STÄDLER, E., ÅHMAN, I., and CARTER, M. 1989. Effects of plant odor on oviposition by the black swallowtail butterfly, *Papilio polyxenes* (Lepidoptera: Papilionidae). *J. Insect Behav.* 2:803–827.

FORMACEK, V., and KUBECZKA, K.-H. 1982. Essential Oils Analysis by Capillary Gas Chromatography and Carbon-13 NMR Spectroscopy. John Wiley & Sons, New York.

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.

GUERIN, P.M., STÄDLER, E., and BUSER, H.-R. 1983. Identification of host plant attractants for the carrot fly, *Psila rosae*. *J. Chem. Ecol.* 9:843–861.

HAMILTON-KEMP, T.R., ANDERSEN, 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.

HANSSON, B.S., VAN DER PERS, J.N.C., and LÖFQVIST, 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.

LANDOLT, P.J. 1989. Attraction of the cabbage looper to host plants and host plant odour in the laboratory. *Entomol. Exp. Appl.* 53:117–124.

LEATHER, S.R. 1987. Pine monoterpenes stimulate oviposition in the pine beauty moth, *Panolis flammea*. *Entomol. Exp. Appl.* 43:295–303.

MA, W.-C., and SCHOONHOVEN, L.M. 1973. Tarsal contact chemosensory hairs of the large white butterfly, *Pieris brassicae* and their possible role in oviposition behavior. *Entomol. Exp. Appl.* 16:343–357.

MASADA, Y. 1976. Analysis of Essential Oils by Gas Chromatography and Mass Spectrometry. John Wiley & Sons, New York.

MCLAFFERTY, F.W., and STAUFFER, D.B. 1989. The Wiley/NSB Registry of Mass Spectral Data. John Wiley & Sons, New York.

NISHIDA, R., and FUKAMI, H. 1989. Oviposition stimulants of an Aristolochiaceae-feeding swallowtail butterfly, *Atrophaneura alcinous*. *J. Chem. Ecol.* 15:2565–2575.

NISHIDA, R., OHSUGI, T., KOKUBO, S. and FUKAMI, H. 1987. Oviposition stimulants of citrus-feeding swallowtail butterfly, *Papilio xuthus* L. *Experientia* 43:342–344.

PALANISWAMY, P., GILLOTT, C., and SLATER, G.P. 1986. Attraction of diamondback moths, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), by volatile compounds of canola, white mustard, and faba bean. *Can. Entomol.* 118:1279–1285.

PAPAJ, D.R. 1986. Conditioning of leaf-shape discrimination by chemical cues in the butterfly, *Battus philenor*. *Anim. Behav.* 34:1281–1288.

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.

RAMASWAMY, S.B. 1988. Host finding by moths: Sensory modalities and behaviours. *J. Insect Physiol.* 34:235–249.

RAUSHER, M.D. 1978. Search image for leaf shape in a butterfly. *Science* 200:1071–1073.

REED, D.K., MIKOLAJCZAK, K.L., and KRAUSE, C.R. 1988. Ovipositional behavior of lesser peachtree borer in presence of host-plant volatiles. *J. Chem. Ecol.* 14:237–252.

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.* 13:1771–1775.

SAXENA, K.N. and GOYAL, S. 1978. Host-plant relations of the Citrus butterfly *Papilo demoleus* L.: Orientational and ovipositional responses. *Entomol. Exp. Appl.* 24:1–10.

SIMON, P.W., LINDSAY, R.C., and PETERSON, C.E. 1980. Analysis of carrot volatiles collected on polymer traps. *J. Agric. Food Chem.* 28:549–552.

SOUTHWELL, I.A., and STIFF, I.A. 1989. Ontogenetical changes in monoterpenoids of *Melaleuca alternifolia* leaf. *Phytochemistry* 28:1047–1051.

STÄDLER, E. 1974. Host plant stimuli affecting oviposition behavior of the eastern spruce budworm. *Entomol. Exp. Appl.* 17:176–188.

STÄDLER, E. 1984. Contact chemoreception, pp. 3–35, *in* W.J. Bell and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman & Hall, London.

STÄDLER, E. 1992. Behavioral responses of insects to plant secondary compounds, pp. 45–88, *in* M.R. Berenbaum and G.A. Rosenthal (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

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

TOLLSTEN, L., and BERGSTRÖM, G. 1988. Headspace volatiles of whole plants and macerated plant parts of *Brassica* and *Sinapis*. *Phytochemistry* 27:4013–4018.

VAIDYA, V.G. 1969. Investigations on the role of visual stimuli in the egg-laying and resting behaviour of *Papilio demoleus* L. (Papilionidae, Lepidoptera). *Anim. Behav.* 17:350–355.

VARO, P.T., and HEINZ, D.E. 1970. Volatile components of cumin seed oil. *J. Agric. Food Chem.* 18:234–238.

VISSER, J.H. 1986. Host odour perception in phytophagous insects. *Annu. Rev. Entomol.* 31:121–144.

WALLBANK, B.E. and WHEATLEY, G.A. 1976. Volatile constituents from cauliflower and other crucifers. *Phytochemistry* 15:763–766.

WHITMAN, D.W., and ELLER, F.J. 1990. Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1:69–75.

WIKLUND, C. 1984. Egg-laying patterns in butterflies in relation to their phenology and visual apparency and abundance of their host plants. *Oecologia* 63:23–29.

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INFLUENCE OF LOW-INTENSITY ULTRAVIOLET RADIATION ON EXTRUSION OF FURANOCOUMARINS TO THE LEAF SURFACE

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Abstract—Exposure of *Ruta graveolens* leaves to low intensity 366-nm radiation led to a ca. 20% increase in concentrations of the furanocoumarins psoralen, xanthotoxin and bergapten, as compared to leaves kept in darkness. Both direct and, even more, scattered UV radiation produced increases in total concentrations. Changes in the concentrations of individual coumarins were generally parallel. Extrusion to the surface was increased, especially in lower, older leaves exposed to the scattered radiation, where it exceeded the control by factors of eight or nine. It is suggested that this response could enhance shielding of leaves against penetration of UV into the cells and that irradiation, by exciting the furanocoumarins, could augment protection against potential microbial invaders.

Key Words—Furanocoumarins, psoralens, *Ruta graveolens*, ultraviolet radiation.

INTRODUCTION

It has been theorized that the current thinning ozone layer will favor survival of only those organisms able to adapt readily to it, reducing the diversity of species on the planet (Biggs et al., 1981; Caldwell et al., 1989; Bornman, 1991). In this context interest has risen in the effects, both detrimental and beneficial, of augmented UV irradiation on plants (Tevini and Teramura, 1989).

UV irradiation is one of several environmental influences that increase the synthesis of linear furanocoumarins (psoralens, Figure 1) in plants that elaborate

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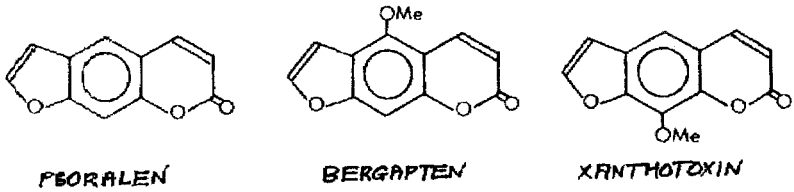

FIG. 1. Structures of psoralen, bergapten, and xanthotoxin.

them (Beier and Oertli, 1983; Beier et al., 1983; Zangerl and Berenbaum, 1987; Douglas et al., 1987). Other aromatic compounds are also known to increase in concentration under UV irradiation (Hadwiger and Schwochau, 1971; Bridge and Klarman, 1972; Pate, 1983; Mohle et al., 1985; Barnby et al., 1989), and enzymes involved in pathways to aromatic compounds (Tietjen and Matern, 1983), as well as the genes coding for these enzymes (Chappell and Hahlbrock, 1984; Lois et al., 1989; Schulze-Lefert et al., 1989; Staiger et al., 1989), were activated by UV.

Acid and salt sprays (Zobel et al., 1991) and extremes of temperature (Zobel and Brown, 1990) also caused dramatic increases of furanocoumarin concentrations in *Ruta graveolens*, both inside the leaves and on the surface. Increases in total concentration of furanocoumarins were observed as the plant's response to air pollution (Dercks et al., 1990) and to mechanical damage during storage (Chaudhary et al., 1985). Ionizing radiation can also trigger the production of phenolic compounds, since phenylpropanoid metabolism in *Citrus* flavedo was enhanced after gamma irradiation (Dubery, 1992). All these findings suggest that increased concentrations of aromatic compounds, and, as we have proposed, especially their extrusion to the surface, may be common reactions of plants to extreme environmental conditions and even to lesser variations (Zobel, 1991).

The response of green plants to different wavelengths of light is very complex (Klein, 1978; Jagger, 1985; Barnes et al., 1987; Bornman, 1989), owing to the buffering reaction of white light (Tyrrell and Peak, 1978; Caldwell and Warner, 1982; Mirecki and Teramura, 1984). Different wavelengths of light can react synergistically or antagonistically (Tyrrell, 1978; Hermsmeier et al., 1991), making the influence of a specific wavelength very difficult to assign. This has led to a greater emphasis recently on studies with monochromatic UV (Adamse et al., 1988; Bogenrieder, 1982) or even with a laser beam, which can precisely target irradiation to a small area (Weber et al., 1991).

If heightened UV irradiation leads to an increased synthesis of furanocoumarins, the question arises as to how the plant copes with suddenly increased concentrations of these hazardous compounds and also whether any of the increased amount is exported to the surface. If so, it would thicken the epicu-

ticular layer, and these compounds would further decrease the small amount of UV normally penetrating into the underlying mesophyll cell layer (Martin et al., 1991), both by dispersion and as strong UV absorbers. Furthermore, being photoactive, these coumarins could then be activated (Potapenko, 1991) and eventually degraded by UV (Trumble et al., 1991).

The aim of the present work was to evaluate the effect of UV radiation on the furanocoumarin concentrations of *R. graveolens* leaves, with a distinction between the surface of the plant and the interior of the leaf.

METHODS AND MATERIALS

Potted plants of *Ruta graveolens*, subshrubs 30 cm high containing ca. 30 branches each with 15–20 leaves, were grown in a greenhouse under conditions previously described (Zobel and Brown, 1989) from November 1, 1990 until January 26, 1991. In this study the upper and lower leaves were investigated separately on the basis of our earlier demonstration that the concentrations of furanocoumarins in the upper and lower leaves differ, as do the responses of these two kinds of leaf to changing conditions (Zobel and Brown, 1990; Zobel, 1991).

On the latter date experiment I was commenced. Two plants were designated for four days of growth under 366 nm irradiation in the absence of visible light, and two for growth in darkness over the same period. The furanocoumarin concentrations in two plants left in the greenhouse did not change after four days. Before the treatment, as a control, leaves from each of the first two plants (Tables 1 and 2, control 1) were collected (cf. Figure 2), with the upper leaves (third from the top) being distinguished from the lower (third from the axillary shoot base). Duplicate samples were collected from each of the upper and lower positions. In each case five leaves taken from each of the two plants were combined to form one sample. Also as a control, analogous samples were collected from the two plants that were to be kept in darkness (Tables 1 and 2, control 2).

Figure 2 shows diagrammatically the arrangement of plant and illumination source for the irradiation experiments. The source was a 366-nm, 18-W model UVL-21 Blak-Ray lamp (Ultra-Violet Products, Inc., San Gabriel, California 91778) with a 45- × 70-mm glass filter. Available dosimetry equipment was not sensitive enough for accurate measurement of the radiation intensity at the distances employed with this lamp (10–15 cm), but extrapolation from measurements at ca. 2 cm indicated values on the order of 0.01 $W/m^2/sec$ at these distances directly beneath the lamp. Measurements made with a 254-nm lamp of the same type on a plane 10 or 15 cm below the filter showed that the intensities diminished by a factor approximating 2–5 at a lateral distance of 10

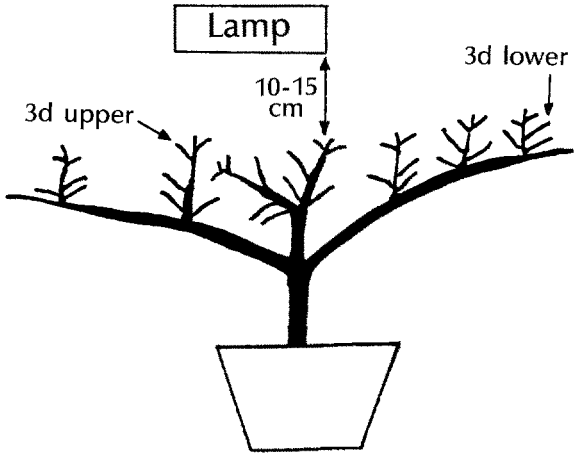

FIG. 2. Arrangement of plant and illumination source for the irradiation experiments. Positions of the collected leaves are denoted by arrows.

cm from the center of the filter, depending on its geometry, and were 1–2 orders of magnitude lower at 20 cm.

Two plants were kept under this lamp for four days, and samples were then collected from the area of direct radiation, defined as ≤ 5 cm laterally from the center of the filter, and, separately, from surrounding shoots receiving indirect, scattered irradiation, defined as that falling ≥ 15 cm laterally from the center of the filter (Tables 1 and 2). In each case duplicate samples were collected from upper and lower leaves, both of them mature, current year's growth as shown in Figure 2. After collection, the plants were returned to the greenhouse.

Experiment II was commenced on February 28, on the same plants, and samples analogous to those described above were collected (experiment II).

All samples, containing 10 leaves each, were weighed and dipped in hot (96°C) water by the procedure described previously (Zobel and Brown, 1988) for removing, together with melted waxes, any other compounds deposited on the surface. After dipping, the leaves were extracted for 2 hr in boiling 20% ethanol preparatory to evaluating the concentrations of psoralens in the interior of the leaves (Tables 1 and 2, interior). The total concentration of furanocoumarins was the sum of the surface and interior concentrations. Sample purification was accomplished by a procedure involving high-performance liquid chromatography as described earlier (Thompson and Brown, 1984; Zobel and Brown, 1989). The error in this experiment did not exceed $\pm 10\%$.

RESULTS

We used continuous darkness or continuous irradiation and collected samples immediately after treatment, in order to preclude the possible repair or recovery in light, or in darkness, known to occur over a period of time after

TABLE 1. EFFECT OF UV IRRADIATION ON FURANOCOUMARIN CONCENTRATIONS[a] IN *Ruta graveolens* LEAVES

	Control 1	Direct UV	%[b]	Indirect UV	%[b]	Control 2	Darkness	%[b]
Experiment I								
Upper leaves								
Surface	395 ± 10	660 ± 50	170	620 ± 50	160	380 ± 20	345 ± 20	91
Interior[c]	150 ± 20	78 ± 10	52	260 ± 10	170	110 ± 5	65 ± 10	59
Total (surface + interior)	545	740	135	880	160	490	410	84
Lower leaves								
Surface	180 ± 20	400 ± 20	220	1650 ± 200	910	300 ± 15	210 ± 25	70
Interior	56 ± 8	140 ± 20	250	205 ± 10	370	35 ± 5	30 ± 6	86
Total (surface + interior)	240	540	225	1860	775	335	240	72
Experiment II								
Upper Leaves								
Surface	1200 ± 150	2200 ± 200	180	1800 ± 100	150	760 ± 40	690 ± 70	91
Interior	400 ± 40	250 ± 10	63	800 ± 60	200	220 ± 15	130 ± 15	59
Total (surface + interior)	1600	2450	150	2600	160	980	820	84
Lower leaves								
Surface	500 ± 40	1200 ± 60	240	4000 ± 300	800	910 ± 40	620 ± 50	68
Interior	180 ± 12	400 ± 20	220	800 ± 70	440	99 ± 10	90 ± 10	91
Total (Surface + interior)	680	1600	235	4800	705	1010	710	70

[a] μg/g fresh weight; mean value ± standard deviation
[b] % of control.
[c] Furanocoumarins extracted from interior of leaves after removal of surface coumarins

TABLE 2. EFFECT OF UV IRRADIATION ON PSORALEN (P), XANTHOTOXIN (X), AND BERGAPTEN (B) CONCENTRATIONS[a] IN *Ruta graveolens* LEAVES

		Control 1	Direct UV	Indirect UV	Control 2	Darkness
Experiment I						
Upper leaves						
Surface	P	120 ± 5	250 ± 30	270 ± 20	130 ± 10	120 ± 10
	X	205 ± 3	335 ± 10	300 + 15	187 ± 5	172 ± 8
	B	70 ± 2	78 ± 10	54 ± 15	63 ± 5	52 ± 2
	ΣP + X + B	395 ± 10	660 ± 50	620 ± 50	380 + 20	345 ± 20
Interior[b]	P	41 ± 4	18 ± 2	88 ± 4	42 ± 2	19.5 ± 1
	X	88 ± 15	51 ± 6	130 ± 4	43 ± 2	35 ± 8
	B	21 + 1	9.1 ± 2	38 ± 2	28 ± 1	10 ± 1
	ΣP + X + B	150 ± 20	78 ± 10	260 ± 10	110 ± 5	65 ± 10
Total (surface + interior)	P	161	268	360	170	140
	X	293	386	430	220	210
	B	91	81	92	91	62
	ΣP + X + B	545	740	880	490	410
% on surface	P	75	93	75	76	86
	X	70	87	70	81	83
	B	77	96	59	69	89
	ΣP + X + B	72	89	71	78	84
Lower leaves						
Surface	P	36 ± 6	130 ± 5	530 ± 70	90 ± 8	65 ± 10
	X	120 ± 10	215 ± 5	850 ± 100	165 ± 5	98 ± 10
	B	24 ± 4	60 ± 10	270 ± 30	47 ± 2	44 ± 5
	ΣP + X + B	180 ± 20	400 ± 20	1650 ± 200	300 ± 15	210 ± 25

Interior	P	13 ± 3	44 ± 10	53 ± 3	9.5 ± 1	7 ± 1
	X	35 ± 5	77 ± 6	120 ± 5	19 ± 3	17 ± 4
	B	8.1 ± 2	20 ± 4	32 ± 2	5.5 ± 1	6 ± 1
	ΣP + X + B	56 ± 8	140 ± 20	205 ± 10	35 ± 5	30 ± 6
Total (surface + interior)	P	46	174	580	100	72
	X	155	282	970	185	115
	B	32	80	300	52.5	50
	ΣP + X + B	240	540	1900	335	240
% on surface	P	73	75	91	90	90
	X	77	76	88	89	85
	B	75	75	90	89.5	88
	ΣP + X + B	76	74	89	89.5	87.5
Experiment II						
Upper leaves						
Surface	P	300 ± 25	810 ± 30	785 ± 40	215 ± 10	200 ± 30
	X	690 ± 110	1100 ± 160	830 ± 55	430 ± 20	385 ± 30
	B	210 ± 15	270 ± 10	185 ± 5	110 ± 10	105 ± 10
	ΣP + X + B	1200 ± 150	2200 + 200	1800 ± 100	760 ± 40	690 ± 70
Interior	P	96 ± 4	57 ± 2	320 ± 30	57 ± 3	39 ± 10
	X	220 ± 32	165 ± 7	380 ± 10	130 ± 10	70 ± 3
	B	84 ± 4	30 ± 1	100 ± 30	30 ± 2	21 ± 2
	ΣP + X + B	400 ± 40	250 ± 10	800 ± 60	220 ± 15	130 ± 15
Total (surface + interior	P	400	870	1170	270	240
	X	910	1260	1200	560	455
	B	300	300	235	140	130
	ΣP + X + B	1600	2450	2600	980	820

TABLE 2. Continued

		Control 1	Direct UV	Indirect UV	Control 2	Darkness
% on surface	P	76	93	67	80	83
	X	76	89	69	77	85
	B	80	90	79	79	81
	ΣP + X + B	75	90	69	77.5	84
Lower leaves						
Surface	P	100 ± 10	285 ± 50	1150 ± 100	250 ± 5	200 ± 25
	X	330 ± 35	815 ± 5	2200 ± 140	525 ± 30	300 ± 20
	B	72 ± 5	100 ± 5	670 + 10	135 ± 5	120 ± 5
	ΣP + X + B	500 ± 40	1200 ± 60	4000 ± 300	910 ± 40	620 ± 50
Interior	P	32 ± 2	135 ± 5	350 ± 20	25 ± 3	20 ± 3
	X	80 ± 2	210 ± 10	230 ± 30	60 ± 5	58 ± 3
	B	68 ± 8	55 ± 5	220 ± 20	14 ± 2	12 ± 4
	ΣP + X + B	180 ± 12	400 ± 20	800 ± 70	99 ± 10	90 ± 10
Total (surface + interior)	P	132	395	1500	280	220
	X	410	1000	245	590	360
	B	140	180	890	150	130
	ΣP + X + B	680	1600	4800	1000	710
% on surface	P	76	72	77	89	91
	X	80	81	90	89	83
	B	51	65	75	90	92
	ΣP + X + B	73.5	75	83	90	87

[a] μg/g fresh weight; mean value ± standard deviation.
[b] Furanocoumarins extracted from interior of leaves after removal of surface coumarins.

irradiation (Jagger, 1981, 1985; McLennan, 1987). To observe cytogenetic effects, Bondar and Popova (1989) used continuous irradiation over *Vicia* plants. In this way we tried to investigate the direct effect of 366-nm irradiation.

Table 1 summarizes the results of the two experiments. The following points are emphasized:

1. The total concentrations of three furanocoumarins in the control plants for UV irradiation (control 1) and darkness (control 2) were similar for the upper leaves, but showed larger differences in the lower leaves owing to higher concentrations on the surface of the lower leaves.

2. The influence of UV was, in general, opposite to that observed in darkness. In all cases concentrations of furanocoumarins were lower in darkness, ranging from 59 to 91% of the control values. Except in the interior of the upper leaves of experiment I, where decreases were observed, UV irradiation led to increases of 150–910% of the control values.

3. In all cases the concentrations of furanocoumarins in experiment II were greater (at least double) than those in experiment I. The changes effected by darkness, and by direct and scattered UV, expressed as a percentage of the respective controls, were in almost all cases highly reproducible between the two experiments.

4. Without exception, the effect of scattered UV was to increase furanocoumarin levels, both in the interior and on the surface of upper and lower leaves.

5. Although there was a similar trend, the effect of direct UV irradiation was less consistent. Higher concentrations of furanocoumarins were located on the surface of all leaves, as well as in the interior of the lower leaves; however, in the interior of the upper leaves the concentrations were lower compared to those of the control.

6. On the upper leaf surface, the increases due to both direct and scattered UV were of similar magnitude. On the lower leaf surface, direct UV caused much less increase over the control than the scattered radiation.

7. Changes in concentrations of the individual coumarins, psoralen, xanthotoxin and bergapten (Table 2), were generally parallel.

DISCUSSION

Darkness in each experiment, in both younger and older leaves of *R. graveolens*, led to a decrease in the total furanocoumarin concentration, by 16% in the upper leaves and by 28–30% in the lower, due both to lowered extrusion, at least in the lower leaves, and decreased concentration inside the leaves. No information is available on the extent to which biosynthesis of furanocoumarins can proceed in darkness, but it would not be expected to continue for any

prolonged period in view of the diminution in the energy supply in the absence of visible light. The lower concentrations prevailing in darkness rendered the pronounced stimulatory effect of UV more striking.

UV increased both total concentration and, more importantly, extrusion, but under direct irradiation the concentration inside the upper, younger leaves was lowered, emphasizing the large percentage extrusion values. With respect to total production of furanocoumarins, the smaller percentage increases over the control in these leaves suggest that overall formation was lowered by the treatment, in comparison to that of the lower, older leaves. The thickness of the outside cell wall of the epidermis was found to influence the amount of light penetrating it and reaching the mesophyll (Robberecht and Caldwell, 1978; Thoma and Tevini, 1982; Lee et al., 1990; Bornman and Vogelmann, 1991).

An unexpected finding of the present study was the markedly greater effect of the indirect, and therefore less intense, radiation in stimulating the production of furanocoumarins, as shown by the higher totals of these compounds isolated in almost all cases. The fact that concentrations in the most exposed area, the upper leaf surfaces, were, if anything, higher under direct irradiation than indirect suggests that photodegradation is not an important factor and that the explanation is more deep-seated. Although we cannot readily account for the phenomenon on the basis of present data, there may well be an optimum level of radiation, which was exceeded, at least under the direct radiation, in the present experiments. It is also possible that some chemical signal from the most highly irradiated areas led to greater synthesis in more remote leaves and to the even more accelerated extrusion to the surface so prominent in the indirectly irradiated lower leaves.

Increased concentrations of furanocoumarins have been observed in other plants after UV irradiation (Beier et al., 1983), but, as now indicated by our present findings in *R. graveolens*, caution must be exercised in the interpretation of this response in view of the existence of two compartments where these compounds accumulate, outside the plant and inside the leaves. The stage of leaf development clearly must also be taken into account. Differences in response to UV irradiation dependent on the developmental stage of the plant have been observed in soybeans (Mirecki and Teramura, 1984; Teramura and Sullivan, 1987). Increased extrusion seems to be the immediate response of *R. graveolens* to UV irradiation, followed by a parallel increase in production.

Extrusion of furanocoumarins to the surface as a primary response to stress (in this case UV irradiation) seems explicable on the basis that accumulation of active furanocoumarins in an exterior compartment of the plant (Städler and Buser, 1984; Zobel and Brown, 1989) constitutes part of its defense system (Harborne, 1985, 1988; Trumble et al., 1990; Zangerl and Berenbaum, 1990). Increased concentrations of furanocoumarins due to UV will modify enzymes in the guts of insects that detoxify these compounds (Ivie et al., 1983; Bull et

al., 1986; Cohen et al., 1989; Lee and Berenbaum, 1989, 1990; Berenbaum, 1991). Thus, by altering the relationship between plant and animal (Berenbaum et al., 1986, 1989, 1991), phenolic compounds will have an ecological impact (Klocke and Kubo, 1991).

We suggest three possible modes of action of extruded furanocoumarins to the advantage of the plant: First, they could protect cells producing these compounds in high concentrations, because the alternative of storage of phenolic compounds in the vacuoles could pose a grave risk of killing the cells by precipitation of the cytoplasmic proteins, as noted in pine needles exposed to acid or salt (Zobel and Nighswander, 1990, 1991). A second function of irradiation is that it could excite surface furanocoumarins (Potapenko, 1991), thus enhancing their antimicrobial action (Trumble et al., 1991). A third could be to improve the shielding against penetration of UV irradiation into the epidermal cells. The thickened shield of externally deposited furanocoumarins, by providing increased protection for the underlying cells, may preserve their normal physiology.

As an additional contribution to survival, we suggest that 366-nm radiation could be transduced by *Ruta* furanocoumarins into usable wavelengths of light by this plant, because, after UV irradiation, furanocoumarins emit blue, green, and orange light (Zobel and March, 1993). The utilization of wavelengths below 400 nm by higher plants is not a well-explored area, but they may be beneficial (Murali and Teramura, 1986; Sullivan and Teramura, 1988; Tevini and Teramura, 1989), as pine needles assimilate under 366-nm radiation (Jagger, 1985). In the algae, *Alternaria alternata* and *Fusarium oxysporum*, biomass can increase under UV irradiation (Osman and Metwally, 1991).

Two further points require study: (1) whether UV irradiation exerts any direct influence on the biosynthetic pathway, and (2) the manner in which the intensity of the irradiation separately influences extrusion and biosynthesis. It is hoped to address these questions in future investigations.

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REFERENCES

ADAMSE, P., KENDRICK, R.E., and KOORNNEEF, M. 1988. Photomorphogenetic mutants of higher plants. *Photochem. Photobiol.* 48:833–841.

BARNBY, M.A., YAMASAKI, R.B., and KLOCKE, J.S. 1989. Biological activity of azarachtin, three derivatives, and their ultraviolet radiation degradation products against tobacco budworm (Lepidoptera: Noctuidae) larvae. *J. Econ. Entomol.* 1982:58–63.

BARNES, P.W., FLINT, S.D., and CALDWELL, M.M. 1987. Photosynthesis damage and protective pigments in plants from latitudical arctic/alpine gradient exposed to supplemental UV-B radiation in the field. *Arct. Alp. Res.* 19:21–27.

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

BEIER, R.C., IVIE, G.W., and OERTLI, E.H. 1983. Psoralens as phytoalexins in food plants of the family Umbelliferae. Significance in relation to storage and processing, pp. 295–310, *in* J.W. Finley and D.E. Schwass (eds.). Xenobiotics in Foods and Feeds. American Chemical Society Symposium Series No. 234. American Chemical Society, Washington, D.C.

BERENBAUM, M.R. 1991. Comparative processing of allelochemicals in Papilionidae (Lepidoptera). *Arch. Insect Biochem. Physiol.* 17:213–221.

BERENBAUM, M., ZANGERL, A.R., and NITAO, J.K. 1986. Constraints on chemical coevolution: The parsnip webworm and the wild parsnip. *Evolution* 40:1215–1228.

BERENBAUM, M., ZANGERL, A.R., and LEE, K. 1989. Chemical barriers to adaptation by a specialist herbivore. *Oecologia* 80:501–506.

BERENBAUM, M.R., NITAO, J.K., and ZANGERL, A.R. 1991. Adaptive significance of furanocoumarin diversity in *Pastinaca sativa*. *J. Chem. Ecol.* 17:207–216.

BIGGS, R.H., KOSSUTH, S.V., and TERAMURA, A.H. 1981. Response of 19 cultivars of soybeans to ultraviolet-B irradiation. *Physiol. Plant.* 53:19–26.

BOGENRIEDER, A. 1982. Action spectra for the depression of photosynthesis by UV irradiation in *Lactuca sativa* L. and *Rumex alpinus* L., pp. 132–139, *in* H. Bauer, M.M. Caldwell, M. Tevini, and R.C. Worrest (eds.). Biological Effects of UV-B Radiation. Proceedings of the workshop held in Munich–Neuherberg. Gesellschaft für Strahlen- und Umweltforschung mbH, Munich.

BONDAR, L.M., and POPOVA, O.N. 1989. Cytogenetic analysis of the effect of chronic irradiation on natural *Vicia cracca* L. populations. *Radiobiologiya* 29:310–314.

BORNMAN, J.F. 1989. Target sites of UV-B radiation in photosynthesis of higher plants. *J. Photochem. Photobiol., B: Biol.* 4:145–158.

BORNMAN, J.F. 1991. UV radiation as an environmental stress in plants. *J. Photochem. Photobiol. B: Biol.* 8:337–342.

BORNMAN, J.F., and VOGELMANN, T.C. 1991. Effect of UV-B radiation on leaf optical properties measured with fiber optics. *J. Exp. Bot.* 42:547–554.

BRIDGE, M.A., and KLARMAN, W.L. 1972. Soybean phytoalexin, hydroxyphaseolin, induced by ultraviolet irradiation. *Phytopathology* 63:606–609.

BULL, D.L., IVIE, G.W., BEIER, R.C., PRYER, N.W., and OERTLI, E.H. 1986. In vitro metabolism of linear furanocoumarin (8-methoxycoumarin, xanthotoxin) by mixed-function oxidases of larvae of black swallowtail butterfly and fall armyworm. *J. Chem. Ecol.* 12:885–892.

CALDWELL, M.M., and WARNER, C.W. 1982. UV-B sensitivity of plant photosynthesis as influenced by visible irradiation, pp. 117–120, *in* H. Bauer, M.M. Caldwell, M. Tevini, and R.C. Worrest (eds.). Biological Effects of UV-B Radiation. Proceedings of the workshop held in Munich–Neuherberg. Gesellschaft für Strahlen- und Umweltforschung mbH, Munich.

CALDWELL, M.M., TERAMURA, A.H., and TEVINI, M. 1989. The changing solar ultraviolet climate and the ecological consequences for higher plants. *Trends Ecol. Evol.* 4:363–366.

CHAPPELL, J., and HAHLBROCK, K. 1984. Transcription of plant defense genes in response to UV light or fungal elicitor. *Nature* 311:76–78.

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.

COHEN, M.B., BERENBAUM, M.R., and SCHULER, M.A. 1989. Induction of cytochrome P-450-mediated detoxification of xanthotoxin in the black swallowtail. *J. Chem. Ecol.* 15:2347–2356.

DERCKS, W., TRUMBLE, J., and WINTER, C. 1990. Impact of atmospheric pollution on linear furanocoumarin content in celery. *J. Chem. Ecol.* 16:443–454.

DOUGLAS, C., HOFFMANN, H., SCHULZ, W., and HAHLBROCK, K. 1987. Structure and elicitor- or UV-light-stimulated expression of two 4-coumarate: CoA ligase genes in parsley. *EMBO J.* 6:1189–1195.

DUBERY, I.A. 1992. Elicitation of enhanced phenylpropanoid metabolism in *Citrus* flavedo by gamma radiation. *Phytochemistry* 31:2659–2662.

HADWIGER, L.A., and SCHWOCHAU, M.E. 1971. Ultraviolet light-induced formation of pisatin and phenylalanine ammonia lyase. *Plant Physiol.* 47:588–590.

HARBORNE, J.B. 1985. Phenolics and plant defense. *Ann. Proc. Phytochem. Soc. Eur.* 25:393–408.

HARBORNE, J.B. 1988. Introduction to Ecological Biochemistry, 3rd ed. Academic Press, London.

HERMSMEIER, D., MALA, E., SCHULZ, R., THIELMANN, J., GALLAUD, P., and SENGER, H. 1991. Antagonistic blue- and red-light regulation of cab-gene expression during photosynthetic adaptation in *Scenedesmus obliquus*. *J. Photochem. Photobiol. B: Biol.* 11:189–202.

IVIE, G.W., BULL, D.L., BEIER, R.C., PRYER, N.W., and OERTLI, E.H. 1983. Metabolic detoxification: Mechanism of insect resistance to plant psoralens. *Science* 221:374–376.

JAGGER, J. 1981. Near-UV radiation effects on microorganisms. *Photochem. Photobiol.* 34:761–768.

JAGGER, J. 1985. Solar-UV Actions on Living Cells. Praeger, New York.

KLEIN, R.M. 1978. Plant and near-ultraviolet radiation. *Bot. Rev.* 44:1–127.

KLOCKE, J.A., and KUBO, I. 1991. Defense of plants through regulation of insect feeding behavior. *Fla. Entomol.* 74:18–23.

LEE, K., and BERENBAUM, M.R. 1989. Action of antioxidant enzymes and cytochrome P-450 monooxygenases in the cabbage looper in resistance to plant phytotoxins. *Arch. Insect Biochem. Physiol.* 10:151–162.

LEE, K., and BERENBAUM, M.R. 1990. Defense of parsnip webworm against phototoxic furanocoumarins: Role of antioxidant enzymes. *J. Chem. Ecol.* 16:2451–2460.

LEE, W.G., HODGKINSON, I.J., and JOHNSON, P.N. 1990. A test for ultraviolet reflectance from fleshy fruits of New Zealand plant species. *N.Z. J. Bot.* 28:21–24.

LOIS, R., DIETRICH, A., HAHLBROCK, K., and SCHULZ, W. 1989. A phenylalanine ammonia-lyase gene from parsley: Structure, regulation and identification of elicitor and light responsive cis-acting elements. *EMBO J.* 8:1641–1648.

MARTIN, G., MYERS, D.M., and VOGELMANN, T.C. 1991. Characterization of plant epidermal lens effects by a surface replica technique. *J. Exp. Bot.* 42:581–588.

MCLENNAN, A.G. 1987. The repair of ultraviolet light-induced DNA damage in plant cells. *Mutat. Res.* 181:1–7.

MIRECKI, R.M., and TERAMURA, A.H. 1984. Effects of ultraviolet-B irradiation on soybean. V. The dependence of plant sensitivity on the photosynthetic photon flux density during and after leaf expansion. *Plant Physiol.* 74:475–480.

MOHLE, B., HELLER, W., and WELLMAN, E. 1985. UV-induced biosynthesis of quercetin 3-O-β-D-glucuronide in dill cell cultures. *Phytochemistry* 24:465–467.

MURALI, N.S., and TERAMURA, A.H. 1986. Effectiveness of UV-B radiation on the growth and physiology of field-grown soybean (*Glycine max*) modified by water stress. *Photochem. Photobiol.* 44:215–219.

OSMAN, M., and METWALLY, M. 1991. Influence of UV-A irradiation on growth and lipid composition in *Alternaria alternata* and *Fusarium oxysporum*. *Microbios* 68:147–155.

PATE, D.W. 1983. Possible role of ultraviolet radiation of Cannabis chemotypes. *Econ. Bot.* 37:396–405.

POTAPENKO, A.Y. 1991. Mechanisms of phytodynamic effects of furocoumarins. *J. Photochem. Photobiol., B: Biol.*, 9:1–33.

ROBBERECHT, R., and CALDWELL, M.M. 1978. Leaf epidermal transmittance of ultraviolet radiation and its implications for plant sensitivity to ultraviolet induced injury. *Oecologia* 32:277–287.

SCHULZE-LEFERT, P., DANGL, J.L., BECKER-ANDRE, M., HAHLBROCK, K., and SCHULZ, W. 1989. Inducible in vivo DNA footprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. *EMBO J.* 8:651–656.

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.

STAIGER, D., KAUTEN, H., and SHELL, J. 1989. A CACGTG motif of the *Antirrhinum majus* chalcone synthase promoter is recognized by an evolutionarily conserved nuclear protein. *Proc. Natl. Acad. Sci. U.S.A.* 86:6930–6934.

SULLIVAN, J.H., and TERAMURA, A.H. 1988. Effects of ultraviolet-B irradiation on seedling growth in Pinaceae. *Am. J. Bot.* 75:225–230.

TERAMURA, A.M., and SULLIVAN, J.H. 1987. Soybean growth responses to enhanced levels of ultraviolet-B radiation under greenhouse conditions. *Am. J. Bot.* 74:975–979.

TEVINI, M., and TERAMURA, A.M. 1989. UV-B effects on terrestrial plants. *Phytochem. Photobiol.* 50:479–487.

THOMA, V., and TEVINI, M. 1982. Effect of enhanced UV-B irradiation on leaf surface and anatomy, pp. 83–92, *in* H. Bauer, M.M. Caldwell, M. Tevini, and R.C. Worrest (eds.). Biological Effects of UV-B Radiation. Proceedings of the workshop held in Munich–Neuherberg. Gesellschaft für Strahlen- und Umweltforschung mbH, Munich.

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

TIETJEN, K.G., and MATERN, U. 1983. Different response of cultured parsley cells to elicitors from two non-pathogenic strains of fungi. 2. Effects on enzyme activities. *Eur. J. Biochem.* 131:409–413.

TRUMBLE, J.T., DERCKS, W., QUIROS, C.F., and BEIER, R.C. 1990. Host plant resistance and linear furanocoumarin content of *Apium accessious*. *J. Econ. Entomol.* 83:519–525.

TRUMBLE, J.T., MOAR, W.J., BREWER, M.J., and CARSON, W.G. 1991. Impact of UV radiation on activity of linear furanocoumarins in the *Bacillus thuringiensis* var. Kurstaki against *Spodoptera exigna*: Implications for tritropic interactions. *J. Chem. Ecol.* 17:973–988.

TYRRELL, R.M. 1978. Radiation synergism and antagonism. *Phytochem. Phytobiol. Rev.* 3:35–113.

TYRRELL, R.M., and PEAK, M.J. 1978. Interactions between UV radiation of different energies in the interaction of bacteria. *J. Bacteriol.* 136:437–440.

WEBER, G., MONAJEMBASKI, F., GREULICH, K.O., and WOLFRUM, J. 1991. Genetic changes induced in higher plants by a UV laser microbeam. *Isr. J. Bot.* 40:115–122.

ZANGERL, A.R., and BERENBAUM, M.R. 1987. Furanocoumarins in wild parsnip: Effect 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.

ZOBEL, A.M. 1991. Effect of the change from field to greenhouse environment on the linear furanocoumarin levels of *Ruta chalepensis*. *J. Chem. Ecol.* 17:21–27.

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. 1990. Seasonal changes of furanocoumarin concentrations in leaves of *Heracleum lanatum*. *J. Chem. Ecol.* 16:1623–1634.

ZOBEL, A.M., and MARCH, R.E. 1993. Autofluorescence reveals different histological localizations of furanocoumarins in fruits of some Umbelliferae and Leguminosae. *Ann. Bot.* 70:In press.

ZOBEL, A.M., and NIGHSWANDER, J.E. 1990. Accumulation of phenolic compounds in the necrotic areas of Austrian and red pine needles due to salt spray. *Ann. Bot.* 66:629–640.

ZOBEL, A.M., and NIGHSWANDER, J.E. 1991. Accumulation of phenolic compounds in the necrotic areas of Austrian and red pine needles due to sulphuric acid spray as a bioindicator of air pollution. *New Phytol.* 117:565–576.

ZOBEL, A.M., BROWN, S.A., and NIGHSWANDER, J.E. 1991. Effect of simulated acid spray on furanocoumarin concentrations on the surface of *Ruta graveolens* leaves. *Ann. Bot.* 67:213–218.

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SPATIAL DISCRIMINATION BETWEEN SOURCES OF PHEROMONE AND AN INHIBITOR BY THE LIGHT-BROWN APPLE MOTH *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae)

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Abstract—Inhibition of the behavioral responses of male *Epiphyas postvittana* moths by the *Z* isomer of the major component of the sex pheromone, *E*11–14:OAc, was studied in a wind tunnel. Inhibition was detectable at a *Z* isomer concentration of 10% that of the major component and was greatest when it was added to the same source as the pheromone. Inhibition declined rapidly as the inhibitor was moved across-wind away from the pheromone source but was largely maintained when the sources were separated upwind or downwind. The results showed that the insects possess a high degree of temporal resolution for odor plumes of different composition.

Key Words—Sex pheromone, inhibitor, temporal resolution, spatial resolution, wind tunnel, light-brown apple moth, *Epiphyas postvittana*, lepidoptera, tortricidae.

INTRODUCTION

During investigations to identify the sex pheromone components of *Epiphyas postvittana*, Bellas et al. (1983) recorded strong electroantennogram (EAG) responses from (*E*)-11-tetradecadecen-1-yl acetate (*E*11–14:OAc) and (*Z*)-11-tetradecadecen-1-yl acetate (*Z*11–14:OAc). Behavioral and chemical experiments showed that *E*11–14:OAc was the major component of the sex pheromone blend. Addition of 5% of a minor component, (*E*,*E*)-9,11-tetradecadien-1-yl

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acetate (*E*9,*E*11–14:OAc), provided a blend attractive to the males. *Z*11–14:OAc had no attractive properties and was not present in the female gland. Subsequently (Rumbo, unpublished) found specific receptors for both *E*11–14:OAc and *Z*11–14:OAc on the male antenna. It was postulated that this ability to detect *Z*11–14:OAc might help the males to differentiate more effectively between *E. postvittana* females and those from sympatric species, which contain both the *E* and *Z* isomers in their pheromone blend, e.g., *Epiphyas ashworthana* (Whittle and Bellas, unpublished). A trapping experiment in which varying amounts of *Z*11–14:OAc were added to pheromone lures showed that *Z*11–14:OAc reduced catches when present in amounts of 10% of *E*11–14:OAc or greater (Bartell, 1982). It was assumed that the presence of *Z*11–14:OAc caused the male moths not to initiate or to reduce their upwind progress and as a result failed to reach the traps. This effect will be referred to here as "inhibition."

Bartell's (1982) experiment suggested that mating in *E. postvittana* might be disrupted by permeating the atmosphere with *Z*11–14:OAc in a similar manner to the more usual technique of releasing a synthetic blend similar to the natural pheromone. Rumbo (unpublished) carried out a trial in an apple orchard with a resident wild population of *E. postvittana*. Sticky delta traps containing the attractive pheromone blend were each surrounded by six symmetrically placed *Z*11–14:OAc sources at distances of 2m from the traps. Regardless of what concentration of *Z*11–14:OAc (up to 100 times that of *E*11–14:OAc in the central trap) was used, males still managed to reach the central trap in numbers not significantly different from a control. Similar experiments have been carried out before (Kaae et al., 1974; McLaughlin et al., 1974; Daterman et al., 1975; Mitchell, 1976) with identical results. To explain them, it was postulated (McLaughlin et al., 1974; Sower et al., 1974) that the inhibitory compound is detected by specific olfactory receptors and do not act by interfering with the pheromone binding sites of one or more components in the pheromone, as suggested by an alternative hypothesis (Roelofs and Comeau, 1971). The presence of *Z*11–14:OAc receptors on the antenna of *E. postvittana* suggested that the separate receptor hypothesis would apply to this insect.

Murlis and Jones (1981) and Murlis (1986) showed that odor plumes are not diffuse and relatively homogeneous entities (cf. Bossert and Wilson, 1963) but consist of a tangle of high concentration filaments separated by areas of low or zero concentration. Moreover, the eddy currents in the air that are responsible for this filamentous structure in odor plumes are distributed randomly in time and space. Thus, not only does the odor in a plume arrive at a downwind point as a series of sharp pulses, but those pulses arrive at different times when plumes originate from spatially separated sources. Kaissling (1986) and Rumbo and Kaissling (1989) have shown that the receptors in *Antherea polyphemus* can detect pheromone pulses at a rate of up to 10 per second. The receptors in *E.*

postvittana have not been measured but may perform similarly. The insect's ability to discriminate between plumes of different composition would then arise from a capacity in the central nervous system to compare the time of arrival of sensory responses to concentration pulses from different sets of receptor types (Priesner and Witzgall, 1984; Kramer, 1986). This hypothesis has not been developed to the quantitative stage. It cannot predict how far apart sources have to be before the temporal pattern of odor pulses will be perceived as distinct and thus alter the behavior of the insect. The ability of moths to discriminate between separate plumes has to be determined experimentally. Measurements were therefore carried out in a wind tunnel by exposing *E. postvittana* males to two sources simultaneously, one a complete-blend pheromone source and the other the *Z* isomer inhibitor. The approach is similar to that of two other recent studies: Witzgall and Priesner (1991) and Liu and Haynes (1992).

METHODS AND MATERIALS

Sources. All measurements were carried out using as an attractant a three-component blend containing 10 μg of *E*11–14:OAc (*Z*11–14:OAc as a possible impurity was not detectable at a resolution level of 0.5% of *E*11–14:OAc by GLC) and 0.5 μg of *E*9,*E*11–14:OAc, the two components originally identified by Bellas et al. (1983), together with 1 μg of a third component, tetradecyl acetate (*n*14:OAc) subsequently found to be also present in the female pheromone gland (Bellas, unpublished). The role, if any, played by this third component has not been elucidated, but this blend and concentration elicits optimal behavioral responses from *E. postvittana* in our wind tunnel. Sources containing this blend were prepared by spreading 10 μl of a solution containing a 20:1:2 mixture of the three components in cyclohexane over a 20-mm-long piece of 5-mm-OD surgical rubber threaded through a piece of wire. *Z*11–14:OAc sources were made up in the same way. An aluminum T-shaped holder with holes drilled at different distances was used to support the sources in the tunnel at half the height of the tunnel and 15 cm from the upwind diffusing screen.

Wind Tunnel. The wind tunnel was of square cross section, sides 0.43 m, and 1.8 m long. The back, floor, and top were translucent white Perspex, with clear Perspex for the front. A household fan was used to drive air through the tunnel, at approximately 0.35 m/sec, with two sheets of muslin cloth acting as dampers. The air was exhausted to outside the room by a fan placed 0.5 m from the open end.

Insects. The insects were from a laboratory culture maintained on an artificial diet (Shorey and Hale, 1965). Pupae were sexed and male insects kept at 25°C in an artificial light regime in a lightbox, providing 14 hr of daylight with the time for commencement of observations, just after lights out, set to a con-

venient time during the day. Two hours before lights out, the insects were taken out of the lightboxes and placed in cylindrical glass containers, 8 cm length and diameter, with press-fit wiremesh ends, and left on a bench in the wind-tunnel room at 25°C. Three insects were placed inside each cage and 60 insects measured for each treatment. One hour later an air conditioner was turned on and the temperature slowly lowered to 21°C over the next hour. To approximate natural dusk conditions, light intensity was reduced progressively over 20 min by switching off three banks of fluorescent lamps at 5-min intervals and finally reducing the output from a set of incandescent lamps using a Variac until the light intensity inside the tunnel was reduced to approximately 5 lux.

Procedure. The effect of Z11–14:OAc on the behavior of insects exposed to the three-component pheromone described above was studied by placing a single Z11–14:OAc source across-wind, upwind, or downwind from the pheromone source. Takeoffs, oriented flights (to the halfway point down the tunnel), approaches (10 cm from source), and landings on the pheromone source were recorded. The experiments may be described as belonging to four different groups:

1. Z11–14:OAc was added to sources already made up with the three-component pheromone. Five different amounts of the Z11–14:OAc were used, corresponding to 0.1, 1, 10, 100, and 1000% the amount of E11–14:OAc in the pheromone. A pheromone source to which only solvent was added served as a control. The sources were positioned in the center of the wind tunnel.

2. Separate pheromone and Z11–14:OAc sources were placed 1, 3, or 10 cm apart symmetrically about the midline of the tunnel across the direction of the airflow. The same amounts of Z11–14:OAc as in (1) were used, together with a solvent only control.

3. Pheromone and Z11–14:OAc sources were both positioned on the midline of the tunnel, 10 cm behind each other: (i) with pheromone downwind, and (ii) with pheromone upwind of the Z11–14:OAc source. Sources were as in (2). This arrangement was introduced to measure the insect response to a combined plume arising from one source being inside the plume from the other.

4. Pheromone and Z5–14:OAc sources were placed 3 cm apart as in (2). This was a control experiment to check that inhibition was not caused merely by the presence of a foreign compound in the tunnel. Z5–14:OAc was used as the alternative to Z11–14:OAc because it elicits a low EAG response (Bellas et al., 1983), and no single cell responses (Rumbo, unpublished) have been recorded from it. Four different amounts of Z5–14:OAc were tested, corresponding to 1, 10, 100, and 1000% of the E11–14:OAc in the pheromone source. A source loaded with solvent only was again included as a control.

For experiments (2) and (4), the two sources were exchanged between measurements to cancel any flight bias to the right or left in the tunnel. Such a bias had been observed in a previous experiment (Rumbo, 1993). To reduce the

possibility of observer bias, experiments (1), (2), (3, ii) and (4) were carried out "blind." The sources were prepared and given to a colleague to scramble and relabel. This procedure was not used for (3, i) because in that case it was considered important to prevent contamination of the source in front by the one behind. We especially did not want to follow a high-strength Z11–14:OAc source with a low one. To achieve this in a blind situation would have meant replacing the pheromone source after each measurement, requiring a large number of sources. We decided to forgo the advantages of a blind trial and to minimize any possible contamination by carrying out the measurements using sources in order of increasing amount of Z11–14:OAc. Six insects were measured for each Z11–14:OAc strength and, when the highest strength was reached, the pheromone source was replaced with a fresh one and the sequence repeated. For (3, ii), 10 sets of scrambled Z11–14:OAc sources were made up and each used to measure six insects.

RESULTS

Flights, approaches and landings for experiments (1) and (2) are shown in Figure 1. With all the components together on the one source, Figure 1(i), inhibition becomes significant at a Z11–14:OAc concentration of 10% of the *E* isomer. The 0.1 and 1% amounts of Z11–14:OAc had no detectable effect. As the separation between the pheromone and the Z11–14:OAc sources increases [Figure 1(ii), 1(iii), 1(iv)] the amount of Z11–14:OAc required to stop the males from reaching the source increases rapidly with distance.

With the pheromone and Z11–14:OAc sources separated by 10 cm in the wind direction [experiment 3, Figure 2(i) and 2(ii)], the inhibitory effect was much greater than when they were separated by the same distance across-wind. One small complication arose with the solvent-only control and the two lower-strength Z11–14:OAc sources, i.e., 0.1 and 1% of *E*11–14:OAc. When they were placed upwind of the pheromone source, experiments 3(i), some insects (13 of 180) landed on them instead of the pheromone source. It was assumed that this was probably due to the visual influence of the first object reached rather than any chemical attraction, and these insects were counted as having landed on the source. Placing the Z11–14:OAc sources upwind or downwind of the pheromone source produced similar effects on the insects with landings reduced by 50% at a Z11–14:OAc strength of approximately 10% of *E*11–14:OAc.

Experiment 4 [Figure 2(iii)] indicated that inhibition was not due to the presence of a foreign compound showing some chemical similarity to the pheromone components, but was due to inherent inhibitory properties of Z11–14:OAc.

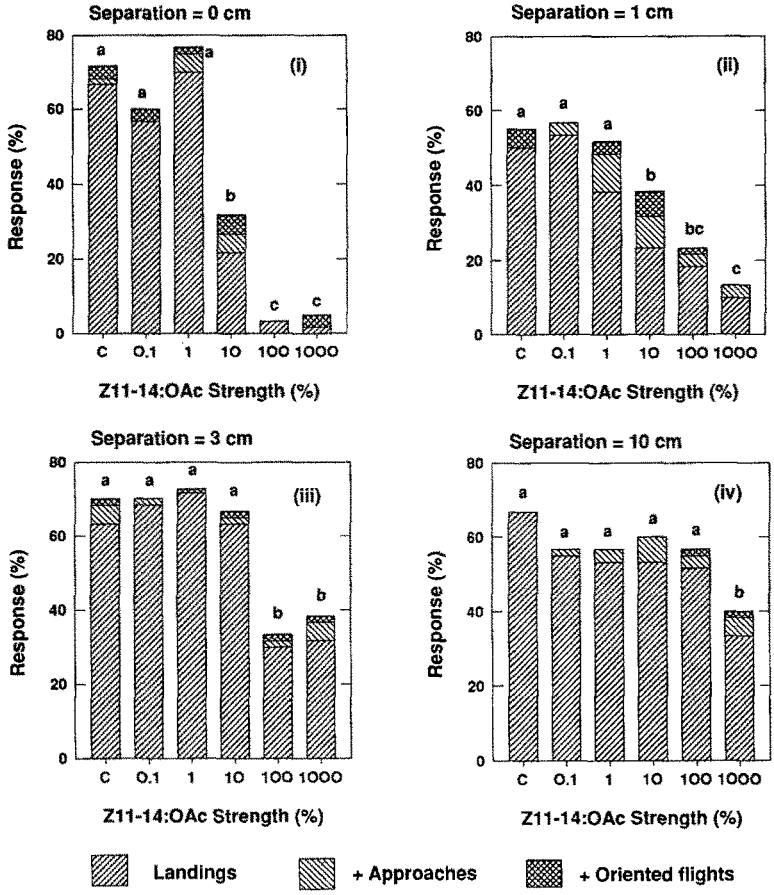

FIG. 1. Oriented flights, approaches, and landings on a pheromone source in the presence of inhibitor Z11–14:OAc, expressed as percentage of E11–14:OAc in the source, and solvent-only control, C: (i) inhibitor added to pheromone source, (ii–iv) inhibitor across-wind of pheromone source. Landings for bars sharing the same letter in each graph are not significantly different at 5% level of significance (Duncan's multiple-range test).

DISCUSSION

The results obtained are consistent with current ideas of the distribution of material within odor plumes and the temporal resolution of pheromone sensory cells as discussed in the Introduction. They show, as did Witzgall and Priesner (1991) and Liu and Haynes (1992), that insects possess a highly developed ability to discriminate between odor plumes of different composition. This is most probably a reflection of the complexity of odors that a male moth expe-

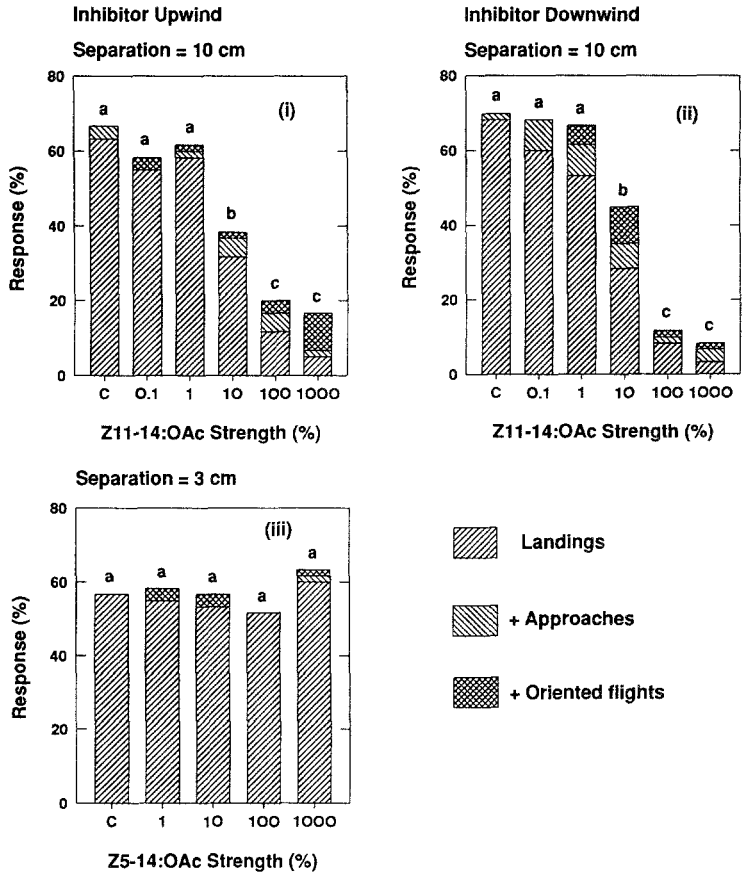

FIG. 2. Oriented flights, approaches, and landings on a pheromone source (i, ii) in the presence of inhibitor $Z11$–14:OAc, expressed as percentage of $E11$–14:OAc in the source, and solvent-only control, C: (i) inhibitor upwind, (ii) inhibitor downwind, (iii) in the presence of $Z5$–14:OAc sources placed acrosswind. Landings for bars sharing the same letter in each graph are not significantly different at 5% level of significance (Duncan's multiple-range test).

riences in the wild and the necessity to ignore most of them when searching for conspecific females.

The degree to which the filamentous structures of two separate plumes differ will depend on the amount of turbulence in the atmosphere. There would be no structure at all if turbulence were totally absent. Compared to an open environment, turbulence in the wind tunnel is probably low, yet some insects were able to discriminate between two sources only 1 cm apart when the con-

centration of the inhibitor was 10% or greater than that of the major component in the pheromone source. It would be expected, therefore, that the insect would have little difficulty in duplicating this performance in its natural environment. This would account for the failure of Z11-14:OAc to prevent males reaching the pheromone sources in the disruption experiment described in the Introduction.

The decline in the inhibitory effect of Z11-14:OAc sources was much less pronounced when they were moved upwind or downwind 10 cm away from the pheromone source, than when they were moved across-wind. It appears, therefore, that the composite plume arising from having one source behind the other contains some of the characteristics of a single plume, making it more difficult for the insect to detect them as separate. In contrast, Liu and Haynes (1992) found that with *Trichoplusia ni* the inhibitory effect was completely eliminated. This could mean that the two species possess different abilities to discriminate between the pheromone and inhibitor. Alternatively, it is possible that the turbulence in the two wind tunnels could have different characteristics so that the two results may not be directly comparable.

As previously discussed, small-scale attempts to suppress trap catches by releasing inhibitors in the vicinity of the traps have been unsuccessful. In spite of this, the prospect of using inhibitors, often single compounds, remains an attractive proposition in mating disruption. It is worthwhile therefore to consider whether it is possible to devise a release strategy that might succeed. Both experiment and theory indicate that insects are able to ignore the inhibitor because the pulses of odor from it and the pheromone arrive at the antenna at different times. Processing in the central nervous system is then able to extract the information contained in the pheromone odor and discard the rest. Any successful strategy involving an inhibitor will need to interfere with this process.

To disrupt the insect's flight behavior, the individual pulses of pheromone in the plume must also simultaneously contain the inhibitor above a certain threshold level. This might be achieved by permeating the area as uniformly as possible with a sufficiently large number of sources. Thus the air passing over the insect and picking up the pheromone would already be loaded with an above-threshold amount of the inhibitor. Unfortunately this would also mean that the gaps between the arrival of the pulses of pheromone would also contain high levels of the inhibitor. This might be just as effective in allowing the insects to again detect the pheromone pulses as distinct. Uniform permeation may also cause adaptation of the inhibitor receptors so that a much reduced inhibitory signal would then reach the central nervous system. The experiments of McLaughlin et al. (1974) indicated that preexposure to the inhibitor did indeed suppress its influence, suggesting that adaptation was taking place. It therefore appears that the prospects for using inhibitors to disrupt mating are not good.

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REFERENCES

BARTELL, R.J. 1982. Problems in identifying multicomponent sex pheromones, *in* R.A. Galbraith (ed.). Insect Pheromones and Their Application. DSIR Entomology, New Zealand, Report 2.

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.

BOSSERT, W.H., and WILSON, E.O. 1963. The analysis of olfactory communication among animals. *J. Theor. Biol.* 5:443–469.

DATERMAN, G.E., DAVES, G.D., and SMITH, R.G. 1975. Comparison of sex pheromone versus an inhibitor for disruption of pheromone communication in *Rhyacionia buoliana*. *Environ. Entomol.* 4:944–946.

KAAE, R.S., SHOREY, H.H., GASTON, L.K., and HUMMEL, H.H. 1974. Sex pheromones of Lepidoptera: Disruption of pheromone communication in *Trichoplusia ni* and *Pectinophora gossypiella* by permeation of the air with nonpheromone chemicals. *Environ. Entomol.* 3:87–89.

KAISSLING, K.-E. 1986. Temporal characteristics of pheromone receptor cell responses in relation to orientation behaviour of moth, pp. 193–200, *in* T.L. Payne, M.C. Birch, and C.E.J. Kennedy (eds.). Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

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.

LIU, Y.-B., and HAYNES, K.F. 1992. Filamentous nature of pheromone plumes protects integrity of signal from background chemical noise in cabbage loop moth, *Trichoplusia ni*. *J. Chem. Ecol.* 18:299–307.

MCLAUGHLIN, J.R., MITCHELL, E.R., CHAMBERS, D.L., and TUMLINSON, J.H. 1974. Perception of *Z*-7-dodecen-1-ol and modification of the sex pheromone response of male loopers. *Environ. Entomol.* 3:677–680.

MITCHELL, E.R. 1976. Inhibition of pheromone perception by male cabbage loopers and beet armyworms: Proximity vs. atmospheric permeation. *Environ. Entomol.* 5:770–772.

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.

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.

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.

ROELOFS, W.L., and COMEAU, A. 1971. Sex pheromone perception: synergists and inhibitors for the red-banded leaf roller attractant. *J. Insect Physiol.* 17:435–448.

RUMBO, E.R. 1993. Interactions between male moths of the lightbrown apple moth, *Epiphyas postvittana* (Walker) (Lep.: Tort.), landing on synthetic sex pheromone sources in a wind-tunnel. *Physiol. Entomol.* In press.

RUMBO, E.R., and KAISSLING, K.-E. 1989. Temporal resolution of odour pulses by three types of pheromone receptor cells in Antherea polyphemus. *J. Comp. Physiol. (A)* 165:281–291.

SHOREY, J.J., 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.

SOWER, L.L., VICK, K.W., and BALL, K.A. 1974. Perception of olfactory stimuli that inhibit the responses of male phycitid moths to sex pheromones. *Environ. Entomol.* 3:277–279.

WITZGALL, P., and PRIESNER, E. 1991. Wind-tunnel study on attraction inhibitor in male *Coleophora laricella* Hbn. (Lepidoptera: Coleophoridae). *J. Chem. Ecol.* 17:1355–1362.

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SOURCE OF SEX PHEROMONE OF THE EGG-LARVAL PARASITOID, *Ascogaster reticulatus* WATANABE (HYMENOPTERA: BRACONIDAE)

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Abstract—A sex pheromone of *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae), identified as (*Z*)-9-hexadecenal, elicits males' antennal mate-searching behavior. In order to localize the source of sex pheromone production, each body part was separated and extracted with hexane and then subjected to 9-cm Petri dish bioassay. The highest activity was found in thorax extract. Among legs, wings, and thorax, legs have the highest activity. Among fore, middle, and hind legs, hind legs have the highest activity. Among coxa and trochanter, femur, tibia, and tarsus, tibia has the highest activity. From these results, the presence of a tibial sex pheromone gland was suggested.

Key Words—Sex pheromone gland, mating behavior, *Ascogaster reticulatus*, Hymenoptera, Braconidae, egg-larval parasitoid, *Adoxophyes* sp., Lepidoptera, Tortricidae, tibia.

INTRODUCTION

The mating behavior of *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae), an egg-larval parasitoid of the smaller tea tortrix, *Adoxophyes* sp. (Lepidoptera: Tortricidae) was analyzed by us, and the characteristic antennal response by male parasitoids was found to be elicited by the presence of sex pheromone deposited on the substrate by walking females (Kamano et al., 1989). The female hexane extract treated in a line on the bottom of a 9-cm Petri dish

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elicited males' following along the line with antennal searching behavior. By using this bioassay, a sex pheromone of this species was analyzed and characterized as (*Z*)-9-hexadecenal (Kainoh et al., 1991). Further, we analyzed the source of this sex pheromone production to locate the pheromone gland in females.

METHODS AND MATERIALS

Insects. The parasitoids and their hosts were from our stock culture based on the rearing method of Kainoh and Tamaki (1982) and Kainoh (1986). Emerged adults were sexed and 10–20 insects of each sex were kept in a transparent plastic container (15 cm diam., 12 cm high) in which honey and wet cotton were provided. The rearing of insects and bioassay were conducted at 25 ± 1°C and 50 ± 20% relative humidity with a 16:8 hr light–dark photoperiod.

Bioassay. Bioassay of the extract or samples was modified from Kainoh et al. (1991). Five microliters of the sample was applied in a 9-cm-long straight line across the center of a 9-cm glass Petri dish. After the solvent was air-dried, a 3- to 4-day-old virgin male parasitoid was released into the dish, which was then capped. The activity was scored based on the total walking distance within 3 min while responding with antennal searching along the treated line, with 9-cm distance given a score of 1. Five to seven males were used for each sample, and the average score was used to evaluate activity.

Head, Thorax, and Abdomen. Thirty virgin females (2 days old) of *A. reticulatus* were collected and killed in a freezer (−20°C). The bodies were separated with microscissors into head, thorax (with wings and legs), and abdomen. Each part was separately dipped in 0.3 ml hexane in a small vial for ca. 20 min, and the extract was serially diluted [10^{-5}–10^{-2} FE (female equivalent)/μl] for bioassay. Seven virgin males (3 days old) were used for each sample in this bioassay.

Thorax, Legs, and Wings. Similar extraction was performed with legs, wings, and thorax. Two-day-old virgin females were killed by freezing (−20°C) and each body part was separated with microscissors and dipped into 0.1 ml hexane for 5 min for one female (10^{-2} FE/μl). Another female body was separated similarly and dipped into 1 ml hexane, and the extract was serially diluted (10^{-5}–10^{-3} FE/μl). This series was replicated twice, and a total of four females was used for the preparation of extracts. Seven virgin males (3 days old) were used for each sample in this bioassay.

Fore, Middle, and Hind Legs. Activity of fore, middle, and hind legs was compared with an extraction procedure similar to the previous one. Legs were separated from 2-day-old virgin females after being killed by freezing (−20°C) and each legs part was dipped into 0.1 ml hexane for 5 min for one female

(10^{-2} FE/μl). Another female's legs were separated similarly and dipped into 1 ml hexane, and the extract was serially diluted (10^{-5}–10^{-3} FE/μl). This procedure was replicated twice, and a total of four females was used for preparing the extracts. Seven males (3 days old) were used for each sample.

Coxa and Trochanter, Femur, Tibia, Tarsus. Activity of each part of the hind leg was compared with a method similar to the previous one. Each part of the hind legs was separated from 2-day-old virgin females and was dipped into 0.1 ml hexane for 5 min for one female (10^{-2} FE/μl). Other females' hind legs were separated similarly and dipped into 1 ml hexane, and the extract was serially diluted (10^{-5}–10^{-3} FE/μl). This procedure was replicated twice, and a total of four females was used for preparing the extracts. Five males (3 days old) were used for each sample.

RESULTS

Head, Thorax, and Abdomen. The dose–response relationship of the extract of female body parts is shown in Figure 1. The cone-shaped curve of female extract was shown previously by Kamano et al. (1989) and Kainoh et al. (1991). The highest activity of diluted extract (5×10^{-4} FE/line) of thorax showed that the thorax, including wings and legs, contains the largest amount of sex pheromone. More than 90% of the pheromone of the whole body is in thorax.

Thorax, Legs, and Wings. Among the thorax, legs and wings, the highest activity was observed in the diluted extract (5×10^{-4} FE/line) of the legs (Figure 2.) These curves showed more than 90% of the pheromone of the thorax is included in the legs.

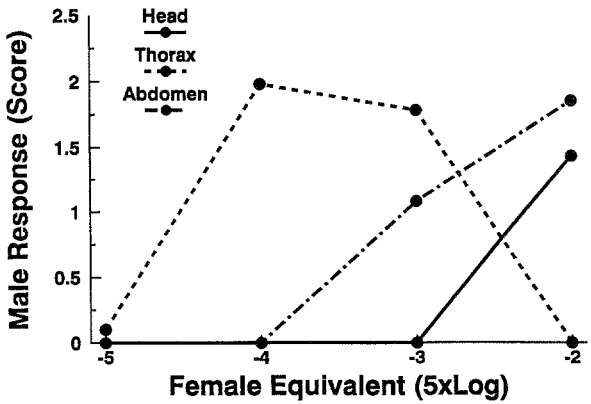

FIG. 1. Dose–response relationship between hexane extract of female body parts (head, thorax, abdomen) and male response in *A. reticulatus*.

Fore, Middle, and Hind Legs. Among fore, middle, and hind legs, the diluted extract (5×10^{-4} FE/line) of hind legs had the highest activity (Figure 3). From these curves, more than 90% of the pheromone of the leg is included in the hind legs.

Coxa and Trochanter, Femur, Tibia, Tarsus. Among coxa and trochanter, femur, tibia, and tarsus, the highest activity was observed in the diluted extract of the tibia (5×10^{-4}–10^{-3} FE/line) (Figure 4). These curves showed that ca. 90% of the pheromone of the hind leg is in the tibia.

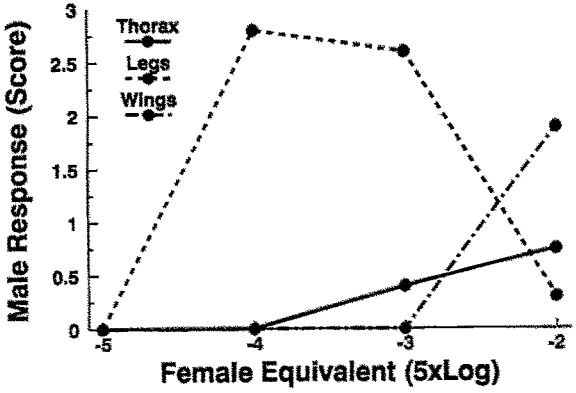

FIG. 2. Dose–response relationship between hexane extract of female body parts (thorax, legs, wings) and male response in *A. reticulatus*.

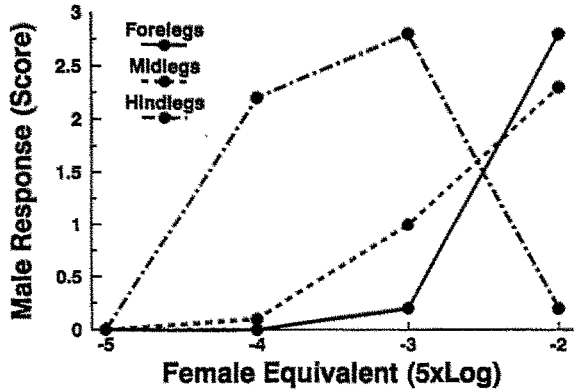

FIG. 3. Dose–response relationship between hexane extract of female body parts (fore, middle, hind legs) and male response in *A. reticulatus*.

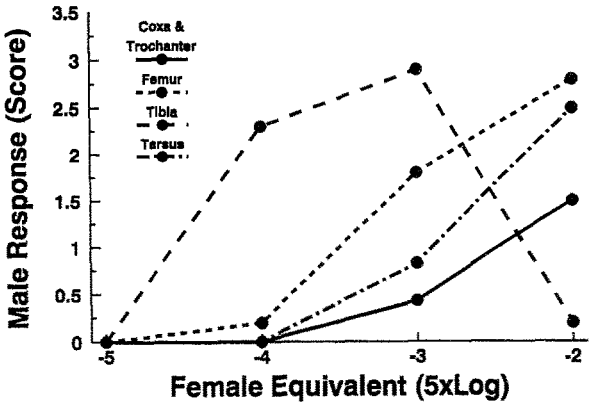

FIG. 4. Dose-response relationship between hexane extract of female body parts (coxa and trochanter, femur, tibia, tarsus) and male response in *A. reticulatus*.

DISCUSSION

The source of a sex pheromone of *A. reticulatus* was proved to be in the tibia of the hind legs. Our preliminary experiments with GLC also indicated that (Z)-9-hexadecenal was most abundant in the legs rather than head, thorax without legs and wings, or abdomen. These data suggest that the sex pheromone gland exists in the tibia and secretes sex pheromone outside the cuticle; then it may disperse through the wax layer and/or by grooming behavior. The smaller amount of pheromone present in the head and abdomen (Figure 1), the thorax without legs and wings (Figure 2), and fore and middle legs (Figure 3) might be from contamination by grooming behavior. To verify this, a similar bioassay is necessary with females soon after emergence and having no experience of grooming behavior.

The presence of pheromone glands in the legs is proved with several ant species such as the tibial gland in *Crematogaster peringueyi* (Fletcher and Brand, 1968), *C. scutellaris* (Billen, 1984), and the tarsi gland in *Onychomyrmex* sp. (Hölldobler and Palmer, 1989). These worker ants secrete trail pheromone from their glands and deposit it on the ground to inform other ants of the same species of the route toward the food collection site. Their characteristic trail-laying behavior may be by placing their hind legs close together and drumming vigorously on the substrate with the tips of the tarsi, as in *C. peringueyi* (Fletcher and Brand, 1968), or dragging the distal end of the tarsi of the hind legs over the ground, as in *Onychomyrmex* sp. (Hölldobler and Palmer, 1989). However, there is no such characteristic pheromone-laying behavior in *A. reticulatus*. Furthermore, when we put a male parasitoid into a 9-cm Petri dish after observing female behavior for a while and removing the female, there was no corre-

lation between the place where the female parasitoid stayed longer by grooming and the place of antennal searching by males introduced later. These observations show that *A. reticulatus* does not actively deposit the pheromone, but rather just leaves it. This behavior may be related to the unwillingness of females to be mated and their escaping from the males' pursuit (Kamano et al., 1989).

Some dipterous insects seem to release contact sex pheromone from the legs as in the unicellular gland of tarsi and tibia in the housefly, *Musca domestica*, and the tsetse fly, *Glossina morsitans* (Schlein et al., 1980). In the mosquito, *Culiseta inornata*, sex pheromone remains restricted to the legs, and it does not spread over the body surface as in other flies (Lang, 1977).

Tagawa (1977) reported the sex pheromone gland in the second valvifer of the female abdomen in a braconid, *Apanteles glomeratus* L. In this species, the female releases volatile sex pheromone produced at the last abdominal segment, and it elicits wing vibration of the male (Obara and Kitano, 1974). Mounting and copulation behaviors of males were accomplished by visual stimuli from females in the presence of sex pheromone (Kitano, 1975). Even when the high dosage of identified sex pheromone, (Z)-9-hexadecenal, for *A. reticulatus* was applied on the bottom of a Petri dish, males showed only antennal searching behavior and did not show other components of mating behavior, such as wing vibration or mounting in response to the treated line (Kainoh et al., 1991). The lack of behavioral components may indicate that stimuli other than (Z)-9-hexadecenal are necessary to elicit the whole mating behavior. In our preliminary experiment, the crude hexane extract of virgin females, treated on a small piece of black paper, did elicit wing vibration behavior of a male. Such wing vibration behavior was not observed toward the black paper impregnated with (Z)-9-hexadecenal. The source of the sex pheromone that elicits the final step of the behavioral sequence may exist in some part of female body other than the tibia, perhaps as in the abdominal tip of *A. glomeratus*. In order to examine the whole sex pheromone secretion complex in *A. reticulatus*, the chemical component(s) eliciting these behaviors must be elucidated, and morphological studies of the sex pheromone glands must be performed.

REFERENCES

BILLEN, J.P.J. 1984. Morphology of the tibial gland in the ant *Crematogaster scutellaris*. *Naturwissenschaften* 71:324–325.

FLETCHER, D.J.C., and BRAND, J.M. 1968. Source of the trail pheromone and method of trail laying in the ant *Crematogaster peringueyi*. *J. Insect Physiol.* 14:783–788.

HÖLLDOBLER, B., and PALMER, J.M. 1989. A new tarsal gland in ants and the possible role in chemical communication. *Naturwissenschaften* 76:385–386.

KAINOH, Y. 1986. Mating behavior of *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae), an egg-larval parasitoid of the smaller tea tortrix moth, *Adoxophyes* sp. (Lepidoptera: Tor-

tricidae). I. Diel patterns of emergence and mating, and some conditions for mating. *Appl. Entomol. Zool.* 21:1–7.

KAINOH, Y., and TAMAKI, Y. 1982. Searching behavior and oviposition of the egg-larval parasitoid, *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae). *Appl. Entomol. Zool.* 17:194–206.

KAINOH, Y., NEMOTO, T., SHIMIZU, K., TATSUKI, S., KUSANO, T., and KUWAHARA Y. 1991. Mating Behavior of *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae), an egg-larval parasitoid of the smaller tea tortrix, *Adoxophyes* sp. (Lepidoptera: Tortricidae). III. Identification of a sex pheromone. *Appl. Entomol. Zool.* 26:543–549.

KAMANO, Y., SHIMIZU, K., KAINOH, Y., and TATSUKI, S. 1989. Mating Behavior of *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae), an egg-larval parasitoid of the smaller tea tortrix, *Adoxophyes* sp. (Lepidoptera: Tortricidae). II. Behavioral sequence and a role of sex pheromone. *Appl. Entomol. Zool.* 24:372–378.

KITANO, H. 1975. Studies on the courtship behavior of *Apanteles glomeratus* L. 2. Role of the male wing during courtship and the releaser of mounting and copulatory behavior in the males. *Kontyu* 43:513–521.

LANG, J.T. 1977. Contact sex pheromone in the mosquito *Culiseta inornata* (Diptera: Culicidae). *J. Med. Entomol.* 14:448–454.

OBARA, M., and KITANO, H. 1974. Studies on the courtship behavior of *Apanteles glomeratus* L. 1. Experimental studies on releaser of the wing-vibrating behavior in the male. *Kontyu* 42:208–214.

SCHLEIN, Y., GALUN, R., and BEN-ELIAHU, M.N. 1980. The legs of *Musca domestica* and *Glossina morsitans* females as the site of sex pheromone release. *Experientia* 36:1174–1175.

TAGAWA, J. 1977. Colonization and histology of the female sex pheromone-producing gland in the parasitic wasp, *Apanteles glomeratus*. *J. Insect Physiol.* 23:49–56.

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IDENTIFICATION OF THE SEX PHEROMONE OF THE GUERNSEY CARPET BEETLE, *Anthrenus sarnicus* MROCZKOWSKI (COLEOPTERA: DERMESTIDAE)

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Abstract—It has been confirmed that adult virgin females of *Anthrenus sarnicus* Mroczkowski exhibit a characteristic headstand posture that is associated with the release of a sex pheromone. Volatiles trapped on filter papers suspended above calling females were attractive to adult virgin males when tested in a two-choice target bioassay. Separate aeration extracts of males and females were analyzed by gas chromatography–mass spectrometry and showed that decanol and decyl *n*-butyrate were released by females only. These components were present in approximately equal amounts and accounted for about 90% of the total area of the chromatogram. Decyl butyrate produced an electroantennogram response with a larger response from males than females. Behaviorally, a mixture of 10 μg of decanol and 10 μg of decyl butyrate attracted 88% of males and 10 μg of decyl butyrate alone attracted 82% of males in the bioassay. The role of decyl butyrate as a sex pheromone is convincing, but this is not the case for decanol.

Key Words—*Anthrenus sarnicus*, Coleoptera, Dermestidae, pheromone, decyl butyrate, decanol, volatile components, EAG, bioassay.

INTRODUCTION

Several species of carpet beetles, genus *Anthrenus* (Coleoptera: Dermestidae), have been reported to cause considerable damage to a wide range of plant and animal materials (Nair, 1991). The beetles thrive in households, warehouses, and museums, with the larval stage causing the greatest amount of damage since it is a relatively long period in the life-cycle (Hall, 1988).

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The Guernsey carpet beetle, *Anthrenus sarnicus* Mroczkowski, was first described from specimens found in a domestic infestation in Guernsey in 1961 (Mroczkowski, 1962). The rise in the number of reported infestations, especially within museums, indicates an increase in the importance of this pest in the UK (Halstead, 1975; Armes, 1988). There is now a growing demand for methods to monitor and control the spread of this beetle (Pinniger, 1990).

Monitoring of pest populations with pheromones provides an efficient and effective method for the determination of population trends (Burkholder and Ma, 1985). The information can then be used to provide a basis for the use of further pest management strategies (Chambers, 1991).

Relatively little is known about the behavior of *A. sarnicus*. However, in two related species, the varied carpet beetle, *A. verbasci* (Kuwahara and Nakamura, 1985), and the furniture carpet beetle, *A. flavipes* (Burkholder et al., 1974), sex pheromone-releasing behavior is characterized by the females assuming a headstand posture. Burkholder et al. (1974) showed that female *A. flavipes* produce a sex pheromone that is attractive to males. This was identified as an unsaturated carboxylic acid, (*Z*)-3-decenoic acid (Fukui et al., 1974). Kuwahara and Nakamura (1985) identified the sex pheromone of the varied carpet beetle, *A. verbasci*, as a mixture of (*Z*)- and (*E*)-5-undecenoic acid.

Female *A. sarnicus* have been reported (Armes, 1985) to assume a headstanding posture similar to that shown by female *A. flavipes* and *A. verbasci*. Volatiles captured on filter papers held above calling female *A. sarnicus* were found to be attractive to conspecific virgin males (Armes, 1985). The purpose of this study was to identify and characterize the chemicals involved.

METHODS AND MATERIALS

Insect Handling. Adult *A. sarnicus* were obtained from a stock culture held in the Central Science Laboratory and maintained at 20 ± 2°C and 70% relative humidity in constant darkness. This culture was originally prepared from descendants of larvae collected from a dead pigeon at a military camp in Wiltshire, England in 1966 (Woodroffe, 1967). The insects were reared on a diet of fishmeal, yeast, and cholesterol in the ratio 100:25:2 (w/w/w). The sex of the insects was determined at the pupal stage; females were identified by conspicuous forklike parameres and males by a more rounded structure (Burkholder et al., 1974). Males and females were kept individually in 2-in. by 1-in. glass tubes containing a piece of pleated filter paper (20 × 20 mm) as a crawling surface. The photoperiod was 8:16 hr light–dark (usually light between 0800 and 1600 hr). Upon emergence from the last larval skin, the beetles were termed 0 days old.

Procedures for Obtaining Insect-Produced Chemicals. Batches of 25 male

and 25 female *A. sarnicus* (3–9 days old) were placed in different glass aeration chambers (105 × 25 mm ID) without food. They were aerated, by suction, for 24 hr with purified air (flow rate 750 ml/min, cleaned through activated charcoal), which was humidified (water bubbler) in one set of separate male and female aerations. In another set of aerations no artificial humidification was used. In both sets of aerations, the volatiles were trapped on fresh Porapak Q (5 g, 50/80 mesh, Waters). The efficiency of the procedure was investigated using the same apparatus. Filter paper disks treated with decanol (100 μg) or decyl butyrate (100 μg) were separately aerated for 3 hr.

The volatile materials trapped on the Porapak were recovered (separately) by Soxhlet extraction with diethyl ether (HPLC grade, 250 ml) for 24 hr. The extracts were concentrated to about 5 ml by Dufton distillation. Control extracts were prepared from Soxhlet extraction of fresh Porapak.

The association of headstand posture with pheromone release was investigated by collection of insect volatiles using the method described by Armes (1985). Armes (1985) observed the peak in female calling behavior (the greatest number of calling females) between 1300 and 1500 hr. This appeared to be the case in the present study, and collection was started at 1330 hr.

Calling females were usually found close to the top of the pleated filter paper. Filter paper disks (20 mm diam., Whatman No. 1) were held 10 mm (suspended from a cork) above stationary females (3–7 days old) for 30 min. A note was made of the females that assumed the calling posture, and these disks were immediately assayed against a blank control 20-mm disk for attractancy. Calling usually averaged about 20 min. Filter paper disks were held above males for 30 min.

In a comparative experiment, filter paper disks (20 mm diameter) were exposed to individual males and individual females (50 insects of both sexes) for 2 hr according to the method of Armes (1985). The volatile materials were recovered from the filter paper by Soxhlet extraction as described above. A control extraction of 50 blank filter paper disks was also performed.

Separate extracts of male and female beetles were prepared by macerating 25 beetles in pentane using a hand-held homogenizer (Uniform). The resulting suspensions were filtered to remove insoluble fragments and concentrated to about 3 ml by Dufton distillation.

Bioassay Procedure. Test material was assayed using a disk bioassay technique similar to that described by Burkholder et al. (1974). Individual males or females (3–7 days old) were placed in an arena, which comprised a 10-cm-diameter aluminum ring that was placed on a piece of filter paper and covered with a 12-cm-diameter Petri dish. The insects were allowed to settle for 1 hr. With the beetle at the perimeter of the arena, a 20-mm-diameter filter paper treated with test material and a 20-mm filter paper control (both were allowed to air for 1 min) were placed in the arena. Filter paper disks were placed along

a diameter of the arena perpendicular to the point of observation and about 3 cm from either edge of the aluminum ring. The time taken for the beetle to respond (touch the filter paper) was recorded as was its behavior (for example, increased antennal movements and amount of turning). The bioassays were started at 1430 hr each day and were run for 10 min. The beetles were tested once only. The tests were carried out at 20 ± 2°C under normal intensity lighting.

Bioassays Conducted. For experiment 1a, the attractancy to males (N = 40) of filter paper disks exposed to calling females was compared with blank disks.

For experiment 1b, the attractancy to females (N = 40) of filter paper disks exposed previously to the same calling females was compared with blank disks.

For experiment 2, the attractancy to males (N = 40; experiment 2a) and females (N = 35; experiment 2b) of disks treated with decyl butyrate and decanol (10 μg each) in 10 μl of diethyl ether was compared with disks treated with 10 μl of diethyl ether alone.

For experiment 3, the attractancy to males (N = 35; experiment 3a) and females (N = 35; experiment 3b) of disks treated with decyl butyrate (10 μg) in 10 μl of diethyl ether was compared with disks treated with 10 μl of diethyl ether alone.

For experiment 4, the attractancy to males (N = 20; experiment 4a) and females (N = 20; experiment 4b) of filter paper disks exposed to males was compared with blank disks.

Electroantennogram (EAG) Procedure. The EAG system was similar to that described previously (White and Chambers, 1989; Chambers et al., 1990). The sex of adult beetles, five of each sex, was determined by dissection of the genitalia. Tests were carried out using isolated heads with the recording electrode implanted into the terminal club segment and the indifferent electrode into the head via a "neck" hole. The airflow across the insect was 1 liter/min. Decyl butyrate was presented at six log doses in ascending order from 1 ng to 1 mg. Prior to each test stimulus, a blank cartridge (solvent only) was presented, and the EAG recorded was subtracted from the subsequent test response to allow for the response to solvent and the mechanical disruption of the airflow.

Identification of Volatile Components. Structure elucidation of the chemical components of all the extracts was carried out using gas chromatography–mass spectrometry (GC-MS) on a HP 5890 series II GC coupled to a VG Trio 1 mass spectrometer operating in EI+ mode (70 eV) with helium as the carrier gas. Chemical ionization mass spectrometry was performed using isobutane as the reagent gas. Compounds were separated using a splitless injection technique (split valve opened after 1 min) on a 50-m CPsil 5 CB column (0.25 mm ID, 0.12 df, Chrompack UK Ltd). The flow rate through the column was 1 ml/min. A temperature program of 50°C for 1 min, then 15°C/min to 250°C was used;

the GC-MS transfer line was maintained at 255°C and the EI source temperature was 200°C.

Octyl hexanoate, decyl butyrate, and dodecyl acetate were prepared by refluxing a 10-fold excess of the appropriate carboxylic acid (Aldrich) with the corresponding alcohol (Aldrich) for 18 hr followed by base extraction and partition into diethyl ether with removal of the solvent in vacuo. Confirmation of structure of the esters was carried out by proton nuclear magnetic resonance on a 60-MHz R-1500 Hitachi Spectrometer using TMS as the internal standard. Purity of the synthetic esters was confirmed by GC-MS.

RESULTS

Aeration of the empty apparatus showed that no volatiles were present on the aeration equipment. Figure 1 shows the total ion current obtained from the mass spectrometer of a male aeration extract and a female aeration extract carried out with humidification of the incoming air. The most significant differences are the two large peaks of approximately equal size in the female aeration extract, which are not present in the male aeration extract. These two peaks account for over 90% of the total area of the female extract.

Mass spectral analysis (EI+) of the earlier-eluting component, retention time (R_t) = 8.97 min, gave the following mass-to-charge ratios with relative abundance, *m/z*: 140 (0.5), 112 (13), 97 (26), 83 (62), 70 (100), 55 (92), and 41 (71). The later eluting component had a retention time of 11.93 min: *m/z*

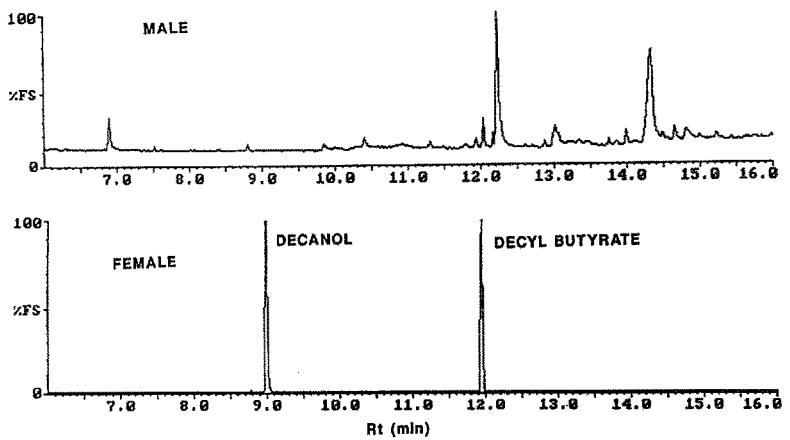

FIG. 1. GC-MS total ion current traces from aerations of male and female *A. sarnicus* showing the presence of decyl butyrate from females (the female trace 10× larger than the male).

185 (1.2), 140 (9), 111 (12), 97 (21), 89 (78), 83 (28), 71 (100), and 43 (79). Chemical ionization mass spectrometry with isobutane gave signals at 159 and 229 for the two components, respectively, suggesting their molecular masses to be 158 and 228, respectively. Assignment of the *m/z* values suggested the first component was an alcohol, decanol, and the second component was a butyrate ester, decyl butyrate.

Authentic decanol gave the same retention time as the first component from the aeration extract and a similar mass spectrum: *m/z*, 140 (0.4), 112 (20), 97 (29), 83 (58), 70 (100), 55 (85), and 41 (77). Synthetic decyl butyrate eluted at the same retention time as the second component in the insect extract and gave a similar mass spectrum (Figure 2): *m/z*, 229 (0.2), 185 (1.0), 140 (10), 111 (15), 97 (20), 89 (85), 83 (31), and 71 (100). Octyl hexanoate and dodecyl acetate have the same molecular weight as decyl butyrate. GC-MS analysis showed that the mass spectra of octyl hexanoate and dodecyl acetate did not resemble the mass spectrum of the insect-derived material.

The aerations without humidification showed that the difference between the male and female extracts was the presence of a large amount of decyl butyrate in the female extract.

GC-MS analysis of the Soxhlet extract obtained from filter papers that had been exposed to males or females showed that the significant difference between the extracts was the presence of a large amount of decyl butyrate in the filter papers that had been exposed to calling females. No significant amount of decanol was found. The other main component, R_t = 12.18 min, was present in both extracts and the control (extract of untreated filter paper) (Figure 3).

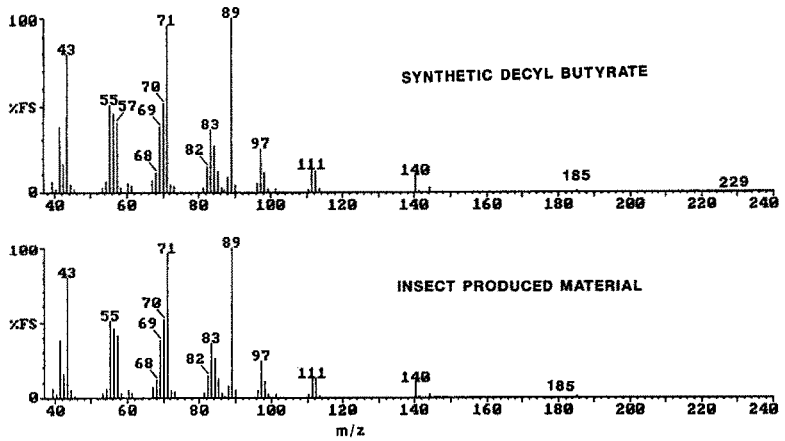

FIG. 2. Comparison of the mass spectra of synthetic decyl butyrate and material collected by aeration of female *A. sarnicus*.

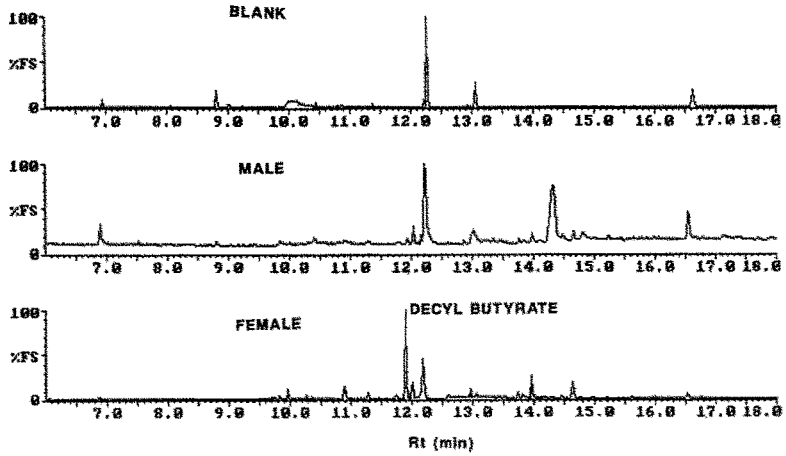

FIG. 3. GC-MS total ion current traces from Soxhlet extraction of filter paper disks exposed to males or calling females or neither (blank) to show the presence of decyl butyrate in the female extract.

Mass spectral assignment suggested this was a substituted aromatic compound, but it was not identified since it probably originated from the filter paper.

The aeration of decyl butyrate to quantify the efficiency of the trapping and extraction gave a value of 92% and showed no degradation of the ester. The efficiency of the trapping and extraction procedure for decanol was 88%.

GC-MS comparison of a standard solution of decyl butyrate with an aeration extract of 25 females for 65 hr (at 1 liter/min) showed that the average release rate per female of decyl butyrate per 24 hr was approximately 1130 ng and for decanol was approximately 980 ng, taking account of the trapping and extraction efficiency.

Figure 4 shows the comparison of the homogenate extract of 25 females and 25 males. The average amount of decyl butyrate per female was found to be approximately 455 ng; however, no significant amount of decanol was found in the homogenate, thus not permitting quantification. No decyl butyrate or decanol was found in the homogenate of male beetles.

EAG Experiments. The EAG response of both sexes to synthetic decyl butyrate is shown in the Figure 5. The results showed that there was a difference in the magnitude of the response from the males and the females—the males produced the larger response. However, the responses were small, and it was not until fairly high amounts of the ester were presented that the difference in the response became significant.

Bioassay Experiments. Responses quoted are the percentage number of beetles that responded to the disk divided by the total number of beetles tested.

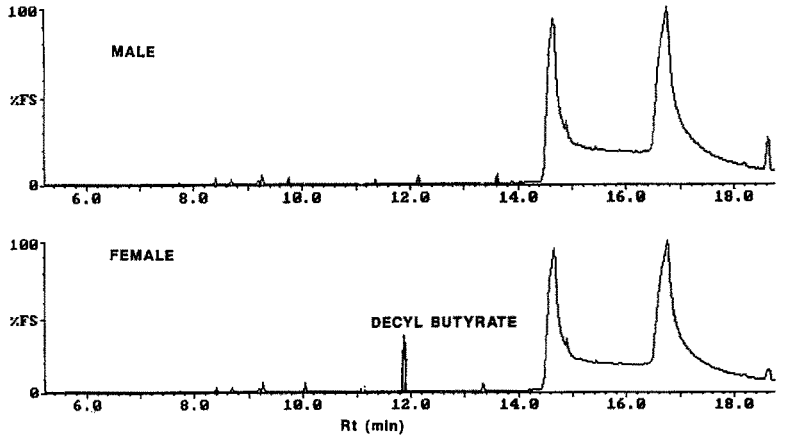

FIG. 4. GC-MS total ion current traces from homogenates of male and female *A. sarnicus* showing the presence of decyl butyrate from females.

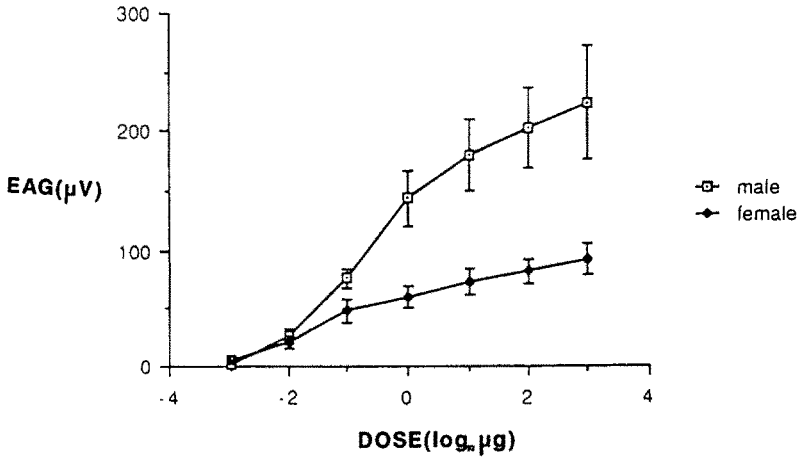

FIG. 5. EAG dose–response curve of *A. sarnicus* to decyl butyrate.

The results of all the bioassay experiments are presented in Table 1. Overall there was a difference in the response of the sexes; more males than females responded to the test disks. However, the difference in the time taken for males or females to respond to the treated disk was not statistically significant (*t*-test). It is unlikely that errors due to incorrect determination of the sex of the beetles contributed to the finding since, if there was any doubt in the determination, the beetle was discarded.

TABLE 1. RESPONSE OF *A. sarnicus* TO NATURAL PHEROMONE AND SYNTHETIC MIXTURES IN LABORATORY BIOASSAYS

Experiment	Test response	Time to touch disk (sec)	Control response	Time to touch disk (sec)
1a. Males vs. disks exposed to females and control disks	73	191 ± 21	NR[a]	NR
1b. Females vs. disks exposed to females and control disks	8	286 ± 104	6	298 ± 142
2a. Males vs. 10 μg decyl butyrate and decanol or 10 μl diethyl ether	88	144 ± 25	NR	NR
2b. Females vs. 10 μg decyl butyrate and decanol or 10 μl diethyl ether	10	250 ± 110	8	278 ± 117
3a. Males vs. 10 μg decyl butyrate or 10 μl diethyl ether	82	177 ± 33	NR	NR
3b. Females vs. 10 μg decyl butyrate or 10 μl diethyl ether	6	234 ± 59	10	287 ± 67
4a. Males vs. disks exposed to males and control disks	6	264 ± 82	NR	NR
4b. Females vs. disks exposed to males and control disks	NR	NR	10	344 ± 127

[a]NR = no response.

The behavior of males and females during the test was different. Males showed directional attraction towards the disk and increased turning near the test filter paper disk. Furthermore, once they had climbed onto the filter paper, all of the respondents explored the disk (rapid movement of antennae), spent on average 12 sec on the disk, and returned to the disk 2–10 times.

Females did not show directional attractancy towards either the control disk or the treated disk. The difference in the time taken for the females to respond either to the control or to the test disk was not statistically different (*t*-test). Those that responded walked across the disk (either control or test) and did not return to it. During the assay, some of the females assumed a headstanding posture, but the behavior of these beetles was not affected by the presence of

the test and the control filter paper disks. Female movement was usually close to the perimeter of the test arena.

DISCUSSION

The results from the bioassay of filter paper disks that had been exposed to calling females show that there is an attractant chemical released by females. This confirms the more detailed study of Armes (1985) and demonstrates that female *A. sarnicus* produce a sex pheromone.

Aeration of the batches of males and females was carried out to obtain a large quantity of material for chemical identification. The aeration of females (both with and without humidification), the extraction of filter papers exposed to females, and the female homogenate all showed the presence of a large quantity of decyl butyrate. Decyl butyrate was not found in any of the male extracts. Additionally, the aerations of beetles using incoming air that had been artificially humidified (to stop the beetles desiccating) showed the presence of decanol, in almost equal amount to the decyl butyrate.

Behaviorally the mixture of decyl butyrate and decanol elicited an attraction in males similar to that produced when males had been presented with filter paper disks that had been exposed to calling females. The attraction was characterized by directional movement towards the source by the males and, when close to the source, an increase in general activity was noted, characterized by turning and rapid movement of the antennae. Many of the males lowered their abdomen, which may have been copulation attempts with the filter paper. From the EAG results it was decided to test 10 μg of decyl butyrate in the bioassay since this dose produced a relatively large difference in electrical activity between the sexes. The bioassay results showed that males were attracted to decyl butyrate, whereas females did not show a preference between decyl butyrate and the control. Furthermore, the attractancy elicited in males by decyl butyrate alone was virtually no different from that with when it was mixed with an equal amount of decanol.

While the evidence for decyl butyrate acting as a pheromone is convincing, the same is not the case for decanol. The absence of decanol from the female aeration extracts without humidification, from extracts of filter papers exposed to females, and from homogenates of females suggests that it may not have been produced by the insects. In view of its close similarity in chemical structure to decyl butyrate, it was felt that it may have been formed by degradation of the ester. However, this was probably not so since none was found during the aeration of authentic decyl butyrate. Tests to establish the occurrence and precise role of decanol are under way and will be reported later.

In contrast to the pheromones produced by *A. verbasci* and *A. flavipes*, which are unsaturated carboxylic acids, the pheromone released by *A. sarnicus* is a saturated ester. Ma et al. (1980) estimated the release of pheromone from single *A. flavipes* at 200–300 ng/female/day using GC quantification of the pentafluorobenzyl derivative of (*Z*)-3-decenoic acid. Female *A. sarnicus* released about three to four times more per day, approximately 1130 ng/female/day. Armes (1985) found the female calling period was between 0800 and 1800 hr, with the greatest number of females calling between 1300 and 1500 hr. These periods corresponded to the period of maximum male response. It seems likely that the release rate of this pheromone may be controlled by a diel periodicity.

Previous studies with *Anthrenus* spp. (Burkholder et al., 1974; Armes, 1985) used more than one beetle per test or used the beetles more than once. These tests would not take account of beetle-to-beetle interactions or habituation to the pheromone source. In this study the use of individual insects rather than batches for bioassay allowed the response time and more detailed behavioral observations to be made readily.

This relatively simple chemical compound is unusual among stored-product beetle pheromones. Thistlewood et al. (1989) identified butyl butyrate and hexyl butyrate as major sexually dimorphic volatile components in the head and thorax of the mullein bug, *Campylomma verbasci*. However, neither ester alone nor in combination was found to be as attractive as a caged virgin female, suggesting the importance of minor components.

The only other report, to the best of our knowledge, of decyl butyrate and decanol being tested for attractancy (both individually and together) was to the rose chafer, *Macrodactylus subspinosus* (Coleoptera: Scarabaeidae) (Williams et al., 1990). They tested more than 60 compounds, including esters, aldehydes, and alcohols and found no significant difference between the attractancy of decyl butyrate and decanol during the early part of the test when compared to a standard mixture of equal amounts of valeric acid, hexanoic acid, and octyl butyrate. However, decyl butyrate became more attractive during the latter part of the test, and it was the only compound to improve its ranking during the test. Its relative attractancy went from 26% of the standard at the beginning of the test to 77% after three weeks. The authors suggested that this result may have been due to a seasonal variation in response of the beetles to the attractant.

Further work will be needed to establish the bioassay dose–response curve to decyl butyrate and thereby determine the male threshold level of behavioral response. The EAG results suggest the threshold for electrical response is between 1 ng and 10 ng of decyl butyrate. Determination of the period of male attractancy and behavioral studies to investigate the responses of the beetles to distant sources should be carried out. The results from these studies should indicate whether the material could be a practical monitoring aid for adult *A. sarnicus*.

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REFERENCES

ARMES, N.J. 1985. Biology and control of *Anthrenus sarnicus* Mroczkowski (Coleoptera: Dermestidae). PhD thesis. University of London.

ARMES, N.J. 1988. The seasonal activity of *Anthrenus sarnicus* Mroczkowski (Coleoptera: Dermestidae) and some other beetle pests in the museum environment. *J. Stored Prod. Res.* 24:29–37.

BURKHOLDER, W.E., and MA, M. 1985. Pheromones for monitoring and control of stored-product insects. *Annu. Rev. Entomol.* 30:257–272.

BURKHOLDER, W.E., MA, M., KUWAHARA, Y., and MATSUMURA, F. 1974. Sex pheromone of the furniture carpet beetle, *Anthrenus flavipes* (Coleoptera: Dermestidae). *Can. Entomol.* 106:835–839.

CHAMBERS, J. 1991. Overview on stored-product insect pheromones and food attractants. *J. Kans. Entomol. Soc.* 63:400–499.

CHAMBERS, J., MORGAN, C.P., WHITE, P.R., MORI, K., FINNEGAN, D.E., and PINNIGER, D.B. 1990. Rust-red grain beetle, *Cryptolestes ferrugineus*, and flat grain beetle, *Cryptolestes pusillus*: Antennal and behavioral responses to synthetic components of their aggregation pheromones. *J. Chem. Ecol.* 16:3353–3372.

FUKUI, H., MATSUMURA, F., MA, M., and BURKHOLDER, W.E. 1974. Identification of the sex pheromone of the furniture carpet beetle *Anthrenus flavipes* (Leconte). *Tetrahedron Lett.* 40:3563–3566.

HALSTEAD, D.G.H. 1975. Changes in the status of insect pests in storage and domestic habitats. Proceedings, First International Conference on Stored Product Entomology, Savannah, Georgia, 1974, pp. 142–153.

HALL, A.V. 1988. Pest control in herbaria. *Taxon* 37:855–907.

KUWAHARA, Y., and NAKAMURA, S. 1985. (*Z*)-5- and (*E*)-5-undecenoic acid: Identification of the sex pheromone of the varied carpet beetle *Anthrenus verbasci* (L.) (Coleoptera: Dermestidae). *Appl. Entmol. Zool.* 20:354–356.

MA, M., HUMMEL, H.E., and BURKHOLDER, W.E. 1980. Estimation of single furniture carpet beetle (*Anthrenus flavipes*, Leconte) sex pheromone release by dose-response curve and chromatographic analysis of pentafluorobenzyl derivative of (*Z*)-3-decenoic acid. *J. Chem. Ecol.* 6:597–607.

MROCZKOWSKI, M. 1962. *Anthrenus sarnicus* sp. n. (Col. Dermestidae) from the Island of Guernsey. *Ann. Mag. Nat. Hist.* 13(5):697–700.

NAIR, S.M. 1991. Problems of biodeterioration in natural history museums. *In* O.P. Agrawal and D. Shastic, (eds.). Biodeterioration of Cultural Property. Proceedings of the International Conference. February 20–25, 1991, Lucknow, India. Macmillan India Limited, New Dehli, pp. 35–44.

PINNIGER, D.B. 1990. Insect Pests in Museums. Archetype Publications, Denbigh, Clwyd.

THISTLEWOOD, H.M.A., BORDEN, J.H., SMITH, R.F., PIERCE, H.D., and MCMULLEN, R.D. 1989. Evidence for a sex pheromone in the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). *Can. Entomol.* 121:737–744.

WHITE, P.R., and CHAMBERS, J. 1989. The saw-toothed grain beetle *Oryzaephilus surinamensis*

(Coleoptera: Silvanidae): Antennal and behavioral responses to individual components and blends of aggregation pheromone. *J. Chem. Ecol.* 15:1015–1031.

WILLIAMS, R.N., MCGOVERN, T.P., KLEIN, M.G., and FICKLE, D.S. 1990. Rose chafer (Coleoptera: Scarabaeidae): Improved attractants for adults. *J. Econ. Entomol.* 83:111–116.

WOODROFFE, G.E. 1967. *Anthrenus sarnicus*. Mroczkowski (Coleoptera: Dermestidae) in Britain. *J. Stored Prod. Res.* 3:263–265.

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IDENTIFICATION OF COMPOUNDS IN AN HPLC FRACTION FROM FEMALE EXTRACTS THAT ELICIT MATING RESPONSES IN MALE SCREWWORM FLIES, *Cochliomyia hominivorax*[1]

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Abstract—When hexane extracts of mature screwworm females were chromatographed on a silica gel column, mating stimulant activity was concentrated in a fraction that eluted with hexane–ether (94:6, v/v). Separation of this fraction with HPLC (acetonitrile–acetone; 60:40, isocratic) resulted in a chromatogram of some 20 peaks. Only peaks 4–11 elicited mating responses. Peaks 5–10 had most of the activity, with peak 8 producing the highest response. Sixteen compounds were characterized from peak 8 by gas chromatography–mass spectrometry: six unbranched secondary acetates ($C_{31}H_{62}O_2$); seven previously unreported methyl-branched secondary acetates ($C_{32}H_{64}O_2$); one unbranched ketone ($C_{31}H_{62}O$); and one methyl-branched ketone ($C_{32}H_{64}O$). The isomeric acetates were not completely resolved from each other by capillary gas chromatography (CGC) on methyl silicone columns. The sixteenth compound was an aldehyde ($C_{30}H_{60}O$) that was present only in occasional peak 8 preparations. These compounds and several derivatives were characterized by capillary gas chromatography–mass spectrometry (CGC-MS). The position of the acetate group was ascertained by conversion to a keto group or by replacement of the acetate with a methyl group. Pheromone activity was not observed in peaks trapped either from CGC or by recombination of the trapped CGC peaks from HPLC peak 8. This apparent loss of activity from CGC peaks or from TLC cannot currently be explained.

Key Words—Screwworm, *Cochliomyia hominivorax*, Diptera, Calliphoridae, contact mating stimulant, sex pheromone, insect lipid, nonacosanyl-1-acetate,

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[1] Mention of a proprietary or commercial product does not constitute an endorsement by the U.S. Department of Agriculture.

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methylnonacosanyl acetate, triacontanone, methyltriacontanone, triacontanal, methyl-branched secondary acetate, methyl-branched ketone, aldehyde, gas chromatography, mass spectrometry.

INTRODUCTION

The screwworm (*Cochliomyia hominivorax*, Coquerel) is a serious pest of livestock, and infestation of animal wounds by screwworm larvae results in a condition called myiasis (Laake et al., 1936; Richardson et al., 1982). Although this insect is considered eradicated in the United States and Mexico as a result of the sterile male release program, it is still a major problem in Central and South America (Snow et al., 1985; Krafsur et al., 1987). Recent reports also placed the screwworm in North Africa, where both animal and human myiasis had been observed (El-Azazy, 1989; Gabaj et al., 1989). Successful application of the sterile insect technique has led to eradication in North Africa also.

Fletcher et al. (1966) described a male pheromone that stimulated wing fluttering, preening, and searching movements by females and body contacts among females, but had no effect on virgin males. Although the male pheromone has received no further attention, there is strong evidence for a female-produced sex recognition pheromone that stimulates copulatory attempts from males on contact (Mackley and Broce, 1981; Hammack, 1986). This behavior is elicited by hexane extracts of females. The female pheromone is required for mating, although there is strain variation in both pheromone production and reception, apparently correlated with duration of colonization (Hammack, 1987, 1991). Pheromone communication is relevant to control of the screwworm via sterile insect releases because success of the release program depends on the ability of mass-reared sterile males to mate wild females (Krafsur et al., 1987). Our study reports the isolation and characterization of chemicals in biologically active fractions purified from hexane surface washes of the adult female screwworm fly.

METHODS AND MATERIALS

Biological

Insects. The screwworm flies used for this study originated from a single egg mass collected in Chiapas, Mexico, in 1986 (FC96 strain). They were reared as previously reported (Hammack, 1987) and separated by sex within 24 hr after adult emergence to insure virginity.

Extraction and Bioassay of Pheromone Activity. Pheromone activity was extracted by surface washing 200 4- to 6-day-old adult females for 15 sec with 10 ml *n*-hexane in a 60-ml separatory funnel. Eleven such washes were pooled

to produce extracts containing 2200 female equivalents (FE). A 200-FE aliquot was removed from each extract, air-dried, and used routinely as a reference standard in bioassays to corroborate responsiveness of males tested one to two weeks after adult emergence. The remainder of each extract, consisting of 2000 FE, was similarly dried and retained for chemical separation and analyses. Samples were brought to the laboratory in hexane for bioassays and stored below −60°C between tests.

The bioassay was described previously (Hammack, 1987). It measured the presence or absence of copulatory attempts by individual test males paired in glass test tubes for 1 min with hexane-washed male decoys. These male decoys were treated with female extract or fractions isolated from the extract. At least five decoys were bioassayed per test sample, each with 10 males. Washed males treated only with hexane elicit no responses (Hammack, 1987).

Chemistry

Instrumentation. Infrared (IR) spectra were obtained with a Nicolet model 5MX spectrometer. CGC procedures used a cross-linked methylsilicone fused silica column (12.5 m × 0.2 mm ID, 0.33 μm film) in a Varian model 3700 with a flame ionization detector. Samples were injected as solutions via a Grob injector (fitted with a glass frit insert) in the split mode. The carrier gas was He at a flow rate of 0.77 ml/min (20 cm/sec) and a back-pressure valve gauge reading of 12.5 psig. Preparatory CGC (PREPCGC) utilized a cross-linked methylsilicone fused silica megabore column (15 m × 0.53 mm ID, 1.2 μm film) in the same instrument. Samples were injected directly onto the column using He as carrier (3 ml/min), and the column effluent was split using a Scientific Glass and Engineering Co. variable splitter (90% collected, 10% to the flame). Electron impact (EI) mass spectra were obtained with a Hewlett-Packard model 5970 MSD interfaced to a model 5890 gas chromatograph and a model 59970 Chem Station (EI-CGC-MS). A cross-linked methylsilicone CGC column (12.5 m × 0.2 mm, 0.33 μm film) was used with a Grob injector (split mode), a He flow rate of 10 ml/min and with a back-pressure valve gauge showing 10 psig. Direct-probe EI and direct-probe chemical ionization mass spectra (DCI-MS) were obtained on a Varian-MAT CH5 double-focusing mass spectrometer using isobutane or ammonia as the reagent gases.

Nuclear magnetic resonance spectra (NMR) were obtained on a Brucker model AM400 spectrometer. All NMR spectra were determined from a sample prepared from 3500 FE of HPLC peak 8 rechromatographed on a C-8 RPHPLC column and dissolved in 35 μl $CHCl_3$. Fully coupled proton spectra (PMR) were obtained at a spectrometer frequency of 400.134 MHz, an observation frequency of 6000.00 Hz with a 5000 Hz sweep width at 21°C. Carbon-13 spectra (CMR) were obtained at a spectrometer frequency of 100.614 MHz, an observation

frequency of 4000.00 Hz with a sweep width of 20,833 Hz at 21°C. The CMR 135° DEPT (multiplicity experiment) was obtained from the same sample at 100.622 MHz at a sweep width of 20,000.00 Hz and observation frequency of 4000.00 Hz.

Isolation of Pheromone Activity. Typically, a crude extract containing 2000 FE in hexane (1.6 ml followed by 2 × 1.6 ml washes) was chromatographed on a heat-activated silica gel column (150°C for 90 min; 63–100 μm mesh, 8 × 100 mm, 4.4 ml void volume). The column was eluted with a step gradient of hexane–diethyl ether (100:0, 98:2, 96:4, 94:6, · · · , 80:20) then finally hexane–ethanol 90:10 in 4.4-ml fractions. Each fraction was bioassayed at 1.0 FE within two weeks of fractionation. Assays at 2.0 and 5.0 FE were completed within six weeks. The 94:6 fraction contained nearly all of the activity and was further fractionated on a reverse phase 5-μm C-18 Nova-Pak (Waters Assoc.) column using a RPHPLC system with 60:40 acetonitrile–acetone as a solvent at 1.0 ml/min. A refractive index detector (RID) indicated approximately 20 RPHPLC peaks that were each bioassayed within five weeks of the initial silica gel separation at 12 FE. Two-dimensional high-performance thin-layer chromatography (HPTLC) of biologically active RPHPLC peak 8 separated the acetates from the ketones and from the aldehyde (94:6 hexane–ether once in the first dimension; and five passes with 94:6 hexane–benzene in the second dimension).

RESULTS AND DISCUSSION

When a hexane extract of adult screwworm females was eluted from silica gel, mating activity was concentrated in fraction 4 (hexane–ether, 94:6; v/v; Table 1). The mass of fraction 4 is 4.3% of the total mass of the surface waxes. When fraction 4 was further fractionated using C-18 RPHPLC, activity was mostly in peaks 5–10 (Figure 1). For this study, we confined our efforts to isolation and identification of the compounds in RPHPLC peak 8, which elicited the highest male responses (Figure 1). The solutes had no useful ultraviolet absorbance, limiting RPHPLC to isocratic conditions and to refractive index monitoring of the effluent. Rechromatography of RPHPLC peak 8 on the same C-18 column or on a C-8 column using the same solvent system or with small incremental increases of the acetonitrile did not improve resolution. RPHPLC peak 8 had a mass of 400 μg from 500 females or 800 ng per fly. CGC of peak 8 gives several peaks the mass equivalents of which are listed in Table 2.

A direct-probe mass spectrum of RPHPLC peak 8 obtained after it was first isolated is shown in Figure 2. The spectrum showed an apparent molecular ion of 420 and a fragment ion series suggested olefinic characteristics. DCI-MS of peak 8 using either isobutane or ammonia as the reagent gas gave conflicting

TABLE 1. MALE RESPONSE TO CRUDE EXTRACT OF FEMALES AND TO FRACTIONS PRODUCED BY ELUTING CRUDE EXTRACT FROM A SILICA GEL COLUMN WITH INCREASINGLY POLAR SOLVENT

Test sample		μg/Fly	Mean (± SE)[a] percentage attempting copulation			
			Test dose (FE)			
			0.5	1.0	2.0	5.0
Crude extract			77 ± 4			
Fr 1	100% H[b]	40.6		0		0
Fr 2	2% E/H	4.6		0		0
Fr 3	4% E/H	0.6		0		0
Fr 4	6% E/H	3.4		68 ± 9	94 ± 4	84 ± 5
Fr 5	8% E/H	0.6		0		0
Fr 6	10% E/H	0.8		0		0
Fr 7	12% E/H	0.8		2 ± 2	2 ± 2	30 ± 9
Fr 8	14% E/H	0.8		0	0	10 ± 3
Fr 9	16% E/H	1.6		0		0
Fr 10	18% E/H	0.8		0		0
Fr 11	20% E/H	24.8		0		0
Fr 12	10% Et/H			0		0

[a] Variation among responses to a minimum of five decoys, each bioassayed with 10 males.
[b] H = hexane; E = diethylether; Et = ethanol, all volume-volume.

data. Isobutane provided a spectrum with an apparent molecular ion of *m/z* 421 (420 + 1; 100%) whereas ammonia gave an apparent ion of *m/z* 498 (either 497 + 1 or 480 + 18, i.e., 480 + NH_4; 100%).

The IR spectrum of peak 8 was fairly simple in appearance: the spectrum showed bands for methyl group at 2955, 2870, 1450, 1377 cm^{-1} (all *m*); for methylene at 2924 (*s*), 2852 (*s*), 1456 (*m*), and 722 (*w*) cm^{-1}; for carbonyl at 1742 (*m-s*), 1242 (*m-s*), and 1108 (*w*) cm^{-1} (Colthup et al., 1964; Silverstein et al., 1981). These data suggest an ester and, more specifically, an acetate ester with more than four adjacent methylene groups. Upon reexamination of the solid probe mass spectrum (Figure 2), a small peak at *m/z* 61 (<3%) along with a peak at *m/z* 43 (100%) were found. Both ions are diagnostic of acetate (Budzikiewicz et al., 1967; Silverstein et al., 1981). However, primary acetates yield fairly intense ions at *m/z* 61 (42% for docosanyl-1-acetate), suggesting that peak 8 contained compounds having either secondary or tertiary acetates. We synthesized a series of secondary acetates (2-, 3-, · · · , 8-pentadecanyl acetates) and found that indeed the *m/z* 61 ion for these compounds was always weak (<5% of base). The MS of the synthetic secondary acetates showed a lack of a molecular ion and did have an M−60 ion as the highest mass ion in

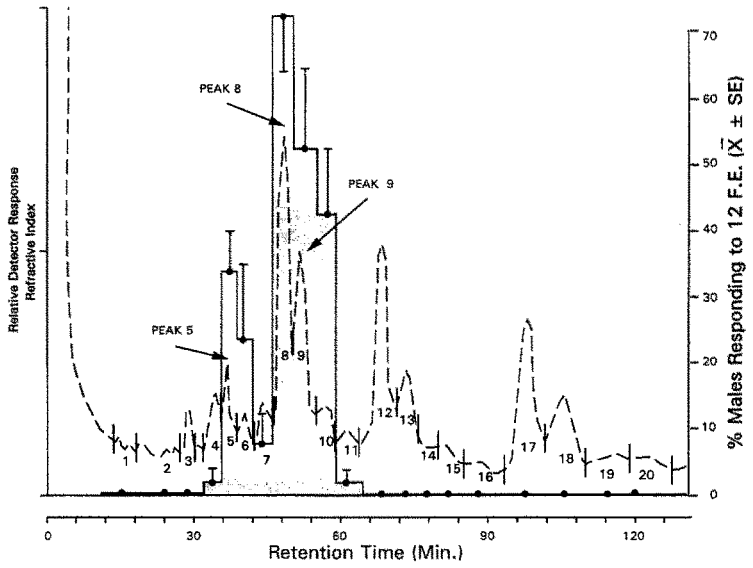


FIG. 1. Separation of fraction 4 into 20 RPHPLC peaks and bioassay responses to the peaks. The refractive index trace (dotted) resulted from 1500 FE injected at sensitivity setting of 4×. The solvent was 60:40 (v/v) acetonitrile–acetone at 1 ml/min using a 5 μm Nova-pak column. For each peak, the solid line indicates the mean (±SE) mating response to five decoys, each bioassayed with 10 males. The vertical slashes on the RPHPLC trace indicate where fractions were cut.

TABLE 2. ESTIMATED QUANTITIES OF MASS OF CGC PEAKS OF RPHPLC PEAK 8 OF SILICA COLUMN FRACTION 4[a]

Entry[b]	Mass (ng/fly)
I-XIII	611
XVI	126
IV	42
XV	23

[a] Based on detector response to 1 μg of hydrocarbon standards with approximately the same retention times.
[b] From Table 4.

the spectrum, suggesting that the *m/z* 420 peak in the MS of peak 8 was possibly also an M−60 peak.

A series of chemical transformations, summarized in Scheme 1, was performed on RPHPLC peak 8 in order to characterize the chemical nature of the

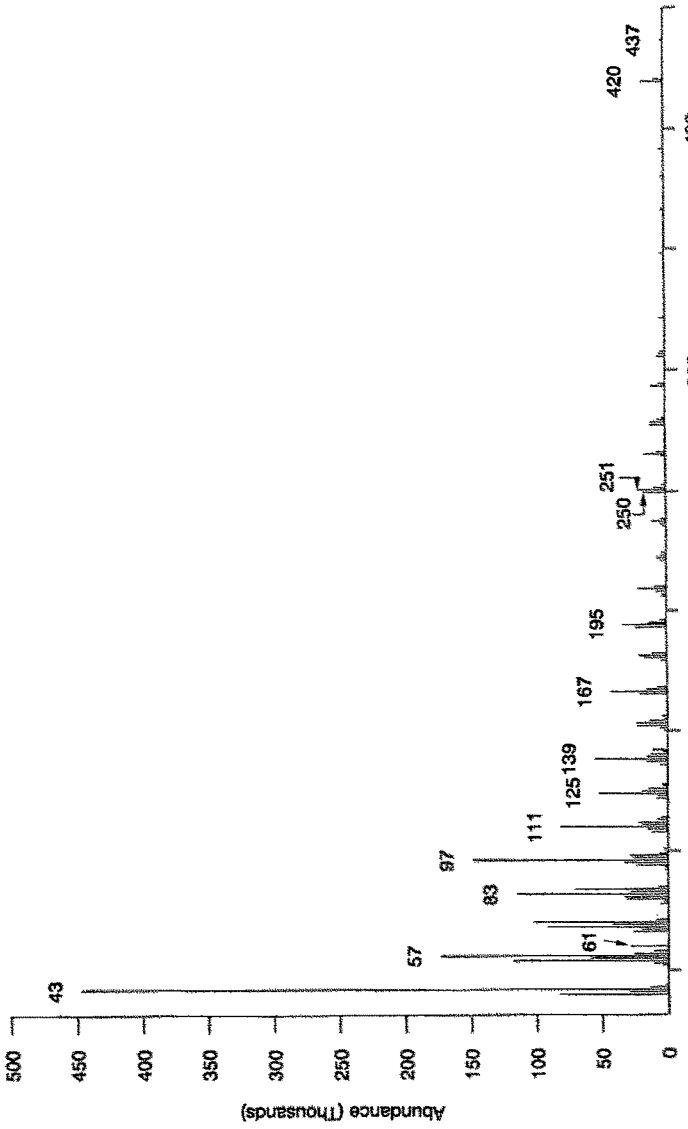
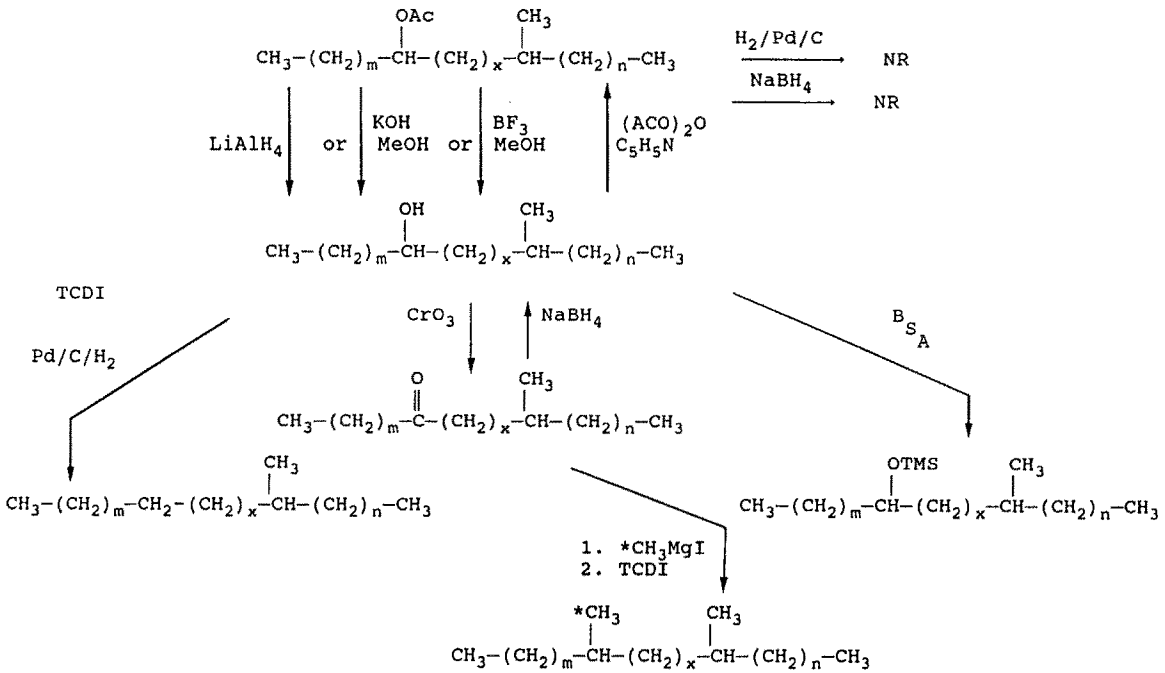


FIG. 2. Direct-probe 70-eV EI-MS of RPHPLC peak 8.



m = 1, 2, 3, 4, 5
x = variable (dependent on m & n)
n = 7, 9, 11, 13
* = either ^{12}C or ^{13}C
TCDI = thiocarbonyldiimidazole
NR = no reaction

SCHEME 1.

compounds in the peak (although the peak is a mixture of three classes of compounds, the transformations are shown only for the acetates since they were generally present in the largest quantity). $LiAlH_4$ reduction of peak 8 gave a GC-MS profile nearly identical to the unreduced substance, although the HPTLC profiles were entirely different. The reduced substance migrated much slower on silica HPTLC (benzene) having an R_f of 0.23 as compared to 0.64 for peak 8 (unreduced substance). Reduction with $NaBH_4$ or hydrogenation with palladium–carbon did not significantly change the original substance as determined by HPTLC or CGC-MS. The reaction products of peak 8 with BF_3/MeOH, or potassium hydroxide hydrolysis, or reduction with $LiAlH_4$ gave identical mass spectra and also had the same R_f on silica gel plates (R_f = 0.23, benzene). Acetylation of the products generated a product whose mass spectrum was identical to the starting material.

The IR and CGC-EI-MS spectral data [including selected ion monitoring (SIM)] as well as the width of the main CGC peak of peak 8 suggested that this peak is a mixture of long-chain secondary alcohol acetates. The ion at m/z 420 is not the molecular ion but the M−60 ion by analogy to the MS of the synthetic acetates. The NH_4^+ CI-MS also corroborated a molecular ion of 480 for the unknown. The reagent gas isobutane provides a stronger Lewis acid, which favors the deacetylation process over the adduct with the parent molecule. However, with ammonia as reagent gas, the NH_4^+ ion (a much weaker Lewis acid) gives an adduct of 18 mass units greater than the molecular weight at 480 + 18 = 498 as essentially the only peak at high mass in the CI-MS. Assuming a molecular weight of 480 and the presence of only one acetate group, we proposed a molecular formula of $C_{32}H_{64}O_2$ for the mixture of acetates.

Thermal deacetylation or dehydration is known to occur in gas chromatography and mass spectrometry of acetates or alcohols, respectively (Budzikiewicz et al., 1967). This fact explains the ion at m/z 420 and the similarities in the MS of the unknown and the $LiAlH_4$ reduced product as well as the BF_3/MeOH transesterification product. Deacetylation of the acetates and dehydration of the corresponding alcohols would give nearly identical mass spectra and the series of unsaturated hydrocarbon ions in the MS mentioned earlier.

The 400-MHz proton magnetic resonance (PMR) and ^{13}C magnetic resonance (CMR) spectra of rechromatographed (RPHPLC) peak 8 (3500 FE) clarified the structural nature of the mixture of acetates. Although an aldehyde and at least two ketones were also present in the mixture, they were not observed, probably due to their low concentration. Relevant features in the PMR were: a doublet at 0.81 ppm, which integrated to three protons (3 p), suggesting a methyl branch in the chain; a triplet at 0.83 ppm (6 p), suggesting two terminal methyl groups; a large peak at 1.23 ppm (44 p), suggesting a number of methylene units (cf., the infrared above); a multiplet observed at 1.47 ppm (4 p), indicating methylene protons adjacent to a methine of the secondary alcohol acetate (off

resonance decoupling of this peak verified that it was coupled to the methine proton bearing the acetate); a singlet (2.01 ppm, 3 p) for a methyl of an acetyl group; and a multiplet (4.82 ppm, 1 p) for a methine of the acetylated secondary alcohol.

The CMR of rechromatographed peak 8 was recorded as both the proton-decoupled spectrum and the multiplicity (DEPT) spectrum. The results are summarized in Table 3. The assignment of the chemical shifts for the above PMR and CMR resonances were based on literature references and to those of synthetic model compounds (Pomonis et al., 1989). Notable in the CMR spectra were resonances that could be assigned to acetyl methyl, terminal methyl, and to a branched methyl (methyl attached to methine).

Microscale oxidation (CrO_3/celite) (Schwartz and Osman, 1977) of the secondary alcohols from either saponification or $LiAlH_4$ reduction of the acetates gave a series of isomeric and homologous ketones whose structures could be determined by CGC-MS (Scheme 1; column 4 in Table 3; Figure 3). The position of the ketone substituent could be ascertained from the mass spectra and hence, by inference, the original position of the acetate. Unfortunately, in the compounds of the mixture, the relative positions of the methyl branch, which was shown to be present by PMR and CMR, could not be determined from the ketone mass spectra (Silverstein et al., 1981). Likewise, the TMS derivatives of the alcohols from reduction or hydrolysis of the acetates yield MS that fix only the position of the hydroxyl group and not the methyl groups.

Reduction (Scheme 1) of the alcohols (from saponification or reduction of the acetates) to the methylene group with thiocarbonyldiimidazole (TCDI) followed by hydrogenation with palladium on carbon (Rasmussen et al., 1981) yield methylalkanes whose CGC-MS profiles characteristically locate the position of the methyl substituent (Pomonis et al., 1980; Pomonis, 1989).

Although the positions of the acetate and the methyl substituents were established by the preceding experiments, the position of the two substituents relative to each other remained ambiguous because the substance was a mixture of isomeric methyl-branched acetates. To establish their relative positions, peak 8 was reduced with $LiAlH_4$ to produce the secondary alcohols that were then oxidized with CrO_3. The resulting ketones were then reacted with either [^{13}C]methylmagnesiumbromide or the corresponding unlabeled Grignard reagent. The product, which contained a mixture of tertiary alcohols was reduced with TCDI and H_2/Pd to yield the dimethyl alkanes (column 6 in Table 4). The position of the acetate is thus established relative to the "native" methyl group by substitution with an unlabeled or a [^{13}C]methyl group. The ^{13}C-labeled methyl group has a mass defect of +1 in the mass spectra and allowed us to differentiate between those fragments containing the native methyl and the labeled methyl group. The use of both [^{12}C]- and [^{13}C]methyl substituents was necessary in those instances when the methyl group was introduced in positions 5–8. The

TABLE 3. CARBON MAGNETIC RESONANCES (CMR) AND THEIR STRUCTURAL ASSIGNMENTS FROM 3500 FEMALE EQUIVALENTS OF HPLC PEAK 8 FROM SCREWWORM EXTRACT

Peak	Chemical shift (ppm)	DEPT experiment[a]	Assignment	
1	14.13	−	CH_3-CH_2-	Terminal methyl
2	19.74	−	$CH_3-\underset{\displaystyle |}{CH}-$	Methine methyl
3	21.30	−	$CH_3-\overset{\displaystyle \|\|}{\underset{\displaystyle O}{C}}-$	Acetyl methyl
4	22.71	+	$-CH_2-CH_2-CH_3$	Methylene adjacent to terminal methyl
5	25.34	+	?	Methylene
6	29.23	+	$-CH_2-CH_2-CH_2$	Methylene
7	29.38	+	$CH_2-CH_2-CH_2-$	Methylene
8	29.52	+	$-CH_2-CH_2-CH_2-$	Methylene
9	29.57	+	$-CH_2-CH_2-CH_2-$	Methylene
10	29.60	+	$-CH_2-CH_2-CH_2-$	Methylene
11	29.68	+	$-CH_2-CH_2-CH_2-$	Methylene
12	29.78	+	$-CH_2-CH_2-CH_2-$	Methylene
13	30.06	+	$-CH_2-CH_2-CH_2-$	Methylene
14	31.76	+	$-CH_2-\underset{\displaystyle OAc}{\underset{\displaystyle \|}{CH}}-CH_2-$	Methylene α to methine-bearing acetate
15	31.95	+	$-CH_2-CH_2-CH_3$	Methylene β to methyl
16	32.77	−	$-CH_2-\underset{\displaystyle CH_3}{\underset{\displaystyle \|}{CH}}-CH_2-$	Methine-bearing methyl
17	34.14	+	?	?
18	37.12	+	$-CH_2-\underset{\displaystyle CH_3}{\underset{\displaystyle \|}{CH}}-CH_2-$	Methylene α to methine-bearing methyl
19	74.48	−	$-CH_2-\underset{\displaystyle OAc}{\underset{\displaystyle \|}{CH}}-CH_2-$	Methine-bearing acetate
20	170.95	+	$-O-\overset{\displaystyle \|\|}{\underset{\displaystyle O}{C}}-CH_3$	Carbonyl carbon

[a] Direction of resonance: + = positive y, indicates carbon bearing 0, 2, 4 hydrogens; − = negative y, indicates carbon bearing 1, 3 hydrogens.

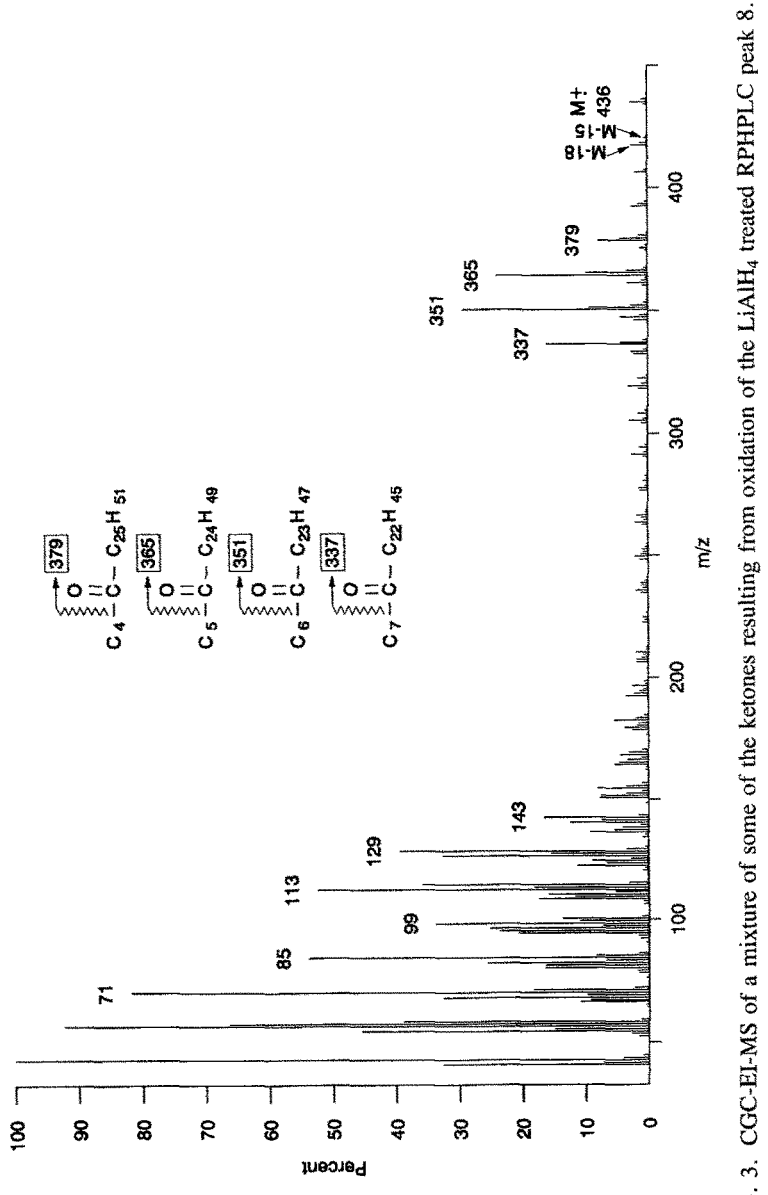


FIG. 3. CGC-EI-MS of a mixture of some of the ketones resulting from oxidation of the $LiAlH_4$ treated RPHPLC peak 8.

TABLE 4. SUMMARY OF PARENT COMPOUNDS AND THEIR DERIVATIVES PRESENT IN FEMALE SCREWWORM (*Cochliomyia hominivorax*) HPLC PEAK 8[a]

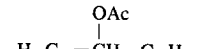
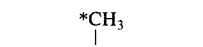
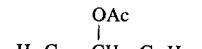
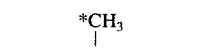
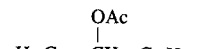
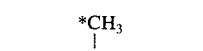
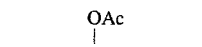
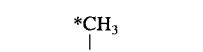
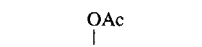
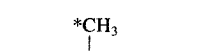
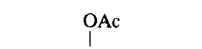
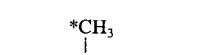
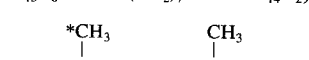
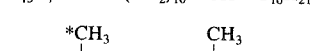
Entry	Parent compound in pheromone mixture[b]		Ketone[c]		Methyl alkane[d]	
I		$(C_{31}H_{62}O_2)$		$(C_{29}H_{58}O)$		$(C_{30}H_{62})$
II		$(C_{31}H_{62}O_2)$		$(C_{29}H_{58}O)$		$(C_{30}H_{62})$
III		$(C_{31}H_{62}O_2)$		$(C_{29}H_{58}O)$		$(C_{30}H_{62})$
IV		$(C_{31}H_{62}O_2)$		$(C_{29}H_{58}O)$		$(C_{30}H_{62})$
V		$(C_{31}H_{62}O_2)$		$(C_{29}H_{58}O)$		$(C_{30}H_{62})$
VI		$(C_{31}H_{62}O_2)$		$(C_{29}H_{58}O)$		$(C_{30}H_{62})$
VII		$(C_{32}H_{64}O_2)$		$(C_{30}H_{60}O)$		$(C_{31}H_{64})$
VIII		$(C_{32}H_{64}O_2)$		$(C_{30}H_{60}O)$		$(C_{31}H_{64})$
IX		$(C_{32}H_{64}O_2)$		$(C_{30}H_{60}O)$		$(C_{31}H_{64})$

TABLE 4. Continued

Entry	Parent compound in pheromone mixture[b]		Ketone[c]		Methyl alkane[d]	
X	$H_{11}C_5-CH(OAc)-(CH_2)_{12}-C(CH_3)-C_{10}H_{21}$	$(C_{32}H_{64}O_2)$	$H_{11}C_5-C(=O)-(CH_2)_{12}-CH(CH_3)-C_{10}H_{21}$	$(C_{30}H_{60}O)$	$H_{11}C_5-CH(*CH_3)-(CH_2)_{12}-CH(CH_3)-C_{10}H_{21}$	$(C_{31}H_{64})$
XI	$H_9C_4-CH(OAc)-(CH_2)_{13}-CH(CH_3)-C_{10}H_{21}$	$(C_{32}H_{64}O_2)$	$H_9C_4-C(=O)-(CH_2)_{13}-CH(CH_3)-C_{10}H_{21}$	$(C_{30}H_{60}O)$	$H_9C_4-CH(*CH_3)-(CH_2)_{13}-CH(CH_3)-C_{10}H_{21}$	$(C_{31}H_{64})$
XII	$H_9C_4-CH(OAc)-(CH_2)_{17}-CH(CH_3)-C_6H_{13}$	$(C_{32}H_{64}O_2)$	$H_9C_4-C(=O)-(CH_2)_{17}-CH(CH_3)-C_6H_{13}$	$(C_{30}H_{60}O)$	$H_9C_4-C(*CH_3)-(CH_2)_{17}-CH(CH_3)-C_6H_{13}$	$(C_{31}H_{64})$
XIII	$H_5C_2-CH(OAc)-(CH_2)_{19}-CH(CH_3)-C_6H_{13}$	$(C_{32}H_{64}O_2)$	$H_5C_2-C(=O)-(CH_2)_{19}-CH(CH_3)-C_6H_{13}$	$(C_{30}H_{60}O)$	$H_5C_2-CH(*CH_3)-(CH_2)_{19}-CH(CH_3)-C_6H_{13}$	$C_{31}H_{64})$
XIV	$H_{31}C_{15}-C(=O)-C_{15}H_{31}$[e]	$(C_{31}H_{32}O)$			$H_{31}C_{15}-CH(*CH_3)-C_{15}H_{31}$	$(C_{32}H_{66})$
XV	$H_{13}C_6-C(=O)-(CH_2)_{13}-CH(CH_3)-C_{10}H_{21}$[e]	$(C_{32}H_{64}O)$			$H_{13}C_6-CH(*CH_3)-(CH_2)_{13}-CH(CH_3)-C_{10}H_{21}$	$(C_{33}H_{68})$
XVI	$C_{29}H_{59}-CHO$[f]	$(C_{30}H_{60}O)$				

[a] HPLC peak 8 consistently showed activity in the pheromone bioassay.
[b] Pheromone activity resides in this fraction (HPLC peak 8).
[c] The keto derivative was obtained by first reducing the natural product with $LiAlH_4$ to the secondary alcohol, which was then oxidized to the ketone.
[d] The asterisk (*) in the structure indicates the methyl carbon added by Grignard reaction to the ketone. The alkane was obtained from the Grignard product after reduction of the alcohol to the methine. Both the carbon-12 and carbon-13 methyl derivatives were synthesized to establish the ketone position relative to any other functional group or substituent, i.e., another methyl substituent.
[e] The ketones indicated by this footnote were present in the natural product.
[f] The aldehyde appears in occasional RPHPLC peak 8 samples.

MS cleavage about the newly introduced [^{13}C]methyl branch point provided fragment ions that were not easily differentiated from the neighboring peaks, especially in the low mass region of the spectrum (*m/z* 40–115 amu range, see discussion below).

The mass spectra of three of several screwworm methyl-branched acetates and their methylalkane derivatives are shown in Figures 4A and 5A. Interpretation of these spectra was aided by determining the spectra of several synthetic methyl-branched acetates (Figure 6 shows only one of these). The molecular ion is not observed by EI but is observed by ammonia CI for the screwworm acetates (mol wt 480; $C_{32}H_{64}O_2$). Proposed structures for the fragment ions found in the EI-MS for a synthetic reference (Figure 6) and these three acetates (Figures 4A and 5A) are summarized in Table 5. The base peak in all cases (acetates) is due to a combination of the acylium and the C_3H_7 ions at nominal *m/z* 43. The protonated acetic acid ion at *m/z* 61 is typically weak for secondary acetates and may be easily overlooked. The ion due to fragmentation of the carbon–carbon bond internal to the acetate-bearing carbon yields an ion whose intensity rapidly decreases with increasing size of the mass of the fragment, i.e., intensity decreases as the acetate substituent is changed progressively from the 2 position to the 7 position and may not be visible in many cases beyond the 7 position (unpublished observations by the authors from MS studies of the entire series of 2- to 8-pentadecanol acetates). These ions appear at *m/z* 129 in Figure 4A, at *m/z* 143 in Figure 5A, and at *m/z* 227 at low intensity for the synthetic acetate (Figure 6). The position of the native methyl substituent in the screwworm acetates is suggested by the ions at *m/z* 167, 278, and 294 but is delineated with confidence only after conversion to the dimethyl derivatives and study of the spectra of several synthetic methyl-branched acetates.

The mass spectra of the dimethyl derivatives (Figures 4B, C and 5B, C) clearly show the positions of the native methyl branch relative to the position of the acetate, which was converted to the new [^{12}C]-/[^{13}C]methyl group (Scheme 1). The original methyl group is found to be in position 19 (numbering, by convention, from the terminal methyl group nearest the acetate group) in the dimethylalkanes because the diagnostic peaks at *m/z* 168 and *m/z* 169 do not show a mass shift in the ^{13}C-derived spectrum (compare spectra in Figure 4B and 4C). In the spectrum in Figure 4B the introduced methyl group is shown to be in position 5. The ions due to scission about the introduced methyl substituent appear at *m/z* 84 and *m/z* 85 with an enhanced *m/z* 84. In the ^{13}C derivative (spectrum in Figure 4C) the ions appear at *m/z* 85 and *m/z* 86 with an enhanced *m/z* 86. In the spectrum in Figure 5, which is from a mixture of two compounds, the peaks due to the introduced methyl groups appear at *m/z* 98 and *m/z* 99 and at *m/z* 112 and *m/z* 113 for the unlabeled derivatives and at *m/z* 99 and *m/z* 100 and *m/z* 113 *m/z* 114 for the ^{13}C-labeled methyl derivatives, respectively. Often these groups of peaks may go unnoticed or may not be obvious without the aid of both the carbon-12 and carbon-13 derivatization.

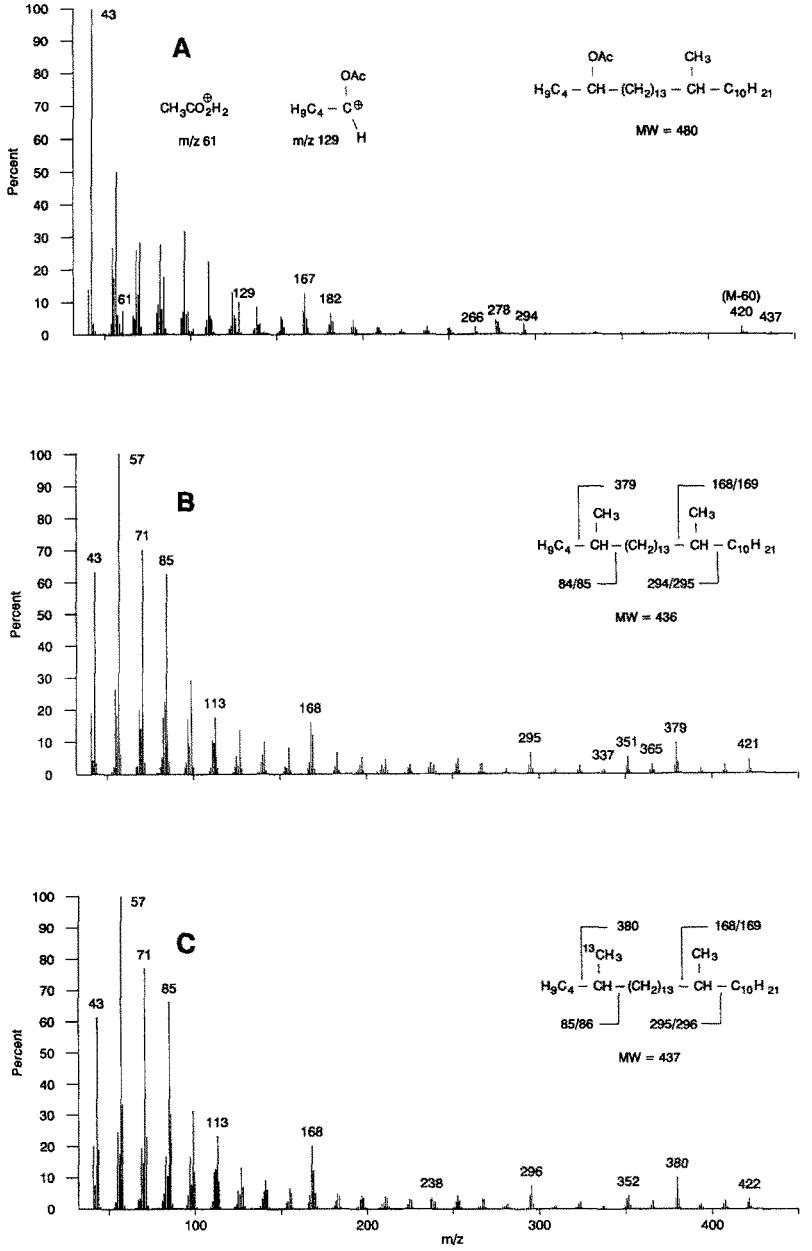


FIG. 4. CGC-EI-MS of: (A) 5-acetoxy-19-methyl-nonacosane (RPHPLC peak 8). Retention time (R_t) = 21.24 min (Figure 8A). (B) The ^{12}C derivative of 5,19-dimethylnonacosane. (C) The ^{13}C derivative of 5,19-dimethylnonacosane.

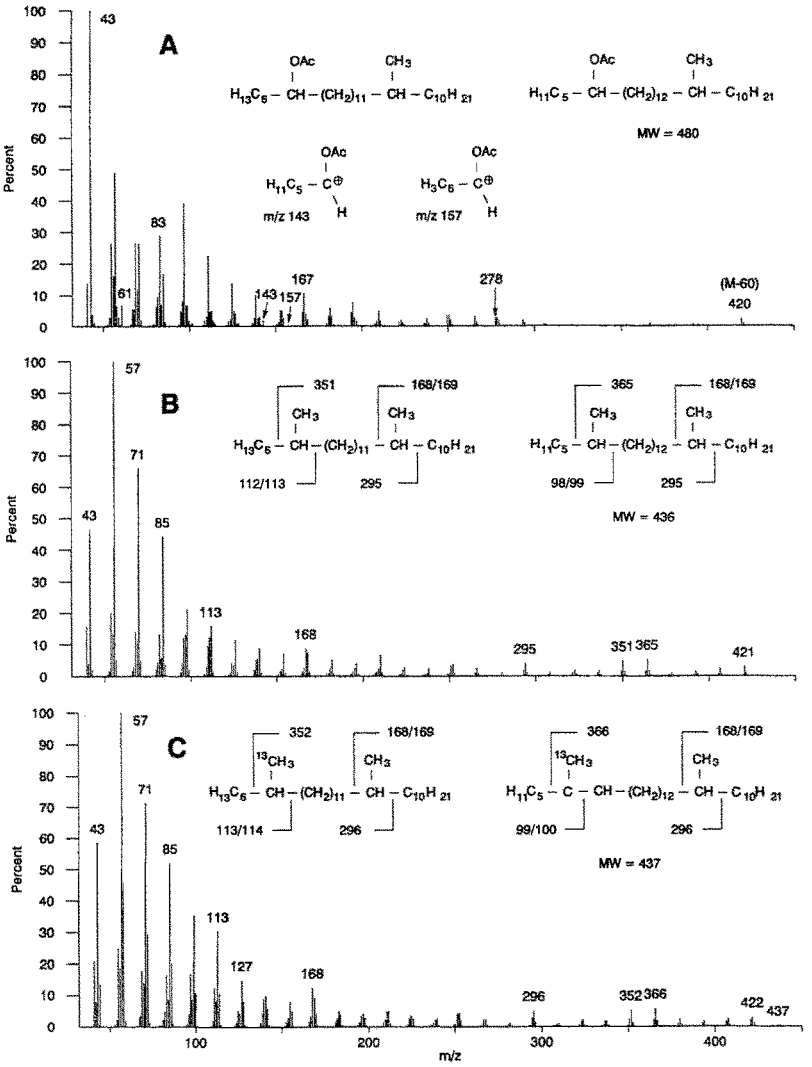


FIG. 5. CGC-EI-MS of: (A) Mixture of 6- and 7-acetoxy-19-methylnonacosane (HPLC peak 8). R_t = 21.02 min (Figure 8A). (B) The ^{12}C derivatives of Figure 5A. (C) The ^{13}C derivatives of Figure 5A.

Other compounds associated with active RPHPLC peak 8 were identified. There were several unbranched acetates (entries I–VI, Table 4) found in the leading edge (20.65–21.05 min) of the main peak in the total ion chromatogram (TIC) shown in Figure 8A. Some ketones (Figure 7A,B) were associated with

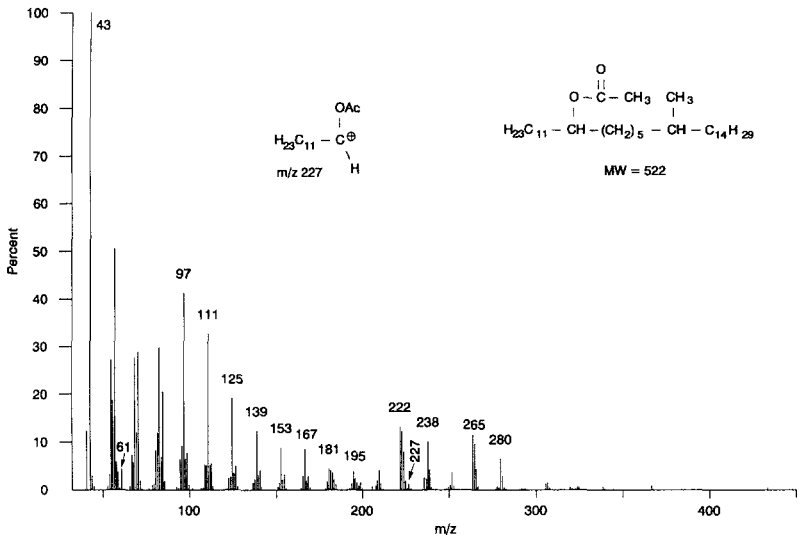
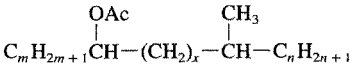


FIG. 6. EI-MS of a synthetic methyl-branched secondary acetate: 12-acetoxy-18-methyldotriacontane ($C_{35}H_{70}O_2$) (Table 4).

the minor peaks such as in Figure 8A (21.8–22.45 min). One ketone was a long-chain symmetrical ketone (16-hentriacontanone; entry XIV, Table 4, Figure 8A) and the second was a methyl-branched ketone: 21-methyl-7-hentriacontanone (XV, Figure 8B). Entry XVI, an aldehyde, was detected in occasional extracts, often in substantial amounts. A TIC of a sample containing the aldehyde accompanied by the selector ion monitor (SIM) chromatogram is shown in Figure 8 and the CGC-EI-MS in Figure 9. The presence of the aldehyde in occasional extracts is interesting and cannot be presently explained. It has been suggested that this compound may have been transferred to the female by male flies before the flies were separated by sex and may act as a pheromone inhibitor. Male-produced inhibitors of pheromones have been demonstrated to be transferred during mating in other insects (e.g., Carlson and Mackley, 1985; Scott et al., 1988; Carlson and Schlein, 1991). We do not believe that this is so in our case because silica column fraction 4 of surface washes of 500 males do not contain detectable RPHPLC peaks similar to those found in analogous female extracts, nor is there recoverable activity in the other column fractions (Pomonis, unpublished). Hammack (private communication) has also shown that 1.0 equivalent of male extract, when combined with 1.0 FE and applied to bioassay decoys, fails to diminish responses. In addition, both virgin females and those inseminated 24–48 hr previously are equally stimulatory (Hammack, 1987).

Hydrocarbons are the compounds most frequently implicated as dipteran

TABLE 5. STRUCTURES[a] AND MASSES OF SELECTED IONS FROM ELECTRON IMPACT MASS SPECTRA OF LONG-CHAIN METHYL-BRANCHED SECONDARY ACETATES FROM SCREWWORM



	Entry			
	Synthetic standard[b]	IX[c]	X[c]	XI
	$m = 11$	$m = 6$	$m = 5$	$m = 4$
	$x = 5$	$x = 11$	$x = 12$	$x = 13$
	$y = 14$	$n = 10$	$n = 10$	$n = 10$
$CH_3C{\equiv}O^+$	m/z 43(100%)	m/z 43(100%)	m/z 43(100%)	m/z 43(100%)
$CH_3COOH_2^+$	61(5)	61(6)	61(6)	61(7)
$C_mH_{2m+1}-\underset{\displaystyle OAc}{\underset{\vert}{CH}}\oplus$	227(1)	157(< 1)	143(< 1)	129(10)
$C_nH_{2n-1}-\underset{\displaystyle CH_2}{\underset{\vert}{CH}}\oplus$	223(13)	167(10)	167(10)	167(12)
$C_nH_{2n+1}-\underset{\displaystyle CH_2}{\underset{\vert}{CH}}-CH_2\overset{\bullet}{\oplus}$	238(10)	182(6)	182(6)	182(6)
$C_mH_{2m+1}-\underset{\displaystyle \cdot H_3C-C}{\underset{\vert}{C}}\oplus(CH_2)_x$	264(12)	278(3)	278(3)	278(4)
$C_mH_{2m+1}-\underset{\displaystyle \cdot CH-CH-CH_3}{\underset{\vert\quad\vert}{\overset{\oplus}{CH}-(CH_2)_x}}$	280(8)	294(2)	294(2)	294(3)
$M-CH_3COOH$ $(M-60)$	462(3)	420(2)	420(2)	420(2)

[a] We do not mean to imply that the structures of the ions are those shown. The structures shown are only convenient representations for that fragment and are consistent with the nominal masses. OAc = acetate.

[b] Synthesized from the corresponding acylated alkylthiophene by Raney Ni desulfurization and subsequent hydrogenation.

[c] Entries IX and X are both present in one spectrum. Thus the percent of base peak intensities results from contribution by both compounds to a common ion fragment.

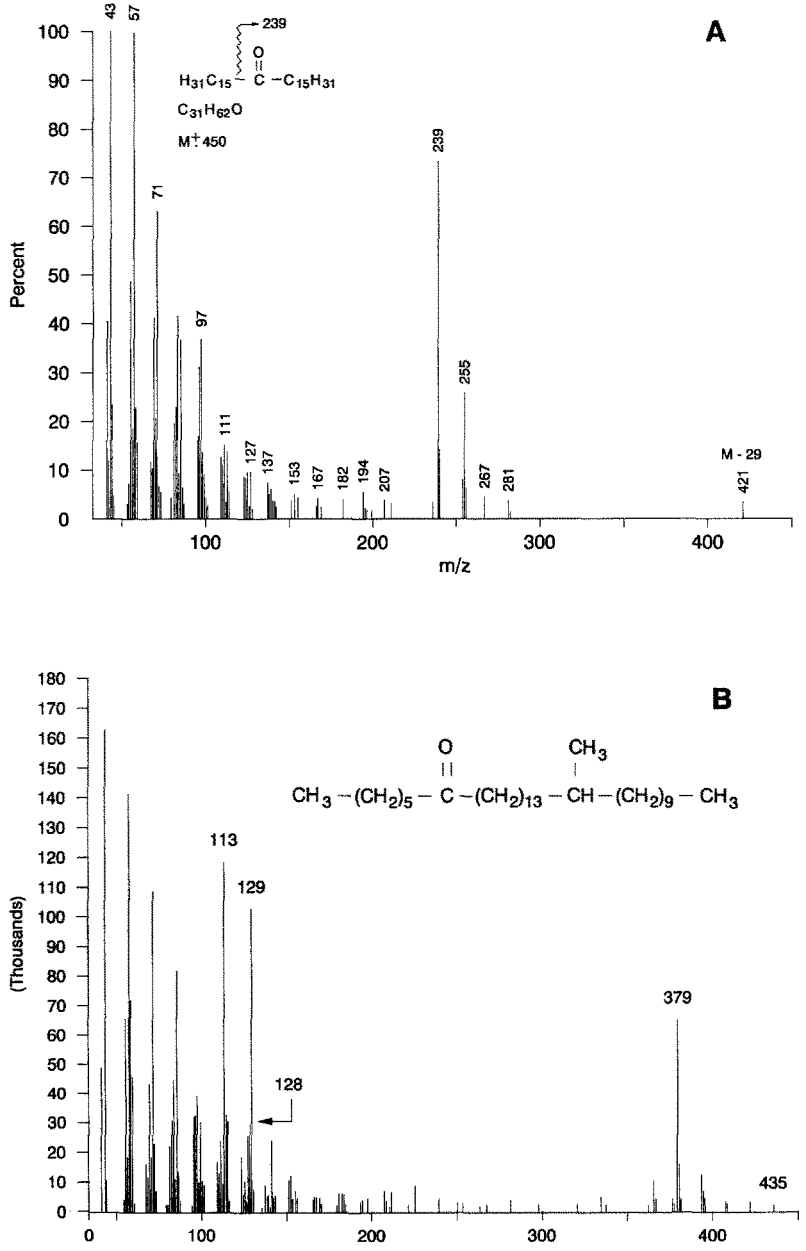


FIG. 7. CGC-EI-MS of: (A) 16-hentriacontanone (XIV, R_t = 21.90 min, Figure 8A) from HPLC peak 8. (B) 21-methyl-7-hentriacontanone (XV, $R_t \approx$ 22.12 min, Figure 8A) from HPLC peak 8.

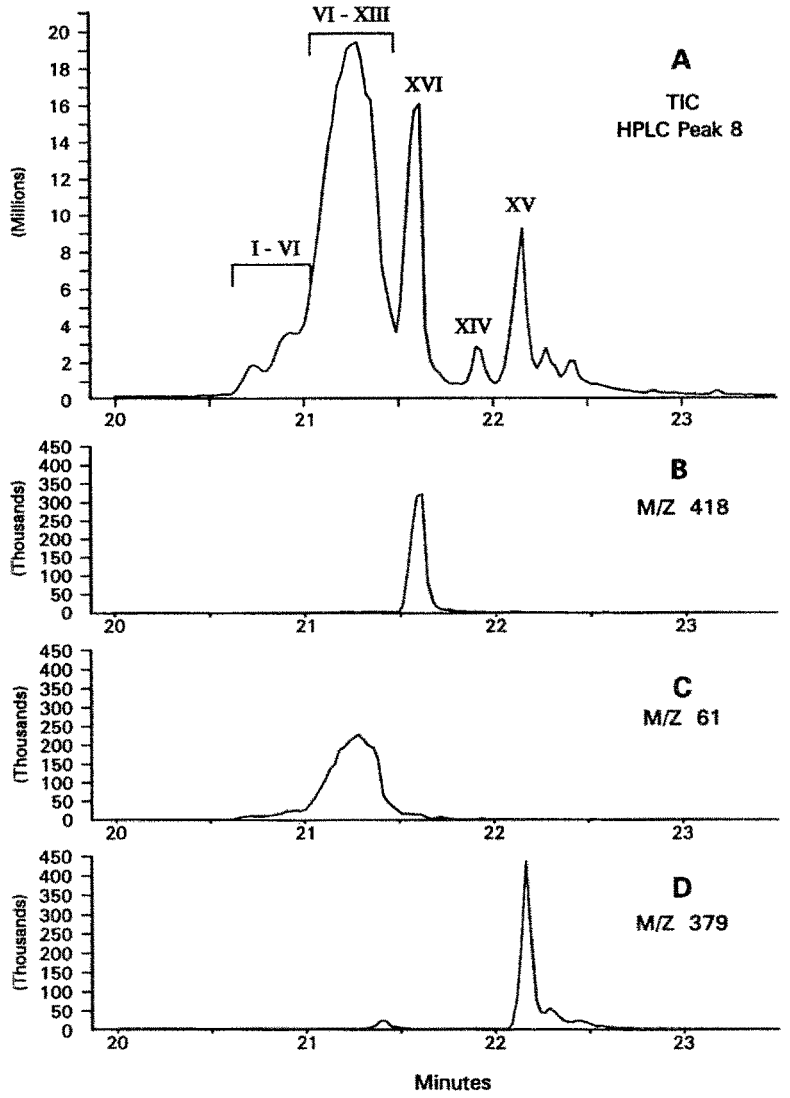


FIG. 8. (A) Total ion chromatogram (TIC) of RPHPLC peak 8 from an extract containing the aldehyde (XVI). (B) Selected ion monitor (SIM) for *m/z* 418 ion in EI-MS of triaconatanal (XVI, R_t = 21.62 min, Figure 9). This ion is due to the loss of H_2O from molecular ion at *m/z* 436. (C) SIM for *m/z* 61 ion ($CH_3CO_2H_2^+$ from acetates (I–XIII). (D) SIM for *m/z* 379 acylium ion ($C_{25}H_{51}C{\equiv}O^+$) from ketone XV.

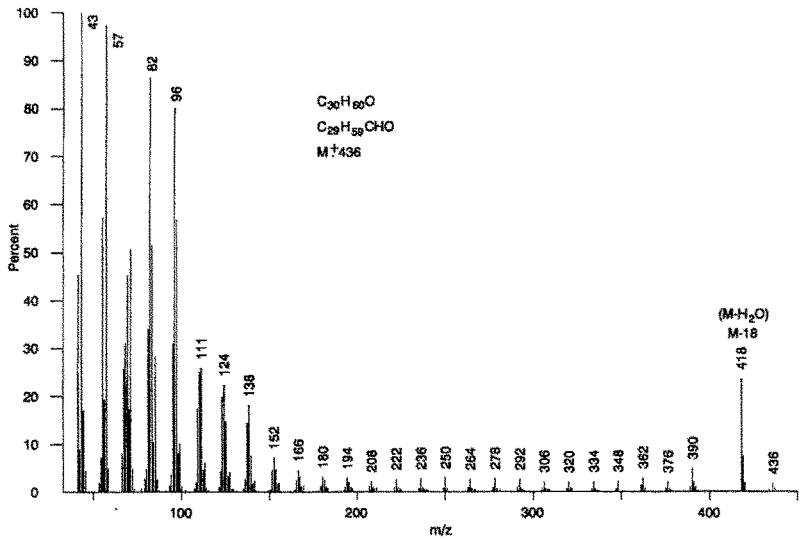


FIG. 9. CGC-EI-MS of triacontanal (XVI, R_t = 21.62 min, Figure 8A).

sex pheromones. Howard and Blomquist (1982) in their in-depth review stressed the hydrocarbon nature of many dipteran pheromones and discussed their complex behavioral activity as attractants and aphrodisiacs. Blomquist et al. (1987) reviewed the more recent literature and listed 44 dipteran species with sex pheromones but only 16 in which the pheromones had been chemically identified. Most are hydrocarbons but several nonhydrocarbon dipteran sex pheromones are known. Screwworms also probably use a nonhydrocarbon sex pheromone because mating stimulant activity was found in a relatively polar silica column fraction, i.e., fraction 4 eluting in hexane–ether; 94:6. RPHPLC of fraction 4 produced several bioactive peaks. The following compounds were identified from bioactive RPHPLC peak 8: 5-, 6-, 7-, 11-, 13-, and 15-acetoxynonacosanes; 3-acetoxy-23-methyl-, 5-acetoxy-23-methyl-, 5-acetoxy-19-methyl-, 6-acetoxy-19-methyl-, 7-acetoxy-19-methyl-, 8-acetoxy-19-methyl-, and 7-acetoxy-15-methylnonacosanes; 16-hentriaconatanone; 21-methyl-7-hentriacontanone; and triacontanal. Many of these compounds are probably optically active. We did not measure the optical rotation of any of the isolated fractions. Additional tests are necessary to establish which of these compounds, if any, are responsible for the mating-stimulant activity isolated in RPHPLC peak 8. We were not successful in obtaining consistent activity upon further fractionation of peak 8 either by CGC or HPTLC. Quarantined colonies of the screwworm are no longer kept in the United States, therefore material for bioassays and for further studies is no longer available.

SUMMARY

Mating response is elicited in the male screwworm fly by extracts of the female screwworm. Activity is associated with an RPHPLC chromatographic fraction that contains a group of compounds that were identified as long-chain branched and unbranched acetates and ketones as well as an aldehyde.

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REFERENCES

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, Orlando, Florida.

BUDZIKIEWICZ, H., DJERASSI, C., and WILLIAMS, D.H. 1967. Mass Spectrometry of Organic Compounds. Holden-Day, San Francisco, xiv + 690 pp.

CARLSON, D.A., and MACKLEY, J.W. 1985. Polyunsaturated hydrocarbons in the stable fly. *J. Chem. Ecol.* 11:1485–1496.

CARLSON, D.A., and SCHLEIN, Y. 1991. Unusual polymethyl alkenes in tsetse flies acting as abstinon in *Glossina morsitans*. *J. Chem. Ecol.* 17:267–284.

COLTHUP, N.B., DALY, L.H., and WIBERLEY, S.E. 1964. Introduction to Infrared and Raman Spectroscopy. Academic Press, New York, xii + 511 pp.

EL-AZAZY, O.M.E. 1989. Wound myiasis caused by *Cochliomyia hominivorax* in Libya. *Vet. Rec.* 124:103.

FLETCHER, L.W., O'GRADY, J.J., JR., CLABORN, H.V., and GRAHAM, O.H. 1966. A pheromone from male screwworm flies. *J. Econ. Entomol.* 59:142–143.

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.

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 fly, *Cochliomyia hominivorax*. *J. Chem. Ecol.* 13:1419–1430.

HAMMACK, L. 1991. Sex pheromone communication in the screwworm. Ontogenetic and strain effects. *Cochliomyia hominivorax*. *J. Chem. Ecol.* 17:2143–2154.

HOWARD, R.W., and BLOMQUIST, G.J. 1982. Chemical ecology and biochemistry of insect hydrocarbons. *Annu. Rev. Entomol.* 27:149–172.

KRAFSUR, E.S., WHITTEN, C.J., and NOVY, J.E. 1987. Screwworm eradication in North and Central America. *Parasitol. Today* 3:131–137.

LAAKE, E.W., CUSHING, E.C., and PARISH, H.E. 1936. Biology of the primary screwworm fly, *Cochliomyia americana*, and a comparison of its stages with those of *C. macellaria*. U.S. Department of Agriculture Technical Bulletin No. 500, 24 pp.

MACKLEY, J.W., and BROCE, A.B. 1981. Evidence of a female sex-recognition pheromone in the screwworm fly. *Environ. Entomol.* 10:406–408.

POMONIS, J.G. 1989. Cuticular hydrocarbons of the screwworm, *Cochliomyia hominivorax* (Diptera: Calliphoridae). Isolation, identification, and quantification as a function of age, sex, and irradiation. *J. Chem. Ecol.* 15:2301–2317.

POMONIS, J.G., NELSON, D.R., and FATLAND, C.L. 1980. Insect hydrocarbons 2. Mass spectra of dimethylalkanes and effect of the number of methylene units between methyl groups on fragmentation. *J. Chem. Ecol.* 6:965–972.

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.

RASMUSSEN, J.R., SLINGER, C.L., KORDISH, R.J., and NEWMAN-EVANS, D.D. 1981. Synthesis of deoxy sugars. Deoxygenation by treatment with *N,N'*-thiocarbonyldiimidazole/tri-*n*-butylstannane. *J. Org. Chem.* 46:4843–4846.

RICHARDSON, R.H., ELLISON, J.R., and AVERHOFF, W.W. 1982. Autocidal control of screwworms in North America. *Science* 215:361–370.

SCHWARTZ, D.P., and OSMAN, S.F., 1977. Applications of chromic acid-celite columns to micro- and semimicro preparations of fatty aldehydes. *Anal. Biochem.* 80:70–75.

SCOTT, D., ROLLIB, C.R., and CARLSON, D.A. 1988. Pheromones exchanged during mating: A mechanism for mate assessment in *Drosophila*. *Anim. Behav.* 36:1164–1173.

SILVERSTEIN, R.M., BASSLER, G.C., and MORRILL, T.C. 1981. Spectrometric Identification of Organic Compounds, 4th ed. John Wiley & Sons, New York, 442 pp.

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 Darilen Gap in Panama. *J. Med. Entomol.* 22:353–360.

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SEX PHEROMONE OF THE WESTERN HEMLOCK LOOPER, *Lambdina fiscellaria lugubrosa* (HULST) (LEPIDOPTERA: GEOMETRIDAE)

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Abstract—The sex pheromone of the western hemlock looper (WHL), *Lambdina fiscellaria lugubrosa* (Hulst), comprises three methylated hydrocarbons: 5,11-dimethylheptadecane (5,11), 2,5-dimethylheptadecane (2,5), and 7-methylheptadecane (7). Compounds extracted from female pheromone glands were identified by coupled gas chromatographic-electroantennographic (GC-EAD) analysis and coupled GC-mass spectroscopy in selected ion monitoring mode. In trapping experiments, (5,11) alone attracted male moths, but addition of either (7) or (2,5) significantly enhanced attraction. (5,11) combined with both (7) and (2,5) was significantly most attractive. (5,11) and (2,5) are also sex pheromone components of the eastern hemlock looper (EHL), *Lambdina fiscellaria fiscellaria* (Guen.). Although (7) is produced by the EHL, it is a pheromone component only in the WHL. It constitutes the first behaviorally active monomethyl-branched hydrocarbon to be found in a geometrid

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and is a novel lepidopteran sex pheromone component. The different 2- versus 3-component sex pheromone supports taxonomic division of EHL and WHL.

Key Words—Lepidoptera, Geometridae, *Lambdina fiscellaria fiscellaria*, *Lambdina fiscellaria lugubrosa*, sex pheromone, 5,11-dimethylheptadecane, 2,5-dimethylheptadecane, 7-methylheptadecane, 5-methylheptadecane.

INTRODUCTION

The western hemlock looper (WHL), *Lambdina fiscellaria lugubrosa* (Hulst), is found in Alaska, British Columbia, Washington, Idaho, and Oregon (Furniss and Carolin, 1977). Throughout most of its range, the preferred host is western hemlock, *Tsuga heterophylla* (Raf.). True firs, *Abies* spp. are favored in northern Idaho and western Montana. In south-central British Columbia western red cedar, *Thuja plicata* Donn., is as readily attacked as western hemlock. During outbreaks, WHL will feed on a wide variety of coniferous trees and associated deciduous trees and shrubs (Dewey et al., 1972; McGuffin, 1987; Turnquist, 1991). Fourteen outbreaks mostly in mature, old-growth forests occurred in British Columbia between 1928 and 1991 (Krannitz, 1992). In 1991, outbreaks in the Cariboo, Nelson, and Kamloops forest regions covered more than 86,000 ha (Wood and Van Sickle, 1992). Completely defoliated trees die rapidly. Significantly defoliated, but surviving trees are predisposed to attack by secondary insects, such as *Tetropium velutinium* Lec., and *Pseudohylesinus tsugae* Sw., leading to further mortality in the second and third year after outbreak (Turnquist, 1991).

The standard tree-beating method (Harris et al., 1972) is currently applied by Forestry Canada's Insect and Disease Survey to detect rising western hemlock looper populations. It involves laborious collection, taxonomic determination, and counting of looper larvae. Correlating numbers of WHL pupae "trapped" in burlap strips at the base of trees (Otvos, 1974) to the number of healthy WHL eggs on the sample tree constitutes another method of assessing WHL populations (Shore, 1989, 1990). Pheromone-based monitoring with nonsaturating traps would be the most efficient and sensitive method of monitoring WHL populations, providing early indication of significant changes in looper abundance.

The sex pheromone of the eastern hemlock looper (EHL) *L. f. fiscellaria* (Guen.) is known (Gries et al., 1991a,b), and attraction of male WHLs to virgin females has been demonstrated (Ostaff et al., 1974). We report the identification of the sex pheromone of the WHL.

METHODS AND MATERIALS

Laboratory Analyses. Fourth- and fifth-instar WHL larvae field collected near Downie Creek, 70 km east of Revelstoke, British Columbia, were reared in the laboratory to adults on western hemlock and western red cedar (20°C,

60% relative humidity, 14 : 10 hr light–dark). Male and female pupae were kept in separate Petri dishes to avoid mating of emergent moths. Four to 5 hr into the scotophase (Shepherd, 1979), abdominal tips of 2- to 3-day-old virgin females were removed and extracted for 5 min in hexane. Aliquots of one female equivalent (1 FE) of pheromone extract were subjected to gas chromatographic-electroantennographic (GC-EAD) analysis (Arn et al., 1975) on two fused silica columns with different retention characteristics (DB-210, DB-1, each 30 m × 0.25 mm ID). Reciprocal analyses (Struble and Arn, 1984), involving female pheromone extract and male antennae of both WHL and EHL, were carried out to increase selectivity and sensitivity of pheromone identification. Coupled GC-mass spectrometry (MS) (Hewlett Packard 5985B) in full-scan and selected ion monitoring (SIM) mode was conducted to confirm the presence of pheromone components in gland extracts. For GC-MS-SIM, full-scan electron impact spectra of synthetic candidate compounds were obtained to select ions indicative of methyl branch positions (Pomonis et al., 1980) (Figure 2). In sequence, 200 pg of synthetic compounds, hexane, and a concentrated pheromone gland extract were chromatographed, each time scanning for the diagnostic ions.

Synthesis. The four candidate pheromone components 5,11-dimethylheptadecane (5,11), 2,5-dimethylheptadecane (2,5), 7-methylheptadecane (7), and 5-methylheptadecane (5) were synthesized as previously described (Gries et al., 1991a). Chemical purity of each compound was >97%. None of the contaminants elicited antennal responses in GC-EAD recordings. According to syntheses, the four stereoisomers of (5,11), and the two enantiomers of each (7), (5), and (2,5) occurred at approximately equal amounts.

Field Bioassay. Field experiments from mid-August to mid-October 1991 were conducted at Downie Creek, 70 km north of Revelstoke, and at Finn Creek, 26 km south of Blue River, both within the interior cedar hemlock biogeoclimatic zone of British Columbia (Meidinger and Pojar, 1991). Experiments were set up in randomized, complete blocks with traps and blocks at 15- to 20-m intervals. Green Unitraps (Phero Tech Inc., Delta, British Columbia) were suspended 1–2 m aboveground 1–4 m within the forest margin, and baited with rubber septa (Sigma Chemical Co, St. Louis, Missouri 63178) impregnated with candidate pheromone components in HPLC-grade hexane. A Dichlorvos cube (1 cm^3) (Green Cross Co., Mississauga, Ontario) in each trap ensured rapid killing and thus preservation of captured moths.

The first experiment tested the four candidate pheromone components (5,11), (2,5), (7) and (5) alone at 100 μg each and in quaternary combination. The second experiment tested the four components in all binary, ternary, and quaternary combinations. The third experiment tested (5,11) alone and in all combinations with the other three candidate pheromone components. A final dose–response experiment tested (5,11) alone and the quaternary blend at a 1 : 1 : 1 : 1 ratio at the following doses for each compound: 0.01, 0.1, 1, 10, 100, and 1000 μg.

Statistical Analysis. Statistical analyses were conducted with SAS statistical package (SAS Institute Inc., Cary, North Carolina 27513). To ensure homogeneity of variance, data were transformed by $\log_{10}(x + 1)$ and subjected to analysis of variance followed by Scheffé test.

RESULTS

Laboratory Analysis. GC-EAD analyses of female WHL gland extracts on two GC columns revealed four EAD-, but no FID-detectable compounds with retention indices identical to those found in the EHL (Gries et al., 1991). Response to (5) was often missing and is also not visible in Figure 1. GC-MS-SIM of 250 FE of gland extract, monitoring for *m/z* 168, 169, 196, and 197

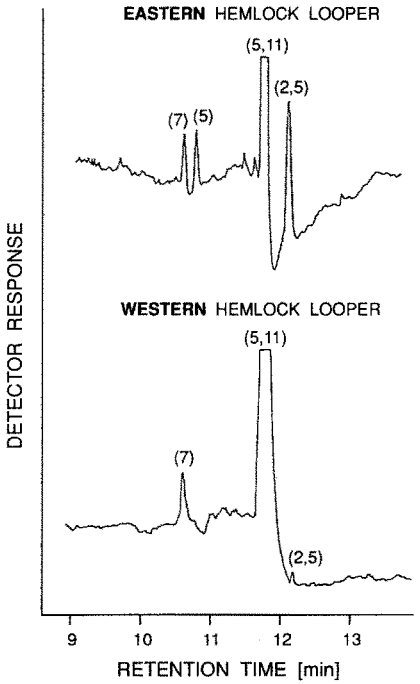

FIG. 1. EAD responses by male WHL antenna to female WHL gland extract compared to EAD responses by male EHL antenna to female EHL gland extract (adapted from Gries et al., 1991a,b). As candidate pheromone components were EAD- but not FID-detectable, corresponding FID traces are omitted. DB-210 column (30 m × 0.25 mm ID): 1 min at 70°C, 20°C/min to 130°C, 2°C/min to 220°C [(5,11) = 5,11-dimethylheptadecane; (2,5) = 2,5-dimethylheptadecane; (7) = 7-methylheptadecane; (5) = 5-methylheptadecane].

for (7), (5), and (2,5), and *m/z* 183 and 211 for (5,11) (Figure 2), resulted in exact retention time and good ion ratio matches of synthetic versus female-produced compounds for all components except (5). (5) was detected by GC-MS-SIM in gland extracts of the EHL, but was only tentatively identified in the WHL. Antennal responses by male WHL to female WHL gland extract, female EHL gland extract, and synthetic (5) coincided on DB-1 and DB-210 columns.

Trapping Experiment. Of the four candidate pheromone components tested individually, only (5,11) was attractive on its own (Figure 3). Blends of (7), (2,5), and (5) without (5,11) were not attractive (Figure 4). Adding (7) or (2,5) to (5,11) significantly enhanced attraction (Figure 5). (5,11) combined with both (7) and (2,5) was significantly most attractive (Figure 4 and 5). In the dose-response test, increasing the amount of pheromone from 0.01 μg to 1000 μg increased the number of captured loopers (Figure 6). At the two higher doses

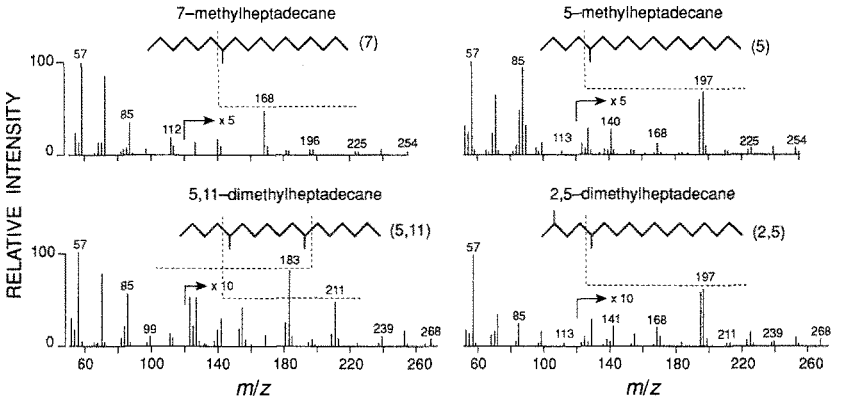

FIG. 2. Mass spectra of synthetic (7), (5), (5,11), and (2,5).

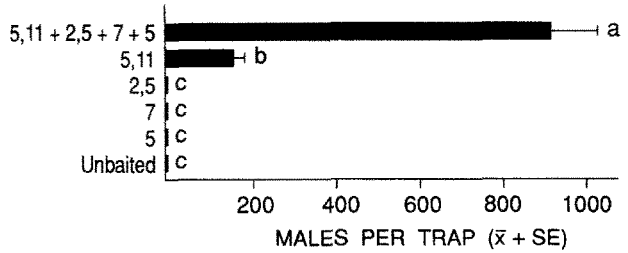

FIG. 3. Captures of WHL in Unitraps baited with candidate pheromone components alone at 100 μg each and in quaternary combination. Downie Creek, British Columbia, August 25–27, 1991, $N = 30$. Bars superscripted by the same letter are not significantly different, $P < 0.05$; compound abbreviations as in Figure 1.

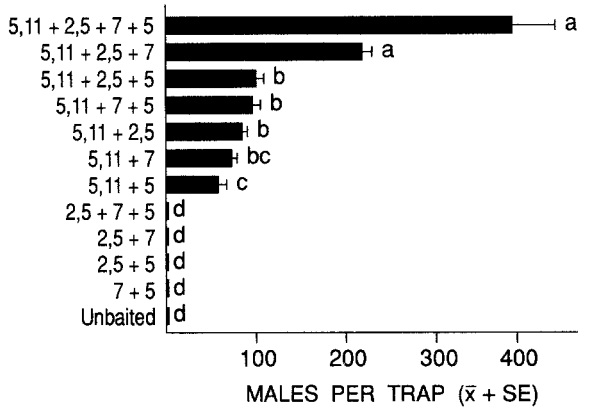

FIG. 4. Captures of WHL in Unitraps baited with candidate pheromone components at 100 μg each in all binary, ternary, and quaternary combinations. Downie Creek, British Columbia, August 27–29, 1991, $N = 30$. Bars superscripted by the same letter are not significantly different, $P < 0.05$; compound abbreviations as in Figure 1.

attraction to the quaternary blend significantly exceeded that to (5,11) alone (Figure 6).

DISCUSSION

Sex pheromones and/or attractants in geometrid moths comprise tetraene hydrocarbons (Roelofs et al., 1982; Bestmann et al., 1982; Underhill et al., 1987) and triene hydrocarbons (Millar et al., 1987) as single pheromone components, combinations of polyenic hydrocarbons (Wong et al., 1984; Bogenschütz et al., 1985; Szöcs et al., 1984; Millar et al., 1990a, 1992), and monoene or diene monoepoxides alone and in combination with corresponding hydrocarbons (Mayer and McLaughlin, 1991; Hansson et al., 1990; Millar et al., 1990b–d, 1992). Methyl-branched hydrocarbons have only recently been identified as a new group of geometrid pheromones with (5,11) and (2,5) occurring in the EHL (Gries et al., 1991a,b). The same two components are part of the pheromone blend in the WHL. (7) is present in the EHL but is behaviorally inactive (Gries et al., 1991a). As a third pheromone component in WHL, (7) constitutes a novel lepidopteran sex pheromone component, and the first behaviorally active monomethyl-branched hydrocarbon in a geometrid.

A three-component pheromone blend has been reported for one other geometrid moth, the fall canker worm, *Alsophila pometaria* (Harris). Wong et al. (1984) identified (*Z*3, *Z*6, *Z*9)-nonadecatriene, (*Z*3,*Z*6,*Z*9,*E*11)-nonadecatraene, and (*Z*3,*Z*6,*Z*9,*Z*11)-nonadecatetraene in pheromone gland extracts from

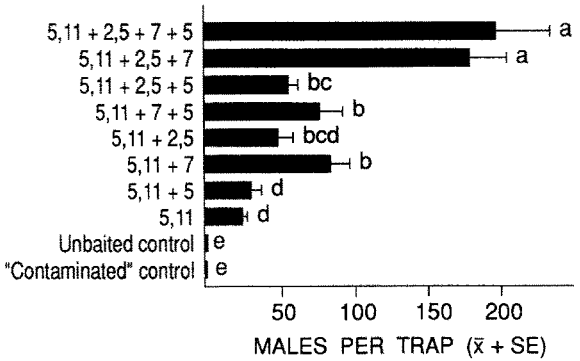

FIG. 5. Captures of male WHL in Unitraps baited with (5,11) alone and in binary, ternary, and quaternary combination with (2,5), (7), and (5) at 100 μg each. All traps had been previously used (Figures 3 and 4) and were thoroughly cleaned (soap, ammonia, bleach, hexane) prior to this experiment. "Contaminated" control traps were previously used (Figures 3 and 4), but did not undergo the cleaning procedure. Finn Creek, British Columbia, September 17–19, 1991, $N = 30$. Bars superscripted by the same letter are not significantly different, $P < 0.05$; compound abbreviations as in Figure 1.

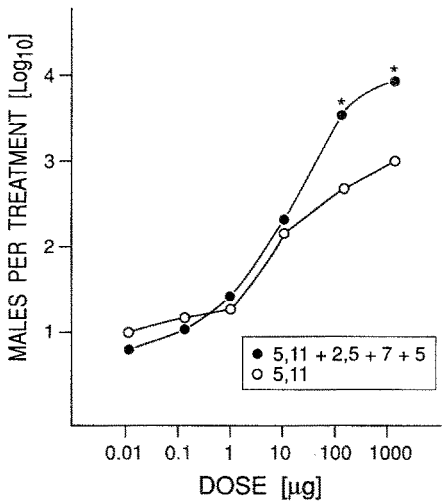

FIG. 6. Captures of male WHL in Unitraps baited with (5,11) alone and in quaternary combination (1 : 1 : 1 : 1) with (2,5), (7), and (5) at increasing doses. Finn Creek, British Columbia, September 28–30, 1991. $N = 10$. Attraction to the single- versus four-component pheromone lure was significantly different at 100- and 1000-μg doses, $P < 0.05$; compound abbreviations as in Figure 1.

female *A. pometaria* and demonstrated that a ternary combination of these compounds was as effective as the whole female extract in eliciting wing fanning, pheromone-oriented flight initiation, and landing on the source.

An exceedingly small, EAD- but not FID-detectable quantity of pheromone in the effluvia of calling female EHLs (Gries et al., unpublished) and in gland extracts of both female EHLs and WHLs is consistent with the low natural attraction of virgin female EHLs (Otvos, 1972; Gries et al., 1991) and WHLs (Ostaff et al., 1974). This low attraction of female looper did not warrant their use in trapping experiments with candidate pheromone components. The natural ratio of the three pheromone components released by female WHLs is still unknown, but GC-MS-SIM indicates that (5,11) is the most abundant pheromone component in pheromone glands. Therefore, calling WHL females are likely to release at least as much of the major pheromone component (5,11) as of synergistic (7) and (2,5).

Comparative morphology indicated subspecies separation of EHL and WHL (McGuffin, 1987). The fact that WHL sex pheromone is comprised of three components [(5,11), (2,5) and (7)], while (7) is behaviorally inactive in EHLs, furthers taxonomic separation of EHL and WHL. It supports the contention that pheromone biology in addition to morphometric, isozyme, and ecological analyses should become an integral part of insect taxonomy (Roelofs and Comeau, 1969; Roelofs and Brown, 1982).

Superiority of the three-component blend over (5,11) alone (Figures 3–6) emphasizes the importance of identifying all pheromone components in a chemical communication system. If pheromone-based disorientation of males is achieved by false trail following rather than sensory adaptation, central nervous system habituation, camouflage, or imbalance in the pattern of sensory input (Rothschild, 1981; Bartell, 1982; Minks an Cardé, 1988), the complete pheromone blend at an appropriate dose should exceed attraction to virgin females and therefore be effective in reducing mating. Increased costs for synthesis and formulation of multi- versus single-component lures are likely to be offset by decreased amounts of synthetic chemical required (Roelofs, 1978; Linn et al., 1986, 1987). For example, in the WHL, 100 μg (per compound) of the four-component lure significantly exceeded attraction to 1000 μg of (5,11) alone (Figure 6). Even if a single pheromone component can be employed for the management of lepidopteran pests (Flint et al., 1988; Charmillot, 1990), the efficacy in mating disruption of single- versus multicomponent lures at various doses should be compared. Identification of all pheromone components in a target species will make these comparisons possible and will allow the development of possibly more effective and/or cheaper management systems.

Chirality of multicomponent methylated hydrocarbon pheromones, such as that of the WHL, offers a wide variety of possible pheromone blends. There are four stereoisomers of (5,11) and two enantiomers each of (7) and (2,5).

Discounting ratio and inhibitory effects of isomers, and (conservatively) assuming that only one isomer each is used of (5,11), (7), and (2,5), there are possibly 44 different and unique blends with either one, two, or three components. As pheromone chirality has already been demonstrated for (5,11) (Li et al., 1993a) and (7) and (2,5) (Li et al., 1993b), any one of these blends may attract a specific moth species when chirally pure stereoisomers and/or enantiomers are field tested in various combinations and in various geographic locations.

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REFERENCES

ARN, H., STÄDLER, 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.

BARTELL, R.J. 1982. Mechanism of communication disruption by pheromones in the control of Lepidoptera: A review. *Physiol. Entomol.* 6:889–892.

BESTMANN, H.J., BROSCHE, T., KOSCATZKY, K.H. MICHAELIS, K., PLATZ, H., ROTH, K., SUSS, J., VOSTROVSKY, O., and KNAUF, W. 1982. Pheromone-XLII. 1,3,6,9-Nonadecatraen, das Sexualpheromon des Frostspanners *Operophtera brumata* (Geometridae). *Tetrahedron Lett.* 23:4007–4010.

BOGENSCHÜTZ, H., KNAUF, W., TRÖGER, E.J., BESTMANN, H.J., and VOSTROVSKY, O. 1985. Pheromone 49: Freilandfänge von Geometriden-Männchen mit C_{19}-Polyenen in Kiefernbeständen. *Z. Angew. Entomol.* 100:349–354.

CHARMILLOT, P.-J. 1990. Mating disruption technique to control codling moth in western Switzerland, pp. 165–182, *in* R.L. Ridgway, R.M. Silverstein, and M.N. Inscoe (eds.). Behavior-Modifying Chemicals for Insect Management: Application of Pheromones and Other Attractants. Marcel Dekker, New York.

DEWEY, J.E., CIESLA, W.M., and LOOD, R.C. 1972. Status of the western hemlock looper in the Northern Region, 1972: A potentially devastating forest pest. USDA Forest Service Technical Report 72-10.

FLINT, H.M., CURTICE, N.J., and YAMAMOTO, A. 1988. Pink bollworm (Lepidoptera: Gelechiidae): Further tests with (*Z,Z*)-isomers of Gossyplure. *J. Econ. Entomol.* 81:679–683.

FURNISS, R.L., and CAROLIN, V.M. 1977. Western Forest Insects. USDA Forest Service Miscellaneous Publication No. 1339.

GRIES, G., GRIES, R., BORDEN, J.H., JIANXIONG LI, SLESSOR, K.N., KING, G.G.S., BOWERS, W.W., WEST, R.J., and UNDERHILL, E.W. 1991a. 5,11-Dimethylheptadecane and 2,5-dimethylheptadecane: Sex pheromone components of the geometrid moth, *Lambdina fiscellaria fiscellaria*. *Naturwissenschaften* 78:315–317.

GRIES, G., JIANXIONG LI, GRIES, R., SLESSOR, K.N., BOWERS, W.W., WEST, R.J., KING, G.G.S., UNDERHILL, E.W., and BORDEN, J.H. 1991b. Method and Composition for attracting and controlling hemlock looper. Canadian Patent Application #2,041,529-1.

HANSSON, B.S., SCÖCS, G., SCHMIDT, F., FRANCKE, W., LÖFSTEDT, C., and TÓTH, M. 1990.

Electrophysiological and chemical analysis of the sex pheromone communication system of the mottled umber, *Erannis defoliaria* (Lepidoptera: Geometridae). *J. Chem. Ecol.* 16:1887–1897.

HARRIS, J.W.E., COLLIS, D.G., and MAGAR, K.M. 1972. Evaluation of the tree beating method for sampling defoliating forest insects. *Can. Entomol.* 104:723–729.

KRANNITZ, S.H. 1992. A synthetic sex pheromone for the western hemlock looper, *Lambdina fiscellaria lugubrosa* (Hulst) (Lepidoptera: Geometridae). MPM thesis. Simon Fraser University, Burnaby, British Columbia, Canada.

LI, J., GRIES, R., GRIES, G., SLESSOR, K.N., KING, G.G.S., BOWERS, W.W., and WEST, R.J. 1993a. Chirality of 5,11-dimethylheptadecane, the major sex pheromone component of the hemlock looper, *Lambdina fiscellaria* (Lepidoptera: Geometridae). *J. Chem. Ecol.* In press.

LI, J., GRIES, G., GRIES, R., BIKIC, J., and SLESSOR, K.N. 1993b. Chirality of synergistic sex pheromone components of the western hemlock looper, *Lambdina fiscellaria lugubrosa* (Hulst) (Lepidoptera: Geometridae). *J. Chem. Ecol.* Submitted.

LINN, C.E., JR., CAMPBELL, M.G., and ROELOFS, W.L. 1986. Male moth sensitivity to multicomponent pheromones: 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., JR., 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.

MCGUFFIN, W.C. 1987. Guide to the Geometridae of Canada (Lepidoptera). II. Subfamily Ennominae. 4. *Mem. Entomol. Soc. Can.* 138:64–65.

MAYER, M.S., and MCLAUGHLIN, J.R. 1991. Handbook of Insect Pheromones and Sex Attractants. CRC Press, Boca Raton, Florida.

MEIDINGER, D., and POJAR, J. 1991. Ecosystems of British Columbia. British Columbia Ministry of Forestry Special Report Series No. 6.

MILLER, 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-heptatriene and two monoepoxydiene analogs. *J. Chem. Ecol.* 13:1371–1383.

MILLAR, J.G., GIBLIN, M., BARTON, D., REYNARD, D.A., NEILL, G.B., and UNDERHILL, E.W. 1990a. 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. 1990b. (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. 1990c. (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. 1990d. 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., and UNDERHILL, E.W. 1992. Sex pheromone components of the geometrid moth *Lobophora nivigerata* and *Epirrhoe sperryi*. *J. Chem. Ecol.* 18:1057–1068.

MINKS, A.K., and CARDÉ, R.T. 1988. Disruption of pheromone communication in moths: Is the natural blend really most effective? *Entomol. Exp. Appl.* 49:25–36.

OSTAFF, D.P., SHEPHERD, R.F., and BORDEN, J.H. 1974. Sex attraction and courtship behavior in *Lambdina fiscellaria lugubrosa* (Lepidoptera: Geometridae). *Can. Entomol.* 106:493–501.

OTVOS, I.S. 1972. Sex attraction in the eastern hemlock looper. *Bimon. Res. Notes* 28:22.

OTVOS, I.S. 1974. A collection method of pupae of *Lambdina fiscellaria fiscellaria* (Lepidoptera: Geometridae). *Can. Entomol.* 106:329–331.

POMONIS, J.G., NELSON, D.R., and FLATLAND, C.L. 1980. Insect hydrocarbons. 2. Mass spectra

of dimethylalkanes and the effect of the number of methylene units between methyl groups on fragmentation. *J. Chem. Ecol.* 6:965–987.

ROELOFS, W.L. 1978. Threshold hypothesis for pheromone perception. *J. Chem. Ecol.* 4:685–699.

ROELOFS, W.L., and BROWN, R.L. 1982. Pheromones and evolutionary relationships of Tortricidae. *Annu. Rev. Ecol. Syst.* 12:395–422.

ROELOFS, W.L., and COMEAU, A. 1969. Sex pheromone specificity: Taxonomic and evolutionary aspects in Lepidoptera. *Science* 165:395–400.

ROELOFS, W.L., HILL, A.S., LINN, C.E., JR. MEINWALD, J., JAIN, S.C., HERBERT, H.J., and SMITH, R.F. 1982. Sex pheromone of the winter moth, a geometrid with unusually low temperature precopulatory responses. *Sciences* 217:657–659.

ROTHSCHILD, G.H.L. 1981. Mating disruption of lepidopteran pests: current status and future prospects, pp. 207–228, *in* E.R. Mitchell (ed.). Management of Insect Pests with Semiochemicals: Concepts and Practice. Plenum, New York.

SHEPHERD, R.F. 1979. Comparison of the daily cycle of adult behavior of five forest lepidoptera from Western Canada, and their response to pheromone traps. *Mitt. Schweiz. Entomol. Gesell.* 52:157–168.

SHORE, T.L. 1989. Sampling western hemlock looper pupae (Lepidoptera: Geometridae) using burlap traps. *J. Entomol. Sci.* 24:348–354.

SHORE, T.L. 1990. Recommendations for sampling and extracting the eggs of the western hemlock looper, *Lambdina fiscellaria lugubrosa* (Lepidoptera: Geometridae). *J. Entomol. Soc. B.C.* 87:000.

STRUBLE, D.L., and ARN, H. 1984. Combined gas chromatography and electroantennogram recording of insect olfactory responses, pp. 161–178, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research. Springer, New York.

SZÖCS, G., TÓTH, M., BESTMANN, H.J., and VOSTROWSKY, O. 1984. A two-component sex attractant for males of the geometrid moth *Alsophila quadripunctata. Entomol. Exp. Appl.* 36:287–291.

TURNQUIST, R. 1991. Western hemlock looper in British Columbia. Forestry Canada Pacific and Yukon Region, FIDS Report 91-8.

UNDERHILL, E.W., MILLAR, J.G., RING, R.A., WONG, J.W., BARTON, D., and GIBLIN, M. 1987. Use of a sex attractant and an inhibitor for monitoring winter moth and Bruce spanworm populations. *J. Chem. Ecol.* 13:1319–1329.

WONG, J.W., PALANISWAMY, P., UNDERHILL, E.W., STECK, W.F., and CHISHOLM, M.D. 1984. Novel sex pheromone components for the fall cankerworm moth, *Alsophila pometaria. J. Chem. Ecol.* 10:463–473.

WOOD, C.S., and VAN SICKLE, G.A. 1992. Forest insect and disease conditions: British Columbia and Yukon—1991. Forestry Canada, Pacific and Yukon Region, Information Report BC-X-334.

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EFFECT OF APIGENINIDIN ON THE GROWTH OF SELECTED BACTERIA

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Abstract—Apigeninidin, a 3-deoxyanthocyanidin present in sorghum, was chemically synthesized and effectively purified by extracting impurities from aqueous 2 N HCl into ethyl acetate. Apigeninidin (50 μmol) applied to filter paper disks inhibited the growth on agar plates of selected gram-positive bacteria (*Bacillus cereus, Staphylococcus aureus, Staphylococcus epidermidis*, and *Streptococcus faecalis*) and to a lesser extent gram-negative bacteria (*Escherichia coli, Serratia marcens*, and *Shigella flexneri*). Apigeninidin was not mutagenic based on the Ames assay. The ability of apigeninidin to precipitate proteins from plant extracts could be physiologically important if it can be shown to bind to specific plant proteins.

Key Words—apigeninidin, 3-deoxyanthocyanidin, phytoalexin, bacteria, protein precipitation, sorghum, sudangrass, mutagen.

INTRODUCTION

Proanthocyanidins (condensed tannins) are commonly present in edible grain of *Sorghum bicolor* (Butler et al., 1984), but are not found in sorghum leaves (Watterson and Butler, 1983). Possible physiological roles of proanthocyanidins include structural support (Zucker, 1983), informational polymers (Stafford, 1988), and defenses against herbivores. The ability of proanthocyanidins to function as defense compounds has been attributed to their ability to precipitate proteins, making them unavailable for digestion.

While proanthocyanidins are present only in the grain, apigeninidin or

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apigeninidin glycosides have been detected in both grain and leaves of sorghum varieties (Stafford, 1966; Nip and Burns, 1969). Apigeninidin inhibits the growth of certain fungi (Schutt and Netzly, 1991) and accumulates in response to *Helminthosporium maydis* and *Colletotrichum graminicola* infection in sorghum mesocotyls (Nicholson et al., 1987; Snyder and Nicholson, 1990). From these reports, we hypothesized that apigeninidin might have a role as a fungal-specific defense compound. Therefore, we wanted to test the effect of apigeninidin on the growth of other microbes such as bacteria.

The ability of apigeninidin to polymerize with its own tautomeric pseudobases under acidic conditions (Ribéreau-Gayon, 1972) might be likened to the condensation reactions for the formation of proanthocyanidins. Due to this similarity, we hypothesized a possible mode of action involving the precipitation of proteins, thereby serving a defensive role in the leaves of sorghum similar to that of proanthocyanidins in the grain. In addition, since several flavonoids have been reported to be mutagens (Chun-Li et al., 1986) and demographic studies by Morton (1970) have correlated ingestion of tannins with esophageal cancer, we wanted to investigate possible mutagenic activity of apigeninidin.

METHODS AND MATERIALS

Synthesis and Purification of Apigeninidin. Apigeninidin was synthesized from naringenin (Sigma) by the method of Sweeny and Iacobucci (1981). Two ethyl acetate extractions of the 2 N HCl solution containing apigeninidin effectively removed impurities. Apigeninidin was considered nearly pure when its 468-nm absorbance peak was 1.5 times that of the 276-nm peak and further extractions did not yield a greater ratio. This is based on the fact that phenols absorb in the 270- to 290-nm region, but not in the 468-nm region. Therefore, when the 276-nm peak of apigeninidin could no longer be reduced relative to its 468-nm peak, the solution was considered nearly free of phenolic impurities. Aqueous solutions were evaporated to dryness while being stirred on a hotplate. Apigeninidin was scraped off the flask and stored in a vial at room temperature.

Bacteria Bioassay. Shigella flexneri, Serratia marcens, Escherichia coli, Streptococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, and *Bacillus cereus* were grown at 37°C in nutrient broth. New cultures were made each day to ensure viability. Bacteria lawns were made by spreading 100 μl broth culture onto nutrient agar plates.

Apigeninidin (94.6 mg) was dissolved in 2 ml of absolute methanol. The oversaturated solution was centrifuged at top speed in an IEC Clinical centrifuge for 5 min to remove undissolved apigeninidin solids. Apigeninidin was quantitated spectrophotometrically in 2 N HCl (MEC = 18,000) (Stafford, 1966) at 468 nm. Apigeninidin (50, 5, and 0.5 μmol) was applied (270 μl) to filter paper

disks (1.2 cm diameter). Control disks had an equal volume (270 μl) of absolute methanol applied. After the methanol had evaporated, the disks were placed on the center of bacteria lawns, and plates were incubated at 37°C overnight. Diameters of growth-inhibition rings were measured with a ruler in three directions and averaged.

Ames Assay. Salmonella typhimurium strains TA98 and TA100 (provided by Dr. J.M. Gentile, Hope College) were maintained as frozen stock at −80°C. Master plates were prepared from these cultures (Maron and Ames, 1983). Genetic integrity of each strain was confirmed in every experiment (Zieger et al., 1981).

Protein Precipitation Assays. Roots (1 g) or shoots (1 g) of 7-day-old sorghum sudangrass (Trudan-8ACP) seedlings were homogenized in 8 ml of deionized water. Homogenates were centrifuged at high speed (IEC Clinical, 5000 *g*) for 15 min. Supernatants were used as the source of water-soluble proteins.

Stock solutions of apigeninidin (mol wt 255, 1.5 mg) and tannic acid (mol wt 1701, 10 mg; Fisher Scientific Co., Lot 775712) were separately dissolved in 10 ml of absolute methanol (580 μM). To 0.5 ml of these stock solutions and to 0.5 ml of the methanol control was added 0.5 ml of sodium phosphate buffers, 100 mM, pH 2, 4, 6, and 8. These 1-ml solutions were separately added to the following solutions: (1) 2 ml of water-soluble (1 mg protein/ml) proteins from roots or shoots, (2) 2 ml of BSA (1 mg protein/ml deionized water, positive control), and (3) 2 ml of deionized water (negative control). These mixtures were vortexed once every 5 min for 15 min before being centrifuged at high speed (IEC Clinical, 5000 *g*) for 15 min. After centrifugation, pelleted materials were dissolved in 0.1 ml of 3% (w/v) NaOH, then 1 ml of 100 mM phosphate buffer (pH 6.0) was added. Amount of protein present in the pellet was determined by the method of Bradford (1976).

Statistical Analysis. When appropriate, data were analyzed using Student's *t*-test and were significant at $P < 0.05$.

RESULTS

Biological Activity of Apigeninidin. Apigeninidin was found to inhibit the growth of gram-positive and gram-negative bacteria (Figure 1). Gram-positive bacteria appeared to be more sensitive to apigeninidin than gram-negative bacteria.

Mode of Action of Apigeninidin. Apigeninidin was not mutagenic for *salmonella* strains TA98 and TA100. The number of revertant colonies on plates containing apigeninidin were slightly lower than those of control colonies (Table 1).

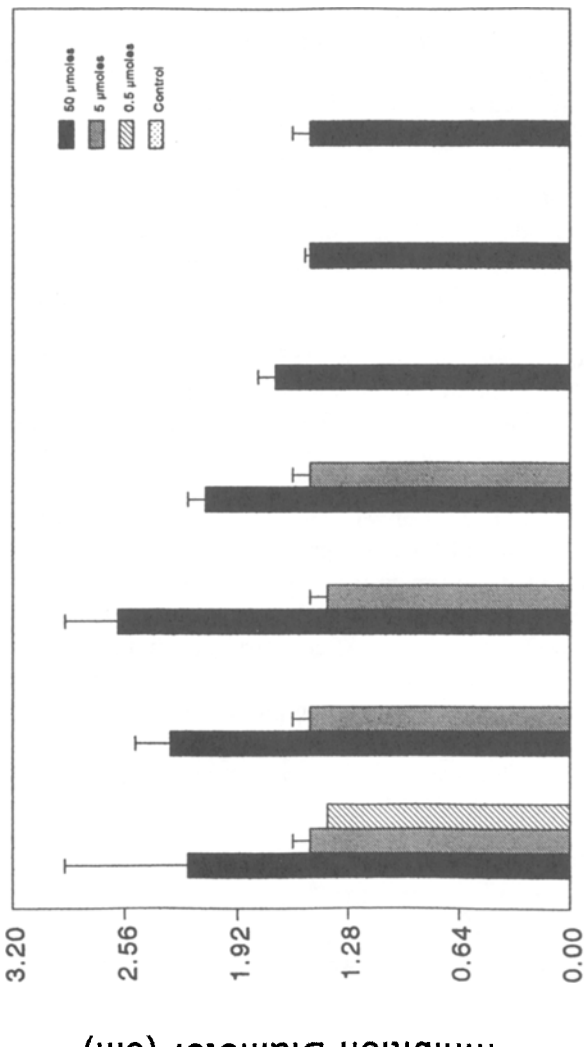

FIG. 1. Effect of apigeninidin on the growth of gram-positive and gram-negative bacteria. Inhibition diameters were determined by subtracting the controls (methanol only) from the test samples (apigeninidin dissolved in methanol). Values shown are an average ± SD of two to five experiments.

Apigeninidin was 95% less effective at precipitating BSA (pH 4) than relatively equal molar amounts of tannic acid (Figure 2). On a per weight basis, the precipitating ability of apigeninidin at pH 4 is only about 12 of tannic acid.

Compared to BSA, apigeninidin was about 20 times more effective at precipitating water-soluble proteins (pH 4) from root or shoot extracts of sorghum sudangrass seedlings (Figure 2). Changing the pH at which the precipitation

TABLE 1. TEST FOR MUTAGENICITY OF APIGENINIDIN USING *Salmonella* STRAINS TA100 AND TA98[a]

Apigeninidin concentration (mM)	Average number of colonies/plate	
	TA100	TA98
21.7	13	14
2.17	102	54
0.217	101	36
0.0217	111	39
0.0	116	51

[a]Solvent was DMSO. Marker plates were normal.

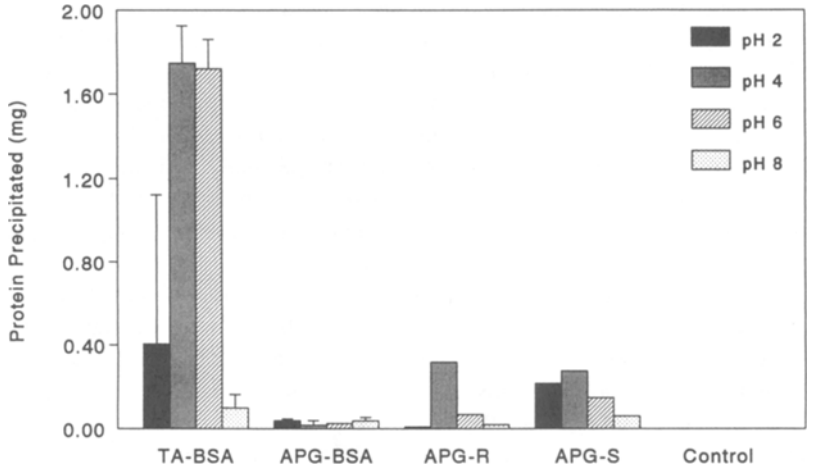

FIG. 2. Ability of apigeninidin (APG) to precipitate bovine serum albumin (BSA) as well as proteins extracted from roots (R) and shoots (S) of sorghum seedlings at differing pH. Protein precipitated was determined by subtracting control (50% methanol) values from the test (apigeninidin in 50% methanol) samples. Tannic acid (TA) in 50% methanol was used as a positive control. Values are an average ± SD of two to four experiments.

assays were performed had a greater effect on the precipitation of plant proteins than on BSA.

DISCUSSION

Apigeninidin is a phytoalexin of sorghum (Nicholson et al., 1987). It also inhibits the growth of certain fungi (Schutt and Netzly, 1991). We now report that the growth of certain nonpathogenic bacteria is also inhibited by apigeninidin. It is not known if apigeninidin levels in sorghum increase in response to specific bacterial pathogens of sorghum. However, the potential biological activity of apigeninidin is not fungal specific.

The mode of action of apigeninidin remains unknown, and we have no evidence for its mutagenic activity. The reduction in the number of revertant colonies in the presence of higher concentrations of apigeninidin indicates that it may be toxic to *Salmonella*, which would support our results from Figure 1. Another possibility is that apigeninidin may have antimutagenic properties. Of the many plant compounds, including flavonoids, that have antimutagenic properties (Wall et al., 1988), the authors are not aware of any anthocyanidins reported to be antimutagens.

Compared to tannic acid, apigeninidin was ineffective at precipitating BSA. Our reason for using tannic acid as a standard precipitant as opposed to proanthocyanidins was due to our interest in apigeninidin as a possible defense compound against herbivores. It is hypothesized that hydrolyzable tannins (tannic acid) are more likely to be involved in herbivore interactions than the proanthocyanidins (Zucker, 1983). Furthermore, our assays were done at various pH ranges for two reasons. First, we wanted to test the protein precipitation ability of apigeninidin under acidic conditions (where self-polymerization of apigeninidin is likely) (Ribéreau-Gayon, 1972) rather than those of more alkaline conditions. Second, we wanted to simulate the different pH ranges possible in the digestive tracts of various herbivores.

The ability of apigeninidin to precipitate proteins from plant extracts could be physiologically important if it can be shown to bind to specific plant proteins. For example, at pH 2, there is a significant difference between apigeninidin binding to root vs. shoot proteins. A pH of 4 was most effective for precipitation of plant extract proteins. Since the precipitation ability of apigeninidin decreases with increasing pH and since acidic conditions favor self-polymerization of apigeninidin, it is assumed that apigeninidin is most effective as a precipitant in a polymeric form. Monomeric phenolic compounds are noted for their ability to complex with proteins and inhibit their activity (Zucker, 1983), but only the polymeric hydrolyzable tannins and proanthocyanidins are effective in precipitating them.

Acknowledgments—This work was supported in part by NSF-REU grant BBS-9100398 and a Hope College Faculty Development Grant. The authors also wish to thank Dr. Vicki Isola for supplying the various bacteria cultures and Dr. James Gentile for the *Salmonella* strains and use of equipment for doing the Ames assay.

REFERENCES

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.

BUTLER, L.G., RIEDL, D.J., LEBRYK, D.G., and BLYTT, H.J. 1984. Interaction of proteins with sorghum tannin: Mechanism, specificity and significance. *J. Am. Oil Chem. Soc.* 61:916–920.

CHUN-LI, Y., SWAMINATHAN, B., BUTLER, L.G., and PRATT, D.E. 1986. Isolation and identification of rutin as the major mutagen of red wine. *Mutat. Res.* 170:103–113.

MARON, D.M., and AMES, B.N. 1983. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* 113:173–215.

MORTON, J.F. 1970. Tentative correlations of plant usage and esophageal cancer zones. *Econ. Bot.* 24:217–226.

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.

RIBÉREAU-GAYON, P. 1972. The anthocyanins, pp. 135–168, *in* Plant Phenolics. Oliver & Boyd, Edinburgh.

SCHUTT, C., and NETZLY, D. 1991. Effect of apiforol and apigeninidin on the growth of selected fungi. *J. of Chem. Ecol.* 17:1123–1125.

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.

STAFFORD, H.A. 1966. Regulatory mechanisms in anthocyanin biosynthesis in first internodes of sorghum vulgare: Effect of presumed inhibitors of protein synthesis. *Plant Physiol.* 41:953–961.

STAFFORD, H.A. 1988. Proanthocyanidins and the lignin connection. *Phytochemistry* 27:1–6.

SWEENY, J.G., and IACOBUCCI, G.A. 1981. Synthesis of anthocyanidins—III. Total synthesis of apigeninidin and luteolinidin chlorides. *Tetrahedron* 37:1481–1483.

WALL, M.E., WANI, M.C., MANIKUMAR, G., ABRAHAM, P., TAYLOR, H., HUGHES, T.J., WARNER, J., and MCGIVNEY, R. 1988. Plant antimutagenic agents. 2. Flavonoids. *J. of Nat. Prod.* 51:1084–1091.

WATTERSON, J.J., and BUTLER, L.G. 1983. Occurrence of an unusual leucoanthocyanidin and absence of proanthocyanidins in sorghum leaves. *J. of Agric. Food Chem.* 31:41–45.

ZEIGER, E., PAGANO, D.A. and ROBERTSON, I.G.C. 1981. A rapid and simple scheme for confirmation of *Salmonella* tester strain phenotype. *Environ. Mutagen.* 3:205–209.

ZUCKER, W.V. 1983. Tannins: Does structure determine function? An ecological perspective. *Am. Nat.* 121:335–365.

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Book Review

Ecological Roles of Marine Natural Products. Valerie J. Paul (ed.). Ithaca and London: Explorations in Chemical Ecology Series, Comstock Publishing Associates (a Division of Cornell University Press), 1992. 245 pp. ISBN 0-8014-2727-4.

Although chemical ecology in terrestrial environments is now a firmly established discipline, analogous studies in marine systems are still in their infancy. This edited volume is the first to summarize the current knowledge of the proposed functions of marine secondary metabolites. It provides a solid overview of most areas of current study and is highly recommended as an authoritative synopsis of this growing subdiscipline of marine science.

This book is the second volume of the series, *Explorations in Chemical Ecology*, edited by Thomas Eisner and Jerrold Meinwald from Cornell University. By in large, the chapters are comprehensive, fully referenced, and provide effective overviews of most aspects of this field. After forward comments by Meinwald and Eisner, and preface remarks by Paul, the text is separated into an informative introduction and six chapters of scientific review. In the introduction, a short history of the development of marine natural products chemistry and its interface with ecology is presented.

The six main chapters are reviews presented by five of the major experts in marine chemical ecology. Roughly organized into three areas of interest, marine plant predator–prey interactions, invertebrate predator–prey interactions, and chemical induction of larval settlement, these chapters illustrate, by comparison with terrestrial environments, the relatively small numbers of studies within this field. Chapter one focuses upon the defensive chemistry of marine plants in tropical reef environments. The chapter discusses the major herbivores present and summarizes, in tabular form, the algal metabolites shown to produce feeding deterrent effects in both laboratory and field oriented bioassays.

Chapter two, which focuses primarily upon the effects of brown algal phlorotannins, provides an interesting comparison of the geographical variation of brown algal defense in both tropical and temperate marine environments. Included are discussions of chemical and algal predator variability between North America and Australasia.

Chapter three continues the theme of algal predator–prey chemical ecology, but delves more deeply into secondary aspects of chemical defenses, such as the importance of generalist mezograzers, the evolution of invertebrate feeding

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specialization, and the acquisition of dietary-derived defenses. Additional discussions of associational refuges and the synergistic effects of secondary metabolite mixtures are also presented.

In chapter four, a detailed discussion of the chemical defenses of selected marine mollusks is presented. The chapter focuses on the opisthobranchs, a group of shell-less mollusks that are both herbivorous and carnivorous. This subject, summarized comprehensively in the chapter, is perhaps the oldest area of marine chemical ecology. Opisthobranchs, which include the herbivorous sea hares and ascoglossans, are known to acquire dietary-derived chemical defenses. Similarly, the nudibranchs described as completely carnivorous, acquire the majority of their chemical defenses from their chemically rich sponge, ascidian, and bryozoan prey.

Chapter five provides a summary of numerous studies of the chemical defenses of a variety of chemically rich invertebrates. Included are references to novel feeding deterrents from alcyonarian soft-corals, sponges, ascidians, and bryozoans. Discussions of the evidence for defenses against competition and surface fouling are also presented.

Chapter six deals with an important aspect of marine chemical ecology, the utilization of chemical cues as attractants for invertebrate larval settlement. This field is growing in importance, since aggregation and the formation of monospecific colonies is a common phenomenon in marine habitats. Likewise, associative settlement, the specific settlement of larvae on other plant and invertebrate taxa, is also a very common observation. The chapter summarizes this active and often controversial field and provides chemical data defining several confirmed settlement inducers.

Overall, this book is highly recommended for the student and researcher interested in following the growth of marine chemical ecology. Judging by the topics discussed and their importance in marine science, this small field is likely to experience significant growth over the next decade.

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ERRATUM

There are a series of typographical errors in the legends to Figures 1, 2, 4, 5, and 6 of Seybold et al. 1992. The Role of Lanierone in the Chemical Ecology of *Ips pini* (Coleoptera: Scolytidae) in California. *J. Chem. Ecol.* **18:** 2305–2329. The inequalities assigned to the null and alternative hypotheses in each paired comparison should be mutually exclusive.

Thus, pairwise comparisons in the legend of Figure 1 (p. 2312) are correctly written as follows:

Comparison	Type of test	Significant
H_O: Ipsdienol $\geq$ ipsdienol + 20 μg lanierone H_A: Ipsdienol $<$ ipsdienol + 20 μg lanierone	One-sided	Yes ($P = 0.0055$)
H_O: Ipsdienol $\leq$ ipsdienol + 2 mg lanierone H_A: Ipsdienol $>$ ipsdienol + 2 mg lanierone	One-sided	Yes ($P = 0.0004$)
H_O: Ipsdienol $\leq$ male-infested log H_A: Ipsdienol $>$ male-infested log	One-sided	Yes ($P = 0.0003$)

Pairwise comparisons in the legend of Figure 2 (p. 2313) are correctly written as follows:

Comparison	Type of test	Significant
H_O: Ipsdienol = male-infested log H_A: Ipsdienol $\neq$ male-infested log	Two-sided	No ($P = 0.3209$)
H_O: Ipsdienol $\geq$ ipsdienol + 20 μg lanierone H_A: Ipsdienol $<$ ipsdienol + 20 μg lanierone	One-sided	No ($P = 0.0202$)
H_O: Ipsdienol + 20 μg lanierone $\geq$ male-infested log H_A: Ipsdienol + 20 μg lanierone $<$ male-infested log	One-sided	No ($P = 0.6942$)
H_O: Ipsdienol + 2 mg lanierone $\leq$ ipsdienol + 0.1 μg lanierone H_A: Ipsdienol + 2 mg lanierone $>$ ipsdienol + 0.1 μg lanierone	One-sided	No ($P = 0.7242$)

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Pairwise comparisons in the legend of Figure 4 (p. 2316) are correctly written as follows:

Comparison	Type of test	Significant
H_O: Ipsdienol $\leq$ blank	One-sided	Yes ($P = 0.0005$)
H_A: Ipsdienol $>$ blank		
H_O: Ipsdienol $=$ male-infested log	Two-sided	No ($P = 0.8557$)
H_A: Ipsdienol $\neq$ male-infested log		
H_O: Ipsdienol $=$ ipsdienol + 20 μg lanierone	Two-sided	No ($P = 0.2775$)
H_A: Ipsdienol $\neq$ ipsdienol + 20 μg lanierone		

Pairwise comparisons in the legend of Figure 5 (p. 2317) are correctly written as follows:

Comparison	Type of test	Significant
H_O: Ipsdienol $=$ blank	Two-sided	No ($P = 0.0833$)
H_A: Ipsdienol $\neq$ blank		
H_O: Lanierone $=$ blank	Two-sided	Could not test
H_A: Lanierone $\neq$ blank		
H_O: Ipsdienol + 20 μg lanierone $\leq$ blank	One-sided	Yes ($P < 0.0001$)
H_A: Ipsdienol + 20 μg lanierone $>$ blank		
H_O: Ipsdienol + 20 μg lanierone $=$ male-infested log	Two-sided	No ($P = 0.1786$)
H_A: Ipsdienol + 20 μg lanierone $\neq$ male-infested log		

Pairwise comparisons in the legend of Figure 6 (p. 2318) are correctly written as follows:

Comparison	Type of test	Significant
H_O: Male-infested log $\leq$ blank	One-sided	Yes ($P < 0.0001$)
H_A: Male-infested log $>$ blank		
H_O: Male-infested log $\leq$ ipsdienol	One-sided	Yes ($P < 0.0001$)
H_A: Male-infested log $>$ ipsdienol		
H_O: Male-infested log $\leq$ ipsdienol + 20 μg lanierone	One-sided	Yes ($P < 0.0001$)
H_A: Male-infested log $>$ ipsdienol + 20 μg lanierone		

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TOXICITY OF ALLYL ISOTHIOCYANATE-AMENDED SOIL TO *Limonius californicus* (MANN.) (COLEOPTERA: ELATERIDAE) WIREWORMS

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Abstract—Acute toxicity of soil amended with allyl isothiocyanate (AITC) to three size classes of *Limonius californicus* (Mann.) wireworms was determined in the laboratory. Wireworms were exposed to AITC at initial concentrations of 120–300 nmol/g soil for one day. During this time, extractable AITC concentrations decreased by 66 to 93%. Probit analysis estimated LC_{50} values of 238 and 226 nmol/g soil at one day posttreatment for medium and large wireworms, respectively. For small wireworms, LC_{50} values decreased from 211 to 157 nmol/g soil during 1–137 days posttreatment. Sublethal concentrations of AITC significantly reduced feeding activity of treated wireworms at three posttreatment times and over the entire 137 days. Wireworm weight was not significantly affected by AITC. The potential exists to use glucosinolate-containing plant tissue as an isothiocyanate (ITC) source to reduce crop damage caused by *L. californicus* wireworms.

Key Words—Rapeseed, *Brassica* spp., allyl isothiocyanate, glucosinolates, allelochemicals, *Limonius californicus* (Mann.), Coleoptera, Elateridae, toxicity, sublethal effects.

INTRODUCTION

Recent environmental and economic concerns over synthetic organic pesticides have spurred interest in the development of safer and less expensive pest control strategies, including the use of chemicals occurring naturally in plants. One such

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group of allelochemicals are glucosinolates, organic anions possessing a β-D-thioglucose moiety, a sulfonated oxime, and any one of a variety of aliphatic or aromatic R groups. Biological activity of glucosinolates is limited. However, damage to plant tissue leads to enzymatic degradation of glucosinolates by thioglucoside glucohydrolase (EC 3.2.3.1) (Underhill, 1980), resulting in the formation of many compounds with allelochemical activity (Lewis and Papavizas, 1971; Hicks, 1974; Fenwick et al., 1983; Toba, 1984; Jiménez-Osornio and Gleissman, 1987; Landis and Gould, 1988; Bialy et al., 1990; Košťál, 1992).

Brassica spp. (primarily canola, winter rapeseed, and mustard) are gaining popularity in the inland Pacific Northwest as viable alternative crops. These biennials are often grown in rotation with cereals and grain legumes and, in addition to seed yield, produce dense foliage, which may limit soil erosion and water loss. Allyl glucosinolate, present in relatively high concentrations in *Brassica oleracea* L., *B. juncea* L. (Sang et al., 1984), *B. carinata* L., and *B. nigra* (L.) Koch, is expected to produce allyl isothiocyanate (AITC) on hydrolysis (Kjaer, 1976). Isothiocyanates (ITC) include some of the most toxic glucosinolate degradation products, having general biocidal properties as a result of interactions with protein (Wood, 1975).

In the Pacific Northwest, allelochemical properties of *Brassica* may be best exploited against soil-borne pests. Incorporation of *Brassica* tissue into the plow zone would initiate production of ITC and may lead to lethal and/or sublethal (e.g., repellency and inhibition of feeding) effects on pest populations, thus conferring protection to the following crop. Suppression of *Aphanomyces euteiches* Drechs. root rot of peas (Papavizas, 1966; Papavizas and Lewis, 1971; Chan and Close, 1987) and root-knot nematodes, *Meloidogyne chitwoodi* Golden et al. (Mojtahedi et al., 1991), has been achieved by green manuring *Brassica* tissues into the soil. Methyl isothiocyanate (MITC) is used as a soil fumigant or is generated in situ from sodium *N*-methyldithiocarbamate and is capable of providing complete soil sterilization.

We studied the toxicity of AITC on *Limonius californicus* (Mann.) wireworms, a widespread and important soil-borne crop pest (Stone, 1941; Toba et al., 1985). Our objectives were to evaluate lethal and sublethal effects of AITC-amended soil on *L. californicus* in the laboratory and to monitor the disappearance of AITC during wireworm exposure.

METHODS AND MATERIALS

Insects. Limonius californicus (Mann.) wireworms were collected in April–May 1990 and 1991 in an agricultural field in Walla Walla County, southeastern Washington, USA (Williams et al., 1992). Wireworms were sorted into three size classes (small, medium, and large) based on estimation of length, placed

in plastic vials with soil from which they were sieved, and held at 4°C until preconditioning. Small, medium, and large wireworms were estimated to be ≤8, 8–13, and 13–18 mm in length, respectively.

The following preconditioning regime was used to obtain satiated wireworms for experimentation. Sixty hours before an experiment, wireworms of the desired size class were transferred to 23° ±2°C, each provided with at least five germinated wheat seeds, and held in darkness. Twelve hours before the experiment, wheat seeds were removed from the vials. Small wireworms were then individually weighed to the nearest 0.1 mg on a Mettler AE100 top-loading balance. This was done to establish baseline weights for study of sublethal effects. Thus, small wireworms were redefined to be 3.0–15.0 mg ($N = 280$, $\bar{X} = 10.4$, SE = 0.18). Wireworms were placed individually and without wheat seeds into labeled 33-ml vials with about 30 g soil moistened to a water content of 15% (w/w), and held in darkness at 23° ±2°C until experimentation.

Voucher specimens are deposited in the W.F. Barr Entomological Museum, University of Idaho, Moscow, Idaho, and in the personal collection of P.J. Johnson, University of Wisconsin, Department of Entomology, Madison, Wisconsin.

Toxicity. A single aqueous suspension of AITC was prepared in the following manner for each wireworm size class. A 0.01 M stock suspension was made by adding 50.1 μl AITC (Aldrich, Milwaukee, Wisconsin) to 50 ml deionized water. The mixture was covered tightly with a Parafilm seal and sonified in a water bath for 30 min. Appropriate dilutions of the stock suspension were made to obtain AITC solutions, which, when added at the rate of 1.5 ml/10 g soil, provided 300, 270, 240, 210, 180, 150, and 120 nmol AITC/g soil. All solutions were prepared with deionized water and sonified for 10 min. Controls were prepared by substituting deionized water for the AITC solution.

Wireworm bioassays were conducted in a Latahco silt loam soil (Argiaquic Xeric Argialbolls). The top 25 cm of field-collected soil was air-dried and crushed sufficiently to pass through a 2-mm sieve. Soil analysis of total C and N was 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; 10 min equilibration). The 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.

A separate bioassay was conducted for each wireworm size class. We used a completely randomized design with 20 replicates per treatment for experiments with medium and large wireworms, and 40 replicates per treatment for the experiment with small wireworms. Therefore, the number of replicates equaled the number of experimental units. Each replicate consisted of one wireworm in a 20-ml glass scintillation vial with 10 g of soil and 1.5 ml of solution, resulting in about 15% soil moisture. This soil moisture is within the range preferred by

L. californicus wireworms tested in Walla Walla silt loam soil (Jones, 1951). Each replicate was prepared by adding to a vial: 0.75 ml of AITC solution, followed by 5 g of soil, a wireworm, 5 g soil, and 0.75 ml solution. The vial was then quickly covered with a Parafilm seal, capped, and held in darkness at 22° ±1°C. At one day posttreatment, mortality was assessed by examining each wireworm for about 30 sec with a 30× Panasonic Light Scope FF-393E. A wireworm larva that responded to gentle prodding and squeezing with forceps was considered "alive"; otherwise, it was categorized as "dead."

Because of their growth potential, small wireworms still alive at one day posttreatment were maintained to observe the influence of time on mortality and sublethal effects. After the one-day mortality assessment, individuals were immediately placed into 33-ml plastic vials with 30 g of soil moistened with distilled water to 15% soil moisture, and held in darkness at 25° ±3°C. The status (mortality, weight, and occurrence of feeding and molting) of each wireworm was recorded 25 days posttreatment and continued on a weekly basis for about 15 weeks. Wireworms were held without food for the first 25 days, but after this interval two to four seeds of germinated wheat, along with fresh soil, were provided to each wireworm at each weekly examination.

AITC Concentrations in Soil. AITC concentrations of 120, 210, and 300 nmol/g soil were monitored at 0, 1, 2, 4, 6, 8, 12, 16, and 24 hr to estimate the concentration of AITC present throughout the bioassays. Experimental procedures and conditions were maintained as closely as possible to those of the wireworm bioassays, but wireworms were not included. Two separate trials were run, each with a freshly prepared stock suspension, and each concentration–time combination was replicated three times per trial. Controls were treated with deionized water and were not replicated.

Each soil sample (10 g) was extracted in a scintillation vial with 7.1 (trial 1) or 6.5 (trial 2) ml of hexane to which 5.5 ml of 0.005 M $CaCl_2$ had been added. Samples were shaken vigorously for 1 min, and centrifuged at 500*g* for 8 min. The hexane layer was removed and, using a syringe filter-holder, passed through a GN-6 (0.45 μm) membrane filter (Gelman, Ann Arbor, Michigan) into an autosampler vial and capped. Extracts were then analyzed for AITC on a Hewlett Packard 5890 series II gas chromatograph using flame photometric detection with a sulfur filter. A DB-Wax capillary column (30 m, 0.32 ID, 0.25 μm film; J & W Scientific, Folsom, California) was used. The initial oven temperature of 100°C was increased by 8°/min to 168°C and held for 1.5 min. Injector and detector temperatures were 200° and 210°C, respectively.

Statistical Analyses. Mortality data were analyzed using PROC PROBIT (SAS Institute 1989, pp. 1325–1350), using a separate probit analysis for each size class. For small wireworms, separate analyses were conducted for each time of mortality assessment, and control mortality was adjusted for by setting $C = 0.025$ in the PROC PROBIT statement. Feeding data were organized into

2 × 2 cross-tabulation tables (controls versus combined treatments) from which likelihood ratio chi-square statistics were calculated using PROC FREQ (SAS Institute 1988, pp. 519–548). The influence of AITC on wireworm weight was assessed using PROC REG and PROC GLM (SAS Institute 1988, pp. 773–875 and 549–640, respectively). Due to heteroscedasticity over time, a weighted regression (reciprocal of the variance) was calculated. PROC REG and PROC GLM were also used to determine if data from the two AITC dissipation trials were different. Contrasts of lines for 120, 210, and 300 nmol/g soil were not significant ($F = 1.55$, $df = 1$, $P > 0.2183$; $F = 1.10$, $df = 1$, $P > 0.3004$; and $F = 0.10$, $df = 1$, $P > 0.7499$, respectively). Therefore, extracted quantities of AITC for each concentration–time combination were averaged over both trials (six replicates). For each concentration–time combination, the measured AITC value was adjusted with the appropriate extraction efficiency as determined at time 0. Extraction efficiency at time 0 was about 68, 70, and 68% for the 120, 210, and 300 nmol/g soil concentrations, respectively. These values are similar to those (71%) obtained in our previous studies with other soils (unpublished).

RESULTS AND DISCUSSION

Toxicity. AITC concentrations of 150–300 nmol/g soil caused 0–90% mortality of medium and large wireworms at one day posttreatment (Table 1). Probit analysis revealed that both populations were homogeneous and that LC_{50} values of 238 and 226 nmol/g soil were estimated for medium and large wireworms, respectively. For small wireworms, one day posttreatment mortality ranged from 0 to 100% at AITC concentrations of 120–270 nmol/g soil (Table 2). The population was homogeneous, and the LC_{50} value estimated was 211 nmol/g soil. Comparison of 95% confidence intervals showed that LC_{50} values between medium and large wireworms did not significantly differ (Table 1). Extractable AITC decreased rapidly during the one-day treatment period, indicating that wireworms were exposed to decreasing concentrations of AITC (Figure 1). However, AITC concentrations appeared to stabilize between 16 and 24 hr posttreatment for the three amended concentrations.

Mortality of small wireworms with time is presented in Table 2. It is important to note that the bioassay involved a one-day wireworm exposure to AITC. Mortality after this one-day period occurred as a result of the initial exposure, but in soil that did not contain AITC. Mortality increased considerably during the 1- to 137-day posttreatment period, and progressed in one of three ways, depending on AITC concentration. Mortality increased only slightly at the lowest concentration (120 nmol/g soil), ranging from 0 to 7.5%. At AITC concentrations of 150 and 180 nmol/g soil, mortality increased considerably

TABLE 1. TOXICITY OF AITC TO *Limonius californicus* (Mann.) WIREWORMS 1 DAY POSTTREATMENT

Size class[a]	Mortality (%) with AITC (nmol/g soil)							LC_{50} (95% CI)	Pearson χ^2	*df*	$P > \chi^2$	Slope	SE
	0[b]	150	180	210	240	270	300						
Medium	0	0	5.0	20.0	55.0	80.0	NA	238 (227–252)	0.2516	4	0.9927	0.0289	0.0053
Large	0	0	20.0	45.0	65.0	80.0	90.0	226 (212–240)	2.4288	5	0.7872	0.0204	0.0032

[a] Medium and large wireworms approximately 8–13 and 13–18 mm in length, respectively. Twenty replications per treatment per size class.
[b] Controls treated with deionized water.

TABLE 2. CUMULATIVE TOXICITY OF AITC TO SMALL (3.0–15.0 mg) *Limonius californicus* (Mann.) WIREWORMS

Days post treatment	Mortality (%) with AITC (nmol/g soil)[a]							LC_{50} (95% CI)	Pearson[c] χ^2	$P > \chi^2$	Slope	SE
	0[b]	120	150	180	210	240	270					
1	0	0	2.5	17.5	45.0	80	100	211 (204–218)	1.8090	0.8749	0.0334	0.0037
25	0	0	22.5	52.5	92.5	100	100	175 (168–181)	2.4829	0.7791	0.0398	0.0048
32	0	2.6	27.5	52.5	92.5	100	100	172 (165–179)	2.5302	0.7719	0.0356	0.0042
39	2.5	2.6	27.5	60.0	92.5	100	100	172 (164–178)	1.4188	0.9222	0.0379	0.0048
46	2.5	5.1	27.5	70.0	92.5	100	100	168 (161–175)	0.5167	0.9915	0.0373	0.0048
53	2.5	5.1	30.0	75.0	95.0	100	100	165 (158–172)	0.4182	0.9948	0.0395	0.0052
60	2.5	5.1	37.5	75.0	95.0	100	100	163 (155–170)	0.9565	0.9660	0.0375	0.0048
67	2.5	5.1	40.0	77.5	97.5	100	100	160 (153–167)	0.7904	0.9776	0.0403	0.0054
74	2.5	5.1	40.0	82.5	97.5	100	100	159 (152–165)	0.7326	0.9811	0.0421	0.0057
81	2.5	5.1	40.0	82.5	97.5	100	100	159 (152–165)	0.7326	0.9811	0.0421	0.0057
88	2.5	5.1	40.0	82.5	97.5	100	100	159 (152–165)	0.7326	0.9811	0.0421	0.0057
95	2.5	7.5	40.0	82.5	97.5	100	100	158 (151–165)	0.2208	0.9989	0.0402	0.0054
102	2.5	7.5	40.0	82.5	97.5	100	100	158 (151–165)	0.2208	0.9989	0.0402	0.0054
137	2.5	7.5	40.0	82.5	100	100	100	157 (150–164)	0.5822	0.9888	0.0445	0.0064

[a] Forty replications per treatment, except 39 replications for lowest AITC concentration (120 nmol/g soil).
[b] Controls treated with deionized water.
[c] $df = 5$.

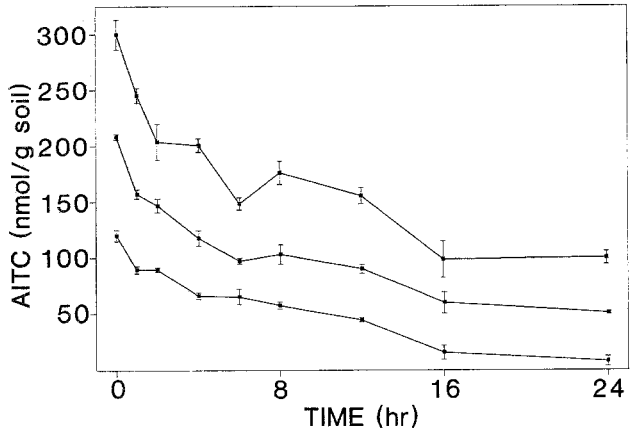

FIG. 1. Decrease in extractable AITC concentrations (120, 210, and 300 nmol/g soil) in a Latahco silt loam soil during a one-day period ($\bar{X} \pm$ SE; $N = 6$).

between days 1 and 67 posttreatment. After this, additional mortality at these concentrations was minimal. At concentrations of 210 and 240 nmol/g soil, mortality was greatest during the first 25 days posttreatment. Comparison of 95% confidence intervals showed that LC_{50} values decreased significantly from 211 to 157 nmol/g soil during the 137-day posttreatment period (Table 2). The greatest decrease per unit time occurred during the initial 25-day posttreatment period. These data show the trend of small wireworm mortality with time, providing a more realistic view of AITC toxicity than that possible from a one-time mortality assessment.

There are few related studies available to compare with our work. Toba (1984) reported metham-sodium-induced mortality of *L. californicus* wireworms buried at depths of 15–90 cm in agricultural fields in the northwestern United States. Metham-sodium degrades in situ to MITC (Miller, 1988), the compound responsible for insecticidal activity. Mortality after the three-week treatment period ranged from 0 to 100%, but decreased as depth increased. Fifty-five percent mortality was achieved at a depth of 60 cm. Assuming a 60-cm depth of incorporation, soil bulk density of 1.4 g/cm^3, and 100% conversion of metham-sodium to MITC (146.5 kg AI/ha was applied), a mean of 238 nmol/g soil MITC led to 55% mortality. Brown et al. (1991) reported that defatted rapeseed (*B. napus* L.) meal (RSM) amended at a rate of 30 g/kg soil to the same soil as used in the current experiments was not toxic to *L. infuscatus* Mots. wireworms after a 17-day treatment period. Glucosinolates contained in that amount of plant tissue could potentially produce ITC concentrations of 1600 nmol/g soil; however, the maximum extractable ITC concentration determined

2 hr after amendment was only 301 nmol/g soil. This amount dissipated by nearly 90% after 24 hr, compared with the present work where the AITC concentration of 300 nmol/g soil decreased by about 60% in the same time (Figure 1). The ITC concentration reported by Brown et al. (1991) (301 nmol/g soil) exceeds the amount of AITC found to be acutely toxic to wireworms in our work and also the amount of MITC estimated to be toxic to wireworms from the data of Toba (1984).

There are distinct differences between the studies, which may explain the variability in results. First, glucosinolates contained in *B. napus* meal are expected to produce 3-butenyl and 4-pentyl isothiocyanate and not AITC or MITC. The acute toxicities of each of these compounds to wireworms have never been compared. Second, in addition to glucosinolates, RSM contains a high concentration of protein, which may react irreversibly with ITC, reducing the effective concentrations (Wood, 1975). Finally, bioassays with RSM were conducted in uncovered containers where volatile losses of ITC were possible. This was not the case in assays with AITC, in which capped vials were used, and in the field where diffusion may limit volatile losses.

Influence of AITC on Wireworm Feeding Activity. Results of feeding observations are presented in Table 3 and Figure 2. Wireworms that had molted during the interval preceding observation were omitted from analysis, because

TABLE 3. TESTS FOR ASSOCIATION BETWEEN AITC TREATMENT AND FEEDING FOR SMALL (3.0–15.0 mg) *Limonius californicus* (Mann.) WIREWORMS

Time post treatment (days)	Likelihood ratio[a]		
	N untreated, treated	χ^2	$P > \chi^2$
32	39, 74	0.594	0.441
39	32, 59	9.782	0.002
46	24, 58	16.382	0.0001
53	29, 63	2.275	0.132
60	32, 62	7.975	0.005
67	31, 63	0.554	0.457
74	38, 53	0.152	0.697
81	29, 49	0.001	0.972
88	22, 49	2.008	0.156
95	26, 44	0.803	0.370
102	23, 50	15.603	0.0001
137	6, 10	3.063	0.080
Overall	331, 634	5.805	0.016

[a] $df = 1$.

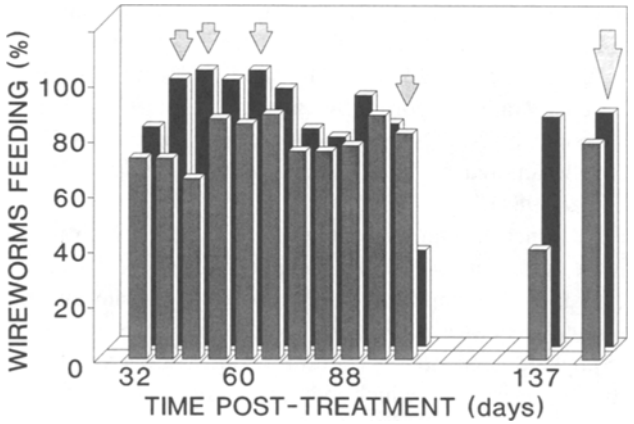

FIG. 2. Feeding activity (percent wireworms feeding) of untreated and AITC-treated wireworms during a 137-day posttreatment period (untreated = solid, treated = hatched). Arrows indicate significant differences; the large arrow indicates overall values.

our data indicated that *L. californicus* wireworms generally did not feed for about seven days prior to molting, an observation made for other wireworm species (Evans and Gough, 1942; Evans, 1944; Zacharuk, 1962; Burrage, 1963). Likelihood ratio tests for association between treatment and feeding activity indicated that wireworms treated with AITC fed significantly less often than untreated (control) wireworms at 39, 46, and 60 days posttreatment and overall (Table 3 and Figure 2). In contrast, untreated wireworms fed less often than treated wireworms at 102 days posttreatment. This was due to reduced feeding activity of untreated wireworms, not to increased feeding activity of treated wireworms (Figure 2). Overall feeding frequency of treated and untreated wireworms was 79 and 85%, respectively (Figure 2).

These results indicate that sublethal effects of AITC may have potential from a pest management standpoint. For most crops, wireworm damage is inflicted during seedling establishment; susceptibility to damage decreases as vegetative growth proceeds (Stone, 1941; Hall, 1990). We did not collect feeding data corresponding to the seedling period (i.e., 0–32 days posttreatment). However, feeding frequencies of treated wireworms at the first three times of assessment (32, 39, and 46 days posttreatment) were slightly less than at later times (Figure 2), suggesting that feeding prior to 32 days posttreatment may be reduced as well. This is supported by a subsequent study with ionic ITC, where feeding frequency increased from a low of 31% at seven days posttreatment to a high of 67% at 56 days posttreatment (unpublished). Therefore, reduced plant damage may be achieved at a treatment concentration considerably lower than that necessary for 50% acute mortality in a wireworm population. Villani and

Gould (1985) screened crude extracts of 78 plant species on the feeding behavior of late-instar *Melanotus communis* Gyll. wireworms. Of the four crucifers initially screened, none led to behavior (arrestant, attractant, or deterrent) of sufficient magnitude to warrant further testing. Because concentrations and identities of allelochemicals in the extracts were unknown, it is impossible to compare their study to ours. Landis and Gould (1988) found that a 1 M concentration of AITC was toxic to larvae of southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber. Corn seeds treated with 0.0001, 0.01, and 0.1 M AITC concentrations had significantly reduced feeding after 72 hr.

Influence of AITC on Wireworm Weight. A linear relationship between weight and time (days) was observed for treated and untreated wireworms (treated $y = -7.49 + 0.716(x)$, $R^2 = 0.99$; untreated $y = -7.67 + 0.721(x)$, $R^2 = 0.99$), but AITC treatment did not have a significant impact on wireworm weight. Contrasts were not significant for slopes or lines ($F = 0.97$, $df = 1$, $P > 0.3243$; and $F = 0.62$, $df = 1$, $P > 0.4330$, respectively). However, weights of treated wireworms were usually lower than those of untreated wireworms for the initial 60 days posttreatment, after which weights of treated wireworms seemed to rebound. This nonsignificant trend corresponds to periods of reduced feeding by treated wireworms (Table 3 and Figure 2) and may represent a sublethal treatment effect.

CONCLUSION

AITC amendment to soil had both lethal and sublethal effects on *L. californicus* wireworms, indicating that using glucosinolate-containing plant tissue as a source of ITC may reduce crop damage caused by these soil-borne insects. Gould (1991) suggested that insects exposed to compounds possessing both toxic and antifeedant properties may be increasingly deterred over time. We believe that future investigations should emphasize sublethal effects (e.g., repellency and inhibition of feeding) of ITC on wireworm populations, rather than on acute lethal effects. Our results showed that AITC reduced feeding by wireworms from 32 to 137 days posttreatment; nevertheless, overall feeding frequency was substantial (79%). Demonstration that naturally occurring levels of AITC suppress wireworm feeding during the time of seedling establishment would give more promise to using *Brassica* spp. as a soil amendment for crop protection. If wireworms could be temporarily repelled or otherwise deterred from feeding on germinating seeds and seedlings, subsequent feeding on older, less vulnerable tissue could be tolerated, while minimizing selective pressure on the wireworms to develop resistance. Gould (1991) proposed such a scenario for managing toxins in genetically engineered plants.

Although few data are available, approximate concentrations of AITC

expected in soil from green manure crops can be estimated. Davis (1988) grew five cultivars of *B. carinata* and 12 cultivars of *B. nigra* in the greenhouse. Allyl glucosinolate concentrations in leaf tissues ranged from 3.9 to 27.9 μmol/g dry tissue when sampled at full bloom just prior to pod set. If we then assume a dry biomass yield of 4 t/ha, soil bulk density of 1.3 g/cm^3, tissue incorporation to a depth of 15.24 cm in the soil, and complete conversion of allyl glucosinolate to AITC, expected AITC concentrations range from 7.8 to 56.3 nmol/g soil. These calculations do not include below-ground tissues, the possibility of ITC produced from root exudates, and the additive impact of other allelochemicals. Although the rate of release of AITC from the tissues is unknown, a biological impact seems possible.

Further investigations with AITC and other glucosinolate degradation products are clearly needed to better understand the interactions of lethal and sublethal effects, release rates from plant tissues, and the influence of soil variables on allelochemical dissipation. Exploring the effects of *Brassica*-amended soil on oviposition preference and subsequent survival of eggs and neonate wireworms may also be fruitful.

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REFERENCES

BIALY, Z., OLESZEK, W., LEWIS, J., and FENWICK, G.R. 1990. Allelopathic potential of glucosinolates (mustard oil glycosides) and their degradation products against wheat. *Plant Soil* 129:277–281.

BROWN, P.D., MORRA, M.J., MCCAFFREY, J.P., AULD, D.L., and WILLIAMS, L., III. 1991. Allelochemicals produced during glucosinolate degradation in soil. *J. Chem. Ecol.* 17:2021–2034.

BURRAGE, R.H. 1963. Seasonal feeding of larvae of *Ctenicera destructor* and *Hypolithus bicolor* (Coleoptera: Elateridae) on potatoes placed in the field at weekly intervals. *Ann. Entomol. Soc. Am.* 56:306–313.

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.

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.

EVANS, A.C. 1944. Observations on the biology and physiology of wireworms of the genus *Agriotes* Esch. *Ann. Appl. Biol.* 31:235–250.

EVANS, A.C., and GOUGH, H.C. 1942. Observations on some factors influencing growth in wireworms of the genus *Agriotes* Esch. *Ann. Appl. Biol.* 29:168–175.

FENWICK, G.R., HEANEY, R.K., and MULLIN, W.J. 1983. Glucosinolates and their breakdown products in food and 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, Agronomy Monograph 9, 2nd ed., Part 1. ASA and SSSA, Madison, Wisconsin.

GOULD, F. 1991. Arthropod behavior and the efficacy of plant protectants. *Annu. Rev. Entomol.* 36:305–330.

HALL, D.G. 1990. Stand and yield losses in sugarcane caused by the wireworm *Melanotus communis* (Coleoptera: Elateridae) infesting plant cane in Florida. *Fla. Entomol.* 73:298–302.

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.

JIMÉNEZ-OSORNIO, J.J., and GLEISSMAN, 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.

JONES, E.W. 1951. Laboratory studies on the moisture relations of *Limonius* (Coleoptera: Elateridae). *Ecology* 32: 284–293.

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.

KOŠŤÁL, V. 1992. Orientation behavior of newly hatched larvae of the cabbage maggot, *Delia radicum* (L.) (Diptera: Anthomyiidae), to volatile plant metabolites. *J. Insect Behav.* 5:61–69.

LANDIS, D.A., and GOULD, F. 1988. Screening for phyto-protectants to guard corn seeds/seedlings from southern corn rootworm feeding injury. *J. Entomol. Sci.* 23:201–211.

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.

MILLER, D.B. 1988. Determination of methylisothiocyanate by an HPLC trapping and derivatization procedure. *LC-GC* 6:842–846.

MOJTAHEDI, H., SANTO, G.S., HANG, A.N., and WILSON, J.H. 1991. Suppression of root-knot nematode populations with selected rapeseed cultivars as green manure. *J. Nematol.* 23:170–174.

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.

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.

SAS Institute Inc. 1988. SAS/STAT User's Guide, Release 6.03 Edition. Cary, North Carolina.

SAS Institute Inc. 1989. SAS/STAT User's Guide for personal computers, version 6. SAS Institute, Cary, North Carolina.

STONE, M.W. 1941. Life history of the sugar-beet wireworm in southern California. USDA Technical Bulletin, 744, 88 pp.

TOBA, H.H. 1984. Metham-sodium for control of wireworm larvae. *J. Agric. Entomol.* 1:392–396.

TOBA, H.H., O'KEEFFE, L.E., PIKE, K.S., PERKINS, E.A., and MILLER, J.C. 1985. Lindane seed treatment for control of wireworms (Coleoptera: Elateridae) on wheat in the Pacific Northwest. *Crop Prot.* 4:372–380.

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.

VILLANI, M., and GOULD, F. 1985. Screening of crude plant extracts as feeding deterrents of the wireworm, *Melanotus communis*. *Entomol. Exp. Appl.* 37:69–75.

WILLIAMS, L., III, SCHOTZKO, D.J., and MCCAFFREY, J.P. 1992. Geostatistical description of the spatial distribution of *Limonius californicus* (Mann.) (Coleoptera: Elateridae) wireworms in the northwestern United States, with comments on sampling. *Environ. Entomol.* 21:983–995.

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.

ZACHARUK, R.Y. 1962. Distribution, habits, and development of *Ctenicera destructor* (Brown) in western Canada, with notes on the related species *C. aeripennis* (Kby.) (Coleoptera: Elateridae). *Can. J. Zool.* 40:539–552.

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HOW HERBIVORES TRACK VARIABLE ENVIRONMENTS: RESPONSE TO VARIABILITY OF PHYTOTOXINS[1]

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Abstract—The concentration of toxins in plants can vary with plant phenology and environmental factors. Changes in toxicity may or may not be associated with changes in plant flavor. These factors may make it difficult for herbivores to avoid toxic plants. We conducted three experiments concerning animals' ability to detect toxins. In experiment 1, we examined whether animals familiar with a tastable toxin on food would adjust intake when toxin concentrations changed. Lambs were initially offered oats with either a low, medium, or high concentration of lithium chloride (LiCl; a salty-tasting toxin). Later, when all lambs were offered oats with the medium concentration of LiCl, lambs that were conditioned with the low LiCl concentration decreased intake while lambs conditioned with the high LiCl concentration increased intake. In experiment 2, we examined flavor aversions formed when animals sampled food with two flavor levels prior to illness. Lambs initially ate the same amount of barley with a low and with a high concentration of either sodium saccharin (a sweet flavor) or aluminum sulfate (a bitter flavor). Lambs then received a mild dose of LiCl. Later, when lambs were offered a choice between barley with either a low or high flavor concentration, lambs preferred the barley with the low flavor concentration. Experiment 3 examined how variable toxicity affected intake when the flavor remained constant. Lambs were initially offered oregano-flavored barley and then received a dose of LiCl. Lambs received either a medium dose, a high dose, or a dose that varied randomly among low, medium, or high amounts of LiCl. Lambs in the medium dose

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group ate more oregano-flavored barley at the end of the trial than did lambs in either of the other groups.

Key Words—Conditioned flavor aversions, diet selection, grazing behavior, phytotoxins, poisonous plants, ruminants, sheep, *Ovis aries*.

INTRODUCTION

Herbivores face many challenges in selecting a nutritionally adequate diet. They must overcome chemical plant defenses and select among plants that vary in nutrient value and toxicity in time and space (Provenza and Balph, 1990; Laca and Demment, 1991). Toxicity and nutrient value vary with plant phenology, age, and plant part (McKey, 1979; Bryant et al., 1992) and with environmental conditions such as drought, soil nutrient levels, and shade (Harborne, 1991; Bryant et al., 1992). Toxicity may also depend on animal age, sex, and hormonal or nutritional state (Freeland and Janzen, 1974; Brattsten, 1979). Diet composition and conditions in the gut, such as pH, temperature, and solution polarity, can also influence the toxicity of plant compounds (Freeland and Janzen, 1974; McArthur et al., 1991). Experience may also affect toxicity. Sustained intake of a compound may increase an animal's ability to detoxify the chemical (Robbins et al., 1987).

It is recognized that plant toxicity varies, depending on plant and animal characteristics, but few theories have been advanced to explain how herbivores avoid lethal doses of phytotoxins in variable environments. A diverse diet may limit an herbivore's exposure to a single phytotoxin (Freeland and Janzen, 1974; Laycock et al., 1988). Flavor–consequence relationships, based on postingestive feedback, may also help herbivores recognize changes in toxicity (Provenza et al., 1988, 1992a). Neither of these mechanisms has been rigorously tested. The following three experiments were conducted to examine the ability of sheep, a generalist herbivore, to adjust intake when: (1) toxicity changes were associated with flavor changes, (2) foods with varying flavor concentration were sampled, and (3) flavor was not related to toxicity.

Animals identify plant toxins by associating food flavor (odor and taste) with postingestive feedback (Garcia, 1989; Provenza and Balph, 1990). The flavor of many plant allelochemicals may impart a distinct flavor to plants. For instance, some tannins presumably are astringent to herbivores, and some flavanones and alkaloids presumably taste bitter (Laycock et al., 1988; Harborne, 1991). When flavor and toxicity are highly correlated, herbivores might first learn to regulate food intake based on postingestive feedback and then simply adjust intake on the basis of flavor intensity. Our first experiment examined whether animals, familiar with a tastable toxin at one concentration, adjust intake when the concentration of that toxin changes.

In experiment 1, sheep experienced a food with a single flavor concentration and constant toxicity prior to testing. However, it may be more common for herbivores to gain experience while sampling individual plants or plant parts that vary in both flavor and toxicity. In experiment 2, we examined the influence of sampling foods with varying intensities of a flavor, prior to illness, on the formation of a conditioned flavor aversion (CFA). We studied this effect for a sweet (sodium saccharin) and a bitter (aluminum sulfate, i.e., alum) flavor.

Flavor may not always be a good indicator of toxicity. For instance, minute changes in stereochemistry, which may be difficult for herbivores to detect through taste or smell, can dramatically change the toxicity of many phytochemicals (Bryant et al., 1992; Provenza et al., 1992a). Some compounds may be present in such minute amounts that they add little or nothing to overall plant flavor (Manners et al., 1992). Moreover, changes in toxicity involving a group of phytochemicals may not be well correlated with changes in plant flavor (Bryant et al., 1992). Finally, factors such as diet selection or an animal's nutritional state may alter plant toxicity but not affect plant flavor (Freeland and Janzen, 1974; Brattsten, 1979). This experiment examined how variability of toxicity influenced food ingestion when flavor remained constant.

METHODS AND MATERIALS

Experiment 1. The experiment involved 60 orphan lambs that were crosses of Columbia, Rambouillet, Suffolk, Targhee, and Finnish Landrace breeds. Lambs, six months old, were penned individually for 10 days and offered alfalfa pellets (*Medicago sativa*) ad libitum for 1 hr every morning. They had access to water and salt at all times. Pens were set side by side to allow social contact between lambs.

For 30 min each afternoon of the 10-day conditioning period, each lamb was offered 500 g of a novel food, oats (*Avena sativa*). The lambs were assigned at random to one of four LiCl (an emetic salt that causes conditioned flavor aversions in most mammals) treatments: (1) control, oats with no lithium chloride (LiCl); (2) low, oats with 0.75% LiCl; (3) medium, oats with 1.5% LiCl; or (4) high, oats with 2.25% LiCl. LiCl was dissolved in water, making a saturated solution, mixed thoroughly with oats, and then the mixture was dried at 60°C for 12 hr. Daily consumption of oats or LiCl-treated oats was recorded.

All lambs were offered 500 g of oats with 1.5% LiCl (the medium treatment level) for 30 min on the first afternoon after the conditioning period (day 11 of the trial). On this day, lambs were again offered alfalfa pellets in the same manner as during conditioning.

Data concerning intake on the first day of the conditioning period were subjected to an analysis of variance (ANOVA). Data on daily intake throughout

conditioning were analyzed with a repeated measures ANOVA. Intake of LiCl-treated oats on the test day was analyzed with a separate ANOVA with Fisher's LSD procedure to separate treatment means. A paired *t* test was used to compare intake on the last day of conditioning (day 10) with that on the test day (day 11).

Experiment 2. Forty-eight 5-month-old lambs were individually penned with continuous access to water and salt. Lambs were divided into two groups. Each lamb in one group received 100 g of barley (*Hordeum vulgare*) grain containing 0.5 and 2% saccharin in separate feed boxes. Each lamb in the other group was offered 100 g of barley containing 1 and 2.5% alum in separate feed boxes. Lambs had access to the foods for 15 min. Sixteen of the lambs in each group received a single, low dose of LiCl (125 mg/kg body weight) (DuToit et al., 1991). The remaining eight lambs in each group served as controls. To ensure that each lamb ate a similar amount of feed with each flavor level, lambs that consumed less than 90 g of either feed were removed from the trial. This resulted in 8 lambs in the saccharin-control, 12 lambs in the saccharin–LiCl treatment, 7 lambs in the alum-control, and 12 lambs in the alum–LiCl treatment.

The following day, each lamb was offered 150 g of the flavored feeds it consumed the previous day. Lambs were allowed to eat for 15 minutes or until all of one of the feeds was eaten. We quantified preference by dividing the amount of the less intensely flavored feed by the total amount of feed consumed (high and low flavor concentration). This yielded a preference index between 0 and 1; 0 indicated complete consumption of the more intensely flavored feed, 1 indicated complete consumption of the less intensely flavored feed, and 0.5 indicated equal consumption of both feeds.

A two-factor ANOVA was used to compare the consumption of barley flavored with saccharin and alum at low and high concentrations and to determine how LiCl influenced the preference for flavored barley.

Experiment 3. Thirty 7-month-old lambs were penned individually for the 10-day trial. Each lamb was offered alfalfa pellets ad libitum for 1 hr every morning and had access to water and salt at all times. For 30 min each afternoon, each lamb was offered an amount of ground barley equivalent to 1% of its body weight. Barley contained ground oregano at 1.5% concentration by weight. Following exposure to oregano-flavored barley, animals received LiCl in gelatin capsules administered orally. The amount of LiCl administered was as follows: (1) medium dose, LiCl equal to 1.5% of barley consumed; (2) high dose, LiCl equal to 2.25% of barley consumed; (3) variable dose, LiCl equal to 0.75, 1.5, or 2.25% of barley consumed, administered on a random schedule. The LiCl dose administered to lambs in the variable treatment averaged 1.5% of the barley consumed over the 10-day trial. The actual dosing schedule is presented in Table 1.

TABLE 1. SCHEDULE OF LiCl DOSE (% OF AMOUNT BARLEY EATEN) RECEIVED BY LAMBS IN VARIABLE TREATMENT DURING EXPERIMENT 3

	Day of experiment									
	1	2	3	4	5	6	7	8	9	10
Dose	1.50	0.75	2.25	1.50	0.75	1.50	2.25	2.25	0.75	none

We compared intake of lambs in the variable dose group with the intake of lambs receiving the high and medium dose. Data from the last six days of the trial were analyzed. The first four days were considered an adjustment period. Data were analyzed with a repeated measures ANOVA with day as the repeated measure. Fisher's LSD test was used to compare treatment means.

RESULTS

Experiment 1. On the first day of conditioning lambs in the medium treatment ate less than other groups ($P < 0.05$; Figure 1) for no obvious reason, but by day 10, the concentration of LiCl on oats strongly affected intake ($P <$

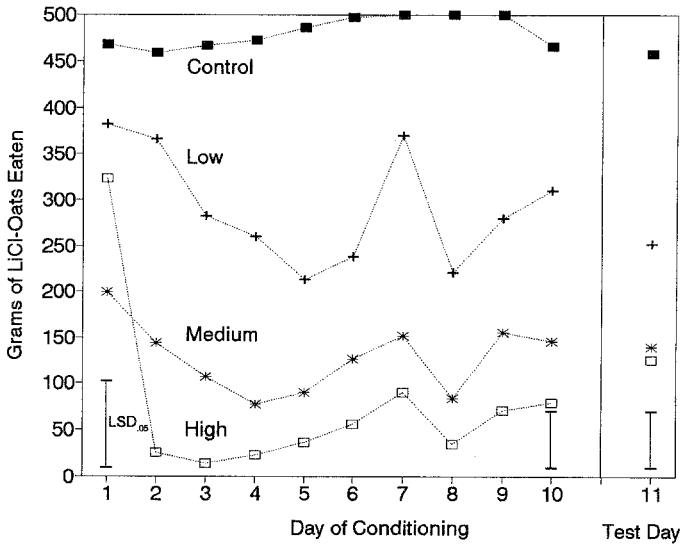

FIG. 1. Daily intake of oats by lambs in the control and LiCl-oats by lambs in the low, medium, and high treatments during the conditioning period, and intake of LiCl-oats by all lambs on the test day of experiment 1.

0.001; Figure 1). Lambs offered untreated oats ate the most while lambs in the low, medium, and high treatment groups ate less, respectively (Figure 1). Although consumption of LiCl-treated oats differed by treatment, the amount of LiCl ingested on day 10 was the same for all treatments (excluding the control; Table 2).

When all lambs, including controls, were offered oats with 1.5% LiCl, lambs in the medium and control groups ate similar amounts as they had on the last day of the conditioning period ($P < 0.05$; Figure 1). However, lambs in the high treatment group increased intake while lambs in the low treatment group decreased intake ($P < 0.05$).

Experiment 2. Compared to lambs in the control group, lambs that received

TABLE 2. AMOUNT OF LiCl-OATS AND LiCl INGESTED BY LAMBS ON DAY 10 OF CONDITIONING PERIOD OF EXPERIMENT 1

Treatment	LiCl-oats eaten (g ± SE)	LiCl ingested (mg/kg ± SE)
Low	310.0a[a] ± 25.6	68.5a ± 5.1
Medium	145.6b ± 18.6	66.6a ± 6.9
High	79.5c ± 1.73	52.5a ± 11.1

[a]Means for treatment followed by a different letter are different at $P < 0.05$.

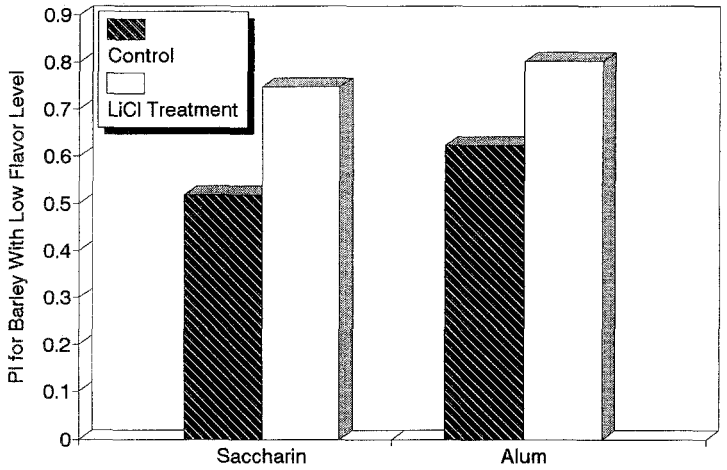

FIG. 2. Preference indices (PI) for barley with the low flavor level of saccharin or alum by control and treatment lambs in experiment 2. Preference equals the amount of barley eaten with the low flavor divided by the total amount of flavored barley eaten.

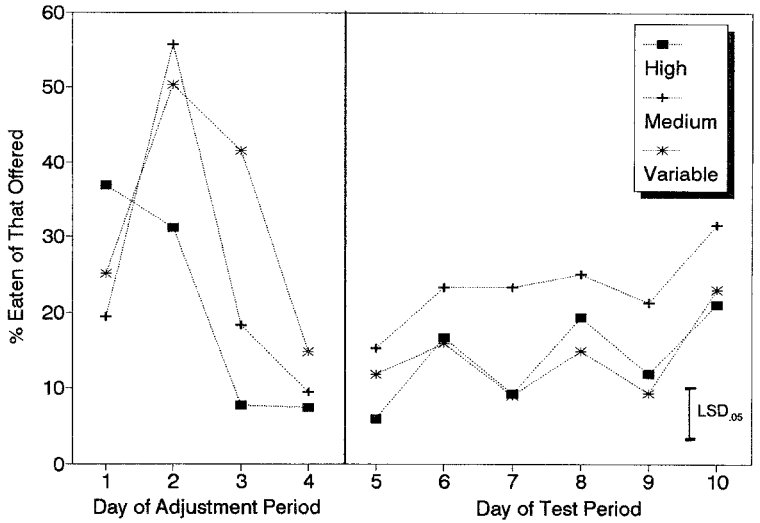

FIG. 3. Percent eaten of oregano-flavored barley offered to lambs in the high, medium, and variable treatments during the adjustment and test periods of experiment 3.

LiCl after eating flavored barley showed a higher preference for barley with a less intense flavor ($P = 0.003$), whether the barley was flavored with saccharin or alum ($P = 0.214$; Figure 2). There was no flavor by treatment interaction ($P = 0.696$).

Experiment 3. LiCl dose strongly influenced intake during the trial ($P < 0.001$; Figure 3). Lambs in the medium-dose group ate more than did lambs in the high or variable treatment, but intake of the high and variable groups was indistinguishable (Figure 3). Intake in all groups increased throughout the trial (i.e., significant day effect; $P < 0.001$). There was no treatment by day interaction ($P = 0.585$).

DISCUSSION

Experiment 1. Lambs adjusted their intake of LiCl-treated oats as LiCl concentration changed, which is consistent with research on rat subjects concerning the generalization of a flavor aversion across flavor concentration gradients (Nowlis, 1974; Scott and Giza, 1987; Spector and Grill, 1988). Lambs' intake may have been influenced by gastrointestinal feedback during the 30-min exposure period on the test day (Provenza et al., 1992b), but this is not likely because the addition of LiCl to oats on the test day did not influence intake of lambs in the control group. Further, lambs usually ate LiCl-treated oats in 10–12 min even though 30 min were allowed for feeding.

These results suggest that sheep can adjust intake of phytotoxins based on flavor alone, provided the concentration of the toxin is highly correlated with plant flavor. This is likely to occur when a plant is defended by a single or a few phytochemicals that have a detectable flavor. Ecological studies that reveal a correlation between the concentration of a toxin and palatability may be examples of the above relationship. For example, the amount of phenolic resin on the leaves and stems of creosote bush (*Larrea tridentata*) is directly related to herbivore (*Neotoma lepida*) acceptance (Meyer and Karasov, 1991).

It is important to note that lambs limited their intake of LiCl to an average of 62.7 mg/kg (SE = 4.5) regardless of the concentration of LiCl on oats. This agrees with speculation that animals do not avoid most toxic foods, but maintain intake below some toxic threshold (Freeland and Janzen, 1974). DuToit et al. (1991) calculated voluntary intake of LiCl by sheep, based on a study by Burritt and Provenza (1989), as 39 mg/kg when oats were treated with 2% LiCl. This is substantially less than the level recorded in the present study. The difference may reflect that LiCl-treated oats was offered to lambs separately from the basal ration (alfalfa pellets), while Burritt and Provenza (1989) offered LiCl-treated oats at the same time as the basal ration. Thus, the presence of an alternative food may have influenced the consumption of LiCl.

Experiment 2. Experiment 2 showed that CFA's act by altering preference along a flavor gradient; once averted to a flavor, lambs showed a greater preference for foods with less intense levels of that flavor. This was true even when animals initially sampled feeds with various flavor concentrations and after only one exposure to the flavor.

This could explain the variation in intake of old growth and current-season growth blackbrush (*Coleogyne ramosissima*) by goats reported by Provenza et al. (1990). On day 1, goats ate nearly equal amounts of old growth and current-season growth blackbrush. However, on day 2 goats preferred old growth, which has a lower concentration of condensed tannins. Differences in the amount of condensed tannins may have created differences in flavor. Thus, when animals became ill, they preferred blackbrush that contained less condensed tannins.

Sweet flavors are generally considered feeding attractants, while bitter flavors are usually considered feeding deterrents (Garcia and Hankins, 1977). The results of experiment 2 suggest that an aversion is as easily formed to a sweet flavor as to a bitter flavor. Sweetness or bitterness may influence the initial acceptance of a food but may not determine the food's subsequent palatability; sweet flavors may become aversive and bitter flavors may become highly preferred (Molyneux and Ralphs, 1992).

Experiment 3. The average dose of LiCl administered to lambs in the variable treatment group was equal to the dose administered to lambs in the medium dose group. However, the aversion acquired by lambs in the variable

treatment was equivalent to that developed by lambs in the high treatment. This response reduced the risk of lambs over-ingesting LiCl.

SUMMARY

Generalist herbivores, such as sheep, apparently have several mechanisms to regulate their intake of toxic plants regardless of whether or not toxic variation can be detected through flavor changes. When changes in flavor did correspond to changes in toxicity, animals adjusted their intake based on flavor concentration. When toxicity could not be detected via flavor, the amount consumed by animals was based on the maximum dose of toxin they had ingested. These results are a step toward understanding how generalist herbivores deal with variability in herbage toxicity.

REFERENCES

BRATTSTEN, L.B. 1979. Biochemical defense mechanisms in herbivores against plant allelochemicals, pp. 200–270, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

BRYANT, J.P., REICHARDT, P.B., and CLAUSEN, T.P. 1992. Chemically mediated interactions between woody plants and browsing mammals. *J. Range Manage.* 45:18–24.

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.

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.* 30:35–46.

FREELAND, W.F., and JANZEN, D.H. 1974. Strategies in herbivory by mammals: The role of plant secondary compounds. *Am. Nat.* 108:269–289.

GARCIA, J. 1989. Food for Tolman: Cognition and cathexis in concert, pp. 45–85, *in* T. Archer and L. Nilsson (eds.). Aversions, Avoidance and Anxiety. Lawrence Erlbaum Assoc., Hillsdale, New Jersey.

GARCIA, J., and HANKINS, W.G. 1977. On the origin of food aversion paradigms, pp. 3–26, *in* L. Barker, M. Domjam, and M. Best (eds.). Learning Mechanisms in Food Selection. Baylor University Press, Waco, Texas.

HARBORNE, J.B. 1991. The chemical basis of plant defense, pp. 45–59, *in* R.T. Palo and C.T. Robins (eds.). Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, Florida.

LACA, E.A., and DEMMENT, M.W. 1991. Herbivory: The dilemma of foraging in a spatially heterogeneous food environment, pp. 29–44, *in* R.T. Palo and C.T. Robins (eds.). Plant Defenses against Mammalian Herbivory. CRC Press, Boca Raton, Florida.

LAYCOCK, W.A., YOUNG, J.A., and UECKERT, D.N. 1988. Ecological status of poisonous plants on rangelands, pp. 27–42, *in* L.F. James, M.H. Ralphs, and D.B. Nielson (eds.). The Ecology and Economic Impact of Poisonous Plants on Livestock Production. Westview Press, Boulder, Colorado.

MANNERS, G.D., PFISTER, J.A., RALPHS, M.H., PANTER, K.E., and OLSEN, J.D. 1992. Larkspur chemistry: Toxic alkaloids in tall larkspurs. *J. Range Manage.* 45:63–66.

MCARTHUR, C., HAGERMAN, A.E., and ROBBINS, C.T. 1991. Physiological strategies of mammalian

herbivores against plant defenses, pp. 103–114, *in* R.T. Palo and C.T. Robbins (eds.). Plant Defenses against Mammalian Herbivory. CRC Press, Boca Raton, Florida.

MCKEY, D. 1979. The distribution of secondary compounds within plants, pp. 56–133, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, New York.

MEYER, M.W., and KARASOV, W.H. 1991. Chemical aspects of herbivory in arid and semiarid habitats, pp. 167–187, *in* R.T. Palo and C.T. Robbins (eds.). Plant Defenses against Mammalian Herbivory. CRC Press, Boca Raton, Florida.

MOLYNEUX, R.J., and RALPHS, M.H. 1992. Plant toxins and palatability to herbivores. *J. Range Manage.* 45:13–18.

NOWLIS, G.H. 1974. Conditioned stimulus intensity and acquired alimentary aversion in the rat. *J. Comp. Physiol. Psychol.* 86:1173–1184.

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.). Behavioral Mechanisms of Food Selection. Springer-Verlag, Berlin.

PROVENZA, F.D., BALPH, D.F., DWYER, D.D., RALPHS, M.H., and PFISTER, J.A. 1988. Toward understanding the behavioral responses of livestock to poisonous plants, pp. 407–424, *in* L.F. James, M.H. Ralphs, and D.B. Nielsen (eds.). The Ecology and Economic Impact of Poisonous Plants on Livestock Production. Westview Press, Boulder, Colorado.

PROVENZA, F.D., BURRITT, E.A., CLAUSEN, T.P., BRYANT, J.P., REICHARDT, P.B., and DISTEL, R.A. 1990. Conditioned flavor aversions: 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. 1992a. Mechanisms of learning in diet selection with reference to phytotoxicosis in herbivores. *J. Range Manage.* 45:36–45.

PROVENZA, F.D., LYNCH, J.J., and NOLAN, J.V. 1992b. Food aversion conditioned in anesthetized sheep. *J. Comp. Psychol.* Submitted.

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.

SCOTT, T.R., and GIZA, B.K. 1987. A measure of taste intensity discrimination in the rat through conditioned taste aversions. *Physiol. Behav.* 41:315–320.

SPECTOR, A.C., and GRILL, H.J. 1988. Differences in the taste quality of maltose and sucrose in rats: Issues involving the generalization of conditioned taste aversions. *Chem. Senses* 13:95–113.

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CHIRALITY OF 5,11-DIMETHYLHEPTADECANE, THE MAJOR SEX PHEROMONE COMPONENT OF THE HEMLOCK LOOPER, *Lambdina fiscellaria* (LEPIDOPTERA: GEOMETRIDAE)

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Abstract—Of the four possible stereoisomers of 5,11-dimethylheptadecane, the major sex pheromone component of the eastern hemlock looper (EHL), *Lambdina fiscellaria fiscellaria* Guen., and the western hemlock looper (WHL), *Lambdina fiscellaria lugubrosa* Hulst, (5*R*,11*S*)-5,11-dimethylheptadecane was the only stereoisomer eliciting electrophysiological responses by male EHL and WHL antennae. In field bioassays with EHL and WHL populations, traps baited with (5*R*,11*S*)-5,11-dimethylheptadecane caught as many males as did traps baited with all four stereoisomers combined or a synthetic mixture of 5,11-dimethylheptadecanes. Catches in traps baited with the other three stereoisomers did not significantly differ from those in the unbaited control traps. We conclude that male antennae lack chemoreceptors for the other three stereoisomers of 5,11-dimethylheptadecane and hypothesize that only (5*R*,11*S*)-5,11-dimethylheptadecane is produced by female EHLs and WHLs.

Key Words—Lepidoptera, Geometridae, eastern hemlock looper, western hemlock looper, sex pheromone, pheromone chirality, dimethylated hydrocarbons, field trapping, electrophysiological recordings, (5*R*,11*S*)-5,11-dimethylheptadecane, (5*S*,11*R*)-5,11-dimethylheptadecane, (5*R*,11*R*)-5,11-dimethylheptadecane, (5*S*,11*S*)-5,11-dimethylheptadecane.

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INTRODUCTION

5,11-Dimethylheptadecane(5,11-dime-17Hy) is the major sex pheromone component of both the eastern hemlock looper (EHL), *Lambdina fiscellaria fiscellaria* Guen. (Gries et al., 1991a,b) and the western hemlock looper (WHL), *Lambdina fiscellaria lugubrosa* Hulst (Gries et al., 1993). A synthetic mixture of all stereoisomers attracted both EHLs and WHLs, but pheromone chirality and behavioral activity of the four stereoisomers were not investigated. We report antennal responses and field attraction to all four stereoisomers of 5,11-dime-17Hy.

METHODS AND MATERIALS

Chemicals. The four stereoisomers of 5,11-dime-17Hy were synthesized from methyl (3*R*)- or methyl (3*S*)-3-methylnonanoate (88% ee each) and methyl (3*R*)- or methyl (3*S*)-3-methylheptanoate (90% ee and 86% ee, respectively). All four starting esters were obtained from methoxybornyl sulfoxides, and their enantiomeric purity was determined by GC analysis of the corresponding (1*R*)-1-phenylethylamides (Li, 1993; Carpita et al., 1989). Chiral purity of 5,11-dime-17Hy at C-5 and C-11 is estimated to be equal to that of the component chiral methyl 3-methylheptanoate and methyl 3-methylnonanoate. Accordingly, synthetic (5*R*,11*S*)-5,11-dime-17Hy was composed of 89.3% (5*R*,11*S*)-, 5.7% (5*R*,11*R*)-, 4.7% (5*S*,11*S*)-, and 0.3% (5*S*,11*R*)-5,11-dime-17Hy; synthetic (5*R*,11*R*)-5,11-dime-17Hy was composed of 5.7% (5*R*,11*S*)-, 89.3% (5*R*,11*R*)-, 0.3% (5*S*,11*S*)-, and 4.7% (5*S*,11*R*)-5,11-dime-17Hy; synthetic (5*S*,11*S*)-5,11-dime-17Hy was composed of 6.6% (5*R*,11*S*)-, 0.4% (5*R*,11*R*)-, 87.4% (5*S*,11*S*)-, and 5.6% (5*S*,11*R*)-5,11-dime-17Hy; synthetic (5*S*,11*R*)-5,11-dime-17Hy was composed of 0.4% (5*R*,11*S*)-, 6.6% (5*R*,11*R*)-, 5.6% (5*S*,11*S*)-, and 87.4% (5*S*,11*R*)-5,11-dime-17Hy. The stereoisomeric mixture of 5,11-dime-17Hy was synthesized as previously described (Gries et al., 1991a).

Experimental Insects. EHL pupae were collected near St. John's, Newfoundland and WHL larvae near Revelstoke, British Columbia. Insects were reared to adults in the laboratory at 20°C, 70% relative humidity, and a photoperiod of 15:9 hr light–dark. Two- to 3-day-old males were used for gas chromatographic–electroantennographic analysis (GC-EAD) (Hewlett Packard 5890A, DB-210 column) (Arn et al., 1975), recording electrophysiological responses by male antennae to 1 pg of the separately injected stereoisomers of 5,11-dime-17Hy. Electrophysiological activity of each stereoisomer was assessed in 6–10 GC-EAD recordings for EHLs and two to three recordings for WHLs.

Field Experiments. Field experiments were set up near St. John's, Newfoundland, and Blue River, British Columbia, in randomized complete blocks

with traps and blocks at 15- to 20-m intervals. Multipher traps in Newfoundland (Biocontrol Services, Ste-Foy, Quebec) and Unitraps in British Columbia (Phero Tech Inc., Delta, British Columbia), were suspended 1.5–2 m above ground and baited with rubber septa (Aldrich Chemical Co., Milwaukee, Wisconsin 53233) impregnated with test chemicals in HPLC-grade hexane. In Newfoundland, the experiment comprised 14 replicates of seven treatments: (1–4) 20 μg of either (5*R*,11*S*)-, (5*S*,11*R*)-, (5*S*,11*S*)-, or (5*R*,11*R*)-5,11-dime-17Hy; (5) all four compounds combined at 20 μg each; (6) a synthetic mixture (80 μg) with approximately equal proportions of each stereoisomer; and (7) an unbaited control trap. The same experimental design was used in British Columbia, except that individual stereoisomers were tested at 2.5 μg each and the quaternary combination and synthetic mixture at 10 μg.

RESULTS

(5*R*,11*S*)-5,11-Dime-17Hy was the only stereoisomer eliciting strong electrophysiological responses by antennae of male EHLs (Figure 1) and WHLs. In field bioassays with EHLs and WHLs, traps baited with (5*R*,11*S*)-5,11-dime-

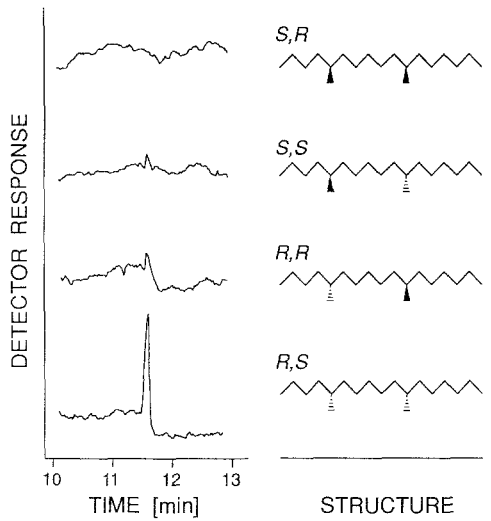

FIG. 1. Representative GC-EAD recordings of male *Lambdina fiscellaria fiscellaria* antennae responding to 1 pg of separately injected stereoisomers of 5,11-dime-17Hy (Hewlett Packard 5890A, DB-210 column, 1 min at 70°C, 20°C/min to 130°C, 2°C/min to 220°C). Small EAD responses to (5*S*,11*S*)- and (5*R*,11*R*)-5,11-dime-17Hy may be attributed to small impurities of the *R*,*S*-stereoisomer. Antennal recordings with male *Lambdina fiscellaria lugubrosa* antennae were essentially identical.

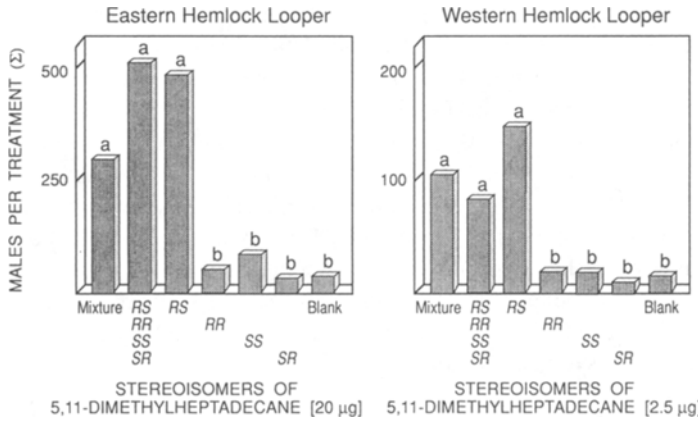

FIG. 2. Total catches of (a) male *Lambdina fiscellaria fiscellaria* in Multipher traps (Biocontrol Services, Ste Foy, Quebec) and (b) male *Lambdina fiscellaria lugubrosa* in Unitraps (Phero Tech Inc. Delta, British Columbia) baited with either (5*R*,11*R*)-, (5*R*,11*S*)-, (5*S*,11*S*)-, or (5*S*,11*R*)-5,11-dime-17Hy alone, the quaternary combination thereof, and a synthetic mixture of 5,11-dime-17Hy: October 13–21, 1991, St. John's, Newfoundland, $N = 4$; September 28–October 2, 1991, Blue River, British Columbia, $N = 14$. Bars superscripted by the same letter are not significantly different (ANOVA, followed by Duncan's multiple range test, $P < 0.05$).

17Hy caught as many males as did those baited with all four stereoisomers combined or the synthetic mixture of 5,11-dime-17Hy (Figure 2). Catches in traps baited with the other three stereoisomers did not significantly differ from those in unbaited control traps.

DISCUSSION

Field bioassays in Newfoundland and British Columbia clearly indicated that (5*R*,11*S*)-5,11-dime-17Hy is the major sex pheromone component of both EHLs and WHLs. The other three stereoisomers were inactive, neither synergizing nor inhibiting attraction to (5*R*,11*S*)-5,11-dime-17Hy (Figure 2). As they did not elicit antennal responses, we conclude that male antennae lack the respective chemoreceptors and hypothesize that (5*R*,11*S*)-5,11-dime-17Hy is the only stereoisomer produced by female EHLs and WHLs.

Behavioral activity of only one of all possible stereoisomers of dimethylated hydrocarbon pheromones has been reported in two other insects. In the mountain-ash bentwing, *Leucoptera scitella* (Zeller) (Lepidoptera: Lyonitiidae), only (5*S*,9*S*)-5,9-dimethylheptadecane attracts males (Tóth et al., 1989), whereas in the tsetse fly, *Glossina pallidipes* (Austen) (Diptera: Muscidae), only (13*R*,23*S*)-

13,23-dimethylpentatriacontane induces copulatory response by male flies (McDowell et al., 1985).

Chiral-specific production and behavioral activity of pheromones have been well documented in scolytid beetles (Borden, 1985; Vanderwel and Oehlschlager, 1987), but relatively few lepidopteran sex pheromones or attractants in lymantrids, noctuids, and geometrids are known to be chiral (Mayer and McLaughlin, 1991 and references therein; Millar et al., 1990a,b) or to comprise a nonracemic mixture of enantiomers (Millar et al., 1991). Chiral-specific attraction or inhibition of pheromones is observed in sympatric species competing for the same pheromone communication channel. In the geometrid moths *Itame occiduaria* and *I. bruneata*, e.g., either enantiomer of (6*Z*,9*Z*)-*cis*-3,4-epoxy-6,9-heptadecadiene (*R,S; S,R*) combined with 3*Z*,6*Z*,9*Z*-heptadecatriene attracted one of the two species while inhibiting response by the other (Millar et al., 1990b). The fact that male antennae of EHLs and WHLs responded only to (5*R*,11*S*)-5,11-dime-17Hy and that none of the stereoisomers attracted sympatric moths suggests that moths with the same seasonal and circadian activity pattern as *Lambdina fiscellaria* are unlikely to utilize 5,11-dime-17Hy as a major pheromone component.

From a management perspective, isomeric 5,11-dime-17Hy can be utilized as part of a pheromonal blend to monitor EHL and WHL populations. To develop the most effective lure, we are currently investigating the chirality of the synergistic pheromone component 2,5-dime-17Hy in both EHL and WHL and 7-me-17Hy in WHL populations.

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REFERENCES

ARN, H., STÄDLER, 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.

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, New York.

CARPITA, A., DE MAGISTRIS, E., and ROSSI, R. 1989. The racemic form and the two enantiomers of 4-methyl-1-nonanol, a sex attractant of the yellow mealworm, *Tenebrio molitor* L. *Gazz. Chim. Ital.* 119:99–105.

GRIES, G., GRIES, R., BORDEN, J.H., JIANXIONG LI, SLESSOR, K.N., KING, G.G.S., BOWERS, W.W., WEST, R.J., and UNDERHILL, E.W. 1991a. 5,11-Dimethylheptadecane and 2,5-dimethylheptadecane: Sex pheromone components of the geometrid moth, *Lambdina fiscellaria fiscellaria. Naturwissenschaften* 78:315–317.

GRIES, G., JIANXIONG LI., GRIES, R., SLESSOR, K.N., BOWERS, W.W., WEST, R.J., KING, G.G.S., UNDERHILL, E.W., and BORDEN, J.H. 1991b. Method and composition for attracting and controlling hemlock looper. Canadian Patent Application #2,041,529-1.

GRIES, G., GRIES, R., KRANNITZ, S.H., LI, J., KING, G.G.S., SLESSOR, K.N., BORDEN, J.H., BOWERS, W.W., WEST, R.J., and UNDERHILL, E.W. 1993. Sex pheromone of the western hemlock looper, *Lambdina fiscellaria lugubrosa* (Hulst). *J. Chem. Ecol.* in press.

LI, J. 1993. Identification and chiral synthesis of sex pheromone components in geometrid moths (Lepidoptera: geometridae). PhD thesis. Simon Fraser University, Burnaby, British Columbia, Canada.

MAYER, M.S., and MCLAUGHLIN, J.R. 1991. Handbook of Insect Pheromones and Sex Attractants. CRC Press, Boca Raton, Florida.

MCDOWELL, P.G., HASSANALI, A., and DRANSFIELD, R. 1985. Activity of diastereoisomers of 13,23-dimethylpentatriacontane, the sex pheromone of *Glossina pallidipes*, and comparison with the natural pheromone. *Physiol. Entomol.* 10:183–190.

MILLAR, J.G., GIBLIN, M., BARTON, D., and UNDERHILL, E.W. 1990a. (3Z,6Z,9Z)-Nonadecatriene and enantiomers of (3Z,9Z)-*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., MORRISON, A., and UNDERHILL, E.W. 1990b. Synthesis and field testing of enantiomers of 6Z,9Z-*cis*-3,4-epoxydienes as sex attractants for geometrid moth. Interactions of enantiomers and regioisomers. *J. Chem. Ecol.* 16:2317–2339.

MILLAR, J.G., GIBLIN, M., BARTON, D., and UNDERHILL, E.W. 1991. Chiral lepidopteran sex attractants: blends of optically active C_{20} and C_{21} diene epoxides as sex attractants for geometrid and noctuid moths (Lepidoptera). *Environ. Entomol.* 20:450–457.

TÓTH, M., HELMCHEN, G., LEIKAUF, U., SZÖCS, G., and SZÖCS, G. 1989. Behavioral activity of optical isomers of 5,9-dimethylheptadecane, the sex pheromone of *Leucoptera scitella* L. (Lepidoptera: Lyonetiidae). *J. Chem. Ecol.* 15:1535–1543.

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, Florida.

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ALLELOPATHIC POTENTIALS OF ALFALFA (*Medicago sativa*) SAPONINS: THEIR RELATION TO ANTIFUNGAL AND HEMOLYTIC ACTIVITIES

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Abstract—A wheat seedling bioassay was used to indicate the relationship between the chemical structure of alfalfa saponins and their allelopathic activity. None of the tested saponins significantly influenced wheat germination. Seedling growth bioassays indicated significant differences among the activities of individual glycosides. The most active were medicagenic acid, its glycosides substituted at the C-3 position with glucose, and hederagenin monoglycoside. Medicagenic acid glycosides, substituted at the C-3 position with glucuronic acid, and zahnic acid tridesmoside were less active. No significant correlation was found among the allelopathic, hemolytic, and antifungal activities of the individual glycosides.

Key Words—*Medicago sativa* L.; *Triticum aestivum* L.; Leguminosae; saponins; triterpenes; biological activity; allelopathy.

INTRODUCTION

Saponins of alfalfa are triterpenoid glycosides found in roots, shoots, seeds, and flowers. Their antifungal, hemolytic, growth-retarding, and surface activities have attracted increasing interest (Price et al., 1987). Most studies have focused on antifungal and hemolytic activities since these assays are widely used for saponin determinations in alfalfa. However, all these studies were done on multicomponent saponin mixtures because individual glycosides were not available (Gestetner et al., 1971). The first attempted studies of individual compounds were undertaken by Nonaka (1986), who suggested that activity of individual alfalfa saponins against *Trichoderma viride* vary depending on the chemical structure. However, no structure–activity relationships were presented

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since chemical formulae of the compounds tested were not established. Recently, we isolated a number of individual glycosides from alfalfa roots and aerial parts and determined their structure-dependent hemolytic and antifungal activities (Oleszek, 1990; Oleszek et al., 1990, 1992a). These studies showed that the activity of individual saponins differs, and these differences may strongly influence the results of saponin determination through biological methods.

The allelopathic functions of alfalfa saponins are not fully understood. There also are divergent opinions on their possible release into the environment by leaching or secretion from the intact plants (Mishustin and Naumova, 1955; Gorski et al., 1991). Despite these divergences, there is evidence that saponins may harm plants when introduced into the soil with alfalfa plant material (Oleszek and Jurzysta, 1987; Wyman-Simpson et al., 1991; Oleszek et al., 1992b). The toxic effect may be disadvantageous when crop plants are affected or desirable when weeds are suppressed. Wyman-Simpson et al. (1991) showed that alfalfa root saponins inhibited wheat and cheat (*Bromus secalinus*) and implied that these compounds have potential as herbicides. These authors compared the inhibitory activities of saponins isolated from alfalfa varieties under divergent seasonal conditions and concluded that the amount, structure, and type of saponins present in alfalfa roots vary with time.

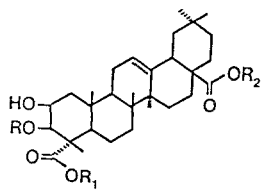
Similar differences in the inhibition of cottonseed germination by saponins isolated from aerial parts of five varieties of alfalfa were reported by Pedersen (1965), who proposed to use this phytotoxic effect as a biotest for saponin determination. No attempt, however, was made to establish whether these varietal differences in activity resulted from variation in the total saponin levels or from chemical structural diversity.

The present report shows the effect of individual saponins on wheat germination, on the growth of wheat roots and shoots, and on seedling appearance. The work was aimed at furthering our understanding of the relationship between alfalfa saponin structure and allelopathic (herbicidal) activity and determining whether this relationship is correlated with other activities.

METHODS AND MATERIALS

Plant Material. Roots and shoots of 2-year-old alfalfa (*Medicago sativa* L. var. Kleszczewska) were collected in June 1988 at Pulawy, Poland.

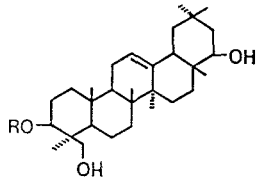
Isolation of Compounds. Glycosides (Figure 1) of medicagenic acid **(1, 2, 3, 4, 5),** hederagenin **(7),** and soyasapogenol B **(6)** were isolated from alfalfa roots and zanhic acid tridesmoside **(8)** from tops (Oleszek et al., 1990, 1992a). Crude saponins were extracted from plant material with 35% (v/v) methanol and partially purified on a short (6 × 10 cm, 55 μm C-18, Waters Assoc.) column. Individual glycosides were separated by high-resolution preparative



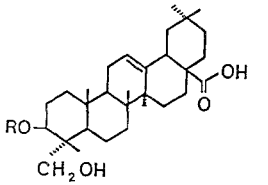
R $-R_1$ $-R_2$

Medicagenic acid

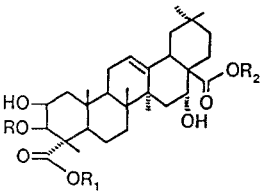
MaNa	-H	-Na	-Na
1	-GlcA	-H	-H
2	-GlcA	-H	-Ara-Rha-Xyl
3	-Glc	-H	-H
4	-Glc	-H	-Glc
5	-Glc	-H	-Ara-Rha-Xyl



Soyasapogenol B
6 -GlcA-Gal-Rha



Hederagenin
7 -Ara-Glc-Ara



Zahnic acid

8	-Glc-Glc-Glc	-Ara	-Ara-Rha-Xyl-Api
9	-Glc-Glc-Glc	-H	-H

FIG. 1. Chemical formulae of tested compounds.

liquid chromatography (Lichroprep Si60 or Lichroprep C-18, 15–25 μm, 30-cm $\times$ 1.2-cm column). Identities were confirmed with TLC, HPLC, NMR, and FAB-MS. Compound **9,** was obtained by alkaline hydrolysis of **8,** i.e., 300 mg **8** were heated for 3 hr at 100°C in 5% KOH. The precipitate was filtered, washed with distilled water, and dissolved in methanol. Evaporation of methanol yielded 150 mg of an amorphous compound with chromatographic and spectral characteristics identical to zanhic acid monoglycoside (Oleszek et al., 1992a).

The mixtures of alfalfa root saponins (RS) and alfalfa shoot saponins (SS) were obtained by a cholesterol precipitation method (Jurzysta et al., 1984).

Thin-layer chromatography (TLC) of saponins was performed on Merck silica gel 60 plates developed with either ethyl acetate–water–acetic acid (system 1, 7:2:2, v/v) or butanol–acetic acid–water (system 2, 4:1:1, v/v), and on Merck C-18 plates developed with methanol–water–acetic acid (system 3, 60:40:0,5, v/v). High-performance liquid chromatography (HPLC) was per-

formed with an analytical HPLC unit (Knauer gradient system with computer control).

^{13}C and ^{1}H NMR spectra were measured on a Jeol GX-400 spectrometer operated at frequencies of 100.4 and 399.65 MHz, respectively. The samples were examined as solutions in pyridine-d_5 (30 mg/0.5 ml) in 5-mm-OD tubes at 27°C.

FAB mass spectra was recorded on a Kratos MS/50TC spectrometer. The samples were dissolved in glycerol and bombarded with a 9-kV (nominal) beam of xenon atoms produced by an ION Tech 11NF atom gun. The spectra were recorded in both positive and negative modes on a UV galvanometer recorder.

Allelopathic Activity. Bioassays were designed to measure the early growth of wheat seedlings treated with purified alfalfa saponins. Individual saponins were dissolved in distilled water at concentrations of 100, 200, 300, 400, and 500 ppm. The solution pH for all compounds ranged from 6.8 to 7.0.

Twenty wheat (*Triticum aestivum* L., var. Boja) seeds were placed in glass Petri dishes (100 × 15 cm) lined with a 9.0-cm-diam. Whatman No. 1 filter paper, and 4 ml of a given test solution was applied to each plate. Each treatment was replicated four times. The dishes were incubated in natural light at 20°C for 96 hr.

The number of germinated seeds were counted. The lengths of the central root and of the coleoptile of each seedling were measured in millimeters. Any changes in the appearance of the seedlings, e.g., root tip browning and necrosis, were also noted. Means per dish across concentration, as well as across structure, were statistically analyzed with ANOVA and regression analyses. The data are presented as percent growth with respect to the control grown in distilled water.

RESULTS AND DISCUSSION

Ten individual saponins were isolated from alfalfa roots and tops. Their structural identities were confirmed by chromatographic and spectral techniques. These compounds, together with the cholesterol-precipitable saponins from alfalfa roots (RS) and shoots (SS) were used in wheat germination/seedling growth tests. Individual compounds were dissolved in distilled water to make concentrations of 100–500 ppm. All compounds except medicagenic acid (Ma) and soyasaponin I **(6)** were readily soluble in water. Minimally soluble medicagenic acid and **6** were rendered soluble by conversion to their sodium salts.

Counts of the number of germinating wheat seeds after a four-day treatment showed that none of the compounds tested significantly influenced germination. In all treatments, including the DI water controls, 94–96% of seeds germinated.

The saponins influenced growth of seedling roots (Table 1) and shoots

TABLE 1. GROWTH OF ROOT OF WHEAT SEEDLINGS TREATED WITH INDIVIDUAL ALFALA SAPONINS[a]

Concentration of saponins (ppm)	Wheat root length (% of control)												
	MaNa	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	RS	SS	LSD
100	29![a]	108	97	55	76	37!	97	34!	82	43	68	75	7.7
200	22!	111	69	35!	54	27!	114	28!	63	28!	55	68	8.7
300	22!	111	58	26!	57	22!	113	21!	44	27!	49	50	7.3
400	12!	98	44!	21!	49	16!	131	20!	34!	21!	39	42!	6.2
500	16!	43!	36!	13!	46	14!	124	15!	30!	17!	29!	33!	7.2
LSD	6.2	5.6	5.5	9.2	14.4	4.3	16.2	5.7	8.3	2.9	4.0	11.0	
Linear regression equation coefficients													
x coeff. $\times 10^{-3}$	−33	−144	−147	−85	−62	−57	+73	−45	−134	−60	−94	−111	
Constant	29	137	105	56	74	40	94	37	91	45	76	87	
(95% CI)	(6)	(31)	(10)	(11)	(16)	(5)	(19)	(5)	(11)	(6)	(4)	(9)	
R	−0.80	−0.78	−0.97	−0.96	−0.72	−0.95	+0.87	−0.91	−0.96	−0.93	−0.99	−0.96	

[a] R = correlation coefficient (significant at $P < 0.05$) for linear regression where x corresponds to saponin concentration and y to seedling root length; ! = treatments where the browning of root tips was observed; LSD = least significant difference at $P \leq 0.05$.

(Table 2), and these effects were dependent on both the saponin concentration and the saponin structure. For all compounds, except **6,** there were significant negative linear correlations between saponin concentration and seedling root length (Table 1). The only compound for which the stimulatory effect increased with increasing concentration was soyasaponin I **(6)** and, thus, this saponin should be considered separately.

The levels of observed root growth inhibition were different for structurally divergent compounds. Examination of the linear regression analyses (root growth inhibition against the saponin concentration) shows that the slope of regression curves and *y* intercept values can be used as parameters, greatly helping to differentiate compounds according to their inhibitory activity (Table 3).

As can be seen from Tables 1 and 3, the highest activity was that of medicagenic acid sodium salt. At a concentration of 100 ppm, this sapogenin salt retarded seedling root growth 70%. This inhibition was slightly higher than that reported for *Lepidium sativum* and *Amaranthus caudatus* (Gorski et al., 1991). These differences may arise from divergent species sensitivities or from the conditions under which experiments were performed.

Among the glycosides of medicagenic acid, the most active were compounds **5** and **3** followed by less active **4,** and much less active **2** and **1.** Relating these activities to the structural features shows that medicagenic acid glycosides having glucuronic acid attached at the C-3 position had lower allelopathic activities than their 3-*O*-glucose analogs, e.g., **1** versus **3** and **2** versus **5.** The data are in good agreement with the results obtained for hemolytic and antifungal activities (Oleszek, 1990; Oleszek et al., 1990, 1992a). No clear relationship was apparent between the number of sugars attached to the aglycone and the allelopathic activity. The four-sugar bisdesmoside **5** had activity equal to that of medicagenic acid and higher activity than the one-sugar monodesmoside **3** or the two-sugar bisdesmoside **4.** Similarly, four-sugar bisdesmoside **2** was much more active than monodesmoside **1,** a quite unexpected difference. Compound **1** at the three lowest concentrations was slightly stimulatory of root growth, and an inhibitory effect was observed only at highest (500 ppm) concentration. In hemolytic and fungal tests, **1** was always slightly less active than **3** but more active than bisdesmoside **2.** This suggests that some factor other than the number of sugars present, e.g., steric orientation, might determine the observed effects.

High allelopathic activity was expressed by hederagenin monodesmoside **7;** this retarded wheat root growth at the same level as highly active MaNa and its glycoside **5.** The data correlate with previous reports of hederagenin monoglucoside activity against *T. viride* and in the hemolytic index test (Oleszek, 1990; Oleszek et al., 1990).

Two zanhic acid glycosides tested had different levels of allelopathic activity, although the tridesmoside **8** retarded wheat seedling growth but lacked inhibitory effect on *T. viride* growth (Oleszek et al., 1992a). Monodesmoside

TABLE 2. GROWTH OF SHOOT OF WHEAT SEEDLINGS TREATED WITH INDIVIDUAL ALFALFA SAPONINS[a]

Concentration of saponins (ppm)	Wheat shoot length (% of control)												
	MaNa	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	RS	SS	LSD
100	56	105	108	96	87	81	98	77	90	103	101	88	15.8
200	52	110	95	69	80	64	111	70	96	84	93	83	9.2
300	47	111	95	60	80	63	108	55	90	77	89	81	13.3
400	43	110	91	59	78	51	115	48	84	66	68	85	12.0
500	42	94	79	50	77	54	114	44	77	67	56	73	13.4
LSD	11.3	7.6	13.2	7.5	12.8	21.4	24.0	17.3	19.9	11.9	7.0	17.1	
Linear regression equation coefficients													
x coeff. $\times 10^{-3}$	−39	−23	−63	−99	−22	−68	+19	−67	−39	−84	−115	−29	
Constant	59	113	113	97	87	80	104	60	99	106	116	90	
(95% CI)	(7)	(12)	(12)	(13)	(10)	(20)	(23)	(13)	(14)	(11)	(8)	(9)	
R*	−0.77*	−0.45	−0.82*	−0.90*	−0.52	−0.67*	+0.22	−0.87*	−0.54	−0.88*	−0.96*	−0.78	

[a] R* = correlation coefficient (significant at $P < 0.05$) for linear regression where x corresponds to saponin concentration and y to seedling shoot length; LSD = least significant difference at $P < 0.05$.

TABLE 3. CLASSES OF SAPONINS SHOWING SIMILAR INHIBITORY ACTIVITY AGAINST WHEAT ROOTS CALCULATED FROM LINEAR REGRESSION ANALYSES AND BROWNING SYMPTOMS

Saponin	Slope $\times 10^{-3}$	y intercept	Browning symptoms[a]
Highly inhibitory			
MaNa	−33 (20)[b]	29 (6)	!!!!!
7	−45 (17)	37 (5)	!!!!!
5	−57 (14)	40 (5)	!!!!!
Less inhibitory			
9	−60 (18)	45 (6)	!!!!
3	−85 (38)	56(11)	!!!!
Moderately inhibitory			
4	−62 (48)	74 (16)	
RS	−94 (12)	76 (4)	!!
SS	−111 (28)	87 (9)	!!
8	−134 (33)	91 (11)	!!
Low inhibitory			
2	−147 (32)	105 (10)	!!
1	−144 (30)	137 (31)	!

[a]Number of exclamation marks corresponds to levels of concentration at which browning occurred.
[b]95% confidence intervals.

9 was much more active than **8**, supporting more general findings that monodesmosidic saponins are more active than bis- or tridesmosides (Mahato et al., 1988; Oakenfull and Sidhu, 1989).

Soyasaponin I, the only glycoside of soyasapogenol B tested in this trial, slightly stimulated seedling root growth, compared to the control grown in distilled water. This observation correlated with the lack of activity of **6** against *T. viride* and in the hemolytic test (Oleszek, 1990; Oleszek et al., 1990).

No significant differences were found between allelopathic potentials of alfalfa root (RS) and shoot (SS) saponin mixtures. This agrees with the hemolytic data but does not correlate with antifungal activities (Oleszek, 1990; Oleszek et al., 1990) where RS were more active than SS. Explanation of these differences requires examination of the compositions of the RS and SS mixtures. Both samples were obtained from alfalfa by precipitation with cholesterol, and they are composed of medicagenic acid glycosides. Quantitative data on the content of individual compounds in these mixtures are not available in the literature. In

preliminary studies, however, it was revealed that the predominant saponin of both the RS and SS fractions is compound **2.** Based on the HPLC peak area, compound **2** made up 70–80% of total saponins (Oleszek, 1991). Thus, compound **2** is the primary contributor to the activity of the fractions. The presence of compounds **3, 5,** and **7** in RS slightly modifies the allelopathic and hemolytic activities of the fraction, but their activities are not drastically different from that of **2.** In the *T. viride* test, compound **3,** which shows high antifungal activity, has greater significance. Fungal inhibition comparable to that of compound **3** requires 30, 16, and 8 times higher concentrations of **2, 7,** and **5,** respectively. This may explain why RS is much more active than SS in the *T. viride* tests.

Interpretation of bioassay data for coleoptile length measurements (Table 2) are less clear-cut than those from roots. Coleoptiles, in general, were less sensitive than roots to the presence of saponins, a finding that agrees with previous reports (Rice, 1984). For all compounds, excluding **6** again, a negative correlation was found between saponin concentration and coleoptile length. Some correlations were not statistically significant. Compound **6** slightly stimulated coleoptile growth. When we again consider the *y* intercept as one of the indicators of activity, compounds MaNa, **7,** and **5** were most active, agreeing with the root data. The *y* intercept of shoots for the other compounds did not differ from the control. At higher saponin concentrations, e.g., 400–500 ppm, shoot elongation was more differentiated, and based on this, the sequence of activity of particular compounds was almost identical to that in the root data.

The presence of saponins in growth medium also affected wheat root system appearance. In some treatments the browning of the root tips, followed by the decay of the whole root system, was observed. This response has been marked in Tables 1 and 3 with an exclamation mark. Close examination of the data shows that the lowest concentrations at which root browning appeared are closely correlated with the activity parameters by which compounds were sequenced, as discussed above. Based on the browning effect, compounds could again be grouped into highly active MaNa, **5,** and **7,** followed by less active **9** and **3,** moderately active **2, 8,** RS and SS, and least active **1.** Exceptions were compounds **4** and **6,** which did not cause browning. Although the absence of browning effect of stimulatory **6** may be understandable, the lack of this effect with saponin **4** was unexpected. However, the behavior of saponin **4** in the hemolytic test was also different from all other compounds having medicagenic acid as aglycone (Oleszek, 1990). The saponin **4** was the only known medicagenic acid glycoside without hemolytic activity for reasons not understood at present.

The browning symptoms observed in the meristemic areas of seedling roots support the hypothesis that these effects arose from the detergentlike properties of saponins. Such detergentlike effects were observed previously by Marchaim et al. (1974). They documented that treatment of cotton seeds with alfalfa sapo-

nins caused structural changes to the membranes, which resulted in increased swelling at the fringe of living cell walls, and might have affected on membrane O_2 permeability. In the present study, the mechanism of saponin action was not researched. However, the absence of browning after treatment by the surface-active compounds **4** and **6** suggests that some other features beyond detergency are responsible for allelopathic activity of saponins. The supposition is supported by previous data (Oleszek and Jurzysta, 1987) showing that alfalfa saponins are inhibitory in soil and that soil texture has significant influence on this effect. Thus, in soil, saponins behaved more like pesticides than detergents.

Some general conclusions on the structure–activity relationships can be drawn. First, glycosides of medicagenic acid, hederagenin, and zanhic acid were inhibitory, whereas soyasapogenol B glycoside was slightly stimulatory for wheat seedling growth. Among the group of medicagenic acid glycosides, those having glucose in C-3 position showed higher activity than similar compounds substituted with glucuronic acid. Zanhic acid glycosides also were more active than glycosides of medicagenic acid glucuronides. In general, monodesmosides **(7, 3, 9)** were more active than bis- **(2, 4)** or tridesmosides **(8);** but the high activity of bisdesmoside **5** and very low activity of monodesmoside **1** are evident exceptions. The allelopathic potentials of individual saponins did not correlate with their hemolytic or antifungal activities. This lack of correlation suggests that the mechanisms by which alfalfa saponins express their hemolytic, antifungal and allelopathic activities are different. In general, these mechanisms are not fully understood (Mahato et al., 1988; Oakenfull and Sidhu, 1989).

The consequences of differing hemolytic and antifungal activities of individual saponins, in relation to the biological tests widely used for saponin determination, have been discussed previously (Oleszek, 1990; Oleszek et al., 1990, 1992a). Similar problems are encountered if the allelopathic potential of the saponin mixture is used for quantification. Any varietal or seasonal variations that alter the relative content of the most active compounds may influence the bioassay results and provide misleading information.

The results here indicate that the allelopathic potentials of the most active alfalfa saponins are not much different from the activities of other groups of plant secondary metabolites (Bialy et al., 1990; Rice, 1984). Thus, these saponins seem to have little potential as herbicides. However, their possible protective functions in the plant can not be ignored. Seeds of alfalfa contain only glycosides of soyasapogenol B, mostly soyasaponin I (Jurzysta, 1970), which showed no antifungal or allelopathic activity. Soon after the germination process begins, biologically active compounds are rapidly synthesized, reaching levels of 9–10% DM (dry matter) (Oakenfull and Sidhu, 1989; Gorski et al., 1991). These concentrations may be overestimates since, in the juvenile seedling stage, saponins contain the monodesmosides **3** and **7** (unpublished), which are highly active against *T. viride* in the bioassay used in the quantification. The high

content of these monodesmosides at the beginning of seedling development might be advantageous during plant establishment. In the roots of mature alfalfa, the highly active compounds **1, 3, 5,** and **7** are still present in considerable amounts, together with the slightly active **2** (Massiot et al., 1988; Oleszek et al., 1990; Oleszek, 1991). Mature alfalfa tops contain predominantly the low activity saponins **2** and **8** (Massiot et al., 1991; Oleszek et al., 1992a); the highly active monodesmosides **3** and **7** are not present. Whether this distribution is the result of localization of saponin synthesis and/or restricted transport in the plant or whether the protective function of saponins is a secondary phenomenon remain open to question.

REFERENCES

BIALY, Z., OLESZEK, W., LEWIS, J., and FENWICK, G.R. 1990. Allelopathic potential of glucosinolates (mustard oil glycosides) and their degradation products against wheat. *Plant Soil* 129:277–281.

GESTETNER, B., ASSA, Y., HENIS, Y., BIRK, Y., and BONDI, A. 1971. Lucerne saponins. IV. Relationship between their chemical constitution, and haemolytic and antifungal activities. *J. Sci. Food Agric.* 22:168–172.

GORSKI, P.M., MIERSCH, J., and PLOSZYNSKI, M. 1991. Production and biological activity of saponins and canavanine in alfalfa seedlings. *J. Chem. Ecol.* 17:1135–1143.

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., OLESZEK, W., and SZYMANEK, B. 1984. Medicagenic acid glycosides as a standard for determination of toxic saponin content in alfalfa. *Proc. Ses. Eucarpia*, Brno. p. 339.

MAHATO, S.B., SARKAR, S.K., and PODDAR, G. 1988. Triterpenoid saponins. *Phytochemistry* 27:3037–3067.

MARCHAIM, U., WEKER, E., and THOMAS, W.D.E. 1974. Changes in the anatomy of cotton seed coats by lucerne saponins. *Bot. Gaz.* 135:139–146.

MASSIOT, G., LAVAUD, C., LE MEN-OLIVIER, L., VAN BINST, G., MILLER, S.F., and FALES, H.M. 1988. Structural elucidation of alfalfa root saponins by MS and NMR analysis. *J. Chem. Soc. Perkin. Trans.* 1988:3071–3079.

MASSIOT, G., LAVAUD, C., BESSON, V., LE MEN-OLIVIER, L., and VAN BINST, G. 1991. Saponins from aerial parts of alfalfa (*Medicago sativa*). *J. Agric. Food Chem.* 39:78–82.

MISHUSTIN, B.N., and NAUMOVA, A.N. 1955. Secretion of toxic substances by alfalfa and their effect on cotton and soil microflora. *Izv. Akad. Nauk USSR, Ser. Biol.* 6:3–9.

MORRIS, R.J., DEY, W.B., and GISLER, D.S. 1961. Isolation, purification and structural activity of an alfalfa root saponins. *J. Org. Chem.* 26:1241–1243.

NONAKA, M. 1986. Variable sensitivity of *Trichoderma viride* to *Medicago sativa* saponins. *Phytochemistry* 25:73–75.

OAKENFULL, D., and SIDHU, S. 1989. Saponins, pp. 97–141, *in* P.R. Cheeke (ed.), Toxicans of Plant Origin. Vol. II. Glycosides. CRC Press, Boca Raton, Florida.

OLESZEK, W. 1990. Structural specificity of alfalfa saponins hemolysis and its impact on two hemmolysis-based quantification methods. *J. Sci. Food Agric.* 53:477–485.

OLESZEK, W. 1991. Atlas of chromatograms. *J. Chromatogr. Sci.* 29:128.

OLESZEK, W., and JURZYSTA, M. 1987. The allelopathic potential of alfalfa root medicagenic acid glycosides and their fate in soil environment. *Plant Soil* 98:67–80.

OLESZEK, W., PRICE, K.R., COLQUHOUN, I.J., JURZYSTA, M., PLOSZYNSKI, M., and FENWICK, G.R. 1990. Isolation and identification of alfalfa root saponins: their activity in relation to a fungal bioassay. *J. Agric. Food Chem.* 38:1810–1817.

OLESZEK, W., JURZYSTA, M., PLOSZYNSKI, M., COLQUHOUN, I.J., PRICE, K.R., and FENWICK, G.R. 1992a. Zanhic acid tridesmmoside and other dominant saponins from alfalfa (*Medicago sativa*) tops. *J. Agric. Food Chem.* 40:191–196.

OLESZEK, W., JURZYSTA, M., and GORSKI, P. 1992b. Saponins—the allelopathic agents, pp. 151–167, *in* S.J.H. Rizvi and V. Rizvi (ed.), Allelopathy: Basic and Applied Aspects. Chapman and Hall, London.

PEDERSEN, M.W. 1965. Effect of alfalfa saponin on cottonseed germination. *Agron. J.* 57:516–517.

PRICE, K.R., JOHNSON, I.T., and FENWICK, G.R. 1987. The chemistry and biological significance of saponins in foods and feedingstuffs. *CRC Crit. Rev. Food Sci. Nutr.* 26:27–135.

RICE, E.L. 1984. Allelopathy. Academic Press, New York.

WYMAN-SIMPSON, C.L., WALLER, G.R., JURZYSTA, M., MCPHERSON, J.K., and YOUNG, C.C. 1991. Biological activity and chemical isolation of root saponins of six cultivars of alfalfa (*Medicago sativa* L.). *Plant Soil* 135:83–94.

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DITERPENE RESIN ACIDS: MAJOR ACTIVE PRINCIPLES IN TALL OIL AGAINST VARIEGATED CUTWORM, *Peridroma saucia* (LEPIDOPTERA: NOCTUIDAE)

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Abstract—Tall oil, a by-product of the kraft process for pulping softwood, has been shown to have insecticidal properties. In the present study, the active principles in tall oil against the variegated cutworm, *Peridroma saucia* Hübner, were investigated. GC-MS analysis showed that abietic, dehydroabietic, and isopimaric acids were major resin acid components of crude tall oil and depitched tall oil. When crude tall oil samples of differing resin acid composition were incorporated into artificial diet at a concentration of 2.0% fresh weight, they suppressed larval growth by 45–60% compared to controls. This suppression was significantly ($P \leq 0.05$) correlated with the equivalent contents of abietic, dehydroabietic, isopimaric, and total resin acids. These results were also evident from a diet choice test, showing that the second-instar larvae obviously selected diets with low levels of resin acids when different diets were randomly arranged in a Petri dish. Bioassays with pure resin acids (abietic, dehydroabietic, and isopimaric acids) demonstrated that all individual chemicals have similar bioactivity against this insect. Comparison of the bioactivities of depitched tall oil and an equivalent mixture of pure resin acids in the *Peridroma* chronic growth bioassay indicated that pure resin acids and depitched tall oil share a common mode of action to this insect. This study confirms that resin acids are major active principles in tall oil against the variegated cutworm, but other chemicals likely also contribute to the bioactivity of tall oil.

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Key Words—Tall oil, resin acids, abietic acid, dehydroabietic acid, isopimaric acid, *Peridroma saucia*, Lepidoptera, Noctuidae, variegated cutworm, bioactivity, natural insecticide.

INTRODUCTION

It is well known that higher plants are a potential source of new insecticides (Arnason et al., 1989), and a few natural products, such as rotenone from *Derris* species, and pyrethrins from *Chrysanthemum* species, are commercially used as insecticides (Isman et al., 1991). In a recent exploration for new sources of natural pesticides, tall oil, a by-product of the kraft process for pulping softwood ("tall" is the Swedish word for pine), has attracted attention. Because tall oil is produced abundantly in the softwood kraft pulp industry, the concept of developing a pest control product from this precursor is very attractive.

Cousin (1989) first reported that tall oil neutrals have insecticidal activities. More recently, crude tall oil and two derivatives were found to have toxic and feeding deterrent activities against the variegated cutworm, *Peridroma saucia* Hübner. These materials significantly reduced growth, feeding, and dietary utilization by variegated cutworm larvae in chronic larval growth bioassays, choice and no-choice feeding tests, and nutritional experiments (Xie and Isman, 1992). However, the active principles associated with the insecticidal properties of tall oil are unknown.

The objectives of the present study are to determine the major chemical constituents in typical crude tall oil and depitched tall oil collected in central British Columbia, Canada, to assess the impact of chemical variation in tall oil on bioactivity to the variegated cutworm, and to evaluate the role of several pure commercial resin acids as deterrents to feeding and growth of the variegated cutworm. Our study aims to identify the active principles in tall oil associated with toxic and feeding deterrent activities against the variegated cutworm.

METHODS AND MATERIALS

Insects. The variegated cutworms used in this study were obtained from a laboratory culture maintained at 25 ± 1°C and a photoperiod of 16L:8D, and reared on artificial diet (No. 9795, BioServ Inc., Frenchtown, New Jersey).

Test Materials. Dehydroabietic and isopimaric acids were obtained from Helix Biotech Corporation, Richmond, British Columbia, Canada. Their purities were stated to be >99%. Abietic acid was purchased from Sigma Chemical Company, St. Louis, Missouri (purity 85%), and purified by crystallization. The purified chemical was analyzed by gas chromatography–mass spectrometry (GC-MS), and the purity was >90%. Crude tall oil and depitched tall oil

supplied by B.C. Chemicals Limited, Prince George, British Columbia, Canada, were used for chemical analysis and bioassay.

Chemical Analysis of Tall Oil Samples. Crude tall oil and depitched tall oil samples were analyzed by GC-MS. GC-MS analyses were performed on a Hewlett-Packard 5890-II gas chromatograph equipped with a HP 5971 Mass Selective Detector. The column employed was a 30-m × 0.25-mm DB-1 (J&W Scientific, Inc., Folsom, California). Ultra high pure (UHP) helium was used as the carrier gas at a head pressure of 15 psi. The injector part was maintained at 325°C, and the oven temperatures were programmed from 160°C to 350°C at a rate of 7°C/min. The temperature of the GC-MS interface was 320°C. Samples and reference chemicals were dissolved in ether–methanol (9:1) solution, and methylated with diazomethane. The resin acid esters were identified by comparing GC retention times and mass spectra to those of reference chemicals purchased from Helix Biotech Corporation. The acid concentrations were quantified by an internal standard, tricosanoic acid methyl ester.

Bioassays of Crude Tall Oil Samples of Differing Diterpene Resin Acid Composition. Several dozen crude tall oil samples, collected from four different pulp mills on a monthly basis over 13 months in central British Columbia, Canada, were analyzed for their chemical composition by GC-MS. Based on these results, six crude tall oil samples, two with high, two with medium, and two with low total resin acid contents, were used for bioassay via incorporation of the test materials into artificial diet. The test materials were dissolved in solvent (isopropyl alcohol) and added to the dry diet constituents at a concentration of 2.0% fresh weight of diet. Control diets were treated with the carrier solvent alone. Bioassays performed were a chronic larval growth test and a diet choice test. In the former case, the bioassay was conducted by placing 20 neonate larvae individually in compartments in a plastic tray with approximately 1 g of treated or control diet. Larvae were maintained in a growth chamber at 25°C and a photoperiod of 16L:8D. After seven days, all larvae were individually weighed, and the mean weights for each treatment group were expressed as a percentage of controls.

The diet choice test was performed to determine insect response to diets of differing chemical contents by randomly placing six pieces of treated artificial diet with the six different crude tall oil samples (2.0% fresh weight of diet) in a plastic Petri dish (9 cm diameter). Twenty second-instar larvae were introduced into the center of each dish. Ten replicates were prepared. After 24 hr in complete darkness (25°C), the number of larvae on each diet was recorded.

Bioassay of Commercial Resin Acids. Bioassays with commercial resin acids (abietic, dehydroabietic, and isopimaric acids) were conducted to verify the importance of individual resin acids on bioactivity of tall oil. The individual test chemicals, dissolved in methanol, were added to the dry diet constituents at concentrations of 0.5–2.0% fresh weight of diet. A tertiary mixture, main-

taining total resin acid concentrations of 0.5–2.0% fresh weight, was incorporated into artificial diet to determine if any additive or synergistic action is involved. Chronic larval growth bioassays were performed as described above.

Comparative Bioassays of Depitched Tall Oil and Commercial Resin Acids. A further set of bioassays was conducted to compare the action of depitched tall oil and a mixture of commercial resin acids on the variegated cutworm. Depitched tall oil was incorporated into artificial diet at concentrations of 0.5–2.5% fresh weight. The three diterpene resin acids, which were mixed together based on their proportions present in the depitched tall oil sample, were incorporated into artificial diet at a total concentration of 0.27–1.09% fresh weight. This concentration range represents the equivalent contents of the three resin acids present in depitched tall oil at concentrations of 1.0–4.0% fresh weight. Chronic larval growth bioassays were performed as described before.

Data Analysis. Linear regression analysis was applied to define all dose–response relationships (Figures 1–4 below) when correlation was found to be significant. Analysis of covariance (ANCOVA) was used to test equality of regression coefficients in Figure 3, and a t test was used to compare regression coefficients (slope and intercept) of depitched tall oil and the mixture of pure resin acids in Figure 4 (Zar, 1984). An arcsin transformation was performed for percentage data before analysis because this transformation would make small or large percentages nearly normal in distribution (Zar, 1984).

RESULTS

GC-MS analyses revealed that representative samples of crude tall oil and depitched tall oil typically contained 34.3% and 40.4% fatty acids and 27.0% and 37.2% diterpene resin acids, respectively. Abietic, dehydroabietic, and isopimaric acids were the major resin acid components of crude tall oil and depitched tall oil. These three resin acids account for 66.1% and 72.8% of total diterpene resin acids in crude tall oil and depitched tall oil, respectively (Table 1).

When the test materials (six selected crude tall oil samples) were incorporated into artificial diet at a concentration of 2.0% fresh weight, they suppressed larval growth by 45–60% compared with controls (treated with carrier solvent alone). This suppression was significantly ($P \leq 0.05$) correlated with the equivalent contents of abietic ($r = -0.96$, $P = 0.0017$), dehydroabietic ($r = -0.97$, $P = 0.0010$), isopimaric ($r = -0.80$, $P = 0.0507$), and total resin acids ($r = -0.96$, $P = 0.0019$) (Figure 1).

The diet choice test revealed that the second-instar larvae selected diets with low levels of resin acids when different diets were randomly arranged in a Petri dish, showing that the number of larvae on diet was significantly ($P < 0.05$) and negatively correlated with the equivalent dietary concentrations of

TABLE 1. RESIN ACID COMPOSITION OF CRUDE TALL OIL AND DEPITCHED TALL OIL[a]

Component	Concentration (weight %)	
	Crude tall oil	Depitched tall oil
Abietic acid	9.1	15.4
Dehydroabietic acid	4.7	6.7
Isopimaric acid	4.0	5.0
Palustric and levopimaric acids	3.7	3.5
Neoabietic acid	2.1	2.2
Pimaric acid	1.7	2.1
Sandaracopimaric acid	0.6	0.7
Dehydrodehydroabietic acid	0.2	0.5
Other resin acids	0.9	1.1
Total resin acids	27.0	37.2

[a] Data represent results from GC-MS analysis for typical crude tall oil and depitched tall oil samples.

abietic ($r = -0.86$, $P = 0.0272$), dehydroabietic ($r = -0.86$, $P = 0.0272$), and total resin acids ($r = -0.94$, $P = 0.0051$) (Figure 2).

Although our results (Figures 1 and 2) suggest that resin acids may in some instances be responsible for antifeedant and growth inhibitory action of tall oil against the variegated cutworm, bioassays with commercial pure resin acids (abietic, dehydroabietic, and isopimaric acids) could verify the importance of individual resin acids to the bioactivity of tall oil. Each of the test chemicals inhibited larval growth in a dose-dependent manner. Significant ($P < 0.05$) negative correlations between larval growth and resin acid concentration in diet were found by regression analysis (Figure 3). ANCOVA indicated that regression slopes of all individual chemicals and their tertiary mixture were not significantly different ($P > 0.05$)($F = 0.15$, $F_{0.05(1),3,8} = 4.07$), suggesting that each of the test chemicals inhibits larval growth in a similar fashion, and no synergistic action was involved.

These conclusions received further support from another experiment aimed at comparing the action of a mixture of pure resin acids and depitched tall oil on bioactivity to the variegated cutworm. Our results clearly indicated that the resin acid mixture and depitched tall oil share a common mode of action to the variegated cutworm because no significant ($P > 0.05$) difference in slopes of the growth curves was found (Figure 4). However, the regression analyses indicate that the intercepts differ significantly ($P < 0.05$), with depitched tall oil showing as much as a 50% stronger growth suppression than the pure resin acid mixture at the same concentrations (Figure 4), suggesting that chemicals other than resin acids substantially contribute to the bioactivity of depitched tall oil to this insect.

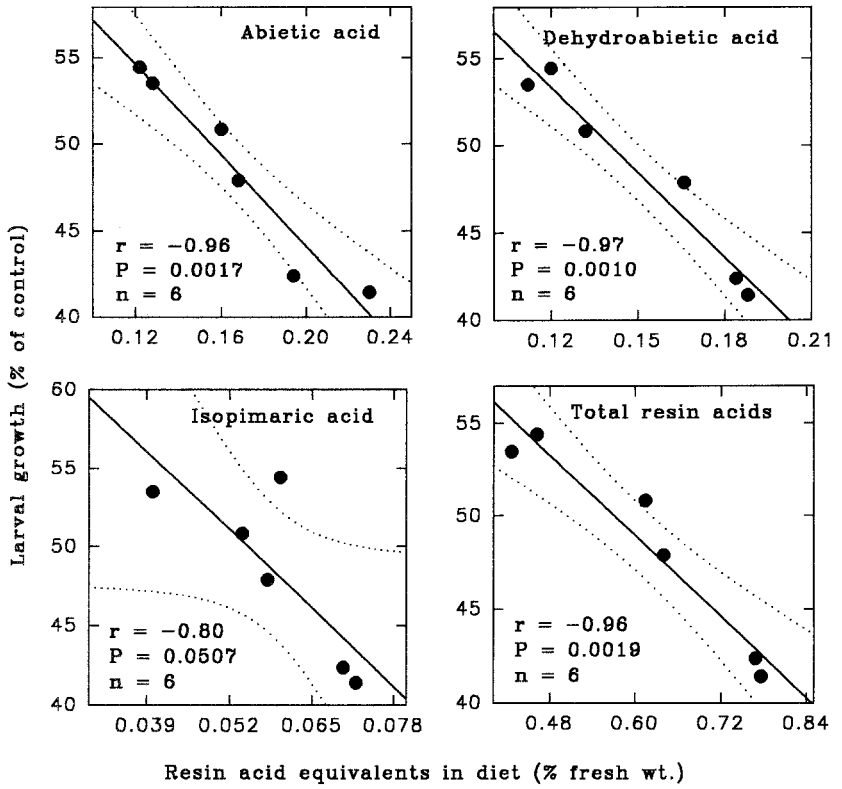

FIG. 1. Relationship between abietic, dehydroabietic, isopimaric, and total resin acids content present in artificial diet and *P. saucia* larval growth (% of control) in a chronic growth test. Dotted lines represent 95% confidence intervals.

DISCUSSION

It is well known that terpenoids are one of the largest groups of plant secondary compounds known to have insecticidal and repellent properties (Beek and de Groot, 1986). Among the terpenoids, diterpenes have been demonstrated to have antifeedant and/or deterrent action against the pink bollworm, *Pectinophora gossypiella* (Elliger et al., 1976), and to deter larval feeding, growth, and dietary utilization of the sawflies, *Pristiphora erichsonii*, *Neodiprion dubiosus*, and *N. rugifrons* (Wagner et al., 1983; Schuh and Benjamin, 1984). Depitched tall oil, in which diterpenic acids (i.e., resin acids) account for over 37% of weight, has been demonstrated to be toxic to neonate *P. saucia* and inhibitory to larval growth (Xie and Isman, 1992). In the present study, we have

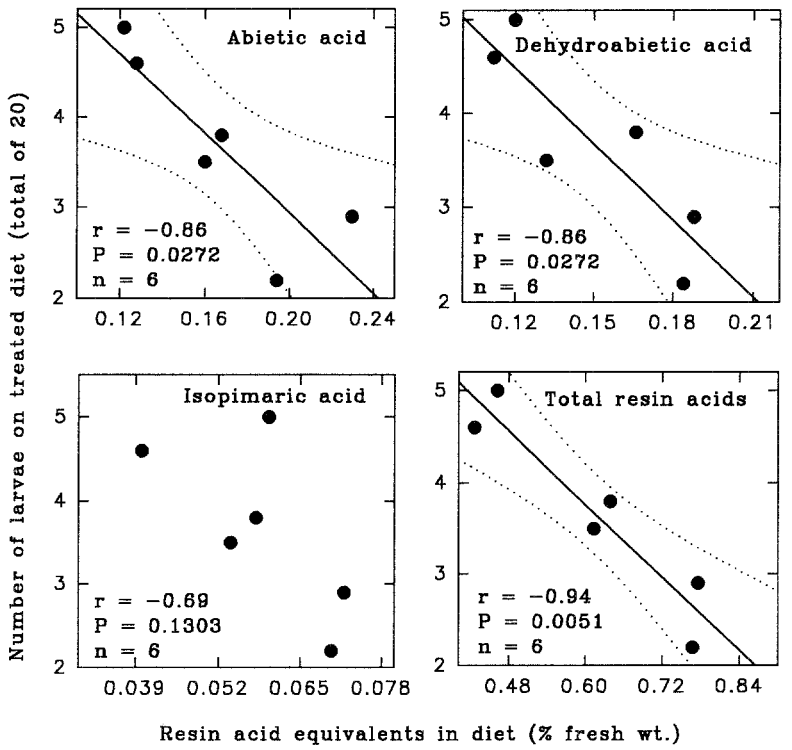

FIG. 2. Relationship between abietic, dehydroabietic, isopimaric, and total resin acids content present in artificial diet and the number of *P. saucia* larvae in a choice test. Dotted lines represent 95% confidence intervals.

confirmed that these diterpenic acids are the major active principles in tall oil against *P. saucia*.

The major basis for this conclusion is the strong correlation between resin acid content in the samples and their bioactivity on larval growth of *P. saucia*. Correlation coefficients for bioactivity versus resin acid contents in tall oil samples indicate that 60–94% of the variation in bioactivity of tall oil can be accounted for by individual or total resin acid contents. This fact clearly suggests that resin acids are largely responsible for the growth-deterrence (physiological) activity of tall oil. This conclusion is further supported by the choice tests, which are useful in detecting small difference in food acceptability (Schoonhoven, 1982). Food acceptability of *P. saucia* larvae was reduced possibly by chemosensory effects. This reduction was also correlated with resin acid contents in tall oil samples, indicating that resin acids likely account for the antifeedant (behavioral) effects of tall oil. Isman et al. (1990) similarly reported that bioac-

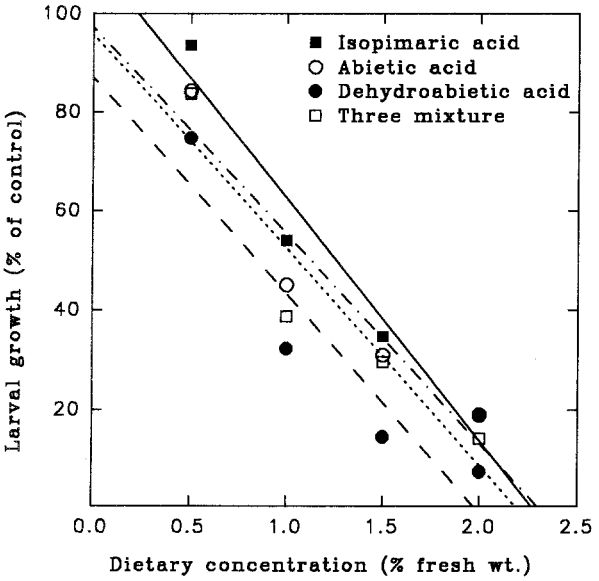

FIG. 3. Dose–response relationship between dietary resin acid content and *P. saucia* larval growth (% of control), comparing bioactivity of each test resin acids and their tertiary mixture on larval weight of *P. saucia* (_____ isopimaric; – · – · – abietic; – – – dehydroabietic; ..... mixture).

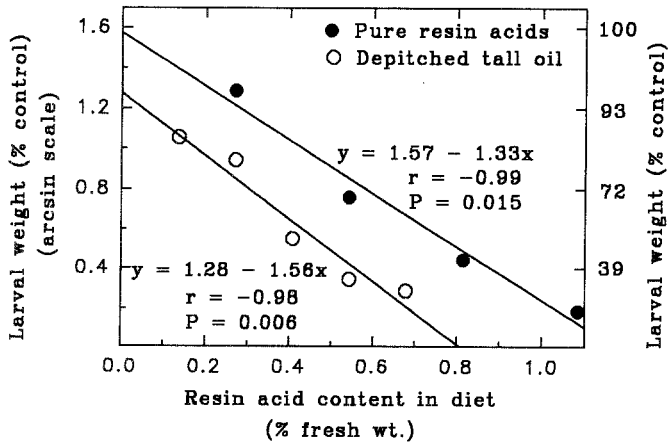

FIG. 4. Dose–response relationship between dietary resin acid content and *P. saucia* larval growth (% of control), comparing bioactivity of a mixture of pure resin acids and depitched tall oil.

tivities of neem oils with various azadirachtin levels on *P. saucia* were significantly ($P < 0.05$) correlated to their azadirachtin content, confirming that azadirachtin was the major active component in neem oil against this insect.

Berenbaum (1985) reported that a natural mixture of six furanocoumarins from parsnip was more toxic to the corn earworm (*Heliothis zea*) than an equivalent dose of xanthotoxin, the most active of the six compounds when tested individually. This indicated that synergistic action was involved. However, our results for resin acids with the variegated cutworm revealed that the tertiary mixture has bioactivity similar to each of the individual chemicals, suggesting a lack of synergistic action.

Depitched tall oil and pure resin acids share a common mode of action to *P. saucia*. The significantly greater bioactivity of depitched tall oil to *P. saucia* (cf. pure resin acids) indicates that resin acids are not the only chemicals in depitched tall oil that contribute substantially to bioactivity. The role of other components of tall oil in suppression of larval growth of *P. saucia* is under investigation.

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REFERENCES

ARNASON, J.T., PHILOGÈNE, B.J.R., and MORAND, P. (eds.). 1989. Insecticides of Plant Origin. ACS Symposium Series 387. American Chemical Society, Washington, D.C.

BEEK, T.A. VAN, and DE GROOT, A. 1986. Terpenoid antifeedants, part I. An overview of terpenoid antifeedants of natural origin. *Recl. Trav. Chim. Pays-Bas*. 105:513–527.

BERENBAUM, M. 1985. Brementown revisited: Interactions among allelochemicals in plants. *Recent Adv. Phytochem.* 19:139–169.

COUSIN, M.J. 1989. Tall oil neutrals to protect plants from insects and the like. U.S. Patent No. 4874610.

ELLIGER, C.A., ZINKEL, D.F., CHAN, B.G., and WAISS, A.C., JR. 1976. Diterpene acids as larval growth inhibitors. *Experientia* 32:1364–1366.

ISMAN, M.B., KOUL, O., LUCZYNSKI, A., and KAMINSKI, J. 1990. Insecticidal and antifeedant bioactivities of neem oils and their relationship to azadirachtin content. *J. Agric. Food Chem.* 38:1046–1411.

ISMAN, M.B., KOUL, O., ARNASON, J.T., STEWART, J., and SALLOUM, G.S. 1991. Developing a neem-based insecticide for Canada. *Mem. Entomol. Soc. Can.* 159:39–47.

SCHOONHOVEN, L.M. 1982. Biological aspects of antifeedants. *Entomol. Exp. Appl.* 31:57–69.

SCHUH, B.A., and BENJAMIN, D.M. 1984. Evaluation of commercial resin acids as feeding deterrents against *Neodiprion dubiosus*, *N. lecontei*, and *N. rugifrons* (Hymenoptera: Diprionidae). *J. Econ. Entomol.* 77:802–805.

WAGNER, M.R., BENJAMIN, D.M., CLANCY, K.M., and SCHUH, B.A. 1983. Influence of diterpene

resin acids on feeding and growth of larch sawfly, *Pristiphora erichsonii* (Hartig). *J. Chem. Ecol.* 9:119–127.

XIE, Y.S., and ISMAN, M.B. 1992. Antifeedant and growth inhibitory effects of tall oil and derivatives against the variegated cutworm, *Peridroma saucia* Hübner (Lepidoptera: Noctuidae). *Can. Entomol.* 124:861–869.

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

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INHIBITIONS OF COTTON SEEDLING GROWTH BY VOLATILE KETONES EMITTED BY COVER CROP RESIDUES[1]

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Abstract—Low-input, sustainable agriculture (LISA) systems can include soil incorporation of residues of weeds and legume cover crop species. However, both seed germination and seedling growth of crops, including cotton (*Gossypium hirsutum* L.), are inhibited by volatile organic compounds emitted by these residues during decomposition. A cotton seedling-growth assay was used to demonstrate that C_4 through C_9 ketones released by decomposing legume residues significantly inhibited root and shoot elongation, root and shoot fresh and dry weight gain, and cotyledon expansion. Seedling root and shoot water relationships were relatively insensitive to those components of plant residue volatile emissions. Concentration (0.0, 0.5, 1.0, 1.5, or 2.0 $\mu M/cm^3$ sterile greenhouse soil mix) and root-zone temperature (20 or 30°C) altered the activity of the more inhibitory ketones. Molecular weight (carbon chain length), carbon chain branching, and position of the carbonyl group also influenced ketone inhibitory activity.

Key Words—Allelopathy, allelochemicals, *Gossypium hirsutum*, seedling, growth inhibition, volatiles, cover crops, sustainable agriculture, LISA, cotton, ketones, alkanones.

INTRODUCTION

During decomposition in soil, residues of both aerial and root tissue of cover crop and weed species emit mixtures of structurally simple volatile organic compounds (Connick et al., 1987; Bradow and Connick, 1990; Bradow, 1991c).

[1] Mention of a commercial or proprietary product does not constitute an endorsement by the U.S. Department of Agriculture.

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The components of the volatile mixtures emitted by residues of several weeds (Connick et al., 1987, 1989; Bradow and Connick, 1990) and cover crops (Bradow and Connick, 1990) have been identified. The vapor phase of some of those C_3–C_{11} hydrocarbons, alcohols, aldehydes, ketones, and of low molecular-weight esters, furans, and monoterpenes identified in the residue emission mixtures were shown to be potent inhibitors of seed germination (Bradow and Connick, 1988a,b, 1990; Bradow, 1991c). The relative germination-inhibitory activity was dependent on both chemical structure and concentration (Bradow and Connick, 1988a,b, 1990; Bradow, 1991c).

In vapor-phase germination assays (Bradow and Connick, 1988b), small-seeded crop species, e.g., onion, carrot, tomato, and lettuce, were particularly sensitive to the inhibitory volatiles. Species with larger seeds, e.g., cucumber, cantaloupe, mung bean, and maize, were unaffected by either 2-heptanone and 2-heptanol. However, both root and shoot growth were inhibited when cotton (*Gossypium hirsutum*, L., var. Coker 315) seedlings were grown in 20 or 30°C soil containing 2 $\mu M/cm^3$ (and lower) levels of C_4–C_9 ketones emitted by residues of Berseem clover (*Trifolium incarnatum* L.), hairy vetch [*Vicia hirsuta* (L.) S.F. Gray], and crimson clover (*Trifolium incarnatum* L.) (Bradow, 1990c, 1991b).

These three legumes are recommended for use in low-input, sustainable agriculture (LISA) and as winter cover crops for cotton, although decomposing cover crop residues have been implicated in reductions in seedling survival and growth (Buntley, 1986; Dabney et al., 1986; Bauer et al., 1991; Bradow and Bauer, 1992), in reduced cotton yields (Rickerl et al., 1988), and lower fiber maturity (Bradow and Bauer, 1992). Decomposing residues of one or more of the three legumes released at least one of the following ketones: 2-butanone, 3-pentanone, 3-methyl-2-butanone, 2-heptanone, 5-methyl-2-heptanone, 3-octanone, and 2-nonanone (Bradow and Connick, 1990). Plant residues decomposing under hypoxic conditions also emitted the highly toxic methyl ketone, 2-octanone (Connick et al., 1987).

The vapor phase of all of the ketones listed above inhibited seed germination under specific conditions of temperature and concentration (Bradow and Connick, 1988a, 1990; Bradow, 1991c). Further, this inhibitory activity was related to chain length and branching, as well as to relative volatility and hydrophilicity (Bradow and Connick, 1988a,b, 1990; Bradow, 1991c).

In the current study, a sterile-soil cotton-seedling growth assay (Bradow, 1991a) has been used to investigate the effects on postemergent cotton seedling root and shoot growth and water relationships of root zone temperature (20 or 30°C) and five concentrations (0.0, 0.5, 1.0, 1.5, or 2.0 $\mu M/cm^3$) of eight C_4–C_9 ketones associated with legume residues. This paper describes the combinations of ketone structure and concentration and root zone temperature that produce, in postemergent cotton seedlings, root and shoot growth inhibitions

comparable to the seed germination reductions previously attributed to ketones emitted by decomposing plant residues.

METHODS AND MATERIALS

The cotton (*Gossypium hirsutum* L., var. Coker 315) seeds were a gift from Dr. P.J. Bauer, USDA, ARS, Florence, South Carolina. The seed germination protocol was that reported earlier (Bradow, 1990a,b, 1991a,d). Unsterilized, fungicide-treated seeds were soaked for 1 hr in deionized (DI) water at room temperature and then rolled in the upper 3 cm of 20-cm-tall paper towel scrolls. The individual scrolls were moistened with DI water and placed upright in 400-ml glass beakers containing 250 ml DI water. The scrolled seeds were germinated for 48 hr at 32°C in the dark.

On day 3, a morphologically homogeneous population of undamaged, disease-free seedlings was selected on the basis of radicle length. The selected seedlings were planted, one seedling per test tube, in glass tubes (200 mm × 23 mm ID) that contained 75 cm^3 of sterile Pro-Mix–Perlite mixture (1 : 1 v/v) wet to field capacity with deionized water. A volume equivalent to 0.5, 1.0, 1.5, or 2.0 $\mu M/cm^3$ of a given ketone was introduced below the soil surface by injection from an adjustable-volume Eppendorf pipet with a disposable tip that was sunk 4.5 cm into the soil during the injection. Care was taken that the seedling roots did not come in direct contact with the pipet tip or the liquid phase of the ketone. Two concentrations of a single ketone were assayed at one time, and each pair of concentration treatments was accompanied by an uninjected DI water control treatment.

The temperature treatment consisted of placing control and treatment tubes randomly in test tube racks sunk in a water bath of appropriate temperature to maintain the soil temperature at either 20 or 30°C. Temperature fluctuations and evaporative loss from the baths were minimized by an insulating 2-cm layer of hollow plastic balls around the treatment tubes. The seedlings were grown for 12 days under cool white fluorescent tubes delivering a photon flux density at cotyledon level of 52–68 $\mu mol/sec/cm^2$; relative humidity was ca. 60% inside the ventilated bench-top growth chamber that was operated at room ambient temperature (25 to 29°C).

The seedlings were harvested on day 14, 12 days after treatment commenced. Seedling root and shoot growth and water relationships (relative water content) were evaluated separately as described by Bradow (1990a, 1991d). Each seedling was eased from the growth tube and the soil was gently washed away from the root with DI water. The root was separated from the hypocotyl at the transition zone, and the lengths of the primary root and of the shoot to the cotyledonary node were measured with a ruler. Fresh weights of the entire

root and entire shoot were also determined. Root and cotyledon dry weights were obtained by microwave dehydration to constant weight (20 min of 675 W at 2450 MHz), a method used for rapid tissue fixation (Walsh et al., 1989). Relative water contents (RWC) of roots and cotyledons were determined, using an adaptation of the method of Weatherley (1950), in which microwave dehydration was substituted for oven-drying. Turgid weights of detached cotyledons and intact radicles were determined after incubation at 31°C for 24 hr in the dark. Although the original application was limited to cotton leaf disks, this method proved to be appropriate and reproducible for both intact 14-day-old cotyledons and nonwoody roots (Bradow, 1990a). Cotyledon areas were estimated by measuring the lengths and widths and applying the formula for the area of an ellipse. When a cotyledon had not expanded sufficiently to permit measurement, an area of zero was recorded.

Each concentration treatment or control consisted of 20 replicate seedlings, and the entire experiment (eight ketones × five concentrations plus DI water control × two temperatures) was performed twice. As an internal check of reproducibility and seed vigor, random combinations of concentration and ketone were also repeated with sets of 20 seedlings during the 18-month duration of the experiment. Variances in the data from the two experiments allowed combination of length and fresh weight data (total treatment $N = 40$ or 60, the higher N representing the DI water controls and the one to two random repetitions per experiment). Variances from the 20 and 30°C DI water controls allowed pooling of those data sets (separately pooled 30 and 20°C DI water controls $N = 60$).

All length and weight data were subjected to the natural logarithm transformation in order to stabilize the variances (Sokal and Rohlf, 1981). The RWC percentages were transformed, as the ratios, using the angular transformation. In RWC determinations, the small seedling roots were weighed and dehydrated in groups of five and the cotyledons in groups of 10 to facilitate handling (total treatment $N = 2$ or 3; pooled DI water controls $N = 3$). The dry weights for groups of five roots or 10 cotyledons were obtained as part of the RWC determinations (total treatment $N = 4$ or 6; pooled DI water control $N = 6$).

Root and shoot growth parameter and water relationship responses to ketone structure, concentration, and root zone temperature were compared via separate three-way analyses of variance (eight ketone structures × five concentrations × two temperatures). Length, fresh-weight, dry-weight, and RWC data for the roots and the shoots were subjected to separate one-way analyses of variance and linear regression analysis. Means separations were made using Tukey's honestly significant difference procedure modified (Sokal and Rohlf, 1981) to accommodate unequal sample sizes and multiple means comparisons (significance level, $\alpha = 0.01$).

RESULTS

Root and Shoot Elongation. Structure and concentration of the ketones (Table 1) were the important factors in both root and shoot elongation; the effect of root zone temperature was small. The only significant first-order interactions were those between ketone structure and concentration. No interactions involving temperature were significant.

The ketones most inhibitory of root elongation on a cumulative basis across concentrations (Table 2) were 2-octanone, 3-octanone, 2-heptanone, and 2-nonanone, followed by 5-methyl-2-heptanone. The 30 and 20°C DI water controls were not significantly different. In the 30°C treatments, the five C_7–C_9 ketones inhibited root growth at the lowest assay concentration; in the 20°C treatment, the lowest concentration of 3-octanone did not inhibit root growth. The linear regression component of the concentration effect was significant ($a = 0.001$) for all assayed ketones except in the case of 3-methyl-2-butanone and 3-pentanone, for which the concentration effects were nonsignificant. The concentration (C) effects at 30°C of 2-heptanone, 2-octanone, 3-octanone, and 2-nonanone were best described by quadratic equations. At 30°C,

$$\text{2-heptanone: cm root length} = 13.3 + 1.3C + 0.04C^2$$

$$(\text{ln transform } r^2 = 0.983)$$

$$\text{2-octanone: cm root length} = 13.0 - 1.7C + 0.07C^2$$

$$(\text{ln transform } r^2 = 0.976)$$

TABLE 1. MEAN SQUARES FROM ANALYSES OF VARIANCE OF KETONE STRUCTURE, CONCENTRATION, AND ROOT-ZONE TEMPERATURE EFFECTS ON COTTON ROOT AND SHOOT ELONGATION

Source	dF	Mean square	
		Root	Shoot
Temperature (temp.)	1	4.42*[a]	5.25*
Structure	7	148.51***	16.34***
Concentration (conc.)	4	123.50***	8.11***
Temp. × structure	7	1.27 NS	0.49 NS
Temp. × conc.	4	0.04 NS	0.10 NS
Structure × conc.	28	13.88 ***	1.63 ***
Temp. × strcture × conc.	28	1.15 NS	0.17 NS
Within subgroups	2661	1.10	0.24

[a] *, **, *** = significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

TABLE 2. EFFECTS OF KETONE STRUCTURE, CONCENTRATION, AND ROOT-ZONE TEMPERATURE ON COTTON SEEDLING ROOT ELONGATION[a]

Structure	Root length (cm)				Slope, linear regression[b]
	0.5 μM/cm^3	1.0 μM/cm^3	1.5 μM/cm^3	2.0 μM/cm^3	
Root-zone temperature: 30°C					
2-Butanone	13.3 c	13.4 bc	9.2 c	8.5 c	−0.33
3-Methyl-2-butanone	13.9 bc	14.9 ab	15.2 ab	16.2 a	NS
3-Pentanone	17.5 a	14.5 a–c	13.9 b	14.0 b	NS
2-Heptanone	7.8 de	5.8 de	1.9 f	1.4 f	Quadratic
2-Octanone	6.3 e	3.1 f	2.7 f	1.5 f	Quadratic
3-Octanone	8.1 de	4.2 ef	2.0 f	1.8 f	Quadratic
5-Methyl-2-heptanone	9.2 d	7.2 d	5.6 de	4.7 d	−0.55
2-Nonanone	6.4 e	4.0 ef	3.9 ef	2.3 f	Quadratic
DI water control, 30°C	13.5 c	13.5 bc	13.5 b	13.5 b	
Root-zone temperature: 20°C					
2-Butanone	12.3 c	12.4 c	7.5 cd	8.4 c	−0.39
3-Methyl-2-butanone	15.0 b	13.8 a–c	16.8 a	15.0 ab	NS
3-Pentanone	15.9 ab	15.8 a	15.6 ab	13.8 b	NS
2-Heptanone	7.0 de	6.0 de	2.2 f	1.8 f	Quadratic
2-Octanone	6.0 e	4.3 ef	2.0 f	1.7 f	Quadratic
3-Octanone	12.4 c	5.9 de	2.5 f	2.4 ef	Quadratic
5-Methyl-2-heptanone	9.3 d	6.6 d	6.9 cd	6.7 c	Quadratic
2-Nonanone	7.5 de	5.9 de	5.2 de	4.3 de	Quadratic
DI water control, 20°C	14.0 bc	14.0 a–c	14.0 b	14.0 b	

[a] Lengths are means of 40 or 60 replications. Means associated with a given concentration and followed by the same letter are not significantly different ($\alpha = 0.01$). Standard errors of means were < 1.3 cm.

[b] y-intercept range of linear regressions = 14.1–14.3 cm, independent of temperature.

$$\text{3-octanone: cm root length} = 13.5 - 1.6C + 0.05C^2$$

$$(\text{ln transform } r^2 = 0.999)$$

$$\text{2-nonanone: cm root length} = 12.9 - 1.6C + 0.06C^2$$

$$(\text{ln transform } r^2 = 0.951)$$

(In these and subsequent response descriptions via nonlinear equations, the coefficients and intercepts are shown as the antilogs to facilitate comparisons with the linear response equations.) Quadratic equations also best described the concentration dependence of the root elongation effects at 20°C of those four ketones and 5-methyl-2-heptanone. At 20°C,

$$\text{2-heptanone: cm root length} = 13.5 + 1.5C + 0.05C^2$$

$$(\text{ln transform } r^2 = 0.961)$$

$$\text{2-octanone: cm root length} = 13.4 + 1.8C + 0.07C^2$$

$$(\text{ln transform } r^2 = 0.969)$$

$$\text{3-octanone: cm root length} = 14.9 - 1.3C + 0.03C^2$$

$$(\text{ln transform } r^2 = 0.936)$$

$$\text{5-methyl-2-heptanone: cm root length} = 13.8 - 1.3C + 0.05C^2$$

$$(\text{ln transform } r^2 = 0.979)$$

$$\text{2-nonanone: cm root length} = 13.4 - 1.4C + 0.05C^2$$

$$(\text{ln transform } r^2 = 0.956)$$

Significant deviation from linear response and quadratic regression equations with $r^2 < 0.900$ were consistent characteristics of the concentration response data for the most inhibitory ketones.

Cotton seedling shoot elongation (Table 3) was less sensitive to the assay ketones than was root elongation, and shoot growth was inhibited by 2-octanone, 3-octanone, 5-methyl-2-heptanone, 2-heptanone, and 2-nonanone, in decreasing order of activity. Again, the 30 and 20°C DI water controls were not significantly different. The shoots of seedlings grown in the 30°C root zone were inhibited by the lowest assay concentration of 2-heptanone, 2-octanone, 3-octanone, and 5-methyl-2-heptanone. Inhibition by 2-octanone required 1.0 μM/cm^3 or higher when the root-zone temperature was 20°C, and concentrations of 1.5 μM/cm^3 were required for shoot growth inhibition by 2-heptanone at that temperature. The lowest 2-nonanone concentration treatment inhibited at 20°C only. Shoot elongation was promoted by 2.0 μM/cm^3 3-methyl-2-butanone at 20 and 30°C and by 1.5 μM/cm^3 of that ketone at 20°C. The 1.5 and 2.0 μM/

TABLE 3. EFFECTS OF KETONE STRUCTURE, CONCENTRATION, AND ROOT-ZONE TEMPERATURE ON COTTON SEEDLING SHOOT ELONGATION[a]

Structure	Shoot length (cm)				Slope, linear regression[b]
	0.5 μM/cm^3	1.0 μM/cm^3	1.5 μM/cm^3	2.0 μM/cm^3	
Root-zone temperature: 30°C					
2-Butanone	8.7 a	8.0 bc	7.6 d	7.0 de	−0.08
3-Methyl-2-butanone	8.4 ab	9.2 a	9.2 ab	9.4 a	0.08
3-Pentanone	8.7 a	8.9 ab	9.6 a	9.4 a	0.09
2-Heptanone	6.8 d–g	6.7 d–f	5.8 e	5.4 fg	−0.17
2-Octanone	5.9 g	5.2 g–i	4.1 g	2.9 i	Quadratic
3-Octanone	6.7 e–g	4.6 hi	4.1 g	4.0 h	Quadratic
5-Methyl-2-heptanone	6.7 e–g	6.1 f–h	5.4 ef	4.9 f–h	−0.20
2-Nonanone	7.3 c–f	6.4 e–g	6.0 e	6.0 ef	−0.14
DI water control, 30°C	8.2 a–c	8.2 a–c	8.2 b–d	8.2 bc	
Root-zone temperature: 20°C					
2-Butanone	7.5 b–e	7.5 c–e	6.3 e	5.8 f	−0.10
3-Methyl-2-butanone	7.7 b–d	8.2 a–c	8.9 a–c	9.0 ab	0.10
3-Pentanone	7.5 b–e	7.7 cd	8.1 cd	8.2 bc	NS
2-Heptanone	7.3 c–f	6.8 d–f	5.4 ef	4.9 f–h	−0.17
2-Octanone	7.5 b–e	5.3 g–i	3.7 g	2.9 i	Quadratic
3-Octanone	6.5 fg	5.4 g–i	4.7 fg	4.4 gh	−0.20
5-Methyl-2-heptanone	6.0 g	5.9 f–h	5.9 e	5.6 f	−0.11
2-Nonanone	6.3 g	6.1 f–h	5.7 ef	5.4 fg	−0.12
DI water control, 20°C	7.5 b–e	7.5 c–e	7.5 d	7.5 cd	

[a] Lengths are means of 40 to 60 replications. Means associated with a given concentration and followed by the same letter are not significantly different ($\alpha = 0.01$). Standard errors of means were < 1.1 cm.
[b] y-intercept ranges of linear regressions = 8.0–8.3 cm at 30°C and 7.2–7.7 cm at 20°C.

cm^3 treatments with 3-pentanone also promoted shoot elongation at 30°C, but that compound had no effect at the lower root-zone temperature. The shoot elongation responses to increasing concentrations of all eight ketones contained significant linear components, regardless of temperature. At 30°C, 2-octanone and 3-octanone concentration response curves were best expressed by quadratic equations. At 30°C,

$$\text{2-octanone: cm shoot length} = 8.0 - 0.4C + 0.007C^2$$

$$(\ln \text{ transform } r^2 = 0.980)$$

$$\text{3-octanone: cm shoot length} = 8.3 - 0.6C + 0.018C^2$$

$$(\ln \text{ transform } r^2 = 0.980)$$

At 20°C, all shoot elongation responses to ketone concentration were linear except those to 3-pentanone (non-significant) and to 2-octanone (quadratic). At 20°C,

$$\text{2-octanone: cm shoot length} = 6.7 - 0.6C + 0.02C^2$$

$$(\ln \text{ transform } r^2 = 0.980)$$

Root and Shoot Fresh Weights. All three main effects (Table 4), including that of temperature, were significant in the analyses of variance of both root and shoot fresh weight data. The temperature × concentration interaction in the root fresh weight data was nonsignificant. The fresh weight second-order interactions

TABLE 4. MEAN SQUARES FROM ANALYSES OF VARIANCE OF KETONE STRUCTURE, CONCENTRATION, AND ROOT-ZONE TEMPERATURE EFFECTS ON COTTON ROOT AND SHOOT FRESH WEIGHT ACCUMULATION

Source	dF	Mean square	
		Root	Shoot
Temperature (temp.)	1	32100.07 ***[a]	276548.10 ***
Structure	7	21103.52 ***	599984.30 ***
Concentration (conc.)	4	11473.12 ***	252260.40 ***
Temp. × structure	7	1133.27 ***	281759.00 ***
Temp. × conc.	4	273.69 NS	310558.40 ***
Structure × Conc.	28	2336.09 ***	339446.30 ***
Temp. × structure × conc.	28	668.91 ***	315948.10 ***
Within subgroups	2661	237.27	32810.16

[a] *, **, *** = significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

were significant, the only significant three-way interactions observed in this study.

Regardless of treatment temperature, 2-octanone was the most inhibitory of root fresh weight accumulation (Table 5). Root fresh weight accumulation was less sensitive than root elongation to the lower concentrations of the assay ketones. In the 30°C treatment, the order of root fresh weight inhibitory activity was 2-octanone > 3-octanone > 2-nonanone > 2-heptanone > 5-methyl-2-heptanone. At that temperature, 2-butanone and 3-pentanone had no effect on root fresh weight accumulation. Root fresh weight accumulation responses at 30°C to 3-methyl-2-butanone, 2-octanone, and 3-octanone were best expressed by quadratic equations.

At 30°C,

$$\text{3-methyl-2-butanone: mg root fresh weight} = 192.3 + 7.7C - 0.5C^2$$

$$(\ln \text{ transform } r^2 = 0.664)$$

$$\text{2-octanone: mg root fresh weight} = 182.9 - 20.0C + 0.7C^2$$

$$(\ln \text{ transform } r^2 = 0.995)$$

$$\text{3-octanone: mg root fresh weight} = 198.8 - 11.9C + 0.3C^2$$

$$(\ln \text{ transform } r^2 = 0.853)$$

The root fresh weights of seedlings exposed to all levels of 2-methyl-2-butanone exceeded both the pooled mean for the 30°C DI water control (185.6 mg) and the mean of the 30°C DI water controls (195.9 mg) run in parallel with the 3-methyl-2-butanone treatments. This skewed parabolic response accounts for the low r^2 value of the quadratic equation for that compound. The mean of the 30°C DI water controls associated with the 3-octanone treatments was 181.3 mg, less than the pooled mean.

At 20°C, the inhibitory activity series was: 2-octanone > 2-heptanone = 2-nonanone = 3-octanone > 5-methyl-2-heptanone > 2-butanone > 3-methyl-2-butanone = 3-pentanone. There was no significant 3-methyl-2-butanone concentration effect in the 20°C treatment, although levels of 1.0, 1.5, and 2.0 $\mu M/cm^3$ apparently increased root fresh weights at the lower treatment temperature. Concentration response curves at 20°C were linear, except in the case of 3-pentanone and 3-octanone. At 20°C,

$$\text{3-pentanone: mg root fresh weight} = 236.5 + 26.4C + 1.8C^2$$

$$(\ln \text{ transform } r^2 = 0.605)$$

$$\text{3-octanone: mg root fresh weight} = 246.1 - 0.9C - 0.05C^2$$

$$(\ln \text{ transform } r^2 = 0.828)$$

TABLE 5. EFFECTS OF KETONE STRUCTURE, CONCENTRATION, AND ROOT-ZONE TEMPERATURE ON COTTON SEEDLING ROOT FRESH WEIGHT ACCUMULATION[a]

Structure	Root fresh weight (mg)				Slope, linear regression[b]
	0.5 μM/cm^3	1.0 μM/cm^3	1.5 μM/cm^3	2.0 μM/cm^3	
Root-zone temperature: 30°C					
2-Butanone	178.9 ef	183.1 d–f	170.9 c–e	166.3 ef	NS
3-Methyl-2-butanone	231.9 bc	215.1 cd	211.3 bc	197.1 b–e	Quadratic
3-Pentanone	202.9 c–e	204.9 c–e	163.0 c–f	231.5 b	NS
2-Heptanone	173.3 ef	171.3 ef	118.3 fg	105.4 hi	−5.23
2-Octanone	108.8 ef	69.2 i	51.3 h	43.7 jk	Quadratic
3-Octanone	188.1 d–f	100.9 hi	85.8 gh	80.8 ij	Quadratic
5-Methyl-2-heptanone	179.4 ef	154.9 fg	132.6 e–g	122.3 h–i	−4.25
2-Nonanone	155.3 f	131.2 gh	129.2 e–g	103.0 hi	−4.91
DI water control, 30°C	185.6 d–f	185.6 d–f	185.6 b–d	185.6 c–e	
Root-zone temperature: 20°C					
2-Butanone	258.6 b	224.2 c	190.9 b–d	184.9 de	−3.33
3-Methyl-2-butanone	241.5 bc	279.6 b	282.0 a	323.4 a	NS
3-Pentanone	319.3 a	381.3 a	227.5 b	229.3 bc	Quadratic
2-Heptanone	170.0 ef	154.6 fg	155.0 d–f	108.7 hi	−6.48
2-Octanone	173.9 ef	95.8 hi	84.3 gh	35.9 k	−11.99
3-Octanone	252.2 b	151.2 fg	97.6 gh	95.0 hi	Quadratic
5-Methyl-2-heptanone	208.7 c–e	183.8 d–f	178.5 b–e	161.9 e–g	−4.00
2-Nonanone	182.3 ef	152.6 fg	131.7 e–g	129.1 f–h	−6.35
DI Water control, 20°C	225.8 b–d	225.8 c	225.8 b	225.8 b–d	

[a] Fresh weights are means of 40 to 60 replications. Means associated with a given concentration and followed by the same letter are not significantly different ($\alpha = 0.01$). Standard errors of means were <7.7 mg/seedling.

[b] y intercept ranges of linear regressions were 188.7–192.0 mg at 30°C and 216.0–217.7 mg at 20°C.

Again, treatment effects that raised root fresh weights above the DI water control means reduced the r^2 values. The 20°C DI water control mean associated with the 3-pentanone treatments was 229.7 mg and that associated with the 3-octanone treatments was 228.9 mg. The root fresh weight of the 20°C DI water control was not significantly greater than that of the 30°C control.

Shoot fresh weight accumulation (Table 6) was more sensitive than that of the root to the lowest concentrations of 2-heptanone, 2-octanone, 3-octanone, and 5-methyl-2-heptanone. This sensitivity and the shoot fresh weights of the DI water controls did not differ with root-zone temperature. The shoot fresh weight accumulation inhibitory activity series was 2-octanone > 3-octanone = 2-heptanone > 5-methyl-2-heptanone = 2-nonanone > 3-pentanone = 2-butanone > 3-methyl-2-butanone. There was no significant 3-pentanone concentration effect at either treatment temperature. At 30°C, the shoot fresh weight accumulation responses to 2-butanone, 2-heptanone, and 2-octanone were best expressed by quadratic equations. At 30°C,

$$\text{2-butanone: mg shoot fresh weight} = 928.4 + 27.7C - 2.2C^2$$

$$(\text{ln transform } r^2 = 0.807)$$

$$\text{2-heptanone: mg shoot fresh weight} = 879.0 - 47.7C + 1.5C^2$$

$$(\text{ln transform } r^2 = 0.926)$$

$$\text{2-octanone: mg shoot fresh weight} = 893.5 - 100.7C + 3.6C^2$$

$$(\text{ln transform } r^2 = 0.994)$$

Again, responses to the lower concentrations of 2-butanone that increased the treatment shoot fresh weight means above those of the 30°C DI water controls reduced the r^2 value of the quadratic equation. The unpooled 30°C DI water control mean associated with the 2-butanone treatments was 906.7 mg.

At 20°C, all significant responses were linear except those to 2-octanone.

$$\text{2-octanone: mg shoot fresh weight} = 885.9 - 92.9C + 3.6C^2$$

$$(\text{ln transform } r^2 = 0.981)$$

Exposure to 3-methyl-2-butanone at both treatment temperatures increased shoot fresh weight accumulation, and the 30 and 20°C DI water controls were not significantly different.

Root and Cotyledon Dry Weights. All three main effects significantly influenced dry weight accumulation (Table 7) of cotton roots and cotyledons. However, the four first-order interactions involving temperature were of low to no significance. The root and cotyledon structure × concentration interactions were significant.

In comparison to the appropriate DI water control, the lowest ketone con-

TABLE 6. EFFECTS OF KETONE STRUCTURE, CONCENTRATION, AND ROOT-ZONE TEMPERATURE ON COTTON SEEDLING SHOOT FRESH WEIGHT ACCUMULATION[a]

Structure	Shoot fresh weight (mg)				Slope, linear regression[b]
	0.5 $\mu M/cm^3$	1.0 $\mu M/cm^3$	1.5 $\mu M/cm^3$	2.0 $\mu M/cm^3$	
Root zone temperature: 30°C					
2-Butanone	1034.1 a	1031.9 a	875.3 bc	827.1 bc	Quadratic
3-Methyl-2-butanone	1035.0 a	1067.7 a	1120.1 a	1184.9 a	16.44
3-Pentanone	880.0 b–d	827.0 c	903.2 b	880.2 bc	NS
2-Heptanone	647.6 g	605.0 de	565.4 d–f	467.8 e–g	Quadratic
2-Octanone	514.2 h	327.8 f	226.1 g	193.2 h	Quadratic
3-Octanone	704.2 fg	519.0 e	509.8 ef	425.2 g	−30.22
5-Methyl-2-heptanone	765.2 d–f	634.2 d	492.6 f	491.9 e–g	−28.22
2-Nonanone	802.2 c–f	660.3 d	579.0 d–f	575.8 d–f	−22.73
DI water control, 30°C	908.9 bc	908.9	908.9 b	908.9 b	
Root-zone temperature: 20°C					
2-Butanone	981.1 ab	989.2 ab	784.6 c	763.3 c	−10.59
3-Methyl-2-butanone	912.2 bc	1031.1 b	1060.9 a	1121.1 a	14.19
3-Pentanone	821.7 c–e	833.3 c	847.4 bc	857.8 bc	NS
2-Heptanone	740.2 c–e	622.2 de	523.9 d–f	454.2 g	−28.61
2-Octanone	535.5 h	363.9 f	339.1 g	298.7 h	Quadratic
3-Octanone	701.8 fg	611.4 de	486.1 f	465.2 fg	−28.09
5-Methyl-2-heptanone	750.5 e–g	682.3 d	621.3 de	583.3 de	−20.17
2-Nonanone	827.4 c–e	698.7 d	626.7 d	616.7 d	−19.76
DI water control, 20°C	905.8 bc	905.8 bc	905.8 b	905.8 b	

[a] Fresh weights are means of 40 or 60 replications. Means associated with a given concentration and followed by the same letter are not significantly different ($\alpha = 0.01$). Standard errors of means were <8.5 mg/seedling.

[b] y intercept ranges of the linear regressions were 889.1–905.3 mg at 30°C and 873.2–894.3 mg at 20°C.

TABLE 7. MEAN SQUARES FROM ANALYSES OF VARIANCE OF KETONE STRUCTURE, CONCENTRATION, AND ROOT-ZONE TEMPERATURE EFFECTS ON COTTON ROOT AND COTYLEDON DRY WEIGHT ACCUMULATION.

Source	dF	Mean square	
		Root	Shoot
Temperature (temp.)	1	8769.67 ***[a]	8634.09 ***
Structure	7	3380.61 ***	30133.63 ***
Concentration (conc.)	4	3808.44 ***	24712.75 ***
Temp. × structure	7	117.70 NS	1255.77 *
Temp. × conc.	4	78.81 NS	216.33 NS
Structure × conc.	28	418.74 ***	3397.15 ***
Temp. × structure × conc.	28	119.89 NS	564.11 NS
Within subgroups	2661	111.89	522.53

[a] *, **, *** = significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

centration had no effect on root dry weight (Table 8). The root dry weight of the 20°C DI water control was not significantly greater than that of the corresponding 30°C control. Increasing concentrations of 2-octanone, 3-octanone, 2-nonanone, 2-heptanone, and 5-methyl-2-heptanone reduced root dry weights in a linear fashion. There was no effect of exposures at 30 or 20°C to 2-butanone, 3-methyl-2-butanone, or 3-pentanone except the apparent dry weight increase in seedling exposed at 20°C to 1.0 $\mu M/cm^3$ 3-pentanone. This outlier may arise from experimenter error during the indirect measurement of pooled radicle dry weights during determinations of relative water contents. In the 20 and 30°C treatments, increasing concentrations (>0.5 $\mu M/cm^3$) of 2-octanone, 2-heptanone, 3-octanone, and 2-nonanone reduced root dry weights linearly. At 20°C, the highest concentration only of 5-methyl-2-heptanone was inhibitory.

Dry weights of the cotyledons (Table 9), not the entire seedling shoot above the transition zone, were obtained as part of the relative water content determination. Again, 2-octanone was the most inhibitory assay ketone at both treatment temperatures. In the 30°C treatment, the two heptanones were equally inhibitory, followed by 3-octanone and 2-nonanone. The concentration response curves at 30°C for these five ketones were all linear. The C_4 and two C_5 ketones had no effect on cotyledon dry weight at either treatment temperature. The response to 2-octanone at 20°C was again quadratic.

$$\text{2-octanone: mg dry weight per 20 cotyledons} = 363.6 - 31.8C + 1.2C^2$$

$$(\text{ln transform } r^2 = 0.995)$$

TABLE 8. EFFECTS OF KETONE STRUCTURE, CONCENTRATION, AND ROOT-ZONE TEMPERATURE ON COTTON SEEDLING ROOT DRY WEIGHT ACCUMULATION[a]

Structure	Root dry weight (mg/10 seedlings)				Slope, linear regression[b]
	0.5 μM/cm^3	1.0 μM/cm^3	1.5 μM/cm^3	2.0 μM/cm^3	
Root-zone temperature: 30°C					
2-Butanone	90.2 c-f	82.5 b-f	99.0 a-d	87.6 a-f	NS
3-Methyl-2-butanone	109.3 a-e	109.2 bc	101.8 a-c	104.3 a-c	NS
3-Pentanone	92.4 b-f	90.9 b-e	83.8 a-f	90.1 a-e	NS
2-Heptanone	82.0 d-f	80.4 b-f	59.2 c-g	49.3 e-h	−2.93
2-Octanone	62.7 f	41.9 f	32.4 g	28.9 h	−5.12
3-Octanone	95.3 b-f	50.0 ef	47.2 fg	37.7 gh	−4.03
5-Methyl-2-heptanone	91.7 c-f	74.7 c-f	59.6 c-g	57.4 d-h	−2.38
2-Nonanone	76.9 ef	69.7 c-f	58.9 d-g	46.8 f-h	−3.21
DI water control, 30°C	94.6 b-f	94.6 b-d	94.6 a-e	94.6 a-d	
Root-zone temperature: 20°C					
2-Butanone	129.8 a-c	109.1 bc	92.8 a-e	109.5 ab	NS
3-Methy-2-butanone	140.1 a	118.6 ab	118.4 a	125.7 a	NS
3-Pentanone	136.0 ab	161.2 a	115.8 a	113.0 ab	NS
2-Heptanone	83.8 d-f	79.4 b-f	72.0 b-g	55.4 d-h	−4.34
2-Octanone	111.7 a-e	58.6 e-f	45.4 fg	32.5 h	−6.05
3-Octanone	123.2 a-d	79.3 b-f	52.1 e-g	46.5 gh	−4.95
5-Methyl-2-heptanone	97.3 a-f	97.4 b-d	91.8 a-e	79.1 b-g	−2.68
2-Nonanone	94.7 b-f	77.1 b-f	76.1 a-f	64.4 c-h	−3.90
Di water control, 20°C	106.6 a-e	106.6 bc	106.6 ab	106.6 a-c	

[a] Dry weights are means of four or six replications. Means associated with a given temperature and followed by the same letter are not significantly different ($\alpha = 0.01$). Standard errors of means were <7.0 mg/10 roots.

[b] y intercept ranges of the linear regressions were 96.6–97.1 mg at 30°C and 120.3–121.8 mg at 20°C.

TABLE 9. EFFECTS OF KETONE STRUCTURE, CONCENTRATION, AND ROOT-ZONE TEMPERATURE ON COTTON COTYLEDON DRY WEIGHT ACCUMULATION[a]

Structure	Cotyledon dry weight (mg/20 cotyledons)				Slope, linear regression[b]
	0.5 μM/cm^3	1.0 μM/cm^3	1.5 μM/cm^3	2.0 μM/cm^3	
Root-zone temperature: 30°C					
2-Butanone	361.6 a–c	362.3 ab	313.8 a–d	305.3 a–e	NS
3-Methyl-2-butanone	435.9 a	451.4 a	409.9 a	436.9 a	NS
3-Pentanone	294.6 bc	301.3 bc	341.0 a–c	337.5 a–d	NS
2-Heptanone	277.8 bc	259.1 b–d	237.4 c–f	192.7 e–g	−10.74
2-Octanone	277.9 bc	159.6 d	143.2 f	116.3 g	−17.69
3-Octanone	312.2 a–c	256.9 b–d	253.9 b–f	236.4 c–g	−8.67
5-Methyl-2-heptanone	307.0 a–c	269.3 b–d	186.6 d–f	183.9 e–g	−12.02
2-Nonanone	317.9 a–c	294.2 bc	245.9 c–f	236.5 c–g	−8.27
DI water control, 30°C	347.5 a–c	347.5 a–c	347.5 a–c	347.5 a–d	
Root-zone temperature: 20°C					
2-Butanone	375.3 a–c	367.1 ab	310.2 a–d	292.1 b–e	NS
3-Methyl-2-butanone	408.6 ab	363.8 ab	409.2 a	437.1 a	NS
3-Pentanone	301.6 a–c	321.6 a–c	339.0 a–d	371.4 a–c	NS
2-Heptanone	316.1 a–c	296.9 bc	281.1 a–e	225.5 d–g	−8.89
2-Octanone	250.9 c	190.5 cd	161.7 ef	154.0 fg	Quadratic
3-Octanone	388.7 ab	351.2 ab	252.8 b–f	216.8 d–g	−8.71
5-Methyl-2-heptanone	337.4 a–c	321.8 a–c	277.1 a–f	248.9 b–g	−7.68
2-Nonanone	362.0 a–c	327.3 ab	298.6 a–d	277.5 b–f	NS
DI water control, 20°C	385.7 ab	385.7 ab	385.7 ab	385.7 ab	

[a] Dry weights are means of four or six replicatons. Means associated with a given temperature and followed by the same letter are not significantly different ($\alpha = 0.01$). Standard errors of means were <6.5 mg/20 cotyledons.

[b] y intercept ranges of linear regressions were 357.3–358.2 mg at 30°C and 372.1–376.2 mg at 30°C

The cotyledon dry weights of the two DI water controls were not significantly different.

Cotyledon Areas. The three main effects were all significant factors in determining cotyledon expansion (Table 10), and the structure × concentration first interaction alone was highly significant.

At both treatment temperatures, 2-octanone was the most inhibitory (Table 11), and all assay concentrations of 2-octanone, 2-heptanone, 5-methyl-2-heptanone, and 3-octanone were inhibitory. Cotyledon areas were also reduced by exposure to 2-nonanone at concentrations greater or equal to 1.0 μM/cm^3. These concentration response curves all contained significant linear components, and the responses to 2-butanone, 3-methyl-2-butanone, and 2-octanone at 30°C were best expressed by quadratic equations.

$$\text{2-butanone: cm}^2\text{ cotyledon area} = 31.3 + 0.3C - 0.04C^2$$

$$(\text{ln transform } r^2 = 0.818)$$

$$\text{3-methyl-2-butanone: cm}^2\text{ cotyledon area} = 31.3 + 1.3C - 0.05C^2$$

$$(\text{ln transform } r^2 = 0.947)$$

$$\text{2-octanone: cm}^2\text{ cotyledon area} = 31.3 - 3.8C + 0.13C^2$$

$$(\text{ln transform } r^2 = 0.988)$$

The 30°C DI water control associated with the 2-butanone treatments was 32.5 cm^2. At both 20 and 30°C, only 1.5 and 2.0 μM/cm^3 2-butanone inhibited

TABLE 10. MEAN SQUARES FROM ANALYSES OF VARIANCE OF KETONE STRUCTURE, CONCENTRATION, AND ROOT-ZONE TEMPERATURE EFFECTS ON COTTON COTYLEDON AREA

Source	dF	Mean square: Cotyledon area
Temperature (temp.)	1	70.69 ***[a]
Structure	7	472.86 ***
Concentration (conc.)	4	426.00 ***
Temp. × structure	7	5.90 NS
Temp. × conc.	4	6.87 NS
Structure × conc.	28	51.27 ***
Temp. × structure × conc.	28	8.78 NS
Within subgroups	2661	9.25

[a] *, **, *** = significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

TABLE 11. EFFECTS OF KETONE STRUCTURE, CONCENTRATION, AND ROOT-ZONE TEMPERATURE ON COTTON COTYEDON AREA

Structure	Cotyledon area (cm^2)				Slope, linear regression[b]
	0.5 $\mu M/cm^3$	1.0 $\mu M/cm^3$	1.5 $\mu M/cm^3$	2.0 $\mu M/cm^3$	
Root-zone temperature: 30°C					
2-Butanone	32.6 a–c	32.1 b–d	27.1 de	26.4 d	Quadratic
3-Methyl-2-butanone	36.4 a	38.1 a	39.0 a	40.1 a	Quadratic
3-Pentanone	29.4 c–f	28.0 de	25.4 e	24.9 de	−0.39
2-Heptanone	25.1 f	18.7 h	17.5 f–h	13.4 h	−1.06
2-Octanone	19.7 g	7.2 i	4.8 i	3.2 i	Quadratic
3-Octanone	25.8 ef	19.5 h	15.3 gh	13.1 h	−1.15
5-Methyl-2-heptanone	25.7 ef	19.8 h	13.3 h	13.5 h	−1.17
Nonanone	27.3 ef	23.7 e–h	18.0 fg	17.3 fg	−0.90
DI water control, 30°C	32.3 a–d	32.3 b–d	32.3 bc	32.3 c	
Root-zone temperature: 20°C					
2-Butanone	34.8 ab	33.6 a–c	27.3 de	25.3 de	Quadratic
3-Methyl-2-butanone	32.2 a–d	37.9 a	37.7 a	40.3 a	0.44
3-Pentanone	27.5 d–f	28.6 c–e	30.9 cd	32.4 c	Quadratic
2-Heptanone	26.4 ef	22.4 f–h	19.9 fg	14.4 gh	−1.15
2-Octanone	17.3 g	7.3 i	7.1 i	6.7 i	Quadratic
3-Octanone	29.7 c–f	25.2 e–g	18.0 fg	15.6 g–h	−1.22
5-Methyl-2-heptanone	26.6 ef	21.4 gh	20.5	18.3	Quadratic
2-Nonanone	30.3 b–e	26.5 ef	25.9 e	22.0 e	−0.72
DI water control, 20°C	36.4 a	36.4 ab	36.4 ab	36.4 b	

[a] Cotyledon areas are means of 80 to 120 replications. Means associated with a given temperature and followed by the same letter are not significantly different ($\alpha = 0.01$). Standard errors of means were <3.6 cm^2.

[b] y intercept ranges of linear regressions were 30.0–30.6 cm^2 at 30°C and 32.9–33.5 cm^2 at 20°C.

cotyledon expansion. Cotyledon areas of seedlings exposed to 3-methyl-2-butanone increased, particularly in the 30°C treatment. The 1.5 and 2.0 $\mu M/cm^3$ levels of 3-pentanone inhibited cotyledon expansion at 30°C, and the 0.5 and 1.0 $\mu M/cm^3$ concentrations were inhibitory in the 20°C treatment. At the lower temperature, responses to 2-butanone, 3-pentanone, 2-octanone, and 5-methyl-2-heptanone were best fitted by quadratic expressions.

$$\text{2-butanone: cm}^2\text{ cotyledon area} = 34.6 + 0.1C - 0.05C^2$$

$$(\ln \text{ transform } r^2 = 0.926)$$

$$\text{3-pentanone: cm}^2\text{ cotyledon area} = 30.7 - 4.7C - 0.19C^2$$

$$(\ln \text{ transform } r^2 = 0.822)$$

$$\text{2-octanone: cm}^2\text{ cotyledon area} = 33.7 - 4.7C + 0.19C^2$$

$$(\ln \text{ transform } r^2 = 0.987)$$

$$\text{5-methyl-2-heptanone: cm}^2\text{ cotyledon area} = 33.9 - 2.0C + 0.07C^2$$

$$(\ln \text{ transform } r^2 = 0.986)$$

The unpooled 20°C DI water control mean associated with the 3-pentanone treatments was 32.7 cm^2; the concave parabolic response to that ketone resulted from the reduced cotyledon areas in the 0.5 and 1.0 $\mu M/cm^3$ treatments. The cotyledon areas of the 30 and 20°C DI water controls were not significantly different.

Root and Shoot Relative Water Contents. Ketone structure was the only significant main effect in the analyses of variance (Table 12) of the seedling root and shoot RWC data. One first-order interaction, that between structure and concentration, was significant in the root RWC data. That significant interaction as well as the structure main effect were due to an increase of 6–9 percentage points in the root RWC of seedlings exposed to 2-butanone at either treatment temperature (data not shown). An increase of the same magnitude was observed in the shoot RWC of seedlings exposed to all four concentrations of 2-butanone at 20°C. The temperature treatments had no effect on the RWC percentages of any of the root and shoot DI water controls.

DISCUSSION

Cover crop residues and other plant debris have been reported to cause significant reductions in seedling growth and survival under field conditions (Buntley, 1986; Dabney et al., 1986; Menges, 1987; Bradow and Bauer, 1992). Soil containing decomposing crop residues also inhibited seedling growth under laboratory conditions (Bradow and Bauer, 1992). Prior to this study in which

TABLE 12. MEAN SQUARES FROM ANALYSES OF VARIANCE OF KETONE STRUCTURE, METHYL KETONE CONCENTRATION, AND ROOT-ZONE TEMPERATURE EFFECTS ON COTTON ROOT AND SHOOT RELATIVE WATER CONTENT

Source	dF	Mean square	
		Root	Shoot
Temperature (temp.)	1	4.66 NS	0.61 NS
Structure	7	61.90 ***[a]	78.65 ***
Concentration (conc.)	4	3.53 NS	22.27 NS
Temp. × structure	7	0.59 NS	22.27 NS
Temp. × conc.	4	2.19 NS	12.99 NS
Structure × conc.	28	6.22 ***	15.74 NS
Temp. × structure × conc.	28	1.66 NS	12.56 NS
Within subgroups	2661	1.66	11.86

[a] *, **, *** = significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

plant residue volatiles were injected into a sterile soil mix, demonstrations and determinations of the activities of volatiles emitted by crop residues have been limited to soilless seed germination assays in vitro (Bradow and Connick, 1988a–c, 1990; Connick et al., 1989; Bradow, 1991c). Sterile greenhouse mix was used in an effort to avoid interference from the reported activities of these ketones as stimulants of fungal spore germination (French, 1984; French et al., 1986) and, possibly, sclerotial growth (King and Coley-Smith, 1968; Linderman and Gilbert, 1969) and mycelial growth (Menzies and Gilbert, 1967). In an earlier study (Bradow, 1991a) of the effects of a soil-incorporated biological fungicide on cotton seedling–disease–complex pathogens, this soil mix proved adequate as a control growth substrate. Comparative studies in which sterile soil and soil from the current field studies are injected with plant residue volatiles are in progress.

Two root-zone temperatures (20 and 30°C) were selected on the basis of other studies of the effects of suboptimal temperatures on postemergent cotton seedling growth (Bradow, 1990a, 1991d). The lower temperature represents probable cool root-zone conditions in the field after cover crop incorporation and cotton planting in the field (Bradow, 1990a, 1991d). Moreover, 20°C inhibited Coker 315 root and shoot growth, in comparison to 30°C (Bradow, 1991d). Early results in this study (Bradow, 1991b) and visual inspection of the growing seedlings had suggested that the lower root-zone temperature decreased seedling growth rate and the inhibitory effects of the injected ketones. No significant growth parameter differences were observed between the 20 and 30°C DI water controls, however, and most temperature interactions with ketone structure and

concentration were of no or low significance. The interactions between ketone structure and concentration were consistently more important. The size differential in the seedlings grown at 20 and 30°C that is apparent through visual observation during the treatment period may arise from the more rapid growth of the 30°C root-zone seedlings early in the treatment period, a difference that disappears or is minimized at harvest on day 14 by the effects of the ambient air temperature on the shoots of seedlings in both root-zone-temperature treatments.

Concentrations in the vapor phase of 34.4 μM/liter of the eight plant residue ketones discussed here inhibited seed germination to some degree in a sealed system consisting of a 2.5-liter glass desiccator (Bradow and Connick, 1988a, 1990). Germinating seeds were inhibited significantly after a three-day exposure to the vapor phase of the eight ketones. The lowest ketone concentration (0.5 μM/cm^3) used is the equivalent of 40 μM/liter when injected into a glass tube containing 75 cm^3 of soil mix with a surface area of 3.8 cm^2.

In this study, the seedlings were grown in a ventilated growth chamber that permitted the ketones to escape from the soil surface, particularly from the 30°C treatments. Despite the increased ventilation and the higher root-zone temperature in the seedling growth assays, the 0.5 μM/cm^3 (or 40 μM/liter) levels of 2-heptanone, 2-octanone, 3-octanone, 5-methyl-2-heptanone, and 2-nonanone all inhibited cotton seedling root elongation. With the exception of 2-nonanone, the lowest treatment of these ketones also reduced shoot elongation and fresh weight accumulation, as well as cotyledon expansion. These seedling responses to 40 μM/liter ketone concentrations were equal to or greater than those predicted from the seed germination assay results.

When the effects of these ketones on all seedling growth parameters were considered together, 2-octanone most consistently reduced seedling growth. Significant levels of this ketone were observed only in the emission profiles of plant residues that were decomposing under hypoxic or anaerobic conditions (Connick et al., 1987), and 2-octanone was highly inhibitory of seed germination (Bradow and Connick, 1988a; Bradow, 1991c). The cumulative inhibitory-activity series for 2-octanone and the seven ketones that were released during aerobic decomposition of legume cover crop residues was: 2-octanone $>>$ 3-octanone $=$ 2-heptanone $>$ 2-nonanone $=$ 5-methyl-2-heptanone $>$ 2-butanone $>$ 3-pentanone $>$ 3-methyl-2-butanone. The order of the activity series was not affected by root-zone temperature.

In the seed studies using 34 μM/liter, 2-octanone, 3-octanone, 2-heptanone, 5-methyl-2-heptanone, and 2-nonanone totally inhibited germination (Bradow and Connick, 1988a, 1990). In those assays, 3-pentanone was much less inhibitory than 2-butanone and 3-methyl-2-butanone (Bradow and Connick, 1988a,b). In the germination assays, C_4–C_6 compounds were less inhibitory than compounds with seven and eight carbons but more inhibitory than those with nine

(Bradow and Connick, 1988a,b, 1990; Bradow, 1991c). The activity series obtained in the seedling growth assays also shows the C_7 and C_8 ketones to be most inhibitory, but the activity of the C_9 ketone was roughly equivalent to those of the two heptanones. The absence of a root-zone temperature effect and the higher activity of the ketones of higher molecular weight suggests that relative volatility was not as important a factor in the ventilated experimental system as it was in the sealed seed germinations system. Chain length remained an important factor in seedling growth inhibition, as it had proven to be in seed germination. The position of the carbonyl group seemed less important than molecular weight in the structure–activity relationships elucidated by the seedling growth assays.

The absence of any root-zone temperature effect upon seedling root and shoot fresh weights and, particularly, upon the root and shoot water relationships was unexpected since earlier paper-towel scroll studies of seedling chilling sensitivity (Bradow, 1990a,b, 1991d) predicted that air temperatures below 30°C would significantly decrease the fresh weight of both roots and shoots. Exposure to the inhibitory volatiles did have this effect, and interactions between temperature and ketone structure were significant, but the difference between the shoot fresh weights of the 30 and 20°C DI water controls was less than expected from the paper-towel scroll studies. The fresh weight of the 20°C DI water control increased, in comparison to that of the 30°C control, an effect observed in seedlings recovering from chilling stress in the paper-towel scroll studies (Bradow, 1990a,b, 1991d). This recovery effect was smaller in Coker 315 than in two other cotton varieties that were more sensitive to suboptimal temperatures (Bradow, 1991d).

The higher air temperature in this soil-grown seedling study may explain the absence of the predicted temperature effect on the fresh weights of the DI water controls and the lack of significant effects upon root and shoot RWC. In the paper-towel scroll studies, treatment temperatures below 30°C increased Coker 315 root RWC and decreased shoot RWC (Bradow, 1991d). The photon flux density in the growth chambers used in those studies was also somewhat higher than that in the ventilated chamber used here. In another as yet unreported greenhouse study, low light levels interfered with normal stomatal opening and reduced the transpiration rate, damping out temperature effects on root and shoot RWC percentages and leaf water potentials. Cotyledons of growth-inhibited seedlings in the two seedling-based studies discussed here were too small and fragile to permit successful porometric measurements.

Previously, components, including ketones, of the volatile organic compounds released by decomposing cover crop and weed residues have been identified (Connick et al., 1987, 1989; Bradow and Connick, 1990) and the inhibitory activity of those compounds demonstrated in seed germination assays (Bradow and Connick, 1988a,b; 1990). The current study shows that the ketones emitted

by legume cover residues inhibit root and shoot growth of postemergent cotton seedlings. Inhibitions similar to those observed under laboratory conditions have been reported in a field study of the effects of crimson clover residues on cotton field establishment and yield (Bradow and Bauer, 1992). Parallel greenhouse and field experiments designed to examine those similarities are in progress. Structure–activity relationships in the seedling growth assays were similar to those observed in the seedling assays, strengthening earlier conclusions (Bradow and Connick, 1990) that volatile emissions from plant residues have a decisive role in the success of any agricultural practice that involves incorporation of large quantities of plant material into the soil.

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REFERENCES

BAUER, P.J., ROACH, S.H., and GREEN, C.C. 1991. Cotton genotype response to green-manured annual legumes. *J. Prod. Agric.* 4:626–627.

BRADOW, J.M. 1990a. Chilling sensitivity of photosynthetic oil-seedlings. 1. Cotton and sunflower. *J. Exp. Bot.* 233:1585–1593.

BRADOW, J.M. 1990b. Chilling sensitivity of photosynthetic oil-seedlings. 1. Cucurbitaceae. *J. Exp. Bot.* 233:1595–1600.

BRADOW, J.M. 1990c. Inhibition of cotton seedling growth by LISA cover crop residues. Proc. 1990 Beltwide Cotton Prod. Res. Conf., p. 845.

BRADOW, J.M. 1991a. Cotton growth in the presence of a seedling-disease-complex biocontrol and sub-optimal temperatures. Proc. 1991 Beltwide Cotton Prod. Res. Conf., pp. 820–824.

BRADOW, J.M. 1991b. Inhibition of cotton seedling growth by LISA cover crop residues. Proc. 1991 Beltwide Cotton Prod. Res. Conf., p. 845.

BRADOW, J.M. 1991c. Relationships between chemical structure and inhibitory activity of C_6 through C_9 volatiles emitted by plant residues. *J. Chem. Ecol.* 17:2193–2122.

BRADOW, J.M. 1991d. Cotton cultivar response to suboptimal postemergent temperatures. *Crop Sci.* 31:1595–1599.

BRADOW, J.M., and BAUER, P.J. 1992. Inhibition of cotton seedling growth by soil containing LISA cover crop residues. Proc. 1992 Beltwide Cotton Prod. Res. Conf., p. 1175.

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 volatiles 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. Proc. 1988 Beltwide Cotton Prod. Res. Conf., pp. 90–91.

BRADOW, J.M., and CONNICK, W.J., JR. 1990. Volatile seed germination inhibitors from plant residues. *J. Chem. Ecol.* 16:645–666.

BUNTLEY, G.J. 1986. Tennessee no-tillage update, pp. 100–102, *in* R.E. Phillips (ed.). Proc.

Southern Region No-Tillage Conf., Southern Region Series Bulletin #319, University of Kentucky, Lexington, Kentucky.

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.). Proc. Southern Region No-Tillage Conf., Southern Region Series Bulletin #319. University of Kentucky, Lexington, Kentucky.

FRENCH, R.C. 1984. Stimulation of uredospore germination of *Puccinia helianthi* and *Uromyces vignae* by aromatic nitriles and related flavor compounds. *J. Agric. Food Chem.* 32:556–561.

FRENCH, R.C., KUJAWSKI, P.T., and LEATHER, G.R. 1986. Effect of various flavor-related compounds on germination of curly dock seed (*Rumex crispus*) and curly dock rust (*Uromyces rumicis*). *Weed Sci.* 34:398–402.

KING, J.E., and COLEY-SMITH, J.R. 1968. Effects of volatile products of *Allium* species and their extracts on germination of sclerotia of *Sclerotium cepivonem* Berk. *Ann. Appl. Biol.* 61:407–414.

LINDERMAN, R.G., and GILBERT, R.G. 1969. Stimulation of *Sclerotium rolfsii* and its antagonists by volatile components of alfalfa hay. *Phytopathology* 59:1366–1372.

MENGES, R.M. 1987. Allelopathic effects of Palmer amaranth (*Amaranthus palmeri*) and other plant residues in soil. *Weed Sci.* 35:339–347.

MENZIES, J.D., and GILBERT, R.G. 1967. Response of the soil microflora to volatile components of plant residues. *Soil Sci. Soc. Am. Proc.* 1:495–496.

RICKERL, D.H., GORDON, W.B., CURL, E.A., and TOUCHTON, J.T. 1988. Winter legume and tillage effects of 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.

WALSH, G.E., BOHANNON, P.M., and WESSINGER-DUVALL, P.B. 1989. Microwave irradiation for rapid killing and fixing of plant tissue. *Can. J. Bot.* 67:1272–1274.

WEATHERLEY, P.E. 1950. Studies in the water relations of the cotton plant. I. The field measurements of water deficits in leaves. *New Phytol.* 49:81–97.

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QUANTIFICATION OF INSECT GROWTH AND ITS USE IN SCREENING OF NATURALLY OCCURRING INSECT CONTROL AGENTS

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Abstract—In studies of the effects of allelochemicals or other factors on the development of different insect species, comparison of growth-inhibiting activities is difficult using the parameters currently employed. We introduce two new parameters, growth index (GI) and relative growth index (RGI), which can unify the quantification of insect development. This quantification can also eliminate the effects of different growth characteristics due to the genetic differences between insect species. By measuring growth-inhibiting effects of two phytochemicals, chaparrin and chaparrinone, on the tobacco budworm, *Heliothis virescens*, and the beet armyworm, *Spodoptera exigua*, bioassay procedures and GI and RGI calculations are demonstrated.

Key Words—Bioassay, tobacco budworm, *Heliothis virescens*, beet armyworm, *Spodoptera exigua*, Lepidoptera, Noctuidae, insect growth inhibitors, allelochemicals, quassinoids, chaparrin, chaparrinone.

INTRODUCTION

Bioassays are indispensable in many biological studies. Commonly used bioassays for specific goals include acute toxicity, growth regulation (Berenbaum, 1986), feeding preference (Lewis and van Emden, 1986), and oviposition preferences (Singer, 1986). The diversity of bioassays ranges from the population level to the molecular level and from in vivo to in vitro studies. The design and implementation of bioassay strategies are issues of considerable attention (Sin-

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den et al., 1988; Wolfson, 1988). However, the parameters commonly used present problems in describing, interpreting, and comparing the results of different bioassays. For example, LD_{50} (the dose which causes 50% mortality in a population) is commonly used to determine the activity of chemicals that have acute toxicity. It can not be used for those chemicals that do not have acute toxicity but disturb insect growth by inhibiting feeding or altering normal development.

Different parameters have been used to study insect growth inhibition of many chemicals, such as the effects of allelochemicals on weight gain, pupal weight, duration of development, daily survival, or mortality. Other parameters such as relative growth rate (RGR: weight gain/day/average body weight) and food consumption index (CI: food eaten/day/body weight) have also been used to study the effect of nutrients and other factors on insect feeding and growth (Waldbauer, 1968). These parameters, however, vary with the insect species and even with instars within the same species. Therefore, unlike LD_{50}, which can be used to compare the acute toxicity of a compound to different insect species, the parameters currently used to measure insect growth are usually species- or stage-specific. The diversity among insect life histories makes it difficult to compare the effect of a chemical (or a factor) on the development of different insect species using the above parameters.

To overcome these limitations, we introduce two new parameters: growth index (GI) and relative growth index (RGI). We suggest that an insect population consuming a food containing chemicals that depress its growth rate usually shows a different distribution of individuals within each instar than does a control population consuming a normal food. At a given point in time, the growth-inhibited population will have more individuals in earlier developmental stages than will the control population. Furthermore, the extent of growth inhibition will be related to the concentration of these chemicals in the food. These characteristics of growth in response to a growth inhibitor are depicted in Figure 1.

Molting is a consistent characteristic of insect development. Therefore, instar can be used for rating the insect growth. For the purpose of the bioassay,

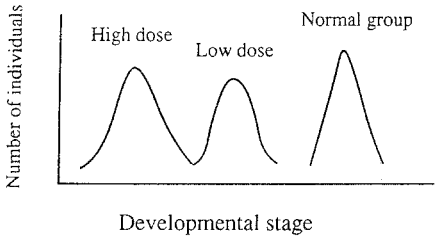


FIG. 1. Effects of a hypothetical insect growth inhibitor on the rate of insect development (see text for discussion).

we define the growth of an insect as the ability to molt and develop into next instar. The number of molts indicates the progression of development. If an insect did not molt, we assume it did not grow. Therefore, we use instar as a parameter and propose a growth index (GI) to represent the rate of insect growth: GI is defined as the sum of stages attained by the individuals under experimental conditions divided by the sum of the highest stages that would be reached in a control population. For example, to bioassay insect development from the first instar through the fifth instar to the pupal stage, using stage 1 to represent the first instar and stage 6 for the pupa, the GI is represented as follows:

$$\mathrm{GI} = \frac{n_1 \times \text{stage 1} + n_2 \times \text{stage 2} + \cdots + n_6 \times \text{stage 6}}{N \times \text{stage 6}} \tag{1}$$

where $n_1, n_2, \ldots, n_6$ are the number of individuals in stage 1, stage 2, . . . , stage 6, respectively. $N = n_1 + n_2 + \cdots + n_6$.

Following the above equation, the practical calculation of GI is:

$$\mathrm{GI} = \frac{\sum_{i=1}^{i_{\max}} [n_{(i)} \times i] + \sum_{i=1}^{i_{\max}} [n'_{(i)} \times (i-1)]}{N \times i_{\max}} \tag{2}$$

where i is the stage number; $n_{(i)}$ is the number of live larvae at i; $n'_{(i)}$ is the number of dead larvae at i; $i_{\max}$ is the highest attainable stage of the insect (here we define $i_{\max} = 6$, i.e. pupal stage); and N is the total number of larvae in the group.

When GI for each tested group and the control group are obtained, the RGI for each tested group can be determined using the following calculation:

$$\mathrm{RGI} = \frac{\text{GI of the tested group}}{\text{GI of the control group}} \tag{3}$$

The dead larvae are also included in the above calculation of GI where $i - 1$ is used to represent the stage of a larva found dead in stage i. This is based on the fact that if a larva is dead at stage i, it must have been alive in an earlier stage $(i - 1)$. For example, if a first-instar larva is found dead, $i - 1$ would be 0 (i.e., no growth); if a fourth-instar larva is found dead, $i - 1$ would be 3.

By including the dead larvae in the calculation of GI, one can distinguish between test compounds in terms of the severity of growth inhibition within a species. For example, if compounds A and B have the same LD_{50}, but compound A kills 50% in an early instar and the remaining larvae develop normally, its GI calculated from equation 2 would be different from that of compound B, which also kills 50% but does so in a later instar while the remaining larvae develop normally.

Based on this calculation, we now define GI as the extent of growth of the

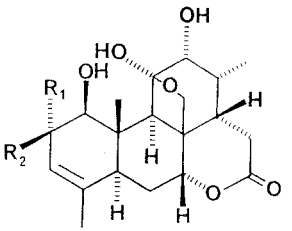
individuals with respect to their theoretical optimum growth. RGI, which is obtained from the GI of the treated group divided by the GI of the control group, is defined as the extent of growth of the individuals in a treated group with respect to the growth of the control group. Using this quantification procedure, it is possible to compare the impact of variables (e.g., allelochemicals, nutrients, environmental factors, etc.) on the development of different insect species by comparing the RGI of these species, analogous to using the LD_{50} to measure the acute toxicity of a chemical.

METHODS AND MATERIALS

Materials. Solvents used were purchased from Fisher Scientific (Santa Clara, California). The insect diet ingredients were purchased from ICN Biomedicals, Inc. (Costa Mesa, California). Chaparrin **(1)** and chaparrinone **(2)** used for the bioassay were previously isolated from a Mexican medicinal plant known as "chaparro amargo," *Castela tortuosa* (Simaroubaceae) (Kubo et al., 1992).

Insects. Heliothis virescens eggs were kindly provided by Dow Chemical Research Center (Walnut Creek, California). *Spodoptera exigua* eggs were a gift from ISK Mountain View Research Center, Inc. (Sunnyvale, California). Larvae were reared individually in 30 ml polyethylene vials in an incubator at 28 ± 1°C and 16:8 hr light–dark. The diet was prepared as described by Chan et al. (1978).

Bioassay. The compounds to be tested were dissolved in a small amount of methanol and mixed into the solid ingredients of the artificial diet. After methanol was evaporated in air, these solid ingredients were incorporated into the diet (Chan et al., 1978). A newly hatched larva was introduced into a



1 R_1=OH, R_2=H

2 R_1, R_2=O

SCHEME 1.

30-ml polyethylene vial containing 4 g of artificial diet with an aliquot of test compound. The control larvae were treated in the same way except that no test compound was added to the diet. Twenty to 30 larvae were used for each concentration.

When >95% of the control larvae pupated, all larvae were classified into defined stages. In this experiment the stage of a larva was defined based on its instar. In *H. virescens* and *S. exigua*, stages 1–5 represented five larval instars, while the pupal stage was defined as stage 6. The growth index (GI) and relative growth index (RGI) of each group were calculated from equations 2 and 3, respectively.

RESULTS

Table 1 records the effect of chaparrinone on the development of individual *H. virescens* larvae. As the concentration of chaparrinone in the diet increased, larval development was slower and mortality was higher. By placing the data in Table 1 into equations 2 and 3, the GI and RGI can be calculated. For example, for *H. virescens* larvae fed on diet containing 80 ppm of chaparrinone, the GI is:

$$GI_{80\ ppm} = \frac{(3 \times 1) + (8 \times 2) + (5 \times 3) + [2 \times (1 - 1)] + [2 \times (2 - 1)]}{20 \times 6}$$

$$= 0.30$$

$$GI_{control} = \frac{(19 \times 6) + (1 \times 5)}{20 \times 6} = 0.99$$

Therefore, RGI of *H. virescens* at 80 ppm of chaparrinone is:

$$RGI_{80\ ppm} = \frac{GI_{80\ ppm}}{GI_{control}} = \frac{0.30}{0.99} = 0.30$$

The effects of chaparrin and chaparrinone on GI and RGI of *H. virescens* and *S. exigua* are summarized in Table 2. When the RGI is plotted against the concentrations of the tested compounds, dose–response curves can be established (see Figure 2). Through linear regression analysis (Wardlaw, 1985), EC_{50} (concentration of the compound that caused 50% reduction in RGI) was interpolated for both tested compounds (Table 3).

Although the modes of action of chaparrin and chaparrinone are not known, antifeeding activity may be the major cause of the growth disruption (Lidert et al., 1987). We have observed that food consumption of *H. virescens* larvae decreased as the concentration of these compounds in the diet increased.

TABLE 1. EFFECT OF CHAPARRINONE ON GROWTH AND SURVIVAL OF *H. virescens* LARVAE[a]

Conc. (ppm)	1st instar		2nd instar		3rd instar		4th instar		5th instar		Pupa, alive
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	
10									7		13
20									20		
40		1	2	1	9		7				
80	3	2	8	2	5						
160		12	5	2	1						
320		20									
Control									1		19

[a]Twenty larvae were used for each concentration.

TABLE 2. RELATIONSHIPS OF TESTED COMPOUNDS AND CONCENTRATIONS TO GI, RGI, AND MORTALITY OF *H. virescens* AND *S. exigua*

Compounds	Conc. (ppm)	*H. virescens* GI	*H. virescens* RGI	*H. virescens* Death (%)	*S. exigua* GI	*S. exigua* RGI	*S. exigua* Death (%)
Chaparrinone	5				0.95	0.98	0
	10	0.94	0.95	0	0.75	0.77	5
	20	0.83	0.84	0	0.62	0.64	0
	40	0.50	0.51	10	0.28	0.29	30
	80	0.30	0.30	20	0.10	0.10	80
	160	0.13	0.13	70	0.00	0.00	100
	320	0.00	0.00	100			
Chaparrin	10	0.98	0.99	0	0.97	1.00	0
	20	0.91	0.92	0	0.98	1.00	0
	40	0.84	0.85	0	0.92	0.95	0
	80	0.76	0.76	0	0.83	0.86	6
	160	0.68	0.68	0	0.73	0.75	11
	320	0.57	0.57	5	0.63	0.65	18
Control		0.99		0	0.97		0

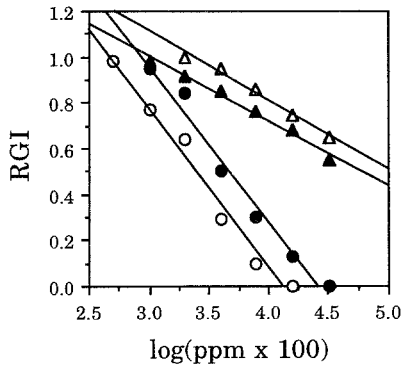


FIG. 2. Dose response of larval development to chaparrinone and chaparrin. Filled circles: response of *H. virescens* to chaparrinone ($y = 2.98 - 0.67x$, $r^2 = 0.98$, $P < 0.01$); open circles: response of *S. exigua* to chaparrinone ($y = 2.84 - 0.69x$, $r^2 = 0.98$, $P < 0.01$); filled triangles: response of *H. virescens* to chaparrin ($y = 1.84 - 0.28x$, $r^2 = 0.98$, $P < 0.01$); open triangles: response of *S. exigua* to chaparrin ($y = 2.00 - 0.30x$, $r^2 = 0.98$, $P < 0.01$).

TABLE 3. GROWTH INHIBITION ACTIVITY OF SOME NATURAL PRODUCTS ON *H. virescens* AND *S. exigua* LARVAE

Compounds	EC_{50} (ppm)[a]	
	H. virescens	*S. exigua*
Chaparrinone	49	25
Chaparrin	611	1000

[a] EC_{50} is the concentration of the compound that caused 50% reduction in RGI. Five to six concentrations were used for each compound and 20–30 larvae were used for each concentration.

DISCUSSION

This is the first application of RGI to estimate the growth inhibition effect of allelochemicals in an insect bioassay. The duration of a bioassay on insect growth generally depends on the life history of the test insect. Consequently, results from different insect species are difficult to compare. The parameter RGI provides a way to stop an experiment at a defined growth stage, i.e., at a time when the control group has reached a particular (or final) stage.

In bioassays of juvenile hormone (JH) and its mimics, quantitative comparison of the developmental status of a test group with that of the control group has been used to assess the activity of JH mimics on insects (Redfern, 1985). However, this method presents difficulties if groups being compared have different numbers of insects. Lidert et al. (1987) used a scoring system based on the larval and pupal developmental status to measure the growth inhibitory effect of different natural products. However, relatively large variations may occur in the results obtained by this method because the variation among individuals in a population responding to the chemical may result in a large spread of scores in a tested group. Application of RGI minimizes the influence of individual variation and allows direct comparisons between species by unifying the calculations used to quantify insect growth. RGI also has advantages over other parameters in the measurement of inhibitory effects on insect development. A discussion of the advantages of RGI and GI follows.

First, by using RGI, the procedure to assay the growth inhibiting effect of allelochemicals can be simplified. In studies of insect growth regulators, it is often found that individuals in the tested group are in different instars at the time of data collection and some larvae die in different instars. Therefore, it is sometimes difficult to analyze the bioassay data using parameters such as mortality or relative growth rate. The above problem can be solved using RGI to measure the growth-inhibiting effects.

In calculating GI, the dead individuals are also considered. In this bioassay

method, the instar when death occurred, and not the specific day within an instar, is required for determination of GI. For example if a larva died during the third instar, it was alive for the entire second instar, therefore, live and dead larvae in the same instar are categorized into different stages for the calculation of GI (see equation 2).

Second, RGI provides information about the age distribution of the individuals in a test group. A smaller RGI indicates that more individuals remained or died in the early instars. Zero RGI means no development (or all died in the first stage) and RGI equal to 1 indicates the full development of individuals in the test group (or no difference from the control group).

Third, RGI provides a quantitative analysis in a way that allows direct comparison of an inhibition factor on the development of different insects species, since RGI is a relative index and independent of varying duration or number of developmental stages for different insect species (see equations 1 and 3).

Fourth, because RGI takes into account not only the number of individuals but also the stages, the sensitivity of the bioassay is increased. This bioassay per se cannot determine the mode of action; however, different types of compounds, independent of modes of action, usually disturb the normal growth processes, and this disturbance can be detected by measuring the changes in RGI. Therefore, application of RGI is particularly useful in bioassays of the crude extracts, which show very weak activities (due to low concentration of active ingredients or to inherently weak activity), as well as in search of active compounds with unknown modes of actions.

The use of RGI is not limited to the analysis of the effects of allelochemicals on insect development. It may be useful in studies of host plant–herbivore interactions, such as host suitability and host resistance. With the same method of calculation, RGI could also be used for groups other than insects, provided that their development could be characterized into consistent stages. Together with the use of other parameters such as LD_{50}, RGI will increase the power of data analysis in bioassays.

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REFERENCES

BERENBAUM, M. 1986. Postingestive effects of phytochemicals on insects: On Paracelsus and plant products, pp. 121–153, *in* J.R. Miller and T.A. Miller (eds.). Insect–Plant Interactions. Plenum Press, New York.

CHAN, B.G., 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.

KUBO, I., MURAI, Y., and CHAUDHURI, S.K. 1992. Structure of Chaparramarin. *Phytochemistry* 31:3262–3264.

LEWIS, A.C., and VAN EMDEN, H.F. 1986. Assays for insect feeding, pp. 95–119, *in* J.R. Miller and T.A. Miller (eds.). Insect–Plant Interactions. Plenum Press, New York.

LIDERT, Z., WING, K., POLONSKY, J., IMAKURA, Y., OKANO, M., TANI, S., LIN, M.Y., KIYOKAWA, H. and LEE, K.H. 1987. Insect antifeedant and growth inhibitory activity of forty-six quassinoids on two species of agricultural pests. *J. Nat. Prod.* 50:442–448.

REDFERN, R.E. 1985. Insect bioassays, pp. 479–516, *in* N.B. Mandava (ed.). CRC Handbook of Natural Pesticides: Methods. Vol. 1, Theory, Practice, and Detection. CRC Press, Boca Raton, Florida.

SINDEN, S.L., SANFORD, L.L., CANTELO, W.W., and DEAHL, K.L. 1988. Bioassays of segregating plants, a strategy for studying chemical defenses. *J. Chem. Ecol.* 14:1941–1950.

SINGER, M.C. 1986. The definition and measurement of oviposition preference in plant-feeding insects, pp. 66–94, *in* J.R. Miller and T.A. Miller (eds.). Insect–Plant Interactions. Plenum Press, New York.

WALDBAUER, G.P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol.* 5:229–288.

WARDLAW, A.C. 1985. Practical Statistics for Experimental Biologists. John Wiley & Sons, Chichester, U.K.

WOLFSON, J.L. 1988. Bioassay techniques, an ecological perspective. *J. Chem. Ecol.* 14:1951–1963.

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EFFECT OF TANNIC ACID CONCENTRATION ON DEVELOPMENT OF THE WESTERN TREEHOLE MOSQUITO, *Aedes sierrensis* (Diptera: Culicidae)

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Abstract—Populations of *Aedes sierrensis* (Ludlow) completed development in tannic acid solutions over a wide concentration range (i.e., 0–1.0 g/liter) in artificial microcosms exposed to field conditions. The most notable effects of high tannic acid concentration were to slow larval developmental rates and to reduce numbers of adults produced; adult size and sex ratio were minimally affected. Vector potential of the western treehole mosquito is discussed in terms of tannin concentration.

Key Words—Treehole mosquito, tannin, *Aedes sierrensis*, Diptera, Culicidae, treehole, insect development, water chemistry.

INTRODUCTION

Aedes sierrensis (Ludlow) is the dominant treehole mosquito of the Pacific coastal range and the foothills of the Sierra Nevada (Bohart and Washino, 1978; Washburn and Anderson, 1986). Adults are aggressive biters and vectors of two filarial worms (Weinman et al., 1973; Walters and Lavoipierre, 1981). Larvae hatch when desiccation-resistant eggs are inundated with treehole water following fall or winter rains, and larvae browse on treehole detritus and filter-feed on microorganisms in the water. Intraspecific competition among larvae for food is often severe (Broadie and Bradshaw, 1991; Washburn et al., 1991) and prolonged development during winter months includes a fourth-instar diapause. Pupation and adult eclosion occur in spring and early summer. After mating and blood-feeding, females lay eggs on and above receding water surfaces as treeholes dry out during the summer.

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Tannins are water-soluble, polyphenolic compounds extractable from oak foliage and bark, major constituents of treehole litter throughout the range of *Ae. sierrensis*. Tannins are structurally diverse but functionally similar in their ability to bind proteins. Tannins may protect plants from vertebrate herbivores (Austin et al., 1989; Mole et al., 1990), invertebrate herbivores (Bernays, 1981; Berenbaum, 1983), and disease (Schlosser, 1980; Walkinshaw, 1989). They may also regulate decay processes and nutrient cycling for the benefit of plants (reviewed by Tiarks et al., 1989; see Haslam, 1988 for an alternative viewpoint). Insect herbivores exploit, tolerate, or succumb to tannin-rich diets, depending upon their adaptations to dietary tannins (Steinly and Berenbaum, 1985; Martin et al., 1987; Schultz, 1989).

Although they are inconsequential to their host trees, inhabitants of water-filled treeholes must survive in varying concentrations of soluble allelochemicals. For example, tannins in treehole waters may limit rates of litter decomposition, bind dissolved proteins, affect growth rates of microorganisms, or directly affect mosquitoes themselves.

Studies suggest that tannin concentration in the larval habitat affects mosquito production. Pospisil and Broche (1981) demonstrated that a laboratory population of *Culex quinquefasciatus*, a foul-water mosquito, did not survive exposure to 0.1 g/liter tannin; development was slowed at lesser tannin concentrations relative to the control. Mitchell and Rockett (1981) found five Great Lakes region mosquito species in treehole waters with tannin–lignin concentrations up to 0.45 g/liter; four of five species were common in treeholes ranging from 45 to 450 mg/liter tannin–lignin. Bradshaw and Holzapfel (1986, 1988) demonstrated high correlations between tannin–lignin concentration (plus eight other chemical and physical properties of treehole habitats) and the distribution of mosquito species in European and Florida treeholes but did not report concentrations. Mercer (1991, 1992) reported that solutions of commercial tannic acid (a hydrolyzable tannin) and solutions of tannin purified from loblolly pine foliage (a mixture of condensed tannins) influenced survivorship of *Ae. sierrensis* populations reared under non-diapause-inducing conditions.

Here I describe a field experiment designed to test the effects of tannic acid on *Ae. sierrensis* growth and survivorship.

METHODS AND MATERIALS

Field Experiment. I measured developmental rates, survivorship, and adult size of *Ae. sierrensis* mosquitoes reared in solutions of commercial tannic acid at five concentrations plus one control. Two of the concentrations exceeded the range of total phenolic concentrations I previously measured in California treeholes (i.e., 0.01–0.5 g/liter). Rainwater, collected as trunk-wash from six oak

trees during early autumn 1988, was filtered and used to prepare the concentration series: 0, 0.1, 0.25, 0.5, 1.0, and 2.0 g/liter tannic acid. Five replicates of each concentration were prepared; 300 ml of the appropriate tannic acid solution were poured into narrow-mouthed 0.5-liter plastic bottles (7 cm diameter) to simulate treeholes. One hundred newly hatched *Ae. sierrensis* larvae and 0.09 g ground rat chow were added to each microcosm, which was then covered with screening. The 30 bottles were arranged haphazardly in a large basin partially filled with water to standardize temperature fluctuations, and the basin was placed in an enclosure under an oak–madrone canopy at the Hopland Field Station (Mendocino County, California) on November 30, 1988.

Each week (except weeks 1, 9, and 26) after beginning the experiment, I emptied the contents of each replicate into an enamel pan and determined numbers and developmental stages of surviving mosquitoes. I noted but did not remove dead mosquitoes or fungal growths. Rat chow was added to each replicate when particulate matter appeared to be limiting in any replicate (0.09 g during weeks 2, 8, and 12; 0.15 g during week 14); I did not inspect the microcosms for microorganisms that serve as food for larvae. Following weekly scoring, the contents of each replicate were returned to the appropriate container and the bottles were arranged haphazardly in the basin and returned to the enclosure. No additional water was added to any microcosm during the course of the experiment.

Emergent adults were aspirated into 70% ethanol for number, gender, and size determinations. Partially eclosed mosquitoes were not included in adult counts or size determinations. Wing length was measured for each adult using a stereo dissecting microscope (30×) with a micrometer scale.

At the end of the experiment, the pH of experimental solutions, original stock solutions (kept refrigerated during the experiment), and fresh solutions of tannic acid prepared with trunk-wash rainwater were measured.

Oviposition Experiment. Gravid female *Ae. sierrensis* were allowed to choose between tannic acid solution or distilled water in a laboratory oviposition choice test. Ten to 15 inseminated and blood-fed females plus 20–25 males were kept in gallon-sized cardboard cylinders and provided with sugar and water. Three cylinders were placed in a ventilated, darkened enclosure. A plastic cup lined with a paper towel wick and containing 15 ml 2.0 g/liter tannic acid solution and an identical cup containing distilled water were placed in each cylinder. Cups with fresh solutions were exchanged and the number of eggs oviposited in each solution determined daily. At the end of the experiment, equal numbers of eggs from each treatment were immersed in 0.1% sodium sulfite solution to stimulate hatching; egg hatch success was determined by counting the number of larvae for each treatment.

Statistical Treatment. Significant differences in survivorship were determined with an epidemiological cohort life-table analysis (Dawson-Saunders and

Trapp, 1990). By using this analysis, I sacrificed variation due to replication and calculated variance instead as a cumulative conditional probability of survival for each treatment. Numbers of surviving mosquitoes were pooled for the five replicates of each treatment; 95% confidence intervals were calculated at intervals for each treatment throughout development.

Developmental rates, proportion of females, and mean male and female wing lengths were determined for each replicate and used to calculate treatment means for each tannic acid concentration. One-way ANOVA was used to determine differences among treatment means if normally distributed or among log-transformed means if treatment variances were unequal (i.e., female wing length) (Sokal and Rohlf, 1981). Multiple comparisons among pairs of means or log-transformed treatment means were done with the T-method (Sokal and Rohlf, 1981). A *t* test for paired comparisons was used to compare oviposition in tannic acid and distilled water (Sokal and Rohlf, 1981).

RESULTS

Field Experiment. Mosquitoes in high tannic acid concentration treatments experienced high initial mortality (Figure 1). All larvae exposed to 2.0 g/liter tannic acid died before the first assessment. Survivors among larval populations exposed to 0.5 and 1.0 g/liter tannic acid solutions were significantly fewer than populations exposed to more dilute solutions by week 2. The number of survivors in the 0.1 g/liter treatment was significantly higher than in the control by week 13 and remained higher for the remainder of the experiment. Survivorship was highest for larvae exposed to 0.25 g/liter tannic acid.

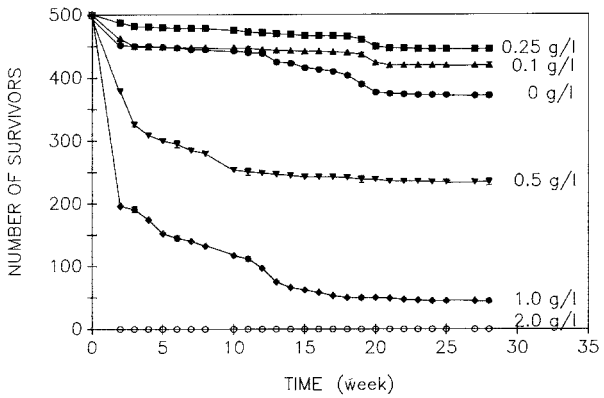

FIG. 1. Survival curves for *Aedes sierrensis* populations developing in six treatments of tannic acid under field conditions. Error bars indicate 95% confidence intervals; treatment error bars that overlap are not significantly different.

Adult eclosion began during week 18 and was completed by week 28 (June 1989). The number of adults produced and the cumulative conditional probability for each treatment are represented, respectively, by end points and error bars of the survival curves in Figure 1. Significantly more adults eclosed from two intermediate tannic acid treatments (0.25 and 0.1 g/liter) than the tannin-free control or the treatments of higher tannic acid concentrations (Figure 1).

Excess food accumulated in the 0.5 and 1.0 g/liter treatments, and fungal clumps appeared within two weeks in the 1.0 g/liter treatment. All populations were minimally affected by freezing of experimental solutions between weeks 8 and 10. Solution volume decreased less than 5% for any microcosm; solutions with tannic acid became increasingly dark through time.

Generally, larval populations developed more slowly as tannic acid concentration increased. Means of median developmental times (i.e., time required for half the surviving population of a replicate to reach a developmental stage) are listed in Table 1 for each tannic acid concentration. Mosquitoes developing in the 0 and 0.1 g/liter tannic acid treatments reached the second and third instars earlier than those in other treatments; likewise, larvae developing in 0.5 or 1.0 g/liter tannic acid reached the third and fourth larval instars significantly more slowly than in other treatments. However, with the exception of the 1.0 g/liter treatment, mosquitoes pupated and emerged as adults at the same time regardless of tannic acid concentration.

Tannic acid concentration had no effect upon the sex ratio of adults produced from experimental populations and had only limited influence upon adult size. Slightly more than half the 1518 emergent adults were female (overall females = 51.3%; range among treatment means = 46.5–59.9% females). The mean proportion of females did not vary significantly among tannic acid treatments ($F_{4,20} = 1.974$, $P = 0.137$). Mean male wing lengths differed significantly among tannic acid treatments ($F_{4,20} = 33.62$, $P < 0.001$), while mean

TABLE 1. MEDIAN DEVELOPMENTAL TIME (MEAN TIME IN WEEKS) FOR MOSQUITO POPULATIONS IN TANNIC ACID SOLUTIONS[a]

Concentration (g/liter)	Second instar	Third instar	Fourth instar	Pupa	Adult
0	2.0a	4.0d	10.4h	19.0k	20.0m
0.1	2.0a	4.8de	10.0h	19.0k	20.0m
0.25	3.0b	6.2e	11.0h	19.0k	20.0m
0.5	3.2b	9.2f	13.6i	19.0k	20.0m
1.0	4.6c	15.0g	18.6j	21.4l	23.0n

[a] Values followed by the same letters within a column are not significantly different (T-method of multiple comparisons; Sokal and Rohlf, 1981).

female wing lengths did not ($F_{4,20} = 1.23$, $P = 0.33$). The smallest males eclosed from the 1.0 g/liter treatment. The relationships between tannic acid concentration and mean male and female wing lengths are illustrated in Figure 2A and B.

The pH values of the experimental tannic acid solutions changed through time. At the end of the experiment, experimental solutions were less acidic than either the original stock solutions or freshly prepared tannic acid solutions. The exception was the experimental 2.0 g/liter treatment, which was removed from the field at week 2 and kept refrigerated until the end of the experiment.

Oviposition Experiment. In three tests, gravid *Ae. sierrensis* females oviposited similarly in 2.0 g/liter tannic acid solution (443 eggs) and distilled water (626 eggs) (*t* test: $t_2 = 0.983$, NS). Equal proportions of eggs hatched following collection from the two solutions: 78.9 and 78.5% for the tannic acid and distilled water treatments, respectively.

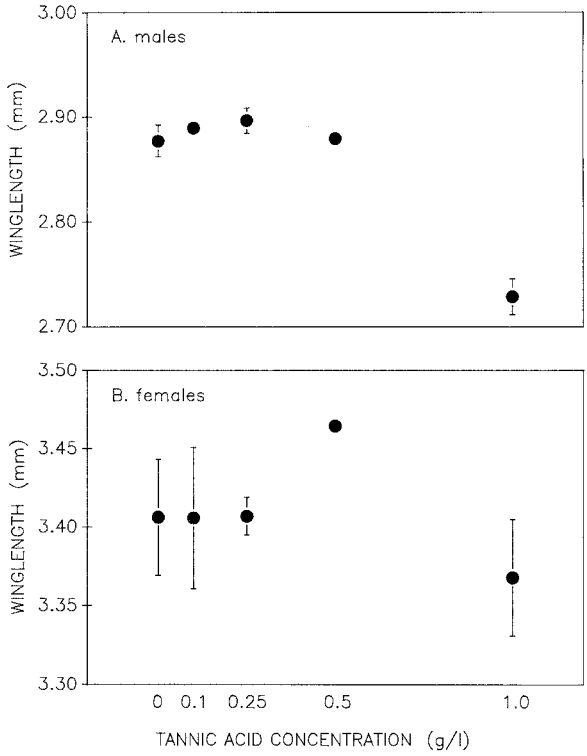

FIG. 2. Mean wing length (± 1 SEM, in mm, $N = 5$) of (A) male and (B) female *Aedes sierrensis* eclosing from solutions of tannic acid.

DISCUSSION

The contribution of each treehole habitat to the biting population of mosquitoes depends upon the number, size, and proportion of females among emergent adults. For many natural populations of mosquitoes, female size is correlated with fecundity and survival, and thereby the opportunity for disease transmission (Hawley, 1985a,b). In this experiment, tannic acid concentration affected numbers of adults produced but neither the proportion nor the size of female mosquitoes. Numbers of adults produced may have been influenced by rates of larval development and intraspecific competition.

Control populations gained no advantage by hastening through larval molts relative to the populations at intermediate concentrations. The end of fourth-instar larval diapause is triggered by a critical photoperiod coincident with warmer treehole water (Jordan and Bradshaw, 1978). Thus all populations that had developed sufficiently produced adults once these external conditions were met. Faster developmental rates in control populations simply meant more time spent in larval diapause.

Tannic acid did not appear to inhibit all decay-causing organisms; fungus accumulated on excess food in the 0.5 and 1.0 g/liter treatments. Food bound by tannins may have been of lower quality for larvae in these treatments. Tannin binding was indicated by the dark tan color of food in these treatments for which mosquito survivorship was low (0.5 and 1.0 g/liter).

Exposure to 1.0 g/liter tannic acid had a pronounced effect on mosquito development; pupation and adult eclosion were significantly later in this treatment than in lower tannin treatments. Slow development caused by tannins may be important in natural treeholes, which may dry before adults are produced. *Aedes sierrensis* are protandrous, and in this experiment males eclosed before females in all treatments (data not shown), although approximately equal numbers of males and females were produced. Delays in eclosion may be reflected in male bias for a given treehole that dries before female eclosion (Washburn et al., 1989). High tannin concentrations may contribute to such delays.

Some early larval mortality may have been due to acidity at high tannic acid concentration. However, most of the initial values fell within the range determined for 81 natural treeholes inhabited by *Ae. sierrensis* (pH range: 3.9–9.4; Washburn and Anderson, 1986). By the end of the experiment, experimental solutions had become less acidic; the greatest pH changes were for the 0.5 and 1.0 g/liter solutions, which had the highest mosquito mortality and the greatest accumulation of food residue and fungus.

Ovipositing *Ae. sierrensis* females did not discriminate against tannic acid solutions sufficiently concentrated (i.e., 2.0 g/liter) to cause complete mortality among their progeny. However, eggs laid in these solutions experienced no loss in viability (e.g., due to tannic acid binding with egg proteins). Gravid *Ae.*

sierrensis females may be unable to predict the quality of treehole habitats during the following developmental season (Hawley, 1985b); through bet-hedging, they may ensure the survival of their progeny by dispersing many, small eggs in different treeholes.

Treehole mosquitoes and other inhabitants must tolerate the variety of tannins in treehole waters and their varying concentrations during the season. Under winter conditions, *Ae. sierrensis* may benefit from intermediate tannin concentrations, thus maximizing adult production. Following infrequent summer rains when opportunistic cohorts of *Ae. sierrensis* develop in warmer, more productive treehole waters without a fourth-instar larval diapause, lower tannin concentrations likewise may allow optimal adult production. In either case, tannin concentration may be an important factor in the high variability in mosquito production from treehole breeding habitats.

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REFERENCES

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(4):1335–1347.

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.

BOHART, R.M., and WASHINO, R.K. 1978. Mosquitoes of California. Division of Agricultural Sciences, University of California, Berkeley. pp. 115–116.

BRADSHAW, W.E., and HOLZAPFEL, C.M. 1986. Habitat segregation among European tree-hole mosquitoes. *Nat. Geograph. Res.* 2(2):167–178.

BRADSHAW, W.E., and HOLZAPFEL, C.M. 1988. Drought and the organization of tree-hole mosquito communities. *Oecologia (Berlin)* 74(4):507–514.

BROADIE, K.S., and BRADSHAW, W.E. 1991. Mechanisms of interference competition in the western tree-hole mosquito, *Aedes sierrensis*. *Ecol. Entomol.* 16:145–154.

DAWSON-SAUNDERS, B., and TRAPP, R.G. 1990. Basic and Clinical Biostatistics. Appleton and Lange, Norwalk. pp. 186–206.

HASLAM, E. 1988. Plant polyphenols (*syn.* vegetable tannins) and chemical defense-a reappraisal. *J. Chem. Ecol.* 14(10):1789–1805.

HAWLEY, W.A. 1985a. A high fecundity aedine: Factors affecting egg production of the western treehole mosquito, *Aedes sierrensis* (Diptera: Culicidae). *J. Med. Entomol.* 22:220–225.

HAWLEY, W.A. 1985b. The effect of larval density on adult longevity of a mosquito, *Aedes sierrensis*: Epidemiological consequences. *J. Anim. Ecol.* 54:955–964.

JORDAN, R.G., and BRADSHAW, W.E. 1978. Geographic variation in the photoperiodic response of the western tree-hole mosquito, *Aedes sierrensis*. *Ann. Entomol. Soc. Am.* 71(4):487–490.

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(3):605–621.

MERCER, D.R. 1991. Tannic acid concentration mediates *Aedes sierrensis* (Diptera: Culicidae) development and parasitism by *Lambornella clarki* (Ciliophora: Tetrahymenidae). *Proc. Calif. Mosquito Vector Control Assoc.* 59:101–107.

MERCER, D.R. 1992. The chemical ecology of *Aedes sierrensis*, the western treehole mosquito. PhD dissertation. University of California, Berkeley. 233 pp.

MITCHELL, L., and ROCKETT, C.L. 1981. An investigation of the larval habitat of five species of tree-hole breeding mosquitoes (Diptera: Culicidae). *Great Lakes Entomol.* 14(2): 123–129.

MOLE, S., BUTLER, L.G., and IASON, G. 1990. Defense against dietary tannin in herbivores: A survey for proline rich salivary proteins in mammals. *Biochem. Syst. Ecol.* 18(4):287–293.

POSPISIL, J., and BROCHE, R.G. 1981. Efectos del tanino sobre las larvas del mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae). Informe Cientifico-Tecnico No. 159. Instituto de Zoologia de la Academia de Ciencias de Cuba, La Habana. 8 pp.

SCHLOSSER, E.W. 1980. Preformed internal chemical defenses, pp. 161–177, *in* J.G. Horsfall and E.B. Cowling (eds.). Plant Disease, Vol. V. Academic Press, New York.

SCHULTZ, J.C. 1989. Tannin-insect interactions, pp. 417–433, *in* R.W. Hemingway and J. Karchesy (eds.). Chemistry and Significance of Condensed Tannins. Plenum Press, New York.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry. W.H. Freeman, New York. 859 pp.

STEINLY, B.A., and BERENBAUM, M. 1985. Histopathological effects of tannins on the midgut epithelium of *Papilio polyxenes* and *Papilio glaucus*. *Entomol. Exp. Appl.* 39:3–9.

TIARKS, A.E., BRIDGES, J.R., HEMINGWAY, R.W., and SHOULDERS, E. 1989. Condensed tannins in southern pines and their interactions with the ecosystem, pp. 369–390, *in* R.W. Hemingway and J. Karchesy (eds.). Chemistry and Significance of Condensed Tannins. Plenum Press, New York.

WALKINSHAW, C.H. 1989. Are tannins resistance factors against rust fungi? pp. 435–446, *in* R.W. Hemingway and J. Karchesy (eds.). Chemistry and Significance of Condensed Tannins. Plenum Press, New York.

WALTERS, L.L., and LAVOIPIERRE, M.M.J. 1981. Vector potential of six mosquito species in landscapes of high heartworm endemicity in northern California. *Proc. Calif. Mosquito Vector Control Assoc.* 49:9.

WASHBURN, J.O., and ANDERSON, J.R. 1986. Distribution of *Lambornella clarki* (Ciliophora: Tetrahymenidae) and other mosquito parasites in California treeholes. *J. Invert. Pathol.* 48:296–309.

WASHBURN, J.O., ANDERSON, J.R., and MERCER, D.R. 1989. Emergence characteristics of *Aedes sierrensis* (Diptera: Culicidae) from California treeholes with particular reference to parasite loads. *J. Med. Entomol.* 26(3):173–174.

WASHBURN, J.O., MERCER, D.R., and ANDERSON, J.R. 1991. Regulatory role of parasites: Impact of host population shifts with resource availability. *Science* 253:185–188.

WEINMAN, C.J., ANDERSON, J.R., LONGHURST, W.M., and CONNOLLY, G. 1973. Filarial worms of Columbian black-tailed deer in California. 1. Observations in the vertebrate host. *J. Wildl. Dis.* 9:213–220.

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ANTIFUNGAL ACTIVITY OF VOLATILE FRACTIONS OF ESSENTIAL OILS FROM FOUR AROMATIC WILD PLANTS IN ISRAEL[1]

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Abstract—Essential oils were extracted from *Majorana syriaca*, *Satureja thymbra*, *Micromeria fruticosa*, and *Salvia triloba*, and their volatile fractions were tested for their antifungal activity against the soil-borne pathogens *Fusarium oxysporum* and *Macrophomina phaseolina* and the foliar pathogens *Botrytis cinerea* and *Exserohilum turcicum*. Results showed a fungistatic effect of 1, 2.5, and 5 μl of the various essential oils on fungal mycelium growth. The most significant effect was exerted by essential oils extracted from *M. syriaca*, which inhibited the growth of *B. cinerea* by 44% and of all the other fungi tested by 100%.

Key Words—Essential oils, antifungal activity, *Majorana syriaca*, *Satureja thymbra*, *Micromeria fruticosa*, *Salvia triloba*.

INTRODUCTION

Essential oils are used in medicinal drugs and for controlling harmful insects (Mansour et al., 1986). Numerous plant extracts are known to possess substances that may be fungicidal or fungistatic in nature, and several investigations have been directed towards the antimicrobial activity of essential oils (Deans and Ritchie, 1987; Jay and Rivers, 1984; Paster et al., 1990). The antimicrobial

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and antifungal activity of 40 essential oils and 32 standard constituents of essential oils were tested by Ross et al. (1980), but their exploration as bactericidal and fungicidal agents received little attention. In a previous study, Reuveni et al. (1984) have reported that volatile fractions of essential oils from *Ocimum basilicum* chemotypes and their components have different inhibition effects against *Fusarium oxysporum* f. sp. *vasinfectum* and *Rhizopus nigricans*. The possible use of volatile fractions of essential oils from several plants as antifungal agents was further tested in this work, with the ultimate aim of using plant essential oils to inhibit growth of soil-borne or foliar plant pathogens.

METHODS AND MATERIALS

Essential oils were hydrodistilled as described by Ravid and Putievsky (1985).

Two soil-borne phytopathogenic fungi, *Fusarium oxysporum* f. sp. *vasinfectum* and *Macrophomina phaseolina*, were isolated from cotton and melon plants, respectively. In addition, two local isolates of the foliar plant pathogens, *Exserohilum turcicum* and *Botrytis cinerea*, were isolated from maize and cucumbers plants, respectively, and grown on potato dextrose agar (PDA; oxoid) at 25°C. Agar squares (0.2 × 0.2 cm) covered with mycelium of the tested fungi were placed in the middle of 9-cm-diam. Petri dishes, containing PDA, and used for the antifungal assays.

The method of application of the volatile fractions, which aimed to prevent a direct contact between the fungal mycelium and the tested oil, has been described by Reuveni et al. (1984). The tested oils (0, 1, 2.5, and 5 μl) were applied by microsyringe on filter paper disks. The disks were previously placed on cover glasses of microscope slides, which were placed 40 mm from the center of the plate in order to prevent direct contact with the substrate. Immediately after oil application, the plates were enfolded in masking tape in order to prevent oil losses by volatilization, and incubated at 25 $\pm$ 1°C in the dark. The diameters of the various fungal colonies of each of five plates for each oil treatment were recorded after 3, 5, 7, 10, and 13 days.

RESULTS AND DISCUSSION

Essential oils from four aromatic wild plants growing in Israel—*Majorana syriaca*, *Satureja thymbra*, *Micromeria fruticosa*, and *Salvia triloba*—were extracted (Ravid and Putievsky, 1985, 1986), and their antifungal activity against phytopathogenic fungi was tested.

Crude extracts of essential oils from *M. syriaca* showed remarkable antifungal activity. An amount of 2.5 μl of extract applied in one Petri dish totally

inhibited mycelium growth of *E. turcicum* (Figure 1, right) and *F. oxysporum* (Figure 2, right). A higher dose of this essential oil (5 μl) was needed to inhibit completely the growth of *M. phaseolina* (Figure 2, left) and to cause a 44% reduction in *B. cinerea* growth (Figure 1, left). The essential oils of *S. thymbra*

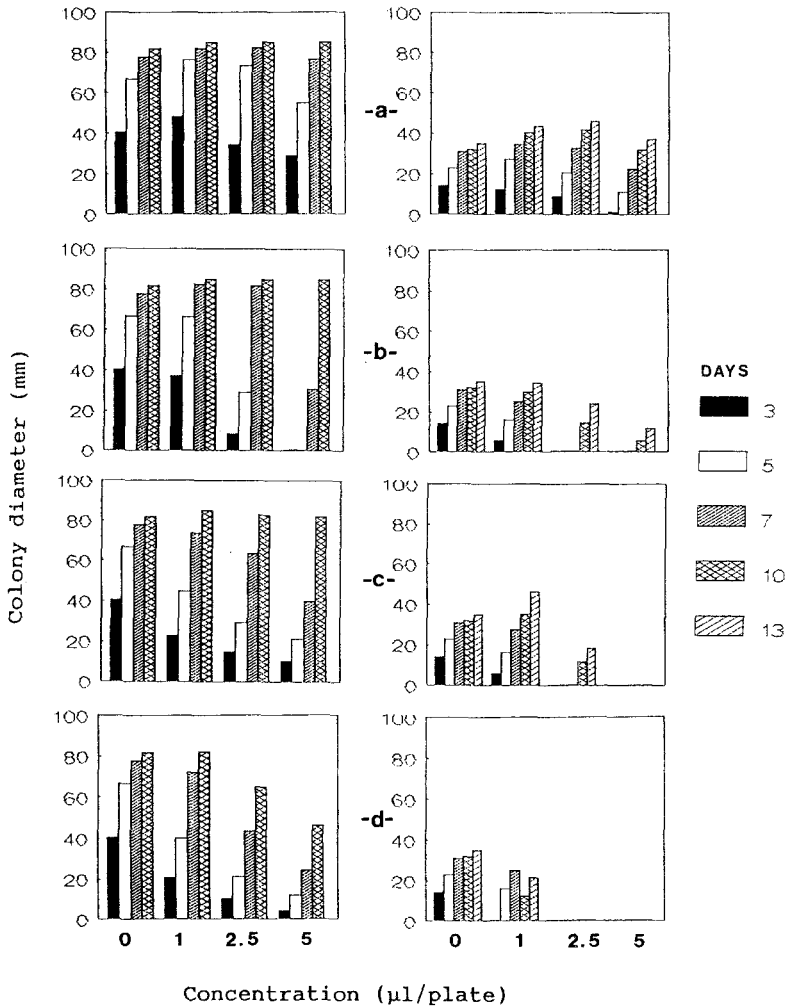

FIG. 1. Antifungal activity of essential oils from four aromatic wild plants: *Salvia triloba* (a), *Micromeria fruticosa* (b), *Satureja thymbra* (c), and *Majorana syriaca* (d), on two foliar pathogens. Left: *Botrytis cinerea*; right: *Exserohilum turcicum*. The effect of 0, 1, 2.5, and 5 μl of the essential oils per plate was monitored 3, 5, 7, 10, and 13 days after inoculation.

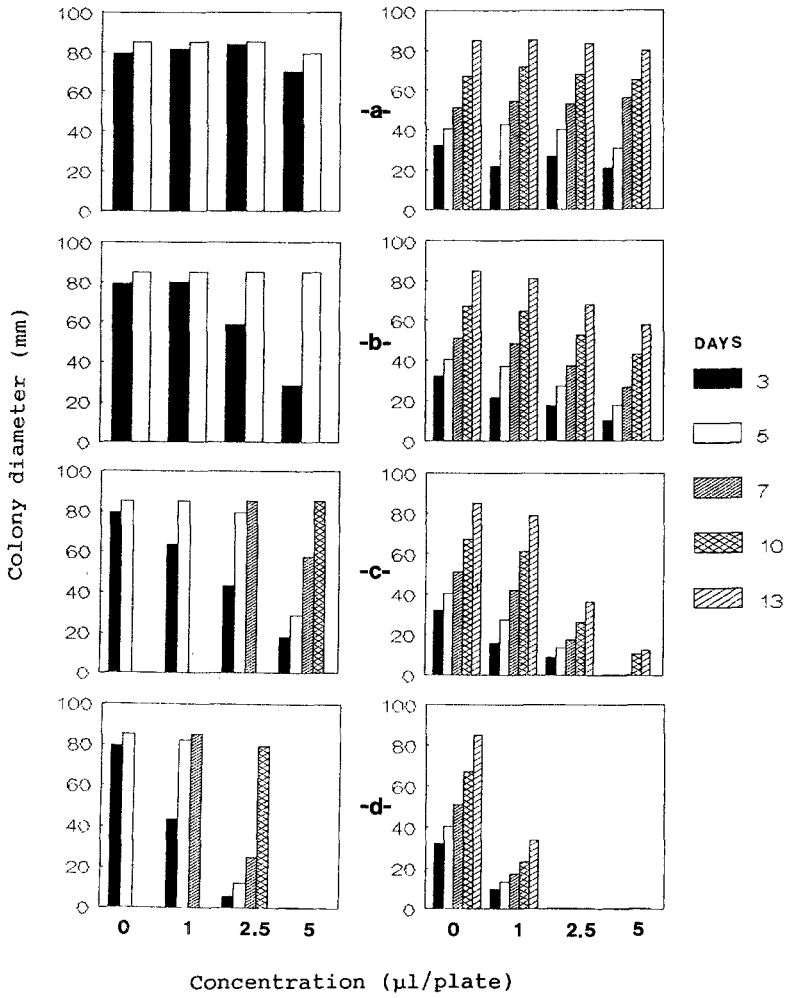

FIG. 2. Antifungal activity of essential oils from four aromatic wild plants: *Salvia triloba* (a), *Micromeria fruticosa* (b), *Satureja thymbra* (c), and *Majorana syriaca* (d), on two oil-borne pathogens. Left: *Macrophomina phaseolina*; right: *Fusarium oxysporum*. The effect of 0, 1, 2.5, and 5 µl of the essential oils per plate was monitored 3, 5, 7, 10, and 13 days after inoculation.

brought about complete inhibition of *E. turcicum* (Figure 1, right) and reduced growth of *F. oxysporum* (85%, Figure 2, right) only at the highest dosage (5 µl). Essential oils of *M. fruticosa* were less effective: at 5 µl reducing the growth of *E. turcicum* and *F. oxysporum* by only 66% and 30%, respectively.

The crude extract had no effect on the mycelial growth of *B. cinerea* or *M. phaseolina*. The essential oils extracted from *S. triloba* had no effect on mycelial growth of the tested fungi. Our results show a remarkable antifungal activity of essential oils extracted from *M. syriaca* and suggest that these oils may serve as antifungal agents for use against *F. oxysporum* and *E. turcicum*. It is likely that some of the "natural" essential oils, such those described in this report, are as toxic as some synthetic pesticides. Therefore, further work is needed to investigate the practical implications and nontoxic dosage of this natural source of antifungal agents in order to reduce the use of synthetic chemicals.

REFERENCES

DEANS, S.G., and RITCHIE, G. 1987. Antibacterial properties of plant essential oils. *Int. J. Food Microbiol.* 5:165–180.

JAY, J.M., and RIVERS, G.M. 1984. Antimicrobial activity of some food flavoring compounds. *J. Food Saf.* 6:129–139.

MANSOUR, F., RAVID, U., and PUTIEVSKY, E. 1986. Studies of the effects of essential oils isolated from 14 species of Labiatae on the carmine spider mite, *Tetranychus cinnabarinus*. *Phytoparasitica* 14:137–142.

PASTER, N., JUVEN, B.J., SHAAYA, E., MENASHEROV, M., NITZAN, R., WEISSLOWICZ, H., and RAVID, U. 1990. Inhibitory effect of oregano and thyme essential oils on moulds and foodborne bacteria. *Lett. Appl. Microbiol.* 11:33–37.

RAVID, U., and PUTIEVSKY, E. 1985. Essential oils of Israeli wild species of Labiatae, pp. 155–161, *in* B. Svendsen and J.J.C. Scheffer (eds.). Essential Oils and Aromatic Plants. Martinus Nijhoff, Dordrecht, The Netherlands.

RAVID, U., and PUTIEVSKY, E. 1986. Carvacrol and thymol chemotypes of East Mediterranean wild Labiatae herbs, pp. 163–167, *in* E.J. Brunke (ed.). Progress in Essential Oil Research. Walter de Gruyter, Berlin.

REUVENI, R., FLEISCHER, A., and PUTIEVSKY, E. 1984. Fungistatic activity of essential oils from *Ocimum basilicum* chemotypes. *Phytopathol. Z.* 110:20–22.

ROSS, A., EL-KELTAWI, N.E., and MEGALLA, S.E. 1980. Antimicrobial activity of some Egyptian aromatic plants. *Fitoterapia* 4:201–206.

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A NEW CUCURBITACIN PROFILE FOR *Cucurbita andreana*: A CANDIDATE FOR CUCURBITACIN TISSUE CULTURE

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Abstract—In addition to previously reported cucurbitacins B, **1,** and D, **2,** cucurbitacin E, **3,** and I, **4,** aglycones and their glucosides 2-*O*-β-glucopyranosyl-cucurbitacin E, **5,** and 2-*O*-β-glucopyranosyl-cucurbitacin I, **6,** were isolated and identified as constituents of *Cucurbita andreana* on the basis of MS, FD-MS, ^{1}H NMR and ^{13}C NMR spectroscopy. Also, 2-*O*-β-glucopyranosyl-cucurbitacin B, **7,** cucurbitacin B glucoside was isolated and identified.

Key Words—*Cucurbita andreana*, cucurbitacins E and I, glucosides, phagostimulant, tissue culture.

INTRODUCTION

Cucurbitacins are highly oxygenated, tetracyclic triterpenes produced in the fruits, roots, and, to a lesser extent, foliage of plants principally in the family Cucurbitaceae. They are notable not only for their extreme bitterness, mammalian toxicity, effects as insect antifeedants, and their purgative, emetic, cytotoxic, antifungal, and antineoplastic properties, but also because of their remarkable effects on chrysomelid beetles in the subtribes Aulacophorina (Old World) and Diabroticina (New World), collectively called diabroticites (Guha and Sen, 1975; Whithouse and Doskotch, 1979; Witkowski and Kanopa, 1981; Sasamori et al., 1983; Fang et al., 1984; Witkowski et al., 1984; Halaweish, 1987; Metcalf and Lampman, 1989; Bar-Nun and Mayer, 1990).

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Cucurbitacins are phagostimulants for diabroticites, which can detect these compounds in plant tissues and on inert substances such as silica gel or filter paper at quantities as low as 0.1 ng (Chambliss and Jones, 1966; Sharma and Hall, 1971; Metcalf, 1985; Tallamy and Halaweish, 1993). This phenomenon has recently been exploited in the development of toxic semiochemical baits (starch granules laced with insecticide and cucurbitacins) to monitor and/or control several pest species of diabroticites (Metcalf et al., 1987; Lance, 1988; Weissling et al., 1989; Lance and Sutter, 1990). Since a number of diabroticites are serious pests of corn, the impact of these beetles on agriculture worldwide is enormous. In the early 1980s, species such as the southern, western, and northern corn rootworms caused well over 1 billion dollars a year in yield loss and insecticide costs in the United States alone (Metcalf, 1986).

The use of cucurbitacins as semiochemical baits for diabroticite control has been so successful in small trials that wide-scale deployment of this methodology is imminent. A principle constraint hindering large-scale production of baits is the lack of economically attractive cucurbitacin sources. To date, all attempts to synthesize these complex molecules have failed, leaving costly and time-consuming extraction from cucurbit tissues as the only proven means of obtaining pure products. Deriving cucurbitacins on a commercial scale from cucurbit gourds has been frustrated by the tough and fibrous tissues of the gourds. Currently, cucurbitacins for diabroticite control products are acquired from pulverized roots of the perennial gourd, *Cucurbita foetidissima*. Unfortunately, the cucurbitacin content of young *C. foetidissima* is often low and variable, and older, more bitter plants take years to develop. Furthermore, acquiring cucurbitacins from field-grown plants is an expensive undertaking requiring compensation for acreage, labor, fertilizer, pesticides, harvesting, and preparative procedures.

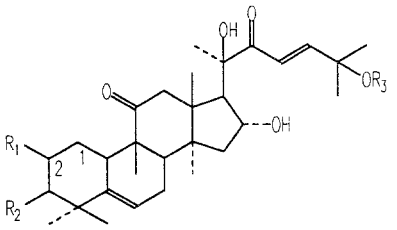
Recent advances in cucurbit tissue culture may provide a viable alternative for achieving the mass production of cucurbitacins (Halaweish, 1987). A critical step toward maximizing cucurbitacin tissue culture productivity is to accurately define the cucurbitacin profile of candidate species under mild extraction conditions. One such species is *C. andreana* Naud, a mesophytic annual from South America that displays rapid growth, prolific fruiting, and extreme bitterness in its fruits and roots. Past quantitative estimates using UV spectrophotometry after TLC separation have yielded large amounts of the aglycones of cucurbitacins B and D in *C. andreana* (Metcalf et al., 1980, 1982), but other aglycones and glycosides have escaped detection with this approach. Here we present new evidence that, in addition to B and D aglycones, *C. andreana* produces substantial quantities of E and I aglycones as well as glucosides of cucurbitacins B, E, and I in its fruits.

METHODS AND MATERIALS

Plant Material. Cucurbita andreana (Cucurbitaceae) was grown to maturity in 25-cm pots in a greenhouse (Agriculture Experiment Station, University of Delaware, Newark, Delaware). Fruits were collected when fully formed and immediately placed in a freezer where they were held until extraction.

Extraction and Fractionation. The fruits (1 kg) were cut into small pieces, homogenized in water (500 ml) for half an hour, filtered, and the filtrate was partitioned with hexane (5 × 300 ml), then chloroform (5 × 300 ml). The chloroform extract was filtered, concentrated, and evaporated to dryness under nitrogen (2.2 g). The residue produced was absorbed onto the top of a silica gel column 60 A A (80 g, 70–230 mesh ASTM) and chromatographed using hexane–ethyl acetate (100, 8:2, 6:4, 4:6, 2:8, 1:8, 600 ml each), ethyl acetate–methanol (9:1, 8:2, 1:1, 600 ml each). Fractions of 100 ml were collected, screened with TLC, and grouped by similarity. Cucurbitacins B (128 mg) and E (28 mg) were isolated from fractions 19–24 and cucurbitacins D (160 mg) and I (34 mg) from fractions 28–40 after further purification through HPLC (described below). Highly polar fractions 49–55 were purified on preparative TLC (MeOH/$CHCl_3$, 1:9), and yielded three glycosides, compounds **5, 6,** and **7** (R_f 0.38, 0.33, and 0.27, respectively) (Scheme 1). These were further purified through HPLC.

TLC, Visualization, and HPLC. Silica gel GF_{254} plates (20 × 10 mm,



	R1	R2	R3	$\Delta^{1,2}$
1	OH	=O	COCH3	–
2	OH	=O	H	–
3	OH	=O	COCH3	+
4	OH	=O	H	+
5	glucose	=O	COCH3	+
6	glucose	=O	H	+
7	glucose	=O	COCH3	–

SCHEME 1.

1-mm thick) were used and developed with toluene–ethyl acetate (25:75) and examined under UV. All compounds were further purified by preparative HPLC. Visualization was achieved using vanillin–phosphoric acid reagent, triphenyltetrazolium chloride reagent (TTC), and ferric chloride reagent.

HPLC separation was performed with a Perkin Elmer S-100 with the UV detector at 229 and 254 nm. The preparative column was Spherisorb-ODS-2 5 μ (20 cm × 10 mm ID) and the gradient separation was achieved using solvent A [acetonitrile–water (ACN/H_2O), 2:8] and solvent B (ACN/H_2O, 45:50) with B 0% to 100% in 35 min and a flow rate of 2 ml/min. UV analysis was performed using a Hewlett Packard 8452A diode array spectrophotometer. IR spectra were analyzed by Perkin Elmer FT-IR spectrometer 1720X. NMR spectra were examined on a Brucker AM 250-MHz and MS spectra on a VG 7070F, GI 70 eV.

Enzyme Hydrolysis. Glycosides **5, 6,** and **7** were hydrolyzed with α-glucosidase (maltase, EC 3.2.1.20, Sigma). Two milligrams of enzyme were added to 1 mg of glycoside and 1 ml of sodium phosphate buffer (pH 6.8), vortexed for 1 min, and the reaction mixture was kept in an incubator for 24 hr at 37°C. The reaction was stopped by warming the reaction mixture in a water bath of 60°C for 5 min. The mixture was then centrifuged. β-Glucosidase was also used (EC 3.2.1.21, Sigma), combining 3 mg of enzyme with 1 mg glycoside and 1 ml of sodium acetate buffer (pH 5.0). It was then treated as was α-glucoside. HPTLC examination of the hydrolysate (solvent I) showed glycoside **5, 6,** and **7** aglycones to be cucurbitacins E, I, and B. Glucose was identified as the sugar part of the three glycosides (Whatman No. 1, BuOH/EtOH/H_2O, 5:1:4). The chromatogram was visualized with aniline phthalate spray reagent and heated at 100°C for 10 min.

Compound **5** (2-*O*-β-D-glucopyranosyl-cucurbitacin E) (26 mg) was obtained as small white crystals (MeOH/$CHCl_3$, 1:1), mp 156–158 [lit. (Hatam et al., 1989) 157–159], IR (KBr) cm^{-1}: 3440, 1720, 1685, 1631; FAB-MS *m/z* (rel. int.), 741 $[M(C_{38}H_{54}O_{13}) + Na]^+$ (4), 497 (aglycone–acetate + H) (6), 479 (aglycone–acetate–H_2O + H) (8), 111 (100).

Compound **6** (2-*O*-β-D-glucopyranosyl-cucurbitacin I) (22 mg) was a white amorphous powder (MeOH), mp 240–243 [lit. (Hatam et al., 1989) 239–240], IR cm^{-1}, 3440, 1685, 1630; FAB-MS *m/z* (rel. int.): 699 $(C_{36}H_{52}O_{12}) + Na]^+$ (4), 497 (aglycone–H_2O + H) (8), 401, 111 (100).

Compound **7** (2-*O*-β-D-glucopyranosyl-cucurbitacin B) (8 mg) was also a white amorphous powder (MeOH) and was identified mainly by enzyme hydrolysis and comparisons of the cucurbitacin aglycone and glucose with standards of cucurbitacin B and glucose, respectively.

RESULTS AND DISCUSSION

In this work the chloroform extract of *C. andreana* was subjected to chromatographic fractionation. In addition to the previously reported cucurbitacins B, **1**, and D, **2** (Metcalf et al., 1982), two other compounds showed spectra

typical of cucurbitacins E, **3**, and I, **4**. This was confirmed by the blue color reaction with $FeCl_3$ (the reagent for diosphenol cucurbitacins) and by comparison with available standards by TLC, HPLC, and cochromatrography. After purification with preparative HPLC, high polar fractions from the column chromatography revealed two major glycosides that were designated compounds **5** and **6** and one minor glycoside, compound **7**.

The ^1H NMR spectrum of compound **5** clearly displayed the presence of a basic cucurbitacin skeleton, including an eight-methyl singlet in the range of 0.90–1.40, a broadened H-6 doublet at δ 5.82, a typical H-16 triplet at δ 4.3, and the characteristic H-12 geminal coupling of the two doublets at δ 2.65 (12 β) and 3.25 (12 α) [J = 15 Hz]. The shift of the AB system also was shown at δ 7.12 and 6.65 of the $\Delta^{23,24}$ in the side chain (Hatam et al., 1989). A doublet at δ 4.98 corresponded to H-2. The upfield shift of H-2 is attributed to the glycosidic linkage at this position as well as the carbonyl in position C-3 (Stupper et al., 1990). An acetate shift at δ 2.02 was assigned at C-25 hydroxyl by comparison (Velde and Lavie, 1983). The glycosidic nature of compound **5** was revealed by: (1) the doublet at δ 4.40 [J = 7.8 Hz] assigned to the β-anomeric proton of the sugar residue and (2) hydrolysis with β-glucosidase enzyme and resistance to hydrolysis with α-glucosidase. ^{13}C NMR showed three downfield shifts at 198.37, 202.64, and 214.48, characteristic of carbonyl shifts at C-3, C-22, and C-11, respectively. Three olefinic centers were also clear and showed the shifts of carbons C-1, C-2, C-5,6, and C-23,24. Assignments of the other carbon shifts as well as the sugar carbons were obtained by comparison with published data (Velde and Lavie, 1983; Jacobs et al., 1990; Hatam et al., 1989). Based on these data, we concluded that compound **5** was cucurbitacin E-2-*O*-β-glucoside. We confirmed our conclusion by hydrolyzing the compound with β-glucosidase and chromatographically comparing the resulting aglycone and sugar with cucurbitacin E and glucose, respectively. Finally, FAB-MS showed a positive ion at 741 $(M + Na)^+$ corresponding to the molecular formula $[(C_{38}H_{54}O_{13}) + Na]^+$ (Hatam et al., 1989).

Compound **6** was also identified as a cucurbitacin glucoside from the ^1H NMR and ^{13}C NMR features mentioned under compound **5**. An acetate shift was not detected in this compound, indicating that it was cucurbitacin I, 2-*O*-β-glucoside. This was confirmed by enzyme hydrolysis and chromatographic identification of the resulting aglycone and its sugar with cucurbitacin I and glucose, respectively. FAB-MS showed a positive ion at m/z 699 $(M + Na)^+$ similar to the mass reported in the literature (Hatam et al., 1983).

Compound **7** was identified as cucurbitacin B-2-*O*-β-glucoside only by enzyme hydrolysis and comparison of the aglycone and the sugar with cucurbitacin B and glucose.

Although others have reported substantial quantities of cucurbitacin B and D in *C. andreana* fruits (Metcalf et al., 1982), ours represents the first isolation of cucurbitacin E and I aglycones and cucurbitacin B, E, and I glucosides from

this species. There are several possible reasons for this discrepancy. Factors such as fruit age, the length of time between harvesting fruits and either freezing or extracting them, and separation techniques all affect results of efforts to quantify cucurbitacins. The fruits examined in our study were relatively young, not yet having produced mature seed. They were frozen within minutes of being separated from the living plant, and they were extracted using mild procedures.

Our results confirm that *C. andreana* is a rich source of cucurbitacins and, as such, is an attractive candidate for tissue culture development. The profile of extractable terpenoids is remarkably simple and consists mainly of cucurbitacins (75% of the total extractable materials). This has applied significance for two reasons. First, cucurbitacin extraction and purification procedures are facilitated under such conditions. Perhaps more important in the context of tissue culture is that interference from structurally related triterpenes is minimal in *C. andreana* compared with that encountered in some other highly bitter *Cucurbita* species such as *C. foetidissima*, the root of which contains a variety of complex triterpenes (Dubois et al., 1988). Furthermore, all of the cucurbitacins detected in *C. andreana* except B glucoside (which has not as yet been tested) have been shown to trigger compulsive feeding in diabroticites (Metcalf et al., 1980; Tallamy, unpublished data). All of these factors elevate *C. andreana* on the list of candidate species for tissue culture screening.

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REFERENCES

BAR-NUN, N., and MAYER, A.M. 1990. Cucurbitacins-repressors of induction of laccase formation. *Phytochemistry* 28:1369–1371.

CHAMBLISS, O.L., and JONES, C.M. 1966. Cucurbitacins: Specific insect attractants in Cucurbitaceae. *Science* 153:1392–1393.

DUBOIS, M., BAUER, R., CAGIOTTI, M.R., and WAGNER, H. 1988. Foetidissimoside A, a new 3,38-didesmosidic triterpenoid saponin, and cucurbitacins from *Cucurbita foetidissima*. *Phytochemistry* 27:881–885.

FANG, X., PHOEBE, C.H., PEZZUTO, J.M., FONG, H.H.S., and FARNSWORTH, N.R. 1984. Plant anticancer agents, XXXIV. Cucurbitacins from *Elaeocarpus dolichostylus*. *J. Nat. Prod.* 47:988–993.

GUHA, J., and SEN, S.P. 1975. The cucurbitacins—a review. *Plant Biochem. J.* 2:12–28.

HALAWEISH, F.T. 1987. Cucurbitacins in tissue cultures of *Bryonia dioica* Jacq. PhD Thesis. University of Wales, Cardiff, U.K.

HATAM, N.A.R., WHITTING, D.A., and YOUSIF, N.J. 1989. Cucurbitacin glycosides from *Citrullus colocynthis*. *Phytochemistry* 28:1268–1271.

JACOBS, H., SINGH, T., REYNOLDS, W., and MCLEAN, S. 1990. Isolation and C-NMR assignments

of Cucurbitacins from *Cayaponia angustiloba*, *C. racemosa*, and *Gurania subumbellata*. *J. Nat. Prod.* 53:1600–1605.

LANCE, D.R. 1988. Potential of 8-methyl-2-decyl popanoate and plant-derived volatiles for attracting corn rootworm beetles (Coleoptera: Chrysomelidae) to toxic baits, *J. Econ. Entomol.* 81:1359–1362.

LANCE, D.R., and SUTTER, G.R. 1990. Field-cage and laboratory evaluations of semiochemical-based baits for managing western corn rootworm (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 83:1085–1090.

LAVIE, D., and GLOTTER, E. 1971. The cucurbitacins, a group of tetracyclic triterpenes. *Fortschr. Chem. Org. Naturst.* 29:307–356.

METCALF, R.L. 1985. Plant kairomones and insect pest control. *Bull. Ill. Nat. Hist. Surv.* 33:175–198.

METCALF, R.L. 1986. Foreward, pp. 7–15, *in* J.L. Krysan and T.A. Miller (eds.). Methods for the Study of Pest *Diabrotica*. Springer-Verlag, New York.

METCALF, R.L., and LAMPMAN, R.L. 1989. The chemical ecology of diabroticites and Cucurbitaceae. *Experientia* 45:240–247.

METCALF, R.L., METCALF, R.A., and RHODES, A.M. 1980. Cucurbitacins as kairomones for diabroticite beetles. *Proc. Nat. Acad. Sci. U.S.A.* 17:3769–3772.

METCALF, R.L., RHODES, A.M., METCALF, R.A., FERGUSON, J.E., METCALF, E.R., and PO-YUNG, L. 1982. Cucurbitacin contents and diabroticite (Coleoptera: Chrysomelidae) feeding upon *Cucurbita* spp. *Environ. Entomol.* 11:931–937.

METCALF, R.L., FERGUSON, J.E., LAMPMAN, R., and ANDERSON, J.F. 1987. Dry cucurbitacin-containing baits for controlling diabroticite beetles (Coleoptera: Chrysomelidae), *J. Econ. Entomol.* 80:870–875.

SASAMORI, H., REDDY, K.S., KIRKUP, M.P., SHABANOWITZ, J., LYNN, D.G., HECHT, S.M., WOODE, K.A., BRYAN, R.F., CAMPBELL, J., LYNN, W.S., EGERT, E., and SHELDRICK, G.M. 1983. New cytotoxic principles from *Datisca glomerata*. *J. Chem. Soc. Perkin Trans. I* 1983:1333–1347.

SHARMA, G.C., and HALL, C.V. 1971. Influence of cucurbitacins, sugars, and fatty acids on cucurbit susceptibility to spotted cucumber beetle. *Am. Soc. Hortic. Sci. J.* 96:675–680.

STUPPER, H., KAHLIG, H., SELIGMANN, O., and WAGNER, H. 1990. Minor cucurbitacin glycosides from *Picrorhiza kurrooa*. *Phytochemistry* 29:1633–1637.

TALLAMY, D.W., and HALAWEISH, F.T. 1993. The effect of age, prior exposure reproductive activity, on sensitivity to cucurbitacins in sex, and southern corn rootworm (Coleoptera: Chrysomelidae), *Environ. Entomol.* Submitted.

VELDE, V.V., and LAVIE, D. 1983. ^{13}C-NMR spectrotoscopy of cucurbitacins. *Tetrahedron* 39:317–321.

WEISSLING, T.J., MEINKE, L.J., TRIMNELL, D., and GOLGEN, K.L. 1989. Behavioral responses of *Diabrotica* adults to plant-derived semichemicals encapsulated in a starch borate matrix. *Entomol. Exp. Appl.* 53:219–228.

WHITHOUSE, M.W., and DOSKOTCH, W.W. 1979. Binding of the cytotoxic and antitumor triterpenes, cucurbitacins, to glucocorticoid receptor of HeLa cells. *Biochem. Pharmacol.* 18:1790–1793.

WITKOWSKI, A., and KONOPA, J. 1981. Selective inhibition of thymidine incorporation into lymphocytes by cucurbitacin B and D. *Biochim. Biophys. Acta* 674:246-250.

WITKOWSKI, A., WOYNAROWSKA, B., and KONOPA, J. 1984. Inhibition of the biosynthesis of desoxyribonucleic acid, ribonucleic acid and protein in HeLa S3 cell by cucurbitacins, glucocorticoid-like cytotoxic triterpenes. *Biochem. Pharmacol.* 33:995–1005.

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ALKALOIDAL RESPONSES TO DAMAGE IN *Nicotiana* NATIVE TO NORTH AMERICA

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Abstract—We performed field tests of alkaloid induction in *Nicotiana attenuata* plants growing in southwestern Utah with mimicry of the two major types of damage inflicted by invertebrate and vertebrate herbivores: leaf damage and stalk removal, respectively. In undamaged plants, seasonal increases in leaf nicotine content occurred at a rate of 0.046% leaf dry mass/day. Leaf damage doubled the accumulation rate to 0.086–0.138% leaf dry mass/day, while stalk removal resulted in a quadrupling of the accumulation rate to 0.206% leaf dry mass/day. These damage-induced increases in nicotine accumulation are significantly larger than between-plant and phenological variations. Leaf damage to the nornicotine-(*N. repanda* and *N. trigonophylla*) and anabasine-accumulating (*N. glauca*) *Nicotiana* species native to North America resulted in 1.5- to 5-fold increases in their principal leaf alkaloid pools. We conclude that alkaloid induction is not limited to nicotine-accumulating *Nicotiana* species and that herbivores feeding on previously damaged plants are likely to encounter tissues with alkaloid titers significantly higher than those of undamaged plants.

Key Words—Damage-induced responses, *Nicotiana attenuata*, *Nicotiana repanda*, *Nicotiana glauca*, *Nicotiana trigonophylla*, nicotine, nornicotine, anabasine, herbivory.

INTRODUCTION

Most of the biosynthetic classes of secondary metabolites are induced by leaf damage (Baldwin, 1993; Rosenthal and Berenbaum, 1991; Tallamy and Raupp, 1991) in at least some plants. These damage-induced metabolites may be part

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of a plant's defensive response to herbivore attack. However, most phytochemical studies of these responses have been performed on plants grown under controlled conditions in greenhouses or field plots: those studies that have examined phytochemical changes induced by damage in native populations of plants have frequently found the between-plant variance in leaf chemistry to be as great as or greater than damage-induced changes (Denno and McClure, 1983; Rossiter et al., 1988; Tallamy and Raupp, 1991). The concentrations of most secondary metabolites are strongly influenced by the availability of nutrients, light, and water (Gershenzon, 1984; Waterman and Mole, 1989) in the plant's environment and by leaf ontogeny and plant genotype (Coleman and Jones, 1991). Thus, it is not clear that the statistically significant damage-induced changes described in greenhouse studies necessarily significantly alter leaf chemistry for herbivores feeding on previously damaged plants growing in the wild. For example, one of the most elegantly documented cases of induced phytochemical responses is the dramatic increase in quinolizidine alkaloids of lupine that occurs within hours of leaf damage. Most of this work has been done on greenhouse-grown plants (Bentley et al., 1987; Johnson et al., 1988; Wink, 1983, 1985, 1987), but an examination of native populations of velvet lupine (*Lupinus lecophyllus*) in Idaho found no induced alkaloidal response (Ralphs and Williams, 1988).

We have documented large (fourfold) changes in the concentrations of nicotine in *Nicotiana sylvestris* Spegazzini and Comes plants in response to leaf damage (Baldwin, 1989; Baldwin et al., 1990) and found that these alkaloidal responses reduce the growth of a tobacco herbivore and protect induced tissues in laboratory feeding trials (Baldwin, 1988b, 1991). *N. sylvestris* is an annual plant native to South America; however, our experiments have used plants grown in the greenhouse and in field plots in New York State. Clearly we need to know whether induced alkaloidal responses can be documented in naturally occurring populations of native plants. To this end, we studied the responses of native populations in southwestern Utah of the summer annual, *N. attenuata* Torrey ex. Watson, to simulations of the two most common types of herbivory found on these plants.

N. attenuata is the only *Nicotiana* species native to North America that accumulates nicotine as its principal leaf alkaloid. The other *Nicotiana* species native to North America accumulate either nornicotine (*N. repanda* and *N. trigonophylla*) or anabasine (*N. glauca*) as their principal alkaloid (Saitoh et al., 1985). In order to determine if the induced alkaloidal responses in North American *Nicotiana* were limited to those species that accumulate nicotine, we studied the alkaloidal changes induced by damage in the other three *Nicotiana* species.

METHODS AND MATERIALS

Alkaloid Quantification. Nicotine, nornicotine, and anabasine were separated and quantified in extracts of leaves by high-pressure liquid chromatography (HPLC) (see description of extraction and HPLC conditions in Baldwin, 1988a).

Two to eight (depending upon the experiment) 0.32-cm^2 circular leaf disks were immediately extracted in a weighed amount of alkaloid extraction solution containing the internal standard, scopolamine (Sigma Chemical Co., St. Louis, Missouri), at a concentration of 50 μg/ml. Two additional leaf disks were removed, weighed (to 0.1 mg), dried for 24 hr at 65°C, and reweighed for calculations of specific leaf mass. Leaf alkaloid concentrations were expressed as percent nicotine, nornicotine, or anabasine of leaf dry mass.

Nicotiana attenuata *Experiments.* Plants from two populations were located in southwestern Utah on the property of the DI Ranch (T40S R19W section 10) and 5 miles to the south on Bureau of Land Management property (T41S R17W section 29). At the latter (BLM) site, approximately 50 plants were growing in a sandy wash. At the former (DI) site, over 300 plants were growing in a gravel wash, which had burned the previous year. Most of these plants were growing among the charred stumps of basin sagebrush (*Artemisia tridentata*), junipers (*Juniperus* spp.), and blackbrush (*Coleogyne ramosissimum*).

During the rosette-stage of growth, which lasts for two to six weeks, *N. attenuata* produces large, ovate basal leaves. Leaves produced during stalk elongation and flowering are smaller and more lanceolate. Herbivory by invertebrates (principally from orthopteran nymphs and adults and lepidopteran larvae) typically results in partially damaged basal leaves. During the 1989 growing season, we monitored (Baldwin, unpublished) damage on 640 basal leaves on 160 plants and recorded partial herbivory for 17.3% of these leaves at the DI site. Herbivory by vertebrates [principally from rock and ground squirrels (*Spermophilus* and *Ammospermophilus* spp.), and black-tailed jack and desert cottontail rabbits (*Lepus californicus* and *Sylvilagus audbonii*, respectively)] typically resulted in the loss of the flowering stalk just above the basal rosette. Of 60 plants monitored at the DI site, 16.8% had the flowering stalk removed. Stalk removal usually results in the regrowth of one to five new stalks.

Our goal was to examine the effects of leaf and stalk damage on the alkaloid concentration of damaged and undamaged basal rosette leaves. These basal leaves represent more than 65% of the plant's total leaf area, and if they are completely lost the plant will frequently (10 of 12 observed natural defoliations) not be able to regrow. To this end, we examined the nicotine concentrations for 18–20 days in: (1) undamaged basal leaves in both flowering- and rosette-stage undamaged plants and plants subjected to simulated leaf herbivory; (2) both damaged and undamaged basal leaves after simulated leaf herbivory in flowering-stage plants; and (3) undamaged basal leaves after simulated browsing, which removed the flowering stalk. In the latter experiment, we also measured the alkaloid concentrations of both the removed stalk and stalks that had regrown after 20 days.

Damage and Sampling Schedules. Herbivory was simulated with scissors. All fully expanded leaves excluding the four to six basal leaves slated for chemical analysis were removed from damaged plants in one cutting. Leaves that

were damaged and also sampled were cut parallel with the midrib, removing the left half of the lamina and leaving the right half for alkaloid analyses. Leaf cuttings were oven dried at 75°C and weighed to the nearest 0.2 mg. Leaf disks (two per sampling period) for alkaloid analysis were removed from basal leaves beginning at the distal portion of the leaf and moving proximally with each sampling. Leaf nicotine (which accounted for more than 98% of the total alkaloid content) concentrations decreased slightly from distal (0.52 ± 0.07% leaf dry mass) to proximal (0.45 ± 0.04% leaf dry mass) portions of the leaf lamina (mean ± SEM of six leaves) on one rosette-stage plant from the DI site on May 23. Thus, we sampled against the natural nicotine gradient within a leaf and hence conservatively estimated the damage-induced alkaloidal response.

Damage-Induced Alkaloid Responses in Undamaged Leaves of Rosette- and Flowering-Stage Plants. On May 20, 50 flowering and 50 rosette-stage plants growing at the DI site without any previous herbivory were labeled and assigned (with a random numbers table) to either undamaged or damaged treatment groups. Samples were taken from the largest leaf occupying the fourth or fifth position from the bottom. Plants were damaged the next day, removing 0.192 ± 0.026 g and 0.916 ± 0.205 g of leaf mass from damaged rosette- and flowering-stage plants, respectively. Undamaged plants were not damaged except for the removal of leaf disks for alkaloid samples. The same leaves were again resampled from all plants on May 25 and 31, and June 6. Herbivores removed the sample leaf or the flowering stalk from six (three undamaged and three damaged) rosette-stage plants and seven (four undamaged, three damaged) flowering-stage plants and were therefore excluded from the analysis.

Damage-Induced Alkaloid Responses in Damaged and Undamaged Leaves on Damaged Flowering-Stage Plants. Ten previously undamaged flowering-stage plants growing at the DI site were cut as in the previous experiment and, in addition, four of the five remaining basal leaves were cut in half, resulting in the removal of 0.63 ± 0.16 g of leaf mass per plant. Two leaf disks were taken from the one undamaged leaf and one from each of the four damaged leaves, which were pooled to estimate the alkaloid concentration of damaged leaves. These five leaves were sampled on May 21, just before damage, and five times after damage (May 22, 24, 26, and 30, and June 5).

Damage-Induced Alkaloid Responses in Undamaged Leaves in Response to Simulated Browsing. Undamaged basal leaves from five previously undamaged, flowering-stage plants growing at the DI site and 15 additional plants growing at the BLM site were sampled on June 11, and their flowering stalks were cut just above the basal rosette of leaves to simulate browsing herbivory. The first 5 cm of stalk from each of the DI plants was weighed and extracted for alkaloids, while the second 5 cm of stalk was weighed, dried at 75°C, and reweighed for wet/dry mass conversions. The previously sampled basal leaves

were sampled again on June 18 and June 27. The flowering stalks, which had regrown since the initial damage, were sampled for a second time on July 1.

Nornicotine- and anabasine-accumulating species: N. repanda, N. trigonophylla, *and* N. glauca. Seeds were obtained from the USDA Agricultural Research Center, Beltsville, Maryland, and germinated in Cornell mix A artificial soil. Experiments with *N. trigonophylla* and *N. repanda* were conducted in the greenhouse; seedlings were grown in 10-cm pots and transplanted into 20-cm pots two weeks before damage to avoid any possible inhibition of alkaloidal responses to damage due to pot-bound growth (Baldwin, 1988a). Two basal rosette leaves were labeled with twist-ties on 10 plants of each species. Five plants of each species were damaged with scissors by cutting all other leaves in half. Two days later, all previously damaged leaves were again cut in half, and 123 hr after the start of damage, the two undamaged sample leaves on damaged and undamaged plants were harvested for alkaloid analysis. *N. glauca* seedlings were grown in the greenhouse for the first year and 28 were transplanted into field plots for their second growing season. On June 11, the first and sixth fully expanded leaves on one stalk of each plant were designated as sample leaves and labeled with rubber bands; four days later, fully expanded leaves in positions 2–5 and 7–12 were cut in half on every other plant. Sample leaves were harvested for anabasine content seven days after damage. Voucher specimens for all species used in this study are deposited at the Bailey Hortatorium at Cornell University.

Statistical Methods. Repeated measures one- and two-way ANOVAs with the damage and growth-stage treatments as main effects were used to analyze the measures of leaf alkaloid concentration of plants that were sampled more than once. A trend analysis over time was performed on the treatment × time interaction (Poorter and Lewis, 1986). If a single DF polynomial treatment contrast was significant for a linear partitioning of the $SS_{interaction}$, a least-squares linear regression was performed on the alkaloid contents over time. One-way analysis of variance was used to analyze singly measured traits except when alkaloid concentrations of plant parts were compared within a plant; these were analyzed with a paired *t* test. Percentages were arcsine transformed. Analysis was performed with the MGLH ANOVA module from Systat Inc. (Evanston, Illinois).

RESULTS

N. attenuata *Experiments.* Leaf nicotine concentrations of undamaged basal leaves on undamaged rosette- and flowering-stage plants increased 1.82- and 2.48-fold, respectively, over the 18-day sampling period. During this same period, the leaf nicotine concentrations of undamaged basal leaves on damaged

rosette-and flowering-stage plants increased 3.45- and 3.95-fold, respectively (Figure 1). The increases in alkaloid contents in response to damage for plants in either stage of growth were highly significant, and no significant growth stage effects or interactions between growth stage and damage were found (Table 1). The linear polynomial contrast for the damage treatment was highly significant

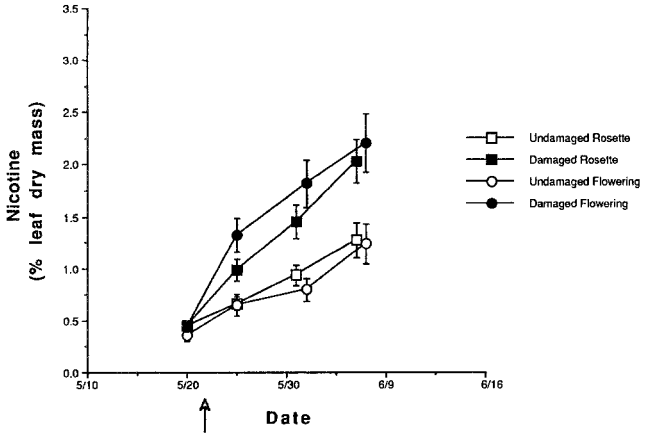

FIG. 1. Mean (±SEM) leaf nicotine concentrations of undamaged basal leaves from undamaged and damaged rosette- (N = 44) and flowering- (N = 33) stage *Nicotiana attenuata* plants growing in southwestern Utah. Arrow indicates time of leaf damage. Refer to Table 1 for statistical analysis.

TABLE 1. REPEATED MEASURES ANOVAS OF ARCSINE-TRANSFORMED LEAF NICOTINE CONCENTRATIONS MEASURED 4–6 TIMES OVER GROWING SEASON FOR (1) BETWEEN-PLANT COMPARISON OF UNDAMAGED BASAL LEAVES ON UNDAMAGED AND DAMAGED ROSETTE- AND FLOWERING-STAGE PLANTS; (2) WITHIN-PLANT COMPARISON OF UNDAMAGED AND DAMAGED BASAL LEAVES ON FLOWERING-STAGE PLANTS

Source of variation	*df*	MS	*F*	*P*
1. Undamaged leaves on rossette- and flowering-stage plants				
Development stage	1	0.000039	0.410	0.524
Damage treatment	1	0.0002222	23.238	0.000
Development stage × damage	1	0.000157	1.639	0.204
Error	73	0.000096		
2. Undamaged and damaged leaves on damaged flowering-stage plants				
Damage treatment	1	0.000326	1.378	0.261
Error	13	0.000236		

($F_{1,73}$ = 18.62; $P < 0.0001$), while the quadratic was not ($F_{1,73}$ = 3.074; P = 0.084), demonstrating that the damage-induced differences between undamaged and damaged plants were maintained throughout the sampling period.

Undamaged basal leaves of flowering-stage plants, which had four of the five basal rosette leaves cut in half, attained nicotine concentrations at the last sampling (2.28 ± 0.25% leaf dry mass; Figure 2) comparable ($F_{1,22}$ = 0.15; P = 0.70; repeated measures ANOVA) to those of plants that did not have the basal rosette leaves damaged (2.20 ± 0.28% leaf dry mass; Figure 1). Damaged leaves attained higher alkaloid concentrations (2.78 ± 0.33% leaf dry mass; Figure 2), representing a 3.73-fold increase in alkaloid content after damage, but the differences between damaged and undamaged leaves on the same plants were not significant (Table 1). Thus, the effect of leaf damage in *N. attenuata* is primarily systemic; the alkaloidal response to damage occurs in damaged and undamaged leaves with equal intensity.

The alkaloidal response in basal leaves was greatest in plants subjected to simulated browsing; basal leaves of these plants attained nicotine concentrations of 5.14 ± 0.56% leaf dry mass (Figure 3). No significant differences were found between the alkaloid responses of plants growing at the DI and the BLM sites ($F_{1,17}$ = 0.004; P = 0.95; repeated measures ANOVA). Stem alkaloid concentrations did not significantly respond (paired t = 2.63; P = 0.06) to damage; the alkaloid concentration of flowering stems that regrew after damage tended to be lower (0.80 ± 0.11% stem dry mass) than those originally damaged (1.63 ± 0.35% stem dry mass).

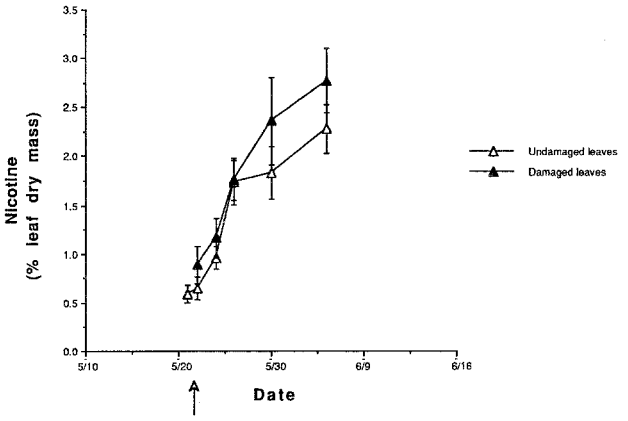

FIG. 2. Mean (±SEM) leaf nicotine concentrations of undamaged and damaged leaves from damaged flowering-stage *Nicotiana attenuata* plants (N = 10) growing in southwestern Utah. Arrow indicates time of leaf damage. Refer to Table 1 for statistical analysis.

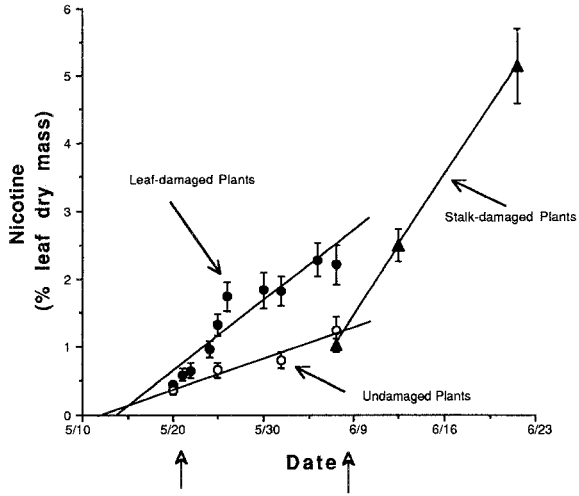

FIG. 3. Means (±SEM) and linear regressions of leaf nicotine concentrations against time of undamaged (○), leaf-damaged (●), and stalk-damaged (Δ) ($N = 20$) *Nicotiana attenuata* plants. First arrow indicates time of damage for plants subjected to simulated leaf herbivory. Second arrow indicates time of stalk removal for plants subjected to simulated browsing. Refer to Table 2 for regression coefficients.

The slopes of the regressions of basal leaf alkaloid concentrations against time summarize the responses in these native populations of *N. attenuata* (Table 2). The normal phenological trend appears to be a linear seasonal increase in leaf alkaloid content at a rate of 0.046–0.047% leaf dry mass/day. This rate of increase is not significantly influenced by the developmental stage of the plant but is doubled (to 0.086–0.138% leaf dry mass/day) if the plants suffer a single bout of leaf herbivory. Simulated browsing damage quadruples the rate of increase (to 0.206% leaf dry mass/day) above the normal phenological pattern found in undamaged plants. The rate of increase in undamaged plants may be an overestimate due to sampling-induced damage. However, given that the initial alkaloid sample of the previously undamaged and unsampled leaves from the browsing experiment falls just below the regression line of the undamaged flowering stage plants that had been sampled thrice previously (Figure 3), the overestimation is likely to be small.

Nornicotine- *and Anabasine-Accumulating Species.* The nornicotine-accumulating species, *N. trigonophylla* and *N. repanda*, had significantly ($t = 3.61$ and 2.60, respectively; $P < 0.04$; $DF = 8$) higher nornicotine concentrations (percent leaf dry mass) in the undamaged leaves of damaged plants (3.41 ± 0.76 and 0.18 ± 0.03, respectively) than of undamaged plants (0.62 ± 0.16 and 0.10 ± 0.01, respectively). Similar results were obtained from the exper-

TABLE 2. REGRESSIONS OF MEAN NICOTINE CONTENTS AGAINST DAYS AFTER SIMULATED HERBIVORY FOR (1) UNDAMAGED BASAL LEAVES ON UNDAMAGED AND DAMAGED ROSETTE- AND FLOWERING-STAGE PLANTS; (2) UNDAMAGED AND DAMAGED BASAL LEAVES ON FLOWERING-STAGE PLANTS; (3)UNDAMAGED BASAL LEAVES ON FLOWERING-STAGE PLANTS SUBJECTED TO SIMULATED BROWSING THAT REMOVED FLOWERING STALK

	Slope (%leaf dry mass/day)	Intercept	R^2
Responses to leaf damage			
1. Between-plant comparisons of undamaged leaves			
Undamaged plants			
Rosette stage	0.046	−0.466	0.999
Flower stage	0.047	−0.570	0.974
Damaged plants			
Rosette stage	0.086	−1.222	0.996
Flowering stage	0.094	−1.240	0.931
2. Within-plant comparison of flowering-stage plants			
Undamaged leaves	0.117	−1.755	0.883
Damaged leaves	0.138	−2.008	0.936
Responses to stalk damage			
3. Undamaged leaves on damaged plants	0.206	−6.808	1.000

iments with the anabasine-accumulating species, *N. glauca*, where damage increased the anabasine contents of undamaged leaves on damaged plants significantly ($F_{1,50} = 16.80$; $P < 0.0001$), in both leaf positions ($F_{1,50} = 3.05$; $P = 0.087$) and no damage × position interaction was found ($F_{1,50} = 0.0001$; $P = 0.987$) in a two-way ANOVA. Undamaged leaves of positions 1 and 6 on damaged plants had anabasine contents (0.47 ± 0.62 and 0.39 ± 0.05% leaf dry mass, respectively) that were 66.5% and 93.5% higher than those of undamaged plants (0.28 ± 0.04 and 0.20 ± 0.02% leaf dry mass).

DISCUSSION

Native populations of *N. attenuata* in southwestern Utah double their normal rate of alkaloid accumulation in response to simulated leaf herbivory and quadruple this rate in response to simulated browsing. Clearly, damage-induced responses in alkaloid accumulation are substantially larger than normal phenological between-plant and between-population variation; moreover, these damage-induced increases were maintained throughout the growing season. This is surprising given the well-described sensitivity of alkaloid accumulation to variations in nitrogen fertilization (Gershenzon, 1984; Waterman and Mole,

1989) and the high spatial variability in nitrogen availability in desert soils (refs. in Gutierrez and Whitford, 1987). It is therefore reasonable to conclude that herbivores feeding on previously damaged *N. attenuata* plants growing in the wild are likely to encounter tissues with alkaloid titers significantly higher than those of undamaged plants. The alkaloidal responses are systemic, and the magnitude of the response appears to be proportional to the severity of the damage: alkaloidal increases in leaves were greatest in plants that had lost their flowering stalks and presumably suffered the greatest immediate fitness loss. One browsed plant growing at the BLM site attained a leaf nicotine concentration of 12.35% leaf dry mass, which is a 14.5-fold damage-induced increase above the prebrowsing levels and the highest induced alkaloid increase we have measured in any nicotine-accumulating species. Moreover, all *Nicotiana* species native to North America are able to increase their species-specific alkaloid pools in response to leaf damage.

Whether these increases in alkaloid contents translate into increased resistance to further herbivory for these previously damaged plants is not clear. Herbivory results not only in "defense"-related changes in secondary metabolism but also in changes in the "civilian" chemistry of a leaf: namely, the changes in primary metabolites that result from a suite of physiological responses that contribute to a plant's resilience to herbivory (Baldwin, 1993). For example, the rapid damage-induced increase in leaf nicotine concentrations in *N. sylvestris* is coordinated with increases in photosynthetic capacity and in protein, nitrate, and total nitrogen contents (Baldwin and Ohnmeiss, 1993) and therefore superficially resembles the process of rejuvenation. The induced alkaloid responses reported here are clearly not a reversal of normal ontogenic trends in alkaloid production or accumulation. Since the food quality of a leaf for an herbivore is determined by the interaction of both its primary and secondary metabolites (Duffey et al., 1986; Johnson and Bentley, 1988; van der Meijden et al., 1984), comparing damage rates or suitability of previously damaged tissues with tissues of undamaged plants is likely to be a poor test of the defensive function of an induced chemical response. Examinations of the functional importance of induced chemical plasticity need to recognize the potentially conflicting demands of defense and regrowth.

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REFERENCES

BALDWIN, I.T. 1988a. Damaged-induced alkaloids in tobacco: Pot-bound plants are not inducible. *J. Chem. Ecol.* 4:1113–1120.

BALDWIN, I.T. 1988b. Short-term damage-induced increases in tobacco alkaloids protect plants. *Oecologia* 75:367–370.

BALDWIN, I.T. 1989. The mechanism of damaged-induced alkaloids in wild tobacco. *J. Chem. Ecol.* 15:1661–1680.

BALDWIN, I.T. 1991. Damage-induced alkaloids in wild tobacco, pp. 47–69, *in* D.W. Tallamy and M.J. Raupp (eds.). Phytochemical Induction by Herbivores. John Wiley & Sons, New York.

BALDWIN, I.T. 1993. Chemical changes rapidly induced by folivory, *in* E.A. Bernays (ed.). Insect-Plant Interactions. CRC Press, Boca Raton, Florida. In press.

BALDWIN, I.T., and OHNMEISS, T.E. 1993. Coordination of photosynthetic and alkaloidal responses to leaf damage in pot-bound and inducible *Nicotiana sylvestris*. Submitted.

BALDWIN, I.T., SIMS, C.L., and KEAN, S.E. 1990. Reproductive consequences associated with inducible alkaloidal responses in wild tobacco. *Ecology* 71:252–262.

BENTLEY, B.L., JOHNSON, N.D., and RIGNEY, L. 1987. Short-term induction in leaf tissue alkaloids in lupines following experimental defoliation. *Am. J. Bot.* 74:646.

COLEMAN, J.S., and JONES, C.G. 1991. A phytocentric perspective of phytochemical induction by herbivores, pp. 3–45, *in* D.W. Tallamy and M.J. Raupp (eds.). Phytochemical Induction by Herbivores. John Wiley & Sons, New York.

DENNO, R.F., and MCCLURE, M.S. 1983. Variable Plants and Herbivores in Natural and Managed Systems. Academic Press, New York.

DUFFEY, S.S., BLOEM, K.A., and CAMPBELL, B.C. 1986. Consequences of sequestration of plant natural products in plant-insect-parasitoid interactions, pp. 31–60, *in* D.J. Boethel and R.D. Eikenbarry (eds.). Interactions of Plant Resistance and Parasitoids and Predators of Insects. Ellis Horwood, Chicester, England.

GERSHENZON, J. 1984. Changes in the levels of plant secondary metabolites under nutrient and water stress. *Recent Adv. Phytochem.* 18:273–321.

GUTIERREZ, J.R., and WHITFORD, W.G. 1987. Chihuahuan desert annuals: Importance of water and nitrogen. *Ecology* 68:2032–2045.

JOHNSON, N.D., and BENTLEY, B.L. 1988. Effects of dietary protein and lupine alkaloids on growth and survivorship of *Spodoptera eridania*. *J. Chem. Ecol.* 14:1391–1403.

JOHNSON, N.D., RIGNEY, L., and BENTLEY, B.L. 1988. Short-term changes in alkaloid levels following leaf damage in lupines with and without symbiotic nitrogen fixation. *J. Chem. Ecol.* 15:2425–2434.

POORTER, H., and LEWIS, C. 1986. Testing differences in relative growth rate: A method avoiding curve fitting and pairing. *Physiol. Plant.* 67:223–226.

RALPHS, M.H., and WILLIAMS, C. 1988. Alkaloid response to defoliation of velvet lupine (*Lupinus leucophyllus*). *Weed Technol.* 2:429–432.

ROSENTHAL, G.A., and BERENBAUM, M.A. 1991. Herbivores, Their Interactions with Secondary Plant Metabolites. Academic Press, New York.

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.

SAITOH, F., NOMA, M., and KAWASHIMA, N. 1985. The alkaloid contents of sixty *Nicotiana* species. *Phytochemistry* 24:477–480.

TALLAMY, D.W., and RAUPP, M.J. 1991. Phytochemical Induction by Herbivores. John Wiley & Sons, New York.

VAN DER MEIJDEN, E., VAN BEMMELEN, M.J., KOOI, R., and POST, B.J. 1984. Nutritional quality and chemical defence in the ragwort-cinnabar moth interaction. *J. Anim. Ecol.* 53:443–453.

WATERMAN, P.G., and MOLE, S. 1989. Extrinsic factors influencing production of secondary metabolites in plants, pp. 107–134, *in* E.A. Bernays (ed.). Insect-Plant Interactions Boca Raton, Florida.

WINK, M. 1983. Wounding-induced increase of quinolizidine alkaloid accumulation in the lupin leaves. *Z. Naturforsch.* 38:905–909.

WINK, M. 1985. Chemical defense of lupins: Biological function of quinolizidine alkaloids. *Plant Syst. Evol.* 150:65–81.

WINK, M. 1987. Chemical ecology of quinolizidine alkaloids, pp. 326–333, *in* G.R. Waller (ed.). Allelochemicals: Role in Agriculture and Forestry. American Chemical Society, Washington, D.C.

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IRIDOID GLYCOSIDE SEQUESTRATION BY *Thessalia leanira* (LEPIDOPTERA: NYMPHALIDAE) FEEDING ON *Castilleja integra* (Scrophulariaceae)[1]

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Abstract—A small population of a polyvoltine checkerspot butterfly, *Thessalia leanira fulvia* (also known as *Chlosyne leanira* ssp. *fulvia*), was found to use *Castilleja integra* as a larval food plant at a localized site (Burnt Mill) southwest of Pueblo, Colorado. Field-captured adult butterflies contained the major iridoid glycosides (catalpol and macfadienoside) of the *Castilleja*. The content of a third iridoid glycoside, methyl shanzhiside, was also relatively high in the collected butterflies even though most individual *Castilleja* plants at Burnt Mill contained little or no methyl shanzhiside. Only a few plants, restricted to a small area, did contain appreciable methyl shanzhiside. Most of the plants that lacked the ester methyl shanzhiside contained shanzhiside, the corresponding free carboxylic acid. *Thessalia* larvae did not normally methylate the acid to produce methyl shanzhiside. Larvae that stopped feeding at an early instar, but yet survived several weeks, did contain major amounts of methyl shanzhiside. It is suggested that only larvae that overwinter or otherwise enter diapause convert shanzhiside to methyl shanzhiside. The *Castilleja* food plant also contained iridoids other than catalpol and macfadienoside, sometimes in major amounts, but these were never found in larvae, pupae, or butterflies.

Key Words—*Thessalia leanira fulvia*, *Chlosyne leanira fulvia*, Lepidoptera, Nymphalidae, *Castilleja integra*, Scrophulariaceae, iridoid glycosides, sequestration, herbivory.

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INTRODUCTION

Iridoid glycoside sequestration by specialist, aposematic butterfly and moth larvae that feed on iridoid-containing plants is now a well-established paradigm (Bowers, 1988; L'Empereur and Stermitz, 1990, and references therein; Boros et al., 1991; Bowers, 1991). One previous study involved consumption of *Castilleja integra* by larvae of the checkerspot butterfly *Euphydryas anicia* at Red Hill, a 2900-m intermontane site in central Colorado (Gardner and Stermitz, 1988). The same food plant was reported (Scott, 1968) to be utilized at southern Colorado sites by another checkerspot, *Thessalia leanira fulvia* (also known as *Chlosyne leanira fulvia*). A small population of adult *Thessalia* was found at Burnt Mill in southern Colorado, where *Castilleja integra* (hereafter *Castilleja*) had *Thessalia* eggs and larvae. Preliminary tests showed that the butterflies indeed contained iridoid glycosides. In order to compare *Thessalia* processing of *Castilleja* with processing of the same larval food plant by *Euphydryas* butterflies, we performed iridoid analyses on the *Castilleja* and on the *Thessalia* butterflies, larvae, larval frass and pupae collected during several of eight seasons we visited Burnt Mill.

METHODS AND MATERIALS

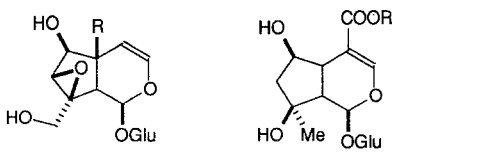
Site and Organisms. The study site was in Pueblo County, Colorado near Burnt Mill Road, 8 miles SW of Pueblo (1550 m; R.66W. T.23S). A diagram of the site is available (Foderaro, 1993). Butterflies were identified by Paul A. Opler, U.S. Fish and Wildlife Service, Fort Collins, Colorado. Adults were collected on the south side of County Road 246 on two small hills, in the area between the hills, and on the north and west slopes of the west hill (a total area of about 400 × 800 m). *Castilleja integra* Gray (voucher FRS 241) from the site was identified by Prof. D. Wilken, Department of Biology, Colorado State University. We visited the Burnt Mill site in April to early July from 1985 to 1992, usually twice each year.

Field-collected butterflies were held in glassine envelopes in a cooler for several hours and frozen upon return to the laboratory. They were removed and dried at 50°C prior to analysis (L'Empereur and Stermitz, 1990). Newly eclosed lab-raised butterflies were held in a vial containing filter paper for 24 hr for meconium collection and then frozen until analysis time. Eggs from the field were brought to the lab, hatched, and raised on field-collected plants that had been analyzed for iridoid glycoside content. Field-collected larvae were similarly raised, with the *Castilleja* food plant being held in the refrigerator or replaced by new field collections from the site. Larvae were either dried at 50°C and weighed prior to analysis or crushed into methanol and analyzed on a wet weight

basis. In the latter case, dry weight was estimated from studies showing that larvae consistently lose 80 ± 2% volatiles upon drying (Mead, 1992).

A series of individual *Castilleja* plants were chosen for iridoid glycoside analysis, some because they held eggs or larvae and others to provide a general view of individual plant iridoid variation throughout the site. Plants were marked with flags and some of the same plants were analyzed in more than one year. Complete details on the plant analyses have been published (Mead and Stermitz, 1993). *Castilleja* are root parasites, and one host plant at the Burnt Mill site was shown to be *Liatris punctata* Hook., which contains pyrrolizidine alkaloids (Mead, 1992). The alkaloids are assimilated into the *Castilleja* and may have an effect on early instar *Thessalia* larvae (Mead, 1992). For the present study, only alkaloid-free *Castilleja* (parasitic on some plant other than *Liatris*) were utilized.

Analytical Methods. Iridoid glycoside isolations and analyses for catalpol (CAT), macfadienoside (MAC), methyl shanzhiside (MSH) and shanzhiside (SH) (Figure 1) were performed mainly as described previously (Gardner and Stermitz, 1988; L'Empereur and Stermitz, 1990). In a modification of the final step (trimethylsilylation of the purified iridoid glycoside mixture), a 1 : 1 mixture of TRI-SIL-Z and TRI-SIL-TBT (both from Pierce Chemical) was used rather than only TRI-SIL-Z since this gave more complete silylation. The reaction tube was heated at 60–70°C for 10 min. GC conditions were changed to the following: injector temp. 275°C, detector temp. 325°C, oven temp. 200°C; initial hold time 1 min, first ramp 2°/min to 250°C, 1 min hold time, second ramp 20°/min until 280°C, final hold 5 min. For the 1992 analyses, the internal standard phenyl glucoside was added just prior to silylation rather than prior to the final extraction since this improved reproducibility. Volumes of final derivatized iridoids and the injected aliquot (Gardner and Stermitz, 1988) were the same in all cases in order to provide comparable results among analyses. This method rarely resulted in an integrator overload for high-content iridoids and such cases are indicated in the Results. The minimum useful detectability was 0.008 mg of a given iridoid per injection. For comparison, single butterflies



catalpol (CAT): R = H
macfadienoside (MAC): R = OH
methyl shanzhiside (MSH): R = Me
shanzhiside (SH): R = H

FIG. 1. Iridoid glycosides of *Castilleja* and *Thessalia*.

(for example) of an average 14 mg dry weight contained an average of 0.6 mg total iridoids.

An HPLC analysis for MSH and SH (or any iridoids bearing a carboxyl or carbomethoxy group) was also developed. An Altex Ultrasphere-ODS analytical (4.6 mm × 25 cm) column was used with 20% aq. MeOH as the mobile phase and detection with a variable wavelength UV detector set at 240 nm.

Iridoid identification was by GC retention time and NMR spectral comparisons with known, previously isolated standards.

RESULTS

General Observations. Butterfly populations were not determined quantitatively, but individual butterfly encounters varied from none to two or three to several dozen in the course of 2-hr, bright morning surveys. In usual years, larvae were first found in late April or early May and adults in late May and early June. These would represent first brood butterflies, whose larvae had overwintered in the third or fourth instar (Scott, 1968). Numbers of eggs and larvae were also highly variable from year to year, with few generally being found. It has been suggested (J. Scott, private communication) that larvae can be found in this area on one in 10 *Castilleja* in "good" years, but on only one in 100 plants in low population years. In the eight years of our study, only 1992 appeared to be a year of high *Thessalia* populations. There was a very early spring and we found eggs on our first visit on May 10, which meant that adults from the first brood (none of which were found) had already mated and oviposited. Eggs and larvae at all instar stages, as well as adults were found on June 21 and July 1, indicating a virtually continuous brooding cycle. This was unusual for the site.

The numbers of *Castilleja*, easily visible because of their bright scarlet blossoms, were also relatively small and varied from a few dozen to about 100. Blooming was highly variable from year to year and is probably governed by spring rain and late snow.

Field-Collected Butterfly Analyses. Because of small butterfly populations, we were only able to collect a total of 35 butterflies for analysis from 1985 to 1991, all from the first brood. All 35 contained catalpol, macfadienoside, and methyl shanzhiside (Table 1; for individual analyses see Gardner, 1987; and Mead, 1992). In 1992, 17 adults, none from the first brood (see above), were captured and analyzed. Of these, eight contained methyl shanzhiside as well as catalpol and macfadienoside (Table 1; for individual analyses see Foderaro, 1993). No other iridoid glycosides were found in the butterflies.

Castilleja Integra Analyses. In May 1988, *Thessalia* eggs were found on three *Castilleja*, so these plants and three others were analyzed for methyl shan-

TABLE 1. CATALPOL (CAT), MACFADIENOSIDE (MAC) AND METHYL SHANZHISIDE (MSH) CONTENT (MEAN PERCENT DRY WEIGHT AND RANGE) OF FIELD-CAPTURED *Thessalia leanira* BUTTERFLIES

	CAT	MAC	MSH	Total
May, June Collections (1985, 1986, 1988, 1990): $N = 23$				
$\bar{X}$	1.7	1.6	0.65	3.9
Range	0–3.0	0–3.7	0.2–1.9	2.0–6.8
May 13, 1991 Collection: $N = 12$				
$\bar{X}$	2.0	1.2	0.59	3.8
Range	1.1–2.8	0.26–3.2	0.17–1.3	3.0–5.8
June 21 and July 1, 1992 Collections: $N = 17$				
$\bar{X}$	0.83	2.5	0.14	3.5
Range	0.16–2.1	0.51–6.0	0.00–0.66[a]	1.0–7.0

[a] Four of five butterflies from the June collection and four of 12 from the July collection contained MSH.

zhiside (MSH) content. The three with eggs contained 0.0, 0.0, and 0.4% MSH, while two of the others had no MSH and one had 1.1%. The same year, six *Castilleja* plants were collected from the montane site (Red Hill) where *Euphydryas anicia* studies had been accomplished (Gardner and Stermitz, 1988; L'Empereur and Stermitz, 1990) and analyzed. None contained MSH. This was in accord with the lack (or very low concentration) of MSH in any adult butterflies captured there during the previous studies. In June 1989, an additional seven *Castilleja* plants (none of which were the same as those analyzed the previous year) were collected and analyzed. None contained MSH, nor did 13 additional plants from the Red Hill site. All these analyses were by the GC method. The same samples were then analyzed by the more sensitive HPLC method. Six of the 13 from Red Hill still had no detectable MSH, while the levels detected in the other seven were 0.02% or lower. Similar results were found for the Burnt Mill plants, where one had no detectable MSH and the others only traces, generally below 0.01%.

In June 1990 an extensive iridoid glycoside analysis was performed on 16 individual *Castilleja* (four from the flagged area where high MSH-containing plants were observed in 1988). The content of eight iridoid glycosides in plant parts was determined (Mead and Stermitz, 1992). A summary of the these analyses for four iridoids (Table 2) showed typical results for catalpol and macfadienoside content. Only two of the plants (the ones from the previously known high MSH area) contained appreciable MSH, but most of the others contained shanzhiside (SH). SH is the carboxylic acid form of the methyl ester MSH (Figure 1).

TABLE 2. IRIDOID GLYCOSIDE CONTENT OF BURNT MILL *Castilleja integra* (1990 SEASON)

Iridoid	Plant part	Average (N = 16)	Range
Catalpol (CAT)	leaf	2.1	0.1–8.6
	bract	3.7	0.4–12.6
	flower	7.1	0.7–14.5
Macfadienoside (MAC)	leaf	13.6	5.7–23.8
	bract	9.6	3.0–16.3
	flower	9.5	4.3–13.7
Methyl shanzhiside[a] (MSH)	leaf	0.5	0.0–5.6
	bract	0.1	0.0–0.9
	flower	0.0	0.0–0.1
Shanzhiside[b] (SH)	leaf	1.1	0.0–4.4
	bract	0.0	
	flower	0.0	

[a] Six of 16 plants contained MSH, of which four had only 0.1%. The two high-content (5.6 and 2.4%) MSH plants contained no MSH.
[b] Ten of 16 plants contained SH. Four of the 16 contained neither MSH or SH.

1992 Field-Laboratory Studies. The high 1992 May–June population of organisms not only yielded more data on the iridoid glycoside content of field-captured adults (Table 1), but also allowed us to perform experiments designed to answer the question: can *Thessalia* methylate the acid SH to produce the ester MSH?

Larvae were collected at Burnt Mill from two *Castilleja* plants that were analyzed and shown to contain SH, but not MSH. Larvae and the same plants were brought to the laboratory, and the larvae were allowed to continue feeding on the same plant material. Six larvae (and their combined frass) from one plant feeding experiment and three larvae (and their combined frass) from the second plant feeding experiment were later analyzed for iridoid glycoside content (Table 3). Catalpol and macfadienoside, but no MSH, were found in any of the larvae. Frass contained no catalpol, but did contain some macfadienoside as well as other iridoids.

A similar experiment was carried out with three other field-collected larvae and plants (also devoid of MSH), but this time analyses were carried out (Table 4) on the resultant pupae (five) and emergent butterflies (two). No MSH was found in pupae, frass, adult butterflies, or the meconium emitted upon eclosion. Again, frass contained no catalpol, small amounts of macfadienoside, and an array of the other iridoid glycosides.

TABLE 3. IRIDOD CONTENT (% DRY WEIGHT) OF *Thessalia* LARVAE AND FRASS FROM *Castilleja*-FED LARVAE (1992 SEASON)

	Plant 1			Plant 2		
Analyte	CAT	MAC	Other[a]	CAT	MAC	Other
Leaf fed	3.7	14.7	5.2	0.28	4.7	2.9
Bract fed	1.2	1.5	0.59	0.27	1.1	2.9
Larva[b]	9.6	2.8	ND[c]	2.7	12.3	ND
Larva	5.7	6.5	ND	1.7	10.8	ND
Larva	3.4	6.6	ND	2.5	22.8	ND
Larva	6.1	4.8	ND			
Larva	7.0	1.3	ND			
Larva	4.6	4.8	ND			
Frass[d]	ND	2.8	0.53	ND	0.94	0.53

[a] Shanzhiside, 8-epiloganic acid, gardoside, adoxosidic acid. No methyl shanzhiside was detected in any samples.
[b] Larvae were collected in the field on the analyzed *Castilleja* and fed for about one week, at which time they appeared to quit eating. They were four-instar larvae at that time.
[c] Not detected.
[d] Combined frass from all larvae.

TABLE 4. IRIDOID CONTENT (% DRY WEIGHT) OF *Thessalia* PUPAE AND BUTTERFLIES FROM *Castilleja*-FED LARVAE (1992 SEASON)

	Experiment 1			Experiment 2			Experiment 3		
Analyte	CAT	MAC	Other[a]	CAT	MAC	Other	CAT	MAC	Other
Leaf fed	0.28	3.5	1.9	0.16	3.6	3.1	0.45	>4[c]	9.2
Bract fed	0.09	0.86	0.68				0.80	2.6	0.5
Pupa	1.1	3.9	ND[b]	5.7	8.7	ND	5.6	>4	>9
Frass	ND	0.17	1.5	ND	1.3	1.7			
Pupa	4.8	16.5	ND				6.9	20.5	ND
Frass	ND	0.92	1.4				ND	0.59	1.8
Butterfly	1.4	2.4	ND	1.2	1.8	ND			
Meconium	2.9	2.5	ND	3.1	3.1	ND			

[a] Shanziside, 8-epiloganic acid, gardoside, adoxosidic acid. No methyl shanzhiside was detected in any samples.
[b] Note detected.
[c] The GC peak was beyond limits of the integrator.

In order to check these data directly from the field, two larvae were taken from two *Castilleja* plants, which were again shown to contain SH but not MSH, and analyzed immediately (Table 5). Again, no MSH was found in the larvae. These larvae had been found on two plants with anomalously low concentrations of macfadienoside and undetectable or only trace amounts of catalpol. Larval content of these iridoids was greatly increased over that of the food plants. Although no catalpol was detected in one of these plants (experiment 1, Table 5), it must be present at a level below the GC detection method. Catalpol is an obligatory biosynthetic precursor of macfadienoside and hence must be present if macfadienoside is present.

An attempt was made to raise organisms from eggs found on *Castilleja* that lacked MSH. Eggs were collected in May 1992 from two such *Castilleja*. The eggs were hatched on their respective plant material in the laboratory, and resultant larvae continued to feed on the same plants. Initially all larvae consumed plant material and grew, but by 12–14 days after hatching larvae from both groups had quit eating, moved little, and appeared to be entering diapause. In one group there were 14 larvae, 2–8 mm in size, and in the other, 16 larvae, 3–9 mm in size. Larval length was used to estimate the instar stage (Scott, 1968). The experiment was terminated after 14 days, the larvae were crushed into methanol and analyzed, as was the frass collected during feeding (Table 6). In this case, the larvae contained appreciable amounts of MSH even though the plant upon which they were feeding did not. A smaller amount was found in the frass. Catalpol and macfadienoside processing was the same as in the other larval feeding experiment (Table 3).

DISCUSSION

General. The *Chlosyne*, sensu lato, group of checkerspots (Ehrlich and Ehrlich, 1961; Scott, 1986), is represented in North America by 17 species (Scott, 1986). These comprise eight species specializing on Compositae as larval food plants, three specializing on Acanthaceae, and four on Scrophulariaceae (Scott, 1986). Food plants have not been described for two species. In his revision of *Chlosyne*, Higgins (1960) stated that one group of taxa represented a "well-characterized compact genus," which he denoted *Thessalia*. Although larval food plant differences were not specifically mentioned by Higgins, the *Thessalia* group consisted of exactly those species that specialize on Scrophulariaceae or species of other families known to contain iridoid glycosides. The present work shows that at least one *Thessalia* is similar to *Euphydryas* and *Poladryas* in that it sequesters iridoid glycosides. Based upon known or suspected chemistry of the larval food plants for *Chlosyne*, sensu stricto, none of this group of butterflies should be iridoid-containing. That *Thessala leanira* is

TABLE 5. IRIDOID CONTENT (%DRY WEIGHT) OF FIELD-COLLECTED *Castilleja* AND *Thessalia* LARVAE FROM SAME PLANTS (1992 SEASON)

	Plant 1			Plant 2		
Analyte	CAT	MAC	Other[a]	CAT	MAC	Other
Leaf	ND[b]	0.30	0.28	ND	1.28	2.2
Bract	ND	1.1	1.4	0.11	0.79	3.1
Larva[c]	0.88	4.0	ND	2.2	2.3	ND
Larva	0.49	4.4	ND	2.3	2.7	ND

[a] Shanzhiside, e.g. 8-epiloganic acid, gardoside, adoxosidic acid. No methyl shanzhiside was found in any samples.
[b] Not detected.
[c] Experiment 1: fourth instar; experiment 2: fifth (penultimate) instar.

TABLE 6. IRIDOID GLYCOSIDE CONTENT (% DRY WEIGHT) OF EARLY INSTAR *Thessalia* LARVAE FED *Castilleja* DEVOID OF MSH

Analyte	CAT	MAC	MSH
Larvae[a]	2.1	10.2	1.1
Frass	ND[b]	3.0	0.66
Larvae[c]	2.1	>6.5[d]	1.3
Frass	ND	2.7	0.20

[a] Combined 14 second to fourth instar larvae.
[b] Not detected.
[c] Combined 16 second to fourth instar larvae.
[d] The GC peak was beyond the limit of the integrator.

not going to be an exception in the genus is evident from the fact that two adult *Thessalia theona* (Ménétriés) collected in Costa Rica were found to contain the rare iridoid glycoside 6-β-hydroxyipolamide (Stermitz, Foderaro, and Opler, unpublished results).

The Burnt Mill site and its *Thessalia leanira–Castilleja integra* herbivore–plant system proved to be suboptimal for a complete quantitative investigation due to the small numbers of butterflies, larvae, and eggs that were usually encountered in spite of an eight-year investigation. Only in the last year (1992) did there appear to be an increased population. Small populations of organisms and those at the edge of their distribution (as *Thessalia* is at Burnt Mill) are, however, of special interest in evolutionary theory (Mayr, 1982) and less than complete results can nevertheless be of importance.

Processing of Iridoid Glycosides. Relatively equal amounts of catalpol and macfadienoside were found, on the average, in field-captured butterflies (Table 1), even though somewhat more macfadienoside than catalpol was generally present in the larval food plant (Table 2). Catalpol was never found in the larval frass (Tables 3 and 4), while some macfadienoside was. When larvae were fed *Castilleja* with low iridoid glycoside concentration (Tables 3–5), the concentration in larvae, pupae, and adult butterflies exceeded that in the food plant, sometimes markedly. Macfadienoside concentration was lower in the butterfly organisms than in the food plant, but only where there was an extremely high concentration in the plant.

The food plant *Castilleja* contained relatively large amounts of other iridoid glycosides, particularly adoxoside, adoxosidic acid, and 8-epiloganic acid (Mead and Stermitz, 1993). These were never found in larvae, pupae, or adult butterflies, but were encountered in larval frass.

The main difference found between the *Euphydryas anicia–Castilleja integra* system at Red Hill (Gardner and Stermitz, 1988; L'Empereur and Stermitz, 1990) and *Thessalia leanira–Castilleja integra* system at Burnt Mill was the consistent and relatively large amount of MSH found in butterflies at Burnt Mill. In our reexamination of individual *Castilleja* from Red Hill, none were found to contain more than traces of MSH. This was also true of most of the *Castilleja* at Burnt Mill, but we did find a few individual plants in one small area of the latter site that contained up to 5.6% MSH. Since all of the 35 adult butterflies captured at Burnt Mill from 1985 to 1991 contained appreciable amounts of MSH, one hypothesis would be that all adults (captured from various parts of the site) originated from larvae that fed on the small subset of MSH-containing plants.

An alternative hypothesis arose from the discovery (Mead and Stermitz, 1993) that *Castilleja* that did not contain MSH did contain SH, the corresponding acid, in appreciable amounts. Thus, *Thessalia* larvae or pupae might be methylating SH to MSH. The results of several feeding experiments (Tables 3–5) showed that this was not normally the case. However, early instar larvae that had stopped feeding and were entering diapause did contain MSH even though the *Castilleja* they were being fed did not (Table 6). *Thessalia* larvae at Burnt Mill enter diapause in the fall and overwinter as such, and hence they too might produce MSH and adult butterflies of the first spring brood would also contain MSH. All adults from the 1985–1991 collections were first brood. It was only in 1992 that adults from subsequent broods were captured and analyzed, and here (Table 1) some that did not contain MSH were encountered for the first time.

It is likely that both the above possibilities contribute to the finding of MSH in Burnt Mill adult *Thessalia* butterflies. Some should indeed arise from larvae that fed on the small subset of MSH-containing plants, while other butterflies

would contain that iridoid because they had overwintered as diapausing larvae. Larvae arising from eggs laid by first brood adults might also enter diapause briefly due to environmental conditions. This could account for finding some adults in the 1992 second brood that contained MSH, although others could have come from larvae feeding on plants in the high-MSH area.

If either of these hypotheses (or a combination) accounts for the MSH presence in *Thessalia* adults at Burnt Mill, how do they apply to the *Euphydryas* case at Red Hill (Gardner and Stermitz, 1988), where only traces of MSH were encountered in the more than 100 butterflies analyzed? The individual plant analyses on *Castilleja* from Red Hill failed to locate any with more than traces of MSH; that is, there were no high-MSH plants located. Thus, *Euphydryas* larvae (which also feed on a second, also MSH-free host plant, *Besseya plantaginea*) have no opportunity to sequester MSH. The Red Hill *Castilleja* did contain shanzhiside, the acid form (Mead, 1992) so *Euphydryas* diapausing larvae would have the opportunity to methylate it to SH, but apparently do not have that capability.

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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. 1991. Iridoid glycosides, pp. 297–325, *in* G.A. Rosenthal and M.R. Berenbaum (eds.). Herbivores. Their Interactions with Secondary Plant Metabolites, Vol. I. Academic Press, San Diego.

BOROS, C.A., STERMITZ, F.R., and MCFARLAND, N. 1991. Processing of iridoid glycoside antirrinoside from *Maurandya antirrhiniflora* (Scrophulariaceae) by *Meris paradoxa* (Geometridae) and *Lepipolys* species (Noctuidae). *J. Chem. Ecol.* 17:1123–1133.

EHRLICH, P.R., and EHRLICH, A.H. 1961. How to Know the Butterflies. W.C. Brown Co., Dubuque, Iowa. pp. 132–140.

FODERARO, T.A. 1993. Chemical analysis of *Penstemon* and *Castilleja* (Scrophulariaceae), *Thessalia* (Lepidoptera) and *Pinus nigra* (Pinaceae). PhD dissertation. Colorado State University.

GARDNER, D.R. 1987. Iridoid chemistry of some *Castilleja* and *Besseya* plants and their hosted checkerspot butterflies. PhD dissertation. Colorado State University.

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.

HIGGINS, L.R. 1960. A revision of the melitaeine genus *Chlosyne* and allied species (Lepidoptera: Nymphalidae). *Trans. R. Entomol. Soc. London* 112:381–465.

L'EMPEREUR, K.M., and STERMITZ, F.R. 1990. Iridoid glycoside content of *Euphydryas anicia* (Lepidoptera: Nymphalidae) and its major host plant, *Besseya plantaginea* (Scrophulariaceae), at a high plains Colorado site. *J. Chem. Ecol.* 16:187–197.

MAYR, E. 1982. The Growth of Biological Thought. Harvard University Press, Cambridge, Massachusetts. pp. 600–606.

MEAD, E.W. 1992. Chemical investigation of a multispecies plant–insect biosystem and an novel *Arctomecon* alkaloid. PhD dissertation. Colorado State University.

MEAD, E.W., and STERMITZ, F.R. 1993. Content of iridoid glycosides in plant parts of *Castilleja integra* (Scrophulariaceae). *Phytochemistry* 32:1155–1158.

SCOTT, J.A. 1968. The life history and habits of *Chlosyne fulvia* (Nymphalidae). *J. Lepid. Soc.* 22:237–241.

SCOTT, J.A. 1986. The Butterflies of North America. Stanford University Press, Stanford, California, p. 300.

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A LONG-RANGE ATTRACTANT KAIROMONE FOR EGG PARASITOID *Trissolcus basalis*, ISOLATED FROM DEFENSIVE SECRETION OF ITS HOST, *Nezara viridula*

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Abstract—A short-chain α,β-unsaturated aldehyde, (*E*)-2-decenal, present in the defensive metathoracic gland of *Nezara viridula* (L.) (Heteroptera: Pentatomidae), stimulates a behavioral response in the egg parasitoid *Trissolcus basalis* (Woll.) (Hymenoptera: Scelionidae). Preliminary studies showed that *T. basalis* are attracted to an area containing adult *N. viridula*, but we also found that female *T. basalis* would examine and probe glass beads coated with an acetone extract of the metathoracic gland from males or females. Using this bioassay, the kairomone was isolated by bioassay directed by preparative gas chromatography and identified by NMR and mass spectrometry as (*E*)-2-decenal. The biological activity of the identified aldehyde was compared with analogs to determine specificity. An unstable *Z* isomer was found to be more active but not present in detectable or behaviorly relevant levels in the host, based on the bell-shaped dose–response curve of the two isomers. An investigation was also designed to determine if the *E* isomer was also responsible for the egg recognition kairomone activity previously reported. However, no 2-decenal isomers were detected in host egg extracts and the chemical characteristics of the 2-decenal isomers differ from the unidentified egg recognition kairomone. The role of the (*E*)-2-decenal in attracting female *T.*

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basalis to *N. viridula* was demonstrated in a Y-tube olfactometer; this alk-2-enal appears to act as a long-range kairomone orienting *T. basalis* to *Nezara* populations.

Key Words—*Trissolcus basalis*, *Nezara viridula*, Hymenoptera, Scelionidae, Heteroptera, Pentatomidae, kairomone, parasitoid, identification, defensive glands, host location, (*E*)-2-decenal.

INTRODUCTION

The importance of kairomones as chemical cues to orient parasitoids to their host has been demonstrated for an increasing number of species of parasitic *Hymenoptera* (Vinson, 1985). Parasitoids often use different kairomones at various stages of the host selection process, so that these chemicals serve as either long-distance attractants, searching stimulants, or host-recognition or host-acceptance factors. Several chemicals involved in the host selection process have been identified (reviewed by Jones, 1981; Kainoh, 1990) from plants, hosts, and host-related sources (food, shelter, associated organisms). The southern green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae) is an important worldwide pest (Clausen, 1978; Jones, 1988); therefore, we have been studying the chemical ecology of its principal solitary egg parasitoid *Trissolcus basalis* (Woll.) (Hymenoptera: Scelionidae). Bin et al. (1987) discussed the host selection behavior of *T. basalis* and reported that female parasitoids were attracted to adult *N. viridula* in an olfactometer. Sales et al. (1980) also reported that *T. basalis* responded to volatiles but from host egg masses in an olfactometer. Bin et al. (1993) provided evidence of a host-recognition factor in the adhesive that holds the eggs together in an egg mass, which elicits strong ovipositor probing by *T. basalis*. We report here the identification of a volatile in the defensive secretion of *N. viridula* that attracts *T. basalis* and the response of *T. basalis* to several structurally related compounds.

METHODS AND MATERIALS

Insect Cultures

The host *Nezara viridula* was reared in plastic cylindrical containers (15 × 20 cm) with screened lids, in an incubator at 28°C, 14:10 hr light–dark and 50% relative humidity, and fed on green beans and sunflower seeds. The colony was derived from individuals collected in soybean fields in south Texas.

The parasitoid *Trissolcus basalis* was maintained in glass test tubes (2 × 9 cm) with screen lids, provided daily with a mixture containing honey (10%), yeast (10%), benzoic acid (1%) in 100 g of sugar (Safavi, 1968). Egg masses of *N. viridula* were glued on strips of cardboard, exposed for parasitization to

three to four *T. basalis* females for 24 hr, and incubated until adult emergence (13–15 days) at 27°C, 14:10 hr light–dark, 70% relative humidity.

Chemical Procedures

Isolation of Kairomone. Adult *Nezara* were frozen at −20°C and their abdomens immediately dissected, leaving the metathoracic gland reservoir attached to the metasternite. The gland contents were collected from the isolated metathoracic reservoir by inserting a fine-pointed glass capillary into the reservoir and taking up the contents by capillary action. The gland contents from either 1 or 10 adults were pooled in a 100-μl cone-bottomed vial with spectral grade *n*-heptane as solvent. The sample was fractionated by preparative GC (Tracor 550) using a 1.83 mm ID glass column with 3% OV-101 on Chromosorb 750 (100–120 mesh) with temperature program from 45°C to 280°C, at 15°C/min. The metathoracic gland contents of one *Nezara* male were separated in seven fractions, each of which was collected by a glass capillary cold trap connected to a splitter at the detection port of the GC (Brownlee and Silverstein, 1968). The collected fractions were bioassayed and the separations and bioassay were repeated twice. Once an active GC fraction was determined, this fraction was reisolated from an extract of 10 *Nezara* male glands and further purified by injecting 25 μl of the corresponding active fraction onto 3% OV-17 on Chromosorb 750, using a similar glass column as above, at 115°C. Three subfractions were collected and bioassayed.

Kairomone Identification. The composition of the metathoracic gland contents of *N. viridula* has already been elucidated (Gilby and Waterhouse, 1965), so we compared the retention time of the single active purified fraction and our mass spectral data with the data of Gilby and Waterhouse (1965). Based on this comparison, we obtained synthetic (*E*)-2-decenal (ICN Biomedicals) and compared the chromatographic, mass, and NMR spectroscope properties with our active fraction. We also conducted and compared bioassay results with both the natural and synthetic material. Since later studies suggested that (*Z*)-2-decenal might be present but unstable, we compared the chromatograms of both (*Z*)- and (*E*)-2-decenal with the chromatograms of the contents of the metathoracic gland removed from live males and immediately injected into the GC.

Synthesis of Analogs. To determine the specificity of the response of *T. basalis* to (*E*)-2-decenal, several analogs were synthesized or purchased and then tested. Six analogs [(*E*)-2-hexenal, *n*-octenal, (*E*)-2-nonenal, *n*-decanal, (*E*)-2-*n*-undecenal, *n*-undecanal] were obtained from commercial sources. The (*E*)-2-decenol was prepared from (*E*)-2-decenal by reaction with excess $NaBH_4$ in methanol at 0°C. The ethyl (*E*)-2-decenoate was prepared by the reaction of triethyl phosphonoacetate with octanal in benzene (Wadsworth and Emmory, 1973). The distilled product was purified by preparative gas chromatography

before testing. (*E*)-2-Decenoic acid was prepared from the ester by reaction with KOH in methanol for 20 min. The solution was washed with ether, acidified, and the acid was extracted with ether.

The *Z* isomer of 2-decenal was prepared as follows: 1-nonyne (4.5 g, 0.036 mol) in 20 ml ether was treated with 0.04 mol (30 ml 1.5 M) CH_3Li. The mixture was stirred for 1 hr, then formaldehyde (3.3 g, 0.11 mol) prepared by heating paraformaldehyde, was bubbled into the solution at 0°C. The solution was poured into 5 vol of water, extracted with two 20-ml portions of ether, and the combined ether extracts were dried over Na_2SO_4, rotary evaporated, and flash distilled (airbath 120°C, 10 mm Hg) to yield 2.4 g (42%) of 2-decyne-1-ol.

A solution of 200 mg (0.0013 mol) of the alcohol in 10 ml of ethyl acetate with 100 mg of 5% palladium on calcium carbonate poisoned with lead was stirred under 1 atm of H_2 gas until the theoretical amount of H_2 had been taken up. The solution was filtered through Celite, rotary evaporated, and treated with 0.4 g (0.002 mol) pyridinium chlorochromate in 4 ml CH_2Cl_2 (Corey and Suggs, 1975). Clean up was conducted in the usual manner followed by final purification using preparative gas chromatography on a 6 ft × 0.4 mm ID glass column packed with 3% SP2340 on Chromosorb G-AW to give material for testing. Approximately 60% isomerization to the *E* isomer took place during the oxidation procedure.

Dose Response. Once the active material was identified, the parasitoids response to different doses of both (*Z*)- and (*E*)-2-decenal and other isomers were determined. Five 10-fold dilutions of (*E*)- and (*Z*)-2-decenal were first bioassayed beginning at 1 $\mu g/\mu l$ in acetone. For the other analogs, bioassay began at 0.01 $\mu g/\mu l$ in acetone using a minimum of ten *T. basalis* females. Five microliters of solution were applied to the three glass beads as described below and the same four behaviors were recorded and evaluated utilizing a minimum of 15 *T. basalis* females.

Because the (*Z*)-2-decenal was unstable, we synthesized, purified, and bioassayed samples containing this isomer within 2 hr of the final synthetic step. Five stock solutions were prepared as follows: 1 $\mu g/\mu l$ (*E*)-2-decenal, 0.1 $\mu g/\mu l$ (*Z*)-2-decenal, 1.08 $\mu g/\mu l$ *E* + 0.27 $\mu g/\mu l$ *Z* (80% *E* to 20% *Z* isomer); 0.675 $\mu g/\mu l$ *E* + 0.675 $\mu g/\mu l$ *Z* (50% *E* to 50% *Z* isomer); and 0.27 $\mu g/\mu l$ *E* + 1.08 $\mu g/\mu l$ *Z* (20% *E* to 80% *Z* isomer). Each were 10-fold serially diluted eight times and 10 μl were applied to three glass beads as described, but only those eliciting antennation and ovipositor unsheathing were recorded. This test was replicated twice with five females per dose. After each bioassay, a stock sample applied to three glass beads as described and held during the bioassay period was extracted and injected into the GC to confirm the isomers in the ratios applied. The results were expressed as the percent of females exhibiting ovipositor unsheathing.

General Bioassay Procedures

Glass Bead Bioassay. Bioassays were conducted in a flat-bottomed depression slide (17 mm diam. × 3 mm deep), covered with a glass cover slip to provide a chamber. Artificial eggs consisting of 1.00-mm-diam. glass beads were cleaned with acetone and oven dried at 150°C for 10 min. The clean glass beads were coated with solvent (control), extract or solution (tests), by placing three beads in a small watch glass with 5 or 10 μl of solvent, extract, or a test fractions. Beads were rolled in the solvent until dry. The three coated beads were placed in the observation chamber on a 10 × 10-mm Parafilm square (American National Can, Greenwich, Connecticut 06836) to hold them in place. Parasitoid females, 72–96 hr after emergence, which had not been exposed to hosts, were used for all bioassays. Each test consisted of 9–15 observations per treatment. Responses were recorded for 10 min after releasing one female into the arena. For all the bioassays, except those involving (*Z*)-2-decenal, responses were recorded with a video camera (RCA model 2000, RCA Video Equipment, New Holland Av., Lancaster, Pennsylvania 17604) connected to a black and white monitor (RCA model TC1110). The duration and frequency of behaviors were recorded using an IBM personal computer XT model 286, programmed as an event recorder with "The Observer" installed as a software package for behavioral research (Noldus, 1991). From the observations, the following behaviors were recorded: (1) encounter: the wasp touched or walked over the beads even if it left in a few seconds; (2) antennation: upon encounter the wasp stopped and examined the beads with its antennae; (3) ovipositor unsheathing: the antennating wasp unsheathed her ovipositor; and (4) acceptance: the female attempted to drill the bead using her ovipositor. After a female was introduced into the arena, the number of times (frequency) she performed each of the four behaviors described and the duration (in seconds) of the period she spent antennating the beads (behavior 2) were recorded.

In tests comparing (*Z*)- and (*E*)-2-decenal, 10 μl of solvent was applied to a group of three glass beads as described, but only the number of females both antennating and unsheathing their ovipositor was recorded. Recording of data was interrupted after 5 min if no encounter occurred or after 5 min of the first encounter or upon acceptance.

Olfactometer Studies. The olfactometer consisted of a 4.0-cm-ID glass Y tube, with the 15-cm-long Y arms connected to a glass sample chamber and a 10-cm common stem. The air flow (100 ml/min) passed through activated charcoal and water before entering sample chambers. Two white neon lamps were placed on the two sides of the system. Ten microliters of a chemical solution applied to rubber septa were placed in one arm of the olfactometer. Solvent treated septa were used as control in the other arm. In studies with (*E*)-2-decenal the concentration used was 0.1 μg/μl in acetone. New septa were used each

time. In each test one *T. basalis* female was placed at the beginning of the common stem and the percentage time spent and the number of times that the insect walked in each arm, during 10 min of bioassay, were measured with the video camera and computer as described above. Fifteen replications, alternating the control arm, were also performed in order to test possible influence of uneven light or other environmental conditions.

Comparison with Egg Recognition Kairomone

Since we used glass beads as a convenient bioassay, we wanted to determine if the volatile kairomone isolated from adult *Nezara* was responsible for the egg recognition behavior reported by Bin et al. (1993) and, if not, to compare the two kairomones (see Discussion for more details concerning the egg recognition kairomone). Bin et al. (1993) demonstrated that a host-recognition kairomone is present in the adhesive covering the eggs, which is used by *N. viridula* to glue the egg mass onto the leaf surface. We determined the presence of the two isomers of 2-decenal in the adhesive. An egg extract was prepared by soaking newly laid eggs in acetone (1 μl/egg, for at least 12 hr) as described by Bin et al. (1993). Ten microliters of the sample was bioassayed for biological activity using glass beads as described above and 100 μl of an active extract was applied to a thin-layer chromatography (TLC) plate (Whatman Kc 18 F, chemically bonded reverse-phase) and developed with 100% acetone as mobile phase. One hundred microliters of (*E*)-2-decenal (the *Z* isomer was not stable enough for this procedure) at 1 μg/μl in acetone was applied to a different lane of the TLC plate to compare the R_f values. The separation was visualized using iodine or long wavelength UV light.

Since (*E*)-2-decenal could be present in the egg but not accumulate in the adhesive, extracts of eggs were prepared from an egg mass (1 μl acetone/egg). Solutions were bioassayed and equivalent active concentrations of acetone egg extract and (*E*)-2-decenal were compared by GC on 3% OV-101 as described. The solvent and fractions from the GC were collected and bioassayed. Equivalent active concentrations were also applied to reverse-phase thin-layer plates and developed in acetone (13 cm). After separation, the plate was divided into 1-cm strips, the plate material was removed from the support with a razor blade, extracted in acetone, and bioassayed.

Egg extracts were also prepared by soaking the eggs in heptane or methylene chloride (24 hr, 1 μl of solvent/egg) or macerating (1 hr, 1 μl solvent/egg). The extracts were immediately injected on a 3% OV-101, Chromosorb 750 column, temperature programmed from 45°C to 280°C at 15°C/min, for the detection of (*E*)- or (*Z*)-2-decenal.

Statistical Analysis

The behavior parameters were analyzed by Kruskall-Wallis ANOVA in order to test the overall variability (comparison with egg recognition kairomone and with analogs) and by the Mann-Whitney U test for significant differences in pairwise comparisons (dose–response test and antennation time in the comparison with analogs) (Siegel, 1956). Bonferroni inequality was applied to make the Mann-Whitney U test reliable for multiple comparisons (Harris, 1975). In the olfactometer bioassay Friedman two-way ANOVA was used (Siegel, 1956).

RESULTS

Previous research (Bin et al., 1987; Sales, 1979) had demonstrated that adult *Nezara viridula* release volatiles that attract female *T. basalis* in an olfactometer. Our preliminary studies indicated the responsible volatiles were associated with the metathoracic glands of adults; in fact, we found that *T. basalis* would respond by rapid location (encounter), antennation, ovipositor extrusion, and even ovipositor drilling (acceptance) of glass beads treated with the diluted contents (0.02–0.001 female equivalents) of the metathoracic gland. Through gas-chromatographic separation and bioassay using glass beads, we found one peak (fraction 5) eliciting equivalent activity to the crude metathoracic gland extract (Figure 1). All other fractions were inactive. Comparison of our gas-chromatographic patterns with published data (Gilby and Waterhouse, 1965) on the identity of the contents of the metathoracic gland of *Nezara viridula* indicated (*E*)-2-decenal as the kairomone responsible for the observed behavior of *T. basalis*. Synthetic (*E*)-2-decenal was found to elicit the same behavior of the crude metathoracic gland extract and had identical chromatographic, NMR, and mass spectral characteristics as the active fraction (Figure 1).

At a concentration less than 5×10^{-4} μg, less than 20% of the females responded with encounters and antennation to (*E*)-2-decenal, while between 5×10^{-3} and 5×10^{-1} μg provided the best response (Figure 2). At 5 μg applied, none of the females unsheathed their ovipositor, but they moved away from the treated beads and there was an increase in preening. Also from Figure 2 it appears that females respond to the different dose-treated beads by increasing their encounter frequency, antennation frequency, and antennation time rather than their frequency of ovipositor unsheathing or acceptance.

Figure 3 shows the response of *T. basalis* to nine of the analogs of (*E*)-2-decenal initially available at 0.01 μg/μl applied. Of these, (*E*)-2-decenoic acid ethyl ester resulted in a prolonged antennation of the few females that responded. While (*E*)-2-decenal resulted in an encounter frequency of over 50, encounter frequencies between 20 and 30 were obtained with all other compounds. Both the 2-decenoic acid ethyl ester and the 2-decenoic acid elicited antennation equal

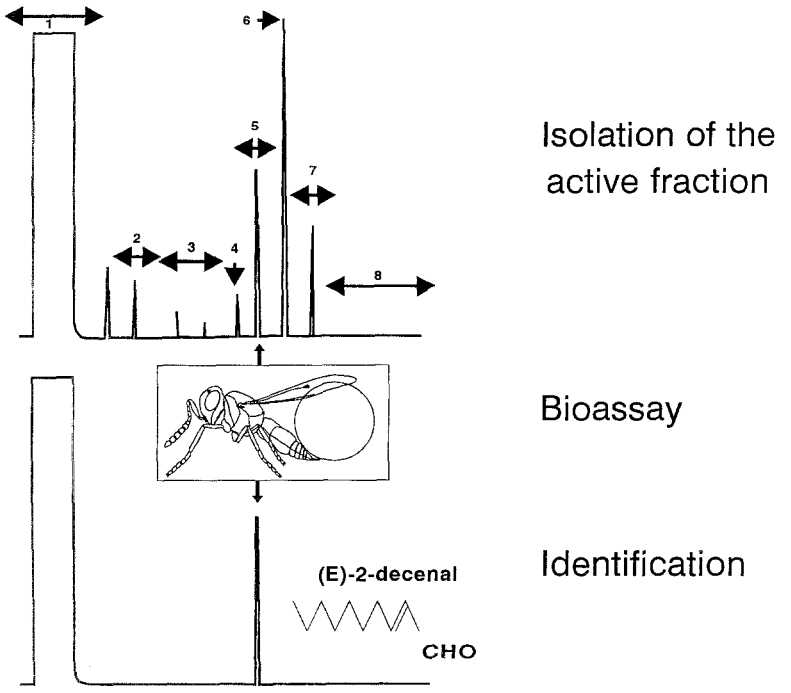

FIG. 1. General scheme for isolation and identification of (*E*)-2-decenal through gas chromatographic separation and a glass bead bioassay: of the fractions obtained by preparative GC from the crude metathoracic gland extract, only fraction 5 elicited acceptance behavior in the wasp.

to (*E*)-2-decenal at 0.01 $\mu g/\mu l$ (Figure 3), but showed no activity at lower doses. Furthermore, none of these compounds was detected in active samples.

(*Z*)-2-Decenal is unstable, converting to the *E* isomer at room temperature over a period of a day. Using freshly synthesized (*Z*)-2-decenal, we found that *T. basalis* is one order of magnitude more sensitive to (*Z*)-2-decenal. Combinations of *Z* and *E* are presented in Figure 4 along with the data of both isomers presented separately on glass beads. Activity of combinations appeared to be additive, the reduction of activity of the (*E*)-2-decenal at higher concentration if *Z* is due to repellency of the higher concentration of the *Z* or the combination.

A comparison of the (*E*)-2-decenal with egg extracts (the host recognition kairomone isolated in acetone from eggs), indicated that both solutions elicited all the behaviors associated with host recognition (Figure 5). The number of encounters is not significantly different for either solution, but the wasps showed a greater response for the egg extract; in fact, antennation time and ovipositor extrusion are significantly higher than for (*E*)-2-decenal or control. Antennation

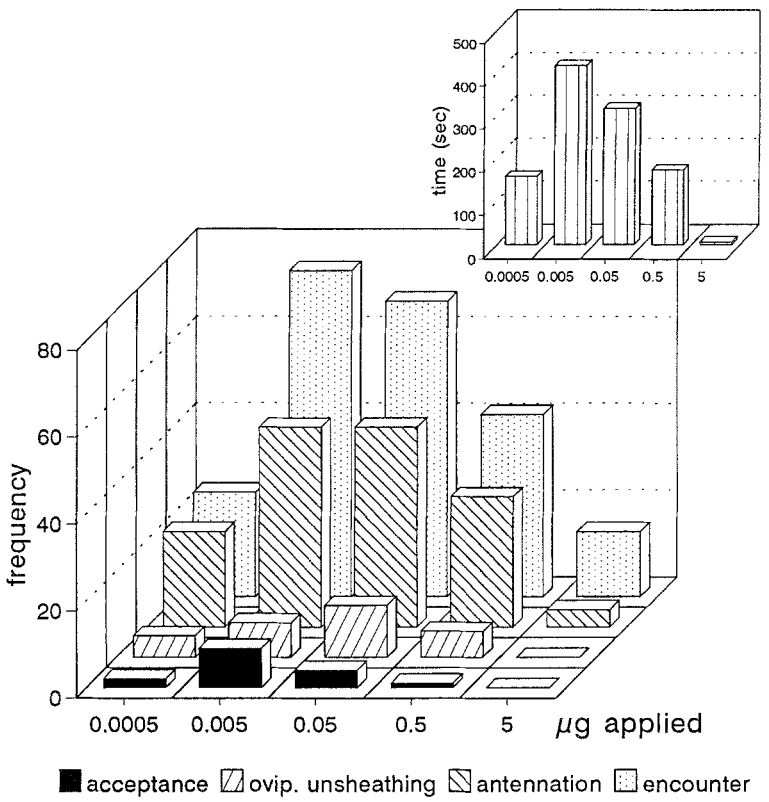

FIG. 2. Frequency of response of female *T. basalis* to different doses of (*E*)-2-decenal applied to glass beads (Kruskall-Wallis ANOVA, $P > 0.05$). The insert figure shows the antennation time (seconds) of females to glass beads treated with different doses.

frequency and acceptance of both kairomone-containing solutions are significantly higher than the solvent, but not different from each other.

(*E*)-2-Decenal or (*Z*)-2-decenal were not detected in heptane extract of egg masses or in active acetone extract of egg masses by gas-chromatographic analysis. We were also unable to detect (*Z*)-2-decenal in the metathoracic gland of either males or females. A response to the GC acetone egg extract was found near the solvent front, while all the analogs of (*E*)-2-decenal eluted much later. Thin-layer chromatography of (*E*)-2-decenal and the active acetone extract of *Nezara* egg revealed only one active band for each, but of different R_f values. These results suggest that the (*E*)-2-decenal is not responsible for the egg recognition activity and, if present on egg masses, it occurs below detectable levels.

The response of female *T. basalis* to (*E*)-2-decenal in a Y-tube olfactometer

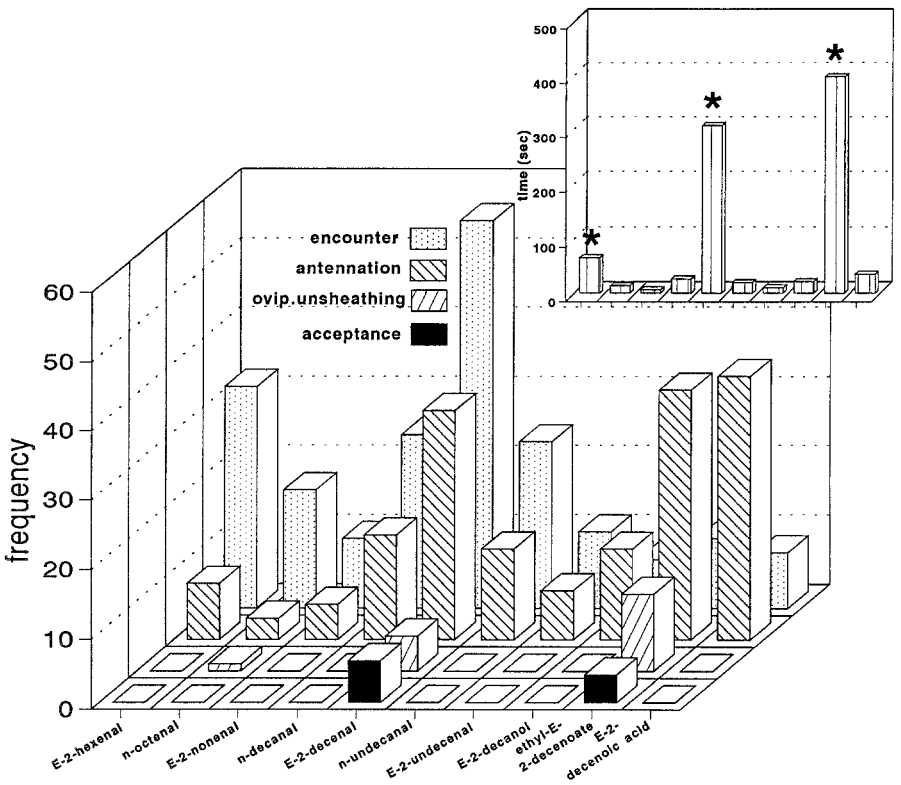

FIG. 3. Response of *T. basalis* to structurally related compounds at 0.01 $\mu g/\mu l$ applied. (*E*)-2-Decenal scored the highest frequency of the four recorded behaviors (the overall variability was tested by Kruskall-Wallis ANOVA at $P > 0.05$). The Mann-Whitney paired comparison showed significant different antennation time for (*E*)-2-hexenal, (*E*)-2-decenal, and ethyl (*E*)-2-decenoate ($*P = 0.05/9 = 0.005$).

(Figure 6) revealed that while the number of times that the wasp walked into the two arms was the same, they spent considerably more time in the (*E*)-2-decenal contaminated air (Friedman two-way ANOVA, $P > 0.01$).

DISCUSSION AND CONCLUSIONS

Several authors (Sales, 1979; Sales et al., 1980; Bin et al., 1987) have provided evidence that *T. basalis* responds to volatiles released by host adults and egg masses. The results of this study demonstrate that (*E*)-2-decenal from the metathoracic gland of *Nezara viridula* is responsible for this attraction and

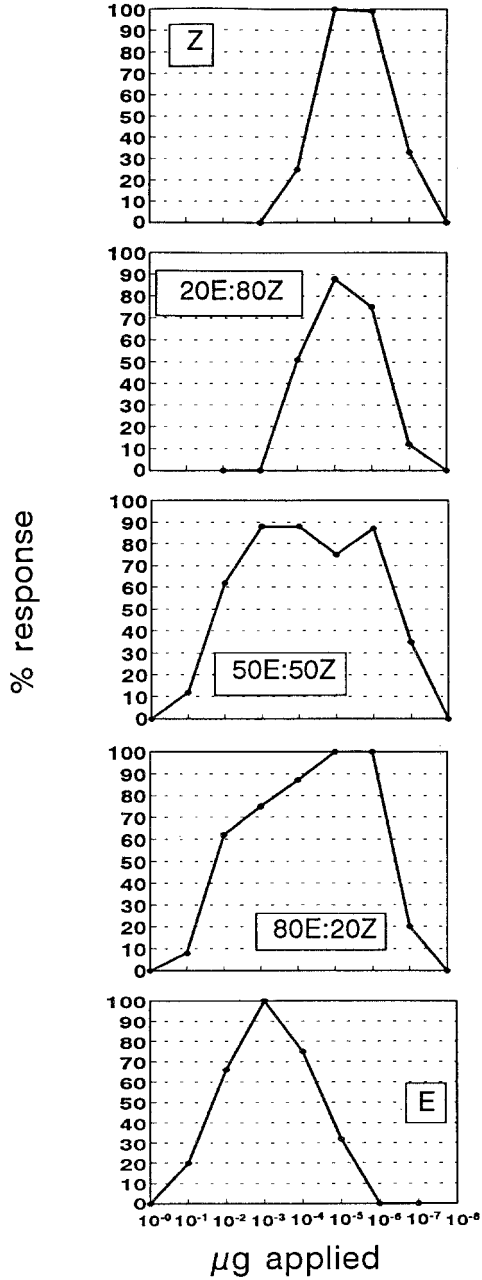

FIG. 4. Response of *T. basalis* to the two isomeric forms of 2-decenal: (*Z*)-2-decenal was found to be active at lower concentrations than (*E*)-2-decenal. The composition in of the mixture is given in the box in each graph.

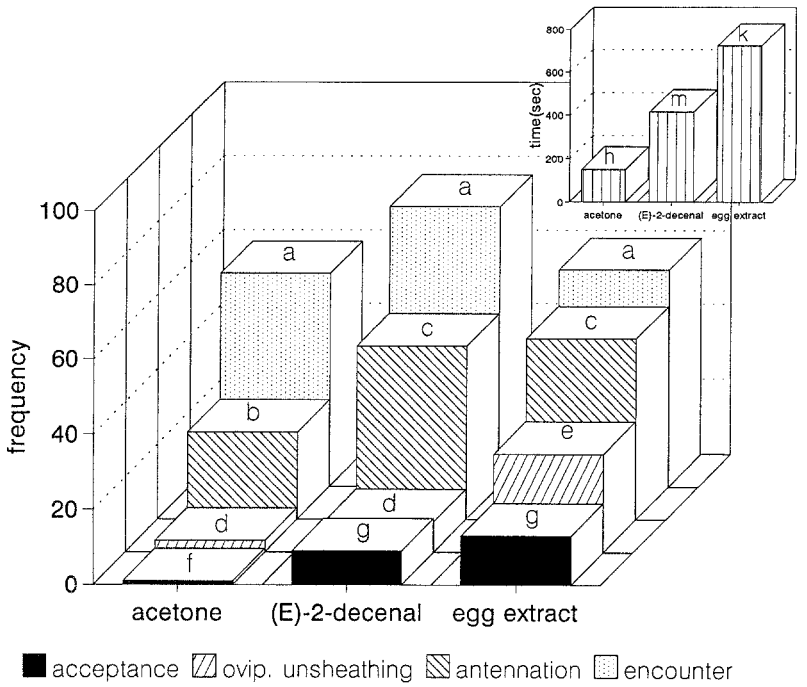

FIG. 5. Comparison of the responses of *T. basalis* between (*E*)-2-decenal and the kairomonally active egg adhesive. Acetone was used as control treatment (Mann-Whitney U test, applying Bonferroni inequality to one-tailed significance level. Different letters mean significant differences at $P = 0.05/3 = 0.017$). The insert figure shows the antennation time (seconds) of females to glass beads treated with the different extracts.

aggregation to adult contaminated areas. None of the other gas-chromatographic regions of the chromatograph eluent elicited activity. Furthermore, since we found that between 0.02 and 0.001 female equivalents were active in our bioassay (each female yielded between 1 and 2 μl of secretion), along with the information from Gilby and Waterhouse (1965) that (*E*)-2-decenal comprises 25.7% of the gland contents, our results with dilutions of pure (*E*)-2-decenal are comparable, suggesting that the other components of the gland are not only inactive, but they are not synergistic. The discovery that *T. basalis* were more sensitive to (*Z*)-2-decenal and the report that traces of the *Z* isomer are present in the defensive secretion of *Nezara viridula* (Gilby and Waterhouse, 1965) suggested the possibility that *T. basalis*, after reaching the community of adult *Nezara*, might respond to females due to the presence of *Z* isomer. However, we were unable to detect (*Z*)-2-decenal at a behaviorally significant level in the defensive secretion of female *Nezara*.

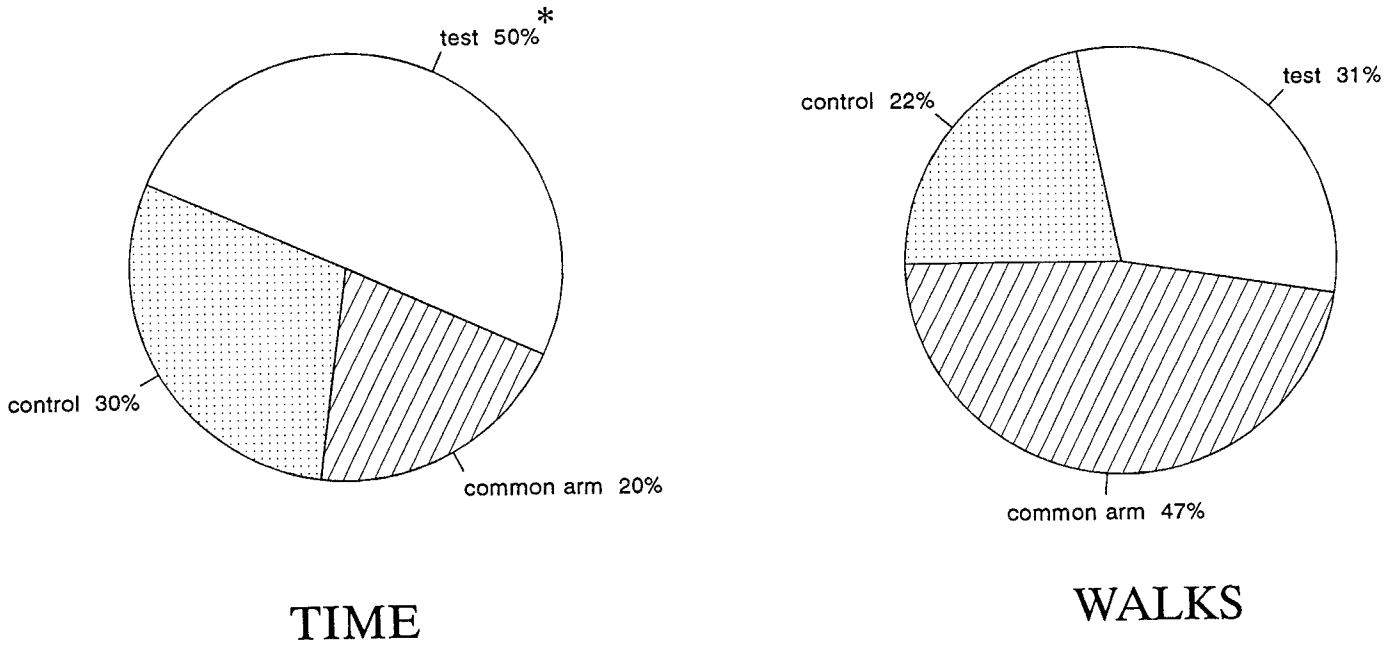

FIG. 6. A chart showing the response of female *T. basalis* in a Y-olfactometer to (*E*)-2-decenal. The "walks" chart shows the number of times a female entered the two arms. The "time" chart shows that females spend significantly more time in (*E*)-2-decenal-contaminated air (Friedman two-way ANOVA, $P < 0.01$).

Although we used glass beads to emulate eggs as a simple and convenient bioassay in the purification process, neither (*Z*)- or (*E*)-2-decenal appears to be present on eggs in sufficient quantities to elicit host-recognition behavior. Furthermore, the activity of an active host recognition extract isolated from eggs (Bin et al., 1993) has a different TLC R_f value than (*Z*)- or (*E*)-2-decenal, and the GC retention times differ. Thus, there appear to be at least two compounds involved in host selection by *T. basalis*. One functions in the possible attraction and aggregation of *T. basalis* female in areas where stink bug adults occur. The second unidentified kairomone appears to be associated with the adhesive used to glue the eggs together and to the substrate on which they are laid (Bin et al., 1993).

The results demonstrate also that the females respond in a dose-dependent way to (*E*)-2-decenal, which is one of the most toxic chemicals within the defensive secretion of *Nezara viridula* (Gilby and Waterhouse, 1965) and it is not tolerated by many insects at high concentrations (Blum, 1981).

T. basalis has a wide host range (14 genera among Pentatomidae and Scutelleridae) (Cumber, 1964; Orr, 1988): it would be interesting to investigate whether (*Z*)-2-decenal is present in any of these species or whether (*E*)-2-decenal is the only attractive compound present in the defensive secretions of all these species. When examining the response of *T. basalis* to related compounds of (*E*)-2-decenal, both 2-decenoic acid and its ethyl ester elicited strong antennation. These compounds were not detected on eggs or in the glue, but they may play a role in the recognition of other species. Aldrich (1988), reported that shield bugs (Heteroptera: Scutelleridae) resemble stink bugs in the arrangement and chemistry of their metathoracic glands and larval dorso abdominal glands. (*E*)-2-Decenal and 4-oxo-(*E*)-2-hexenal are common secretory components. Whether the response of *T. basalis* to Scutelleridae is only due to the (*E*)-2-decenal or also to 4-oxo-(*E*)-2-hexenal would be of interest.

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REFERENCES

ALDRICH, J.R. 1988. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol.* 33:211–238.

BIN, F., VINSON, S.B., and COLAZZA, S. 1987. Responsiveness of *Trissolcus basalis* (Woll.) (Hym: Scelionidae) female to *Nezara viridula* (L.) (Het: Pentatomidae) in an olfactometer. Parasitoids insect. *Colloq. INRA* 48:15–16.

BIN, F., VINSON, S.B., STRAND, M.R., COLAZZA, S., and JONES, W.A. 1993. Source for an egg

kairomone for *Trissolcus basalis*, a parasitoid of *Nezara viridula*. *Physiol. Entomol.* 18(1): 7–15.

BLUM, M.S. 1981. Chemical Defenses of Arthropods. Academic Press, New York.

BROWNLEE, R.G., and SILVERSTEIN, R.M. 1968. Micro-preparative gas-chromatograph and modified carbon skeleton determinator. *Anal. Chem.* 13:2077–2079.

CLAUSEN, C.P. 1978. Introduced parasites and predators of arthropod pests and weeds: A world review. USDA Agriculture Handbook 480. pp. 24–26.

COREY, E.I., and SUGGS, J.W. 1975. Pyridinium chlorochromate. An efficient reagent for oxidation of primary and secondary alcohols to carbonyl compounds. *Tetrahedron Lett.* 1975:2647–2750.

CUMBER, R.A. 1964. The egg parasite complex (Scelionidae: Hymenoptera) of shield bugs (Pentatomidae, Acanthosomidae: Heteroptera) in New Zealand. *N.Z. J. Sci.* 7:536–554.

GILBY, A.R., and WATERHOUSE, D.F. 1965. The composition of the scent of the green vegetable bug, *Nezara viridula*. *Proc. R. Entomol. Soc. B*, 162:105–120.

HARRIS, R.J. 1975. A Primer of Multivariate Statistic. Academic Press, New York. p. 98.

JONES, R.L. 1981. Chemistry of semiochemicals involved in parasitoid–host and predator–prey relationship, pp. 921–927, *in* D.A. Nordlund, R.L. Jones, and W.J. Lewis (eds.). Semiochemicals: Their Role in Pest Control. John Wiley & Sons, New York.

JONES, W.A. 1988. World review of the parasitoids of the green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae). *Ann. Entomol. Soc. Am.* 75(2):262–273.

KAINOH, Y. 1990. Chemicals controlling the behavior of parasitoids and predators. *In* The Use of Natural Enemies to Control Agricultural Pests. FFTC Book Series 40. pp. 212–221.

NOLDUS, L.P.J.J. 1991. The Observer: A software system for collection and analysis of observational data. *Behav. Res. Methods, Instr. Comput.* 23(3):415–429.

ORR, D.B. 1988. Scelionid wasps as biological control agents: A review. *Fla. Entomol.* 71(4):506–528.

SAFAVI, M. 1968. Etude biologique et écologique des Hyménoptères parasites des oeufs des punaises des céréales. *Entomophaga* 13:381–495.

SALES, F.M. 1979. Responsiveness and threshold for host-seeking stimulation of the female *Trissolcus basalis* (Woll) by the eggs of the host *Nezara viridula* (L.) *Fitossanidade* 3:36–39.

SALES, F.M., MCLAUGHLIN, J.R., and SAILER, R.I. 1980. Quantitative analysis of the behavior patterns of the female *Trissolcus basalis* (Wollaston) when stimulated by the kairomonal extract of the host, *Nezara viridula* (L.) *Fitossanidade, Fortaleza* 4(1):43–50.

SIEGEL, S. 1956. Nonparametric Statistics for the Behavioral Science. McGraw-Hill, New York.

VINSON, S.B. 1985. The behavior of parasitoids, pp. 417–469, *in* G.A. Kerkut and L.I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol 9. Pergamon Press, Elmsford, New York.

WADSWORTH, W.S., and EMMORY, W.D. 1973. Pages 547–549, *in* H.E. Baumgartner (ed.). Organic Syntheses Colloquium, Vol. V. John Wiley and Sons, New York.

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SOYBEAN PHYTOALEXIN, GLYCEOLLIN, PREVENTS ACCUMULATION OF AFLATOXIN B_1 IN CULTURES OF *Aspergillus flavus*

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Abstract—The soybean phytoalexin, glyceollin, suppresses the accumulation of aflatoxin B_1 in cultures of *Aspergillus flavus*. At concentrations of 6.25 μg/ml and 62.5 μg/ml, glyceollin causes 70% and 95% decreases in the maximum observed levels of aflatoxin B_1, respectively. In contrast to the dramatic effect on aflatoxin B_1 levels, these concentrations have little effect on fungal growth. For example, at 62.5 μg/ml in liquid culture, glyceollin causes a barely discernible lag in the beginning of growth and a 11.5% decrease in maximum fungal mass. When the same concentration of glyceollin is added to the colony margin on semisolid medium, an inhibition zone is formed and then overgrown in one day. Glyceollin appears to act by inhibiting aflatoxin B_1 synthesis, since the rate of aflatoxin B_1 breakdown is not increased in fungal cultures that have been grown in the presence of glyceollin. Glyceollin does accumulate in viable soybean seeds that have been infected with *Aspergillus flavus*. Such seeds accumulate aflatoxin B_1 at one-third the rate of non-glyceollin-producing, nonviable seeds. These results suggest that the synthesis of glyceollin in infected seeds may explain, at least in part, why aflatoxin contamination of soybeans is not a common problem.

Key Words—*Aspergillus flavus*, fungus, aflatoxin, mycotoxin, phytoalexin, glyceollin.

INTRODUCTION

The aflatoxins are toxic, secondary metabolites produced by strains of the fungi *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare. These compounds

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are both acutely toxic and carcinogenic when consumed by animals. One of the aflatoxins, aflatoxin B_1, is the most potent, naturally occurring carcinogen known (Squire, 1981). While the aflatoxins have the potential to contribute to human health problems, presently, most concerns about aflatoxin contamination in the United States arise from deleterious effects on the productivity of domestic animals (Hamilton, 1987; Pier, 1987). Such animals directly consume those grains and/or oilseeds and commercial feeds most commonly contaminated with aflatoxin. While the aflatoxins occur as natural contaminants in a variety of agricultural commodities worldwide, they are most commonly associated with maize, peanuts, cotton seed, and tree nuts in the United States. Although aflatoxin contamination may result from postharvest infection of grains and/or oilseeds or processed feeds held under improper storage conditions, it more commonly occurs preharvest in the field (Payne, 1992).

In much of the United States, soybeans are grown during the same cropping season as maize. While soybean seeds readily support the growth of *A. flavus* (Abdullah et al., 1984; Pinto et al., 1991; and this study), contamination of soybean seeds by aflatoxin does not appear to be a common problem (Smith and Moss, 1985). Of course, the absence of aflatoxin in soybean seeds could result from differences in seed development between maize and soybeans that prevent infection by *A. flavus*, differences in the way the two crops are managed on the farm or differences in schemes of grain handling after harvest. Alternatively, soybean seeds may possess molecular mechanisms that prevent the accumulation of aflatoxin. In this paper, we report that the soybean phytoalexin, glyceollin, which is made in response to seed infection by *A. flavus*, suppresses accumulation of aflatoxin B_1 at concentrations where the glyceollin has only a transient effect on the growth of the fungus.

METHODS AND MATERIALS

Fungal Strain and Plant Material. Aspergillus flavus, strain 5337, was provided by NCAUR, Peoria, Illinois. Conidia were stored in sterile lyophile tubes. Working cultures were prepared by inoculating PDA slants (Tuite, 1969) with conidia and incubating the cultures at 26°C until the slants were covered with conidia. Slants were stored at 4°C until used. Conidial suspensions were prepared by rinsing spores from the agar surface with a solution of 0.01% (v/v) Triton X-100 in sterile water.

Soybean seeds [*Glycine max* (L.) Merr. cv. Williams 82] were purchased from Missouri Seed Improvement Association. Seeds had a moisture content of 7.6% as determined by drying to a constant weight in an oven at 60°C and a germination rate of 96%. Seeds of cv. Clark 63 (7.7% moisture, 94% germination rate) were provided by Dr. M. Brown, Plant Pathology Department,

University of Missouri. Heat-killed seeds (0% germination) were prepared by heating seeds at 60°C for two days.

Inoculation of Soybean Seeds. Lots of 200 seeds each were surface-sterilized by treatment with 1.2% (v/v) sodium hypochlorite for 5 min followed by washing with three volumes of sterile, deionized water. Seed moisture averaged 10% by weight after sterilization. Seeds were placed in a sterile flask and inoculated with 1×10^3 conidia suspended in enough sterile distilled water to increase the seed moisture content to 21% by weight. Flasks containing the inoculated seeds were placed on a shaker (28°C, 120 rpm) overnight to equilibrate. Seeds were removed from the flasks and placed in sterile Petri dishes, each containing two pieces of sterile Whatman No. 1 filter paper moistened with 1.5 ml of deionized water. Petri plates were placed in an incubator at 28°C. Uninoculated, viable seeds germinated during the incubation period. In these cases only the whole plant was extracted for glyceollin and aflatoxin measurements.

Fungal Cultures. Fungus was cultured on a glucose–salts (N5) medium (Doyle and Marth, 1978), which prevents conidia formation. Typically, 20-ml aliquots of medium were placed in 125-ml Erlenmeyer flasks and inoculated with 0.4 ml of a suspension containing 1×10^8 conidia/ml in sterile distilled water. Cultures were grown stationary or in a rotary shaker (100 rpm) at 28°C. In experiments where the effect of glyceollin on aflatoxin accumulation was measured, glyceollin dissolved in 95% (v/v) ethanol was added to the sterile, cooled medium and an equal volume of ethanol was added to control flasks.

Recovery and Measurement of Glyceollin and Aflatoxin. Glyceollin was extracted from soybean seeds and glyceollin concentrations measured as described previously (Abdullah et al., 1984; Karr et al., 1992). Quantitation of residual glyceollin in fungal mycelium and culture filtrate was carried out coincident with measurement of aflatoxin B_1 (AFB_1) except that glyceollin was measured by A_{280}.

For quantitation of AFB_1 in inoculated soybean seeds, 10-g samples of seeds were taken from each treatment at two-day intervals, dried for two days in an oven at 60°C, and ground in a grinder (type A10 S1, Janke and Kunkel, Breisgau, Germany). A 4-g aliquot of the meal was extracted with 10 ml of acetone in a blender at medium speed for 5 min. Solvent was removed and the sample was fractionated and AFB_1 quantitatively measured after HPLC as described previously (Abdullah et al., 1984) except that the column was eluted with 60% (v/v) methanol to separate AFB_1 from interfering compounds. In the case of quantitation of AFB_1 in mycelium from liquid culture, the mycelial mats were collected on two layers of cheesecloth, oven dried for two days at 60°C and extracted using the procedure described for seeds. Aflatoxin was recovered from the culture filtrate by adjusting the filtrate pH to 6.5 and partitioning two times against two volumes of chloroform. The chloroform was removed on a

rotary evaporator at 45°C under vacuum and the aflatoxin was dissolved in 2 ml of methanol and quantitated after HPLC as described previously (Stubblefield and Shotwell, 1977). AFB_1 was routinely confirmed by conversion to AFB_{2a} and demonstration of chromatographic identity with authentic AFB_{2a} after HPLC (Stubblefield and Shotwell, 1977) or TLC (Przybylski, 1975).

Preparation of Glyceollin. Glyceollin was isolated from sliced, germinating soybean seeds challenged with an incompatible bacterium known to elicit high levels of glyceollin accumulation (unpublished results of this laboratory). Surface-sterilized, germinating seeds were sectioned into 2- to 3-mm-thick slices and inoculated with *Pseudomonas syringae* pv. *tabaci* (1×10^7 cells per gram fresh weight of seed slices) and incubated at 21°C in the dark. After five days, glyceollin was isolated as described previously (Yashikawa et al., 1978), except that hexane–ethyl acetate–methanol (60:40:5 v/v) and 2-mm Silica Gel 60, F_{254} plates (E. Merck) were used for the preparative TLC step. The glyceollin-containing fractions eluted from the TLC plates were combined and further purified by HPLC by elution from a C_{18}-semipreparative column (6.5 × 250 mm, Alltech Associates, Deerfield, Illinois) with 70% (v/v) aqueous methanol. Fractions that contained glyceollin were pooled and the solvent removed, under vacuum, at 45°C on a rotary evaporator. Glyceollin was dissolved in ethanol and stored at −20°C. Manipulations with methanol solutions of glyceollin were carried out in the dark. Glyceollin was confirmed as described previously (Karr et al., 1992). Glyceollin prepared in this way contained a mixture of the glyceollin isomers I, II, and III in a ratio of 4:2:1. Authentic glyceollin was kindly supplied to us by Dr. N. Keen, University of California-Riverside, and Dr. J. Paxton, University of Illinois.

Immunochemical Methods. Antibodies to AFB_1 were prepared in rabbits to bovine serum albumin conjugated aflatoxin B_{2a} and RIA assays conducted as described previously (Lau et al., 1981; Chu, 1984; Fremy and Chu, 1989). Antiserum to *A. flavus* was kindly proved by Dr. J. Wallin, Plant Pathology, University of Missouri-Columbia. Rocket immunoelectrophoresis (RIE) was carried out as described previously (Weeke, 1973). Samples for RIE were prepared by grinding soybean seeds with phosphate-buffered saline (PBS, 0.05 M sodium phosphate and 0.1 M sodium chloride; 10 ml/10 g of seeds) in a blender at high speed. The extract was subjected to centrifugation at 8000 *g* and the supernatant liquid applied to the sample well of the RIE plate. Total fungal antigen was reported as rocket height in centimeters of the most abundant antigen. The most abundant antigen was demonstrated to be immunochemically the same at all growth times (unpublished results of this laboratory) by line rocket immunoelectrophoresis (Weeke, 1973).

RESULTS

Aspergillus flavus grew well on both heat-killed (nonviable) and viable soybean seeds. In nonviable seeds, AFB_1 was detectable by six days postinoculation (PI). AFB_1 could also be detected in viable seeds six days PI but the

initial rate of AFB_1 increase was three times less than was observed in nonviable seeds (Figure 1). Aflatoxins B_2, G_1, and G_2 were not detected. The two types of seeds also differed in that glyceollin levels in the viable seeds reached a maximum of 10 $\pm$ 2 μg/g fresh wt at eight days PI while glyceollin was not detected in an extract of *A. flavus* inoculated, nonviable seeds. Uninoculated control seeds did not contain detectable glyceollin or aflatoxin.

When *A. flavus* was grown in liquid NS medium in stationary culture, AFB_1 was detectable in the external culture medium beginning two days PI and in the mycelium two to three days PI. Total AFB_1 levels (sum of the AFB_1 in the mycelium and the culture filtrate) increased through day 4 and begin to decline when the increase in fungal dry weight ceased (Figure 2A and 2B). The same pattern of AFB_1 accumulation was obtained using the RIA assay, except that AFB_1 levels were consistently 70% of those obtained by HPLC. Cultures also contained much lower levels of AFB_2. AFB_2 levels followed the same pattern of increase and decline as AFB_1 (Figure 2A), but are not provided since AFB_2 was not detected in seeds or in cultures containing glyceollin. The same pattern of AFB_1 accumulation could be seen in soybean seeds, cv. Williams 82, inoculated with *A. flavus*, where aflatoxin accumulation was detectable coincident with the appearance of measurable fungal antigen and began to decline with the cessation of fungal antigen increase (Figure 3). The same results were obtained when cv. Clark 63 was inoculated with *A. flavus*.

Glyceollin is a transient inhibitor of *A. flavus* growth. When a drop of glyceollin (62.5 μg/ml)-containing solution is placed at the advancing edge or growing mycelium on a PDA plate, it causes formation of an inhibition zone that is overgrown in one day. In liquid culture medium, glyceollin at a concentration of 62.5 μg/ml caused a small but reproducible lag in the beginning of rapid fungal growth (presumably reflecting the transient growth inhibition

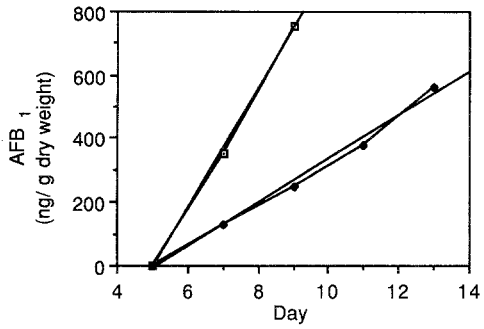

FIG. 1. Accumulation of aflatoxin B_1 in viable (♦) and non-viable (□) seeds of soybean cultivar Williams 82. Initial rates of aflatoxin B_1 accumulation are described by the regression equations (♦) $y = -354.75 + 68.8x$, $R^2 = 0.991$, and (□) $y = -952.9 + 188.8x$, $R^2 = 0.998$, respectively.

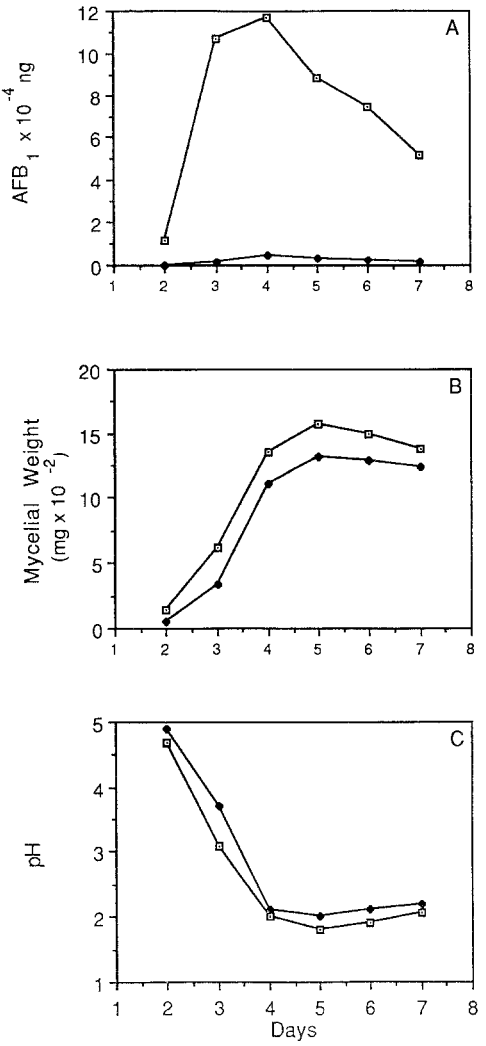

FIG. 2. Growth of *A. flavus* in the presence (♦) and absence (□) of 62.5 μg/ml glyceollin. (A) Sum of the aflatoxin B_1 isolated from fungal mycelium and culture filtrate. (B) Increase in fungal mycelial dry weight. (C) pH of the culture filtrate.

observed on plates) and a slight decrease in the peak mass of fungus (Figure 2B). The average decrease in maximum fungal mass in three experiments caused by this concentration of glyceollin was 11.5 ± 3%. The presence of glyceollin had no effect on the rapid pH decrease of the medium observed during fungal growth (Figure 2C). In contrast, the same level of glyceollin caused a 95%

decrease in the maximum level of total AFB_1 that accumulated in the cultures. The average values for AFB_1 per milligram of fungal dry mass in three experiments are shown graphically in Figure 4. When this experiment was repeated using 6.25 μg/ml of glyceollin (a level nearer that observed in infected seeds), a 70% drop in the maximum average levels of total AFB_1 was observed.

When filtrates were harvested from cultures grown with and without glyceollin four days PI (a time when AFB_1 begins to decline; Figure 2A) and a known amount of authentic AFB_1 was added to each, AFB_1 continued to decline. The rates of AFB_1 disappearance were almost twice as high in filtrates from *A. flavus* grown without 62.5 μg/ml glyceollin (15 ng/hr vs. 8 ng/hr). In addition, when the fungal growth was collected from the same cultures and added to fresh

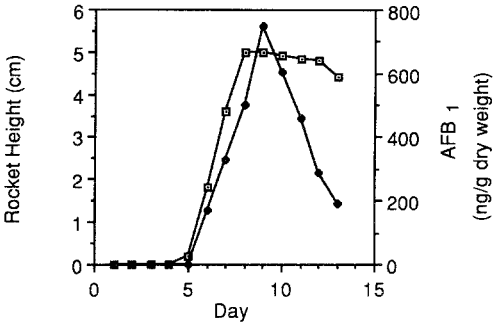

FIG. 3. Growth of *A. flavus* and aflatoxin production in nonviable seeds of soybean cultivar Williams 82. Fungal growth (□) was determined as the height in centimeters of the most abundant antigen after rocket electrophoresis. AFB_1 (♦) was determined as described in Methods and Materials.

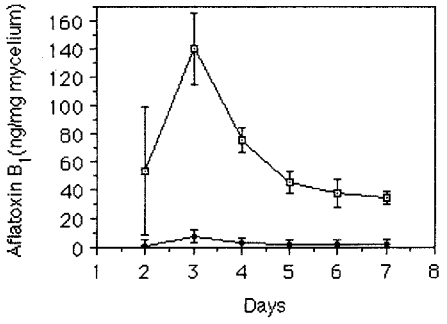

FIG. 4. Total aflatoxin B_1 (ng/mg mycelium dry weight) produced in cultures in the presence (♦) and absence (□) of 62.5 μg/ml glyceollin. Data points are the means from three independent experiments. Error bars show the standard deviation.

culture medium lacking a carbon source but containing exogenously added AFB_1, AFB_1 declined at the same rate regardless of whether the fungus had been grown with or without glyceollin. Growth of *A. flavus* in glyceollin at 62.5 μg/ml did alter the distribution of AFB_1 between mycelium and culture filtrate. Mycelium grown in the presence of glyceollin accumulated only half the percentage of AFB_1 as cells grown in the absence of glyceollin (Figure 5).

Glyceollin levels decreased in filtrates of cultures of *A. flavus* over the seven-day period of these experiments (Figure 6). The decrease was linear over a six-day period reaching a final glyceollin level of less than 5 μg/ml by day 7. The glyceollin that disappeared from the culture filtrate could not be recovered from the fungal mycelium. Glyceollin was stable in the NS medium at pH 5.5 but disappeared at the same rate ($y = 47.6 - 8.6x$) as in cultures (Figure 6)

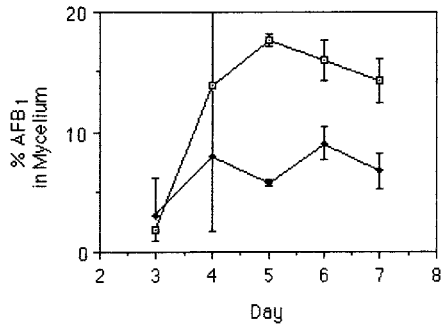

FIG. 5. Percentage of the total aflatoxin B_1 present in the mycelium of *A. flavus* grown in the presence (♦) or absence (□) of 62.5 μg/ml glyceollin. The remainder of the aflatoxin B_1 was present in the culture filtrate. Data points are the means from three independent experiments. Error bars show the standard deviation.

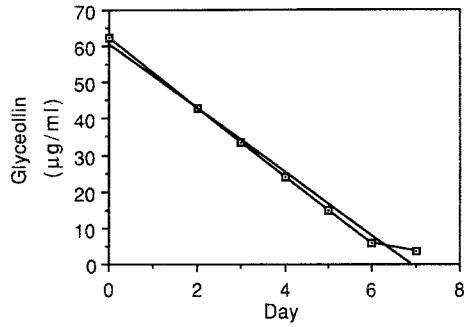

FIG. 6. The rate of disappearance of glyceollin from cultures of *A. flavus* is described by the regression equation $y = 68.57 - 8.7x$, $R^2 = 0.988$.

when introduced into NS medium at pH 2.0, a pH reached by day 4 in growing cultures (Figure 2C). The difference in intercept results from the larger first day decrease in recoverable glyceollin in NS medium at pH 2.0.

DISCUSSION

Aspergillus flavus is capable of growth and AFB_1 production on soybean seeds. The pattern of AFB_1 accumulation in seeds (Figure 3) is the same as that observed in *A. flavus* cultures (Figure 2A). It consists of a rapid increase in AFB_1 coincident with the increase in fungal mass followed by a decrease in AFB_1 when fungal growth ceases. The ability of AFB_1-producing strains of *Aspergillus* to degrade AFB_1 has been reported previously (Doyle and Marth, 1978).

While AFB_1 was found to accumulate in both viable and nonviable seeds, the initial rate of accumulation in viable seeds was approximately three times lower. The lower rate of AFB_1 accumulation in viable seeds was accompanied by the accumulation of the soybean-produced pterocarpan, glyceollin. It is not surprising that glyceollin accumulation is elicited during infection by *A. flavus*. Glyceollin belongs to a class of plant-produced, antimicrobial compounds termed phytoalexins, whose synthesis is elicited by pathogenic organisms (Paxton, 1980). While phytoalexins have been proposed to play a role in plant disease resistance responses (e.g., Ebel and Grisebach, 1988) in vegetative tissue, little information is available about their possible role in limiting diseases in seeds.

Glyceollin was only able to cause a transient inhibition of growth of *A. flavus* in culture. When applied to semisolid medium near the colony margin at a concentration of 62.5 μg/ml, glyceollin caused the formation of an inhibition zone that was overgrown in the subsequent day. In liquid cultures, glyceollin at a concentration of 62.5 μg/ml caused a barely discernible lag in the beginning of mycelial growth and a small decrease in total fungal mass (Figure 2B). At a concentration of 125 μg/ml in liquid cultures, glyceollin caused an easily observed lag in the initiation of mycelial growth, but the effect was still transient (data not given). Glyceollin-induced, transient growth inhibition has been demonstrated for *Bradyrhizobium japonicum* (Parniske et al., 1991; Karr et al., 1992) and *Pseudomonas syringae* pv. *glycinea* (unpublished results of this laboratory). In the case of *B. japonicum*, the transient nature of growth inhibition does not result from breakdown of glyceollin but from a developed resistance to the inhibitory effects of glyceollin (Parniske et al., 1991). This developed resistance can be stimulated by pretreatment of the bacterial cells with the soybean isoflavone, genistein. The glyceollin-induced, transient growth inhibition in cultures of *A. flavus* may be different. In this case, glyceollin disappears from the culture medium. It is unlikely that this reflects a detoxification mechanism

expressed by *A. flavus*, since the disappearance of glyceollin occurs at the same rate in the presence and absence of the fungus at low culture medium pH values. It is more likely that the acidification of medium during growth leads to the acid catalyzed breakdown of glyceollin (Dewick, 1982).

In contrast to the small, transient effect on fungal growth, glyceollin severely depressed the accumulation of AFB_1 in both the culture medium and in the fungal mycelium (Figure 2A). At initial concentrations of 62.5 μg/ml and 6.25 μg/ml, glyceollin caused a 95% and 70% decrease in the maximum levels of AFB_1. The effect of glyceollin on AFB_1 accumulation was not transient and continued after glyceollin had been nearly depleted from the medium.

The mechanism by which glyceollin represses AFB_1 accumulation is not clear. The toxicity of glyceollin has been attributed to disruption in membrane functions (Weinstein et al., 1981) and inhibition of electron transport processes (Boydson et al., 1983). Either activity could lead to a decrease in the synthesis of AFB_1. Whatever the mechanism of action of glyceollin, it does not appear to increase the rate of AFB_1 breakdown since AFB_1 does not disappear faster in cultures grown in the presence of glyceollin.

Soybeans are a major oilseed crop in the United States. They provide not only a source of oil but are processed to produce a variety of human foods and feeds for domestic animals. Unlike maize, soybean seeds in commercial marketing channels are rarely found to be contaminated with aflatoxin (Smith and Moss, 1985). This lack of AFB_1 contamination in soybean seeds is unlikely to result from the absence of the fungus, *Aspergillus flavus*, in soybean fields since both soybeans and maize are grown in the same regions of the country and, indeed, are often grown in rotation in the same fields. In addition, soybeans and maize are generally grown during the same cropping period, eliminating seasonal weather differences as a possible cause of the preferential contamination of maize with AFB_1. Finally, since soybean seeds support the growth of *A. flavus* (Figure 3) (Abdullah et al., 1984; Pinto et al., 1991), it is clear that soybeans do not escape AFB_1 contamination through exclusion of the fungus by some resistance mechanism.

In this paper, we suggest that the antimicrobial secondary metabolite, glyceollin, contributes at least in part to the repression of AFB_1 accumulation in soybean seeds and the lack of AFB_1 contamination of soybean seeds in commercial marketing channels. Such a role has also been suggested for the phytoalexin in peanuts (Wotton and Strange, 1987). If glyceollin does act by inhibiting synthesis of AFB_1, it will be necessary to determine its site of action in the biosynthetic pathway since a number of AFB_1 precursors are also genotoxic (Sakai et al., 1992) and may be accumulating in *A. flavus*-infected soybean seeds.

It should be noted that the glyceollin system has a number of advantages as a natural control system for minimizing AFB_1 contamination. First, glyceollin

represses accumulation of AFB_1 at concentrations that have no effect on the growth of the fungus. Since AFB_1 provides no proven advantage to the fungus that makes it, repression of AFB_1 synthesis in the absence of growth inhibition will not produce selective pressure on the fungus to become glyceollin resistant. Second, glyceollin is not produced constitutively but accumulates only in infected seeds. Such a site-specific delivery of a chemical control agent is ideal in that it decreases the amount of total chemical needed to the minimum required to control AFB_1 contamination.

While AFB_1 contamination of soybean seeds is not presently a problem, these results do provide insight into how to approach the problem of AFB_1 contamination in other crops. It is unlikely that glyceollin could be used directly as a chemical treatment to prevent AFB_1 accumulation in crops such as maize. While we have been able to demonstrate that addition of glyceollin to maize seed that was subsequently infected with *A. flavus* causes a one- to two-day delay in appearance of detectible AFB_1 (unpublished results of this laboratory), the treatment procedure was very inefficient, with less than 0.1% of the added glyceollin being taken up by the seed. In addition, such a chemical treatment procedure would not be useful as a control for AFB_1 contamination that occurs before harvest. In light of the complexity of the biosynthetic pathway for the synthesis of glyceollin, it is also unlikely that scientists will be able (in the near future) to produce recombinant maize plants that have the ability to synthesize glyceollin. These results do suggest that a search for a similar, microbially induced metabolite in maize seeds (and other affected crops) might be one approach to decreasing the problem of AFB_1 contamination.

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REFERENCES

ABDULLAH, A., BALDWIN, R.E., FIELDS, M.L., and KARR, A.L. 1984. Sensory properties and safety aspects of germinated small-seeded soybeans and mungbeans. *J. Food Prot.* 47:434–439.

BOYDSTON, R., PAXTON, J.D., and KOEPPE, D.E. 1983. Glyceollin: A site-specific inhibitor of electron transport in isolated soybean mitochondria. *Plant Physiol.* 72:151–155.

CHU, F.S. 1984. Immunoassays for analysis of mycotoxins. *J. Food Prot.* 47:562–569.

DEWICK, P.M. 1982. Isofavonoids, pp. 535–640, *in* J.B. Harborne and T.J. Mabry (eds.). Flavonoids: Advances in Research. Chapman and Hall, London. New York.

DOYLE, M.P., and MARTH, E.H. 1978. Aflatoxin is degraded by fragmented and intact mycelia of *Aspergillus parasiticus* grown 5 to 18 days with or without agitation. *J. Food Prot.* 41:549–555.

EBEL, J., and GRISEBACH, H. 1988. Defense strategies of soybean against the fungus *Phytophthora megasperma* f. sp. *glycinea*: A molecular analysis. *Trends Biochem. Sci.* 13:23–27.

FREMY, J.M., and CHU, F.S. 1989. Immunochemical methods of analysis for aflatoxin M_1, pp. 97–125, *in* H. Van Egmond (ed.). Mycotoxins in Dairy Products. Elsevier Applied Science, London.

KARR, D.B., EMERICH, D.W., and KARR, A.L. 1992. Accumulation of the phytoalexin, glyceollin, in root nodules of soybean formed by effective and ineffective strains of *Bradyrhizobium japonicum*. *J. Chem. Ecol.* 18:997–1008.

HAMILTON, P.B. 1987. Aflatoxicosis in farm animals, pp. 41–50, *in* M.S. Zuber, E.B. Lillehoj, and B.L. Renfro (eds.). Aflatoxin in Maize: Proceedings of the Workshop. CIMMYT, El Batan, Mexico.

LAU, H.P., GAUR, P.K., and CHU, F.S. 1981. Aflatoxin B_{2a}-hemiglutarate and antibody production. *J. Food Safety* 3:1–13.

PARNISKE, M., AHLBORN, B., and WERNER, D. 1991. Isoflavonoid-inducible resistance to the phytoalexin glyceollin in soybean rhizobia. *J. Bacteriol.* 173:3432–3439.

PAYNE, G.A. 1992. Aflatoxin in maize. *Crit. Rev. Plant Sci.* 10:423–440.

PAXTON, J. 1980. A new working definition of the term "phytoalexin." *Plant Dis.* 64:734.

PIER, A.C. 1987. Aflatoxicosis and immunosuppression in mammalian animals, pp. 58–65, *in* M.S. Zuber, E.B. Lillehoj, and B.L. Renfro (eds.). Aflatoxin in Maize: Proceedings of the Workshop. CIMMYT, El Batan, Mexico.

PINTO, V.E.F., VAAMONDE, G., BRIZZIO, S.B., and APRO, N. 1991. Aflatoxin production in soybean varieties grown in Argentina. *J. Food Prot.* 54:542–545.

PRZYBYLSKI, W. 1975. Formation of aflatoxin derivatives on thin layer chromatographic plates. *J. Assoc. Off. Anal. Chem.* 58:163–164.

SAKAI, M., ABE, K., OKUMURA, H., KAWAMURA, O., SUGUIRA, Y., HORIE, Y., and UENO, Y. 1992. Genotoxicity of fungi evaluated by SOS microplate assay. *Nat. Toxins* 1:27–34.

SMITH, J.E., and MOSS, M.O. 1985. Mycotoxins: Formation, Analysis and Significance. John Wiley & Sons, New York. pp. 83–103.

SQUIRE, R.A. 1981. Ranking animal carcinogens: A proposed regulatory approach. *Science* 214:877–880.

STUBBLEFIELD, R.D., and SHOTWELL, O.L. 1977. Reverse phase analytical and preparative high pressure liquid chromatography of aflatoxins. *J. Assoc. Off. Anal. Chem.* 60:784–790.

TUITE, J. 1969. Plant Pathological Methods. Burgess Publishing, Minneapolis, Minnesota. pp. 12–80.

WEEKE, B. 1973. General remarks on principles, equipment, reagents and procedures, pp. 15–35, *in* N.H. Axelsen, J. Kroll, and B. Weeke (eds.). A Manual of Quantitative Immunoelectrophoresis, Methods and Applications. Blackwell Scientific Publications, Oxford.

WEINSTEIN, L.I., HAHN, M.G., and ALBERSHEIM, P. 1981. Host-pathogen Interactions. XVIII. Isolation and biological activity of glycinol, a pterocarpan phytoalexin synthesized by soybeans. *Plant Physiol.* 68:358–363.

WOTTON, H.R., and STRANGE, R.N. 1987. Increased susceptibility and reduced phytoalexin accumulation in drought-stressed peanut kernels challenged with *Aspergillus flavus*. *Appl. Environ. Microbiol.* 53:270–273.

YASHIKAWA, M., YAMAUCHI, K., and MASAGO, H. 1978. Glyceollin: Its role in restricting fungal growth in resistant soybean hypocotyls infected with *Phytophthora megasperma* var *sojae*. *Physiol. Plant Pathol.* 12:73–82.

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LABORATORY AND FIELD EVIDENCE FOR MALE-PRODUCED AGGREGATION PHEROMONE IN *Rhynchophorus cruentatus* (F.) (Coleoptera: Curculionidae)

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Abstract—Laboratory and field assays were conducted to determine if palmetto weevil, *Rhynchophorus cruentatus* (F.), adults produce an aggregation pheromone. Attraction of females in a Y-tube olfactometer to conspecific males was greater than to clean air. Male and female attraction to conspecific male volatiles combined with host-palm, *Sabal palmetto* (Walter), volatiles was greater than to host-palm volatiles alone. Similarly, more weevils were caught in the field in traps baited with conspecific males plus host-palm tissue than in similar traps baited with only males, or palm tissue, or females, or females plus palm tissue. These results suggest that *R. cruentatus* males produce an aggregation pheromone(s) that is highly attractive to conspecific adults of both sexes when combined with host-palm volatiles. This study is an important step towards understanding the chemical ecology of *R. cruentatus*.

Key Words—Coleoptera, Curculionidae, *Rhynchophorus cruentatus*, *Sabal palmetto*, aggregation pheromone, olfactometer, field trapping.

INTRODUCTION

The genus *Rhynchophorus* (palm weevils) is comprised of 10 species, seven of which are considered major pests of coconut in production areas (Wattanapongsiri, 1966). The only species occurring in North America is the palmetto weevil, *Rhynchophorus cruentatus* (F.). This large (24 to 33-mm-long) beetle ranges

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from the Florida Keys through the coastal regions of the southeastern United States (Wattanapongsiri, 1966) and is not a pest of healthy ornamental palms, but will attack transplanted or stressed palms (Giblin-Davis and Howard, 1989). In Florida, *R. cruentatus* is sympatric with the native cabbage palmetto, *Sabal palmetto* (Walter) (Woodruff, 1967), but can also complete its life cycle in several introduced palm species (Giblin-Davis and Howard, 1988).

Despite recent advances, the chemical ecology of *Rhynchophorus* spp. is not fully understood. Palms that are stressed or damaged are vulnerable to attack and semiochemicals emanating from these trees are attractive to adults (Chittenden, 1902; Wattanapongsiri, 1966; Rochat, 1990; Morin et al., 1986; Weissling et al., 1992). Furthermore, aggregation pheromones have been identified from two *Rhynchophorus* spp. Males of the Neotropical species, *R. palmarum* (L.), produce an aggregation pheromone identified as (2*E*)-6-methyl-2-hepten-4-ol (Rochat et al., 1991). The chirality of this compound is known to be 4(*S*) (Oehlschlager et al., 1992). Rochat et al. (1991) suggest that *R. palmarum* males produce pheromone when they feed on a suitable host plant and thus signal conspecifics to aggregation. In addition, a male-produced aggregation pheromone that is very synergistic with palm volatiles has recently been identified from *R. phoenicis* F., a pest of palms in Africa (Gerhard Gries, personal communication). Information such as this is important in understanding the biology and phylogeny of *Rhynchophorus* spp. and in possibly increasing efficacy of management against pest species.

The objective of this study was to gather evidence to support the presence or absence of an aggregation pheromone in *R. cruentatus* adults.

METHODS AND MATERIALS

Laboratory Bioassay

Insects. Rhynchophorus cruentatus adults used in experiments were either collected in the field or laboratory-reared. Field-collected beetles were trapped in 19-liter black plastic buckets covered with tops made from polyvinyl chloride (PVC) tubes (2.4 cm ID) glued together longitudinally. These traps allowed weevil entry but prevented escape (Weissling et al., 1992). When weevils were required for laboratory bioassays, traps were baited with 2.5 kg of fresh, chopped *S. palmetto* bud tissue and were left for one week in a native stand of *S. palmetto* 12 km south of La Belle, Florida (Hendry County). Trapped weevils were collected, separated by sex, and maintained without food in groups of four on moist tissue paper in 120-ml plastic cups (Giblin-Davis et al., 1989) at 26°C, until used in bioassays. Additional weevils were cultured in the laboratory by the methods of Giblin-Davis et al. (1989).

Olfactometer. The response of *R. cruentatus* adults to volatiles emanating from host-plant and conspecific sources was tested in a Y-tube olfactometer. The Y-tube juncture was flexible PVC (25 mm ID). The Y-tube arms (45 cm long) and holding tube (30 cm long) were 23 mm ID borosilicate glass tubing. Purified compressed air was passed through a Nalgene membrane filter holder filled with moistened toweling for humidification, and split through valves leading to flowmeters. Air flow to both arms of the olfactometer was regulated at 250 ml/min and passed through 500-ml glass flasks (one per Y-tube arm) that held the stimulus to be tested before passing through each arm of the Y tube. Tygon tubing provided all connections between the air source and the Y tube; all remaining connectors were glass or glass wrapped with Teflon tape.

After each test, all components of the olfactometer in contact with weevils or palm were washed in a detergent solution and rinsed with water. Afterwards, all glassware was rinsed in acetone, and the Y-tube juncture was rinsed in absolute ethanol and allowed to air dry. Tygon tubing between the sample flasks and Y tube were replaced after each test. The Y-tube apparatus was housed in a room maintained at 30°C. A black cloth was used to cover the olfactometer to eliminate light as a stimulus.

Fourteen paired treatments were compared in the olfactometer (Table 1). When insects were used as a volatile source, they were placed in sample flasks in groups of five males and/or five females. Finely chopped *S. palmetto* bud tissue (50 g, 24–72 hr old) was added to sample flasks when used as a source of volatiles. Weevils in sample flasks were allowed to interact with palm tissue when both sources were tested simultaneously, except in one comparison where male weevils in contact with palm tissue were compared to male weevils not in contact with palm tissue. For this comparison, an additional flask was added to each "line" so that air passed through both flasks in series before entering a Y-tube arm. When separated, five males were placed in the upstream flask and palm tissue in the downstream flask, preventing weevil exposure to palm volatiles. This treatment was compared to the treatment where five males and palm were placed together in one of the two sample flasks.

For each treatment pair, 10 randomly chosen weevils of each sex were individually released into the olfactometer and checked at 5 min. Weevil response was recorded if an insect walked an arbitrarily chosen distance of at least 3 cm into one arm of the Y tube, otherwise "no response" was recorded. Treatment pairs were tested three separate times in random order ($N = 30$) with fresh weevils.

Percent attractancy was calculated as: (number of insects entering a sample arm/total number responding) × 100. Differences between the number of weevils entering each sample arm for each paired treatment were analyzed by the Kruskal-Wallis test (chi-square approximation) (SAS Institute, 1985).

TABLE 1. RESPONSE OF INDIVIDUAL MALE AND FEMALE *R. cruentatus* ADULTS TO VARIOUS TREATMENTS IN Y-TUBE OLFACTOMETER

Comparison					% Response		
A	B	Sex	*N*	No. of responses	A	B	Pr > chi sq.[a]
Control[b]	Control	male	30	20	45	55	0.5323
		female	30	19	53	47	0.7489
5 Males	Control	male	30	22	59	41	0.2332
		female	30	20	73	27	0.0002
5 Females	Control	male	30	23	43	57	0.3931
		female	30	25	28	72	0.0035
5 Males + 5 Females	Control	male	30	27	70	30	0.0030
		female	30	22	82	18	0.0001
5 Females	5 Males	male	30	23	17	83	0.0001
		female	30	21	14	86	0.0001
Palm[c]	Control	male	30	24	88	12	0.0001
		female	30	23	67	33	0.0087
5 Males	Palm	male	30	26	12	88	0.0001
		female	30	22	23	77	0.0003
5 females	Palm	male	30	23	22	78	0.0001
		female	30	23	26	74	0.0013
5 Males + palm	Palm	male	30	23	74	26	0.0013
		female	30	23	78	22	0.0001
5 Females + palm	Palm	male	30	25	60	40	0.1615
		female	30	21	67	33	0.0328
5 males + palm	5 Males	male	30	26	77	23	0.0001
		female	30	23	87	13	0.0001
5 Females + palm	5 Females	male	30	26	86	14	0.0001
		female	30	25	92	8	0.0001
5 females + palm	5 Males + palm	male	30	26	31	69	0.0060
		female	30	25	32	68	0.0117
Males + palm[d] (together)	Males + palm (separated)	male	30	28	54	46	0.5963
		female	30	25	52	48	0.7795

[a] Pr > chi sq. = probability of obtaining chi square value (Kruskal-Wallis test, chi square approximation).
[b] Control = no source of volatiles.
[c] 50 g fresh, chopped *S. palmetto* bud tissue.
[d] Air source carried volatiles from 50 g of palm tissue and five male weevils. Where separated, weevils were placed in a flask positioned upstream of palm volatiles.

Field Test

Traps similar to that described by Weissling et al. (1992) were constructed and used in the field to test attraction of weevils to live conspecifics in contact with and without *S. palmetto* tissue. Traps consisted of 19-liter black plastic buckets with PVC tops (Figure 1). Tapered, two-part flower pots (15-cm-diam. bottom, 21.5-cm-diam. opening, 18 cm high; displacement volume: 4.8 liters) were used in each bucket to contain the test stimuli. Flower pot openings were covered with hardware cloth cones (6-mm openings), inverted, attached to PVC tops with wire, and suspended in each bucket. Trap tops were held in place by two anchored metal rods (1.3 cm diam.) and with elastic cords. Four holes (5.5 mm diam.) were drilled 8 cm from the bottom of each bucket to drain rainwater. When placed in the field, the bottom of each bucket was flooded with a solution of 50–60 g of Alconox detergent (Alconox, Inc., New York 10003) in 2 liters of water. Trap tops and all internal surfaces of each trap were coated with a fine spray of mineral oil. Weevils entering traps could not escape and presumably drown before releasing volatiles and contributing to attraction of test stimuli.

Treatments tested included traps baited with four water-moistened cellulose sponges (unbaited control), 10 *R. cruentatus* males, 10 *R. cruentatus* females,

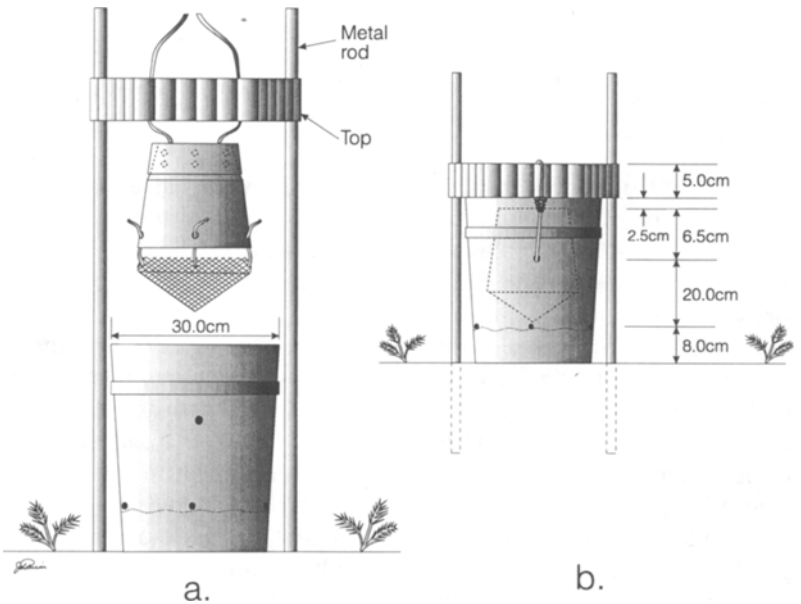

FIG. 1. Baffled top, bucket, and inverted flower pot used to trap and kill *R. cruentatus* adults attracted to *S. palmetto* tissue and/or conspecific insects: (a) unassembled, (b) assembled.

1.5 kg *S. palmetto* tissue, 1.5 kg *S. palmetto* tissue plus 10 *R. cruentatus* males, and 1.5 kg *S. palmetto* tissue and 10 *R. cruentatus* females. Four water-moistened sponges were also added to all traps baited with live weevils only. Sponges were not added to traps containing palm because of lack of space in the inverted flower pots. However, evidence suggests that moist sponges are not attractive or repellent to weevils (R.M.G.-D. unpublished data). Traps were arranged in a randomized complete block design (four blocks) in a 300-ha pasture interspersed with *S. palmetto* and saw palmetto, *Serenoa repens* (Bartr.), located 12 km south of La Belle, Florida. Traps were placed on the ground and spaced approximately 20 m apart within each block. Blocks were separated by at least 300 m. Studies were conducted on June 19–26, and June 26–July 2, 1992. Treatments within each block were rerandomized between sample periods.

The number of male and female *R. cruentatus* adults collected per trap per 24 hr were used for data analysis. Data were subjected to square root $(x + 0.05)$ transformation and treatments were tested for significant differences by analysis of variance using the Statistical Analysis System general linear models procedure (SAS Institute, 1985). Untransformed means are presented. Analyses were conducted for treatment effects over both sample periods and within each sample period. The least significant difference test was used for mean separation where significant ($P < 0.05$) statistical differences occurred.

RESULTS

Laboratory Bioassay

The Y-tube olfactometer used in this study proved to be a discriminating tool for comparing the response of *R. cruentatus* adults to several treatments. Periodic tests where no stimulus was introduced into Y-tube arms revealed no bias for weevils to choose one side over the other, suggesting the lack of volatile desorption onto the plastic Y-tube juncture.

Significantly more females were attracted to five males than to the clean air control (Table 1). When five females as a stimulus were compared to the control, more females entered the control arm. Attraction of both male and female weevils to five males and five females combined in a sample flask was significantly greater than to the control. Weevil attraction to five males was significantly greater than to five females. Significantly more weevils were attracted to chopped palm tissue than to the control, five males, or five females. The addition of five males to palm tissue resulted in significantly greater attraction of weevils than to palm tissue alone or to five males alone. Similarly, female response to five females plus palm tissue was significantly greater than to palm tissue alone, while attraction of males and females to females plus palm tissue was significantly greater than to females alone. Attraction of weevils to

five males plus palm tissue was significantly greater than to five females plus palm tissue. Response of weevils to five males that were separated from palm tissue, and unable to detect palm volatiles, was not different than to five males in contact with palm tissue.

Field Test

Weevils were caught in traps baited with palm tissue and conspecific insects (Table 2). Statistical analysis indicated significant sample period × treatment interactions (male: $F = 3.1$; $df = 3, 14$; $P < 0.04$; females: $F = 5.9$; $df = 3, 14$; $P < 0.01$). Analyses within sample periods indicated significant differences among treatments (June 19–26: male: $F = 8.3$; $df = 5, 15$; $P < 0.01$; female: $F = 34.8$; $df = 5, 15$; $P < 0.01$; June 26–July 2: male: $F = 15.2$; $df = 5, 15$; $P < 0.01$; female: $F = 16.9$; $df = 5, 15$; $P < 0.01$). Significantly more weevils were collected from traps baited with chopped palm tissue and 10 conspecific males than any of the other treatments during both sample periods (Table 2). In addition, significantly more females were collected from traps baited with chopped palm than all other treatments except palm plus 10 males, during the first sample period. During the second sample period, significantly more males were collected from traps baited with palm tissue and 10 conspecific females than all treatments except palm and palm plus 10 males.

TABLE 2. MEAN NUMBER *R. cruentatus* ADULTS CAUGHT IN ATTRACT-AND-KILL TRAPS[a] PLACED IN FIELD AND BAITED WITH 1.5 kg CHOPPED *S. palmetto* TISSUE AND/OR 10 LIVE CONSPECIFIC WEEVILS, OR LEFT UNBAITED, LA BELLE, FLORIDA 1992

	Mean[b] No./trap/day ± SEM			
	June 19–26		June 26–July 2	
Treatment	Male	Female	Male	Female
Unbaited	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
Females	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
Males	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.1 ± 0.1 a
Palm	0.1 ± 0.1 a	0.2 ± 0.1 b	0.1 ± 0.1 ab	0.1 ± 0.1 a
Palm + females	0.1 ± 0.1 a	0.1 ± 0.1 a	0.3 ± 0.2 b	0.3 ± 0.2 a
Palm + males	0.7 ± 0.4 b	1.0 ± 0.2 c	1.5 ± 0.5 c	2.0 ± 0.5 b

[a] $n = 4$.

[b] Means within a column followed by the same letter are not significantly different (least significant difference, $P \leq 0.05$).

DISCUSSION

Weissling et al. (1992) substantiated earlier observations that *R. cruentatus* adults are attracted to volatile compounds emitted by stressed *S. palmetto* trees and suggested that olfaction plays an important role in orientation of adults to their host plants. Results of the present study suggest that *R. cruentatus* males also produce an aggregation pheromone(s). In olfactometer tests, male volatiles were more attractive to females than the control, but palm volatiles were more attractive to both sexes than male volatiles alone. However, the combination of male and host-palm volatiles was more attractive to both sexes than host-tissue alone and males alone. In the field, caged males were not attractive, but when palm and males were combined the grouping was very attractive to *R. cruentatus* adults of both sexes. This suggests that the male-produced aggregation pheromone of *R. cruentatus* is ineffective in the field unless combined with host-plant volatiles. *Rhynchophorus palmarum* (Oehlschlager et al., 1992), *R. phoenicius* (G. Gries, personal communication), *Sitona lineatus* L. (Blight and Wadhams, 1987), and *Pissodes approximatus* Hopkins (Booth et al., 1983) are among the many examples of other weevils that show similar trends for synergistic combinations of species-specific pheromones and host-plant volatiles.

Results of the olfactometer treatment where males in contact with palm were compared to males not exposed to palm volatiles (Table 1) suggest that *R. cruentatus* interaction with host-palm tissue is not a requirement for pheromone production, as suggested by Rochat et al. (1991) for *R. palmarum*. Our field results also indicate that the attractiveness of palm volatiles is greatly enhanced when combined with the male-produced pheromone(s) (Table 2).

In the olfactometer, female choice of the control over females alone was somewhat surprising, especially because females combined with palm were more attractive to responding females than palm alone (Table 1). Data suggest that females may release a female-specific pheromone that is repellent. The repellent effect could have been overcome in laboratory assays where females were placed on palm tissue by a possible increase in host volatiles emanating from feeding scars, as has been observed with *Hylobius abietis* (L.) feeding on pine (Tilles et al., 1986). Further investigation is needed, however, to determine if a female-specific pheromone is produced and what its effect is on perceiving individuals.

The identification of an aggregation pheromone(s) produced by *R. cruentatus* males is an important step towards understanding the chemical ecology of this insect. Field studies with synthetic pheromone(s) should provide needed insight into the host-finding behavior of *R. cruentatus* adults to determine precisely what constitutes a susceptible palm. In addition, synthetic pheromone(s) combined with food-type volatiles could be used in management systems to increase capture of adults in traps used for monitoring or in mass-trapping *R. cruentatus* adults.

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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.

BOOTH, D.C., PHILLIPS, T.W., CLAESSON, A., SILVERSTEIN, R.M., LANIER, G.N., and WEST, J.R. 1983. Aggregation pheromone components of two species of *Pissodes* weevils (Coleoptera: Curculionidae): Isolation, identification, and field activity. *J. Chem. Ecol.* 9:1–12.

CHITTENDEN, F.H. 1902. The palm and palmetto weevils. *USDA Entomol. Bull.* 38:23–28.

GIBLIN-DAVIS, R.M., and HOWARD, F.W. 1988. Notes on the palmetto weevil, *Rhynchophorus cruentatus* (Coleoptera: Curculionidae). *Proc. Fla. State Hortic. Soc.* 101:101–107.

GIBLIN-DAVIS, R.M., and HOWARD, F.W. 1989. Vulnerability of stressed palms to attack by *Rhynchophorus cruentatus* (Coleoptera: Curculionidae) and insecticidal control of the pest. *J. Econ. Entomol.* 82:1185–1190.

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.

MORIN, J.P., LUCCHINI, F., FERREIRA, J.M.S., and FRAGA, L.S. 1986. *Rhynchophorus palmarum* control using traps made from oil palm cubes. *Oleagineux* 41:57–62.

OEHLSCHLAGER, A.C., PIERCE, H.D., JR., MORGAN, B., WIMALARATNE, P.D.C., SLESSOR, K.N., KING, G.G.S., GRIES, G., GRIES, R., BORDEN, J.H., JIRON, L.F., CHINCHILLA, C.M., and MEXZAN, R.G. 1992. Chirality and field activity of Rhynchophorol, the aggregation pheromone of the American palm weevil. *Naturwissenschaften* 79:134–135.

ROCHAT, D. 1990. *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae): Nuevos datos sobre el comport amiento del insecto y su control por trampeo olfactivo. *Rev. Palmas* 11:69–79.

ROCHAT, D., MALOSSE, C., LETTERE, M., DUCROT, P.H., ZAGATTI, P., RENOU, M., and DESCOINS, C. 1991. Male-produced aggregation pheromone of the American palm weevil, *Rhynchophorus palmarum* (L.) (Coleoptera: Curculionidae): Collection, identification, electrophysiological activity, and laboratory bioassay. *J. Chem. Ecol.* 17:2127–2140.

SAS INSTITUTE. 1985. SAS User's Guide: Statistics, 5th ed. SAS Institute, Cary, North Carolina.

TILLES, D.A., NORDLANDER, G., NORDENHEM, H., EIDMANN, H.H., WASSGREN, A.-B., and BERGSTROM, G. 1986. Increased release of host volatiles from feeding scars: A major cause of field aggregation in the pine weevil *Hylobius abietis* (Coleoptera: Curculionidae). *Environ. Entomol.* 15:1050–1054.

WATTANAPONGSIRI, A. 1966. A revision of the genera *Rhynchophorus* and *Dynamis* (Coleoptera: Curculionidae). Department of Agricultural Science Bulletin, Bangkok 1. pp. 1–328.

WEISSLING, T.J., GIBLIN-DAVIS, R.M., SCHEFFRAHN, R.H., and MENDOZA, N.M. 1992. Trap for capturing and retaining *Rhynchophorus cruentatus* (Coleoptera: Curculionidae) adults using *Sabal palmetto* as bait. *Fla. Entomol.* 75:212–221.

WOODRUFF, R.E. 1967. A giant palm weevil, *Rhynchophorus cruentatus* (Fab.) in Florida (Coleoptera: Curculionidae). Florida Department of Agriculture, Division of Plant Industry, Entomology Circular No. 63.

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CHEMICAL CORRELATES OF α-TOCOPHEROL (VITAMIN E) ALTERED *Malacosoma disstria* HERBIVORY IN *Fraxinus pennsylvanica* var. *subintegerrinia*, GREEN ASH

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Abstract—The antioxidant α-tocopherol (vitamin E), applied in a basal trunk band to the green ash tree, *Fraxinus pennsylvanica* var. *subintergerrinia*, elicited an alteration of foliar feeding by *Malacosoma disstria* larvae (Lepidoptera: Lasiocampidae). The bioassayed effects were dependent on the dosage of elicitor, the time after elicitation, and the position in the tree. Leaves for chemical analysis were collected from trees receiving two dosages and at two intervals after elicitation. Compounds in the ethyl acetate extractables from the ash tree leaves were separated by TLC and HPLC. TLC separations showed differences in the nonhydrolyzed extractables attributable to elicitor dosage and time after elicitation. TLC-resolved differences were also evident among acid-hydrolyzed samples. HPLC-resolved profiles revealed eight peaks in the nonhydrolyzed extractables that were quantitatively negatively correlated with larval feeding preference between elicited versus control foliage on at least one of the two sampling dates. Results from this study and other investigations reported in the literature indicate that the antioxidant α-tocopherol (vitamin E) can function as an environmental-stress elicitor of alterable defensive chemistry in green ash and other plants.

Key Words—α-Tocopherol, vitamin E, elicitor, alterable resistance, antiherbivory, ash tree, *Malacosoma disstria*, forest tent caterpillar, Lepidoptera, Lasiocampidae.

INTRODUCTION

Biotic and abiotic entities capable of eliciting increased or decreased antixenosis regarding herbivory (e.g., through an altered synthesis of secondary metabolites) are widely known (Akazawa et al., 1960; Larsson et al., 1986; Chiang et al.,

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1987; Lin et al., 1990a, b; Haukioja, 1991; Benz and Abivardi, 1991; Neupane and Norris, 1991a); however, much less is known of the mechanisms by which elicitors cause such alteration of defensive phytochemistry.

Sulfhydryl–protein-dependent, oxidative–reductive electrochemistry has recently been implicated as an important parameter of the mechanisms by which plants respond to elicitors, and thus alter their chemical defenses against herbivory (Neupane and Norris, 1990, 1991a; Haanstad and Norris, 1992). Based on this involvement of oxidative–reductive chemistry, Neupane and Norris (1991b, 1992) and Haanstad and Norris (1993) have shown that the antioxidants α-tocopherol and L-ascorbic acid can serve as elicitors and alter effectively the sulfhydryl–protein redox chemistry involved in the elicitation of alterable defensive phytochemistry against insect herbivory.

Haanstad and Norris (1993) showed specifically that α-tocopherol (vitamin E), applied to green ash trees (*Fraxinus pennsylvanica* var. *subintegerrinia* (Vahl) Fernald) in a basal-trunk wrap, elicited systemically altered tree antixenosis against the forest tent caterpillar, *Malacosoma disstria* (Hübner), herbivory. Whereas this previous study on ash trees focused on insect behavioral assessments of the antioxidant α-tocopherol as an elicitor of alterable tree antixenosis, this research deals with chemical correlates of that previously characterized alteration of ash nonpreference. Based on existing knowledge, we tested two hypotheses in the current study: (1) that there is no chemistry in the α-tocopherol-elicited green ash trees that is quantitatively related to its alterable antixenosis against forest tent caterpillar; and further (2) that a positive relationship cannot be deduced between the elicited antixenosis in ash trees and the elicited glycosidic content of such trees.

METHODS AND MATERIALS

Experimental Background. The plants utilized were 4.6-m-tall Summit cultivar green ash (*Fraxinus pennsylvanica* var. *subintegerrinia* (Vahl) Fernald), trees, with a 5.1-cm mean trunk diameter. The basal trunk of elicited trees received α-tocopherol, a proven elicitor of alterable plant resistance to herbivory, as a band application at the dosages of 25.0 or 50.0 IU/ml in heavy mineral oil (Haanstad and Norris, 1993). An international unit (IU) equals 1 mg of all-*rac*-α-tocopheryl acetate (U.S. Pharmacopaea, 1980). Sixty milliliters of either concentration of α-tocopherol in mineral oil were placed on a 120-cm^2 bandage; the control trees received only 60 ml mineral oil (i.e., solvent).

Leaves for bioassays were removed from the trees at several intervals after treatment, and two-choice feeding assays were conducted with disks cut from such leaves and with third-instar forest tent caterpillars, *Malacosoma disstria* (Hübner) (Lepidoptera: Lasiocampidae). The insect's feeding option thus was between a comparable leaf disk from an elicited versus a control tree.

Chemical Analysis. Leaves for chemical analyses were collected from three ash trees for each elicitation dosage and the control at 8 and 16 days after treatment. Three randomly chosen leaves from each tree were immediately put individually into a glass jar containing 80% methanol, and then stored in darkness at −20°C until chemical analysis.

Extraction Procedure. All individual leaflets were removed from the compound ash leaves, and the resultant denuded leaf petioles were cut into 0.5-cm-long sections. The petiole sections and intact leaflets were then combined and homogenized in 50 ml of 100% methanol for about 4 min at 50% maximum speed, using an Omni-mixer (Ivan Sorvall, Inc., Norwalk, Connecticut). The resultant homogenate was filtered through Whatman No. 2 paper under vacuum. The storage methanol per leaf was also filtered through Whatman No. 2, and the two filtrates were pooled and reduced to 1 ml by rotoevaporation at 42 ± 2°C. The concentrated methanol extractables were then redissolved in 50 ml of double-distilled (dd) water, ethyl acetate, and ethanol (25:20:5), and partitioned three times in a separatory funnel with 100-ml ethyl acetate. The three ethyl acetate fractions per leaf were combined, rotoevaporated to dryness, and then redissolved in 5–10 ml ethyl acetate. Each sample was then placed in a preweighed screw-cap vial, dried under a stream of nitrogen, and the dry weight determined. Samples in vials were then wrapped in aluminum foil and stored at −20°C in darkness.

Method for Acid Hydrolysis. Acid hydrolysis to break glycosidic bonds (i.e., to separate aglycones from sugars in glycosides) was performed on a 13-mg subsample of the dried ethyl acetate extractables from each sampled leaf. This subsample was hydrolyzed 1.5 hr in 10% hydrochloric acid at 100 ± 5°C. The resultant hydrolyzed subsample was cooled, filtered through Whatman No. 2 paper, and then extracted gradually with 9 ml (3 × 3 ml) of chloroform under vacuum. Finally, the chloroform extractables of each subsample were dried under a nitrogen stream, and their dry weight was determined.

Thin-Layer Chromatographic (TLC) Analyses. Ten milligrams of the dried nonhydrolyzed ethyl acetate fraction of leaf extractables were dissolved in 1 ml 100% acetone. The 1.6–7 μg (the amount remaining after filtration) of the hydrolyzed fraction was dissolved in 800 μl 100% chloroform. Each 20-μl sample was vortexed for 6 sec before being spotted with a glass capillary tube on a silica gel TLC plate (20 × 20 cm, 250 μm thick with 5- to 17-μm particle size and a 254-nm fluorescent indicator) (Sigma Chemicals, St. Louis, Missouri). Plates were then developed in a solvent mixture of chloroform–acetone–acetic acid (90:10:1) in a closed glass tank saturated with solvent vapors. The solvent was run up 65% of the plate area, and then the plate was allowed to air dry at room temperature. Resolved bands were first detected under 254-nm UV light (Mineral Light Lamp, UVSL-25, Ultra Violet Products, San Gabriel, California), and then each plate was photographed.

Resolved and dried TLC plates were next examined for resolved compounds with a TLC scanner under UV light (TLC SCAN II, CAMAG Scientific Inc., Wilmington, North Carolina 28405) combined with a CATS3 computing-integrator software program.

The scanning was performed with the deuterium lamp and a line spectrum, 366 nm, at a scanning speed of 5.0 mm/sec. The scanner was programmed to execute a new zero balance before the first track only. Scanning sensitivity (SENS) of the photomultiplier was 170 units; the signal amplification value was 20 units. The measurement mode was "absorption," where absorbed and reflected initial light were measured (CATS3 Software Manual) (CAMAG Scientific, Inc., 1990).

High-Performance Liquid Chromatographic (HPLC) Analyses. A 10-mg aliquot of each sample was dissolved in 1 ml HPLC-grade methanol (Aldrich Chemical Company), vortexed for 1 min, and then passed through a Gelman Nylaflo 0.20-μm filter. Just before the injection into the HPLC, each sample was vortexed for 1 min, then a 20-μl volume was injected into a Beckman Ultrasphere ODS 5-μm column (4.6 mm $\times$ 25 cm). The eluting solvent system consisted of 2% acetic acid–acetonitrile (90% : 10%) for 18 min, then a linear gradient to 100% acetonitrile in 9 min, and then back to the starting ratio in 3 min. There were three replicate injections per leaf sample. The HPLC system was controlled by an Altex 422 programming microprocessor and two Altex 100A pumps. The UV detector was set at 254-nm absorbance. Data were integrated and peak areas were recorded through a Spectra Physics 4400 computing integrator.

Coinjected reference chemicals included the glucoside, fraxin (0.5 mg/ml, w/v); its aglucone, fraxetin (0.5 mg/ml, w/v); the glucoside, esculin (1 mg/ml, w/v); its aglucone, esculetin (1 mg/ml, w/v); and the aglucone, quercetin (80 ng/μl, w/v). An amount (40–50 μl) of a standard chemical was diluted in 80–100 μl of plant extractables, and the resultant mixture was vortexed 1 min; then 20 μl of the mixture was injected into the HPLC. Fraxetin was coinjected with one representative sample from each elicited and nonelicited tree; the other authentic compounds were randomly injected with only one treatment. There were three replicate injections.

Statistics. The mean areas of eight individual HPLC peaks were analyzed by two-way ANOVA where treatments were taken as one factor and replications within trees as the other. Significant differences between pairs of means were determined using Tukey's method (Statistix, Analytical Software). Samples that originated on different dates were treated as separate groups.

RESULTS

Forest tent caterpillar herbivory (i.e., a behavioral response) was altered on green ash trees by each of two dosages of α-tocopherol (Table 1) (also see Haanstad and Norris, 1993).

TABLE 1. MEAN ± SE ASH LEAF AREA EATEN BY FOREST TENT CATERPILLAR FROM ELICITED VERSUS NONELICITED TREES

Behavioral response	Days[a]	Leaf area eaten (cm^2)[b]	
		Treatment	Control
Nonpreferred[c]	8	0.18 ± 0.04**[e]	0.41 ± 0.04
Preferred 1[c]	16	0.49 ± 0.07*	0.31 ± 0.06
Preferred 2[d]	16	0.46 ± 0.05**	0.16 ± 0.04

[a] Days after elicitation.
[b] Tabular data were taken for reference from Haanstad and Norris (1993), where the net difference between treatment and control is presented in graphic form.
[c] Vitamin E dose of 25.0 IU/ml.
[d] Vitamin E dose of 50.0 IU/ml.
[e] Means followed * or ** are significantly different from their control at $P < 0.05$ or $P < 0.01$, respectively.

TLC Resolution of Leaf Extractables. Nonhydrolyzed and hydrolyzed fractions of the extractables from foliage of both elicited (i.e., α-tocopherol-treated) and nonelicited (i.e., control) trees, as resolved by TLC scanning, revealed the comparative chemical patterns shown in Figure 1A–C.

HPLC Resolution of Leaf Extractables. Analyses of the profiles of HPLC-resolved peaks from leaves of elicited (treated) versus nonelicited (control) trees of Haanstad and Norris (1993) indicated mainly quantitative, rather than qualitative, differences (Figure 2A–C). Five peaks were especially major ones in terms of area, and these were chosen first for more detailed comparisons. Three other integrated peaks with lesser areas also showed changes in the area that were quantitatively relatable to the assayed level of leaf antixenosis to forest tent caterpillar larvae on at least one sampling date (e.g., 16 days after elicitation). The remainder of the resolved peaks showed considerable variation in their area; therefore, further analyses of extractables did not involve these latter peaks. When coinjected with leaf extractables, four authentic reference compounds (i.e., fraxin, fraxetin, esculetin, quercetin) proved to be quantitatively minor constituents, whereas esculin was not detected (Table 2).

The HPLC-resolved areas of the eight further studied peaks provided apparently useful chemical correlates of leaf antixenosis to *M. disstria*. Such areas from leaf samples of elicited (nonpreferred) (25 IU/ml) versus nonelicited (control B) trees at eight days after treatment differed significantly ($P < 0.05$) for peaks 1–6 (Figure 3), but not for peaks 7 and 8.

The areas for peaks 1, 2, 3, 5, 6, and 7 in samples from preferred 1 (25 IU/ml) and preferred 2 (50 IU/ml) versus control A at 16 days after treatment were significantly different at $P < 0.05$ (Figure 4). The area of peak 4 did not

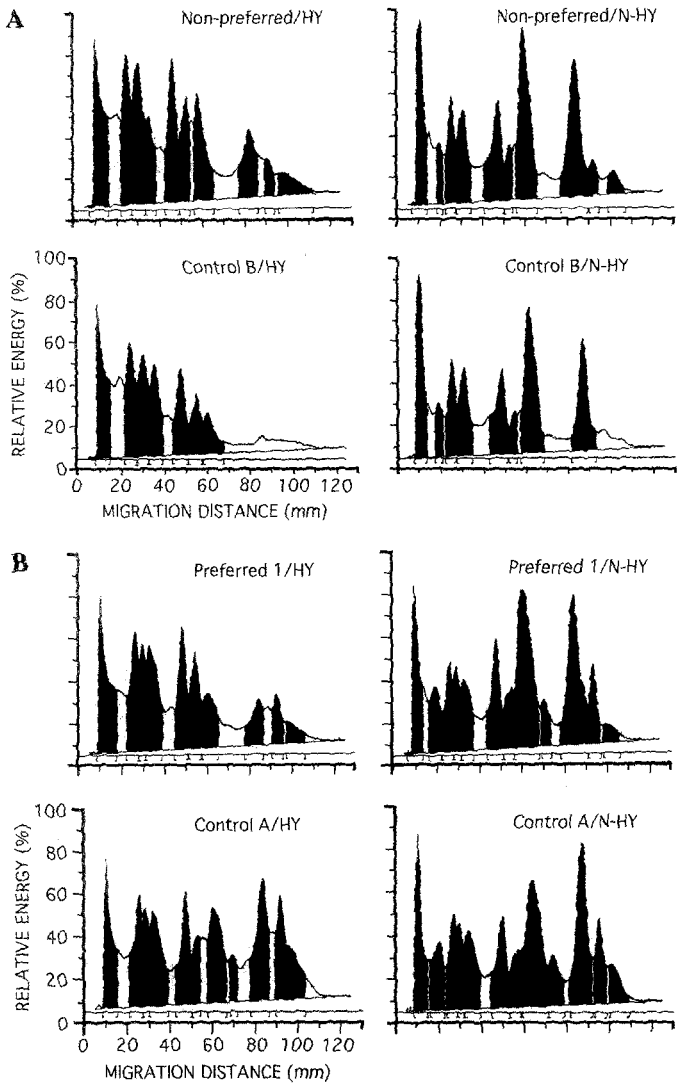

FIG. 1. TLC chromatograms of ethyl acetate extractables of ash tree leaves elicited by α-tocopherol (vitamin E). Profiles are of hydrolyzed (*in* 10% hydrochloric acid) and nonhydrolyzed fractions. (A) *Nonpreferred/HY (hydrolyzed)* and nonpreferred/N-HY (nonhydrolyzed) (elicited with 25 IU/ml α-tocopherol) versus control B/HY (hydrolyzed) and control B/N-HY (nonhydrolyzed); leaves for chemical analyses were collected at eight days after elicitation. (B) Preferred 1/HY (hydrolyzed) and Preferred 1/N-HY (nonhydrolyzed) (elicited with 25 IU/ml α-tocopherol) versus control A/HY (hydrolyzed) and control A/N-HY (nonhydrolyzed); leaves were collected at 16 days after elicitation. (C) Preferred 2/HY (hydrolyzed) and preferred 2/N-HY (nonhydrolyzed) (elicited with 50 IU/ml α-tocopherol) versus control A/HY (hydrolyzed) and control A/N-HY (nonhydrolyzed); leaves were collected at 16 days after elicitation.

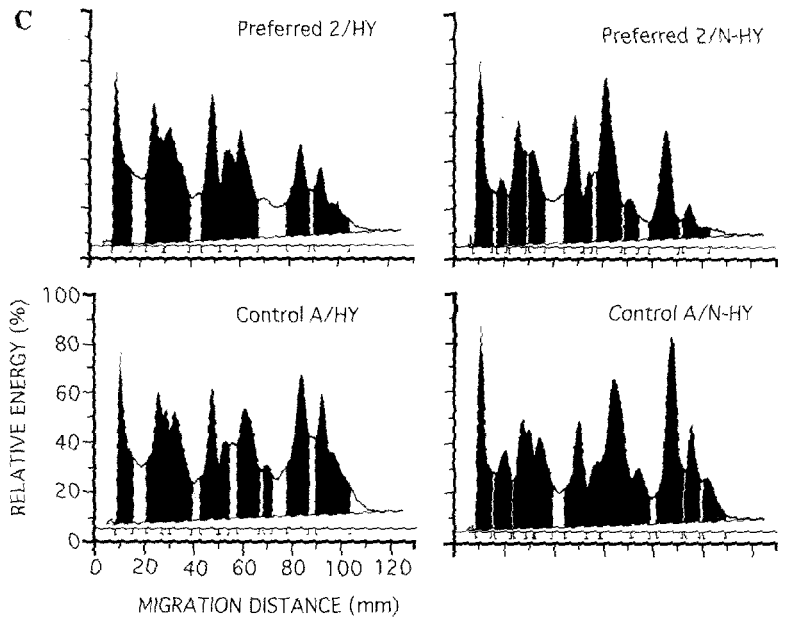

FIG. 1. Continued

differ between preferred 1 and control A. The area of peak 8 was not significantly different between preferred 1 and control A or preferred 2, but was different between the latter two treatments.

Seasonal variation was evident in HPLC profiles of extractables from leaves removed at 8 versus 16 days after elicitation (i.e., control B versus control A) (Figure 5). The areas for the first six of the overall eight studied peaks for control A (eight days after elicitation) versus control B (16 days after elicitation) were significantly different at $P < 0.05$. Areas for peaks 7 and 8 did not differ between the two controls. The area of peak 1 was larger than that of peak 5 in both nonpreferred and control B trees at eight days after elicitation, whereas the area of peak 5 was larger than that of peak 1 in all three leaf extractables (e.g., preferred 1, preferred 2, and control A) at 16 days after elicitation.

DISCUSSION

Based on the present study and other reports (Neupane and Norris, 1991b; Haanstad and Norris, 1993), it is clear that the antioxidant α-tocopherol can elicit an altered chemically based plant antixenosis regarding insect herbivory; therefore we reject the first hypothesis. Regarding the second hypothesis, quantitative changes in elicited glycosides may prove to be significant factors regard-

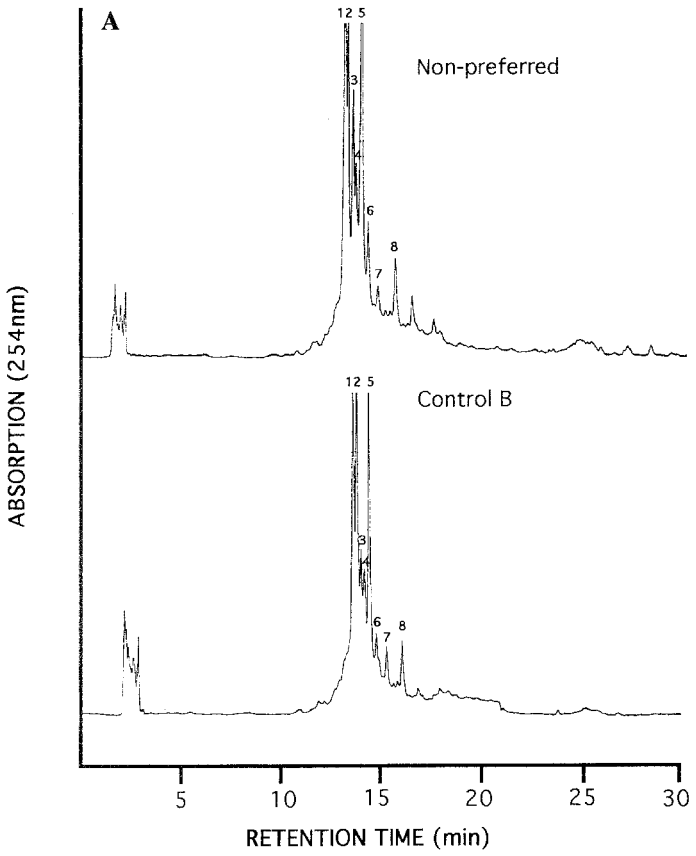

FIG. 2. HPLC-resolved profiles of eight selected peaks (topped by the numbers 1–8) that were used as a basis for comparisons among treatments in the nonhydrolyzed ethyl acetate leaf extractables. (A) Nonpreferred (elicited with 25 IU/ml α-tocopherol) versus control B (non-elicited); leaves were collected at eight days after elicitation. (B) Preferred 1 (elicited with 25 IU/ml α-tocopherol) versus control A (nonelicited); leaves were collected at 16 days after elicitation. (C) Preferred 2 (elicited with 50 IU/ml α-tocopherol) versus control A (nonelicited); leaves were collected at 16 days after elicitation.

ing *M. disstria* herbivory. If this proves true based on further research, the second hypothesis could then be rejected.

Alteration of antixenosis is a very dynamic response influenced by the dosage of elicitor, the time after elicitation, and the position in the plant (Neupane and Norris, 1990, 1991a, b, 1992; Haanstad and Norris, 1992, 1993). Such observed dynamics agree well with our determined changes in the HPLC profiles of the nonhydrolyzed ethyl acetate extractables (Table 1, Figure 2A–C)

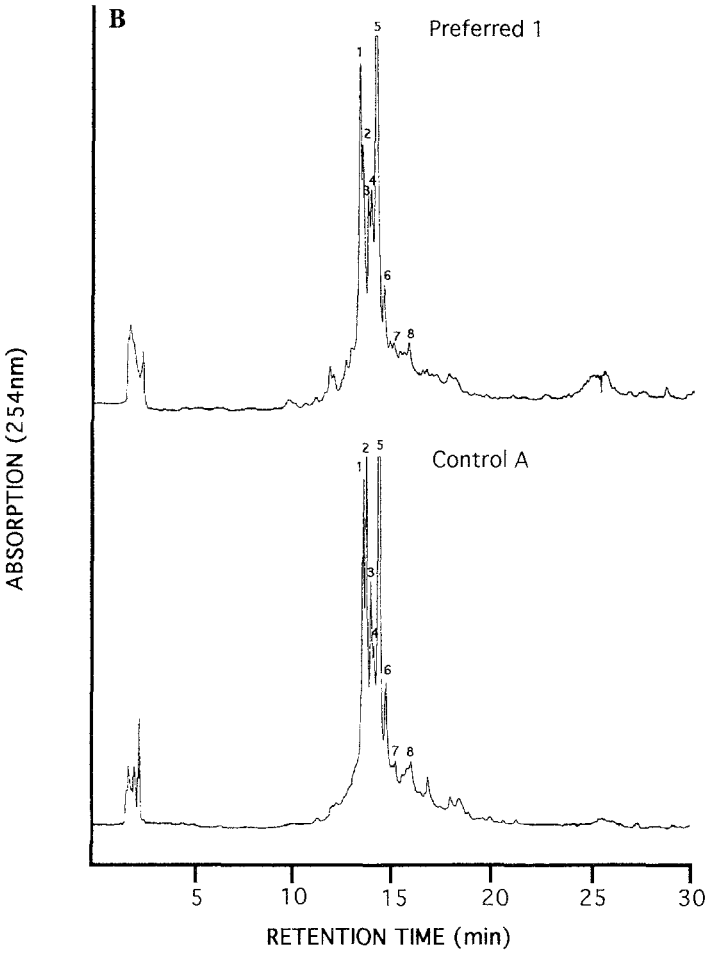

FIG. 2. Continued

and with the amount of overall extractables (total amount of peak area), which was greater with increased antixenosis (nonpreference) and less with increased preference.

Seasonal changes in the herbivory and the HPLC-resolved chemicals over an eight-day period in control (nonelicited) foliage (Figure 5) both indicate the plant's biochemical and physiological dynamics (Haanstad and Norris, 1993). The elicitory antioxidant α-tocopherol affected especially the quantity of defensive phytochemistry.

Regarding dose-dependent tree responses to the elicitory antioxidant

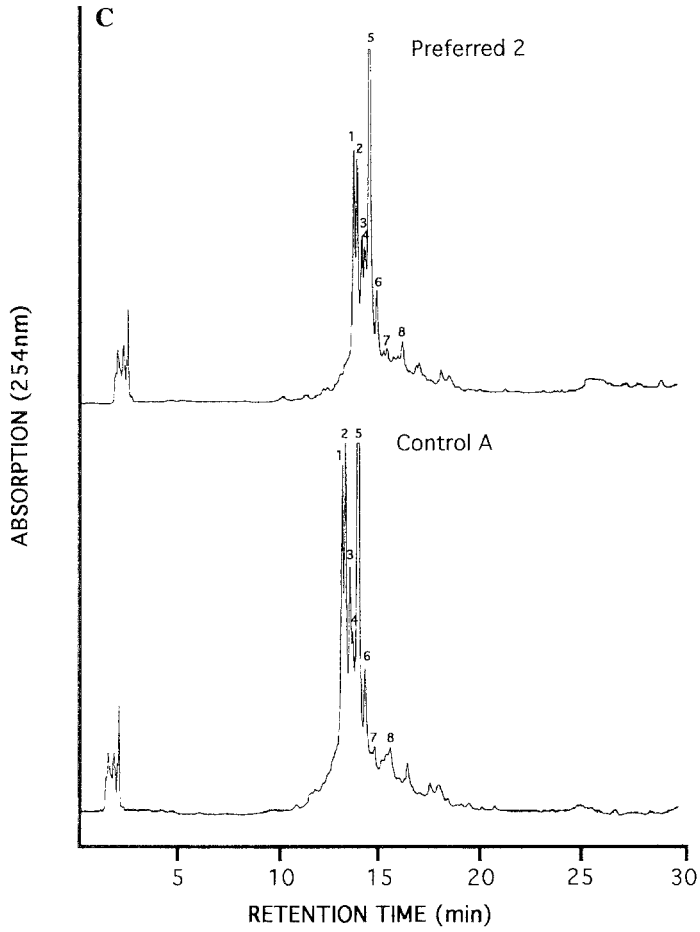

FIG. 2. Continued

TABLE 2. RELATIVE HPLC RESOLVED RETENTION TIMES OF AUTHENTIC REFERENCE COMPOUNDS

Authentic reference compound	Mean retention time (min)
Fraxin	11.00
Fraxetin	12.41
Esculin	6.40
Esculetin	11.47
Quercetin	15.85

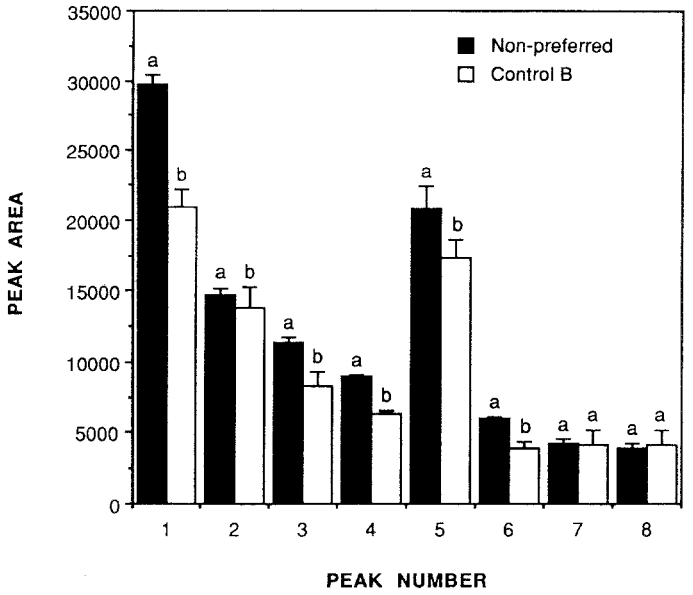

FIG. 3. Comparative mean areas of the indicated peaks in the standardized HPLC analysis of the nonhydrolyzed fraction of a ethyl acetate extractables from the ash tree foliage. Shown are nonpreferred (elicited with 25 IU/ml α-tocopherol) versus control B (non-elicited); leaves were collected at eight days after elicitation. Peak areas not followed by the same letter are significantly different at $P < 0.05$.

α-tocopherol, there was an increase in overall extractables as nonpreference (antixenosis) increased as compared to the control at eight days after a 25 IU/ml elicitation. Conversely, the amount of overall extractables decreased as the feeding preference of the forest tent caterpillar increased as compared to the control at 16 days after either the 25 IU/ml or 50 IU/ml elicitation. In these studies increased antiherbivory was associated only with the lower, i.e., 25 IU/ml, dosage of elicitor.

The observed changes in the HPLC profile of the eight selected peaks seem sufficiently correlated with the increased degree of herbivory to suggest at least a partially causative relationship. These peaks represent quantitatively the majority of the resolved chemicals. The first five selected peaks showed individually, and as a group, consistent quantitative relationships to the recorded level of herbivory at both sampling periods. This was also true for the latter three peaks (i.e., 6, 7, and 8) at 16 days after elicitation; however, at eight days after elicitation only peak 6 of this group showed a positive correlation with the level of antiherbivory. Regarding the other resolved peaks, all were both quantita-

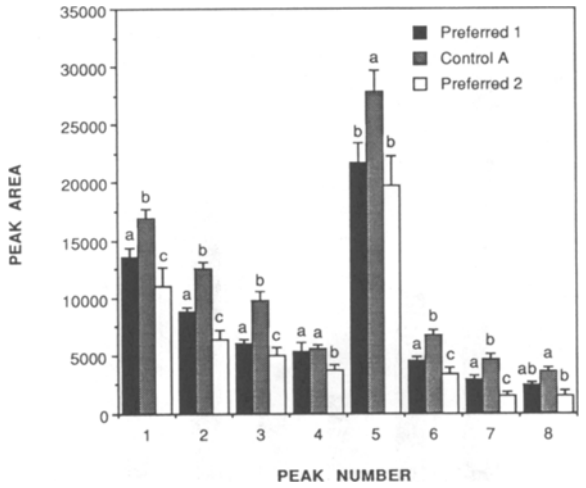

FIG. 4. Comparative mean areas of the indicated peaks in the HPLC analysis of the nonhydrolyzed fraction of ethyl acetate extractables from the ash tree foliage. Shown are preferred 1 (elicited with 25 IU/ml α-tocopherol) and preferred 2 (elicited with 50 IU/ml α-tocopherol) versus control A (nonelicited); leaves were collected at 16 days after elicitation. Peak areas not followed by the same letter are significantly different at $P < 0.05$.

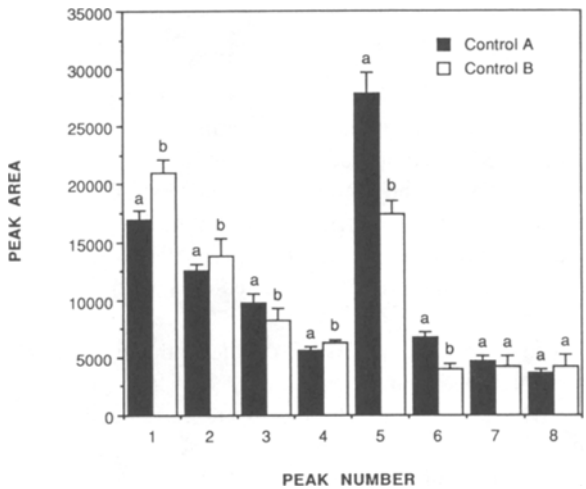

FIG. 5. Comparative mean areas of the indicated peaks in the HPLC analysis of the nonhydrolyzed fraction of ethyl acetate extractables from leaves collected at 8 (control B) versus 16 (control A) days after elicitation. Peak areas not followed by the same letter are significantly different at $P < 0.05$.

tively minor and inconsistent in their relationship to the forest tent caterpillar's feeding preference/nonpreference.

Studies of the chemical identities of the eight peaks chosen for further investigation and their effects on *M. disstria* herbivory, growth, and development are underway.

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REFERENCES

AKAZAWA, T., URITANI, I., and KUBOTA, H. 1960. Isolation of ipomeamarone and two coumarin derivatives from sweet potato roots injured by the weevil, *Cylas formicaris elegantulus*. *Arch. Biochem. Biophys*. 88:150–156.

BENZ, G., and ABIVARDI, C. 1991. Preliminary studies on wound-induced and PIIF-induced resistance in some solanaceous plants against *Spodoptera littoralis* (Boisd.) (Lepidoptera, Noctuidae). *J. Appl. Entomol*. 111:349–357.

CAMAG Scientific Inc. 1990. CATS3 Software Manual. 4, pp. 2–18.

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.

HAANSTAD, J.O., and NORRIS, D.M. 1992. Altered elm resistance to smaller European elm bark beetle (Coleoptera: Scolytidae) and forest tent caterpillar (Lepidoptera: Lasiocampidae). *J. Econ. Entomol*. 85:172–181.

HAANSTAD, J.O., and NORRIS, D.M. 1993. Antioxidant alpha-tocopherol altered green ash antixenosis to forest tent caterpillar (Lepidoptera: Lasiocampidae). *J. Econ. Entomol*. Submitted.

HAUKIOJA, E. 1991. Cyclic fluctuations in density: Interactions between a defoliator and its host tree. *Acta Oecol*. 12:77–88.

LARSSON, S., WIREN, A., LUNDGREN, L., and ERICSSON, T. 1986. Effects of light and nutrient stress on leaf phenolic chemistry in *Salix dasyclados* and susceptibility to *Galerucella lineola* (Coleoptera). *Oikos* 47:205–210.

LIN, H., KOGAN, M., and FISCHER, D. 1990a. Induced resistance in soybean to the Mexican bean beetle (Coleoptera: Coccinelidae): Comparisons of inducing factors. *Environ. Entomol*. 19:1852–1857.

LIN, H., KOGAN, M., and ENDRESS, A.G. 1990b. Influence of ozone on induced resistance in soybean to the Mexican bean beetle (Coleoptera: Coccinelidae). *Environ. Entomol*. 19:854–858.

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.P., and NORRIS, D.M. 1991a. Sulfhydryl-reagent alteration of soybean resistance to the cabbage looper, *Trichoplusia ni*. *Entomol. Exp. Appl*. 60:239–245.

NEUPANE, F.P., and NORRIS, D.M. 1991b. α-Tocopherol alteration of soybean antiherbivory to *Trichoplusia ni* larvae. *J. Chem. Ecol*. 17:1941–1951.

NEUPANE, F.P., and NORRIS, D.M. 1992. Antioxidant alteration of *Glycine max* (Fabaceae) defensive chemistry: Analogy to herbivory elicitation. *Chemoecology* 3:25–32.

U.S. Pharmacopaea 1980. 20:846.

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HOST RECOGNITION BY ENTOMOPATHOGENIC NEMATODES: BEHAVIORAL RESPONSE TO CONTACT WITH HOST FECES

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Abstract—Host recognition by entomopathogenic nematodes may occur through contact with insects' excretory products, cuticle, or gut contents. We analyzed the behavioral responses of four species of entomopathogenic nematodes during contact with feces of natural or experimental hosts. Host recognition by nematodes was manifested in alterations in the frequency and/or duration of one or more search parameters including forward crawling, head-waving, body-waving, stopping, backward crawling, head-rubbing, and head-thrusting. *Heterorhabditis bacteriophora* and *Steinernema glaseri* showed behavioral responses to contact with feces of their natural hosts, *Spodoptera exigua* (Lepidoptera) and *Popillia japonica* (Coleoptera), and to the experimental hosts, *Acheata domesticus* (Orthoptera) and *Blatella germanica* (Blatteria). *Steinernema carpocapsae* responded only to *B. germanica* feces, whereas *S. scapterisci* did not significantly respond to any of the insect species. During contact with cockroach feces, all nematodes, except *S. scapterisci*, showed avoidance behavior. We suggest that ammonia present in cockroach feces is inhibitory to nematodes. Specific host recognition by entomopathogenic nematodes may be an important mechanism to maintain host affinities.

Key Words—Host recognition behavior, entomopathogenic nematodes, feces, *Spodoptera exigua*, Lepidoptera, Noctuidae, *Popillia japonica*, Coleoptera, Scarabaeidae, *Blattella germanica*, Blatteria, Blattellidae *Acheata domesticus*, Orthoptera, Gryllidae.

INTRODUCTION

Host recognition is a vital step in the life cycle of most parasites. In plant (e.g., Grundler et al., 1991) and animal (e.g., Stewart et al., 1987; Granzer and Haas,

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1991) parasitic nematodes, host recognition consists of a sequence of behavioral responses to an array of stimuli associated with host or host-related materials. For instance, on exposure to the root exudates of a host plant, second-stage juveniles of *Heterodera schachtii* exhibit a preinfection exploratory behavior that includes stylet thrusting and head-end bending (Grundler et al., 1991). In response to environmental and host stimuli, infective juveniles of the hookworm *Ancylostoma caninum* initiate host-finding and host recognition in four behavioral phases including: snakelike movement, waving behavior, creeping direction, and penetration behavior (Granzer and Haas, 1991).

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae possess tremendous potential as biological control agents of insects (Gaugler and Kaya, 1990). The soil-inhabiting, infective juveniles sense, search for, and invade suitable host insects. Host search by the infective juveniles is believed to include directed orientation towards host-released stimuli, such as CO_2 (Gaugler et al., 1980), excretory products (Schmidt and All, 1978, 1979), and temperature gradients (Pye and Burman, 1981; Byers and Poinar, 1982). Lewis et al. (1992) reported that infective juveniles of *Steinernema glaseri* shifted from ranging to localized search after contact with feces or cuticle of potential host insects. However, host recognition and its specificity in entomopathogenic nematodes is totally unstudied.

Although entomopathogenic nematodes possess an extremely broad laboratory host range (Poinar, 1979), ecological and behavioral barriers restrict their natural host range (Gaugler, 1988). Owing to its sit-and-wait (ambush) host-seeking behavior (Gaugler et al., 1990; Lewis et al., 1992), *S. carpocapsae* is well adapted to parasitize active, surface-dwelling insect species. Conversely, *S. glaseri* is highly mobile (cruiser) and is therefore best adapted to parasitize sedentary, subterranean insect species (Kaya, 1990; Lewis et al., 1992). In field populations, *S. glaseri* is associated with scarabaeid larvae and *S. carpocapsae* with lepidopteran larvae (Poinar, 1990).

Temporal and spatial factors, such as synchronous life cycles and differing habitat preferences, also appear to determine the host range of entomopathogenic nematodes (Webster and Dunphy, 1988). For example, New Zealand populations of *S. feltiae* occur at the bases of tussock grass, where they have become adapted to parasitize lepidopteran larvae (Noctuidae and Hepialidae) feeding on the roots of these grasses (Poinar, 1990). These strains of *S. feltiae* are ineffective parasites of native scarabaeid larvae living in the same habitat. Danish populations of *S. feltiae* have become adapted to parasitize bibionid flies (Bovien, 1937). Another nematode species, *S. scapterisci* Nguyen and Smart, is well adapted to parasitize mole crickets in Uruguay (Nguyen and Smart, 1990).

Although the mechanism of host recognition in entomopathogenic nematodes is not understood, the above examples of host-specific adaptations suggest that specific host recognition occurs. The response to CO_2 by some nematodes

suggests that volatile cues are general attractants, permitting host habitat location, but providing little information about their source. Host recognition in these nematodes may occur through contact with insect (1) excretory products, (2) cuticle, or (3) gut contents. In this paper, we concentrated on host recognition by entomopathogenic nematodes when in contact with insect feces. Behavioral responses of four nematode species to natural and experimental hosts were compared. We consider a natural host as an insect species from which the nematode has been isolated in the field, while experimental hosts are not normally associated with the nematode species in field collections. We hypothesized that if there is specific host recognition, then entomopathogenic nematodes should respond differently to contact cues from the natural versus experimental hosts.

METHODS AND MATERIALS

Entomopathogenic nematode species originally isolated from different groups of insect species (Poinar, 1990) were selected for testing: *S. carpocapsae* from lepidopteran larvae, *S. glaseri* from scarabaeid larvae, *S. scapterisci* from mole crickets, and *Heterorhabditis bacteriophora* from lepidopteran and scarabaeid larvae. Four insect species were chosen to represent the above groups of hosts: the house cricket *Acheata domesticus* (Orthoptera: Gryllidae), the German cockroach *Blatella germanica* (Blatteria: Blattellidae), the Japanese beetle grub *Popillia japonica* (Coleoptera: Scarabaeidae), and the beet armyworm *Spodoptera exigua* (Lepidoptera: Noctuidae).

Nematode Cultures. Infective juvenile *H. bacteriophora* (HP88), *S. carpocapsae* (All), and *S. glaseri* (NC) were cultured in last-instar wax moth (*Galleria mellonella*), at 25°C, following Dutky et al. (1964). *S. scapterisci* (Uruguay) was reared in adult house cricket. All experiments were performed with infective juveniles, harvested three to five days after initial emergence from the host and washed three times in sterile distilled water.

Preparation of Fecal Extracts. Feces were collected from adult house cricket, adult German cockroach, last-instar Japanese beetle, and last-instar beet armyworm reared on their respective diets: house cricket at 25°C, on rabbit food (Blue Seal Foods, Inc., Lawrence, Massachusetts), German cockroach at 27°C on rat chow 5012 (Purina Mills, Inc., St. Louis, Missouri), Japanese beetle at 25°C on roots of turfgrass, and beet armyworm at 28°C on Lima bean (*Phaseolus vulgaris*) leaves. Fifty insects were collected from the cultures and placed individually in well plates (12.5 × 8.5 cm) where they were held for 24 hr at 25°C before the feces were collected. Immediately after collection, 20 mg of feces were mixed with 2 ml of sterile distilled water, macerated, centrifuged at 3000 rpm for 3 min, and the supernatant was collected.

Nematode Behavior Assay. The assay arena was a microscope slide (7.5

× 3.5 cm) covered with a 3-mm layer of 1.5% agar. Immediately before testing, 20 μl of fecal extract was spread uniformly over the agar with a nylon brush. One infective juvenile was then placed on the agar and, after 15 sec of acclimatization to the new surface, its behavior was monitored for 2 min. Only actively moving infective juveniles were chosen. The frequency and duration of forward crawling, head-waving, body-waving, stopping, backward crawling, head-rubbing, and head-thrusting were recorded using a computerized tabulation software (Observer R, Noldus, Wageningen, The Netherlands). The behaviors were defined as follows: (1) forward crawling—sinusoidal forward movement generated by backward body waves; (2) head-waving—lifting <25% of the anterior of the body from the substrate with side-to-side movement; (3) body-waving—lifting 26–75% of anterior of the body with side-to-side movement [this behavior is different from nictation as described by Ishibashi and Kondo (1990), wherein the nematode stands on its tail, almost straight, and waves in the air]; (4) stopping—still, no visible movement for a time span greater than 2 sec; (5) backward crawling—sinusoidal backward movement generated by forward body waves; (6) head-rubbing—pushing of head against the body making a "pea" shape; and (7) head-thrusting—repeated pushing of head against the substrate.

Every nematode species was exposed to fecal extracts from each insect species tested, comprising 16 combinations in all. Fifteen nematodes (replicates) were studied for each treatment. Five nematodes were studied on each of the three slides. Only exsheathed infective juveniles were used. Control slides were treated with 20 μl of sterile distilled water.

Determination of Deterrent in Cockroach Feces. Cockroach feces are known to contain large quantities of ammonia (Mullins and Cocran, 1973); therefore, we determined the quantity of ammonia in the German cockroach feces following Oser (1954). The effects of pure ammonia (derived from ammonium hydroxide, 30% NH_3, Fisher Scientific, Springfield, New Jersey) at the concentration equivalent to that in feces on the behavior of *H. bacteriophora* were evaluated. As ammonia readily dissolves in boric acid, feces were mixed with boric acid to remove ammonia. The effects of feces thus treated with boric acid on behavior of *H. bacteriophora* were studied. One hundred microliters of 4% boric acid (H_3BO_3, Fisher Scientific, Springfield, New Jersey) was mixed with 100 μl of fecal extract prior to its application to the agar surface. Treatment with sterile distilled water and pure ammonia + boric acid served as controls. Only 20 μl of the test solution was applied to the agar surface, and the frequency and duration of various behaviors were recorded as described above.

Statistical Analysis. The relative duration of various behaviors was converted to percent and then normalized using arcsine transformation. The transformed data and the data on frequency of behaviors were analyzed using analysis

of variance and Tukey's studentized range test (SAS Institute, 1982). All comparisons were at the 0.05 significance level.

RESULTS

Response to Extracts from Feces. Heterorhabditis bacteriophora and *S. glaseri* infective juveniles altered their behavior in response to contact with feces of all four insect species (Figures 1–4). When in contact with feces from a member of its natural group of hosts, the beet armyworm, *H. bacteriophora*, significantly reduced the duration of forward crawling and increased the duration and frequency of stopping and head-thrusting (Figure 1). In response to contact with feces from another natural host, the Japanese beetle, *H. bacteriophora* significantly reduced the duration of forward crawling and increased the duration and frequency of head-thrusting. The response of *H. bacteriophora* to contact

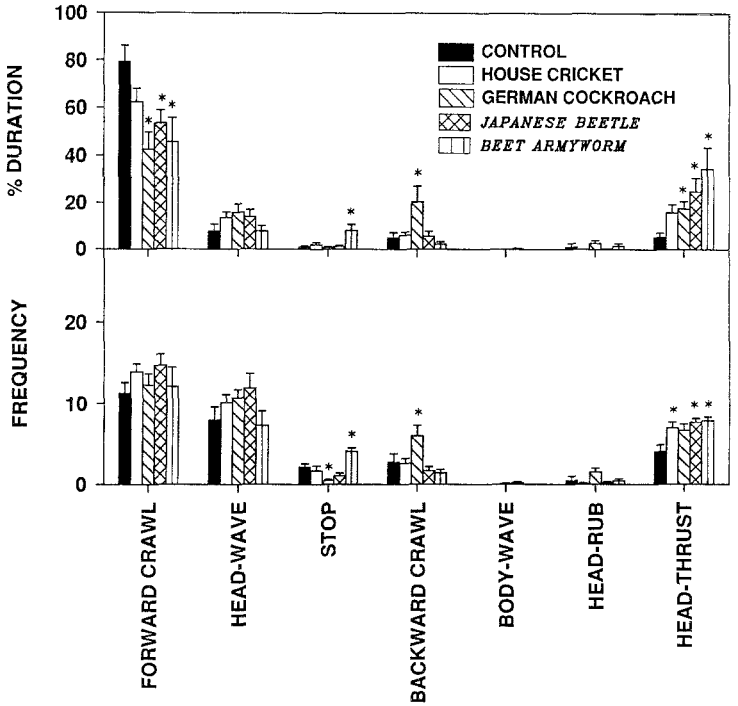

FIG. 1. Duration and frequency (±SE) of various behaviors of *Heterorhabditis bacteriophora* during contact with insect fecal extracts or water. Italicized figure legends represent natural hosts. Asterisks denote values significantly different from controls at 0.05% level.

with feces of the two experimental hosts was different. For example, during contact with feces from the house cricket, *H. bacteriophora* significantly increased only the frequency of head-thrusting, whereas with feces from the German cockroach, the nematodes significantly decreased the duration of forward crawling and increased the duration of backward crawling and the frequency and duration of head-thrusting.

During contact with feces from the natural host, the Japanese beetle, *S. glaseri* significantly reduced the frequency of head-waving and increased the duration and frequency of head-thrusting (Figure 2). Infective juvenile *S. glaseri* also showed different responses to contact with feces of the experimental hosts. For example, during contact with feces from the beet armyworm, *S. glaseri* significantly reduced the duration of forward crawling and increased the duration of head-waving and the frequency and duration of head-thrusting. During contact with feces from the house cricket, *S. glaseri* only significantly increased the frequency and duration of head-thrusting, whereas the nematodes increased the

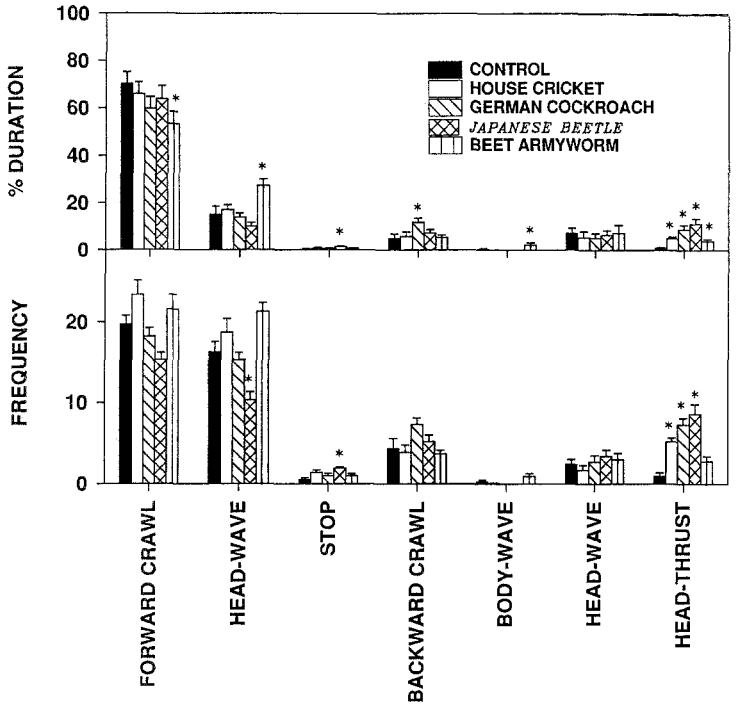

FIG. 2. Duration and frequency (±SE) of various behaviors of *Steinernema glaseri* during contact with insect fecal extracts or water. Italicized figure legends represent natural hosts. Asterisks denote values significantly different from controls at 0.05% level.

duration of backward crawling and frequency and duration of head-thrusting when in contact with feces from the German cockroach.

Infective juvenile *S. carpocapsae* did not show measurable response to contact with feces of their natural hosts (Figure 3). However, the infective juveniles responded to contact with feces of the German cockroach by significantly increasing the frequency and duration of backward crawling. *Steinernema scapterisci* did not significantly respond to feces of any of the insects examined (Figure 4).

Determination of Deterrent in Cockroach Feces. Each milligram of cockroach feces contained 0.9 (±0.02) μl of ammonia. Ammonia at this concentration was inhibitory to *H. bacteriophora* infective juveniles (Figure 5); the nematodes spent 23.5% of the total time backward crawling. The inhibitory effect of ammonia was almost equivalent to that of feces with which nematodes spent about 21.5% of time backward crawling. When boric acid was mixed

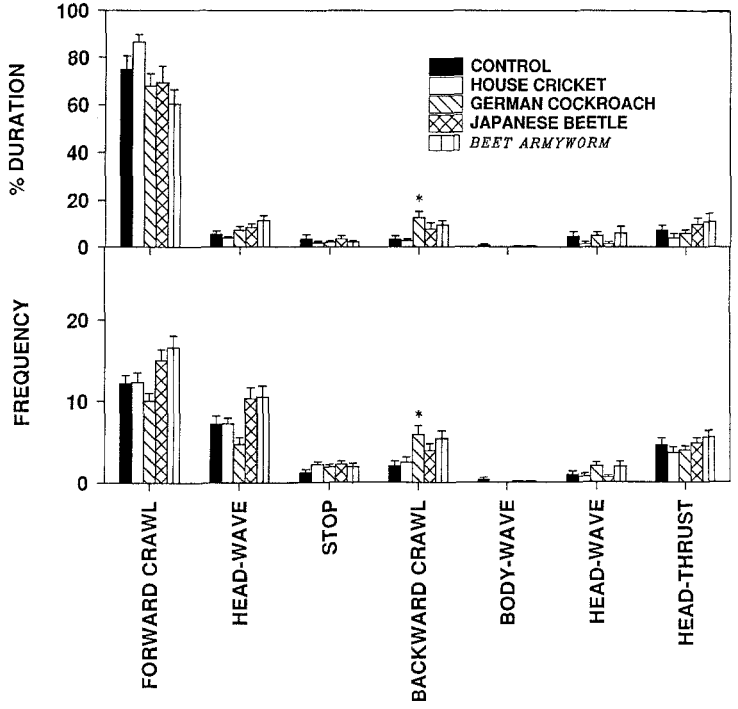

FIG. 3. Duration and frequency (±SE) of various behaviors of *Steinernema carpocapsae* during contact with insect fecal extracts or water. Italicized figure legends represent natural hosts. Asterisks denote values significantly different from controls at 0.05% level.

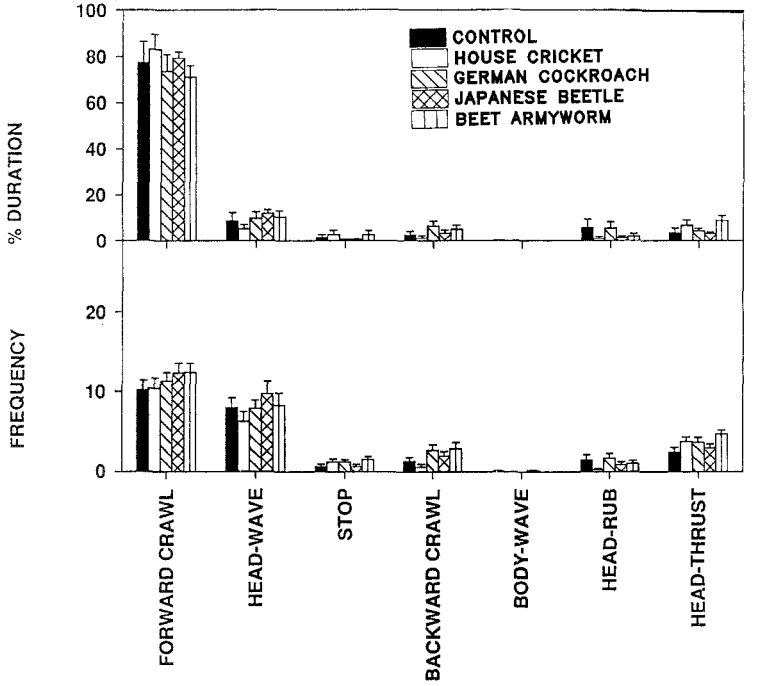

FIG. 4. Duration and frequency (±SE) of various behaviors of *Steinernema scapterisci* during contact with insect fecal extracts or water. Asterisks denote values significantly different from controls at 0.05% level.

with ammonia or fecal extract, the frequency and duration of backward crawling by nematodes did not differ significantly from the control.

DISCUSSION

Not all nematode species altered their behavioral sequence in response to contact with feces from either the natural or experimental hosts. Infective juvenile *H. bacteriophora* and *S. glaseri* changed their behavior in some manner to feces from all four insect species, whereas *S. scapterisci* did not significantly alter its behavior to any of them. *Steinernema carpocapsae* significantly responded to only German cockroach feces. This overall pattern of behavioral modification of nematode species in response to contact cues fits well with their host search behavior. Infective juveniles of *H. bacteriophora* and *S. glaseri* have many of the characteristic behaviors of cruise foragers (Gaugler, 1988), whereas *S. carpocapsae* (Gaugler, 1988) and *S. scapterisci* (Grewal et al.,

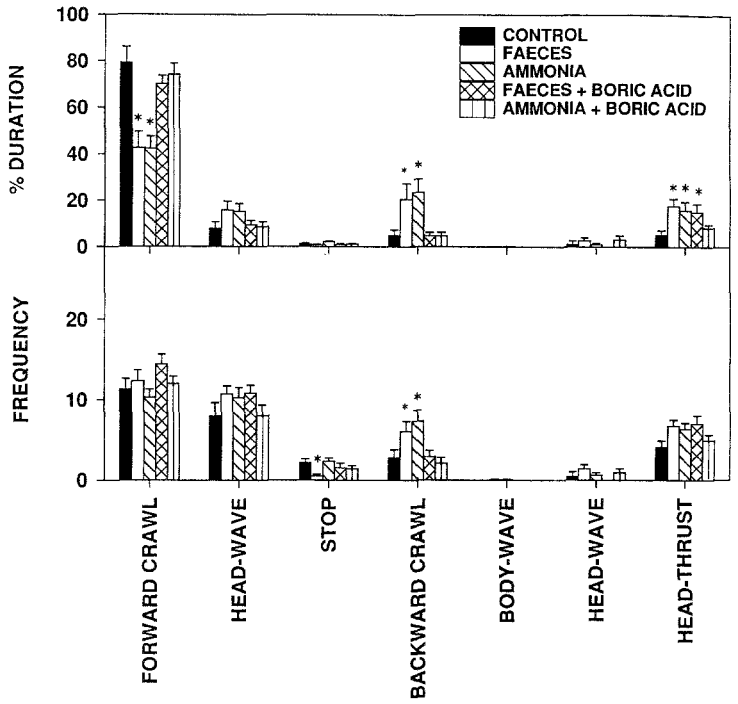

FIG. 5. Effects of fecal extracts of the German cockroach *Blatella germanica* or ammonia on the duration and frequency (±SE) of various behaviors of *Heterorhabditis bacteriophora*. Boric acid was used to absorb ammonia. Asterisks denote values significantly different from controls at 0.05% level.

1993a) are ambush foragers. The relevance of chemical cues in host search by the two types of foragers differ: cruising foragers rely more heavily on chemical cues than ambush foragers (Bell, 1991). In another study, *S. glaseri* responded to selected host cues by shifting from ranging to localized search, characterized by decreased locomotory rate, distance traveled, search area, and the proportion of the test period spent moving, whereas *S. carpocapsae* did not measurably respond to the same host cues (Lewis et al., 1992). Furthermore, *S. carpocapsae* and *S. scapterisci* infective juveniles use an alternative mechanical means of attachment to the host, nictation (Campbell and Gaugler, 1993) and therefore, are less likely to be affected by chemical host cues. Our results further support that cruiser nematode species respond more strongly to the chemical host cues (feces) than the ambush foragers.

Infective juveniles of *H. bacteriophora* and *S. glaseri* exhibited specific behavioral changes after contact with feces of the four insect species examined.

For example, when in contact with feces of the natural host, the beet armyworm, infective juvenile *H. bacteriophora* reduced the time spent forward crawling and increased the frequency and duration of stopping and head-thrusting, whereas with the experimental host, the house cricket, the nematodes increased the duration and frequency of head-thrusting only. *S. glaseri* decreased the frequency of head-waving and increased the duration and frequency of head-thrusting during contact with feces of the natural host, the Japanese beetle, whereas the nematodes reduced the duration of forward crawling and increased the frequency and duration of head-waving and head-thrusting with feces from the experimental host, the beet armyworm. These host-specific alterations in nematode behavior during contact with feces suggest host recognition by entomopathogenic nematodes.

Host affinities seen in field populations have been attributed to ecological similarity and behavioral compatibility between nematodes and their respective host species of insects (Webster and Dunphy, 1988). For example, subterranean insects are unlikely to be parasitized by surface-foraging nematodes, and ambush foragers would rarely encounter sedentary hosts. In addition to ecological mechanisms and search strategies reinforcing the recognized host–parasite pairs, however, there is evidence that *S. glaseri* shifts from ranging to localized search more strongly after contact with feces from their natural host, the Japanese beetle, than with feces from the beet armyworm (Lewis et al., 1992). Our study confirms this observation and supports host recognition as an important mechanism to maintain host affinities of entomopathogenic nematodes.

When in contact with cockroach feces, all the nematode species except *S. scapterisci* significantly increased the frequency and time spent backward crawling. Mixing of cockroach feces with boric acid, which absorbed ammonia, eliminated the backward crawling behavior of *H. bacteriophora*, suggesting that the ammonia present in the feces was inhibitory. Pye and Burman (1981) reported that ammonia at 7.5 mM concentration repelled *S. carpocapsae*. Zervos and Webster (1989) reported that the American cockroach *Periplaneta americana* is not very susceptible to parasitism by *Heterorhabditis zealandica* (= *Heterorhabditis heliothidis* T327); nymphs were only parasitized when forced into prolonged exposure to a damp substrate rich in nematodes, and adults when starved and dehydrated. Cockroaches being externally ammonotellic (Bell and Adiyodi, 1981), excrete ammonia as the major nitrogenous material in feces. The avoidance behavior of nematodes in the presence of ammonia may restrict nematode penetration through the anus from which ammonia is excreted. This may have important implications in the biological control of cockroaches with entomopathogenic nematodes.

Sensitivity of *H. bacteriophora* to contact with German cockroach feces was related to the ineffective parasitism of cockroaches by the nematodes (Grewal et al., 1993b). However, *S. scapterisci* did not show a significant increase

in backward crawling during exposure to cockroach feces, and they effectively parasitized adult cockroaches. The lesser sensitivity of *S. scapterisci* to contact with the German cockroach feces may be due to its adaptation to parasitize cockroaches (Grewal et al., 1993b).

Another interesting aspect of the interaction between nematodes and cockroach feces or ammonia was the increase in the duration of head-thrusting. Besides negative locomotion, *H. bacteriophora* significantly increased the duration of head-thrusting while in contact with ammonia. In the presence of a toxic bacterium, *Bacillus* sp., the free-living nematode *Caenorhabditis elegans* frequently burrowed into the nutrient agar substrate (Grewal, 1990). Although the nematodes were unable to burrow into the 1.5% plain agar we used, the increase in head-thrusting was associated with the increase in nematode reversals. Therefore, the increased head-thrusting may be a part of the nematodes' behavioral repertoire representing avoidance.

The present study has recorded the scanning behaviors of entomopathogenic nematodes in the presence or absence of host excretory products. Scanning is the set of mechanisms by which animals move their receptors and sometimes their bodies or appendages to capture information from the environment efficiently (Evans and O'Brian, 1986). Some insects locate resources by casting their bodies or appendages to the left and right of the path, thereby increasing the arc within which prey can be contacted laterally, e.g., larvae of nectivorous flies (Syrphidae) and lacewings (Chrysopidae) (Bansch, 1966; Chandler, 1969). Nematode attraction to chemical stimuli has often been attributed to klinotactic orientation (Croll, 1970; Dusenberry, 1980, 1983). The side-to-side movements or head-waving of nematodes has been interpreted as sampling stimuli (Gaugler et al., 1980; Green, 1980). Presumably the distance between the amphids is insufficient to permit comparison of the intensity of stimulation without head movement (Wharton, 1986; Ishibashi and Kondo, 1990). Apparently other behaviors including body-waving, frequency of stops (stopping), backward crawling, head-rubbing, and head-thrusting also constitute a nematode's host-scanning mechanism. Although the significance of all the individual behaviors is not yet known, host-specific alterations in nematode behavioral responses suggest specific host recognition by entomopathogenic nematodes.

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REFERENCES

BANSCH, R. 1966. On prey-seeking behavior of aphidophagous insects, pp. 123–128, *in* I. Hodek (ed.). Proceedings of the Prague Symposium on Ecology of Aphidophagous Insects. Academia, Prague.

BELL, W.J. 1991. Searching Behaviour: The Behavioural Ecology of Finding Resources (Animal Behaviour Series). Chapman and Hall, London.

BELL, W.J., and ADIYODI, K.G. 1981. The American Cockroach. Chapman and Hall, London.

BOVIEN, P. 1937. Some types of association between nematodes and insects. *Vidensk. Medd. Dan. Naturhist. Foren. Khobenhavn* 101:1–114.

BYERS, J.A., and POINAR, G.O., JR. 1982. Location of insect hosts by the nematode *Neoplectana carpocapsae* in response to temperature. *Behavior* 79:1–10.

CAMPBELL, J.F., and GAUGLER, R. 1993. Nictation behavior and its ecological implications in entomopathogenic nematode search strategies. *Behaviour* In press.

CHANDLER, A.E.F. 1969. Locomorory behavior of first instar larvae of *Aphidophagous syrphidae* (Diptera) after contact with aphids. *Anim. Behav.* 17:673–678.

CROLL, J. 1990. The Behavior of Nematodes: Their Activity, Senses and Responses. Edward Arnold, London.

DUSENBERRY, D.B. 1980. Behavior of free-living nematodes, pp. 127–158, *in* B.M. Zukerman (ed.). Nematodes as Biological Models. Academic Press, New York.

DUSENBERRY, D.B. 1983. Chemotactic behavior of nematodes. *J. Nematol.* 15:168–173.

DUTKY, S.R., THOMPSON, J.V., and CANTWELL, G.E. 1964. A technique for the mass propagation of the DD-136 nematode. *J. Insect Pathol.* 6:417–422.

EVANS, B.I., and O'BRIAN, W.J. 1986. An analysis of the feeding rate of white crappie. *Develop. Environ. Biol. Fish.* 7:200–306.

GAUGLER, R. 1988. Ecological considerations in the biological control of soil-inhabiting insects with entomopathogenic nematodes. *Agric. Ecosys. Environ.* 24:351–360.

GAUGLER, R., and KAYA, H.K. 1990. Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, Florida.

GAUGLER, R., LEBECK, L., NAKAGAKI, B., and BOUSH, G.M. 1980. Orientation of the entomopathogenic nematode *Neoaplectana carpocapsae* to carbon dioxide. *Environ. Entomol.* 9:649–652.

GAUGLER, R., CAMPBELL, J.F., and MCGUIRE, T. 1990. Selection for host finding in *Steinernema feltiae*. *J. Invertebr. Pathol.* 54:363–372.

GRANZER, M., and HAAS, W. 1991. Host-finding and host recognition of infective *Ancylostoma caninum* larvae. *Int. J. Parasitol.* 21:429–440.

GREEN, C.D. 1980. Nematode sex attractants. *Helminthol. Abstr.* 49B:81–93.

GREWAL, P.S. 1990. Studies on saprobic rhabditid nematodes and their associated bacteria affecting mushroom culture. PhD thesis. University of London.

GREWAL, P.S., GAUGLER, R., KAYA, H.K., and WUSATY, M. 1993a. Infectivity of the entomopathogenic nematode *Steinernema scapterisci* (Nematoda: Steinernematidae). *J. Invertebr. Pathol.* In press.

GREWAL, P.S., GAUGLER, R., and LEWIS, E.E. 1993b. Host recognition behavior of entomopathogenic nematodes during contact with gut contents. *J. Parasitol.* 77:In press.

GRUNDLER, F., SCHNIBBE, L., and WYSS, U. 1991. In vitro studies on the behavior of second-stage juveniles of *Heterodera schachtii* (Nematoda: Heteroderidae) in response to host plant root exudates. *Parasitology* 103:149–155.

ISHIBASHI, N., and KONDO, E. 1990. Behavior of infective juveniles, pp. 139–150, *in* R. Gaugler and H.K. Kaya (eds.). Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, Florida.

KAYA, H.K. 1990. Soil ecology, pp. 93–117, *in* R. Gaugler and H.K. Kaya (eds.). Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, Florida.

LEWIS, E.E., GAUGLER, R., and HARRISON, R. 1992. Entomopathogenic nematode host finding: Response to host contact cues by cruise and ambush foragers. *Parasitology* 105:309–315.

MULLINS, D.E., and COCHRAN, D.G. 1973. Nitrogenous excretory materials from the American cockroach. *J. Insect Physiol.* 19:1007–1018.

NYUGEN, K.B., and SMART, G.C., JR. 1990. *Steinernema scapterisci* n. sp. (Rhabditida: Steinernematidae). *J. Nematol.* 22:187–199.

OSER, B.L. 1954. Hawk's Physiological Chemistry, 14th ed. McGraw-Hill, New York.

POINAR, G.O., JR. 1979. Nematodes for Biological Control of Insects. CRC Press, Boca Raton, Florida.

POINAR, G.O., JR. 1990. Taxonomy and biology of Steinernematidae and Heterorhabditidae, pp. 23–61, *in* R. Gaugler and H.K. Kaya (eds.). Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, Florida.

PYE, A.E., and BURMAN, M. 1981. *Neoaplectana carpocapsae*: Nematode accumulations on chemical and bacterial gradients. *Exp. Parasitol.* 51:13–20.

SAS INSTITUTE. 1982. SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina.

SCHMIDT, J., and ALL, J.N. 1978. Chemical attraction of *Neoaplectana carpocapsae* (Nematoda: Steinernematidae) to insect larvae. *Environ. Entomol.* 7:605–607.

SCHMIDT, J., and ALL, J.N. 1979. Attraction of *Neoaplectana carpocapsae* (Nematoda: Steinernematidae) to common excretory products of insects. *Environ. Entomol.* 8:55–61.

STEWART, G.L., DESPOMMIER, D.D., BURNHAM, J., and RAINES, K.M. 1987. *Trichinella spiralis*: Behavior, structure, and biochemistry of larvae following exposure to components of the host enteric environment. *Exp. Parasitol.* 63:195–204.

WEBSTER, J.M., and DUNPHY, G.B. 1988. Host compatibility of insects to nematodes, pp. 237–245, *in* J.A. Veech and D.W. Dickson (eds.). Vistas on Nematology: A Commemoration of the Twenty-fifth Anniversary of the Society of Nematologists. Society of Nematologists, Inc., Hyattsville, Maryland.

WHARTON, D.A. 1986. A Functional Biology of Nematodes. The Johns Hopkins University Press, Baltimore, Maryland.

ZERVOS, S., and WEBSTER, J.M. 1989. Susceptibility of cockroach *Perilaneta americana* to *Heterorhabditis heliothidis* (Nematoda: Rhabditoidea) in the laboratory. *Can. J. Zool.* 67:1609–1611.

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INSECTICIDAL EFFECT OF ESSENTIAL OILS FROM MEDITERRANEAN PLANTS UPON *Acanthoscelides Obtectus* SAY (COLEOPTERA, BRUCHIDAE), A PEST OF KIDNEY BEAN (*Phaseolus vulgaris* L.)

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Abstract—The bioactivity of 22 essential oils from aromatic and medicinal plants was tested upon *Acanthoscelides obtectus* Say (Coleoptera, Bruchidae), a pest of kidney bean (*Phaseolus vulgaris* L.). The insecticidal effect was evaluated by determination of 24- and 48-hr LC_{50} and LC_{50} (from 1.50 mg/dm^3 to more than 1000 mg/dm^3). Isoprenoids and phenylpropanoids were identified by gas chromatography. The most efficient essential oils were extracted from plants belonging to Labiatae. *Origanum marjorana* and *Thymus serpyllum* essential oils were the most toxic.

Key Words—Essential oils, Labiatae, Umbelliferae, *Lauraceae*, *Citrus limon*, *Cymbopogon nardus*, *Eucalyptus globulus*, *Myristica fragrans*, insecticidal effect, LC_{50}, *Acanthoscelides obtectus* Say, Bruchidae, Coleoptera, terpenoids, benzenoids.

INTRODUCTION

Insect control using plant material is an ancient practice all over the world. As synthetic or petroleum-based insecticides posed serious health hazards for Mammalia, efforts have been devoted to search for new classes of insecticides derived from plants with a lower mammalian toxicity and a lesser persistence in the environment, such as pyrethrinoids or rotenone compounds. However, increased

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insect resistance to pesticides has led to the finding of new molecules from botanicals as alternative pest-control agents, a well-established approach in control strategies for pests. The best known compound of this new class of insecticides is azadirachtin, a limonoid from leaves and fruits of the neem tree, *Azadirachta indica* A. Juss (Saxena, 1989). Recently, the insecticidal and repellent properties of plants or plant-derived substances from Asia, Africa, and America against the main pests of these areas (Anjana et al., 1988; Rajapakse, 1990; Morallo-Rejesus et al., 1990) were tested.

In the south of France, *Acanthoscelides obtectus* Say (Coleoptera, Bruchidae) is one of the most damaging of the bruchid insect family. Its host plant is the kidney bean (*Phaseolus vulgaris* L.), an important food produced and consumed in this area, but French production is now insufficient to supply demand because of storage losses. To protect their stored products, farmers of southwestern France once stored with the beans the seeds of aromatic plants commonly considered as condiments for cooking or herb tea. We tested the efficiency of these practices and observed that the most efficient botanicals belonged to the Labiatae family, and some Gramineae, Myrtaceae, and Lauraceae were also insecticidal (Regnault-Roger and Hamraoui, 1992a). These plants are a source of essential oils. Antifungal, antiseptic, and bactericidal properties of essential oils (Benjilali et al., 1984; Aboutabl et al., 1986; Dube et al., 1989; Richard, 1992) were previously observed. More recently, insecticidal effect on some Curculionidae, Bostrichidae, Cucujidae, Tenebrionidae (Shaaya et al., 1991) and dipterous families were mentioned (Konstantopoulou et al., 1992). We also observed the insecticidal effect of some essential oils on *Acanthoscelides obtectus* Say (Regnault-Roger and Hamraoui, 1991, 1992b).

However, the chemical composition of essential oils depends on several parameters including the degree of maturity of the plant at harvest, and the seasonal geographic, and developmental conditions. In this work, we screened the insecticidal effect of 22 essential oils, extracted mainly from Mediterranean plants, upon *Acanthoscelides obtectus* Say and determined their chemical composition by gas chromatography. According to the literature data, a comparison of structure–activity relationships will be discussed.

METHODS AND MATERIAL

Biological. Insects (*Acanthoscelides obtectus* Say) and beans (*Phaseolus vulgaris* L.) have been cultivated in our Institute for many years. The insects belonged to the same generation. Beetles are kept in a room at 27°C with a moisture level of 65–75% and photoperiod of 12 hr light–12 hr dark. Botanicals come from local markets and the Institute's fields. Essential oils were extracted by steam distillation (Guenther, 1972). The following essential oils were tested

and analyzed: from the Labiatae family—*Thymus vulgaris*, *T. serpyllum*, *Mentha piperita*, *Rosmarinus officinalis*, *Satureia hortensis*, *Lavandula angustifolia*, *Origanum vulgare*, *O. marjorana*, *Ocimum basilicum*, *Salvia officinalis*, *Verbena officinalis*; from the Umbelliferae family—*Petroselinum sativum*, *Cuminum cyminum*, *Apium graveolens*, *Anethum graveolens*, and *Coriandrum sativum*; from the Lauraceae family—*Laurus nobilis*, *Cinnamomum verum*; and also *Citrus limon* (Rutacae), *Myristica fragrans* (Myristicaceae), *Cymbopogon nardus* (Gramineae), and *Eucalyptus globulus* (Myrtaceae).

Bioassay. The insecticidal tests were made on 10 pairs of adults enclosed within an experimental cage (interior volume 106 cm^3). The experiments were conducted in a controlled atmosphere, in a room at 24°C with a moisture level of 65–75% relative humidity and photoperiod of 14 hr light and 10 hr dark. The essential oils were deposited on Whatman filter paper (Aboutabl et al., 1986) in a small fumigation room closed by a sheet of gauze, thus avoiding direct contact between the deposit and the beetles. Quantities of essential oil from 0.5 μl to 20 μl were deposited. Each treatment was replicated three times. Mortality was observed after 24 and 48 hr.

Chemical Analysis. Gas chromatographic analysis to identify the terpenoid and benzenoid compounds was adapted from Zweig and Sherma (1972) and Dey and Harborne (1991) on a Chrompack CP 9000 with a WCOT fused silica column (50 m long, OD 0.45 mm, ID 0.32 mm); liquid phase CP-WAX-52 CB FID detector; nitrogen gas with a 1.32 mL/min flow. Injector temperature: 250°C, detector temperature 250°C, injected amount 0.2 μl with a 1/6 lessening. Temperature program was: isotherm 5 min at 50°C, 2°C/min gradient to 115°C, isotherm 30 min, then 2°C/min gradient to 210°C, isotherm 10 min. This program was chosen for better identification of some terpenoids.

Statistical Studies. The statistical study lies on ascendant hierarchic classification (cluster analysis) using the usual euclidian average distance, calculated on factorial coordinates relative to adult mortality (Roux, 1985).

RESULTS

A linear relation could be defined between mortality of the beetles and the amounts of the essential oils using the Probits method; thus the fumigant effect of the oils might be evaluated calculating LC_{50} (concentration inducing 50% mortality of the insect population) (Figure 1).

Eleven essential oils presented a LC_{50} of less than 10 mg/dm^3 (*O. majorana*, *T. serpyllum*, *C. verum*, *R. officinalis*, *O. basilicum*, *C. cyminum*, *S. hortensis*, *T. vulgaris*, *S. officinalis*, *C. sativum*, and *O. serpyllum*) for 24 hr and 15 for 48 hr (in addition: *L. angustifolia*, *L. nobilis*, *M. piperita*, *V. officinalis*).

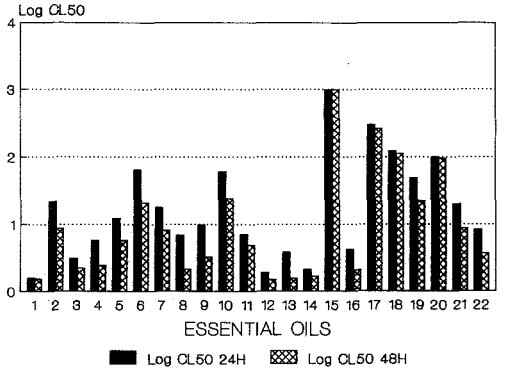

FIG. 1. Adult mortality (LC_{50} at 24 and 48 hr) of *Acanthoscelides obtectus* with regard to some botanicals.

An LC_{50} (both LC_{50} 24 hr and LC_{50} 48 hr) of 10 mg/dm^3 to 100 cm^3 was observed with *L. nobilis*, *V. officinalis*, *M. piperita*, *A. graveolens*, *C. nardus*, *E. globulus*, and *C. limon*, *M. fragrans*, *A. graveolens* and *P. sativum* had an LC_{50} more than 100 mg/dm^3. Consequently, the cluster analysis distinguished eight groups (for LC_{50} 24 hr) in one group alone *P. sativum*; the least active, *Apium graveolens*, *C. limon*, *M. fragrans*; then a group with *Anethum graveolens*, *E. globulus* and *C. nardus*; one with *M. piperita*, *L. angustifolia*, *L. nobilis*, *V. officinalis*; and the others classified in the most active group (Figure 2a). For LC_{50} 48 hr, *Anethum graveolens* was classified with *E. globulus* and *C. nardus*. Then *V. officinalis*, *L. nobilis*, and *M. piperita* were grouped together. The main group included the most efficient: *L. angustifolia*, *O. vulgarè*, *S. officinalis*, *C. sativum*, *T. serpyllum*, *R. officinalis*, *S. hortensis*, *T. vulgaris*, *O. majorana*, *C. verum*, and *C. cyminum*.

Different terpenoids and benzenoids of essential oils of botanicals were identified by gas chromatography (Table 1). The main components of each essential oil were given in Table 2. Figure 3 shows a characteristic GC chromatogram: of *Origanum marjorana*.

DISCUSSION

The effects of essential oils upon insects depend on several parameters including chemical composition and species susceptibility of the insect even though the biochemical target appears to be limited (Casida, 1990).

The species susceptibility of the insect to essential oils has already been noted. Whereas early studies noted a repellent effect from the content of pine needles (Dieter and Geyer, 1970), insecticidal properties were noted against

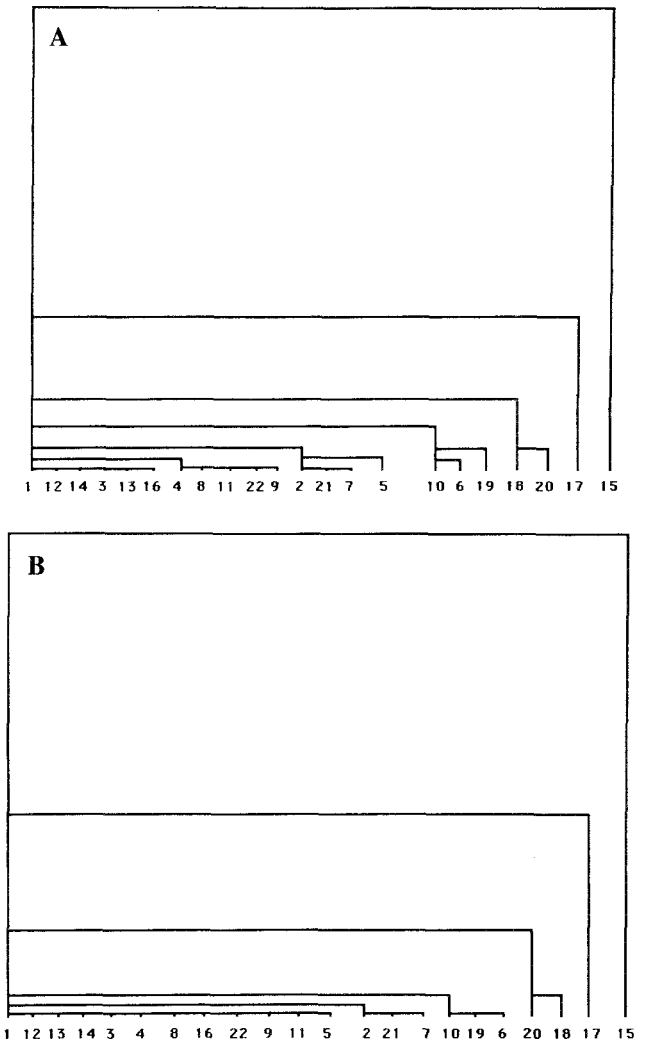

FIG. 2. Branched classification established on the mortality of *A. obtectus* adults with regard to some essential oils. (a) LC_{50} at 24 hr; (b) LC_{50} at 48 hr.

Periplaneta americana (American cockroach) and *Zonocerus variegatus* (a grasshopper) from essential oils of *Denettia tripetala* (Iwuabe et al., 1981). However, the physiological responses of each insect species to the same crude plant extract of essential oil were not the same (Kalemba et al., 1990; Morallo-Rejesus and Tantengco, 1986; Shaaya et al., 1991). We observed that essential oil from *Cymbopogon nardus* was one of the least effective upon *A. obtectus*

TABLE 1. STEAM-VOLATILE CONSTITUENTS OF SOME SPECIES FROM LABIATAE, UMBELLIFERAE, LAURACEAE, RUTACAE, MYRISTICACEAE, MYRTACEAE AND GRAMINEAE FAMILIES

	Thymus serpyllum	*Mentha piperita*	*Rosmarinus officinalis*	*Satureia hortensis*	*Lavandula angustifolia*	*Eucalyptus globulus*	*Laurus nobilis*	*Thymus vulgaris*	*Origanum vulgare*
α-Pinene	0.47	0.23	0.53	4.08	0.20	3.86	7.13	0.15	0.37
Camphene	0.10	0.29	0.29	2.42	0.19	0.01	0.59		0.11
β-Pinene	0.09	0.96	0.01	0.59	0.13	1.61	4.92	0.09	0.20
Sabinene		0.07			0.04	0.27	9.11		
β-Myrcene	0.84	0.14	0.12	1.21	0.38	1.22	1.29	0.60	0.60
Limonene	0.69	1.01	0.18	3.49	0.76		1.98	0.17	0.21
Cineole-β-Pheli.	1.96	0.65	10.60	4.04	0.12	86	48.6	1.24	0.36
γ-Terpinene	3.21	0.08	0.27	1.72	2.33	0.84	0.42	1.74	0.28
para *p*-Cymene	10.00	0.01	0.20	9.60		2.43	1.61	17.3	16.3
Camphor	0.25	0.30	30.6	0.86	0.45	0.09		0.16	0.01
Linalool	2.31	0.12	2.10	6.35	30.8	0.08	4.13	1.50	8.51
t-Caryophyllene	0.33	2.72		0.76	11.10		1.35	6.12	0.52
α-Terpineol		0.51	1.74			0.64			
Borneol			22.1		1.57	0.02	0.14	0.60	
Geraniol			0.91		0.32	0.38	0.19		
Eugenol			0.87		0.19		0.55	0.39	
Thymol	30.4		1.32	13.4	0.12			47.5	26.6
Carvacrol	28.9		2.60	39.9	0.11			2.70	39.4
Linalyl acetate		3.10			34.2				
Menthone		35							
Isomenthone		12.8							
Menthol		18.8							
α-Humulene									
Citronellal									
α + β-Thuyone									
Terpinene 1-OL-4									
Cinnamaldehyde									
Carvone									
Cuminaldehyde									
Apiole									

but it was the most effective upon *Sitrotoga cereallela* Ol. (Krishnarajah et al., 1985). A very toxic effect of essential oil extracted from *Citrus* species was shown upon *Sitophilus zeamais* Motsch, but was less noticeable upon *Tribolium castaneum* (Haubruge et al., 1989) as *C. limon* essential oil had a poor effect upon *A. obtectus*.

Chemical compositions of essential oils vary, depending a lot on extraction

TABLE 1. CONTINUED

Cympobogon nardus	*Salvia officinalis*	*Origanum majorana*	*Ocimum basilicum*	*Cinnamomum verum*	*Petroselinium sativum*	*Cuminum sativum*	*Apium graveolens*	*Myristica fragrans*	*Anethum graveolens*	*Citrus limon*	*Verbena officinalis*	*Coriandrum sativum*
0.02	2.87	0.85	0.49	0.14	15	0.71	0.2	23.2	0.6	1.83	0.91	5.53
0.01	2.35	0.01	0.13		0.13	0.11		0.29	0.01	0.05	0.53	1.13
0.02	2.25	0.378	1.31		11.8	11.4	0.38	15.6	0.05	12.2	1.21	0.41
0.01	0.17	6.93	0.4		0.41	0.31		22.6	0.1	1.86	0.59	
0.06	0.65	1.66	0.55		0.78	0.74	0.15	3.22	11.79	1.15	0.1	0.6
2.14	0.84	1.5	0.37	0.06	0.74	0.94	73.2	3.92	35.9	63.9	18.9	2.54
0.08	6.41	1.75	7.55	0.16	3.44	0.42		0.61	1.59	0.05	2.34	0.13
	0.28	10.3	0.08		0.38	11.4	0.01	3.25		6.73	0.04	5.45
0.05	0.38	3.06	0.81	0.81	0.47	10.2	0.1	0.82	2.28	3.52	0.2	2.93
0.12	6.47		0.92			0.3	0.01		4.52		0.12	5.03
0.80	3.59	15.3	50	0.24	0.25	0.53	0.14	0.31	0.21	0.18	2.76	68.2
0.05	5.04	2.68	0.42		0.17		0.17	4.94		0.04	0.6	0.28
0.09	3.00	3.07			0.35			0.76		0.24		0.49
1.19	2.24	0.45	0.32	0.09	0.01			0.11	0.09		1.69	0.38
21.6	0.01	0.16	0.2							0.01	1.33	1.52
			6.42	3.64	0.08			0.4		0.5		
		0.2			10.2		0.14		0.4			
					3.42		0.78	0.2				
	21.4	3.34	0.17									
	5.15											
33.8												
	24 + 4											
		20.6										
				90.1								
									34.8		32.4	
						42.5						
					43							

techniques (e.g., solvent, pH), the part of the sample studied (flower, leaf, stem), the season (degree of maturity of the plant at the crop period), and ecologic conditions (geography, development, hygrometry, photoperiod). Chemical compounds of the essential oils are closely involved in the secondary metabolism of plants and often are found as precursor and transformation compounds. Among these secondary metabolites, terpenoids are very widely dis-

TABLE 2. MAIN COMPONENTS OF DIFFERENT ESSENTIAL OILS TESTED ON *A. obtectus* SAY

Essential oil	Family	Main components (%)
1 *Thymus serpyllum* L.	*Labiatae*	Thymol (30.4), carvacrol (28.9), *p*-cymene (10), citra (4.2)
2 *Mentha χ piperita* L.	*Labiatae*	Menthone (3.5), menthol (18.8), isomenthone (12.8)
3 *Rosmarinus officinalis* L.	*Labiatae*	Camphor (30.6), borneol (22.1), 1,8-cineole/β-phellandrene (10.6)
4 *Satureia hortensis* L.	*Labiatae*	Carvacrol (39.9), thymol (13.4), linalool (6.4)
5 *Lavandula angustifolia* P. Miller	*Labiatae*	Linalyl acetate (34.2), linalool (31.8), β-Caryophyllene (11.1)
6 *Eucalyptus globulus* Labill.	*Myrtaceae*	1.8-Cineole (86), α-pinene (3.9) *p*-cymene (2.4)
7 *Laurus nobilis* L.	*Lauraceae*	1.8-Cineole (48.6), sabinene (9.1), terpinyl acetate? (8.2), α-pinene (7.1)
8 *Thymus vulgaris* L.	*Labiatae*	Thymol (47.5 and 26.5), *p-cymene (17.3 and 18.4), β-caryophyllene (6.1 and 3.5)*
9 *Origanum vulgare* L.	*Labiatae*	Carvacrol (39.4), thymol (26.6), p-cymene (16.3)
10 *Cympobogon nardus* Wats	*Gramineae*	Citronella (33.8), geraniol (21.6), citronellol (9.2), geranyl acetate (3.4)
11 *Salvia officinalis* L.	*Labiatae*	β & β-Thuyone (28), linalyl acetate (21.4), β-caryophyllerie and α-humulene (5)
12 *Origanum majorana* L.	*Labiatae*	Terpinene-1-ol-4 (20.6), linalool (15.3), γ-terpinene (10.3)
13 *Ocimum basilicum* L.	*Umbelliferae*	Linalool (50), limonene (7.5), eugenol (3.6), estragole (3.2)
14 *Cinnamomum verum* Presl.	*Lauraceae*	Cinnamaldehyde (90), eugenol (3.6)
15 *Petroselinum sativum* L.	*Umbelliferae*	Apiole (43), thymol (10.2)
16 *Cuminum cyminum* L.	*Umbelliferae*	Cuminaldehyde (42.5), β-pinene (11.8), γ-terpinene (11.4), nerol (11.4)
17 *Apium graveolens* Houtt	*Umbelliferae*	Limonene (73.2), sequiterpene (13)
18 *Myristica fragrans* L.	*Myristicaceae*	β Pinene (23.2), sabinene (22.6), myristicine (7.9)
19 *Anethum graveolens*	*Umbelliferae*	Limonene (35.9), carvone (34.8), β-myrcene (11.8)
20 *Citrus limon* (L.) Burm f	*Rutacae*	Limonene (63.9), β-pinene (12.2)
21 *Verbena officinalis* L.	*Labiatae*	Carvone (32.4), limonene (18.9), citral (17.6)
22 *Coriandrum sativum* L.	*Umbelliferae*	Linalool (68.2), α-pinene (5.5), γ-terpinene (5.5)

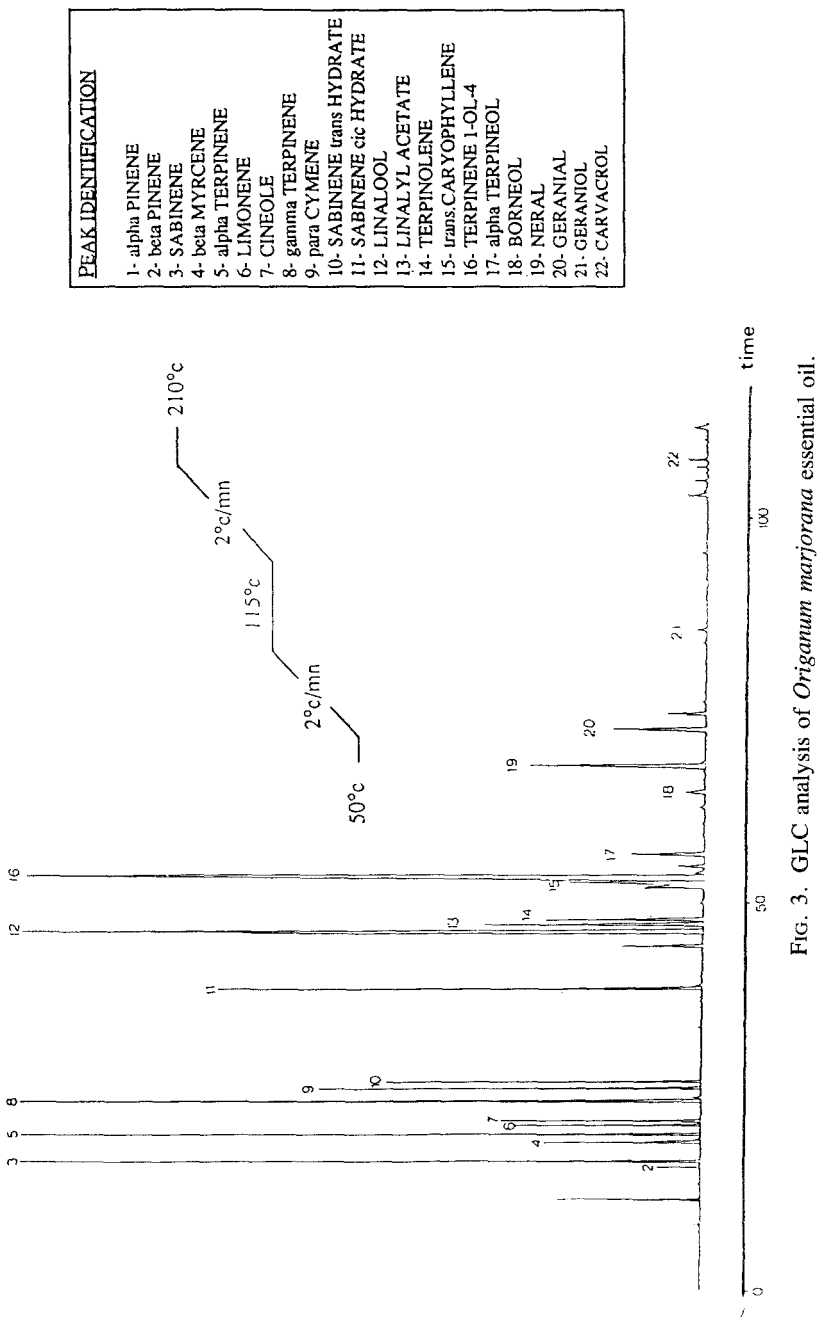

FIG. 3. GLC analysis of *Origanum marjorana* essential oil.

tributed throughout the plant kingdom as monoterpenes, sesquiterpenes, or diterpenes, derivatives of isoprene or phenylpropane skeletons. Several thousand compounds occur and the class of dicotyledons, especially the orders Lamiales and Rutales, are the most prolific in their production of monoterpenes (Banthorpe and Charlwood, 1980): the widespread diversity of terpenoids and benzenoids was illustrated in the essential oils we identified (Table 1).

The different chemotypes of each sample are shown in Table 2. They are in good agreement with results in the literature, although we noted few differences: percentages of camphor and borneol in *R. officinalis* are higher than the norm (Formacek and Kubeczka, 1985). We also tested another sample of thymol-*p*-cymene thyme chemotypes and observed that the most efficient contained more thymol: 47.5/26.5% (unpublished data). It would be interesting to test other thyme chemotypes (Granget and Passet, 1971, 1973). If *C. nardus* and *S. officinalis* are classical chemotypes, the respective percentages of citronellal and linalyl acetate are very high (Formacek and Kubeczka, 1985).

It appears in Table 2 that the main compound of the most effective extracts upon *A. obtectus* present an oxygenated function (hydroxylated or phenolic) within their chemical skeleton. Microsomal cytochrome P-450 monooxygenases play an important role in the detoxification in plant toxins for herbivorous insects, their activities being generally higher in generalists than in semispecialists (Yu, 1987). The susceptibility of *A. obtectus* to the oxygenated structure might be considered to be related to the monooxygenase activity; further experimentation is needed to prove this point. However, both oxygenated and nonoxygenated terpenoids developed toxicity against insects. The bioactivity of terpenoids such as *d*-limonene, α-terpineol, β-myrcene, linalool, and pulegone against insects, including the housefly, German cockroach, rice weevil, or western corn rootworm as neurotoxics has been reviewed (Coats et al., 1991). Toxicity of *p*-cymene or thymol as main constituents of thyme oils to *Rhizoperta dominica* or *Leptinotarsa decemlineata* was observed by Kurowska et al. (1991); as was the activity of 1,8-cineole against *Tribolium castaneum* (Shaaya et al., 1991), mosquitoes (Klocke et al., 1985), or linalool extracted from *Ocimum canum* essential oils against stored product insects (Weaver et al., 1991; Ntezurubanza, 1991). We have also noted that *O. basilicum* (linalool, 50%) is more active upon *A. obtectus* than *C. sativum* (linalool, 68.2%). Two hypotheses may be proposed: the activity of linalool is associated with those of other terpenoids, or the two samples did not contain the same stereoisemeric form of linalool: *l*-linalool (*O. basilicum*) would be more efficient than *d*-linalool (*C. sativum*).

The complexity of the chemical composition of essential oils led to complex activity, probably with synergism or antagonism phenomena between allelochemicals. External factors (geography, seasons) and internal factors (metabolism, maturity of the plant) were involved without definite proof of an ecological function. Essential oils were considered for a long time as excretory products

in plant kingdom. More recent studies have pointed out their involvement in interspecies communication (for example, in pollination) and they also appear to be defenses against predators such as fungi, herbivorous mammals, and insects.

REFERENCES

ABOUTABL, E.A., SOLIMAN, F.M., ELZALABANI, S.M., BRUNKE, and EL KHERSH, T.A. 1986. Essential oil of *Thymus. Bovei Benth. Sci. Pharmacol.* 54(1):43–48.

ANJANA, A., SONE, A.L., and GUPTA, K.C. 1988. Natural products as protectants of pulses against pulse beetles. *Bull. Grain Technol.* 26(2):154–164.

BANTHORPE, D.V., and CHARLWOOD, B.V. 1980. The terpenoids, pp. 185–215, *in* E.A. Bell and B.V. Charlwood (eds.). Secondary Plant Products. Encyclopedia of Plant Physiology. New Series, Vol. 8. Springer-Verlag, Berlin.

BENJILALI, B., TANTAOUI-ELARAKI, A., AYADI, A., and HILAL, M. 1984. Method to study the antimicrobial effect of essential oils. Application to the antifungal activity of six maroccan essences. *J. Food Prot.* 47(10):748–752.

CASIDA, J.E. 1990. Pesticide mode of action: evidence for and implications of a finite number of biochemical targets, pp. 11–22, *in* J.E. Casida (ed.). Pesticides and Alternatives: Innovative Chemical and Biological Approaches to Pest Control. Elsevier Amsterdam.

COATS, J.R., KARR, L.L., and DREWES, C.D. 1991. Toxicity and neurotoxic effects of monoterpenoids, pp. 305–316, *in* Naturally Occurring Pest Bioregulators. ACS Symposium Series 449. American Chemical Society, Washington, D.C.

DEY, P.M., and HARBORNE, J.B. 1991. Methods in Plant Biochemistry VII. Academic Press, London. pp. 23–24.

DIETER, O., and GEYER, W. 1970. Resin and essential oil contents of pine needles for the development of needle feeding insects. *Arch. Forstw.* 19(2):151–167.

DUBE, S., UPADHYAY, P.D., and TRIPATHI, S.C. 1989. Antifungal, physiochemical and insect repelling activity of the essential oil of *Ocinum basilicum. Can. J. Bot.* 67(7):2085–2087.

FORMACEK, V., and KUBECZKA, K.H. 1985. Essential Oils Analysis by Capillary Gas Chromatography and ^{13}C NHR. Wiley, New York.

GRANGET, R., and PASSET, J. 1971. Types chimiques (chémotypes) de l'espèce *Thymus vulgaris* L. *C. R. Acad. Sci. Paris, Série D,* 273:2350–2353.

GRANGET, R., and PASSET, J. 1973. *Thymus vulgaris* spontané de France: Races chimiques et chémotaxinomie. *Phytochemistry* 12:1683–1691.

GUENTHER, E. 1972. The production of essential oils, pp. 87–226, *in* E. Guenther (ed.). The Essential Oils, Vol. 1, 2nd ed., Krieger, R.E., Malebar, Florida.

HAUBRUGE, E., LOGNAY, G., MARLIER, M., DANHIER, P., GILSON, J.C., and GASPAR, C. 1989. The toxicity of five essential oils extracts from *Citrus* species with regard to *Sitophilus zeamais* Motsch (Col. Curculionidae), *Prostephanus truncatus* (Horn) (Col. Bostrychidae) and *Tribolium castaneum* Herbst (Col. Tenebrionidae). *Meded. Fac. Landbouwwet. Rijksuniv. Gent.* 54(36):1083–1093.

IWUABE, M.O.E., OSISIOGU, I.U.W., and AGBAKWURU, E.O.P. 1981. Dennettia oil, a potential new insecticide: Tests with adults and nymphs of *Periplaneta americana* and *Zonocerus variegatus. J. Econ. Entomol.* 74(3):249–252.

KALEMBA, D., GORA, J., KUROWSKA, A., and MAJDA, T. 1990. Studies of essential oils with respect to their effect on insect III. Essential oil of goldenrod (*Solidago canadensis* L.). *Zez. Nank. Politech. Lodz, Technol. Chem. Spozyw.* 609(47):91–97.

KLOCKE, J.A., BALANDRIN, M.F., ADAMS, R.P., and KINGSFORD, E. 1985. Insecticidal chromenes from the volatile oil of *Hemizonia fitchii. J. Chem. Ecol.* 11(5): 701–712.

KONSTANTOPOULOU, I., VASSILOPOULOU, L., MAURAGANI-TSIPIDOV, P., and SCOURAS, Z.G. 1992. Insecticidal effects of essential oils. A study of the effects of essential oils extracted from eleven Greek aromatic plants on *Drosophilia auraria. Experientia* 48(6):535–619.

KRISHNARAJAH, S.R., GANESALINGAM, V.K., and SENANAYAKE, U.M. 1985. Repellency and toxicity of some plants oils and their terpene components to *Sitotroga cerealella* (Olivier), (Lepidoptera, Gelechiidae). *Trop. Sci.* 25(4):249–252.

KUROWSKA, A., KALEMBA, D., GORA, J., MADJA, T. 1991. Analysis of essential oils: Influence on insects. Part IV: essential oil or garden thyme (*Thymus vulgaris* L.). *Pestycydy (Warsaw)* 2:25–29.

MORALLO-REJESUS, B., and TANTENGCO, G.B. 1986. Biological activity of flower extracts as insecticides. *NTSA Technol. J.* 11(1):37–46.

MORALLO-REJESUS, L.B., MAINI, H.A., OHSAWA, K., and YAMAMOTO, I. 1990. Insecticidal actions of several plants to *Callosobruchus chinensis*, pp. 91–100, *in* K. Fujii, A.M.R. Gatehouse, C.D. Johnson, R. Mitchel and T. Yoshida (eds). Bruchids and Legumes, Economics, Ecology and Co-Evolution. Kluwer Academic, Netherlands.

NTEZURUBANZA, L. 1991. Quelques applications d'*Ocimum canum*. Revue de la littérature. Rivista Italiana EPPOS, 10èmes Journées Scientifiques de Digne les Bains. pp. 703–706.

RAJAPAKSE, R.H. 1990. Effect of five botanicals as protectants of greengram against the pulse beetle *Callosobruchus maculatus*, pp. 85–90, *in* K. Fujii et al. (eds.). Bruchids and Legumes, Economics, Ecology and Co-Evolution. Kluwer Academic, Netherlands.

REGNAULT-ROGER, C., and HAMRAOUI, A. 1991. Effects of Mediterranean botanicals and essential oils as protectants of kidney bean (*Phaseolus vulgaris* L.) against the pulse beetle (*Acanthoscelides obtectus* Say). Communication to the VIII ISCE Annual Meeting, Dijon, France.

REGNAULT-ROGER, C., and HAMRAOUI, A. 1992a. Efficiency of plants from south of France used as traditional protectants of *Phaseolus vulgaris* L. against its bruchid *Acanthoscelides obtectus* (Say). *J. Stored Prod. Res.* In press.

REGNAULT-ROGER, C., and HAMRAOUI, A. 1992b. Influence d'huiles essentielles aromatiques sur *Acanthoscelides obtectus* Say, bruche du haricot (*Phaseolus vulgaris* L.). *Actual. Bot.* In press.

RICHARD, H. 1992. Epices et Aromates. Techniques et Documentations. Lavoisier APRIA, Paris. 338 pp.

ROUX, M. 1985. Algorithmes de classification. Masson, Paris, Computer program STAT-ITCF, 1987, 149 pp.

SAXENA, R.C. 1989. Insecticides from neem, pp. 110–135, *in* J.T. Arnason, B.J.R. Philogène, and P. Morand (eds.). Insecticides of Plant Origin. ACS Symposium Series 387. American Chemical Society, Washington, D.C.

SHAAYA, E., RAVID, U., PASTER, N., JUVEN, B., LISMAN, U., and PISSAREV, V. 1991. Fumigant toxicity of essential oils against four major stored-product insects. *J. Chem. Ecol.* 7(3):499–504.

WEAVER, D.K., DUNKEL, F.V., and JACKSON, L.L. 1991. Toxicity of *R,S*-linalool to four species of storage Coleoptera as influenced by degradation and volatilization, pp. 1725–1737, *in* F. Fleurat-Lessard and P. Ducom (eds.). Proceedings of the 5th International Working Conference on Stored-Product Protection, Vol. III.

YU, S.J. 1987. Microsomal oxidation of allelochemicals in generalist (*Spodoptera frugiperda*) and semispecialist (*Anticarsia gemmetalis*) insect. *J. Chem. Ecol.* 13(3):423–436.

ZWEIG, G., and SHERMA, J. 1972. Handbook of Chromatography, Vol. 1. CRC Press, Cleveland. 784 pp.

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EFFECTS OF NITROGEN AND DOUGLAS-FIR ALLELOCHEMICALS ON DEVELOPMENT OF THE GYPSY MOTH, *Lymantria dispar*

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Abstract—Two experiments were conducted to examine the influence of foliar nitrogen, terpenes, and phenolics of Douglas-fir on the development of gypsy moth larvae. In the first experiment, foliar concentrations of nitrogen and allelochemicals were manipulated by fertilizing 3-year-old potted seedlings with 0 or 200 ppm nitrogen. Concentrations of foliar nitrogen (0.33–2.38%) were negatively correlated with the phenolics (15.8–24.4 mg/g). Sixth-instar larvae previously reared on current-year Douglas-fir needles were allowed to feed on these seedlings. Pupal weights (312.8–995.6 mg) were positively correlated with levels of foliar nitrogen, negatively correlated with amounts of foliar phenolics, and uncorrelated with terpene concentrations. In the second experiment, terpene and phenolic extracts from Douglas-fir foliage were incorporated at natural levels into artificial diets with high and low levels of protein nitrogen. Neonate larvae grew faster and were larger on the high nitrogen control diet (4.1–4.5%), however, fourth instars performed better on the control diet with low nitrogen levels (2.5–2.7%). Foliar terpenes incorporated into diet had little effect on neonate fitness, but may induce subtle physiological changes in later instar larvae. Phenolics, alone or in combination with terpenes, excessively suppressed growth and survival, with no individuals living through the fourth instar, regardless of the nitrogen level. Incorporating foliar phenolic extracts into artificial diet caused unnatural levels of toxicity

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and failed to clarify the effects of Douglas-fir phenolics on gypsy moth fitness. Foliar nitrogen is a key factor influencing gypsy moth development on Douglas fir, but may be mitigated to some degree by phenolics.

Key Words—Artificial diet, herbivory, nitrogen, *Lymantria dispar*, Lepidoptera, Lymantriidae, monoterpenes, nutritional ecology, phenolics, *Pseudotsuga menziesii*.

INTRODUCTION

The gypsy moth, *Lymantria dispar* L., is a phytophagous insect that can complete its life cycle on hundreds of plant species (Mosher, 1915; Miller and Hanson, 1989a). Angiosperms are usually preferred over gymnosperms, with oak, poplar, willow, and a few other hardwoods favored when available (Lechowicz and Mauffette, 1986; Barbosa, 1978; Barbosa et al., 1983). The host range can expand and contract, depending on population densities. Newly hatched first instars disperse in search of a suitable host before feeding; they usually remain on their original host until the fourth instar, when a portion of the population switches to other host species (Elkinton and Liebhold, 1990). Larvae may then move from a favored hardwood host to a secondary conifer unsuitable to survival and development of young instars. Larvae switched to pine species may outperform larvae switched to another suitable hardwood or larvae that remain on the original hardwood host through all instars as evidenced by greater mean pupal weight and greater number of eggs/mass (Barbosa et al., 1986; Rossiter, 1987).

Since the introduction of the gypsy moth to North America in 1869, most large-scale forest defoliation by this insect has occurred in the northeastern United States (McManus et al., 1989). Western forests were free of this pest until the late 1970s and early 1980s, when isolated outbreaks were discovered in California and the Pacific Northwest (Czerwinski and Isman, 1986; Oregon State University Extension Service, 1986). Infestations in Oregon were controlled with *Bacillus thuringiensis* (Berlinger), but reestablishment by human-assisted transport of egg masses or larvae from other infested areas remains a threat and requires continued monitoring.

Although the species composition of Pacific Northwest forests (Franklin and Dyrness, 1988) is very different from that of eastern forests (Houston, 1981), numerous western hardwoods have proven to be very or moderately suitable as gypsy moth hosts (Daterman et al., 1986; Miller and Hanson, 1989a), including the abundant Garry oak or Oregon white oak (*Quercus garryana* Dougl.) and red alder (*Alnus rubra* Bong.) (Miller et al., 1991a). Various western conifers can also sustain growth and development through the entire gypsy moth life cycle (the life cycle after hatching normally encompasses five larval instars for males and six for females, followed by a pupal and then an adult

stage) (Miller and Hanson, 1989b). Survival on Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] foliage, one of the most common conifers of this region, strongly depends on the availability of new foliage and temperature (Joseph, 1989; Miller et al., 1991b; Joseph and Kelsey, submitted).

Previously we reported that gypsy moth larvae fed Douglas-fir foliage took about two weeks longer to reach pupation and attained lower pupal weights than did larvae fed white alder (*Alnus rhombifolia* Nutt.) foliage (Joseph et al., 1991). Chemical analysis indicated that nitrogen, phenolics, and, to a lesser extent, terpenes could be affecting larval performance. Since these components always co-occur in Douglas-fir foliage, their individual effects on the developmental and nutritive physiology of gypsy moth could not be clearly determined. In this paper we manipulated the nutritional (nitrogen) and nonnutritional (terpenes and phenolics) components in Douglas-fir seedlings and artificial diets in an attempt to better understand how these dietary factors influence the suitability of Douglas fir to gypsy moth.

METHODS AND MATERIALS

Douglas-Fir Seedlings and Nitrogen Fertilization. Three-year-old Douglas-fir seedlings approximately 40–70 cm tall were obtained from a local nursery. Seedlings were planted in individual 15- × 15-cm pots with a mixture of clean sand and perlite (50:50 v/v) and maintained under greenhouse conditions (natural photoperiod, 25–30°C). Each pot was flushed repeatedly with excess water to leach the potting mixture of available nitrogen. Twelve potted seedlings were randomly selected; six were fertilized with a nitrogen-free (0 ppm) solution and the other six were fertilized with a nitrogen-rich (200 ppm) solution (300 ml/pot). These treatments were applied weekly from April 21 to June 10, 1989. The two solutions were modified from that of Johnson et al. (1957), with ammonium nitrate as the nitrogen source; they contained the same concentrations of all nutrients except nitrogen.

About 2.0 g of sucrose (equivalent to about 1000 kg/ha) was placed around each seedling watered with the 0 ppm nitrogen solution. Sucrose provides a readily available carbon substrate to soil microbes; their subsequent rapid growth immobilizes much of the remaining nitrogen in microbial biomass, enhancing nitrogen stress for the plants (Johnson and Edwards, 1979). Applying sucrose maximized the chances of obtaining a difference in foliar response to the nitrogen treatments. In mid-June, growth response to the fertilizer was visible in new foliage. Needles on seedlings treated with 0 ppm nitrogen were chlorotic, short, and dense, whereas those on nitrogen-treated seedlings were dark green, long, and less dense. Needles 1 year old and older showed no visible response to the treatments and were removed manually, leaving only the new needles. This was done to eliminate any influence of needle age on larval response.

Foliar Analysis. A few current-year needles required for the foliar analysis were collected from each of the 12 seedlings before subjecting them to larval feeding. A subsample of needles was oven dried (60°C) and ground for nitrogen and total phenol analysis. Nitrogen was measured by a micro-Kjeldahl procedure with an automated Technicon Autoanalyzer II (Anonymous, 1975). Total phenols were analyzed by a colorimetric method using Folin-Ciocalteu reagent (Julkunen-Tiitto, 1985). The remaining fresh needles were stored frozen and ground with liquid nitrogen immediately before extraction with pentane–aqueous MeOH (33% H_2O), 1 : 1. Terpenes were quantified by gas chromatography (GC) as previously described (Joseph et al., 1991).

Douglas-fir Foliar Extracts. Douglas-fir needles were removed from a single, well-fertilized, 10-year-old tree from a local tree farm. After air-drying, they were stored in sealed plastic bags at −4°C. Five hundred grams of tissue was frozen with liquid nitrogen and ground with a mortar and pestle. This was extracted overnight with 2000 ml of pentane and filtered through Whatman No. 1 paper. The extraction was repeated three more times to ensure complete removal of the terpenes. The four extracts were combined and concentrated with vacuum (at room temperature) until most of the pentane had evaporated. To isolate the terpenes from other components in the concentrate, additional pentane was added and the solution was steam-distilled for 90 min. Terpenes were recovered from the water condensate by repeated washing with pentane. The washes were combined and concentrated with vacuum; the resulting terpene fraction, containing some residual pentane, was stored at 4°C until incorporated into diet. Terpene composition and quantity in the steam distillate were checked by diluting 200 μl of the concentrate to 10 ml with pentane containing fenchone as internal standard and analyzed by GC (Joseph et al., 1991). Composition of the distilled terpene fraction differed little from a direct solvent extract of Douglas-fir needles.

The pentane-extracted tissue was air-dried and further extracted with acetone–water (7 : 3) to remove phenolics (Hagerman, 1988; Cork and Krockenberger, 1991). After soaking in solvent for 24 hr, the tissue was vacuum-filtered. This procedure was repeated twice to ensure complete removal of the compounds. The three filtrates were combined, the solvent was removed with vacuum, and the concentrate was stored at 4°C. This fraction was not further purified and so contained soluble components in addition to phenolics. Nevertheless, it was a phenolic-rich extract with 250 mg of catechin equivalents/g dry weight, more than 10 times the amount in needles of nitrogen-stressed (0 ppm) Douglas-fir seedlings (Table 1).

Preparation of Artificial Diet. Bio-Serv gypsy moth diet from which casein was omitted was used to prepare the experimental diets. Casein was added in different amounts to vary the nitrogen level: 5 g casein/liter in the low nitrogen diets and 25 g casein/liter in the high nitrogen diets. The high nitrogen diets

TABLE 1. QUALITY OF FOLIAGE FED TO SIXTH-INSTAR GYPSY MOTHS AND LARVAL–PUPAL DEVELOPMENT (AT 24°C)[a]

Seedling/insect variables	Nitrogen level (ppm)[b]	
	0	200
Foliar quality	(N = 5)	(N = 6)
Total nitrogen (% dry wt)	0.40 ± 0.04 a	1.69 ± 0.24 b
Total terpenes (mg/g dry wt)	9.16 ± 1.35 a	13.16 ± 2.52 a
Total phenols (mg/g dry wt)[c]	22.41 ± 0.63 b	16.82 ± 0.39 a
Moisture (% fresh wt)	71.19 ± 0.89 a	72.64 ± 0.57 b
Developmental variables	(N = 17)	(N = 17)
Larval period (days)	48.4 ± 0.6 a	49.2 ± 0.4 a
Pupal weight (mg)[d]	513.07 ± 24.81 a	666.00 ± 4.58 b
Pupal period (days)	10.1 ± 0.3 a	10.1 ± 0.3 a
Fecal weight (mg)[e]	130.0 ± 7.3 a	136.7 ± 12.8 a

[a] Mean ± SE. Within rows, means followed by different letters differ significantly at $P \leq 0.05$ (Fisher's protected LSD).
[b] Nitrogen (200 ppm) was applied to 3-year-old potted seedlings as ammonium nitrate in a nutrient solution modified after that of Johnson et al. (1957). Seedlings at 0 ppm received the same solution without ammonium nitrate.
[c] Milligrams of catechin equivalents/g dry wt.
[d] Fresh weights two days after pupation.
[e] Dry weight of feces of sixth-instar larva.

had the standard amount of casein recommended for rearing gypsy moth larvae (Bell et al., 1981). Fresh diet was prepared for measuring the nutritional indices during the fourth instar.

During preparation, all diets were maintained at 50°C in a water bath until the Douglas-fir extracts were incorporated. This temperature was selected to minimize the loss of terpenes, when present. The terpene and phenolic extracts were incorporated at 2.15 ml and 2.15 g/100 g of wet diet, respectively. These were comparable with the mean terpene (18 mg/g dry weight) and total phenol (30 mg/g dry weight) concentrations measured in Douglas-fir foliage (Joseph et al., 1991). The phenolic extract was dissolved in 15 ml of water before mixing. The terpene and phenolic extracts were added together at the above concentrations to study their combined effect (T + P diet). All diets were stored at 4°C in sealed plastic bags between feedings. Two control diets, one with pentane solvent (2.15 ml/100 g wet diet), and the other with no solvent, were also prepared. One group of larvae was fed 1-year-old Douglas-fir needles, collected fresh as needed from a mature tree in the field; another group was given no food at all and served as a starved control.

Since variation in available water can have multiple effects on the nutri-

tional physiology of larvae (Reese and Beck, 1978; Scriber, 1977), the water content of diets was determined gravimetrically.

Diet Analysis. Subsamples of diet were prepared and analyzed for nitrogen as outlined above for the seedlings. To extract terpenes, approximately 0.5 g of diet was ground in a tissue grinder with 4 ml MeOH–H_2O (1 : 1). The slurry was rinsed into a vial, followed by pentane (2 ml) containing fenchone as internal standard. After shaking for 30 min, the solution was centrifuged (5 min) to settle particulates. The pentane layer was removed and analyzed by GC (Joseph et al., 1991). To extract phenols, approximately 250 mg of diet was macerated in a tissue grinder with acetone–H_2O (7 : 3), shaken 5 min, and centrifuged (5 min) to settle particulates. A 200-μl aliquot was diluted to 2 ml with distilled H_2O and analyzed as described for seedling foliage. To compensate for interference from diet ingredients, values obtained from control and pentane control diets were subtracted from the phenolic and T + P diets, respectively. Diet analyses were conducted in triplicate.

Insects. Gypsy moth egg masses were obtained at the end of January 1988 from an oak woodland in Seneca Creek State Park, Montgomery County, Maryland. The eggs were shipped to our laboratory and stored at 4°C until May. Several egg masses were randomly selected, pooled, dehaired, and surface-disinfected with 10% formaldehyde for 1 min. They were warmed to 25°C to hatch.

Larval Rearing on Seedlings. Sixth-instar females were used to minimize variation in assessing the effects of seedling fertilization on larval development and fecundity. The fifth and sixth instars are responsible for 90–95% of all biomass consumed over all instars (Miller et al., 1991b). First through fifth instars were fed fresh current-year foliage clipped from fertilized Douglas-fir trees at a local tree farm. One hundred fifty larvae were placed into 145-ml cups, 10 larvae per cup for the first three instars and 3 per cup for the remaining instars. Freshly molted sixth-instar females were randomly picked from these cups and weighed; three or four larvae were placed on each potted seedling with only new-growth needles available. A cardboard collar with the same circumference as the pot was placed at the base of each seedling to collect feces. Each seedling was covered with a black mesh cage to prevent larvae from escaping. Parameters measured were days to pupation, live pupal weight (weighed two days after pupation), pupal period, and average fecal dry weight. The experiment was conducted in a quarantine room at 24°C, 45–50% relative humidity, and 16 : 8 hr light–dark.

Larval Rearing on Artificial Diets. Ninety larvae, five per 145-ml cup, were reared from hatch on each of the artificial diets tested. Diet was replaced daily for the first 11 days and then every two days until the experiment was terminated. Replacing the diets frequently kept the diets fresh and prevented contamination. Diet was provided as cubes of 1 cm^3 fresh volume per cup.

Eleven and 20 days after hatching, the number of surviving larvae, the number molted, and fresh weights of five larvae grouped together were measured.

Nutritional indices were measured with 20–25 fourth instars randomly selected from the control, pentane control, and terpene diets during the third instar. The two diets containing phenolics impacted growth and survival so adversely that no fourth instars were available for this experiment. Larvae continued to receive their respective diets until completion of the experiment. Freshly molted fourth instars were weighed and placed individually into a 30-ml cup with a weighed amount of diet. A subsample of five or more freshly molted larvae per diet were oven-dried at 70°C in order to estimate initial dry weights for the remaining individuals. Dry-weight gain was calculated by directly measuring final biomass of each larvae and subtracting the estimated initial dry weight determined at the beginning of the fourth instar. Every time a freshly molted fourth instar was given experimental diet, a sample of the same diet was weighed and dried at 70°C. Dry weight of food eaten was estimated by subtracting the dry weight of uneaten food from the estimated dry weight of food given. Nutritional indices were calculated with the above dry weights (milligrams) as described by Waldbauer (1968).

Statistical Analyses. Differences between mean developmental variables and mean foliar variables were computed by a two-sample t-test at a significance level of 0.05. Developmental variables were correlated with the foliar variables by multivariate correlation analysis. Foliar data were analyzed by regression analysis. Survivorship data for artificial-diet experiments were analyzed with the Z test for weighted proportions (Devore and Peck, 1986). The other developmental data were subjected to a standard two-way ANOVA; means were compared by Fisher's protected least significant differences (FPLSD).

RESULTS

Seedling Response to Nitrogen Fertilization. Just before larvae were placed on the seedlings, the foliar nitrogen concentration of seedlings receiving 200 ppm nitrogen fertilizer was four times that of seedlings receiving 0 ppm nitrogen fertilizer (Table 1). Twenty-four days after placement, just after the larvae had pupated, the percent nitrogen had increased from 0.4 to 1.1% in seedlings receiving the 0 ppm treatment and from 1.69 to 1.85% in seedlings receiving the 200 ppm treatment. The increase in the 0 ppm treatment may have resulted from the internal reallocation of nitrogen from other tissues. Terpene concentrations varied considerably between seedlings within a treatment and did not differ statistically between treatments (Table 1). At both levels of nitrogen, β-pinene was the major component at 38–44% of the terpene fraction; α-pinene contributed about 10%. Phenols were 33% higher in seedlings treated with 0

ppm nitrogen than in seedlings treated with 200 ppm nitrogen, a statistically significant difference (Table 1). The water content was 1.5% greater in seedlings receiving 200 ppm nitrogen, a small but statistically significant difference. The foliar nitrogen level was negatively and nonlinearly correlated with concentrations of phenols (Figure 1). Terpenes showed a positive but statistically insignificant correlation with foliar nitrogen ($r = 0.40$). The ratio of total phenolics to total terpenes was higher in nitrogen-limited seedlings. Percent moisture was negatively correlated with the amount of total phenols ($r = -0.50$).

Development of Sixth Instars Reared on Seedlings. Of the four parameters measured, only fresh pupal weight showed a significant difference between the two treatments (Table 1). Average pupal weights of larvae reared on seedlings at 200 ppm nitrogen were 153 mg (23%) heavier. Larval period and pupal period were not correlated with levels of foliar constituents (Table 2). Thus, larvae did not take longer to grow bigger, suggesting they either ate more, were more efficient, or both. Pupal weight was positively correlated with nitrogen and

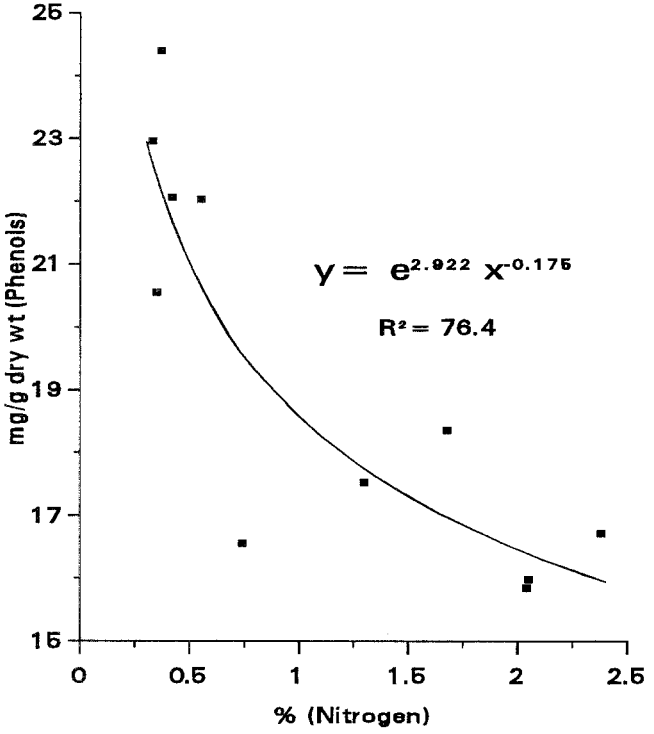

FIG. 1. The relationship between total phenols and nitrogen in the new needles of 3-year-old Douglas-fir seedlings fertilized with 0 and 200 ppm nitrogen solutions.

TABLE 2. CORRELATION COEFFICIENTS (r) AND SIGNIFICANCE LEVELS (P) FOR GYPSY MOTH DEVELOPMENTAL PARAMETERS AS A FUNCTION OF DOUGLAS-FIR FOLIAR QUALITY

	Gypsy moth developmental variables[a]							
	Larval period		Pupal weight		Pupal period		Fecal weight	
Foliar quality	r	P	r	P	r	P	r	P
Total nitrogen	−0.21	NS	0.50	<0.01	0.05	NS	−0.11	NS
Total terpenes	−0.006	NS	−0.02	NS	−0.04	NS	−0.03	NS
Total phenols	0.03	NS	−0.42	<0.05	−0.005	NS	−0.20	NS
Moisture	0.19	NS	0.38	<0.05	−0.08	NS	0.60	<0.05

[a] Developmental parameters were measured for gypsy moth (sixth-instar females) fed on 3-year-old potted Douglas-fir seedlings fertilized at two levels of nitrogen (0 and 200 ppm). NS = not significant at the 0.05 level.

moisture levels, but negatively correlated with total phenols (Table 2). Fecal weight was positively correlated with percent moisture.

Chemical Characteristics of Artificial Diets. Percent nitrogen ranged from 2.5 to 2.7% for the low nitrogen diets and from 4.1 to 4.5% for the high nitrogen diets. The within-level variation was due to plant extracts and incorporation procedures, but was considered too low to affect the results significantly. Terpenes were recovered at 79.5% from the terpene diet and 74.4% from the combined T + P diet. Some were undoubtedly lost by volatilization and some may have bound to materials in the diet. Phenolics were not readily extractable from the diets; only 21.1% of the added amount was recovered from the phenolics diet and 16.7% from the T + P diet. Water content ranged from 83.5 to 84.1% of fresh weight; such differences were considered too small to influence the results.

Development of Neonate Larvae on Artificial Diets. Survival 11 days after hatch was not affected by nitrogen level on any artificial diet (Table 3). Survival of larvae on diet containing terpenes was slightly reduced, whereas the phenolic fraction had no effect. When terpenes and phenols were combined (T + P), however, they dramatically decreased larval survival at both levels of nitrogen. Neonate larvae fed Douglas-fir foliage survived a maximum of eight days and apparently ate very little, as indicated by the small amounts of frass (data not shown).

On all diets, the higher nitrogen levels produced significantly heavier larvae after 11 days (Table 3). Terpenes had a minimal effect on larval weights, in contrast to the large decreases in the larvae receiving phenolics. Combining T + P significantly decreased larval weight, but more severely at the higher nitro-

TABLE 3. DEVELOPMENTAL CHARACTERISTICS OF GYPSY MOTH LARVAE FED DOUGLAS-FIR FOLIAR EXTRACTS IN ARTIFICIAL DIET AT TWO LEVELS OF CASEIN (NITROGEN), 11 AND 20 DAYS AFTER ECLOSION[a]

	Survival (%)[c]		Fresh weight (mg)		Index of development[d]	
Diet/casein level[b]	11 days	20 days	11 days	20 days	11 days	20 days
Control						
L	98.9 c	90.0 e	10.5 ± 1.2 e	49.7 ± 3.8 e	1.5 ± 0.1 c	3.2 ± 0.1 e
H	94.5 bc	90.0 e	15.6 ± 1.7 f	53.1 ± 3.7 e	1.7 ± 0.1 d	3.4 ± 0.1 e
Pentane control[e]						
L	97.8 c	94.6 e	8.5 ± 1.3 de	44.0 ± 4.0 de	1.4 ± 0.1 bc	3.1 ± 0.1 de
H	97.8 c	92.7 e	14.5 ± 1.4 f	46.9 ± 2.6 de	1.6 ± 0.1 d	3.2 ± 0.1 de
Total terpene extracts						
L	94.5 bc	89.9 e	7.4 ± 0.9 d	36.1 ± 4.6 d	1.4 ± 0.1 bc	2.9 ± 0.1 d
H	93.4 b	83.7 de	15.0 ± 1.4 f	39.8 ± 3.0 de	1.7 ± 0.1 d	3.2 ± 0.1 de
Total phenol extracts						
L	98.9 c	72.0 cd	2.5 ± 0.5 b	9.8 ± 2.4 b	1.0 ± 0.1 a	1.8 ± 0.1 b
H	97.8 c	69.1 c	7.5 ± 0.7 de	21.1 ± 2.7 c	1.3 ± 0.1 b	2.6 ± 0.1 c
Total terpene + total phenol extracts						
L	81.2 a	15.0 a	1.5 ± 0.2 a	3.4 ± 0.6 a	1.0 ± 0.1 a	1.0 ± 0.0 a
H	87.8 ab	50.0 b	3.8 ± 0.4 c	11.4 ± 2.0 b	1.1 ± 0.1 a	1.9 ± 0.2 b

[a] Percent survival was analyzed with the Z test for weighted proportions. Fresh weight and instar attained are expressed as means ± SE and analyzed by ANOVA; Fisher's protected LSD was used to separate means. Values in columns followed by different letters differ significantly at $P \leq 0.05$.

[b] Allelochemicals extracted from Douglas-fir foliage were added at approximately natural concentrations to artificial diet containing casein at low (L, 2.5–2.7% N) or high (H, 4.1–4.5% N) levels.

[c] Initial $N = 90$ at 11 days for all treatment groups. At day 20, $N = 40$–60, as 20–25 larvae were randomly picked from the survivors of the original group of 90 for measurement of nutritional indices.

[d] The index of development is the mean of the different larval instars present on the date of sampling.

[e] Pentane control diet contained the same amount of pentane as the terpene-extract diet.

gen level than at the lower nitrogen level. Development, as reflected by the index of development (or mean instar attained) (Table 3), was faster at the higher nitrogen level on all diets except T + P. Total terpenes had no effect on developmental rate. Phenolics and T + P lowered the rate of development, and most larvae failed to molt into the second instar.

Larval survival on various diets after 20 days ranged between 4 and 66% lower than after 11 days. The greatest effect during this period was on diets containing phenolics. Nitrogen levels affected larval survival only for the T + P diet (Table 3); it was lower at low nitrogen than at high nitrogen. Larval weights (Table 3) on control and terpene diets were not affected by nitrogen levels, in contrast to those on day 11. Terpenes still had no effect on larval weights by day 20. Phenolic and T + P diets produced the smallest larvae, but the effect was mediated by the nitrogen level; high nitrogen produced heavier larvae. Combining T + P decreased larval weight by the same amount as the sum of their individual effects regardless of the nitrogen level. Developmental rate was affected by nitrogen levels only when phenolics were present. Most larvae on control and terpene diets were in the third instar by day 20, whereas they were in the first and second instars on the phenolics and T + P diets.

Nutritional Indices in the Fourth Instar. Weight gain and final weights of fourth instars were significantly less at the higher level of nitrogen on control diets with or without pentane (Table 4). Terpenes in the lower nitrogen diet had a subtle negative effect on weight gain, similar to that of high nitrogen concentrations in the control diet (Table 4). Nutritional indices could not be measured on diets containing phenolics because no larvae survived, regardless of the nitrogen level.

Relative consumption rate (RCR) and efficiency of processing digested food (ECD) were reduced at the higher levels of nitrogen in both control diets. Addition of terpenes to the diet at either nitrogen level had no effect on ECD relative to the controls at the respective nitrogen level (Table 4). The effect of nitrogen concentration on fourth instars was different for diets with and without terpenes. Larvae on high nitrogen-terpene diets showed the same RCR, RGR, weight gain, and final weight as larvae on low nitrogen-terpene diets. Larvae fed diets with terpenes and high nitrogen had a higher RCR than control diets with only high nitrogen.

DISCUSSION

Leaf nitrogen is a key nutritional factor for most insect herbivores (Mattson, 1980; Scriber and Slansky, 1981; Mattson and Scriber, 1987). Our observations in this study support our previous finding (Joseph et al., 1991) that gypsy moth larvae survive and develop best when fed Douglas-fir tissue with high nitrogen

TABLE 4. EFFECTS OF DIETARY NITROGEN AND DOUGLAS-FIR EXTRACTS ON FOOD CONSUMPTION, WEIGHT GAIN, FECAL PRODUCTION, INSTAR DURATION, AND NUTRITIONAL INDICES IN GYPSY MOTH FOURTH INSTARS[a]

Larval parameters[b]	Diet[c]					
	Control		Pentane control[d]		Total terpene	
	L (18)	H (16)	L (15)	H (16)	L (16)	H (14)
Total food consumed (mg)	75.2 ± 6.2 d	41.9 ± 4.1 ab	70.1 ± 7.2 cd	36.6 ± 2.8 a	57.9 ± 7.7 bc	40.9 ± 5.2 ab
Total feces (mg)	54.6 ± 4.5 b	26.6 ± 2.5 a	49.5 ± 4.3 b	22.9 ± 1.9 a	42.1 ± 4.6 b	23.6 ± 3.3 a
Weight (mg)						
Initial	10.0 ± 0.6 c	8.2 ± 0.3 b	8.7 ± 0.7 bc	8.3 ± 0.4 b	7.9 ± 0.5 b	6.2 ± 0.4 a
Final	22.8 ± 1.4 b	14.4 ± 1.2 a	20.0 ± 1.7 b	14.2 ± 1.1 a	15.8 ± 1.1 a	12.6 ± 1.4 a
Total gained	12.8 ± 1.2 b	6.2 ± 1.2 a	11.4 ± 1.3 b	5.9 ± 1.0 a	8.0 ± 0.8 a	6.3 ± 1.2 a
Instar duration (days)	8.2 ± 0.4 a	9.3 ± 0.6 a	8.5 ± 0.6 a	9.4 ± 0.4 a	8.3 ± 0.3 a	8.2 ± 0.4 a
Nutritional indices[e]						
RCR	0.96 ± 0.08 b	0.59 ± 0.06 a	1.01 ± 0.09 b	0.50 ± 0.04 a	0.87 ± 0.07 b	0.82 ± 0.08 b
RGR	0.18 ± 0.02 b	0.09 ± 0.02 a	0.16 ± 0.02 b	0.08 ± 0.02 a	0.12 ± 0.01 ab	0.13 ± 0.03 ab
AD	27.7 ± 1.3 b	36.0 ± 1.9 b	26.2 ± 3.1 a	36.9 ± 2.3 bc	24.0 ± 2.4 a	42.8 ± 2.0 c
ECD	63.2 ± 3.8 b	37.8 ± 5.6 a	76.3 ± 10.0 b	42.7 ± 5.1 a	73.8 ± 10.8 b	38.1 ± 5.9 a
ECI	17.0 ± 0.8 a	13.5 ± 2.0 a	16.7 ± 1.2 a	15.3 ± 2.0 a	15.1 ± 1.5 a	15.5 ± 2.2 a

[a] Expressed as mean ± SE. Within rows, means followed by different letters differ significantly at $P \leq 0.05$ (Fisher's protected LSD). No larvae reared on diets containing total phenol or total terpene + total phenol (T + P) extracts survived through the fourth instar.

[b] Dry weights.

[c] L = Low casein (2.5–2.7% N); H = high casein (4.1–4.5% N); numbers in parentheses are initial sample sizes.

[d] *Pentane control diet contained the same amount of pentane as the terpene-extract diet.*

[e] RCR = relative consumption rate (mg food ingested/mg initial larval weight/day); RGR = relative growth rate (mg larval weight gained/initial larval weight/day); AD = approximate digestibility [100 × (mg food ingested − mg feces)/mg food ingested]; ECD = efficiency of conversion of digested food (100 × mg larval weight gained)/(mg food ingested — mg feces); ECI = efficiency of conversion of ingested food (100 × mg larval weight gain/mg food ingested).

concentrations. This response, however, may not result from nitrogen availability alone, because of the inverse correlation between nitrogen and total phenol concentrations in fertilized and unfertilized trees (Joseph et al., 1991) or seedlings (Table 1). Douglas-fir terpenes do not appear to be involved in these larval responses, since mean concentrations were not changed by nitrogen fertilization treatments (Table 1) (Joseph et al., 1991).

Dietary levels of nitrogen affected larval development, especially larval weight, but the response was influenced by age; first-instar development was best on high nitrogen diets, but fourth-instar performance was best on the low nitrogen diets. This reversal with age may be caused by changes in nitrogen utilization as reflected by different rates of nitrogen consumption (NCR) and assimilation (NAR) during the larval life cycle. Montgomery (1982) reports that both of these rates increase between the first and second instars, then steadily decline through the remaining instars until pupation. A declining nitrogen requirement for optimal larval growth appears to be synchronized with the declining nitrogen content in host foliage (Hough and Pimentel, 1978).

Fourth instars on a high nitrogen diet should be able to meet physiological requirements for nitrogen by consuming less than larvae on a low nitrogen diet (Mattson, 1980; Tabashnik, 1982; Ohmart et al., 1985). However, increased efficiencies of digestion and assimilation might be required to optimize growth at reduced consumption rates. In our experiment, fourth instars on high nitrogen diets consumed less and had higher digestion efficiencies (AD) and lower assimilation efficiencies (ECD) than did larvae on low nitrogen diets. Therefore, at high nitrogen levels larvae were able to digest a greater portion of the ingested dry matter, but they were less efficient than larvae on low nitrogen diets at converting this digested material into growth (ECD), indicating a greater metabolic cost associated with catabolizing and excreting excess nitrogen.

Low ECDs can be caused by high metabolic costs resulting from low dietary moisture (Reese and Beck, 1978), higher than optimum levels of protein nitrogen (Schroeder, 1986), low-quality protein (Karowe and Martin, 1989), or the presence of allelochemicals in the diet (Manuwoto et al., 1985). Our results confirm Schroeder's (1986) observation that higher than optimum levels of protein nitrogen in the diet increase metabolic costs associated with its excretion. Adding terpenes to diets with high nitrogen did not further increase metabolic costs (reduce the ECD) compared to the diets with high nitrogen only.

In our seedling experiment, pupal weights of sixth instar females on low nitrogen Douglas-fir foliage (0.40% dry weight N) reached only 77% of the pupal weight of larvae eating high nitrogen foliage (1.69% dry weight N). Although phenolic concentrations in fir foliage are inversely correlated with nitrogen and could have contributed to these reduced pupal weights, the differences were similar in magnitude to those caused by dietary nitrogen levels alone. Lindroth et al. (1991) report that pupal weights of female gypsy moth larvae

reared from the fourth instar on a low nitrogen artificial diet (1.26% dry weight N) were only 78% of the pupal weights attained by larvae on a high nitrogen diet (2.44% dry weight N). The low nitrogen diet used by Lindroth et al. (1991) is similar in nitrogen concentration to Douglas-fir foliage over 1 year old, which ranges between 0.7 and 1.2% dry weight during spring and early summer (Smith et al., 1981).

First instars fed fresh 1-year-old Douglas-fir foliage in our diet experiment produced little or no frass and lived only three days longer than larvae given no food. These larvae probably starved to death. Mortality of first-instar larvae on pine foliage has been reported at 96–100% (Barbosa et al., 1986; Rossiter, 1987). Barbosa et al. (1986) attribute this to leaf toughness that physically interferes with biting and results in starvation. However, first-instar survival was nearly three times greater (72%) on new Douglas-fir foliage than on 1-year-old tissue (24%) (Miller et al., 1991b). The new tissue is less tough (personal observation); it also has a much higher nitrogen level (Smith et al., 1981), a lower tannin concentration (Stafford and Lester, 1981), equal or lower terpenes (Maarse and Kepner, 1970; Joseph and Kelsey, submitted), and 20% higher water content (Joseph and Kelsey, submitted). Neonate larvae can begin development on new foliage and subsequently complete development successfully on old Douglas fir.

The impact of terpenes in artificial diet on survival and development was limited regardless of nitrogen levels. Adding terpenes to the high nitrogen diet of fourth instars did counteract the suppression of high nitrogen levels on RCR, and at low nitrogen levels larval weight gain was lower than controls. Similarly there was no correlation between terpene concentrations and pupal weight or larval and pupal periods of sixth-instar females reared on fertilized seedlings. Larval performance is actually enhanced when late instars switch from oak foliage, completely lacking in monoterpenes, to pine, rich in monoterpenes (Barbosa et al., 1986; Rossiter, 1987). Although monoterpenes in gypsy moth diet do not alter performance markedly, they may still cause physiological changes, such as inducing detoxification enzymes (Brattsten, 1986; Yu, 1986, 1987; Harwood et al., 1990) or increasing resistance to pathogens. For example, gypsy moth larvae eating Douglas-fir foliage are less susceptible to carbaryl than larvae eating white alder (Moldenke et al., 1992), and larvae with pitch pine (*Pinus rigida* Mill.) in their diet are more resistant to nuclear polyhedrosis virus than are larvae fed red oak (Rossiter, 1987).

Phenolics are the major allelochemical component in hardwood hosts preferred by gypsy moth. In oak leaves, for example hydrolyzable tannins make up a significant portion of the phenolics; condensed tannins are present but not very abundant (Keating et al., 1990a). Gypsy moth is considered a tannin-adapted species whose feeding may be stimulated by tannins (Rossiter et al., 1988; Schultz, 1989), but this may not prevent larvae from being adversely

affected by them. Rossiter et al. (1988) report greater red oak defoliation associated with higher levels of total phenols and hydrolyzable tannins, but pupal mass and fecundity were negatively correlated with these compounds. Furthermore, other nontannin phenolics adversely affect gypsy moth. High levels of tremulacin, a glycoside in aspen, reduce the growth of fourth instar larvae, whereas moderate levels have no effect (Lindroth and Hemming, 1990). Juglone, from walnut foliage, greatly reduces survival and larval growth (Lindroth et al., 1990). Any of the condensed tannins and several nontannin phenolics in Douglas fir, alone or in combination, could have a deleterious effect on gypsy moth.

Although phytochemicals are commonly incorporated into artificial diets to assay the effects of allelochemicals on insect feeding (Lewis and van Emden, 1986), potentially serious drawbacks of these methods must be considered when interpreting the results (Berenbaum, 1986), especially when incorporating tannins (Bernays et al., 1989). Of the three components we added to artificial diet, the phenolic fraction affected larval survival and development more severely than did diets of Douglas-fir foliage from trees (Joseph et al., 1991) containing comparable concentrations of phenolics. The aqueous methanol extract of Douglas-fir foliage contains condensed tannins and flavonoid monomers and dimers such as catechin and epicatechin-catechin, respectively (Stafford and Lester, 1981). Chlorogenic acid is also present (Radwan, 1975). Tannins can complex with proteins, amino acids, fats, polysaccharides, and nucleic acids in both natural and artificial diets (Bernays et al., 1989). Phenolic binding did occur in our diet, since only 20% of the amount added could readily be extracted. Under oxidative conditions, dihydroxyphenols such as chlorogenic acid can be converted to quinones that can form covalent bonds with free amino and sulfhydryl groups of proteins and amino acids, altering their solubility, digestion, and absorption (Felton et al., 1992). On the other hand, gypsy moth larvae feeding on high phenolic diets may be partially protected from adverse effects of oxidized phenolics and phenolic–protein complexes by the alkaline conditions and possible surfactants in the gypsy moth midgut (Appel and Martin, 1990; Felton and Duffey, 1991; Keating et al., 1990b). Since our results with artificial diet differ so noticeably from feeding experiments with natural foliar diets, and because the effects of phenolics cannot be separated from nitrogen in foliar diets, the individual and interactive effects of Douglas-fir phenolics on survival and performance of gypsy moth remain unclear. These results also support Bernay's (1989) concerns with incorporating tannins into artificial diet.

In summary, nitrogen appears to be one of the most nutritionally important components in Douglas-fir foliage for gypsy moth. Nitrogen levels required for optimal larval growth may decrease between early and late instars. Terpenes and phenolics, the two major classes of allelochemicals in Douglas fir, may influence its suitability as a gypsy moth host. Terpenes alone appear to have a

minimal effect on gypsy moth fitness, but may cause changes in larval physiology that decrease susceptibility to insecticides and disease. Potential adverse effects from phenolics, alone or in combination with terpenes, remain unresolved.

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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.

APPEL, H.M., and MARTIN, M.M. 1990. Gut redox conditions in herbivorous lepidopteran larvae. *J. Chem. Ecol.* 16:3277–3290.

BARBOSA, P. 1978. Distribution of an endemic larval gypsy moth population among various tree species. *Environ. Entomol.* 7:526–527.

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.

BELL, R.A., OWENS, C.D., SHAPIRO, M., and TARDIF, J.R. 1981. Development of mass-rearing technology, pp. 599–633, *in* C.C. Doane and M.L. McManus (eds.). The Gypsy Moth: Research Toward Integrated Pest Management. Science Education Agency Technical Bulletin 1584. USDA, Forest Service, Washington, D.C.

BERENBAUM, M. 1986. Postingestive effects of phytochemicals on insects: On paracelsus and plant products, pp. 121–153, *in* J.R. Miller and T.A. Miller (eds.). Insect–Plant Interactions. Springer-Verlag, New York.

BERNAYS, E.A., COOPER DRIVER, G., and BILGENER, M. 1989. Herbivores and plant tannins. *Adv. Ecol. Res.* 19:263–302.

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.

CORK, S.J., and KROCKENBERGER, A.K. 1991. Methods and pitfalls of extracting condensed tannins and other phenolics from plants: Insights from investigations on *Eucalyptus* leaves. *J. Chem. Ecol.* 17:123–134.

CZERWINSKI, C., and ISMAN, M.B. 1986. Urban pest management: Decision-making and social conflict in the control of gypsy moth in west-coast cities. *Bull. Entomol. Soc. Am.* 22:36–41.

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.

DEVORE, J., and PECK, R. 1986. Statistics, the Explanation and Analysis of Data. West Publishing Co., St. Paul, Minnesota.

ELKINTON, J.S., and LIEBHOLD, A.M. 1990. Population dynamics of gypsy moth in North America. *Annu. Rev. Entomol.* 35:571–596.

FELTON, G.W., and DUFFEY, S.S. 1991. Reassessment of the role of gut alkalinity and detergency in insect herbivory. *J. Chem. Ecol.* 17:1821–1836.

FELTON, G.W., DONATO, K.K., BROADWAY, R.M., and DUFFEY, S.S. 1992. Impact of oxidized plant phenolics on the nutritional quality of dietary protein to a noctuid herbivore, *Spodoptera exigua*. *J. Insect Physiol.* 38:277–285.

FRANKLIN, J.F., and DYRNESS, C.T. 1988. Natural Vegetation of Oregon and Washington. Oregon State University Press, Corvallis.

HAGERMAN, A.E. 1988. Extraction of tannin from fresh and preserved leaves. *J. Chem. Ecol.* 14:453–461.

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.

HOUSTON, D.R. 1981. Forest stand relationships, pp. 267–293, *in* C.C. Doane and M.L. McManus (eds.). The Gypsy Moth: Research Toward Integrated Pest Management. Science Education Agency Technical Bulletin 1584. USDA, Forest Service, Washington, D.C.

JOHNSON, D.W., and EDWARDS, N.T. 1979. The effects of stem girdling on bio-geochemical cycles within a mixed deciduous forest in eastern Tennessee. II. Soil nitrogen mineralization and nitrification rates. *Oecologia (Berlin)* 40:259–271.

JOHNSON, G.M., STOUT, P.R., BROYER, T.C., and CARLTON, A.B. 1957. Comparative chlorine requirements for different plant species. *Plant Soil* 8:337–353.

JOSEPH, G. 1989. Host suitability studies of Douglas-fir and white alder to the gypsy moth. Masters thesis. Oregon State University, Corvallis.

JOSEPH, G., MILLER, J.C., BERRY, R.E., WERNZ, J., MOLDENKE, A.F., and KELSEY, R.G. 1991. White alder and Douglas-fir foliage quality and interegg-mass influences on larval development of gypsy moth, *Lymantria dispar*. *J. Chem. Ecol.* 17:1783–1799.

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.

KAROWE, D.N., and MARTIN, M.M. 1989. The effects of quantity and quality of diet nitrogen on the growth, efficiency of food utilization, nitrogen budget, and metabolic rate of fifth-instar *Spodoptera eridania* larvae (Lepidoptera: Noctuidae). *J. Insect Physiol.* 35:699–708.

KEATING, S.T., HUNTER, M.D., and SCHULTZ, J.C. 1990a. Leaf phenolic inhibition of gypsy moth nuclear polyhedrosis virus. Role of polyhedral inclusion body aggregation. *J. Chem. Ecol.* 16:1445–1457.

KEATING, S.T., SCHULTZ, J.C., and YENDOL, W.G. 1990b. The effect of diet on gypsy moth (*Lymantria dispar*) larval midgut pH and its relationship with larval susceptibility to a baculovirus. *J. Invert. Pathol.* 56:317–326.

LECHOWICZ, M.J., and MAUFFETTE, Y. 1986. Host preferences of the gypsy moth in eastern North America versus European forests. *Rev. Entomol. Que.* 31:43–51.

LEWIS, A.C., and VAN EMDEN, H.F. 1986. Assays for insect feeding, pp. 95–119, *in* J.R. Miller and T.A. Miller (eds.)., Insect–Plant Interactions. Springer-Verlag, New York.

LINDROTH, R.L., and HEMMING, J.D.C. 1990. Responses of the gypsy moth (Lepidoptera: Lymantriidae) to tremulacin, an aspen phenolic glycoside. *Environ. Entomol.* 19:842–847.

LINDROTH, R.L., ANSON, B.D., and WEISBROD, A.V. 1990. Effects of protein and juglone on gypsy moths: Growth performance and detoxification enzyme activity. *J. Chem. Ecol.* 8:2533–2547.

LINDROTH, R.L., BARMAN, M.A., and WEISBROD, A.V. 1991. Nutrient deficiencies and the gypsy moth, *Lymantria dispar*: Effects on larval performance and detoxication enzyme activities. *J. Insect Physiol.* 37:45–52.

MAARSE, H., and KEPNER, R.E. 1970. Changes in composition of volatile terpenes in Douglas fir needles during maturation. *J. Agric. Food Chem.* 18:1095–1101.

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 (Berlin)* 67:1–7.

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. John Wiley & Sons, New York.

MCMANUS, M., SCHNEEBERGER, N., REARDON, R., and MASON, G. 1989. Gypsy moth. Forest Insect & Disease Leaflet 162. USDA, Forest Service.

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.

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. 1991a. Development of the gypsy moth (Lepidoptera: Lymantriidae) on garry oak and red alder in western North America. *Environ. Entomol.* 20:1097–1101.

MILLER, J.C., HANSON, P.E., and KIMBERLING, D.N. 1991b. Development of the gypsy moth (Lepidoptera: Lymantriidae) on Douglas-fir foliage. *J. Econ. Entomol.* 84:461–465.

MOLDENKE, A.F., BERRY, R.E., MILLER, J.C., KELSEY, R.G., WERNZ, J.G., and VENKATESWARAN, S. 1992. Carbaryl susceptibility and detoxication enzymes in gypsy moth (Lepidoptera: Lymantriidae): Influence of host plant. *J. Econ. Entomol.* 85:1628–1635.

MONTGOMERY, M.E. 1982. Life-cycle nitrogen budget for the gypsy moth, *Lymantria dispar*, reared on artificial diet. *J. Insect Physiol.* 28:437–442.

MOSHER, F.H. 1915. Food plants of the gipsy moth in America. USDA Bulletin 250.

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 (Berlin)* 65:543–549.

OREGON STATE UNIVERSITY EXTENSION SERVICE. 1986. The gypsy moth in Oregon: Potential effects and management options. Oregon State University, Corvallis.

RADWAN, M.A. 1975. Genotype and season influence chlorogenic acid content in Douglas-fir foliage. *Can. J. For. Res.* 5:281–284.

REESE, J.C., and BECK, S.D. 1978. Interrelationships of nutritional indices and dietary moisture in the black cutworm (*Agrotis ipsilon*) digestive efficiency. *J. Insect Physiol.* 24:473–479.

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.

SCHROEDER, L.A. 1986. Protein limitation of a tree leaf feeding lepidopteran. *Entomol. Exp. Appl.* 41:115–120.

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.

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 (Berlin)* 28:269–287.

SCRIBER, J.M., and SLANSKY, F., JR. 1981. The nutritional ecology of immature insects. *Annu. Rev. Entomol.* 26:183–211.

SMITH, R.B., WARING, R.H., and PERRY, D.A. 1981. Interpreting foliar analyses from Douglas-fir as weight per unit of leaf area. *Can. J. For. Res.* 11:593–598.

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.

TABASHNIK, B.E. 1982. Responses of pest and non-pest *Colias* butterfly larvae to intraspecific variation in leaf nitrogen and water content. *Oecologia (Berlin)* 55:389–394.

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.

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ENANTIOSPECIFIC SYNTHESIS FROM D-FRUCTOSE OF (2*S*,5*R*)- AND (2*R*,5*R*)-2-METHYL-1,6-DIOXASPIRO[4.5]DECANE [THE ODOR BOUQUET MINOR COMPONENTS OF *Paravespula vulgaris* (L.)][1]

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Abstract—The synthesis of (2*S*,5*R*)- **(1)** and (2*R*,5*R*)-2-methyl-1,6-dioxaspiro[4.5]decane **(2)** from (2*RS*,5*R*,8*R*,9*R*,10*S*)-8,9,10-trihydroxy-2-methyl-1,6-dioxaspiro[4.5]decane **(8),** obtained in five steps from D-fructose using Wittig's methodology, reduction, and spiroketalation, has been accomplished by a Corey dideoxygenation at C-8,9, followed by a Barton deoxygenation at C-10, of the appropriately protected derivatives.

Key Words—Enantiospecific synthesis, spiroacetal, D-fructose, pheromone, *Paravespula vulgaris*.

INTRODUCTION

The *E*/*Z* mixture of 2-methyl-1,6-dioxaspiro[4.5]decane was identified by Francke et al. (1978) as minor components of the odor bouquet of workers of the common wasp *Paravespula vulgaris* (L.), but no information about the chirality of such compound was given.

Several racemic syntheses (Francke et al., 1978; Phillips et al., 1980; Ley

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[1]Enantiospecific synthesis of spirocetals. Part V. For Part IV, see Izquierdo et al. (1992).

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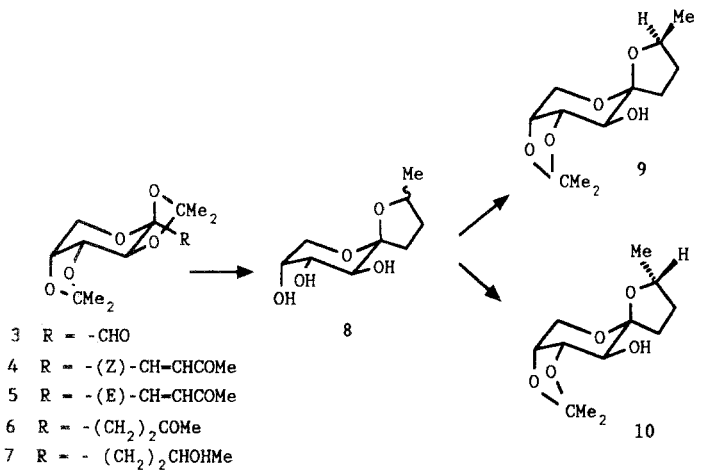
and Lygo, 1982; Doherty et al., 1984; Rosini et al., 1989) as well as diastereomeric mixtures with (2*R*,5*RS*) (Hungerbühler et al., 1980) and (2*S*,5*RS*) (Hintzer et al., 1981) configurations of the above compounds, where the *S*-malic and *S*-lactic acid were used as "chiral pool," respectively, have been reported.

On the other hand, the epimers with (2*R*,5*R*) and (2*R*,5*S*) configurations of 2-methyl-1,6-dioxaspiro[4.5]decane were obtained by Mori et al. (1985) from by-products produced in the synthesis of the minor components of olive fruit fly sex pheromone, from *S*-malic acid. Finally, all isomers have been synthesized (Iwata et al., 1987) by a high stereo-controlled five-membered ring cyclization using sulfoxides as chiral auxiliary.

As a part of our continuing efforts to synthesize optically active pheromones, we report herein on the enantiospecific synthesis of (2*S*,5*R*)- **(1)** and (2*R*,5*R*)-2-methyl-1,6-dioxaspiro[4.5]decane **(2)** using a derivative of D-fructose as chiral starting material (Scheme 1).

METHODS AND MATERIALS

General Methods. Melting points were determined with an Electrothermal melting points apparatus and are uncorrected. Solutions were dried over $MgSO_4$ before concentration under diminished pressure. ^{13}C (75.4 MHz) and ^1H NMR spectra (300, 250, and 80 MHz, internal Me_4Si) were recorded with Bruker AM-300, WM-250, and WP-80 spectrometers for solutions in $CDCl_3$; IR spectra with a Perkin-Elmer 782 instrument; and mass spectra with a Hewlett-Packard HP-5988-A spectrometer. Optical rotations were measured for solutions in chlo-



SCHEME 1.

roform (1-dm tube) with a Perkin-Elmer 141 polarimeter. GC was performed on a Perkin-Elmer 8410 gas chromatograph equipped with a flame-ionization detector, a steel column (2 m × 0.125 in. ID) packed with 5% OV-17 on Chromosorb W (100–120 mesh) at 170° (A), 150° (C), and 115° (D), and a glass column (1.5 m × 0.25 in. ID) packed with 5% OV-101 on Diatomite (100–120 mesh) at 215° (B). The N_2 flow rate was 30 ml/min, the injection port temperature was 250°, and the zone-detector temperature was 250°. TLC was performed on silica gel G (Merck) with detection by charring with sulfuric acid. Column chromatography was performed on silica gel (Merck, 7734). Some of the noncrystalline compounds for which elemental analyses were not obtained were shown to be homogeneous by chromatography and characterized by NMR and mass spectrometry.

(Z)- ***(4)*** *and* (E)-*1,3,4-Trideoxy-5,6:7,8-di*-O-*isopropylidene-β*-D-arabino-*non-3-ene-2,5-diulo-5,9-pyranose* ***(5).*** To a solution of 2,3:4,5-di-*O*-isopropylidene-*β*-D-*arabino-hexaldo-2-ulo-2,6-pyranose* **(3)** (Izquierdo and Plaza, 1990) (5 g, 19.4 mmol) in dry dichloromethane (40 ml), a solution of acetylmethylenetriphenylphosphorane (6.6 g, 20.6 mmol) in the same solvent (20 ml) was added. TLC (ether–hexane, 1:1) revealed the formation of a compound of higher mobility. The reaction was left at room temperature overnight, and then concentrated to a semicrystalline mass that was extracted with hot hexane (4 × 25 ml). Concentration of the extracts, followed by column chromatography (ether–hexane, 2:1) gave a first fraction (1.6 g) that GC analysis (*B*) showed to be constituted by two compounds lately identified as **4** and **5.** The second fraction was pure **5** (4 g) as a colorless syrup, $[\alpha]_D = -30°$ (*c* 1.3); ν^{film} 2994, 2941, and 2911 (C—H), 1706 and 1684 (C=O, conjugated), 1644 (C=C, conjugated), 1384 and 1374 (CMe_2), 1254, 1214, 1175, 1113, 1072, 984, and 899 cm^{-1} (C—O—C and 1,3-dioxolane ring). ^1H NMR (80 MHz): δ 6.71 (d, 1 H, $J_{3,4}$ 16 Hz, H-4), 6.45 (dd, 1 H, H-3), 4.62 (dd, 1 H, $J_{6,7}$ 3, $J_{7,8}$ 8 Hz, H-7), 4.45 (m, 1 H, H-8), 4.42 (d, 1 H, H-6), 3.98 (dd 1 H, $J_{8,9}$ 2, $J_{9,9'}$ 13 Hz, H-9), 3.73 (dd, 1 H, $J_{8,9'}$ 1 Hz, H-9′), 2.28 (s, 3 H, H-1,1,1), 1.55, 1.48, 1.35, and 1.33 (4 s, 12 H, 2 CMe_2). For the ^{13}C NMR, see Table 1. Mass spectrum: *m/z* 299 (0.3, $M^+ + 1$), 284 (1.1, $M^+ + 1$-Me), 283 (6.4, M^+-Me), 241 (2.6, $M^+ + 1$-Me-Ac), 240 (1.4, M^+-Me-Ac), 225 (1.7, M^+-Me-Me_2CO), 223 (0.6, M^+-Me-AcOH), 183 (6.3), 182 (2.1), 165 (2.4, M^+-Me-Me_2CO-AcOH), 113 (3.9), 111 (3.9), 110 (6.8), 98 (10.9), 97 (26.4), 85 (14.9), 69 (14.2), 59 (18.2, Me_2COH^+), and 43 (100, Ac^+).

Analysis: Calc. for $C_{15}H_{22}O_6$: C, 60.39; H, 7.43. Found: C, 58.71; H, 7.20.

Chromatography (ether–hexane, 4:1 to 3:1) of the first fraction gave additional **5** (1.3 g) and pure **4** (240 mg, 4%), $[\alpha]_D = -29.5°$ (*c* 1.7); ν^{film} 2980, 2929, and 2895 (C—H), 1695 (C=O, conjugated), 1378 (CMe_2), 1245, 1208, 1165, 1065, 1045, 988, 890, and 870 cm^{-1} (C—O—C and 1,3 dioxolane ring).

TABLE 1. ^{13}C NMR DATA FOR **4–7**

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	$\underline{C}Me_2$	$C\underline{Me}_2$
4	32.07	204.28	131.36	132.36	111.12	73.87	70.23	70.06	61.18	109.18 109.10	26.16 25.78 24.09 23.96
5	24.05	198.05	130.48	143.94	101.23	73.45	70.30	69.95	61.26	109.10	27.37 26.31 26.08 25.85
6	29.93	208.28	34.64	37.68	103.59	73.92	70.72	70.56	60.96	108.86 107.51	25.80 24.93 24.75 24.02
7	23.14	67.87[a] 67.64	32.67 32.59[a]	37.34[a] 37.12	104.06 103.98[a]	74.16[a] 74.02	70.83	70.64	61.04 60.99[a]	108.99 107.61	26.40 25.84 25.09 24.12

[a]Peaks of lower intensity.

^{1}H NMR: δ 5.91 (d, 1 H, $J_{3,4}$ 13 Hz, H-4), 5.73 (d, 1 H, H-3), 4.68 (dd, 1 H, $J_{6,7}$ 3, $J_{7,8}$ 8 Hz, H-7), 4.18 (d, 1 H, H-6), 4.26–4.11 (m, 1 H, H-8), 3.88 (dd, 1 H, $J_{8,9}$ 2, $J_{9,9'}$ 13 Hz, H-9), 3.70 (d, 1 H, H-9′), 2.36 (s, 3 H, H-1,1,1), 1.47, 1.31, and 1.24 (3 s, 12 H, relative intensities 2:1:1, 2 CMe_2). For the ^{13}C NMR, see Table 1. Mass spectrum:*m/z* 299 (0.5, M^++1), 283 (6.1, M^+-Me), 225 (2.6, M^+-Me-Me_2CO), 223 (1.0, M^+-Me-AcOH), 183 (5.5), 182 (2.1), 165 (5.1, M^+-Me-Me_2CO-AcOH), 143 (9.2), 113 (4.0), 111 (6.9), 110 (9.8), 98 (15.0), 97 (30.6), 85 (16.3), 69 (12.3), 59 (18.9, Me_2COH^+), and 43 (100, Ac^+).

Analysis: Calc. for $C_{15}H_{22}O_6$: C, 60.39; H, 7.43. Found: C, 61.55; H, 7.27.

The total yield of **5** was 5.3 g (92%).

When the reaction of the aldulose (0.5 g, 1.9 mmol) with the above ylide (0.7 g, 2 mmol) was carried out in methanol (8 ml) for 24-hr and the residue (580 mg) analyzed by GLC, the *E*/*Z* ratio was 4:1 (B, retention time 5.76 and 4.66 min for the *E* and *Z* isomer, respectively).

*1,3,4-Trideoxy-5,6:7,8-di-*O*-isopropylidene-β-*D*-*arabino*-non-2,5-diulo-5,9-pyranose* ***(6).*** Compound **5** (3.2 g, 10.7 mmol) in methanol (50 ml) was hydrogenated at 4 atm over 5% Pd-C (0.5 g) for 30 min. GLC (B) then revealed that **5** had disappeared and that a new compound (T 4.87) was present. The catalyst was filtered off and the filtrate concentrated to a residue (3.1 g) that was chromatographed (ether–hexane 1:2) to yield **6** (2.8 g, 87%), $[\alpha]_D = -16°$ (*c* 1.1); ν^{film} 2980 and 2925 (C—H), 1710 (C=O), 1370 (CMe_2), 1250, 1210, 1160, 1070, 980, 900, and 865 cm^{-1} (C—O—C and 1,3-dioxolane ring). ^{1}H NMR (250 MHz): δ 4.55 (dd, 1 H, $J_{6,7}$ 2.4, $J_{7,8}$ 8 Hz, H-7), 4.20 (m, 1 H, H-8), 4.11 (d, 1 H, H-6), 3.83 (dd, 1 H, $J_{8,9}$ 2 Hz, $J_{9,9'}$ 13 Hz, H-9), 3.69 (dd, 1 H, $J_{8,9'}$ 0.7 Hz, H-9′), 2.84 (ddd, 1 H, $J_{3,3'}$ 14.2, $J_{3,4}$ 5.5, $J_{3,4'}$ 9.4 Hz, H-3), 2.71 (ddd, 1 H, $J_{3',4}$ 9.4, $J_{3',4'}$ 5.5 Hz, H-3′), 2.15 (s, 3 H, H-1,1,1), 2.15 (ddd, 1 H, $J_{4,4'}$ 14 Hz, H-4), 1.98 (ddd, 1 H, H-4′), 1.50, 1.49, and 1.34 (3 s, 12 H, relative intensities 1:1:2, 2 CMe_2). For the ^{13}C NMR, see Table 1.

Analysis: Calc. for $C_{15}H_{24}O_6$: C, 59.98; H, 8.06. Found: C, 60.62; H, 7.78.

*1,3,4-Trideoxy-5,6:7,8-di-*O*-isopropylidene-β-*D*-gluco- and -*D*-manno-non-5-ulo-5,9-pyranose* ***(7).*** For method a, compound **5** (3 g, 10 mmol) in methanol (50 ml) was hydrogenated at 1.5 atm over Raney-nickel (10 g) overnight. TLC (ether–hexane, 3:2) then showed the presence of a compound of lower mobility. The catalyst was filtered off and the filtrate concentrated to yield a residue (3 g). GLC (B) of the residue revealed it to be constituted by a main compound (retention time 5.30 min). The same result was reached when a mixture of **4** and **5** (4.5 g) was hydrogenated under the same conditions giving a residue (4.5 g). Both residues were combined and chromatographed (ether–

hexane, 2:3) to afford **7** (6.8 g, 89.5%), $[\alpha]_D = -8.5°$, $[\alpha]_{365} = -14.5°$ (*c* 1); ν^{film} 3473 (OH), 2970, 2939, and 2878 (C—H), 1383 and 1374 (CMe_2), 1254, 1212, 1192, 1176, 1108, 1086, 989, and 900 cm^{-1} (C—O—C and 1,3-dioxolane ring). ^1H NMR (250 MHz): δ 4.57 (dd, 1 H, $J_{6,7}$ 2.4, $J_{7,8}$ 8 Hz, H-7), 4.20 (m, 1 H, H-8), 4.11 (d, 1 H, H-6), 3.90–3.76 (m, 1 H, H-2), 3.87 (dd, 1 H, $J_{8,9}$ 2, $J_{9,9'}$ 13 Hz, H-9), 3.66 (dd, 1 H, $J_{8,9'}$ 1 Hz, H-9′), 2.15 (bs, 1 H, HO-2), 2.10–1.60 (2 m, 4 H, H-3,3′,4,4′) 1.51, 1.48, 1.36, and 1.34 (4 s, 12 H, 2 CMe_2), and 1.19 (d, 3 H, $J_{1,2}$ 6 Hz, H-1,1,1). For the ^{13}C NMR, see Table 1.

Analysis: Calc. for $C_{15}H_{26}O_6$: C, 59, 58; H, 8.67. Found: C, 60.78; H, 8.62.

For method b, compound **6** (105 mg, 0.35 mmol) in methanol (2 ml) was reduced with $NaBH_4$ (20 mg). After 15 min, GLC analysis revealed the absence of **6** and the presence of **7.** The reaction was neutralized (conc. acetic acid), concentrated, and the residue extracted with chloroform (5 ml) and washed with water. Concentration of the chloroformic extracts gave **7** (107 mg, quantitative).

For method c, a 100-ml three-neck flask equipped with a dropping funnel, a stirring bar, and a glass-inlet tube was flushed with nitrogen and charged with **6** (2.5 g, 8 mmol) in anhydrous tetrahydrofuran (25 ml). The content of the flask was cooled to −40°C before L-selectride (M, 18 ml, 18 mmol) was added gradually so that the temperature was maintained at −40°C. Stirring was continued at −40°C for 1 hr, and the solution was then allowed to warm to −10°C before 3 M sodium hydroxide (5 ml) followed by aqueous 30% hydrogen peroxide (15 ml) were added, keeping the temperature below 15°C. The solution was saturated with potassium carbonate and diluted with chloroform, the organic phase separated, and concentrated to yield a residue that was chromatographed as above to afford **7** (2.5 g, quantitative).

*(2*RS*,5*R*,8*R*,9*R*,10*S*)-8,9,10-Trihydroxy-2-methyl-1,6-dioxaspiro[4.5]decane* ***(8).*** A solution of **7** (1.4 g, 4.6 mmol), obtained by method a, in aqueous 70% trifluoroacetic acid (15 ml) was kept at room temperature for 30 min. TLC (chloroform–methanol, 5:1.5) then showed a slower-running spot. The reaction mixture was concentrated, dissolved in absolute ethanol (20 ml), neutralized ($NaHCO_3$), filtered, and concentrated to yield a residue (1.16 g) that was subjected to column chromatography (chloroform–methanol, 9:1) to afford **8** (824 mg, 87%) as a solid foam.

Acetylation of **8** (824 mg, 4 mmol) as usual in dry pyridine (10 ml) and acetic anhydride (5 ml) for 24 hr gave a crystalline mass that was chromatographed (ether–hexane, 2:3 to 1:1) to yield the tri-*O*-acetyl derivative (1.25 g, quantitative). Several recrystallizations (ether–hexane) allowed the isolation of (2*S*,5*R*,8*R*,9*R*,10*S*)-8,9,10-triacetylyoxy-2-methyl-1,6-dioxaspiro[4.5]decane (100 mg), mp 150–151°C, $[\alpha]_D = -137.5°$ (*c* 1); ν^{film} 2969, 2938, 2903, 2876 (C—H), 1748 and 1745 (C=O, acetate), 1449, 1441, 1392, 1373, 1259, 1229,

1104, 1085, 1048, and 938 cm^{-1} (C—O—C). ^1H NMR: δ 5.36 (d, 1 H, $J_{9,10}$ 10.3 Hz, H-10), 5.27 (dt, 1 H, H-8), 5.24 (dd, 1 H, $J_{8,9}$ 3.4 Hz, H-9), 4.21 (sex, 1 H, $J_{2,Me} = J_{2,3} = J_{2,3'} = 6.3$ Hz, H-2), 4.01 (dd, 1 H, $J_{7,8}$ 1.7, $J_{7,7'}$ 13 Hz, H-7), 3.60 (dd, 1 H, $J_{7',8}$ 1.7 Hz, H-7'), 2.09 and 2.03 (2 s, 6 H, Ac at C-9 and C-10), 1.92 (s, 3 H, Ac at C-8), 2.17–1.82 (m, 3 H, H-3,4,4'), 1.33 (ddt, 1 H, $J_{3',4}$ 8.7, $J_{3,3'}$ 11.8 Hz, H-3'), and 1.18 (d, 3 H, Me-2). ^{13}C NMR: δ 170.47 and 170.37 (MeCO at C-9,10), 170.05 (MeCO at C-8), 107.08 (C-5), 75.85 (C-2), 69.44 and 69.40 (C-9,10), 28.51 (C-8), 61.69 (C-7), 34.20 (C-4), 31.57 (C-3), 20.99, 20.78, and 20.68 (3 MeCO and Me-2).

Analysis: Calc. for $C_{15}H_{22}O_8$: C, 54.54; H, 6.71. Found: C, 54.68; H, 6.70.

*(2S,5R,8R,9R,10S)-**(9)** and (2R,5R,8R,9R,10S)-10-Hydroxy-8,9-isopropylidenedioxy-2-methyl-1,6-dioxaspiro[4.5]decane **(10)**.* To a stirred solution of **8** (2.58 g, 12.6 mmol) in dry acetone (35 ml), anhydrous copper sulfate (2 g) and *p*-toluenesulfonic acid (160 mg) were added. The reaction mixture was kept at room temperature for 24 hr. GLC (A) then showed the presence of two peaks (retention time 5.42 and 5.80 min). The reaction mixture was neutralized (K_2CO_3), filtered, and concentrated. The residue (3.03 g), after repeated column chromatography (ether–hexane, 5:1 to 1:2) and recrystallizations (hexane), afforded **9** (1.04 g) as white crystalline needles, mp 81–83°C, retention time 5.42 min, $[\alpha]_D = -162°$ (*c* 1); ν^{KBr} 3449 (OH), 2991, 2974, 2961, and 2940 (C—H), 1384 and 1374 (CMe_2), 1272, 1248, 1223, 1113, 1082, 1071, 1027, 925, 854, and 801 cm^{-1} (C—O—C and 1,3-dioxolane ring). ^1H NMR: δ 4.22 (bsex, 1 H, H-2), 4.14 (dd, 1 H, H-8), 4.04 (dd, 1 H, $J_{8,9}$ 5.7, $J_{9,10}$ 7.3 Hz, H-9), 3.99 (dd, 1 H, $J_{7,8}$ 2.6, $J_{7,7'}$ 13.3 Hz, H-7), 3.88 (d, 1 H, H-7'), 3.59 (dd, 1 H, $J_{OH,10}$ 9.3 Hz, H-10), 2.27 (ddd, 1 H, $J_{3,4}$ 6.1, $J_{3',4}$ 10, $J_{4,4'}$ 12.8 Hz, H-4), 2.09 (ddt, 1 H, $J_{2,3}$ 6.4, $J_{3,4'}$ 8.6, $J_{3,3'}$ 12 Hz, H-3), 2.02 (d, 1 H, HO-10), 1.85 (ddd, 1 H, $J_{3',4'}$ 6 Hz, H-4'), 1.51 and 1.33 (2 s, 6 H, CMe_2), 1.41 (dddd, 1 H, $J_{2,3'}$ 7.2 Hz, H-3'), and 1.21 (d, 3 H, $J_{Me,2}$ 6.1 Hz, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 244 (0.3, M^+), 229 (2.7, M^+-Me), 185 (0.9), 159 (5.7), 157 (3.9), 143 (1.0), 142 (1.2), 114 (11.4), 101 (100, $C_5H_9O_2^+$), 100 (37.0, $C_5H_8O_2^+$), 86 (21.3, $C_5H_9O_2^+$-Me), 85 (52.8, $C_5H_8O_2^+$-Me), 84 (12.5), 83 (12.1), 59 (16.9, Me_2COH^+), and 43 (13.5, Ac^+).

Analysis: Calc. for $C_{12}H_{20}O_5$: C, 59.00; H, 8.25. Found: C, 58.53; H, 8.45.

Eluted second was **10** (1 g), as white crystalline needles, mp 120°C, retention time 5.80 min, $[\alpha]_D = -165°$ (*c* 1); ν^{KBr} 3445 (OH), 2989, 2974, 2958, 2941, and 2884 (C—H), 1384 and 1375 (CMe_2), 1271, 1247, 1221, 1168, 1131, 1113, 1080, 1071, 1058, 1025, 925, 855, and 800 cm^{-1} (C—O—C and 1,3-dioxolane ring). ^1H NMR: δ 4.28 (dquin, 1 H, $J_{Me,2} = J_{2,3} = 6.2$, $J_{2,3'}$ 8.7 Hz, H-2), 4.15 (dd, 1 H, $J_{7,8}$ 2.6, $J_{8,9}$ 5.6 Hz, H-8), 4.07 (dd, 1 H, $J_{7,7'}$ 13.3 Hz, H-7), 4.04 (dd, 1 H, $J_{9,10}$ 7.4 Hz, H-9), 3.91 (d, 1 H, H-7'), 3.60

TABLE 2. ^{13}C NMR DATA FOR **1, 2, 9–14a,** AND **16–17a**

Compound	C-2	C-3	C-4	C-5	C-7	C-8	C-9	C-10	$\underline{C}Me_2$	$C\underline{Me}_2$	Me-2	$PhC\underline{H}_2$	Ph	C=S	Me—Ts
1	73.98	31.86	34.67	105.75	61.37	25.80	20.68	38.35			21.45				
2	76.62	32.18	34.57	105.53	61.19	25.40	20.75	39.66			23.40				
9	78.09	31.62	33.99	107.43	59.73	72.15	73.86	75.91	109.16	28.19 26.19	20.81				
10	79.11	31.37	34.41	107.38	59.39	72.20	74.00	78.26	109.19	28.29 26.28	22.65				
11	78.10	31.50	33.96	107.29	59.52	74.30	75.76	78.15	108.64	28.36 26.41	20.80	73.14	138.68a[a] 127.49b 128.21c 127.89d		
11a	78.74	31.26	34.41	107.04	59.28	74.32	77.99	78.24	108.78	28.34 26.43	22.38	73.16	138.39a 127.60b 128.23c 128.20d		
12	78.39	31.44	34.09	108.27	63.09	70.07	71.09	75.44			20.89	75.16	138.40a 127.95b,d 128.58c		
12a	78.44	31.31	34.58	107.98	62.75	70.08	71.31	78.31			22.56	75.44	138.28a 128.19b 128.07c 128.67d		
13	76.73	31.40	33.74	106.43	58.08	80.72	83.61	75.72			20.69	73.57	136.93a 128.11b 128.45c 128.42d	191.09	

13a	79.72	31.28	34.21	106.24	57.79	80.72	83.81	75.71		22.10	73.61	136.72a 128.26b 128.70c 128.60d	191.07	
14	75.78	31.63	34.34	105.20	61.15	124.12	127.55[b]	72.78		21.07	70.97	138.59a 128.31[b] 127.80[b] 127.67[b]		
14a	78.15	31.77	34.84	104.82	61.07	124.02	127.68[b]	72.87		22.54	71.16	138.59a 128.35[b] 128.04[b] 127.72[b]		
16	79.16	31.91	34.56	105.28	60.51	25.30	26.33	75.59		20.65		144.75a 127.72b 129.85c 134.47d		21.70
16a	78.90	31.53	34.51	105.06	60.08	25.34	26.42	78.26		22.42		144.80a 127.81b 129.87c 134.42d		21.71
17	75.44	31.94	34.70	105.67	60.83	24.73	25.15	80.42		21.06			215.63	
17a	78.08	31.66	34.88	105.43	60.34	24.82	25.16	80.32		22.58			215.80	

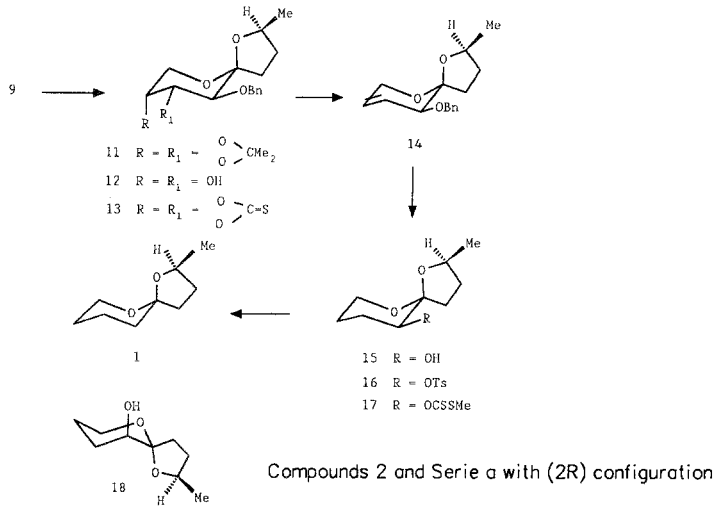
[a] a, C_{ipso}; b, C_{ortho}; c, C_{meta}; d, C_{para}.
[b] Assignments may be interchanged. SMe in **17** and **17a** at 18.82 and 19.02, respectively.

(dd, 1 H, $J_{OH,10}$ 8.8 Hz, H-10), 2.27 (dt, 1 H, $J_{3,4} = J_{3',4}$ = 8.1, $J_{4,4'}$ 12.5 Hz, H-4), 2.11 (d, 1 H, HO-10), 1.99 (dddd, 1 H, $J_{3,4'}$ 1.7, $J_{3,3'}$ 11.9 Hz, H-3), 1.91 (ddd, 1 H, $J_{3',4'}$ 7.2 Hz, H-4'), 1.70 (ddt, 1 H, H-3'), 1.52 and 1.34 (2 s, 6 H, CMe_2), and 1.28 (d, 3 H, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 229 (M^+-Me), 159 (3), 157 (3), 143 (1), 142 (1), 114 (12), 101 (100, $C_5H_9O_2^+$), 100 (35, $C_5H_8O_2^+$), 86 (15, $C_5H_9O_2^+$-Me), 85 (66, $C_5H_8O_2^+$-Me), 83 (15), 59 (33, Me_2COH^+), and 43 (33, Ac^+).

Analysis: Calc. for $C_{12}H_{20}O_5$: C, 59.00; H, 8.25. Found: C, 59.22; H, 8.40.

When acetonation was carried out on a sample of **8** (5 g) obtained by method c, compounds **9** and **10** were isolated at 43% (2.1 g) and 57% (2.7 g), respectively.

(2S,5R,8R,9R,10S)-10-Benzyloxy-8,9-isopropylidenedioxy-2-methyl-1,6-dioxaspiro[4.5]decane ***(11)*** *(Scheme 2).* To a stirred suspension of sodium hydride (400 mg, 12 mmol) (80% oil dispersion), previously washed free of oil with dry hexane by stirring and decantation, in anhydrous tetrahydrofuran (10 ml) and under an inert atmosphere (N_2), imidazole (50 mg) and compound **9** (1.1 g, 4.5 mmol) in the same solvent (10 ml) were added dropwise. The reaction was refluxed for 30 min, cooled, and then benzyl bromide (1 ml, 8 mmol) was added dropwise and the reflux continued for further 30 min. TLC (ether–hexane, 1:2) then revealed the presence of a compound of higher mobility. The excess of hydride was destroyed by addition of ether saturated with water (20 ml) and water. The organic phase was separated and the aqueous



SCHEME. 2.

phase extracted with ether (2 × 10 ml). Concentration of the combined extracts and column chromatography (ether–hexane, 1:4) of the residue gave syrupy **11** (1.26 g, 91%); $[\alpha]_D = -90°$ (*c* 1.2); ν^{film} 3092, 3068, and 3055 (C—H, aromatic), 2987, 2936, and 2880 (C—H), 1382 and 1371 (CMe_2), 1243, 1218, 1169, 1123, 1081, 1022, 1003, 940, 901, 890, and 854 (C—O—C and 1,3-dioxolane ring), 737 and 698 cm^{-1} (aromatic). ^1H NMR: δ 7.37–7.27 (m, 5 H, $\underline{Ph}CH_2$), 4.97 and 4.68 (2 d, 2 H, *J* 12.1 Hz, $Ph\underline{CH}_2$), 4.38 (dd, 1 H, $J_{8,9}$ 5.7, $J_{9,10}$ 7.6 Hz, H-9), 4.20 (dd, 1 H, H-8), 4.19 (sex, 1 H, H-2), 4.05 (dd, 1 H, $J_{7,8}$ 2.7, $J_{7,7'}$ 13.3 Hz, H-7), 3.89 (d, 1 H, H-7′), 3.51 (d, 1 H, H-10), 2.18 (ddd, 1 H, $J_{3,4}$ 6.2, $J_{3',4}$ 10, $J_{4,4'}$ 12.6 Hz, H-4), 2.04 (ddt, 1 H, $J_{2,3}$ 6.3, $J_{3,4'}$ 8.2, $J_{3,3'}$ 12 Hz, H-3), 1.75 (ddd, 1 H, $J_{3',4'}$ 6.2 Hz, H-4′), 1.59 and 1.37 (2 s, 6 H, CMe_2), 1.44 (dddd, 1 H, $J_{2,3'}$ 7.0 Hz, H-3′), and 1.24 (d, 3 H, $J_{Me,2}$ 6.2 Hz, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 334 (0.3, M^+), 320 (0.3, M^++1-Me), 319 (0.3, M^+-Me), 275 (3.2), 243 (5.0, M^+-Bn), 200 (5.9), 143 (68.2, M^+-Bn-$C_5H_8O_2$), 113 (4.5), 101 (9.3, $C_5H_9O^+$), 100 (41.8, $C_5H_8O_2^+$), 92 (9.6), 91 (100, $C_7H_7^+$), 85 (41.2, $C_5H_8O^+$-Me), 65 (9.8), 59 (9.4, Me_2COH^+), 57 (15.7), and 43 (11.6, Ac^+).

(2R,5R,8R,9R,10S)-10-Benzyloxy-8,9-isopropylidenedioxy-2-methyl-1,6-dioxaspiro[4.5]decane ***(11a).*** Compound **10** (1.06 g, 4.34 mmol) was benzylated with sodium hydride (230 mg, 9.5 mmol), and benzyl bromide (0.8 ml, 6.5 mmol) in anhydrous tetrahydrofuran (18 ml) as described above to afford syrupy **11a** (1.36 g, 94%), $[\alpha]_D = -88°$ (*c* 1.2); ν^{film} 3091, 3067, and 3035 (C—H, aromatic), 2985, 2937, and 2876 (C—H), 1381 and 1370 (CMe_2), 1244, 1218, 1169, 1120, 1079, 1023, 992, 939, 884, and 855 (C—O—C and 1,3-dioxolane ring), 738 and 698 cm^{-1} (aromatic). ^1H NMR: δ 7.37–7.27 (m, 5 H, $\underline{Ph}CH_2$), 4.94 and 4.68 (2 d, 2 H, *J* 12 Hz, $Ph\underline{CH}_2$), 4.36 (dd, 1 H, $J_{8,9}$ 5.6, $J_{9,10}$ 7.5 Hz, H-9), 4.31 (dquin, $J_{Me,2} = J_{2,3} = 6.1$, $J_{2,3'}$ 8.7 Hz, H-2), 4.19 (dd, 1 H, $J_{7,8}$ 2.6 Hz, H-8), 4.13 (dd, 1 H, $J_{7,7'}$ 13.3 Hz, H-7), 3.90 (d, 1 H, H-7′), 3.50 (d, 1 H, H-10), 2.23 (dt, 1 H, $J_{3,4} = J_{3',4} = 8.4$, $J_{4,4'}$ 12 Hz, H-4), 1.97 (dddd, 1 H, $J_{3,4'}$ 1.5, $J_{3,3'}$ 11.5 Hz, H-3), 1.79 (ddd, 1 H, $J_{3',4'}$ 7.4, $J_{4,4'}$ 12 Hz, H-4′), 1.65 (ddt, 1 H, H-3′), 1.51 and 1.37 (2 s, 6 H, CMe_2), and 1.27 (d, 3 H, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 334 (0.3, M^+), 319 (0.5, M^+-Me), 243 (2.4, M^+-Bn), 200 (3.5), 143 (48.1, M^+-Bn-$C_5H_8O_2$), 113 (2.6), 101 (10.6, $C_5H_9O_2^+$), 100 (51.5, $C_5H_8O_2^+$), 92 (8.3), 91 (100, $C_7H_7^+$), 85 (45.6, $C_5H_8O_2^+$-Me), 65 (4.7), 59 (6.4, Me_2COH^+), 57 (10.3), and 43 (5.7, Ac^+).

(2S,5R,8R,9R,10S)-10-Benzyloxy-8,9-dihydroxy-2-methyl-1,6-dioxa-spiro-[4.5]decane ***(12).*** A solution of **11** (1.32 mg, 3.9 mmol) in aqueous 60% acetic acid (10 ml) was heated at 50°C for 1.5 hr. TLC (ether) then showed the presence of a slower-running compound. The solution was concentrated and the residue dissolved in absolute ethanol (15 ml), neutralized (Na_2CO_3), filtered, and concentrated again to a syrup that was chromatographed (ether) to afford

12 (1.1 g, quantitative), $[\alpha]_D = -97°$ (*c* 1.2); ν^{film} 3395 and 3374 (OH), 3092, 3068, and 3036 (C—H, aromatic), 2974, 2947, and 2897 (C—H), 1499 (benzyl) 1456, 1274, 1206, 1159, 1120, 1087, 1074, 1028, 1001, 984, and 936 (C—O—C), 734 and 696 cm^{-1} (aromatic). ^1H NMR (80 MHz): δ 7.31 (bs, 5 H, PhCH$_2$), 4.75 (s, 2 H, PhCH$_2$), 4.35–3.45 (m, 6 H, H-2,7,7′,8,9,10), 2.26 (bs, 2 H, HO-8,9), 2.25–1.12 (m, 4 H, H-3,3′,4,4′), and 1.20 (d, 3 H, *J* 6 Hz, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 294 (0.08, M^+), 279 (0.06, M^+-Me), 264 (0.19), 263 (1.11), 203 (6.59, M^+-Bn), 175 (1.17), 173 (1.24), 163 (2.69), 150 (2.51), 130 (1.14), 127 (2.81), 121 (1.35), 114 (1.34), 113 (2.97), 195 (3.34), 103 (30.83), 101 (54.58, $C_5H_9O_2^+$), 91 (100, $C_7H_7^+$), 85 (16.16), 83 (12.30), 65 (17.17), 55 (18.61), and 43 (15.72, Ac^+).

(2R,5R,8R,9R,10S)-10-O-Benzyl-8,9-dihydroxy-2-methyl-1,6-dioxa-spiro-[4.5]decane ***(12a).*** Compound **11a** (1.34 g, 4 mmol) was hydrolyzed as above to yield syrupy **12a** (1.13 g, 96%), $[\alpha]_D = -111°$ (*c* 1.2); ν^{film} 3422 (OH), 3091, 3068, and 3035 C—H, aromatic), 2973, 2933, and 2880 (C—H), 1498, 1455, 1379, 1210, 1165, 1112, 1081, 978, 932, and 881 (C—O—C), 748 and 698 cm^{-1} (aromatic). ^1H NMR (80 MHz): δ 7.31 (bs, 5 H, PhCH$_2$), 4.72 (s, 2 H, PhCH$_2$), 4.25 (m, 1 H, H-2), 4.10–3.45 (m, 4 H, H-7,7′,8,9,10), 2.50 (bs, 2 H, HO-8,9), 2.25–1.42 (m, 4 H, H-3,3′,4,4′), and 1.21 (d, 3 H, $J_{Me,2}$ 6 Hz, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 279 (0.05, M^+-Me), 264 (0.15), 263 (0.74), 203 (5.54, M^+-Bn), 175 (1.70), 163 (2.77), 150 (3.24), 113 (2.93), 101 (55.05, $C_5H_9O_2^+$), 91 (100, $C_7H_7^+$), 85 (17.99), 83 (13.18), 65 (15.43), 55 (18.01) and 43 (14.59, Ac^+).

(2S,5R,8R,9R,10S)-10-Benzyloxy-2-methyl-8,9-thiocarbonyldioxy-1,6-dioxaspiro[4.5]decane ***(13).*** To a stirred solution of **12** (1.1 g, 3.7 mmol) in dry toluene (20 ml), 1,1′-thiocarbonyldiimidazole (810 mg, 4.5 mmol) was added and the mixture refluxed for 1.5 hr. TLC (ether) then revealed the presence of a new compound with higher mobility. The mixture was cooled, washed with water and concentrated to a residue that was chromatographed (ether–hexane, 1:1), to yield crystalline (on standing) **13** (900 mg, 73%), mp 73–75°C $[\alpha]_D = -78.5°$ (*c* 1.2); ν^{KBr} 3068 and 3035 (C—H, aromatic), 2975, 2934, and 2889 (C—H), 1455, 1444, 1367, 1345, 1319, 1291, 1204, 1165, 1116, 1090, 1073, 988, 929, and 890 (C=S and C—O—C), 748 and 699 cm^{-1} (aromatic). ^1H NMR data: δ 7.40–7.28 (m, 5 H, PhCH$_2$), 5.09 (t, 1 H, $J_{8,9} = J_{9,10} = 7.2$ Hz, H-9), 4.90 and 4.66 (2 d, 2 H, *J* 11.8 Hz, PhCH$_2$), 4.87 (dt, 1 H, $J_{7,8}$ 1.8 Hz, H-8), 4.21 (sex, 1 H, $J_{Me,2} = J_{2,3} = J_{2,3'} = 6.5$ Hz, H-2), 4.07 (d, 2 H, H-7,7), 3.57 (d, 1 H, H-10), 2.15 (ddd, 1 H, $J_{3,4}$ 6.2, $J_{3',4}$ 9.8, $J_{4,4'}$ 12.5 Hz, H-4), 2.06 (ddt, 1 H, $J_{3,4'}$ 8.3, $J_{3,3'}$ 12.8 Hz, H-3), 1.75 (ddd, 1 H, $J_{3',4'}$ 6.4 Hz, H-4′), 1.49 (dddd, 1 H, H-3′), and 1.24 (d, 3 H, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 336 (0.2, M^+), 245 (6.3, M^+-Bn), 191 (1.7), 145 (8.9), 101 (16.4, $C_5H_9O_2^+$), 92 (12.2), and 91 (100, $C_7H_7^+$).

Analysis: Calc. for $C_{17}H_{20}O_5S$: C, 60.69; H, 5.99. Found: C, 61.03; H, 5.38.

(2R,5R,8R,9R,10S)-10-Benzyloxy -2-methyl - 8,9 - thiocarbonyldioxy-1,6-dioxaspiro[4.5]decane ***(13a).*** Compound **12a** (1.12 g, 3.8 mmol) was treated with 1,1′-thiocarbonyldiimidazole (880 mg, 4.9 mmol) in dry toluene (20 ml) as above to yield, after column chromatography (ether–hexane, 1 : 1) syrupy **13a** (970 mg, 76%), $[\alpha]_D = -67°$ (*c* 1.5); ν^{film} 3067 and 3036 (C—H, aromatic), 2975, 2937, and 2875 (C—H), 1455, 1443, 1367, 1345, 1294, 1275, 1173, 1157, 1114, 1078, 1059, 989, 928, and 887 (C=S and C—O—C), 737 and 700 cm^{-1} (aromatic). ^1H NMR: δ 7.40–7.28 (m, 5 H, $\underline{Ph}CH_2$), 5.08 (t, 1 H, $J_{8,9} = J_{9,10} = 7.2$ Hz, H-9), 4.90 and 4.65 (2 d, 2 H, *J* 11.7 Hz, $PH\underline{CH}_2$), 4.86 (dd, 1 H, $J_{7,8}$ 2.6 Hz, H-8), 4.33 (dq, 1 H, $J_{Me,2} = J_{2,3} = 6.1$, $J_{2,3'}$ 9 Hz, H-2), 4.17 (dd, 1 H, $J_{7,7'}$ 14.2 Hz, H-7), 4.08 (d, 1 H, H-7′), 3.56 (d, 1 H, H-10), 2.21 (dt, 1 H, $J_{3,4} = J_{3',4} = 8.4$, $J_{4,4'}$ 12.5 Hz, H-4), 2.01 (dddd, 1 H, $J_{3,4'}$ 1.6, $J_{3,3'}$ 11.9 Hz, H-3), 1.78 (ddd, 1 H, $J_{3',4'}$ 7.4 Hz, H-4′), 1.65 (ddt, 1 H, H-3′), and 1.27 (d, 3 H, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 336 (0.12, M^+), 245 (5.94, M^+-Bn), 191 (1.42), 145 (7.84), 101 (14.53, $C_5H_9O_2^+$), 92 (10.73), and 91 (100, $C_7H_7^+$).

(2S,5R,10S)-10-Benzyloxy-2-methyl-1,6-dioxaspiro[4.5]dec-8-ene ***(14).*** A solution of **13** (800 mg, 2.38 mmol) in trimethylphosphite (2.5 ml) was heated under reflux for 8 hr. TLC (ether–hexane, 1 : 1) then showed the presence of a faster-running compound. The mixture was dissolved in dichloromethane (15 ml) and washed with aqueous 15% sodium hydroxide, water, and concentrated. Column chromatography (ether–hexane, 1 : 4) of the residue yielded **14** (550 mg, 88%) as a colorless mobile oil, $[\alpha]_D = +22°$ (*c* 1); ν^{film} 3067 and 3036 (C—H, aromatic), 2975, 2948, 2932, 2888, and 2854 (C—H), 1455, 1387, 1203, 1190, 1113, 1105, 925, and 893 (=C—H and C—O—C), 736 and 697 cm^{-1} (aromatic). ^1H NMR: δ 7.40–7.28 (m, 5 H, $\underline{Ph}CH_2$), 5.88 and 5.82 (2 m, 2 H, H-8,9), 4.80 and 4.57 (2 d, 2 H, *J* 12.2 Hz, $PH\underline{CH}_2$), 4.38–4.25 (m, 2 H, H-2,7), 4.10 (m, 1 H, H-10), 3.99 (2 m, 1 H, H-7′), 2.20–2.07 (m, 2 H, H-3,4), 1.84–1.75 (m, 1 H, H-4′), 1.58–1.44 (m, 1 H, H-3′), and 1.28 (d, 3 H, $J_{Me,2}$ 6.2 Hz, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 261 (0.05, M^+), 161 (2.80), 160 (18.01), 105 (5.20), 92 (22.64), 91 (100, $C_7H_7^+$), 65 (29.48), 55 (17.89), 43 (17.17, Ac^+), and 41 (36.11).

(2R,5R,10S)-10-Benzyloxy-2-methyl-1,6-dioxaspiro[4.5]dec-8-ene ***(14a).*** Compound **13a** (970 mg, 2.88 mmol) was refluxed in trimethylphosphite (3.5 ml) for 15 hr. Work-up of the reaction mixture as above afforded **14a** (704 mg, 94%) as a colorless mobile oil, $[\alpha]_D = +9.5°$, $[\alpha]_{365} = +58°$ (*c* 0.9); ν^{film} 3068 and 3037 (C—H, aromatic), 2973, 2932, 2889, 2870, and 2855 (C—H), 1455, 1448, 1387, 1195, 1165, 1111, 1066, 983, and 884 (=C—H and C—O—C), 736 and 697 cm^{-1} (aromatic). ^1H NMR: δ 7.40–7.22 (m, 5 H, $\underline{Ph}CH_2$), 5.83 (s, 2 H, H-8,9), 4.75 and 4.56 (2 d, 2 H, *J* 12 Hz, $Ph\underline{CH}_2$),

4.38–4.27 (m, 2 H, H-2,7), 4.06 (m, 1 H, H-10), 3.98 (2 m, 1 H, H-7′), 2.19 (dt, 1 H, $J_{3,4} = J_{3',4} = 8.3$, $J_{4,4'}$ 12 Hz, H-4), 2.01 (m, 1 H, H-3), 1.81 (ddd, 1 H, $J_{3,4'}$ 1.5, $J_{3',4'}$ 7.6 Hz, H-4′), 1.72 (ddt, 1 H, $J_{2,3'}$ 9, $J_{3,3'}$ 11.8 Hz, H-3′), and 1.32 (d, 3 H, $J_{Me,2}$ 6 Hz, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 260 (0.08, M^+), 161 (1.16), 160 (9.38), 105 (4.03), 92 (16.71), 91 (100, $C_7H_7^+$), 65 (21.14), 55 (11.99), 43 (9.92, Ac^+), and 41 (25.76).

(2S,5R,10S)-10-Hydroxy-2-methyl-1,6-dioxaspiro[4.5]decane ***(15).*** A solution of compound **14** (500 mg, 1.9 mmol), in anhydrous methanol (10 ml) and a drop of triethylamine, was hydrogenated at 2 atm over Raney-nickel (1 g) for 48 hr. TLC (ether–hexane, 2 : 1) then revealed the conversion of **14** into a slower-moving product. The catalyst was filtered off and the filtrate concentrated to a residue that was chromatographed (ether–hexane, 1 : 1) to afford **15** (260 mg, 79%), retention time 3.38 min (C), $[\alpha]_D = -84°$ (*c* 1.2); ν^{film} 4361 (OH), 2948 and 2880 (C—H), 1094, 1072, 1069, and 962 cm^{-1} (C—O—C). ^1H NMR (80 MHz): δ 4.40–3.92 (m, 1 H, H-2), 3.90–3.00 (m, 3 H, H-7,7′,10), 2.45–1.00 (m, 9 H, H-3,3′,4,4′,8,8′,9,9′, HO-1), and 1.20 (d, 3 H, $J_{Me,2}$ 6 Hz, Me-2).

*(2S,5R,10S)-2-Methyl-10-*p*-toluenesulfonyloxy-1,6-dioxaspiro[4.5]decane* ***(16).*** Compound **15** (260 mg, 1.5 mmol) was treated in cooled pyridine (3 ml) with *p*-toluenesulfonyl chloride (560 mg, 2.9 mmol), the mixture was kept at room temperature for 12 hr. TLC (ether–hexane, 1 : 1) then showed the presence of a faster-running product. The reaction mixture was concentrated, dissolved in dichloromethane (10 ml), washed with aqueous 5% hydrochloric acid, water, saturated sodium hydrogen carbonate solution, water, and concentrated again to a residue that was chromatographed (ether–hexane, 1 : 1) to yield syrupy **16** (480 mg, quantitative), $[\alpha]_D = -48°$ (*c* 1); ν^{film} 2973 and 2884 (C—H), 1601 (tosylate), 1368, 1310, 1177, 1099, 1066, 990, 959, 905, 855, and 835 (sulfonate and C—O—C), and 669 cm^{-1} (aromatic). ^1H NMR: δ 7.78 and 7.33 (2 d, 2 H, *J* 8.3, Ts), 4.45 (dd, 1 H, $J_{9a,10}$ 11.7, $J_{9e,10}$ 4.9 Hz, H-10), 4.20 (m, 1 H, H-2), 3.75 (dt, 1 H, $J_{7a,7e} = J_{7a,8a} = 11.4$, $J_{7a,8e}$ 3.5 Hz, H-7a), 3.45 (ddt, 1 H, $J_{7e,8a}$ 4.7, $J_{7e,8e} = J_{7e,9e} = 1.5$ Hz, H-7e), 2.43 (s, 3 H, MePh), 2.05–1.85, 1.78–1.53, and 1.43–1.31 (3 m, 8 H, H-3,3′,4,4′,8,8′,9,9′), and 1.19 (d, 3 H, $J_{Me,2}$ 6 Hz, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 327 (0.11, $M^+ + 1$), 311 (0.10, M^+-Me), 271 (0.19), 190 (0.17), 172 (1.38), 171 (9.84), 155 (6.61), 139 (2.25), 113 (4.37), 111 (3.60), 110 (8.24), 101 (17.34, $C_5H_9O_2^+$), 91 (42.11, $C_7H_7^+$), 83 (11.35), 71 (100), 65 (21.12), 55 (32.93), 43 (44.53, Ac^+), and 41 (28.74).

(2S,5R,10S)-2-Methyl-10-[(thiomethyl)thiocarbonyloxy]-1,6-dioxaspiro-[4.5]decane ***(17).*** To an ice-water-cooled solution of **15** (290 mg, 1.7 mmol) in anhydrous THF (10 ml), imidazole (20 mg), sodium hydride (102 mg, 3.4 mmol) (80% oil dispersion), carbon disulfide (0.3 ml, 4.5 mmol), and methyl iodide (0.3 ml, 4.7 mmol) were added, under argon. The stirring was continued

for 30 min. TLC (ether–hexane, 1:1) revealed the presence of a faster-running compound. The excess of hydride was destroyed by cautious addition of ether saturated with water and water. The organic phase was separated and the aqueous phase extracted with ether. The combined extracts were washed with brine and water, concentrated, and the residue chromatographed (ether–hexane, 1:4) to afford syrupy **17** (410 mg, 92%), $[\alpha]_D = -82°$ (*c* 0.6); ν^{film} 2971 and 2951 (C—H), 1225 (C=S), 1063, and 958 cm^{-1}. ^1H NMR: δ 5.65 (dd, 1 H, $J_{9a,10}$ 11.3, $J_{9e,10}$ 5 Hz, H-10), 4.28 (sex, 1 H, $J_{2,Me} = J_{2,3} = J_{2,3'} = 6.2$ Hz, H-2), 3.86 (dt, 1 H, $J_{7a,7e} = J_{7a,8a} = 11.4$, $J_{7a,8e}$ 3 Hz, H-7a), 3.55 (ddt, 1 H, $J_{7e,8a}$ 4.7, $J_{7e,8e} = J_{7e,9e} = 1.6$ Hz, H-7e), 2.55 (s, 3 H, SMe), 2.14–1.60 and 1.45–1.38 (2 m, 8 H, relative intensity 7:1, H-3,3′,4,4′,8,8′,9,9′), and 1.26 (d, 3 H, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 264 (0.5, $M^+ + 2$), 263 (0.4, $M^+ + 1$), 262 (2.3, M^+), 229 (0.8, M^+-SH), 183 (0.3), 155 (43.4, M^+-OCSSMe), 154 (18.6, M^+-OCS-MeSH), 101 (9.8, $C_5H_9O_2^+$), 97 (17.0, $C_6H_9O^+$), 91 (38.8, $CSSMe^+$), 71 (100), 55 (30.9), and 43 (26.2 (Ac^+).

(2S,5R)-2-Methyl-1,6-dioxaspiro[4.5]decane ***(1).*** To a solution of **17** (390 mg, 1.48 mmol) in dry toluene (1 ml), azobisisobutyronitrile (10 mg), and tri-*n*-butyltin hydride (0.8 ml, 3 mmol) were added under argon. The mixture was refluxed for 30 min. During this time the pale yellow solution became colorless. The mixture was cooled and chromatographed twice (*n*-pentane to *n*-pentane–ether, 5:1) to afford **1** (130 mg, 57%). Retention time 2.40 min (D), $[\alpha]_D = -81.4°$ (*c* 0.4, *n*-pentane) [lit. (Iwata et al., 1987) $[\alpha]_D = -79.1°$ (*c* 0.392, *n*-pentane)]. ^1H NMR (C_6D_6): δ 4.23 (sex, 1 H, $J_{2,3} = J_{2,3'} = J_{2,Me} = 6.2$ Hz, H-2), 3.95 (dt, 1 H, $J_{7a,7e} = J_{7a,8a} = 11$, $J_{7a,8e}$ 3 Hz, H-7a), 3.57 (ddt, 1 H, $J_{7e,8a}$ 4.7, $J_{7e,8e} = J_{7e,9e} = 1.8$ Hz, H-7e), 2.05–1.14 (m, 10 H, H-3,3′,4,4′,8,8′,9,9′,10,10′), and 1.14 (d, 3 H, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 157 (1, $M^+ + 1$), 156 (6, M^+), 155 (2, M^+-1), 141 (6, M^+-Me), 128 (8, $C_7H_{12}O_2^+$), 112 (18, $C_7H_{12}O^+$), 111 (16, $C_7H_{11}O^+$), 101 (100, $C_5H_9O_2^+$), 100 (32, $C_5H_8O_2^+$), 98 (59, $C_6H_{10}O^+$), 83 (42, $C_6H_{10}O^+$-Me), 56 (22), 55 (34), and 43 (18, Ac^+).

Hydrogenation of ***14a.*** For method a, compound **14a** (680 mg, 2.6 mmol) in anhydrous methanol (10 ml) was hydrogenated at 2 atm over PdO (400 mg) for 15 hr. TLC (ether–hexane, 1:1) showed the absence of **14a** and the presence of a complex mixture. The catalyst was filtered off, and the filtrate concentrated to a residue that was chromatographed (ether–hexane, 1:2) to yield (2*R*,5*R*,10*S*)-10-hydroxy-2-methyl-1,6-dioxaspiro[4.5]decane (**15a,** 90 mg), retention time 2.66 min (C), $[\alpha]_D = -105°$ (*c* 1.2); ν^{film} 3451 (OH), 2970, 2946, and 2879 (C—H), 1444, 1213, 1159, 1118, 1094, 1066, 1031, 984, 961, 890, and 875 cm^{-1} (C—O—C). ^1H NMR (80 MHz): δ 4.46–4.00 (m, 1 H, H-2), 3.92–3.30 (m, 3 H, H-7,7′,10), 2.47–1.42 (m, 8 H, H-3,3′,4,4′,8,8′,9,9′), 1.70 (d, 1 H, $J_{HO,10}$ 11 Hz, HO-10), and 1.25 (d, 3 H, $J_{Me,2}$ 6 Hz, Me-2).

A second fraction was identified as (2*R*,5*S*,10*S*)-10-hydroxy-2-methyl-1,6-

dioxaspiro[4.5]decane (**18,** 46 mg), retention time 3.24 min (C), $[\alpha]_D = +51°$ (*c* 1.2); ν^{film} 3461 (OH), 2968 and 2882 (C—H), 1458, 1381, 1362, 1214, 1197, 1177, 1090, 1050, 1000, 953, 905, and 876 cm^{-1} (C—O—C). ^1H NMR (80 MHz): δ 5.26 (m, 1 H, H-2), 4.00–3.25 (m, 3 H, H-7,7',10), 2.25–1.00 (m, 8 H, H-3,3',4,4',8,8',9,9'), and 1.23 (d, 3 H, $J_{Me,2}$ 6 Hz, Me-2).

By method b, compound **14a** (1.1g, 4.2 mmol) in anhydrous methanol (40 ml) and triethylamine (0.1 ml) was hydrogenated at 4 atm over Raney-nickel (1 g) for 48 hr. TLC (ether–hexane, 1:1), showed the presence of a slower-running compound. The catalyst was filtered off, washed, with methanol, and the combined filtrate and washing concentrated to a residue that was chromatographed (ether–hexane, 1:2) to afford **15a** (370 mg, 52%).

*(2R,5R,10S)-2-Methyl-10-*p*-toluenesulfonyloxy-1,6-dioxaspiro[4.5]decane* ***(16a).*** Treatment of **15a** (80 mg, 0.47 mmol) in cooled dry pyridine (1.5 ml) with *p*-toluenesulfonyl chloride (190 mg, 1 mmol) and work-up of the reaction mixture as above gave syrupy **16a** (97 mg, quantitative), $[\alpha]_D = -60°$ (*c* 1.2); ν^{film} 2971, and 2885 (C—H), 1600 (TsO), 1367, 1190, 1177, 1099, 1066, 985, 904, 857, 836 (C—S and C—O—C), and 669 cm^{-1} (aromatic). ^1H NMR: δ 7.78 and 7.33 (2 d, 4 H, *J* 8.3 Hz, Ts), 4.46 (dd, 1 H, $J_{9e,10}$ 5, $J_{9a,10}$ 11.7 Hz, H-10), 4.28–4.16 (m, 1 H, H-2), 3.79 (dt, 1 H, $J_{7a,7e} = J_{7a,8a} = 11.5$, $J_{7a,8e}$ 3.6 Hz, H-7a), 3.45 (m, 1 H, H-7e), 2.43 (s, 3 H, Me tosylate), 2.08–1.79 and 1.76–1.52 (2 m, 8 H, H-3,3',4,4',8,8',9,9'), and 1.26 (d, 3 H, $J_{Me,2}$ 6.1 Hz, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 327 (0.06, $M^+ + 1$), 311 (0.06, M^+-Me), 271 (0.1), 172 (1.03), 171 (5.85), 155 (4.07), 111 (2.77), 110 (3.26), 101 (8.76, $C_5H_9O_2^+$), 91 (22.27), 71 (100), 55 (21.71), and 43 (37.33, Ac^+).

(2R,5R,10S)-2-Methyl-10-[(thiomethyl)thiocarbonyloxy]-1,6-dioxaspiro-[4.5]decane ***(17a).*** Compound **15a** (370 mg, 2.15 mmol) was treated in anhydrous THF (10 ml) with imidazole (10 mg), sodium hydride (130 mg, 4.5 mmol) (80% oil dispersion), carbon disulfide (0.4 ml, 7 mmol), and methyl iodide (0.2 ml, 3.5 mmol) as above to afford syrupy **17a** (436 mg, 77%), $[\alpha]_D = -81°$ (*c* 1.4): ν^{film} 2990, 2975, and 2895 (C—H), 1235 (C=S), 1070, and 990 cm^{-1} (C—O—C). ^1H NMR: δ 5.64 (dd, 1 H, $J_{9a,10}$ 11.2, $J_{9e,10}$ 5 Hz, H-10), 4.34–4.22 (m, 1 H, H-2), 3.89 (dt, 1 H, $J_{7a,7e} = J_{7a,8a} = 11.3$, $J_{7a,8e}$ 3 Hz, H-7a), 3.54 (ddt, 1 H, $J_{7e,8a}$ 4.7, $J_{7e,8e} = J_{7e,9e} = 1.6$ Hz, H-7e), 2.54 (s, 3 H, SMe), 2.14–2.04 and 2.02–1.61 (2 m, 8 H, relative intensity 1:7, H-3,3',4,4',8,8',9,9'), and 1.32 (d, 3 H, $J_{Me,2}$ 6 Hz, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 264 (0.2, $M^+ + 2$), 263 (0.3, $M^+ + 1$), 262 (1.9, M^+), 229 (0.6, M^+-SH), 183 (1.5), 155 (39.1, M^+-OCSSMe), 154 (21.8, M^+-OCS-MeSH), 101 (9.5, $C_5H_9O_2^+$), 97 (13.9, $C_6H_9O^+$), 91 (42.9, $CSSMe^+$), 71 (100), 55 (27.2), and 43 (24.3, Ac^+).

(2R,5R)-2-Methyl-1,6-dioxaspiro[4.5]decane ***(2).*** To a solution of **17a** (397 mg, 1.5 mmol) in dry toluene (1 ml), azobisisobutyronitrile (10 mg), and tri-*n*-

butyltin hydride (0.8 ml, 3 mmol) was added under argon. The mixture was refluxed for 15 min. TLC (ether–hexane, 1:3) showed a slightly slower-running product. The mixture was cooled and chromatographed (*n*-pentane to *n*-pentane–ether, 10:1) to afford **2** (168 mg, 78%), retention time 2.72 min (D), $[\alpha]_D$ = −98° (*c* 0.3, *n*-pentane) [lit. (Mori et al., 1985) $[\alpha]_D$ = −86° (*c* 0.32, *n*-pentane); lit. (Iwata et al., 1987) $[\alpha]_D$ = −83.4° (*c* 0.728, *n*-pentane)]. ^{1}H NMR (C_6D_6): δ 4.16–4.04 (m, 1 H, H-2), 4.02 (dt, 1 H, $J_{7a,7e} = J_{7a,8a}$ = 11, $J_{7a,8e}$ 4 Hz, H-7a), 3.58 (ddt, 1 H, $J_{7e,8e} = J_{7e,9e}$ = 1.8, $J_{7e,8a}$ 4.7 Hz, H-7e), 2.04–1.14 (m, 10 H, H-3,3′,4,4′,8,8′,9,9′,10,10′), and 1.28 (d, 3 H, $J_{Me,2}$ 6.2 Hz, Me-2). For the ^{13}C NMR, see Table 2. Mass spectrum: *m/z* 157 (0.9, M^++1), 156 (7.1, M^+), 155 (1.3, M^+-1), 141 (5.8, M^+-Me), 128 (6.5, $C_7H_{12}O_2^+$), 112 (13.1, $C_7H_{12}O^+$), 111 (11.8, $C_7H_{11}O^+$), 101 (100, $C_5H_9O_2^+$), 100 (31.7, $C_5H_8O_2^+$), 98 (47.1, $C_6H_{10}O^+$), 83 (37.4, $C_6H_{10}O^+$-Me), 56 (20.8), 55 (35.2), and 43 (22.0, Ac^+).

RESULTS AND DISCUSSION

Reaction of 2,3:4,5-di-*O*-isopropylidene-β-D-*arabino*-hexaldo-2-ulo-2,6-pyranose **(3)** (Izquierdo and Plaza, 1990) with acetylmethylenetriphenylphosphorane in dry dichloromethane gave mainly (*E*)-1,3,4-trideoxy-5,6:7,8-di-*O*-isopropylidene-β-D-*arabino*-non-3-ene-2,5-diulo-5,9-pyranose **(5,** 92%) and a small amount of the *Z* isomer **(4,** 4%). When the reaction was performed in methanol the *E*/*Z* ratio was 4:1. These findings are in agreement with those previously reported (Izquierdo and Plaza, 1988; Aamlid et al., 1990), where the increase of the solvent polarity favors the formation of the *Z* isomer. The configuration at the double bond in **4** and **5** was established on the basis of the $J_{3,4}$ values, 13 and 16 Hz, respectively.

Catalytic hydrogenation of **5** over palladium-on-charcoal afforded the saturated ketone **6.** In order to increase the stereoselectivity in the reduction of the carbonyl group in **6,** different procedures were carried out. Thus, hydrogenation of **5,** or a mixture of **4** and **5,** over Raney-nickel in methanol (method a), caused the reduction of the alkene and carbonyl functions to yield an unresolvable mixture of epimers at C-2 **(7).** Reduction of **6** with sodium borohydride (method b) or L-selectride (method c) gave also **7.** Although a high stereoselectivity was not found in any case, the ^{13}C NMR data showed that the ratio of epimers in **7,** obtained by method a, was reversed compared to that obtained by method c.

Treatment of **7** with trifluororacetic acid hydrolyzed the isopropylidene groups and promoted intramolecular glycosidation to give the spiroacetal **8** as an unresolvable mixture of epimers at C-2. The acetonation of **8** allowed its resolution as (2*S*,5*R*,8*R*,9*R*,10*S*)- **(9)** and (2*R*,5*R*,8*R*,9*R*,10*S*)-10-hydroxy-8,9-isopropylidenedioxy-2-methyl-1,6-dioxaspiro[4.5]decane **(10)** in an ≈1:1 ratio.

The configuration at C-2 in **9** and **10** was assigned on the basis of their NMR data. Thus, the methyl group at C-2 in **10** (see Methods and Materials, and Table 2) suffers a deshielding effect with respect to that in **9,** because of the its *Z* configuration. These results are in accordance to those previously reported (Francke et al., 1980; Mori and Ikunaka, 1984) for analogous compounds. In addition, the configuration at the spiro center in both compounds reflects the disposition of substituents in the pyranose ring (Perron and Albizati, 1989) and the anomeric effect (Deslongchamps et al., 1981).

Dideoxygenation at C-8,9 in **9** and **10** was carried out by protection of the hydroxyl group at C-10 as benzyl ether (**11** and **11a,** respectively) and hydrolysis of the isopropylidene group to give the corresponding diols (**12** and **12a**), which were subjected to Corey and Winter's (1963) elimination to produce the olefins **14** and **14a,** respectively.

Hydrogenation of **14a** over palladium oxide gave a complex mixture from which (2*R*,5*R*,10*S*)-10-hydroxy-2-methyl-1,6-dioxaspiro[4.5]decane **(15a)** and its (2*R*,5*S*,10*S*) epimer **(18)** could be isolated. When hydrogenation of **14** and **14a** was performed over Raney-nickel in the presence of triethylamine, no epimerization at C-5 occurred, and only (2*S*,5*R*,10*S*)-10-hydroxy-2-methyl-1,6-dioxaspiro[4.5]decane **(15)** and **15a** were obtained, respectively.

Attempts to transform **15** and **15a** into the required spiroacetals **1** and **2,** via elimination reactions of the corresponding *p*-toluenesulfonate esters (**16** and **16a**) on alumina (Redlich and Francke, 1984) and subsequent hydrogenation of the resulting olefins failed at the elimination stage, probably due to the equatorial disposition of the *p*-toluenesulfonyloxy group in both compounds (Aamlid et al., 1987). This purpose was finally achieved by application of Barton and McCombie's (1975) method to **15** and **15a,** through the corresponding 10-[(thiomethyl)thiocarbonyloxy] derivatives **17** and **17a** by reduction with tri-*n*-butyltin hydride. Spiroacetals **1** and **2,** obtained by this procedure, showed physical and spectroscopic data in agreement with those previously reported (Francke et al., 1979; Mori et al., 1985; Iwata et al., 1987).

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.

AAMLID, K.H., HOUGH, L., and RICHARDSON, A.C. 1990. Synthesis of 1-deoxy-6-epicastanospermine and 1-deoxy-6,8a-diepicastanospermine. *Carbohydr. Res.* 202:117–129.

BARTON, D.H.R., and MCCOMBIE, S.W. 1975. A new method for the deoxygenation of secondary alcohols. *J. Chem. Soc. Perkin. Trans. I* 1975:1574–1585.

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.

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.

DOHERTY, A.M., LEY, S.V., LYGO, B., and WILLIAMS, D.J. 1984. Synthesis of spiroacetals using organoselenium-mediated cyclisation reactions. X-Ray molecular structure of (2*S*,8*R*)-8-methyl-2-phenyl-1,7-dioxaspiro[5.5]undecan-4(*R*)-ol. *J. Chem. Soc. Perkin Trans. I* 1984:1371–1377.

FRANCKE, W., HINDORF, G., and REITH, W. 1978. Methyl-1,6-dioxaspiro[4.5]decanes as odors of *Paravespula vulgaris* (L.). *Angew. Chem. Int. Ed. Engl.* 17:862.

FRANCKE, W., HINDORF, G., and REITH, W. 1979. Mass spectrometry fragmentation of alkyl-1,6-dioxaspiro[4.5]decanes. *Naturwissenschaften* 66:619–620.

FRANCKE, W., REITH, W., and SINNWELL, V. 1980. Bestimmung der relativen Konfiguration bei Spiroacetalen durch ^{1}H- und ^{13}C-NMR-spektroskopie. *Chem. Ber.* 113:2686–2693.

HINTZER, K., WEBER, R., and SCHURIG, V. 1981. Synthesis of optically active 2*S*-, and 7*S*-methyl-1,6-dioxaspiro[4.5]decane, the pheromone components of Paravespula Vulgaris (L.), from *S*-ethyl lactate. *Tetrahedron Lett.* 22:55–58.

HUNGERBÜHLER, E., NAEF, R., WASMUTH, D., and SEEBACH, D. 1980. Synthese optisch aktiver 2-Methyl- und 2-äthyl-1,6-dioxaspiro[4.4]nonan- und -[4.5]decan-pheromone aus einem gemeinsamen chiralen Vorläufer. *Helv. Chim. Acta* 63:1960–1970.

IWATA, C., MORITANI, Y., SUGIYAMA, K., FUJITA, M., and IMANISHI, T. 1987. Stereoselective synthesis of all isomers of 2-methyl-1,6-dioxaspiro[4.5]decane (insect pheromone) using a chiral sulfoxide auxiliary. *Tetrahedron Lett.* 28:2255–2258.

IZQUIERDO, I., and PLAZA, M.T. 1988. Synthesis of 1,2-dideoxy-3-heptulose derivatives. *Carbohydr. Res.* 173:41–52.

IZQUIERDO, I., and PLAZA, M.T. 1990. An enantiospecific synthesis of (−)-talaromycins A and B from D-fructose. *Carbohydr. Res.* 205:293–304.

IZQUIERDO, I., PLAZA, M.T., RICHARDSON, A.C., and AAMLID, K.H. 1993. Enantiospecific synthesis of (*R*)-1,6-dioxaspiro[4.5]decane from a derivative of D-fructose. *Carbohydr. Res.* In press.

LEY, S.V., and LYGO, B. 1982. Synthesis of methyl-1,6-dioxaspiro[4.5]decanes using organoselenium mediated cyclization reactions. *Tetrahedron Lett.* 23:4625–4628.

MORI, K., and IKUNAKA, M. 1984. Synthesis of all of the four energetically possible stereoisomers of 7-ethyl-2-methyl-1,6-dioxaspiro[4.5]decane. *Tetrahedron* 40:3471–3479.

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.

PERRON, F., and ALBIZATI, K.F. 1989. Chemistry of spiroketals. *Chem. Rev.* 89:1617–1661.

PHILLIPS, C., JACOBSON, R., ABRAHAMS, B., WILLIAMS, H.J., and SMITH, L.R. 1980. Useful route to 1,6-dioxaspiro[4.4]nonane and 1,6-dioxaspiro[4.5]decane derivatives. *J. Org. Chem.* 45:1920–1924.

REDLICH, H., and FRANCKE, W. 1984. Synthesis of enantiomerically pure 1,7-dioxaspiro[5.5]undecanes, pheromone components of olive fly (*Dacus oleae*). *Angew. Chem. Int. Ed. Engl.* 23:519–520.

ROSINI, G., BALLINI, R., and MAROTTA, E. 1989. Functionalized nitroalkanes in synthesis of 1,6-dioxaspiro[4.5]decane components of Paravespula Vulgaris pheromone. *Tetrahedron* 45:5935–5942.

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Book Review

Insect Chemical Ecology. An Evolutionary Approach. Bernard D. Roitberg and Murray B. Isman (eds.). New York and London: Chapman and Hall, 1992. 359 pp. ISBN 0-412-01881-0.

The editors' aim was "to provide a comprehensive treatment of insect chemical ecology from an evolutionary perspective" in which causal and functional aspects are in balance. The contributors have reached that important goal in every respect. Their studies will strongly stimulate further attempts in this field of research.

The first part of the book contains five chapters dealing with general questions of the evolution of intra- and interspecific interactions among organisms mediated by chemicals.

In Chapter 1, B.D. Roitberg emphasizes the necessity of an integrated approach to questions of chemical ecology, namely function, causation, phylogeny, and ontogeny. With the example of marking pheromones, especially with the "self-marking" by parasitoids, the author illustrates the usefulness of such an approach.

In Chapter 2, M.D. Rausher provides a thorough analysis of available data concerning the evolution of insect–plant interactions. Statistical techniques for estimating the magnitude of variation and selection are critically discussed. It is shown that the methods used by animal and plant breeders for predicting evolutionary changes cannot be used for the same predictions in natural populations. After defining the terms "resistance," "defense," and "tolerance" and their relation to plant fitness, the author discusses the theories of coevolution and the evolution of diet breadth. He unequivocally shows how little concrete evidence is available to support widely accepted hypotheses.

M. Berenbaum and D. Seigler (Chapter 3) delineate the presumably evolutionary processes that have resulted in extant secondary metabolism in both plants and insects. Phylogenetic, energetic, and toxicological constraints on the production of secondary metabolites, as well as the role of biotic factors that might select for production of such substances, are discussed in detail. While Rausher (Chapter 2) found no strong evidence for the assumption of a generally occurring reciprocal adaptive evolution between plants and herbivorous insects, Berenbaum and Seigler implicitly regard it as fully proved.

A comprehensive survey of information-conveying chemicals is given by M. Dicke and M.W. Sabelis (Chapter 4). The terminology they use includes a

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number of anthropomorphic terms that turn out to be very useful for expressing complex interactions mediated by "infochemicals." Energetic and evolutionary aspects of information transfer are presented by an example of the tritrophic system consisting of plants, spider mites, and predatory mites.

The physiological basis of the sensory perception of chemicals and the physiological responses to toxins and nutrients are discussed by M.B. Isman (Chapter 5) with special regard to the presumably neural and biochemical targets upon which selection might act. The author emphasizes the absolute necessity of a much broader knowledge of insect physiology and genetics before predictions can be made for the evolutionary responses of insects to semiochemicals.

The second part of the book is a collection of studies on specific problems within insect chemical ecology viewed from an evolutionary perspective.

Evolutionary processes resulting in the insects' resistance to plant toxins are discussed by M.A. Caprio and B.E. Tabashnik (Chapter 6). The authors focus on how new alleles that confer resistance to host-plant toxins are maintained and spread within insect populations. The molecular basis of changes within the insect genome that enhance resistance is dealt with in detail. Although Rausher (Chapter 2) logically distinguishes between plant resistance and plant defense, Caprio and Tabashnik use these terms as synonyms.

M.D. Bowers addresses the problem of the evolution of chemical defense in insects (Chapter 7). The phylogenetic approach shows that unpalatability to predators has evolved independently several times during insect evolution. With a mechanistic approach, the physiological and biochemical backgrounds of the evolution of unpalatability are examined. The evolutionary relationship between sequestration and de novo synthesis of defensive chemicals and the role of kin vs. individual selection are discussed. The necessity of measuring costs of chemical defense is emphasized.

Behavioral plasticity due to motivation and learning is discussed by J. Jaenike and D.P. Papaj (Chapter 8). A computer model based on the assumption of motivation and learning in ovipositing females shows that learning may amplify genetic differences among individuals in host preferences and thus may facilitate host race formation in sympatry. Even modest effects of learning can substantially affect host use.

In a concise study, P.L. Phelan addresses the problem of what processes govern the evolution of mate-signaling systems in insects (Chapter 9). It is concluded that due to the conflict between sexes in maximizing fitness, the communication system is not under strong reciprocal, but rather an asymmetric, selection. Signal shift might result from stochastic processes, which explains the genetic variation found in the sex pheromones of Lepidoptera. Studies on the genetics of variation in sex pheromone production and responses will help in understanding the processes of speciation.

Chapter 10, by M.L. Winston, deals with the role of semiochemicals in

kin and colony recognition, in the maintenance of recognition-based colony function, and in the evolution of sociality in social insects.

In Chapter 11, J.N. McNeil delineates the possibility of using an evolutionary perspective in the application of pheromones in pest management programs. Understanding of mate choice by females and male–male competition might help the interpretation of trap catch data. It is shown that the continued use of pheromones for mating disruption could result in the evolution of "resistant" populations. More efficient lines of communication between researchers in basic and applied disciplines are needed to achieve real progress in application of pheromones.

The volume is closed by taxonomic and subject indices. Unfortunately, the subject index is very scanty; it does not help one's orientation in the volume. Most entries refer only to one page number, e.g., "pheromone 124," "sex pheromone 175," although three chapters deal with these subjects.

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Book Review

Ökologische Biochemie. 2nd. enlarged edition. Dieter Schlee. Jena: Gustav Fischer Verlag, 1992. DM 138.00 hardcover, 587 pp., 243 figures, 61 tables. ISBN 3-334-60393-8. In German.

Ecology and (bio)chemistry: how can they be related? Biochemistry studies the basic molecular mechanisms of life. Ecology is, ever since Haeckel (1866), the discipline that describes the interaction of organisms with their biotic and abiotic surroundings. Thus, obviously, any biochemical process necessarily plays an ecological role. Ecology is much more than the chemistry of life; it allows us to understand interactions and selection on all organismic levels up to those of populations and biocenoses. Clearly, products of biochemical processes steer or control ecological adaptations and are thus essential elements of the ecological interplay.

Schlee covers major biochemical processes that have ecological relevance, beginning with physical factors such as light and temperature and ending with the complex phenomena of symbioses. This compares, in essence, with the condensed "special ecology" book *Strategies of Biochemical Adaptation*, by P.W. Hochachka and G.N. Somero (1973). Both books intend to teach biochemistry to ecologists. The title of Schlee's book is therefore appropriate, but, unfortunately, the terms are usually now used in a much narrower sense. Textbooks, symposium reports, and journals concerned with (bio)chemical ecology (emphasis on ecology, not biochemistry) have operated for some 20 years with the unwritten conception that only biotic (organismic) and not abiotic (climate, light, temperature, gravity) processes belong under such a definition.

The first 60 pages of the book are an introduction to the "Organismus und Umwelt," and consider ecologically relevant influences, cellular metabolism, microbial adaptation, stress. The second part (200 pages) treats biochemical adaptations to abiotic factors in a somewhat surprising sequence: light = photocontrol of gene expression (phytochrome system, accessory pigments; vision); types of photosynthesis (C3, C4, etc., plants); anaerobiosis; air pollution; salinity; food shortage; and wound stress. In the remainder of the book, allelochemical interactions are considered (secondary metabolism, interactions between all kinds of organisms, ecobiochemical relations in connection with aggregations, and social relations).

The latter part of the book is of particular interest for chemical ecologists, but also demonstrates its major difficulty. Many phenomena and technical terms

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are listed, but often one misses the critical evaluation and ecophysiological explanation. One example: Although the author describes the now generally accepted knowledge that the principal "raison d'être" of secondary plant metabolites is defense against other plants, microbes, and herbivores, he still adheres to the early concept of the late master of plant metabolism, Kurt Mothes, that at least the sequestered nitrogen-containing metabolites but also latex tubes, etc., are the result of "inner excretion" and thus a waste product of the plants. There exists no evidence for this opinion. Indeed, Mothes himself could not even be convinced to the contrary when Jerrold Meinwald and I had coffee with him at the Hamburg biochemical congress in 1976.

Any referee must carefully avoid being too critical of those chapters with which he or she is most familiar. Pheromones have been at the center of my interest for many years. The book treats them quite well and broadly, and even touches on the beginning of ecological understanding as shown for bark beetles and lepidopteran pests. Receptor physiology is barely treated, but in all fairness, the biochemical background is still in its infancy. Pyrrolizidine alkaloids and their fate, from the plants that produce them all the way to pheromones of male Lepidoptera, have been of recent interest to me. They exhibit extremely complex relationships, which so far we only partly understand. Schlee reports these phenomena properly, although his report is somewhat outdated. He has missed several essential publications and does not always make the ideal emphasis, but the references given suffice to lead the interested reader to the essential publications. If an extrapolation from the evaluation of this and several other chapters is permitted, one must applaud Schlee's care in reporting appropriately on an enormous variety of phenomena. Unavoidably, in a book of this dimension, there are errors. Just to mention one: monarch and viceroy butterflies are clearly visual mimics but not chemical mimics, such as bolas spiders which mimic noctuid moth pheromones to catch their prey.

The merit of the book is the great amount of data presented. The author deserves much credit for bringing all this material together. The book's 243 figures are mostly chemical formulae and functional processes. There is nearly no "visual aid" to help the reader realize the effects of the biochemical processing. It is, as always, a matter of taste how one follows the selection of data and their presentation. A book that is a kind of encyclopedia deserves a comprehensive index and careful cross-referencing, and both could have been extended. The literature index is particularly critical. On the cover, 2500 entries are claimed and they fill 100 pages. Critical checking revealed some text author's names missing in the references section and some facts reported in the text go without any reference. Since the literature is quoted separately for each chapter (but not with each chapter), redundancy is unavoidable and the search for a given name is often a tricky task.

Schlee is a professor of plant biochemistry at the University at Halle-

Wittenberg and has authored and coauthored textbooks and laboratory manuals. He wrote both editions of Ökologische Biochemie (the first, 1986, was soon sold out) under the sociopolitical constraints of the East German Republic, where access to the international literature was restricted. This revised and expanded second edition must have been just ready for printing when the state borders opened, too late for this edition. Mastering a huge range of literature by a single author is a formidable task even under optimal conditions with a reliable library system at hand. For this author, the conditions must have been less than ideal. Presumably, his major motive to write books was to help his students who necessarily had even greater problems of access to the international literature than their professor. What are my thoughts about another edition? Ideally, I would like to see more ecophysiological examples or at least a critical updating of the facts presented.

The present book demonstrates in a striking way the problems of a truly interdisciplinary approach that is so badly needed in *chemical ecology*. Chemists are interested in compounds and their fates. Schlee is a biochemist and thus emphasizes his science. Physiologists want to understand functional morphology, and ecologists care for interactions on all levels. Chemical and physiological facts are thus only the building stones, not the elements of this branch of ecology. I have yet to see a comprehensive book on chemical ecology.

In summary, the book is a rich reference source of biochemical phenomena that are tentative or even obvious factors in ecological relations. It is not, however, only concerned with "chemical ecology" in the sense of this journal and does not read like a "wonderful little book" as was said of Harborne's essay, "Introduction to Ecological Biochemistry" (1988). Schlee's text is clear, probably also for a foreigner, as I found only a few of the ill-famed German sentences that can fill a paragraph. Beginners in this branch of ecological science might have difficulties with the overwhelming amount of complex facts, yet the academic teacher and researcher will profit from this presentation of biological interactions.

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PHENOLIC ACIDS AFFECT PHOTOSYNTHESIS AND PROTEIN SYNTHESIS BY ISOLATED LEAF CELLS OF VELVET-LEAF[1]

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Abstract—The effects of *p*-coumaric, ferulic, chlorogenic, and vanillic acids on photosynthesis and protein synthesis by isolated leaf cells of velvetleaf (*Abutilon theophrasti* Medik) were investigated. Photosynthesis and protein synthesis were measured in cell suspensions by the incorporation of $^{14}CO_2$ and [^{14}C]leucine, respectively. None of the tested phenolic acids except vanillic reduced photosynthesis by more than 50% at the highest concentration and 30 min of incubation. At 100 μM concentrations and 60-min incubation periods, *p*-coumaric, ferulic, chlorogenic, and vanillic acids inhibited photosynthesis by 33, 37, 57, and 65%, respectively. Ferulic acid was the most inhibitory to protein synthesis and reduced the incorporation of [^{14}C]leucine by 50% at about 1.0 μM after 60 min of incubation. At the highest concentrations tested in this study, vanillic and ferulic acids were inhibitory to photosynthesis and protein synthesis, respectively, whereas chlorogenic and *p*-coumaric acids did not inhibit either physiological process. The maximum inhibition of protein synthesis by chlorogenic acid was 19% and by vanillic acid was 28% at 100 μM concentrations. Chlorogenic, vanillic, and *p*-coumaric acids at 0.1 μM caused increased protein synthesis over the untreated control. Overall, photosynthesis was more sensitive than protein synthesis to the four phenolic acids tested.

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Key Words—Chlorogenic acid, *p*-coumaric acid, ferulic acid, vanillic acid, growth inhibition, velvetleaf, *Abutilon theophrasti*.

INTRODUCTION

Of all the growth-inhibiting compounds released by living or decaying plant parts, phenolics and the associated compounds are the most common (Horsley, 1977; Rice, 1979; Whittaker, 1970). Phenolic compounds are widely found in different plant tissues (Abdul-Wahab and Rice, 1967; Guenzi and McCalla, 1966; Jackson and Willemsen, 1976; Moje, 1966; Wang et al., 1967; Whitehead, 1964) and occur either in a free state or conjugated with sugars as glycosides. Numerous studies have shown that phenolics are allelopathic, as they inhibit seed germination and plant growth (Borner, 1960; Guenzi and McCalla, 1966; Lodhi, 1979a,b). Einhellig et al. (1970) reported that scopoletin, a coumarin derivative, inhibited dry matter production, leaf area expansion, and photosynthesis in tobacco (*Nicotiana tobacum* L.), sunflower (*Helianthus annuus* L.), and redroot pigweed (*Amaranthus retroflexus* L.). Chlorogenic acid also reduced stomatal aperture in tobacco and sunflower (Einhellig and Kuan, 1971), while ferulic acid and *p*-coumaric acid reduced leaf water potential and stomatal diffusive conductance in grain sorghum [*Sorghum bicolor* (L.) Moench.] and soybean [*Glycine max* (L.) Merr.] (Einhellig and Stille, 1979; Einhellig et al., 1985). Patterson (1981) reported that 1000 μM *p*-coumaric, ferulic, and vanillic acids severely reduced the photosynthesis of soybean, whereas 100 μM had no effect. Coumarins and cinnamic acids also suppressed photosynthesis of *Lemna minor* L. at concentrations corresponding to their individual threshold for growth inhibition (Einhellig, 1986).

These studies investigated the impact of phenolics on photosynthesis and other macromolecular processes using whole plants. However, by treating whole plants with phenolic acids and measuring growth responses, a researcher is faced with many inherent limitations and problems. For example, the type of application (root or foliar), the site of uptake, and the pattern of translocation of a given chemical are all variables that could complicate such studies. These limitations encountered in allelopathic research with intact plants can be circumvented by the use of isolated plant cell preparations.

Several researchers (Ashton et al., 1977; Hatzios, 1982; Hatzios and Howe, 1982; Malakonduiah and Fang, 1978) have used plant cells isolated from the leaves of higher plants for determining the mechanism of action of herbicides. In this study, we employed similar techniques to examine the effects of four phenolic acids on stromal-associated CO_2 fixation (Calvin cycle) reactions and protein synthesis. The objectives of this study were to determine the effects of

p-coumaric, ferulic, chlorogenic, and vanillic acid on photosynthesis and protein synthesis of enzymatically isolated leaf cells of velvetleaf.

METHODS AND MATERIALS

Plant Material. Velvetleaf was grown from seed in moist vermiculite in a greenhouse with day and night temperatures of 26 and 21 ± 1°C, respectively. Plants were grown under natural light supplemented with a fluorescent light with 150 $\mu E/m^2/sec$ for two weeks.

Chemicals. The herbicide diuron [*N'*-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea] with 99% purity and the fungicide metalaxyl [*N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-DL-alanine, methyl ester] with 98% purity were used as standard inhibitors for photosynthesis and protein synthesis, respectively. Analytical grade phenolic compounds *p*-coumaric, ferulic acid, chlorogenic, and vanillic acids were then tested. Each compound was prepared by dissolving the appropriate amount in acetone and diluting with distilled water to a final concentration of 0.1, 1.0, 10, and 100 μM in all suspensions. The concentration of acetone in solution was less than 0.5%.

Cell Isolation. Detached mature leaves from velvetleaf plants were rinsed with distilled water, blotted, deveined, and cut into 1 mm × 1 cm strips. Four to five grams of leaf tissue were then vacuum infiltrated with 30 ml of infiltration medium containing 20 mM MES, pH 5.8, 0.1% macerase, 2% pectinase, and 0.3% potassium dextran sulfate (Hatzios, 1982). After vacuum infiltration, the leaf tissue was filtered through a 242-μm mesh nylon net, the filtrate was discarded, and the leaf tissue remaining was transferred to a beaker with 30 ml of a maceration medium containing 20 mM MES, pH 5.8, 0.2% pectinase, 2% cellulase, 0.3% potassium dextran sulfate, and 0.6 M mannitol. The tissue was stirred slowly on a magnetic stirrer for 10 min. The suspension was filtered through the same nylon net and the filtrate discarded. The leaf tissue was transferred to another 30 ml of fresh maceration medium and was stirred for 2 hr. The mesophyll cells released during this period were filtered again through the nylon net and washed three times with 10 ml of incubation medium by centrifugation at 60*g* for 3 min. The incubation medium contained 0.5 M mannitol 5 mM KNO_3, 2 mM $Mg(NO_3)_2$, 1 mM $CaCl_2$, 50 mM HEPES-KOH buffer, at pH 7.8. The cells were resuspended with incubation medium. For chlorophyll determination, three 1-ml samples of the final cell suspension were placed in three 15-ml centrifuge tubes. To each tube, 4 ml of 80% acetone were added and the tubes were stirred using a vortex mixer; then the tubes were kept in the dark for 2 hr. At the end of this time, the tubes were centrifuged at 150*g* for 5 min, and the supernatant fluid was then assayed spectrophotometrically for its chlorophyll content according to the method of Arnon (1949). The chlorophyll

content of cell preparations used in this study was 18–30 μg/chlorophyll/ml photosynthesis assay medium.

Photosynthesis. Two milliliters of the cell preparation suspended in incubation medium were placed into a 25-ml Erlenmeyer flask. To each flask, 0.1 ml containing 0.1 μCi [^{14}C]$NaHCO_3$ and 0.05 ml of diuron or the phenolic acids were added. The flasks were placed on a shaker illuminated with a fluorescent light (145 $\mu E/m^2/sec$) and incubated for 30 and 60 min at 24 $\pm$ 1°C. At the end of the incubation periods, 200 μl of the samples were removed from each flask and placed on a 2.3-cm-diameter filter paper disk (Whatman 3 mm). The disks were acidified with 0.1 ml of 90% formic acid to release any unfixed $^{14}CO_2$ and dried in a hood. Radioactivity was determined by placing the disks in scintillation vials containing 10 ml of scintillation fluid and quantified by liquid scintillation counter (LSC). The counts per minute (cpm) were converted to disintegration per minute (dpm) based on counting efficiency of the counter (>97%). The results were then converted to dpm/mg chlorophyll (chl) and expressed as percent of the untreated control. Data were subjected to analysis of variance and means were separated using LSD (0.05).

Protein Synthesis. Protein synthesis was determined by adding 0.1 μCi of L-[U-^{14}C]leucine (specific activity 290 mCi/mmol), metalaxyl, and the phenolic acids to 2 ml of cell suspension in 25-ml flasks. The flasks were placed on a shaker at 24 $\pm$ 1°C under a fluorescent light (145 $\mu E/m^2/sec$) and incubated for 30 and 60 min. At the end of the incubation periods, a 500-μl sample was collected from each flask and placed in 20-ml vials. To each vial, 2 ml of ice-cold trichloroacetic (TCA) at 10% w/v was added and the vials were left overnight at 4°C in a refrigerator. The TCA-insoluble protein precipitates were then collected by filtering each sample through a 2.1-cm glass fiber filter disk. The disks were then washed successively with 6 ml (3 $\times$ 2 ml) of ice-cold TCA (10% w/v), 6 ml (3 $\times$ 2 ml) of 80% ethanol, 2 ml of acetone, and 4 ml (2 $\times$ 2) of diethyl ether. The disks were then dried, placed into vials with 20 ml of scintillation fluid, and radioassayed.

The results were converted to dpm/mg chl and data were subjected to analysis of variance. There were three replications in each experiment, and all experiments were conducted twice. Although all data were calculated in dpm/pmg of chlorophyll, these values were converted to percentage of control (no chemical present) for uniform graphic presentations of the data.

RESULTS AND DISCUSSION

Photosynthesis. Diuron has been a standard research tool in photosynthesis and was included in this study as a reference. Inhibition of photosynthesis by diuron reached 50% of the untreated control between 0.1 and 1.0 μM after both

30 and 60 min of incubation (Figure 1), which is similar to values reported elsewhere (Fedtke, 1982; Moreland, 1980). The observed responses of the cells to diuron indicate that the cells were physiologically active throughout this study.

None of the tested allelochemicals were as inhibitory as diuron to photosynthesis (Figures 1–5), but the inhibition increased with an increase in the concentration of the phenolic acids at both incubation times. At 100 μM concentrations, *p*-coumaric, ferulic, chlorogenic, and vanillic acids inhibited pho-

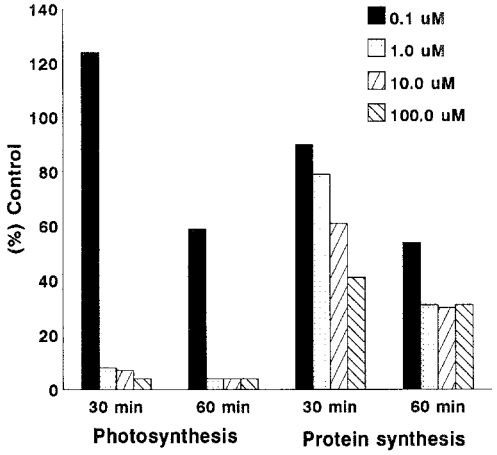

FIG. 1. The effects of diuron on photosynthesis and metalaxyl on protein synthesis.

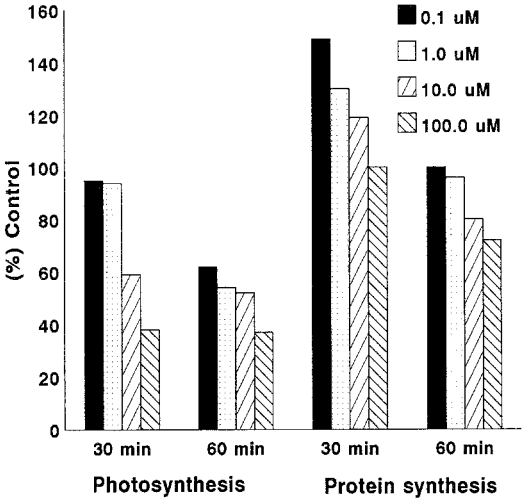

FIG. 2. The effects of coumaric acid on photosynthesis and protein synthesis.

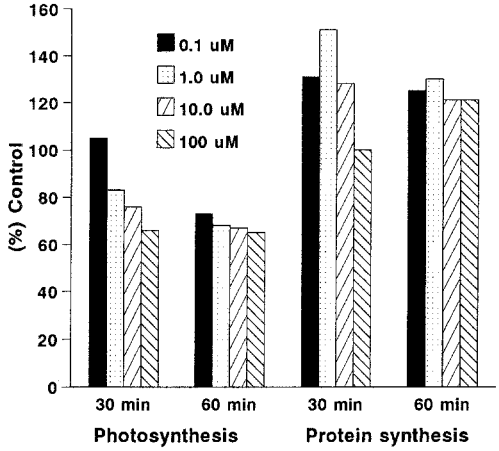

FIG. 3. The effects of ferulic acid on photosynthesis and protein synthesis.

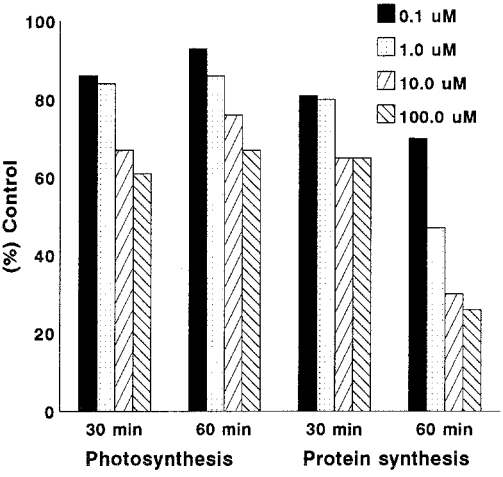

FIG. 4. The effect of chlorogenic acid on photosynthesis and protein synthesis.

tosynthesis by 33, 37, 57, and 65% at 60 min, respectively (Figures 2–5). These four phenolic acids can be compared as to their relative effect on the inhibition of photosynthesis: vanillic > chlorogenic > ferulic > *p*-coumaric acids.

It is not known whether these compounds reach concentrations as high as 100 μM inside mesophyll cells, but bulk soil extractions have indicated soil solution concentrations of 4.9×10^{-5}, 4.2×10^{-5}, and 3.2×10^{-5} M for vanillic, *p*-coumaric, and ferulic acids, respectively (Whitehead, 1964). More-

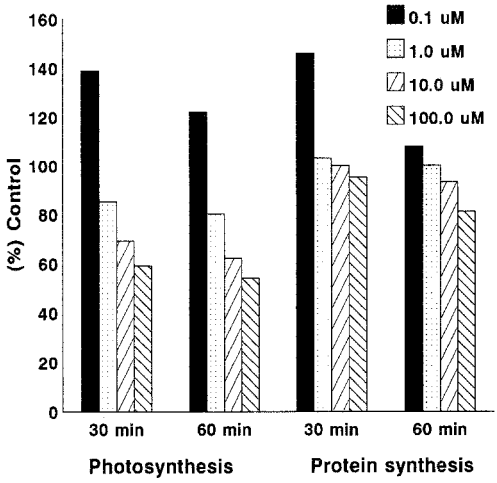

FIG. 5. The effect of vanillic acid on photosynthesis and protein synthesis.

land and Novitzky (1987) reported that several phenolic acids, including vanillic and ferulic acids, inhibited CO_2-dependent oxygen evolution in isolated chloroplasts. However, the I_{50} concentrations ranged between 1 and 10 mM and are much higher than used in this research. Patterson (1981) reported that 1000 μM *p*-coumaric and ferulic acids severely reduced photosynthesis of soybean, whereas 100 μM had no effect. Concentrations above 1000 μM are considered too high to establish a direct effect of a xenobiotic on any physiological process using isolated cells or organelles (Ashton et al., 1977).

Protein Synthesis. The fungicide metalaxyl belongs to the group of chemicals known as acylalanines, and several members of this group are reported to inhibit protein synthesis (Ashton et al., 1977). Metalaxyl was included in this research as a standard inhibition treatment for protein synthesis. Metalaxyl inhibited protein synthesis more at 60 min than at 30 min (Figure 1). At 30 min, there was a progressive reduction in the incorporation of [^{14}C]leucine with an increase in concentration. At 60 min, maximal inhibition occurred at 1.0 μM of metalaxyl, and there was no further reduction at higher concentrations. Protein synthesis was reduced by 50% between 10 and 100 μM at 30 min and between 0.1 and 10 μM after 60 min incubation.

Similar to metalaxyl, the degree of protein inhibition by ferulic and vanillic acids was greater at 60 than 30 min of incubation. This suggests either a delay in induction in absorption or subcellular transport, a delay in the induction of the inhibition, or that the chemicals were inhibiting a metabolic site that supplied an essential factor for protein synthesis rather than affecting it directly. It is not

possible to determine which of these factors is responsible for the delayed inhibition of the chemicals from the data presented.

None of the allelochemicals except ferulic acid inhibited protein synthesis by as much as 50%. Chlorogenic, vanillic, and *p*-coumaric acids at 0.1 μM caused increased incorporation of [^{14}C]leucine above the rate of the untreated cells. The maximum inhibition by chlorogenic acid was 19% and for vanillic acid it was 28%, both at 100 μM concentrations. Ferulic reduced [^{14}C]leucine incorporation by 50% at about the 1.0 μM concentration after 60 min incubation, and this was comparable to the effect of the standard treatment (metalaxyl).

On the whole, photosynthesis was more sensitive than protein synthesis to the allelochemicals tested. Protein synthesis is not considered to be the most sensitive site of action for herbicides (Ashton et al., 1977). Among the phenolics tested, vanillic acid was inhibitory to photosynthesis while ferulic acid reduced the incorporation of [^{14}C]leucine by cells. Chlorogenic and *p*-coumaric acids do not have a direct effect on either photosynthesis or protein synthesis. However, it is important to note that results reported here were for individually applied phenolic acids. Under natural conditions, additive or synergistic inhibitory effects of such allelochemical compounds may become a more determinable event at lower concentrations than the effects of individual compounds. It would be worthwhile if future studies address this by investigating the effects of several allelochemicals alone or in combination on selected plant processes.

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REFERENCES

ABDUL-WAHAB, A.S., and RICE, E.L. 1967. Plant inhibition in old field succession. *Bull. Torrey Bot. Club* 94:486–497.

ARNON, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris* L. *Plant Physiol.* 24:1–7.

ASHTON, F.M., DEVILLIERS, O.T., GLENN, R.K., and DUKE, W.B. 1977. Localization of metabolic sites of action of herbicides. *Pestic. Biochem. Physiol.* 7:122–141.

BORNER, H. 1960. Liberation of organic substances from higher plants and their role in the soil sickness problem. *Bot. Rev.* 26:393–424.

EINHELLIG, F.A. 1986. Mechanisms and modes of action of allelochemicals, pp. 170–188, *in* A.P. Putnam and C.S. Teng (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

EINHELLIG, F.A., and KUAN, L. 1971. Effects of scopoletin and chlorogenic acid on stomatal aperture in tobacco and sunflower. *Bull. Torrey Bot. Club* 98:155–162.

EINHELLIG, F.A., and STILLE, M.L. 1979. Effects of ferulic and *p*-coumaric acids on plant water status. *Abstr. Bot. Soc. Am., Misc. Ser. Publ. No.* 157:40–41.

EINHELLIG, F.A., RICE, E.L., RISSER, P.G., and WENDER, S.H. 1970. Effects of scopoletin on growth, CO_2 exchange rates and concentration of scopoletin, scopolin and chlorogenic acids in tobacco, sunflower and pigweed. *Bull. Torrey Bot. Club* 97:22–23.

EINHELLIG, F.A., MUTH, M.S., and SCHON, M.K. 1985. Effects of allelochemicals on plant-water relationship, pp. 170–195, *in* A.C. Thompson (ed.). The Chemistry of Allelopathy. *American Chemical Society*, Washington, D.C.

FEDTKE, C. 1982. Biochemistry and Physiology of Herbicide Action. Springer-Verlag, New York.

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

HATZIOS, K.K. 1982. Use of isolated leaf cells of *Abutilon theophrasti* to localize the action of two amino-triazinone herbicidal derivatives. *Z. Naturforsch* 370:276–281.

HATZIOS, K.K., and HOWE, C.M. 1982. Influence of the herbicide hexazinone and chlorsulfuron on the metabolism of isolated soybean leaf cells. *Pestic. Biochem. Physiol.* 17:207–214.

HORSLEY, S.B. 1977. Allelopathic interference among plants. II. Physiological modes of action, pp. 93–136, *in* H.E. Wilcos and A.F. Hamer (eds.). Proceedings, Fourth North American Forest Biology Workshop, School of Continuing Education, College of Environmental Science, Suny, Syracuse, New York.

JACKSON, J.R., and WILLEMSEN, R.W. 1976. Allelopathy in the first stages of secondary succession on the piedmont of New Jersey. *Am. J. Bot.* 63:1015–1023.

LODHI, M.A.K. 1979a. Allelopathic potential of *Salsola kali* L. and its possible role in rapid disappearance of weedy stage during revegetation. *J. Chem. Ecol.* 5:429–437.

LODHI, M.A.K. 1979b. Germination and decreased growth of *Kochia scoparia* in relation to its autoalleopathy. *Can. J. Bot.* 57:1083–1088.

MALAKONDUIAH, N., and FANG, S.C. 1978. Influence of monuron on photosystem II and light dependent $^{14}CO_2$ fixation in isolated cells of C_3 and C_4 plants. *Pestic. Biochem. Physiol.* 10:268–274.

MOJE, W. 1966. Organic soil toxins, pp. 533–569, *in* H.D. Chapman (ed.). Diagnostic Criteria for Plants and Soils. University of California Press, Berkeley.

MORELAND, D.E. 1980. Mechanism of action of herbicides. *Annu. Rev. Plant Physiol.* 31:597–638.

MORELAND, D.E., and NOVITZKY, W.P. 1987. Effects of phenolic acids, coumarins and flavonoids on isolated chloroplast and mitochondria, pp. 247–261, *in* G.R. Waller (ed.). Symposium on Allelochemicals: Role in Agriculture, Forestry and Ecology. American Chemical Society, Washington, D.C.

PATTERSON, D.T. 1981. Effects of allelopathic chemicals on growth and physiological responses of soybean (*Glycine max*). *Weed Sci.* 29:53–59.

RICE, E.L. 1979. Allelopathy—an update. *Bot. Rev.* 45:15–109.

WANG, T.S.C., YANG, T.K., and CHUANG, T.T. 1967. Soil phenolic acids as plant growth inhibitors. *Soil Sci.* 103:239–246.

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

WHITTAKER, R.H. 1970. The biochemical ecology of higher plants, pp. 43–70, *in* E. Sondheimer and J.B. Simeone (eds.). Chemical Ecology, Academic Press, New York.

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THE SCARAB BEETLE *Anomala cuprea* UTILIZES THE SEX PHEROMONE OF *Popillia japonica* AS A MINOR COMPONENT

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Abstract—GC-EAD analyses revealed that the scarab beetle *Anomala cuprea*, the cupreous chafer, utilizes, in addition to the previously identified major sex pheromone (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one, a minor component, (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one, which has been previously identified as the sex pheromone of the Japanese beetle. Release of the sex pheromone blend did not significantly differ when collected from feeding or starving female beetles, nor did it differ from volatiles collected in the scoto- and photophase. However, after mating, the amount and the ratio of the two components changed. Field tests revealed that traps baited with the synthetic sex pheromone captured more beetles than traps containing only virgin females. Based on field experiments, 10 mg of a 90:10 blend of the pheromone was suggested as appropriate for monitoring of the cupreous chafer, although the optimal ratio for attractiveness is yet to be established. The occurrence of minor components in the pheromone system of other scarab beetles is also discussed.

Key Words—Japanese beetle, cupreous chafer, GC-EAD, (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one, (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one, sex pheromone, *Anomala cuprea*, *Popillia japonica*, Coleoptera, Scarabaeidae.

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INTRODUCTION

Herbivorous scarab beetles (Coleoptera: Scarabaeidae) include various economically important pests in agriculture, horticulture, and forestry. Both larvae and adults damage crops by feeding on underground and aerial parts, respectively. In Japan, the most important scarab species is *Anomala cuprea* Hope, the cupreous chafer, which is a severe pest of a wide variety of crops, mainly soybean, grape, and strawberry. Due chiefly to the difficulty of controlling this pest with conventional insecticides, there has been an increasing interest in environmentally sound alternative methods of control. Sex pheromones of only a few species of scarab beetles have been identified so far, but they are so successfully applied in the field that they are seriously considered as alternatives in combination with other IPM techniques in order to minimize or even replace hard chemicals. Therefore, identification of these semiochemicals is of utmost importance to investigate their relevance for field application.

Nevertheless, identification of scarab sex pheromones is a hard task, with the lack of uniform and consistent laboratory bioassays being the main obstacle. This difficulty can be exemplified by the fact that sex pheromone evidence has been proven for beetles in the genera *Phyllophaga*, *Lachnosterna*, *Popillia*, *Pachypus*, *Polyphylla*, *Plectis*, *Melolontha*, *Costelytra*, *Rhizotrogus*, *Rhaepaea* (Bestmann and Vostrowsky, 1988), *Cyclocephala* (Potter, 1980), and *Cotinis* (Domek and Johnson, 1987), but until recently sex pheromones of scarabs were identified only in *Costelytra zealandica* (Henzell and Lowe, 1970), *Popillia japonica* (Tumlinson et al., 1977), and *Anomala rufocuprea* (Tamaki et al., 1985).

In order to alleviate the difficulties incurred in isolating sex pheromones by monitoring only with bioassays (either in wind tunnels or in field tests), a GC-EAD technique has been applied (Leal et al., 1992a). That work led to the identification of (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one as a sex pheromone of *Anomala cuprea* (Leal, 1991). Although all the sex pheromones identified from scarab beetles hitherto were single components, we kept trying to identify any possible minor component. Recent findings showed that *Anomala daimiana* utilizes a binary mixture, whose individual components are sex pheromones of other *Anomala* spp. (Leal et al., 1993). This result further stimulated our investigation on the minor components of other scarab species. We describe here the utilization by *Anomala cuprea* of (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one, the sex pheromone of the Japanese beetle, as a minor component. Field evaluations of the binary pheromone blend will also be described.

METHODS AND MATERIALS

Chromatographic and MS Analyses. GC analyses were performed on a Hewlett-Packard 5890 equipped with either an HP-1 column (12 m × 0.2 mm; 0.33 μm) or a DB-wax column (30 m × 0.25 mm; 0.25 μm). The oven was

operated at 50°C for 1 min, programmed at 4°C/min to 180°C, held at this temperature for 1 min, programmed again at 10°C/min to 210°C and held at this temperature for 30 min. Both internal and external standards were used for quantitative analyses. Mass spectra were recorded on a Hewlett-Packard 5891 mass selective detector using either HP-1 or DB-wax columns.

GC-EAD. The responses of *A. cuprea* antennae were recorded with a previously described GC-EAD system (Leal et al., 1992a) utilizing two left antennae of males. The system was tested before and after analysis by the introduction of vapor of the major sex pheromone or air (Burger et al., 1991). This was done by puffing volatiles in a Pasteur pipet into the interface of the GC exit and the EAD glass transfer line.

Rearing of Insects. A. cuprea was raised according to a previously reported method (Hatsukade et al., 1984).

Aeration. The airborne volatiles of either male or female beetles were collected as previously reported (Leal et al., 1992a). In order to investigate the effect of starvation on the release of the sex pheromones, the volatiles of groups of 10–20 virgin female beetles were collected for 24 hr either in the presence of foodstuff (grape leaves) or without it. Aeration was also carried out in both scotophase (1800–0600 hr) and photophase (0900–1700 hr) with virgin females.

Effect of Mating on Pheromone Release. A group of 20 virgin female beetles of nearly the same age (12 days old on average) was placed together with 20 unmated males for three days. Then the mated females were isolated and their pheromones were collected after one, two, and three days.

Syntheses. (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one was synthesized as previously reported (Leal, 1991) and (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one was prepared according to a reported method (Senda and Mori, 1983). Their optical purities were >99%, i.e., >97% ee, as revealed by GC using a Chiraldex GTA column (20 m × 0.25 mm; 0.125 μm) (Leal, 1991).

Field Experiments. Evaluation of the pheromone system in the field was conducted at the National Institute of Sericultural and Entomological Science field in Tsukuba and at Chiba Prefectural Agricultural Experiment Station field in Chiba, Japan in the summer of 1992. The traps used were either funnel traps (Japan Tobacco Inc.), hereafter called JT traps, or water pan traps (Sankei Chemical), hereafter called Sankei traps. The chemicals (10 mg, unless otherwise mentioned), dissolved in hexane, were applied to rubber septa (Daburu Kyappu No. 2, Araki Rubber Co. Ltd., Osaka), and the solvent was allowed to evaporate in a fume hood in an airstream for one day. The baits were then used in the field or stored at −30°C. The traps were set at 1.5 m above the ground at 10-m intervals near Japanese chestnut orchards or sweet potato experimental fields. The experiments were done with at least three replicates, and the positions of the traps were randomized from time to time to avoid any effect of trap location. Virgin females were placed in plastic bottles provided along with

JT traps. Small holes were opened and the individuals were renewed daily. Capture data were transformed to log $(x + 1)$, and differences between means were tested for significance by ANOVA. Throughout this paper, treatments followed by the same letters are not significantly different at a 5% level in the Scheffe F test.

RESULTS AND DISCUSSIONS

Identification of Minor Component. GC-EAD analyses of the airborne volatiles of virgin female beetles fed on grape leaves revealed the occurrence of two EAD-active peaks (Figure 1), which appeared at 40.32 and 46.23 min on a DB-wax capillary column. The peak with the shorter retention time has been previously detected (Leal et al., 1992a) from volatiles of field-captured female beetles. However, the EAD response to the peak at 46.23 min was often missed, most probably due to differences in response of individual antennae rather than due to the origin of samples. In order to improve the system, we used two male antennae in the biodetector to minimize the individual effect. This method gave an improvement in the signal-to-noise ratio, but although the signal of the major component was reproducible, response of the minor component was detected only in less than 8% of the trials with males of different ages and origins. Nevertheless, the signals were generated from volatiles of virgin as well as field-captured female beetles. Two EAD peaks appeared also on a HP-1 capillary column at 24.74 and 30.47 min.

The major EAD-active peak was identified as (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one (Leal, 1991), based mainly on its MS (Figure 2A). The minor peak, on the other hand, gave a similar MS (Figure 2B) displaying the base peak at $m/z = 111$ and the molecular ion peak at $m/z = 224$. This was reasoned to be due to a lactone analog of the major component, having a chain longer by two methylenes. Such a compound, (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one, has been previously identified as the sex pheromone of the Japanese beetle (Tumlinson et al., 1977). In fact, synthetic japonilure gave the same MS and retention times on both columns and was EAD active. Due to the amount of the natural product available, its absolute configuration was not confirmed, but the fact that the Japanese beetle and the cupreous chafer both utilize *R* enantiomers strongly suggested the *R* configuration (later corroborated by bioassay). Regarding the double bond, it was possible to confirm its configuration as *Z* by comparison with synthetic isomers; the *E* isomer appeared at 46.62 min on the DB-wax column.

Sex Pheromone Release. Feeding of the green June beetle, *Cotinis nitida*, has been demonstrated to stimulate aggregation of conspecific males (Domek and Johnson, 1988). Therefore, we examined whether this would also happen

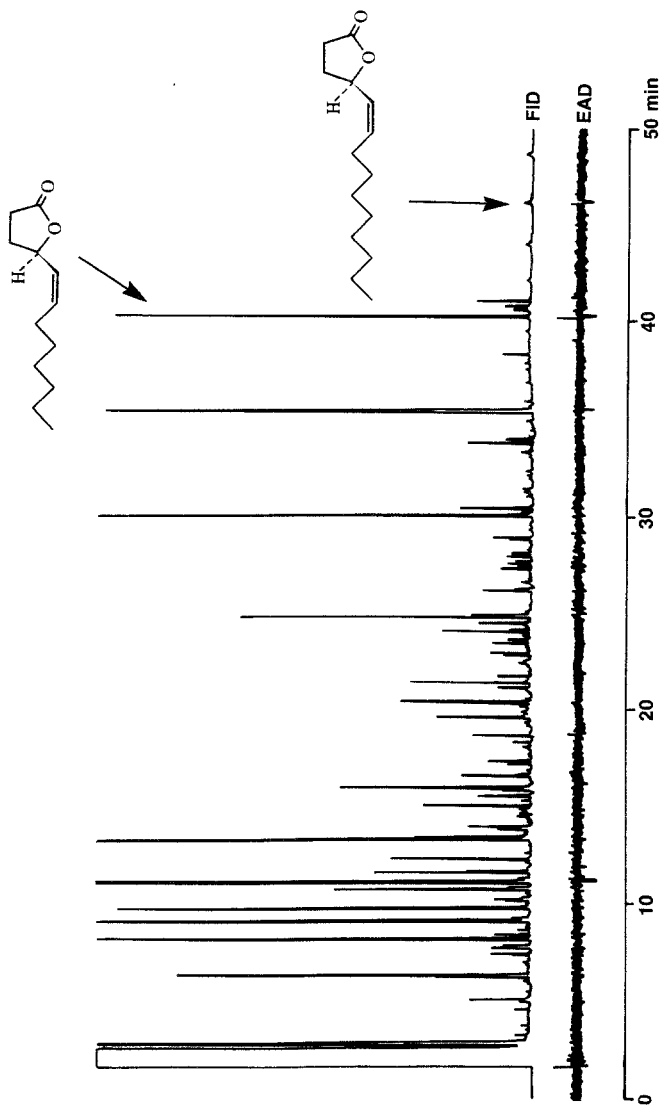

FIG. 1. Coupled GC-EAD response of *A. cuprea* male antennae to the airborne volatiles of virgin female beetles fed on grape leaves.

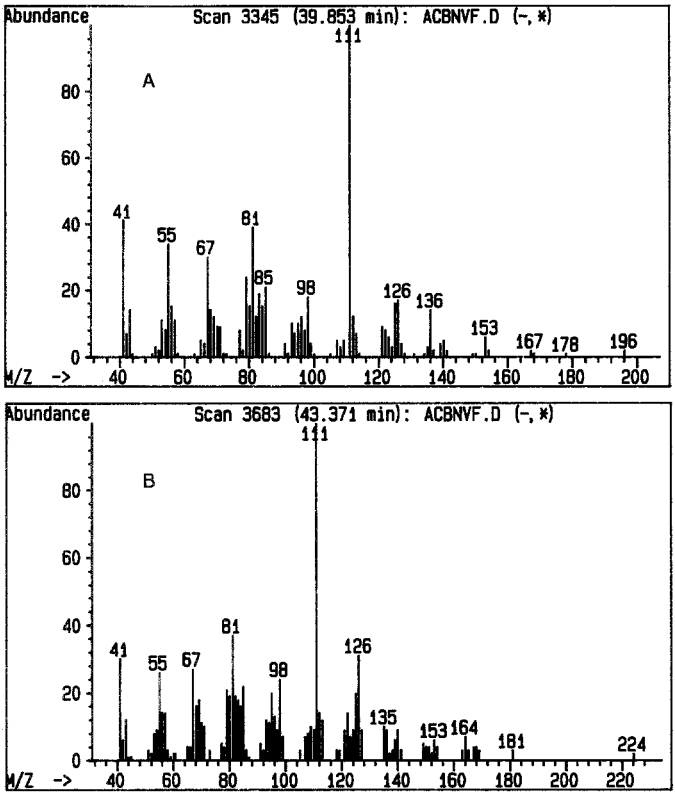

FIG. 2. EI-MS of the two EAD-active peaks. (A) Major peak identified as (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one. (B) Minor component identical to the Japanese beetle sex pheromone.

with sex pheromone release by *A. cuprea*. However, the cupreous chafer fed on grape leaves did not significantly release more pheromone than starving beetles (Figure 3). Furthermore, nearly equal amounts of the semiochemicals were collected when aeration was done in scoto- or photophase (Figure 4). The fact that the total amount of pheromone collected in the latter experiments differs from the one-day long aeration (Figure 3) might be due to the difference in collection times.

One day after mating, the amount of the major pheromone (but not of the minor) significantly decreased. Interestingly, however, as the isolation period increased, the amount of pheromone released increased for the major component and there was a trend to decrease for the minor component (Figure 5). That

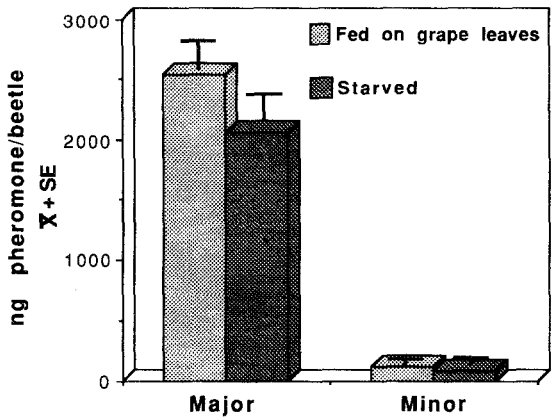

FIG. 3. Amount of (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one (major) and (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one (minor) collected from the headspace of *A. cuprea* virgin females fed on grape leaves and starved females.

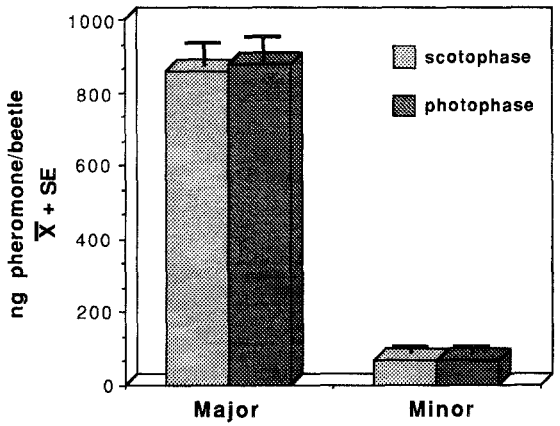

FIG. 4. Amount of sex pheromone released by *A. cuprea* virgin female during scoto- and photophase.

mated females can again produce the major component seems reasonable, since mating occurs several times. However, the reason for a major/minor ratio change remains unclear. One possible explanation would be a selective advantage of virgin females against mated females, giving the former a higher mating probability.

Catches of Cupreous Chafer. Preliminary experiments were carried out with a binary mixture of (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one (92.5%) and (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one (7.5%), which was on aver-

age the ratio detected from virgin females (see Figure 5). A large number of beetles were captured in Tsukuba at the beginning of the flight season (July 2–9). Baited traps caught an average of 52 beetles per trap per day, whereas no beetles were captured in the control traps. Catches in July 8 were limited by the capacity of the trap, i.e., 105 beetles/trap. In Chiba, the synthetic pheromone blend was compared to the catches by two virgin female beetles in tests conducted July 1–6. Pheromone-baited traps caught significantly more beetles than virgin female traps (Figure 6).

A study was carried out to determine the response of the cupreous chafer to different dosages of the 92.5:7.5 pheromone blend. In the experiments of July 18–24, the 100-mg treatment dosage captured significantly more beetles

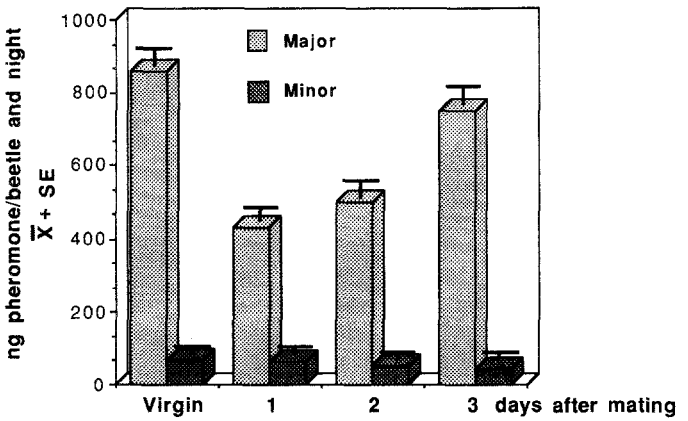

FIG. 5. Effect of mating and isolation on the amount of pheromone released by *A. cuprea*. Major, (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one; minor, (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one.

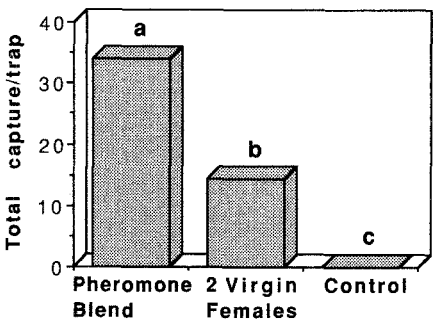

FIG. 6. Response of *A. cuprea* males to traps baited with rubber septa containing the sex pheromone blend, two virgin female beetles, and control.

than the 10-mg dosage (Figure 7). Although later experiments confirmed this trend, the captures at that time were not significantly different, probably due to the decrease of the insect population in the field. Based on these results, as well as economic reasons, the 10-mg dosage was considered to be appropriate for monitoring applications.

In another series of experiments (July 7–12), traps were baited with the pheromone blend in various ratios, in order to establish the optimum for maximum attractiveness to the cupreous chafer. Although traps baited with the pheromone blend containing 10, 15, and 20% of the minor component captured significantly more beetles than those baited with 1 and 5%, there was no significant difference in captures among the 10, 15, or 20% baits (Figure 8). A new series of tests was carried out at the end of the flight season (August 24–September 9), using traps baited with the three ratios of the minor component, namely, 10, 15, and 20%. Again there was no significant difference in captures among the three treatments. This was carried out at two dosages, 10 and 20

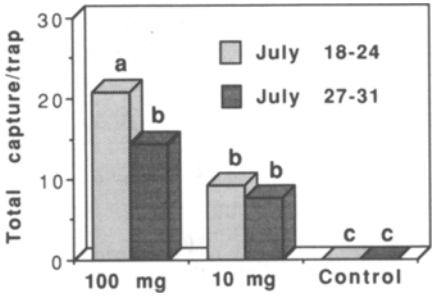

FIG. 7. Effect of the pheromone amount on the capture of *A. cuprea* males.

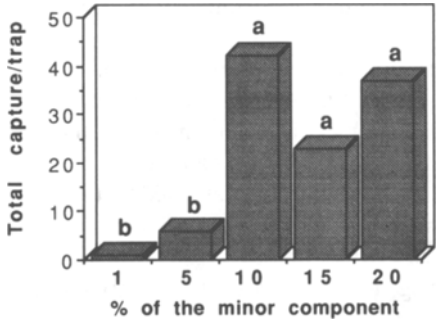

FIG. 8. Response of *A. cuprea* males to traps baited with different ratios of (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one (major)/(*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one (minor).

mg, and there was no significant difference in the catches with these ratios. As demonstrated by the pheromone release experiments, the ratio of the two components undergoes great changes, according to the sexual stage of females. Therefore, it is hard to establish one optimal major/minor ratio. Nevertheless, we propose as a rule of thumb that a mixture of 90% (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one and 10% (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one can be considered a workable ratio for monitoring applications. Further experiments, however, will be carried out during the next season(s) to establish the optimal ratio.

Although it was suggested as early as 1964 (Wright, 1964) that multicomponent pheromones would be widely used because they carry a greater amount of information, only now did we obtain concrete evidence that some scarab beetles utilize binary mixtures. This was confirmed to be the case in two species of the genus *Anomala*, namely, *A. cuprea* and *A. daimiana* (Leal et al., 1993). In both cases, these species utilize a pheromone produced by other scarab beetles as the minor component of their own blend. *Holotrichia parallela* also utilizes a minor component, which was identified as *R*-(−)-linalool, as will be described elsewhere (Leal et al., in preparation). On the other hand, there was no evidence of such minor components in *A. schonfeldti* (Leal et al., 1992c) and *Blitopertha orientalis* (Leal, 1993). The latter utilizes a *Z*/*E* mixture, whose significance has yet to be investigated. Based only on the attractiveness of their single-component pheromone systems in the field, *Costelytra zealandica*, *Popillia japonica*, and *A. rufocuprea* do not seem to possess minor components. However, as already pointed out by other authors (Francke, 1992), satisfactory attractiveness in field tests may mask the occurrence of minor components. Results of field experiments with *H. parallela* stress this point. Traps baited only with the major pheromone, L-isoleucine methyl ester (Leal et al., 1992b), captured 26 male beetles per trap per night, which is a satisfactory figure for most applications. The captures, however, tremendously increased to 86 male beetles per trap per night when the minor component, (*R*)-(−)-linalool, was added (Leal, unpublished data).

Interestingly, results of field applications of the sex pheromones of the Japanese beetle, *P. japonica*, and the soybean beetle, *A. rufocuprea*, simultaneously applied in the same traps demonstrated that the Japanese beetle sex pheromone tremendously inhibits the captures of *A. rufocuprea*, but that the catches of the Japanese beetle were not affected by the soybean beetle pheromone (Ono, personal communication).

In conclusion, *A. cuprea* utilizes a sex pheromone blend that can be utilized in field for monitoring the occurrence of this agricultural pest and, thus, minimize pesticide applications.

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REFERENCES

BESTMANN, H.J., and VOSTROWSKY, O. 1988. Pheromones of the Coleoptera, pp. 95–183, in E.D. Morgan and N.B. Mandava (eds.). CRC Handbook of Natural Pesticides, Vol. IV, Part A. CRC Press, Boca Raton, Florida.

BURGER, B.V., NELL, A.E., and PETERSEN, W.G.B. 1991. Analysis of pheromones: Enrichment of thick film capillary traps and GC detection with a living detector (EAD). *J. High Res.* 14:718–723.

DOMEK, J.M., and JOHNSON, D.T. 1987. Evidence of a sex pheromone in the green June beetle *Cotinis nitida* (Coleoptera: Scarabaeidae). *J. Entomol. Sci.* 22:264–267.

DOMEK, J.M., and JOHNSON, D.T. 1988. Demonstration of semiochemically induced aggregation in the green June beetle, *Cotinis nitida* (L.) (Coleoptera: Scarabaeidae). *Environ. Entomol.* 17:147–149.

FRANCKE, W. 1992. Odour communication: Structural principles in insect–plant and insect–insect interactions. Annual Meeting of the Japan Society for Bioscience, Biotechnology and Agrochemistry, Tokyo, 1992, p. 350.

HATSUKADE, M., YAMADA, K., and LIZUKA, Y. 1984. Rearing method for the production of successive generations of *Anomala cuprea* Hope (Coleoptera: Scarabaeidae). *Jpn. J. Entomol. Zool.* 28:14–19 (in Japanese with an English abstract).

HENZELL, R.F., and LOWE, M.D. 1970. Sex attractant of the grass grub beetle. *Science* 168:1005–1006.

LEAL, W.S. 1991. (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one, the sex pheromone of the scarab beetle *Anomala cuprea*. *Naturwissenschaften* 78:521–523.

LEAL, W.S. 1993. (*Z*)- and (*E*)-Tetradec-7-en-2-one, a new type of sex pheromone from the oriental beetle. *Naturwissenschaften* 80:86–87.

LEAL, W.S., MOCHIZUKI, F., WAKAMURA, S., and YASUDA, T. 1992a. Electroantennographic detection of *Anomala cuprea* Hope (Coleoptera: Scarabaeidae) sex pheromone. *Appl. Entomol. Zool.* 27:289–291.

LEAL, W.S., MATSUYAMA, S., KUWAHARA, Y., WAKAMURA, S., and HASEGAWA, M. 1992b. An amino acid derivative as the sex pheromone of a scarab beetle. *Naturwissenschaften* 79:184–185.

LEAL, W.S., HASEGAWA, M., and SAWADA, M. 1992c. Identification of *Anomala schonfeldti* sex pheromone by high-resolution GC-behavior bioassay. *Naturwissenschaften* 79:518–519.

LEAL, W.S., SAWADA, M., and HASEGAWA, M. 1993. The scarab beetle *Anomala daimiana* utilizes a blend of two other *Anomala* spp. sex pheromones. *Naturwissenschaften* 80:In press.

POTTER, D.A. 1980. Flight activity and sex attraction of northern and southern masked chaffers in Kentucky turfgrass. *Ann. Entomol. Soc. Am.* 73:414–417.

SENDA, S., and MORI, K. 1983. Asymmetric synthesis of (*R*,*Z*)-(−)-5-(1-decenyl)oxacyclopentan-2-one, the sex pheromone of the Japanese beetle. *Agric. Biol. Chem.* 47:2595–2598.

TAMAKI, Y., SUGIE, H., and NOGUCHI, H. 1985. Methyl (*Z*)-5-tetradecenoate, sex-attractant pheromone of the soybean beetle, *Anomala rufocuprea* Motschulsky (Coleoptera: Scarabaeidae). *Appl. Entomol. Zool.* 20:359–361.

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.

WRIGHT, R.H. 1964. After pesticides—what? *Nature* 204:121–125.

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DUFOUR'S GLAND AND POISON GLAND CHEMISTRY OF THE MYRMICINE ANT, *Messor capensis* (Mayr)

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Abstract—The Cape harvester ant, *Messor capensis* (Mayr), is widespread in the more arid regions of southern Africa, where it forms trails many meters long and harvests considerable quantities of seeds. The poison gland contains primarily the alkaloid, anabaseine, with minor amounts of the related alkaloid, anabasine, and an unidentified compound. The Dufour's gland contains predominantly alkanes and alkenes of carbon chain length 12–23. *n*-Pentadecane is the major component, with lesser amounts of *n*-pentadecene, *n*-tridecane, *n*-heptadecane, *n*-tetradecane, *n*-heneicosene, and *n*-tricosene. The dienes, *n*-heneicosadiene and *n*-tricosadiene are rather unusual components of the Dufour's gland of ants.

Key Words—Dufour's gland, poison gland, myrmicine ant, alkanes, alkenes, anabaseine, anabasine, alkaloids, Hymenoptera, Formicidae.

INTRODUCTION

Analyses of the Dufour's gland and poison gland volatiles of many myrmicine ants have shown that the Dufour's gland usually contains a series of hydrocarbons, and other relatively nonpolar compounds, of a carbon chain length of 11–21 (Blum, 1981). In contrast, the poison gland produces a variety of interesting behavioral substances, many of which are nitrogen-containing (Blum, 1981; Wheeler and Duffield, 1988).

For example, the 2,6-dialkylpiperidines (MacConnell et al., 1971; Brand et al., 1972) and the 3,5-dialkylpyrrolidines and pyrrolines (Pedder et al., 1976; Jones et al., 1979) of *Solenopsis* species, and the 2,5-dialkylpyrrolidines of *Megalomyrmex* species (Jones et al., 1991) are generally considered to be used

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in defense. Trail following is elicited by 3-butyl-5-methylindolizidine in *Monomorium pharaonis* (Ritter et al., 1973), by methyl 4-methylpyrrole-2-carboxylate in *Atta texana* (Tumlinson et al., 1971), and various alkylpyrazines in *Tetramorium* (Attygalle and Morgan, 1983), *Pheidole pallidula* and possibly *Acromyrmex* species (Evershed and Morgan, 1983), and *Messor bouvieri* (Jackson et al., 1991).

The alkaloid anabaseine (3,4,5,6-tetrahydro-2,3′-bipyridine) occurs in the poison gland of two *Aphaenogaster* species (Wheeler et al., 1981), and the related alkaloid, anabasine, is the major volatile product in the poison gland of *Messor ebeninus* (Forel) (Coll et al., 1987); these substances may be part of the alarm–defense system of these ants. Anabasine is a well-known alkaloid produced by tobacco plants, and anabaseine has been isolated from certain marine hoplonemertine species (Kem, 1971; Kem et al., 1971).

The Cape harvester ant, *Messor capensis*, is widespread in the drier areas of southern Africa, and these soil dwelling ants follow long distinct trails, harvesting large quantities of seeds. They are fairly slow and deliberate in their movements and only show signs of excitement when severely provoked. Their main defensive behavior seems limited to biting and wiping the poison gland contents on an offending object such as forceps or stalk of grass.

Analysis of the volatiles from single Dufour's glands and poison glands, by solventless sample introduction, has established the presence of a variety of normal alkanes and alkenes from C_{12} to C_{23} in the former gland and anabaseine, anabasine, and a third compound, which is possibly a derivative of octadecenoic acid in the latter gland. While anabaseine is the major component of the poison gland, these two alkaloids have not been reported to occur together in either *Aphaenogaster* species (Wheeler et al., 1981) or *M. ebeninus* (Coll et al., 1987).

METHODS AND MATERIALS

Worker ants were collected from colonies on the University of Fort Hare farm (26°50′E, 32°47′S), from the Alice golf course, and from Adelaide, all in the eastern Cape region of southern Africa. Individual Dufour's glands and poison glands were removed and placed in the cup of a glass capillary as described by Burger et al. (1990) for solventless introduction in a gas chromatograph. The main modification to the method comprised the placement of an on/off toggle valve in the carrier gas line immediately before the injection port. This valve was closed for a period of 2–3 sec while the capillary tube containing the sample was introduced, after which a purge off time of 20 sec was set. GC-MS analyses were run on a Hewlett Packard 5890 GC and 5970 MSD. A HP Ultra 1 column (50 m) was temperature programmed from 50°C to 260°C at 5°C/min, and held at 260°C. Helium was used as carrier gas at 25 cm/sec. A portion of the contents

of the poison glands was reduced with $NaBH_4$ and $NaBD_4$ in ethanol, followed by the addition of water and extraction into *n*-hexane.

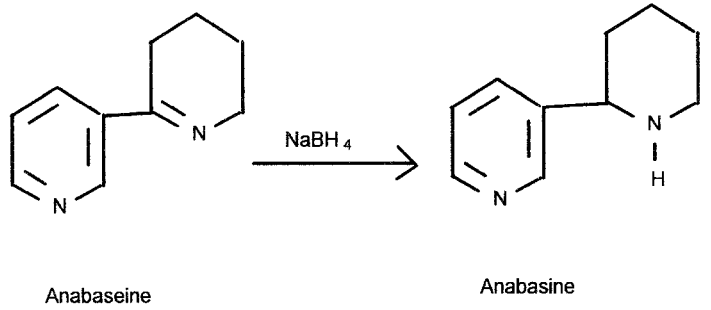
RESULTS

Poison Gland. Chromatograms of the poison gland indicated the presence of one major and two minor compounds. The first of the three peaks (approximately 5 area %) gave a mass spectrum with a molecular ion at *m/z* 162 (40%), a base peak at *m/z* 84 (100%), and characteristic fragment ions at *m/z* 105 (60%) and 133 (45%). Both the retention time and the mass spectrum were identical to those obtained for an authentic sample of anabasine (Sigma Chemical Co., St. Louis, Missouri).

The second and major peak (approximately 92 area %) gave a molecular ion and base peak at *m/z* 160 (100%), with significant fragment ions at *m/z* 159 (70%), 131 (44%), 104 (41%), and 145 (26%). Reduction of a poison gland extract with $NaBH_4$ led to an appreciable reduction in this second peak together with an increase in the anabasine peak. Reduction of a similar extract with $NaBD_4$ gave a mass spectrum for the anabasine containing one atom of deuterium, and in addition, the mass spectrum of this major peak was identical to the published spectrum of anabaseine (Kem, 1971) (Scheme 1).

The third peak (approximately 3 area %) gave a mass spectrum with a typical alkene fragmentation pattern in the low mass region. Significant ions at *m/z* 264 and 265 (40%), however, are suggestive of an octadecenoic acid derivative. A possible molecular ion at *m/z* 324 (20%) was apparent, but no structure has been assigned to this compound.

Dufour's Gland. Chromatograms of single Dufour's glands, introduced by solventless injection, illustrated the presence of numerous compounds. The identities of these compounds are presented in Table 1. The majority of peaks were readily identified as *n*-alkanes and *n*-alkenes. Standards of all the identified



Anabaseine Anabasine

SCHEME 1.

TABLE 1. DUFOUR'S GLAND VOLATILES OF ANT, *M. capensis*[a]

Peak	Retention time (min)	Area (%)	Identity		Evidence
1	21.99	0.1	*n*-Dodecane	*n*-C12	GC, MS
2	24.68	0.1	*n*-Tridec-?-ene	*n*-C13:1	MS
3	25.06	6.4	*n*-Tridecane	*n*-C13	GC, MS
4	27.34	0.2	*n*-Tetradec-?-ene	*n*-C14:1	MS
5	27.56	0.5	*n*-Tetradec-?-ene	*n*-C14:1	MS
6	27.88	3.4	*n*-Tetradecane	*n*-C14	GC, MS
7	29.80	0.3?			
8	30.12	9.4	*n*-Pentadec-?-ene	*n*-C15:1	MS
9	30.39	12.7	*n*-Pentadec-?-ene	*n*-C15:1	MS
10	30.80	42.3	*n*-Pentadecane	*n*-C15	GC, MS
11	31.95	0.1	5-Methylpentadecane	5-Me-C15	MS
12	32.42	0.2	3-Methylpentadecane	3-Me-C15	MS
13	32.60	0.2	*n*-Hexadec-?-ene	*n*-C16:1	MS
14	33.11	0.2	*n*-Hexadecane	*n*-C16	GC, MS
15	34.79	0.4	*n*-Heptadeca-?, ?-diene	*n*-C17:2	MS
16	35.03	1.8	*n*-Heptadec-?-ene	*n*-C17:1	MS
17	35.55	3.9	*n*-Heptadecane	*n*-C17	GC, MS
18	37.32	0.3	*n*-Octadec-?-ene	*n*-C18:1	MS
19	39.27	0.3	*n*-Nonadeca-?, ?-diene	*n*-C19:2	MS
20	39.44	0.5	*n*-Nonadec-?-ene	*n*-C19:1	MS
21	39.53	1.0	*n*-Nonadec-?-ene	*n*-C19:1	MS
22	39.68	0.1	2-Ketone (*m/z* 58 base peak)		
23	39.98	0.6	*n*-Nonadecane	*n*-C19	GC, MS
24	41.62	0.2	*n*-Eicos-?-ene	*n*-C20:1	MS
25	43.43	0.3	*n*-Heneicosa-?, ?-diene	*n*-C21:2	MS
26	43.59	1.2	*n*-Heneicos-?-ene	*n*-C21:1	MS
27	43.72	2.9	*n*-Heneicos-?-ene	*n*-C21:1	MS
28	45.82	0.4	*n*-Docos-?-ene	*n*-C22:1	MS
29	48.20	2.3	*n*-Tricosa-?, ?-diene	*n*-C23:2	MS
30	48.46	7.5	*n*-Tricos-?-ene	*n*-C23:1	MS
31	48.57	0.4	*n*-Tricos-?-ene	*n*-C23:1	MS

[a]Typical results obtained from a single gland.

n-alkanes were available for comparison of retention time and mass spectrum. All other compounds were identified by interpretation of the mass spectrum obtained, as well as by comparison with those in the NBS library of mass spectra available in the data base of the Hewlett Packard 5970 Mass Selective Detector. Standard 1-alkenes from C_{13} to C_{19} were available for comparison of retention times and mass spectra of the identified *n*-alkenes. The trace components, 5-methylpentadecane and 3-methylpentadecane, were identified from the pres-

ence of increased intensities of *m/z* 168 and 169 and *m/z* 196 and 197, respectively.

DISCUSSION

Harvester ants are an important component of ecosystems, and the genus *Messor* consists of more than 100 species worldwide (Robertson, South African Museum, Cape Town, personal communication, 1992). *M. capensis* (Mayr) is a granivorous ant that collects, utilizes, and, to some extent, distributes seeds. As it occurs in semiarid regions, some concern has been expressed about its influence on the seed bank and an investigation of the nest population-density, diaspore collection and utilization, and foraging strategy of *M. capensis* has been conducted (Vorster, 1989).

The success of solventless introduction of small-scale samples from insects for gas chromatographic analysis on capillary columns has been adequately demonstrated by Morgan (1990) and Burger et al. (1990). Our results show that about 92% of the detectable volatiles in the *M. capensis* poison gland is anabaseine, with anabasine being a minor component (approximately 5%). This result is similar to that obtained on *Aphaenogaster* species (Wheeler et al., 1981), where anabaseine comprised approximately 90% of the volatiles of the poison gland. In contrast, Coll et al. (1987), using packed column GC-MS, found that anabasine constituted over 90% of the volatiles in the poison gland secretion of *M. ebeninus* collected in Israel and is the major component of the poison gland of the Mediterranean harvester ant, *Messor bouvieri* (Jackson et al., 1989). The fact that two Mediterranean species of *Messor* have anabasine as a major component of this gland and a southern African species of *Messor* and a North American species of *Aphaenogaster* have anabaseine as a major component may have significant chemotaxonomic value.

The Dufour's gland of *M. capensis* contains *n*-pentadecane (42%) as the major component, as does the secretion of this gland of *M. ebeninus* (Coll et al., 1987). Table 1 lists the various compounds identified in the glandular secretion of *M. capensis* and the area percent given is typical of proportions found in a number of analyses of single glands. Most of the identified compounds are commonly found in Dufour's gland secretions of ants (Blum, 1981). 8,11-Nonadecadiene has been identified in the Dufour's gland secretion of several Attini (Evershed and Morgan, 1981; Salzemann et al., 1992), and this secretion of *M. bouvieri* (Jackson et al., 1989) contains principally 6,9-heptadecadiene, with small amounts of pentadecadiene and nonadecadiene. The secretion of *M. capensis* contains four dienes, C17:2, C19:2, C21:2, and C23:2. The double bond positions in the monoenes and dienes has not been determined.

When individual workers of *M. capensis* are carefully held and the tip of

the abdomen touched, they release a visible amount of secretion. Touching the tip of the abdomen with the capillary used for the solventless introduction of samples resulted in some of the secretion entering the cup of the capillary. Chromatographing this secretion established that only the poison gland contents were discharged, as there was no evidence of any Dufour's gland components in the chromatograms obtained. The solventless sample introduction of individual glands in this study produced extremely good chromatograms and trace components are easily detectable. However, no alkylpyrazines responsible for trail following, as found in *M. bouvieri* (Jackson et al., 1991), have been detected. Certain behavioral studies are in progress but no definitive data are available.

REFERENCES

ATTYGALLE, A.B., and MORGAN, E.D. 1983. Trail pheromone of the ant *Tetramorium caespitum* L. *Naturwissenschaften* 70:264–265.

BLUM, M.S. 1981. Chemical Defenses of Arthropods. Academic Press, New York, 562 pp.

BRAND, J.M., BLUM, M.S., FALES, H.M., and MACCONNELL, J.G. 1972. Fire ant venoms: Comparative analyses of alkaloidal components. *Toxicon* 10:259–271.

BURGER, B.V., MUNRO, Z., SMIT, D., SCHMIDT, U., WU, C.-L., and TIEN, F.-C. 1990. Sample introduction in gas chromatography: Simple method for the solventless introduction of crude samples of biological origin. *J. Chromatogr.* 518:207–214.

COLL, M., HEFETZ, A., and LLOYD, H.A. 1987. Adnexal gland chemistry of *Messor ebeninus* Forel (Formicidae: Myrmicinae). *Z. Naturforsch.* 42C:1027–1029.

EVERSHED, R.P., and MORGAN, E.D. 1981. Chemical investigations of the Dufour glands contents of attine ants. *Insect Biochem.* 11:343–351.

EVERSHED, R.P., and MORGAN, E.D. 1983. The amounts of trail pheromone substances in the venom of workers of four species of attine ants. *Insect Biochem.* 13:469–474.

JACKSON, B.D., WRIGHT, P.J., and MORGAN, E.D. 1989. 3-Ethyl-2,5-dimethylpyrazine, a component of the trail pheromone of the ant *Messor bouvieri. Experientia* 45:487–489.

JACKSON, B.D., WRIGHT, P.J., and MORGAN, E.D. 1991. Chemistry and trail following of a harvester ant. Proceedings Conference on Insect Chemical Ecology, Tabor 1990. Academia Prague and SPB Acad. Publ., The Hague, pp. 109–112.

JONES, T.H., BLUM, M.S., and FALES, H.M. 1979. Synthesis of unsymmetrical 2,5-di-*n*-alkylpyrrolidines: 2-Hexyl-5-pentyl-pyrrolidine from the thief ants *Solenopsis molesta*, *S. texanas* and its homologues. *Tetrahedron Lett.* 20:1031–1034.

JONES, T.H., DEVRIES, P.J., and ESCOUBAS, P. 1991. Chemistry of venom alkaloids in the ant *Megalomyrmex foreli* (Myrmicinae) from Costa Rica. *J. Chem. Ecol.* 17:2507–2518.

KEM, W.R. 1971. A study of the occurrence of anabaseine in *Paranemertes* and other nemertines. *Toxicon* 9:23–32.

KEM, W.R., ABBOTT, B.C., and COATES, R.M. 1971. Isolation and structure of a hoplonemertine toxin. *Toxicon* 9:15–22.

MACCONNELL, J.G., BLUM, M.S., and FALES, H.M. 1971. The chemistry of fire ant venom. *Tetrahedron* 26:1129–1139.

MORGAN, E.D. 1990. Preparation of small-scale samples from insects for chromatography. *Anal. Chim. Acta* 236:227–235.

PEDDER, D.J., FALES, H.M., JAOUNI, T., BLUM, M., MACCONNELL, J., and CREWE, R.M. 1976.

Constituents of the venom of a South African fire ant (*Solenopsis punctaticeps*): 2,5-Dialkyl-pyrrolidines and -pyrrolines, identification and synthesis. *Tetrahedron* 32:2275–2279.

RITTER, F.J., ROTGANS, I.E., TALMAN, E., VERWIEL, P.E., and STEIN, F. 1973. 5-Methyl-3-butyl-octahydro-indolizine, a novel type of pheromone attractive to pharaoh's ant, *Monomorium pharaonis* (L.). *Experientia* 29:530–531.

SALZEMANN, A., NAGNAN, P., TELLIER, F., and JAFFE, K. 1992. Leaf-cutting ant *Atta laevigata* (Formicidae: Attini) marks its territory with colony-specific Dufour gland secretion. *J. Chem. Ecol.* 18:183–196.

TUMLINSON, J.H., SILVERSTEIN, R.M., MOSER, J.C., BROWNLEE, R.G., and RUTH, J.M. 1971. Identification of the trail pheromone of a leaf-cutting ant, *Atta texana*. *Nature* 234:348–349.

VORSTER, M.J. 1989. Diaspore collection and utilization by the granivorous ant *Messor capensis* (Mayr) and its influence on grassveld composition. MSc thesis. University of the Orange Free State, South Africa.

WHEELER, J.W., and DUFFIELD, R.M. 1988. Pheromones of Hymenoptera and Isoptera, pp. 59–206, *in* E.D. Morgan and N.B. Mandava (eds.). Handbook of Natural Pesticides, Vol. IV, Part B, Pheromones. CRC Press, Boca Raton, Florida.

WHEELER, J.W., OLUBAJO, O., STORM, C.B., and DUFFIELD, R.M. 1981. Anabaseine: Venom alkaloid of *Aphaenogaster* ants. *Science* 211:1051–1052.

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QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIPS (QSAR) OF TRIMEDLURE ISOMERS

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Abstract—Trimedlure (*tert*-butyl 4- and 5-chloro-*cis*- and *trans*-2-methylcyclohexane-1-carboxylate), a mixture of eight isomers, is used as an attractant for detecting and monitoring the male Mediterranean fruit fly. This paper reports the quantitative structure–activity relationship (QSAR), via CHEM-X, of the eight purified isomers (racemic mixtures) of trimedlure. The relationship between structure and attractiveness is demonstrated by utilizing male medfly field catch on day 0 of the individual isomers vs. several molecular descriptors: volume, surface area, a torsion angle, and an interatomic distance.

Key Words—Trimedlure, medfly, *Ceratitus capitata*, attractancy, quantitative structure–activity relationship, QSAR, CHEM-X, linear regression.

INTRODUCTION

Trimedlure (TML), *tert*-butyl 4- and 5-chloro-*cis*- and *trans*-2-methylcyclohexane-1-carboxylate, is a synthetic mixture of eight isomers (each of which is a racemic mixture) that is used as an attractant for detecting and monitoring the male medfly, *Ceratitis capitata* (Wiedemann). The medfly is a worldwide pest of fruits, nuts, and vegetables (Hagen et al., 1981; Jackson and Lee, 1985). Commercial TML is prepared by a four-step procedure (Beroza et al., 1961)

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that results in a preponderance (90–95%) of four *trans* isomers (McGovern et al., 1986) where the *trans* designation refers to the diequatorial relationship between the vicinal 1-carboxylic ester group and the 2-methyl group (Figure 1). The four *trans*-TML isomers are arbitrarily designated A, B1, B2, and C (McGovern and Beroza, 1966) with the chlorine atom in the 4- or 5-position being either equatorial or axial (Warthen et al., 1988). The remaining 5–10% of commercial TML consists of four *cis* isomers (Figure 1), arbitrarily designated V, W, X, and Y (McGovern et al., 1986; Leonhardt et al., 1982); V, W, and X are shown with a 1-carboxyl-2-methyl axial–equatorial conformation (McGovern et al., 1986), and Y (McGovern et al., 1986; Warthen et al., 1987) is shown with a 1-carboxyl-2-methyl equatorial–axial conformation.

The gas chromatographic (GC) separation of these eight isomers (Leonhardt et al., 1982; Warthen and McGovern, 1986a; Beroza and Sarmiento, 1964) and the stereochemical, structural assignments of each of the *trans* (McGovern and Beroza, 1966) and *cis* isomers (McGovern et al., 1986) have been reported. The semipreparative high-performance liquid chromatographic (HPLC) separation of the four *trans*-TML isomers (Sonnet et al., 1984) and the analytical HPLC separation of the four *cis* isomers (Warthen and McGovern, 1986b) preceded the subsequent semipreparative HPLC separation of all eight TML isomers (Warthen and McGovern, 1988). These separations made possible the isolation of sufficient quantities of all eight isomers of TML to determine relative attractiveness in the field (McGovern et al., 1990) (Figure 2). Although the most attractive *trans* isomer is TML-C (McGovern et al., 1966, 1987), or more

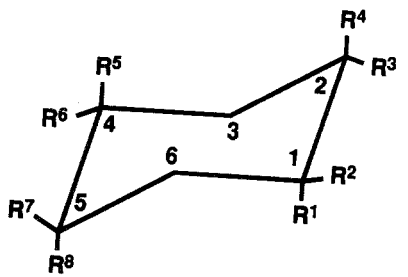
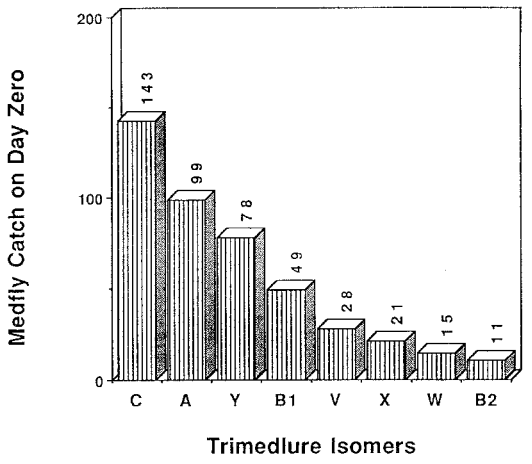


FIG. 1. The *trans*-(A, B1, B2, C) and *cis*- (V, W, X, Y) TML isomers (calculated lowest energy conformers). TML-A: $R^2 = CO_2t\text{-Bu}$, $R^3 = CH_3$, $R^8 = Cl$, $R^1 = R^4 = R^5 = R^6 = R^7 = H$; TML-B1: $R^2 = CO_2t\text{-Bu}$, $R^3 = CH_3$, $R^7 = Cl$, $R^1 = R^4 = R^5 = R^6 = R^8 = H$; TML-B2: $R^2 = CO_2t\text{-Bu}$, $R^3 = CH_3$, $R^6 = Cl$, $R^1 = R^4 = R^5 = R^7 = R^8 = H$; TML-C: $R^2 = CO_2t\text{-Bu}$, $R^3 = CH_3$, $R^5 = Cl$, $R^1 = R^4 = R^6 = R^7 = R^8 = H$; TML-V: $R^1 = CO_2t\text{-Bu}$, $R^3 = CH_3$, $R^7 = Cl$, $R^2 = R^4 = R^5 = R^6 = R^8 = H$; TML-W: $R^1 = CO_2t\text{-Bu}$, $R^3 = CH_3$, $R^5 = Cl$, $R^2 = R^4 = R^6 = R^7 = R^8 = H$; TML-X: $R^1 = CO_2t\text{-Bu}$, $R^3 = CH_3$, $R^6 = Cl$, $R^2 = R^4 = R^5 = R^7 = R^8 = H$; TML-Y: $R^2 = CO_2t\text{-Bu}$, $R^4 = CH_3$, $R^7 = Cl$, $R^1 = R^3 = R^5 = R^6 = R^8 = H$.



Most active	C	A	Y	B1	-	-	-	-
Least active	-	-	-	-	V	X	W	B2
trans	C	A	-	B1	-	-	-	B2
cis	-	-	Y	-	V	X	W	-
1,2,4	C	-	-	-	-	X	W	B2
1,2,5	-	A	Y	B1	V	-	-	-

FIG. 2. Field medfly catch on day 0 (means of six replicates) of TML isomers (racemic mixtures) (McGovern et al., 1990), and groupings of four TML isomers utilized in Table 4 data.

specifically the 1*S*,2*S*,4*R*-TML-C enantiomer, followed by the *trans* enantiomers 1*R*,2*R*,5*S*-TML-A > 1*R*,2*R*,5*R*-TML-B1 (Sonnet et al., 1984; Doolittle et al., 1991), the individual *cis* enantiomers have not been biologically evaluated to complete the study on relative attractiveness of TML enantiomers.

Therefore, this paper reports the quantitative structure–activity relationships (QSAR) of only the eight TML isomers (racemic mixtures) rather than the 16 enantiomers and suggests a relationship between molecular descriptors of the isomers and medfly attractiveness.

METHODS AND MATERIALS

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

Conformational analyses were performed on the TML conformers begin-

ning with the torsion angle (edfg) (Figure 3), then (bcde), and then (abcd) each with an angle interval of every 10°. The MM energy of the lowest energy conformer was optimized. Then the three torsion angles were each varied within the 10° window in the same order; if a decrease in the MM energy was observed, iterations were performed to obtain a lower MM energy. This process was continued until a change of 0.5° for any of the three torsion angles no longer produced any decrease in the MM energy. Dipole moments, interatomic distances, molecular volumes, and surface areas were determined on the final energy-minimized conformers. Correlation analyses (Kilpatrick, 1987; Freund and Walpole, 1980; Hayslett, 1968) of these descriptors vs. medfly catch on day 0 for each isomer (McGovern et al., 1990) were performed for the six groupings of four TML isomers (Figure 2) and the group of all eight TML isomers to discover structure–activity relationships. The linear best fit for each analysis, by the method of least squares, was constructed and the R^2 (Kilpatrick, 1987) was calculated.

RESULTS AND DISCUSSION

Two possible conformers (inverted chairs) for each TML isomer were targets for QSAR. Based upon the MM energies obtained, those in Table 1 are the calculated lowest energy conformers and the following are MM energies

TABLE 1. MOLECULAR DESCRIPTORS AND MEDFLY CATCH OF CALCULATED LOWEST ENERGY MM MINIMIZED TML CONFORMERS

Isomer/ conformation[a]	MM energy (kcal/mol)	Torsion angles (°)[b] (abcd),	(bcde),	(edfg)	Dipole moment (D)	Mol. vol. (Å^3)[c]	Surface area (Å^3)[c]	Medfly catch (on day 0)[d]
1,2,4-Isomers								
C/e,e,a	−2.712	+178.94,	+3.04,	−8.45	0.98	186.39	190.00	143
X/a,e,e	−2.755	−178.49	−3.56,	+0.72	0.95	186.45	189.26	21
W/a,e,a	−2.464	+179.03,	+2.54,	+45.42	1.13	186.36	187.90	15
B2/e,e,e	−3.139	+178.94,	+3.14,	−5.35	1.09	186.15	191.65	11
1,2,5-Iomers								
A/e,e,a	−4.284	−179.14,	−2.70	−1.29	1.86	186.04	190.04	99
Y/e,a,e	−3.097	+179.31,	+1.73,	+27.00	0.88	186.30	191.22	78
B1/e,e,e	−4.309	−179.42,	−1.58,	+5.88	1.22	186.60	191.81	49
V/a,e,e	−4.377	−179.59,	−1.49	+0.33	1.16	186.55	189.21	28

[a] Conformations of Co_2*t*-Bu, CH_3, and Cl: e = equatorial, a = axial.
[b] See Figure 3 for angle designations.
[c] Molecular volume determined with 5 contours/Å, except for B1 at 4.75 contours/Å.
[d] Means from six replicates (McGovern et al., 1990).

(kilocalories per mole) of the calculated highest energy conformers: C/a,a,e −2.087; X/e,a,a −0.329; W/e,a,e −1.946; B2/a,a,a −0.978; A/a,a,e −3.650; Y/a,e,a −2.310; B1/a,a,a −2.078; V/e,a,a −3.118. The calculated lowest energy conformers in Table 1 agree with the proposed conformations in the literature (McGovern and Beroza, 1966; McGovern et al., 1986). Inconclusive data on the conformation of TML-W in solution via NMR was suggested by Warthen and McGovern (1988); for the present study, data for the calculated lowest energy conformer of TML-W was used since it represents the conformation in the vapor phase. The remaining calculated lowest energy TML conformers also represent those conformations of the molecule that would predominate in the vapor phase at room temperature in vacuo. This is particularly important since these same energy-minimized conformations in the vapor phase at approximately the same temperature would be impinging upon the male medfly antennae, in field tests (Keaau, Hawaii; McGovern et al., 1990); whether or not these minimized conformations would be modified (Liljefors et al., 1985) by the receptor to a higher energy state is unknown, but it seems unlikely due to the energy barrier to the higher energy stable chair conformation. Moreover, attractiveness to the receptor site should depend upon an existing minimal energy conformation in the vapor phase. Any one of many equally probable higher energy conformations are equally unlikely to exist in the vapor phase in abundance. Without additional experimental evidence, there is no rational basis for predicting which one of the many higher energy states should interact with the receptor. Since A and B1 are liquids and B2 and C (the most attractive isomer; McGovern et al., 1990) are solids, volatility of the individual TML isomers was not considered because McGovern et al. (1966) indicated that the attractiveness of the *trans*-TML isomers is related more to their stereochemistry than to their volatilities. Volatilities of the *cis*-TML isomers have not been studied, but V is a liquid, Y is a semisolid, and W and X are solids (Warthen and McGovern, 1988); semisolid Y (the most active *cis* isomer) is more attractive than V (a liquid) (McGovern et al., 1990).

Correlation analyses of the data in Tables 1–3 and several averages of pairs of interatomic distances show some possible relationships concerning structure and attractiveness of TML isomers. Although we were familiar with the limitations of using field attractancy data (McGovern et al., 1990) over single cell EAG data (Liljefors et al., 1984), no techniques had been developed for obtaining single cell data. The field attractancy data in this paper are means that resulted from six replicates that were statistically analyzed. There are 14 linear correlations of $R^2 > 0.905$ (Table 4) between physical or molecular descriptors of TML isomers (TML groupings of 4, designated in Figure 2) and medfly attractiveness from 270 attempted correlations or observations. There are two correlations of $R^2 > 0.5$ [HPLC R_f and cosine torsion (edfg); $R^2 = 0.592$ and 0.517, respectively] with all eight isomers from 45 attempted correlations or

TABLE 2. MOLECULAR, HPLC, R_f, AND GLC R_t DESCRIPTORS OF CALCULATED LOWEST ENERGY MM MINIMIZED TML CONFORMERS

Isomer/ conformation[a]	Interatomic distance (Å^2) Cl-2CH$_3$	Cl-/O\[b]	/O\-2CH$_3$	Area (Å^2)[c]	HPLC R_f(min) (silica)[d]	GC R_t (min) DMS[e]	Supelcowax[d]
1,2,4-Isomers							
C/e,e,a	4.552	4.753	3.451	7.443	22.1	17.4	50.7
X/a,e,e	5.220	4.274	3.544	7.540	13.2	17.1	48.6
W/a,e,a	4.553	5.235	3.009	6.824	13.8	17.4	46.7
B2/e,e,e	5.203	6.485	3.525	9.148	18.5	16.6	45.9
1,2,5-Isomers							
A/e,e,a	4.838	5.466	3.592	8.539	20.1	16.7	44.9
Y/e,a,e	4.990	5.382	3.127	7.670	16.7	19.8	56.5
B1/e,e,e	6.059	5.561	3.494	9.582	14.1	16.6	45.8
V/a,e,e	6.062	4.543	3.543	7.999	12.0	17.1	44.5

[a] Conformations of CO_2t-Bu, CH_3, and Cl: e = equatorial, a = axial.
[b] /O\ = alcohol oxygen.
[c] $A = 1/2c\{b^2\text{-}[(c^2 + b^2 - a^2)/2c]^2\}^{1/2}$; interatomic distances in the previous column were used for the scalene triangle.
[d] Warthen and McGovern (1988).
[e] Warthen and McGovern (1986a).

observations. Thirteen correlations in Table 4 would be expected by random selection with at least an $R^2 = 0.905$, $N = 4$ (two for each group of four in Figure 2). Two correlations would be expected with at least an $R^2 = 0.5$, $N = 8$. Thus, although some of the correlations found would occur purely by chance, the majority of them are well above their minimum R^2 for random correlation. These difficulties are in part due to the reasonably small number of stereoisomers that have been synthesized. Nevertheless, the results do aid in more complicated and significant correlations.

It was necessary to consider combinations of descriptors that influence the relationship between the structure of all eight TML isomers and medfly attractiveness. The use of the molecular volume–molecular surface area ratio as a descriptor versus medfly attractiveness showed $R^2 > 0.905$ for groupings of four *trans* isomers, four *cis* isomers, and the most active isomers (CAYB1) (Table 4); even six (four *cis* + 1,2,5-A and -B1) of the eight isomers would correlate ($R^2 = 0.682$). Including the cosine torsion (abcd) as a modulating factor (f1) to the molecular volume–molecular surface area ratio added more 1,2,5-numerical weight (Table 4; AYB1V, $R^2 = 0.925$) to the relationship and allowed seven of the eight TML isomers (all but TML-W) to be correlated ($R^2 = 0.858$).

It was found necessary to include an average interatomic distance, [(tC$\underline{\text{C}}$H$_3$-6C) + (Cl-2C$\underline{\text{C}}$H$_3$)]/2, tC$\underline{\text{C}}$H$_3$ = atom "a" in Figure 3, as a modulating factor

TABLE 3. INTERATOMIC DESCRIPTORS OF CALCULATED LOWEST ENERGY MM MINIMIZED TML CONFORMERS

Interatomic distance (Å)[a]	TML-C (e,e,a)[b]	TML-B2 (e,e,e)	TML-X (a,e,e)	TML-W (a,e,a)	TML-A (e,e,a)	TML-Y (e,a,e)	TML-B1 (e,e,e)	TML-V (a,e,a)
=O−Cl	5.954	6.912	6.170	5.881	4.986	6.242	5.973	5.713
=C−Cl	4.816	6.018	4.968	4.947	4.512	5.222	5.171	4.709
=C−3C(aH)	4.150	2.798	2.816	4.173	4.149	4.128	4.152	2.823
Cl−1C(H)	4.535	4.730	5.608	4.466	2.743	4.379	4.405	4.832
Cl−6C(aH)	2.760	4.423	2.753	4.398	3.674	2.863	2.861	2.837
$t\underline{C}H_3$−6C	5.223	5.169	5.188	5.638	5.105	4.966	5.180	5.178
$t\underline{C}H_3$−2C	5.034	5.039	5.146	4.895	5.126	5.455	5.064	5.185
$t\underline{C}H_3$−2CH_3	5.274	5.371	5.388	4.712	5.473	4.847	5.352	5.409
$t\underline{C}H_3$−6C(aH)	4.636	6.222	6.599	4.545	4.481	4.418	4.577	6.221
/O\[c]−6C	3.069	3.028	3.051	3.432	2.978	2.865	3.042	3.048
/O\−2C	2.955	2.963	3.062	2.835	3.028	3.277	2.971	3.073
/O\−1C(H)	3.344	3.347	3.344	3.242	3.347	3.305	3.347	3.342
tC−6C	4.360	4.330	4.371	4.568	4.299	4.252	4.333	4.354
tC−2C	4.269	4.265	4.347	4.251	4.312	4.471	4.287	4.370
tC−2$\underline{C}H_3$	4.449	4.509	4.499	4.256	4.548	4.215	4.500	4.508
tC−1C(H)	4.453	4.460	4.454	4.393	4.457	4.423	4.459	4.449
2$\underline{C}H_3$−6C(aH)	4.178	4.187	4.160	4.205	4.180	2.798	4.184	4.203

[a] $t\underline{C}H_3$ = atom a in Figure 3; tC = atom b in Figure 3.
[b] Conformations of CO_2t-Bu, CH_3 and Cl: e = equatorial, a = axial.
[c] /O\ = alcohol oxygen.

(f2) to the molecular volume–molecular surface area ratio x (f1) to add more 1,2,4-numerical weight (CB2XW, $R^2 = 0.879$) for TML-W to the relationship. This resulted in a correlation of the grouping of all eight TML isomers. The constants in f1 and f2, which represent the average cosine torsion (abcd) and the average of two interatomic distances, [($t\underline{C}H_3$-6C) + (Cl-2$\underline{C}H_3$)]/2, were optimized. This resulted in an equation of the form $Z = AX^2Y^2$, where A = the volume–surface area ratio, X^2 = f1, and Y^2 = f2. The equation is more specifically designated as

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

When the index or independent variable (Å^3) of the eight TML isomers, determined by the right-hand side of this equation, and the dependent variable (medfly catch on day 0 of the eight TML isomers) are analyzed for simple linear regression (Kilpatrick, 1987; Freund and Walpole, 1980; Hayslett, 1968), a regression line is obtained ($R^2 = 0.939$) with $P < 0.01$ (Figure 4). Logical input of

TABLE 4. CORRELATION ANALYSES OF MOLECULAR/PHYSICAL DESCRIPTORS OF MM MINIMIZED TML CONFORMERS VERSUS MEDFLY CATCH ON DAY 0 OF TML GROUPING (R^2 IN DESCENDING ORDER)

Descriptor[a]	TML grouping (isomers)[b]	R^2	Equation
$N = 4$, $R^2 > 0.905$ from 270 observations, $P = 0.05$			
HPLC R_f (silica)	1,2,5 (AYB1V)	0.983	$Y = -76.213 + 8.8848X$
Average interatomic [(tC−6C) + (tCH$_3$−2C)]/2	*cis* (YVXW)	0.981	$Y = 2555.4 + 542.23X$
Interatomic ⁄O\[c]−1CH	*cis* (YVXW)	0.972	$Y = 4828.3 - 1437.3X$
Average interatomic [(tCH$_3$−6C) + (tC−2CH$_3$)]/2	*cis* (YVXW)	0.962	$Y = 930.71 - 186.2X$
Molecular volume/surface area	*trans* (CAB1B2)	0.961	$Y = -1.1695 \times 10^4 + 1.2060 \times 10^4X$
Interatomic ⁄O\−6C	*cis* (YVXW)	0.957	$Y = 982.52 - 315.88X$
HPLC R_f (silica)	MA (CAYB1)	0.943	$Y = -105.7 + 10.812X$
Average interatomic [(tC−6C) + (tC−2CH$_3$)]/2	*cis* (YVXW)	0.928	$Y = 1291.8 - 286.97X$
Cosine torsion (abcd)	1,2,5 (AYB1V)	0.925	$Y = -444.66 - 538.17X$
GLC R_f(DMS)	*cis* (YVXW)	0.919	$Y = -339.94 + 21.074X$
Average interatomic [(tCH$_3$−6C) + (= C−Cl)]/2	LA (VXWB2)	0.919	$Y = 149.72 - 25.058X$
Molecular volume/surface area	*cis* (YVXW)	0.916	$Y = 3747.6 - 3771.3X$
Molecular volume/surface area	MA (CAYB1)	0.910	$Y = -9473.0 + 9792.8X$
Average interatomic [(tCH$_3$−6C) + (Cl − ⁄γO\)]/2	*trans* (CAB1B2)	0.909	$Y = 924.81 - 158.22X$
$N = 8$, $R^2 > 0.5$ from 45 observations, $P = 0.05$			
HPLC R_f(silica)	(CAYB1VXWB2)	0.592	$Y = -107.97 + 10.021X$
Cosine torsion (edfg)	(CAYB1VXWB2)	0.517	$Y = 76.525 - 60.738X$

[a] Data from Tables 1–3; tC = atom b in Figure 3; tCH$_3$ = atom a in Figure 3.
[b] TML grouping from Figure 2; MA = most active; LA = least active (McGovern et al., 1990).
[c] ⁄O\ = alcohol oxygen.

descriptors based on chemistry was utilized to determine the index or independent variable for the x axis, resulting in a type of principal components analysis. Therefore, since stepwise multiple regression (Topliss and Edwards, 1979; Dunn et al., 1984) was not specifically used, it is not possible (Hansch, 1991) to evaluate the probability for the chance fitting of the final equation even though the simple correlations did play some role in the selection of the descriptors to be used. We believe that the observed increase of R^2 from 0.592 to 0.939 is much greater than what would be expected by chance alone.

The above equation relates molecular descriptors (Å^3) with medfly catch of the eight TML isomers (racemic mixtures). The structure–activity relationship

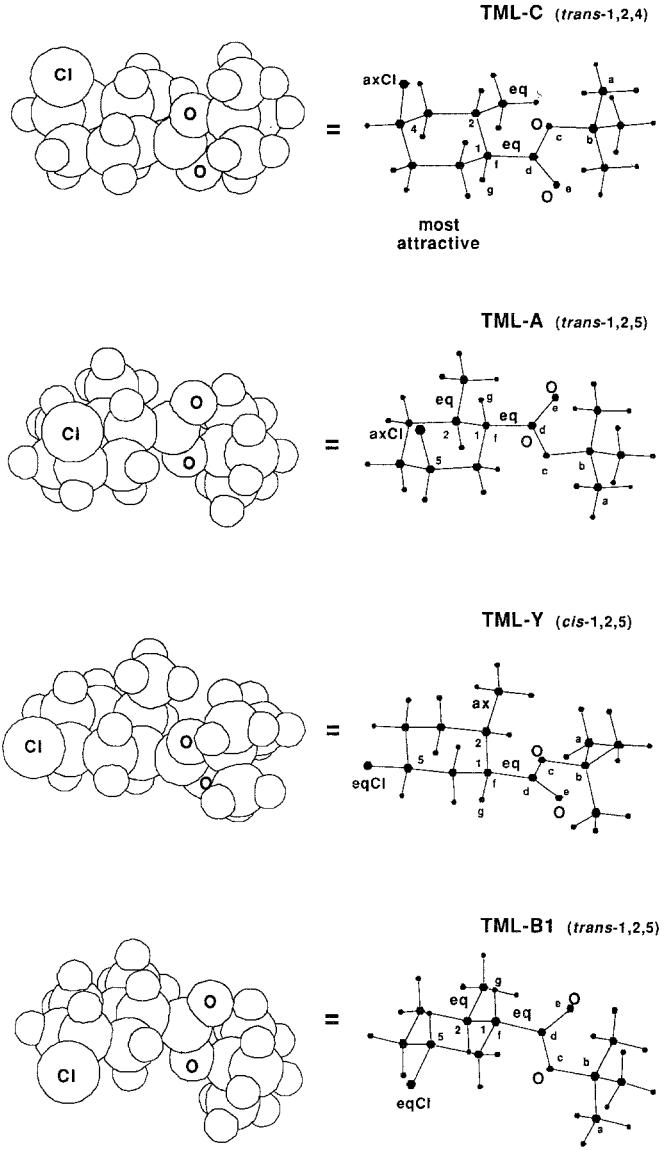


FIG. 3. Calculated lowest energy MM minimized TML conformers with rotated space-filled equivalents; attractiveness to male medfly is C > A > Y > B1 > V > X > W > B2, (McGovern et al., 1990).

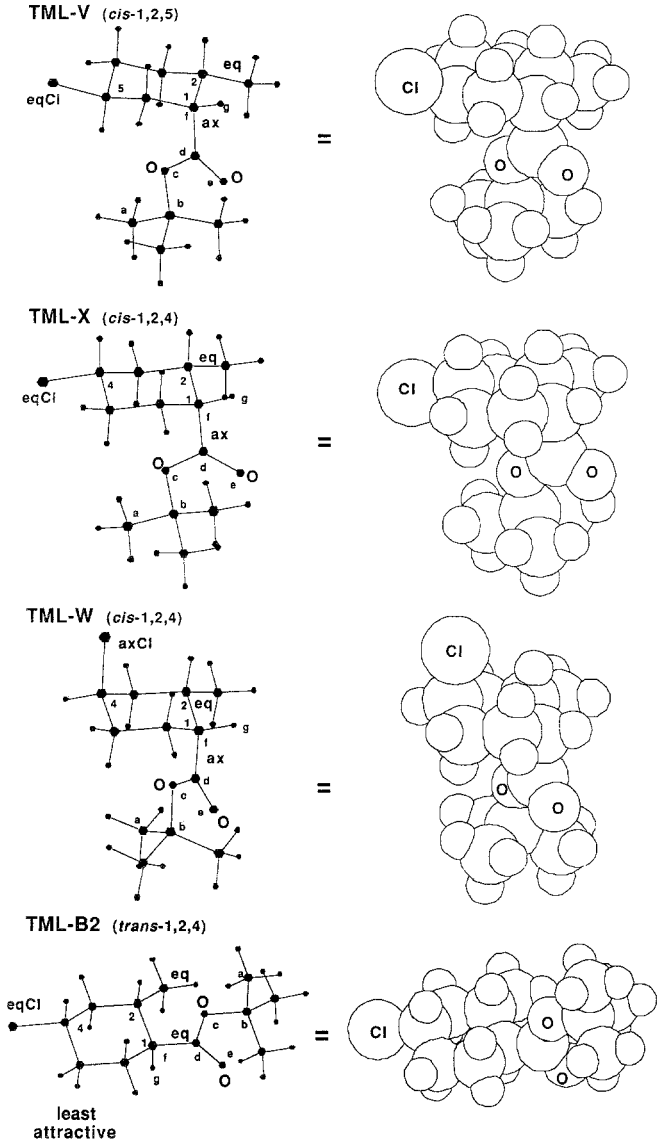


FIG. 3. Continued

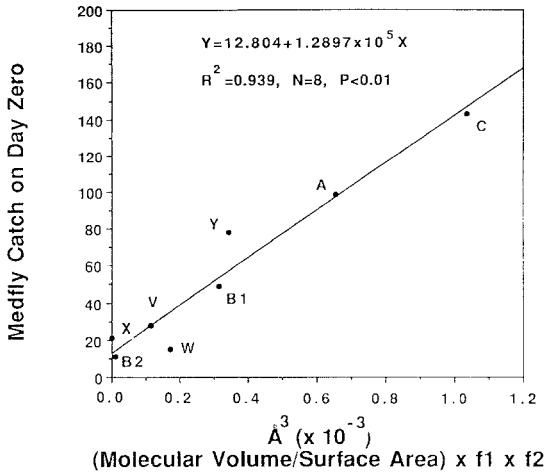


FIG. 4. Linear regression ($R^2 = 0.939$, $N = 8$, $P < 0.01$) of medfly catch on day 0 (means of six replicates) of eight TML isomers (racemic mixtures) (McGovern et al., 1990) versus [(molecular volume/molecular surface area) × f1 × f2] ($Å^3$) of calculated lowest energy MM minimized TML conformers. f1 = $[\cos abcd + 0.895]^2$; f2 = $\{[(t\underline{C}H_3\text{-}6C)+(Cl\text{-}2\underline{C}H_3)/2] - 5.221\}^2$.

of this series of compounds follows: The molecular volume–surface area of this series of compounds represents a molecular size–shape ratio, which, when varied $>$ or <0.981, in combination with f1 and f2, no longer will impinge the medfly antennae correctly for attractancy. The term f1 (cos torsion abcd) and the first distance of f2 ($t\underline{C}H_3$-6C) define the orientation of the *tert*-butyl ester with respect to the C=O moiety and the cyclohexane ring, while the second distance of f2 (Cl-2$\underline{C}H_3$) limits the spatial relationship requirement of these substituents that is necessary for medfly attractancy. These distance descriptors differ for each racemic TML isomer mixture, but the two enantiomers of that mixture have equivalent distance descriptors.

Observation of the rotated space-filled structures in Figure 3 reveals that the axial position of the 4-Cl, the equatorial position of the 2-CH_3, and the 1-equatorial *tert*-butyl formate are extremely important for medfly attraction. When the Cl is no longer axial or in the 4 position as in TML-B1, activity decreases (Figure 4), and as the *tert*-butyl formate becomes axial as in TML-V, TML-X, and TML-W (even with an axial Cl), medfly attraction decreases even further (Figure 4). TML-B2 is least attractive, probably due to the absence of the axial 4-Cl even though a 1-equatorial *tert*-butyl formate and an equatorial 2-CH_3 are present. These visual observations are closely linked to the descriptors of the eight TML isomers (Tables 1–3), which appear in the

equation used to calculate the index or independent variable ($Å^3$) in the simple linear regression analysis.

CONCLUSIONS

QSAR, via CHEM-X, of TML isomers and examination of data resulted in an equation for the relationship between medfly catch on day 0 and four molecular descriptors of the eight TML isomers (racemic mixtures). The equation is used to determine the index or independent variable of distance for simple linear regression analysis with the dependent variable of medfly catch; a linear best-fit line results ($R^2 = 0.939$, $P < 0.01$). Essentially, medfly catch in the eight-isomer TML series of compounds is determined by the ratio of molecular volume to surface area with modulation of the molecular surface area by a torsion angle and an average of two interatomic distances. Future QSAR correlations will involve single-cell studies with racemic TML isomers and enantiomers (when the *cis* enantiomers become available). These future studies will help to confirm the present work and lead to a more refined independent variable in our equation with descriptors that will address the asymmetry of the enantiomers.

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REFERENCES

BEROZA, M., and SARMIENTO, R. 1964. Gas chromatographic analysis of insect attractants: Trimedlure, medlure, siglure, butyl sorbate, cue-lure, methyleugenol. *J. Assoc. Off. Agr. Chem.* 47:838–841.

BEROZA, M., GREEN, N., GERTLER, S.I., STEINER, L.F., and MIYASHITA, D.M. 1961. New attractants for the Mediterranean fruit fly. *J. Agric. Food Chem.* 9:361–365.

BURKERT, U., and ALLINGER, N.L. 1982. Molecular Mechanics. ACS Monograph 177. American Chemical Society, Washington, D.C., p. 339.

CLARK, T. 1985. Computational Chemistry, A Practical Guide to Chemical Structure and Energy Calculations. John Wiley & Sons, New York, p. 332.

DOOLITTLE, R.E., CUNNINGHAM, R.T., MCGOVERN, T.P., and SONNET, P.E. 1991. Trimedlure enantiomers: Differences in attraction for Mediterranean fruit fly, *Ceratitus capitata* (Wied.) (Diptera: Tephritidae). *J. Chem. Ecol.* 17:475–484.

DUNN, W.J., III, WOLD, S., EDLUND, U., HELLBERG, S., and GASTEIGER, J. 1984. Multivariate structure–activity relationships between data from a battery of biological tests and an ensemble of structure descriptors: The PLS method. *Quant. Struct. Act. Relat.* 3:131–137.

FREUND, J.E., and WALPOLE, R.E. 1980. Mathematical Statistics, 3rd ed., Prentice-Hall, Englewood Cliffs, New Jersey, p. 419.

HAGEN, K.S., ALLEN, W.W., and TASSAN, R.L. 1981. Mediterranean fruit fly: The worst may be yet to come. *Calif. Agric.* 35:5–7.

HANSCH, C. 1991. New perspectives in QSAR. *Pharmacochem. Libr.* 16:3–10.

HAYSLETT, H.T., JR. 1968. Statistics Made Simple. Doubleday, Garden City, New York, p. 107.

JACKSON, D.S., and LEE, B.B. 1985. Medfly in California 1980–1982. *Bull. Entomol. Soc. Am.* 31:29–37.

KILPATRICK, N. 1987. Business Statistics Using Lotus 1-2-3. John Wiley & Sons, New York, p. 259.

LEONHARDT, B.A., MCGOVERN, T.P., and PLIMMER, J.R. 1982. Capillary GC analysis of trimedlure, the attractant for the medfly. *J. High Resol. Chromatogr. Chromatogr. Commun.* 5:430–431.

LILJEFORS, T., THELIN, B., and VAN DER PERS, J.N.C. 1984. Structure–activity relationships between stimulus molecule and response of a pheromone receptor cell in turnip moth. *J. Chem. Ecol.* 10:1661–1675.

LILJEFORS, T., THELIN, B., and VAN DER PERS, J.N.C., and LÖFSTEDT, C. 1985. Chain-elongated analogues 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.

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. 1986. *cis*-Trimedlure: Attraction for the Mediterranean fruit fly (Diptera: Tephritidae) and isomeric structural assignments. *J. Econ. Entomol.* 79:98–102.

MCGOVERN, T.P., CUNNINGHAM, R.T., and LEONHARDT, B.A. 1987. Attractiveness 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., WARTHEN, J.D., JR., and CUNNINGHAM, R.T. 1990. Relative attraction of the Mediterranean fruit fly (Diptera: Tephritidae) to the eight isomers of trimedlure. *J. Econ. Entomol.* 83:1350–1354.

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.

TOPLISS, J.G., and EDWARDS, R.P. 1979. Chance factors in studies of quantitative structure–activity relationships. *J. Med. Chem.* 22:1238–1244.

WARTHEN, J.D., JR., and MCGOVERN, T.P. 1986a. GC/FTIR analyses of trimedlure isomers and related esters. *J. Chromatogr. Sci.* 24:451–457.

WARTHEN, J.D., JR., and MCGOVERN, T.P. 1986b. Purification of *cis*-trimedlure isomers by high-performance liquid chromatography. *Chromatographia* 21:651–654.

WARTHEN, J.D., JR., and MCGOVERN, T.P. 1988. Semi-preparative high-performance liquid chromatographic separation of trimedlure isomers. *Chromatographia* 25:811–814.

WARTHEN, J.D., JR., WATERS, R.M., and MCGOVERN, T.P. 1987. ^{13}C and ^{1}H chemical shift assignments and conformation confirmation of trimedlure-Y via 2-D NMR. *Spectrochim. Acta* 43:895–899.

WARTHEN, J.D., JR., WATERS, R.M., and MCGOVERN, T.P. 1988. $^{1}H/^{13}C$ chemical shift correlations in the complex proton pattern of *trans*-trimedlure isomers. *Spectrochim. Acta* 44:175–178.

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DABBING AND SHOOTING OF BENZO- AND NAPHTHOQUINONE SECRETIONS: DEFENSIVE STRATEGIES OF BARK-INHABITING ALEOCHARINE (COL.: STAPHYLINIDAE) AND TENEBRIONID (COL.: TENEBRIONIDAE) BEETLE LARVAE

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Abstract—The abdominal glands of three bark-inhabiting larvae of genera *Bolitochara*, *Leptusa* (Staphylinidae), and *Hypophloeus* (Tenebrionidae) were studied chemically and morphologically. Behavior of the larvae indicated that secretion is emitted only after severe disturbance of the larvae. These mechanical contacts may also occur incidentally with coinhabiting nonpredatory arthropods when the beetle larvae move within small interstices under bark. Depending on the species, the secretions contained 1,4-benzoquinone derivatives and three 6-alkyl-naphthoquinones dissolved within various alkanes, alkenes, ethyl-, isopropyl-, and isoamylesters. More erratically distributed gland constituents also detected were acetophenone, benzyl propionate, and methyl hydroxybenzoate. In the laboratory, synthetic quinone-containing solutions simulating those found in *Leptusa* and *Bolitochara* larvae acted as strong topical irritants and caused further damage to last-stage *Calliphora vomitoria* larvae if hydrocarbons or esters were used as solvents. The natural secretions of *Hypophloeus versipellis* elicited considerable mortality in two subcortical sciarid larvae cooccurring with tenebrionid larvae. Bioassay and secretion chemistry of the Staphylinidae/Tenebrionidae larval secretions indicated that they are typical defensive secretions that act topically. Morphological data characterized Bolitocharini larvae as possessing protuberant abdominal tergites supplied with an interiorly situated gland reservoir. After mechanical contact, the defensive secretion is topically applied to other arthropods by dabbing this tergal protuberance on targets. The defensive gland of *Hypophloeus versipellis* is unusual in possessing a movable reservoir opening situated at the anterior border of tergite IX. By this peculiar gland morphology *Hypophloeus* larvae are capable of shooting secretion droplets frontally from their slightly depressed dorsal abdominal surface without bending their abdominal tips dorsally. This seems an adaptation to the interstitial habitat of the larvae. The types of

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defensive glands and their phylogenetic value in Aleocharinae/Tenebrionidae larvae are discussed.

Key Words—Coleoptera, Staphylinidae, Tenebrionidae, larvae, defensive glands, quinone, naphthoquinone, taxonomy.

INTRODUCTION

During a study dealing with bark-inhabiting arthropods, it became evident that larvae of various species of Aleocharinae and Tenebrionidae bear a median exocrine gland on abdominal segments VIII or IX. The secretion of these glands was always emitted as a droplet or was even shot over the body surface of the larvae when they were strongly mechanically molested. A multitude of fragmentary data in the literature with respect to morphology and distribution of this peculiar structure within the subfamily Aleocharinae has been carefully compiled up to 1984 (Frank and Thomas, 1984). Some further observations focus on external gross morphology of exocrine glands of the tenebrionid genera *Hypophloeus* (= *Corticeus*; Hypophloeinae) (Doyen and Lawrence, 1979) and *Menimus* (Gnathidiinae) (Watt, 1974).

As to the biological significance of the aleocharine larval gland, various observations and speculations have been published: Depending on the species, this secretion was said to form the silken cocoon of the prepupa (for discussions see Ashe, 1981b), to have a locomotory function (Brass, 1914), to have a defensive function (Ashe, 1981b; Frank and Thomas, 1984), or to appease attacking ants in myrmecophilous species (Hölldobler et al., 1982). Nothing is known of the significance of the tenebrionid larval gland system.

Because information on the chemical compositions of these larval secretions is totally lacking, it was the main aim of this study to present initial chemical results from selected staphylinid and tenebrionid species. Combined laboratory bioassays should further elucidate the biological significance of the secretion and indicate whether the identified compounds show any topical irritancy or toxicity against both laboratory and field target organisms. Therefore, it was a further aim of this paper to characterize these glands morphologically, to describe the depletion of the glands especially in the previously unknown shooting species, and to discuss whether the presence of larval glands in other Aleocharinae species can be used for phylogenetic purposes.

METHODS AND MATERIALS

Aleocharinae larvae were sieved from fragments of bark-dwelling fungi in the vicinity of Aachen/Rhineland (*Bolitochara*). Further larvae (*Leptusa*, *Hypophloeus*) were isolated, especially from bark-beetle tunnels under the bark of

pine (*Pinus sylvestris*) and spruce (*Picea abies*) in the vicinity of Bayreuth/Bavaria. *Bolitochara* larvae were found together with adults of *Bolitochara obliqua*; *Hypophloeus* larvae were kept in the laboratory and produced adults of *H. versipellis* Baudi. *Hypophloeus* larvae were also identified by using the key of Klausnitzer (1978). *Leptusa* and *Bolitochara* larvae were additionally identified as genera by using the key of Topp (1978) and by comparing with morphological data of Paulian (1941).

Gland reservoirs were excised from frozen beetles on Dry Ice and deposited in the groove of a cooled wire plunger, which is movable within a needle (0.1 μl Mini Injector, Precision Sampling Corporation). This method allowed injection of single gland reservoirs into the injection port of a gas chromatograph. Capillary gas chromatography–mass spectrometry (GC-MS) of the *Bolitochara* secretion was performed on a Varian 3700 capillary gas chromatograph coupled to a MAT 44 quadrupole mass spectrometer, which operated at 80 eV and was connected with a Varian SS 200 computer system. A 20-m SE 30 glass capillary column was used temperature-programmed from 60°C (2 min isothermal) to 250°C (heating rate 12°C/min).

EI mass spectra (70 eV) of *Leptusa* and *Hypophloeus* secretions were obtained on a Finnigan MAT-EI Iontrap ITD 800 connected to a Carlo Erba Vega GC 6000 gas chromatograph. Gas chromatographic separation was achieved by using a 12.5-m OV-1701 fused silica capillary column programmed from 60°C (isothermal 2 min) to 260°C (heating rate 12°C)/min. CI mass spectra were obtained by using methanol as reactant gas.

For microscopic observations, fragments or whole larvae of *Leptusa* and *Hypophloeus* were macerated in 5% KOH for 24 hr. For scanning electron microscopy (SEM) the specimens were sputter coated with gold and examined in a Stereoscan 90 microscope (Cambridge Instruments).

The topical irritancy of various *Leptusa* compounds with and without *p*-toluquinone was tested on last-instar larvae of *Calliphora vomitoria*. Immediately before the experiment, quinone-saturated solutions were prepared by ultrasonic treatment of 0.5 ml fluid with excess *p*-toluquinone. From each solution or pure solvent 2 μl were placed topically on a crawling *Calliphora* larva ($N = 30$ larvae per test). The time between topical application and contraction of the larva was recorded up to a maximum of 30 sec, and the number of larvae that showed any positive reaction was noted. Larvae tested with water were used as controls. For the synthetic *Leptusa* mixture, the following compounds were mixed: 10 μl each of tridecane, ethyl tetradecanoate, ethyl linoleate; 4 μl each of ethyl dodecanoate, ethyl hexadecanoate, ethyl palmitoleate, isamyl propionate; and 2 μl each of dodecane, benzyl propionate, pentadecane, isopropyl tetradecanoate, ethyl pentadecanoate and ethyl octadecanoate. The fate of *Calliphora* larvae (kept at 22°C) after having been topically treated with 2 μl of the aforementioned fluids and solutions was determined four days later. Notes

were made of the numbers of intact pupae (completely sclerotized), damaged pupae (shrunken or with white spots), and dead larvae (brownish discoloration; reduced internal pressure).

The topical irritancy of the *Hypophloeus* secretion was tested against two unidentified Sciaridae larvae, cooccurring under bark with the tenebrionid larvae. The larger-sized, yellow fly larvae showed body lengths comparable with those of *Hypophloeus* beetle larvae (5 mm). The smaller-sized, white-colored fly larvae reached about 75% of the body length of darkling beetle larvae.

Under a binocular, one fly larva was taken with a forceps and brought near to the gland reservoir opening of a *Hypophloeus* larva. Usually this evokes a complete depletion of the gland reservoir, evident in the distinct contraction of the fly larva several seconds afterwards, which is not due to mechanical stimulation. In each experiment, 20 fly larvae were used; in every case one fly larva was put into contact with an unmolested beetle larva. Mortality rates of fly larvae were recorded between 0 and 96 hr after applying the defensive secretion of the darkling beetle larva. Untreated Sciaridae larvae were used as controls. Significance of mortality differences between treated Sciaridae larvae and untreated controls was estimated by using the Fisher test.

RESULTS

Chemistry of Secretion

By GC-MS techniques last-stage larvae of the two Bolitocharini species *Bolitochara obliqua* and *Leptusa* spec. (Staphylinidae) and of *Hypophloeus versipellis* (Tenebrionidae) have been shown to contain quinones/hydroquinones, aromatics, naphthoquinones, hydrocarbons, and esters within their abdominal glands (Figures 1 and 2).

Quinones. EI-mass spectra of compounds **4, 6, 8,** and **28** exhibited molecular ions of 122 **(4),** 138 **(6),** 152 **(8),** and 136 **(28).** Both consecutive elimination of 28 amu in **4/6/28** and typical fragmentation patterns in **4, 6, 8,** and **28** revealed the presence of the four 1,4-benzoquinones methyl-1,4-benzoquinone (**4,** Figure 2), methoxy-1,4-benzoquinone (**6:** only present in *Leptusa* species), 2-methyl-3-methoxy-1,4-benzoquinone (**8,** Figure 2), and ethyl-1,4-benzoquinone (**28:** only present in *Hypophloeus*; Figure 2). Structure of **4, 8,** and **28** was confirmed by coinjection of synthetic **(4, 28)** or natural compound **8** (from tergal glands of adults of *Leptusa pulchella*) (Steidle and Dettner, 1993).

Hydroquinones. According to their EI mass spectra and comparisons with authentic constituents, compounds **14** (M^+: 124, 107, 105, 95, 77, 67), **15** (M^+: 154, 139, 124, 11, 93, 82, 65; exclusively in *Leptusa* species) and **36** (M^+: 138, 136, 123, 108, 79, 54; *Hypophloeus*) were identified as toluhydroquinone **(14),** methoxytoluhydroquinone **(15),** and ethyl-1,4-hydroquinone **(36).**

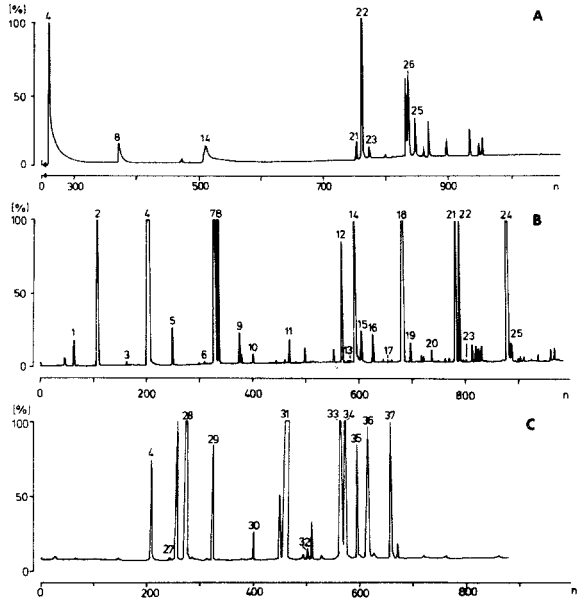

FIG. 1. Total ion current chromatograms of defensive gland secretions of (A) *Bolitochara obliqua* (Staphylinidae, 20 m SE-30 column), (B) *Leptusa* species (Staphylinidae, 12.5 m OV-1701 column) and (C) *Hypophloeus versipellis* (Tenebrionidae, 12.5-m OV-1701 column). For explanation of compound numbers see text.

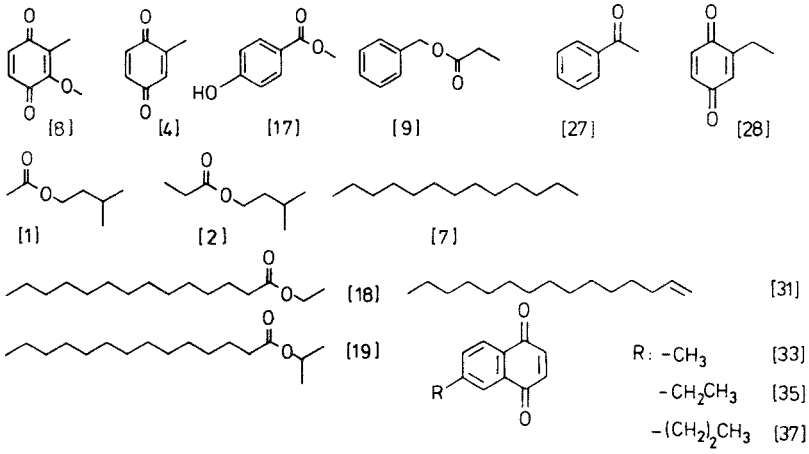

FIG. 2. Typical defensive compounds from defensive glands of Aleocharinae **(1, 2, 4, 7–9, 17–19)** and Tenebrionidae **(4, 27, 28, 31, 33, 35, 37)** larvae. For explanation of compound numbers see text.

Further Aromatics. In *Leptusa* species there were identified two further aromatics exhibiting molecular masses of 164 (**9:** 108, 91, 79, 77, 65, 57) and 152 (**17:** 121, 93, 65, 57, 44), which were indistinguishable from benzyl propionate (**9,** Figure 2) and methyl 4-hydroxybenzoate (**17,** Figure 2). The trace constituent **27** had a molecular ion of 120 (further fragments at *m/z* 105, 77, 51) and was identical (retention time; EI mass spectrum) with authentic acetophenone (**27,** Figure 2).

Naphthoquinones. The three main constituents of *Hypophloeus* larvae had molecular masses 172 **(33),** 186 **(35),** and 200 **(37),** which were confirmed by M+1 fragments (CI). They broke down in a well-defined manner and showed the following EI mass spectra: **33** (172, 157, 144, 118, 116, 115, 90, 89, 74, 633), **35** (186, 171, 158, 143, 130, 129, 118, 115, 102, 90, 89), and **37** (186, 185, 172, 171, 157, 144, 143, 129, 128, 118, 115, 102, 89). The three compounds exhibited prominent fragments at M−28 and M−56 (consecutive elimination of CO), which favored the presence of naphthoquinones (Bowie et al., 1965). Distinct fragments at *m/z* 118 (rel. intensities in **33:** 60%, **35:** 50%, **37:** 15%) and *m/z* 90 (rel. intensities in **33:** 45%, **35:** 40%, **37:** 25%), but missing fragments at *m/z* 104 and *m/z* 76, excluded the location of the alkyl group at C-2 as was evident after comparison with a synthetic sample of 2-methyl-1,4-naphthoquinone [*m/z* 118 and 90 absent, *m/z* 104 (70), *m/z* 76 (78)]. For the remaining possibilities, 5-methyl- and 6-methyl-1,4-naphthoquinone, the 5-isomer could be ruled out because: (1) Fragment at *m/z* 127 (synthetic 5-methyl-1,4-naphthoquinone: rel. int. 20%) was absent in both the larval methyl-1,4-naphthoquinone and the synthetic 6-methyl-1,4-naphthoquinone. (2) Relative intensity of fragment at *m/z* 118 exceeds 50% in the larval (65%) and the synthetic sample of 6-methyl-1,4-naphthoquinone (81%) but is only 18% in synthetic 5-methyl-1,4-naphthoquinone. The presence of 6-methyl-1,4-naphthoquinone in the larval secretion was further confirmed by its retention time (scan number 635), which shows the following values for synthetic samples: 611 scans (5-methyl-1,4-naphthoquinone), 622 (2-methyl-1,4-naphthoquinone), and 637 (6-methyl-1,4-naphthoquinone). Compounds **33, 35,** and **37** were therefore identified as 6-methyl-1,4-naphthoquinone (**33,** Figure 2), 6-ethyl-1,4-naphthoquinone (**35,** Figure 2), and 6-propyl-1,4-naphthoquinone (**37,** Figure 2). Compound **37** showed no presence of a distinct M−15 fragment, which would indicate the presence of isopropyl-1,4-naphthoquinone.

Hydrocarbons. In *Leptusa* species abdominal gland secretions alkanes, including undecane **(3),** dodecane **(5),** tetradecane **(10),** and pentadecane **(11)** were identified as minor constituents; tridecane (**7,** Figure 2) was the only main constituent. Mass spectra and retention times of the five compounds matched those of authentic alkanes. In *Hypophloeus*, a homologous group of straight-chained alkenes has been identified that coeluted with appropriate 1-alkenes. Therefore the presence of 1-tridecene **(29),** 1-tetradecene **(30),** 1-pentadecene

(**31,** main constituent, Figure 2), 1-hexadecene **(32),** and 1-heptadecene **(34)** seems possible (although no alkene references with other double bond positions were available). Other monounsaturated hydrocarbons of the secretion did not coelute with 1-alkene references and are probably characterized by other double bond positions.

Esters: The two Bolitocharini species synthesize a broad array of ethyl-, isopropyl-, and isoamyl esters. Secretions of both Bolitocharini larvae contained several compounds **(12, 16, 18, 20–22, 24–26)** with diagnostic fragments at *m/z* 88 and 101. Molecular ions could be recorded for compounds **12** (228), **16** (242), **18** (256), **20** (270), **22** (284), and **25** (312). A comparison of retention times and EI mass spectra with authentic ethyl esters confirmed the presence of ethyl dodecanoate **(12),** ethyl tridecanoate **(16),** ethyl tetradecanoate (**18,** Figure 2), ethyl pentadecanoate **(20),** ethyl hexadecanoate **(22),** and ethyl octadecanoate **(25).** Of the unsaturated ethyl esters, compound **21** (M^+: 282) coeluted with authentic ethyl palmitoleate, compound **26** (M^+: 310) with authentic ethyl oleate and compound **24** with authentic ethyl linoleate (M^+: 308). Although EI-mass spectroscopic data were indistinguishable for larval unsaturated ethyl esters and authentic samples, an exact determination of double-bond positions was not possible due to the minute amounts of larval secretion available.

Leptusa species larvae additionally contained three compounds [**13, 19, 23** (also in *Bolitochara* larvae)] characterized by diagnostic fragments at *m/z* 60, 61, 102, M−41, M−42 and molecular masses of 242 **(13),** 270 **(19),** and 298 **(23).** Retention times and fragmentation patterns of these compounds were identical with those of isopropyl dodecanoate **(13),** isopropyl tetradecanoate (**19,** Figure 2) and isopropyl hexadecanoate **(23).** EI-MS fragmentations of compounds **1** and **2** in *Leptusa* species (**1:** 98, 95, 85, 81, 70, 57, 55; **2:** 101, 75, 70, 57, 55) and retention times were indistinguishable from those of synthetic isoamylacetate (**1,** Figure 2) and isoamylpropionate (**2,** Figure 2).

Morphology and Depletion of Glands

Both representatives of Bolitocharini larvae (*Bolitochara, Leptusa*) have a dorsoventrally compressed body with a protuberant abdominal tergum VIII extending posteriorly over segment IX (Figure 3A, D). The posterior border of this protuberance bears a transverse slit (Figure 3A). Both Bolitocharini larvae exude gland material by revolving their abdominal tips against any arthropods they contact (predators: ants, adult *Xantholinus* rove beetles; cooccurring insects: bark-beetle and longhorn-beetle larvae, fly larvae). Contacted animals are therefore topically treated with small secretion droplets, which may be applied and distributed by using setae of the abdominal tip and of the protuberance as brushes. The inflexible Bolitocharini reservoir extends anteriorly to the frontal border of tergum VIII (Figure 3D). Its wall consists of circular sclerotized ridges that

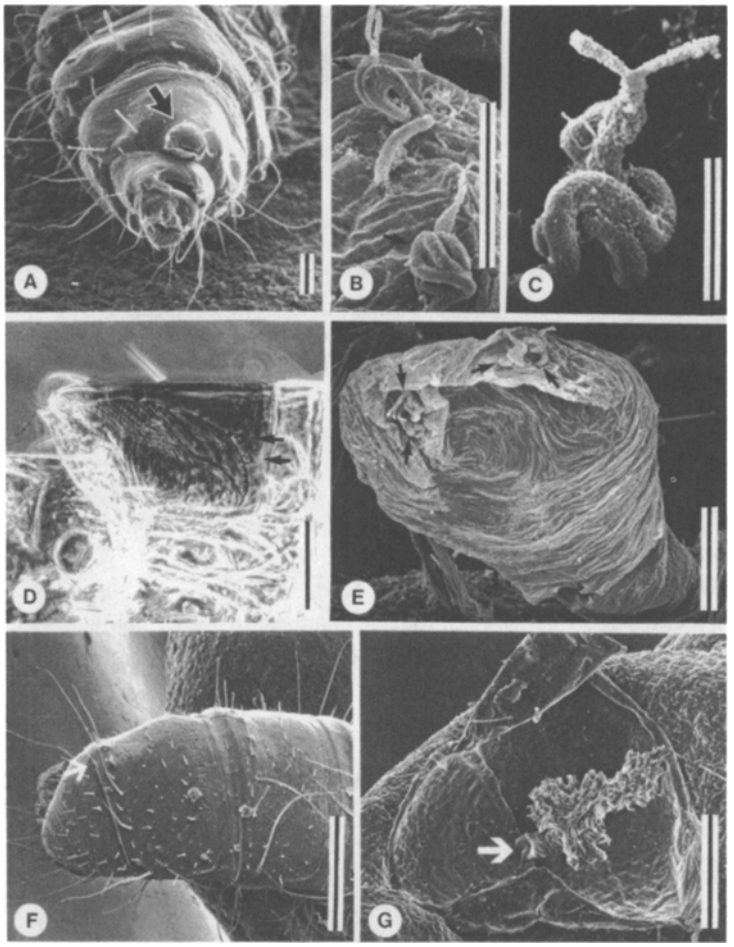

FIG. 3. SEM and light microscopic photographs of abdominal defensive gland of *Leptusa* species (Staphylinidae: Bolitocharini; A–E) and *Hypophloeus versipellis* (Tenebrionidae: Hypophloeini; F, G). (A) Caudal view of abdominal tip with protuberant abdominal tergum VIII (arrow). (B, C) Looped gland tubules with divided apex from macerated glands. (D) Side view (light microscopy) of KOH-macerated tergum VIII with interiorly situated sclerotized gland reservoir and two gland tubules (arrows). (E) Apical surface of KOH-macerated gland reservoir showing four gland tubules (arrows). (F) Dorsolateral view of abdominal tip of *Hypophloeus* larva showing gland reservoir opening located at anterior border of tergite VIII (arrow). (G) Interior view (SEM) of macerated tergites VIII/IX), showing irregularly folded gland reservoir and insertion of efferent duct at anterior border of tergite IX (arrow); pincer-like structures are situated below efferent duct at anterior border of tergite IX. Reference bars: A, F = 250 μm, B, D, E = 50 μm, C = 10 μm, G = 200 μm.

resemble tracheal taenidia (Figure 3D, E). Anteriorly, the gland reservoir bears four voluminous tubule-bearing gland cells (Figure 3D, E). Gland tubules of *Leptusa* are characterized by two loops and show a distinctly divided apex (Figure 3B, C).

In *Hypophloeus* larvae, glandular morphology and exudation of secretion are extremely peculiar and are here described for the first time. Tergum VIII bears no protuberance as in rove-beetle larvae and the reservoir opening of the tergal gland is situated at the anterior border of tergite IX (Figure 3F, 4A, B). This area bears two strongly sclerotized pincers (Figure 4B), which act as a support for the soft efferent duct of the gland reservoir (Figure 3G). Unlike those of Bolitocharini larvae, gland reservoirs of *Hypophloeus* are completely membranous. When completely filled with the reddish secretion, they may extend frontally just beneath the seventh tergite (Figure 3G). Gland cells on the reservoir surface do not survive KOH treatment.

The efferent duct of the reservoir runs posteriorly below the sclerotized hind border of tergum VIII, passes below the intersegmental membrane VIII/IX, and turns up to reach the sclerotized pincers of tergum IX, which represent the external reservoir opening (Figure 3G, 4B).

On molestation, larvae of *Hypophloeus* bend their hind bodies towards the aggressor, and small droplets of the defensive secretion are shot anteriorly. The defensive fluid may spread frontally along a slightly depressed, longitudinal central area of tergites VIII to VI (Figure 4B). At the posterial border of tergite VIII, the setal row is interrupted centrally in order to "clear the field of fire." Opposite to the movable pincers of anterior tergite IX, the centrally situated caudal area of tergite VIII also has a faintly depressed troughlike surface (Figure 4B). This peculiarly structured hind surface of tergite VIII allows the secretion from the slitlike reservoir opening to be shot anteriorly. The sclerotized caudal surface of tergite VIII represents a support for the reservoir opening and functions like the notch of a rear sight. In order to project secretion onto the surfaces of tergites, the frontal part of tergum IX is slightly elevated, and at the same time the pincers are brought near to the depressed posterior border of sclerotized tergite VIII (Figure 4B). The secretion may adhere to the surface of anteriorly situated tergites about several minutes or so, until complete evaporation has been achieved. This peculiar evacuation of a gland reservoir is even possible when the tenebrionid larvae inhabit restricting cavities such as bark beetle frass-galleries, where the abdominal tips cannot be revolved dorsally.

Laboratory Bioassays and Field Observations

Topical Irritancy of Synthetic Quinone Mixtures as Found in Leptusa *Secretion.* Selected constituents of the Bolitocharini secretions were topically tested against full-grown *Calliphora* larvae by recording contraction time and number of reacting specimens. The compounds were tested alone or in admixture with

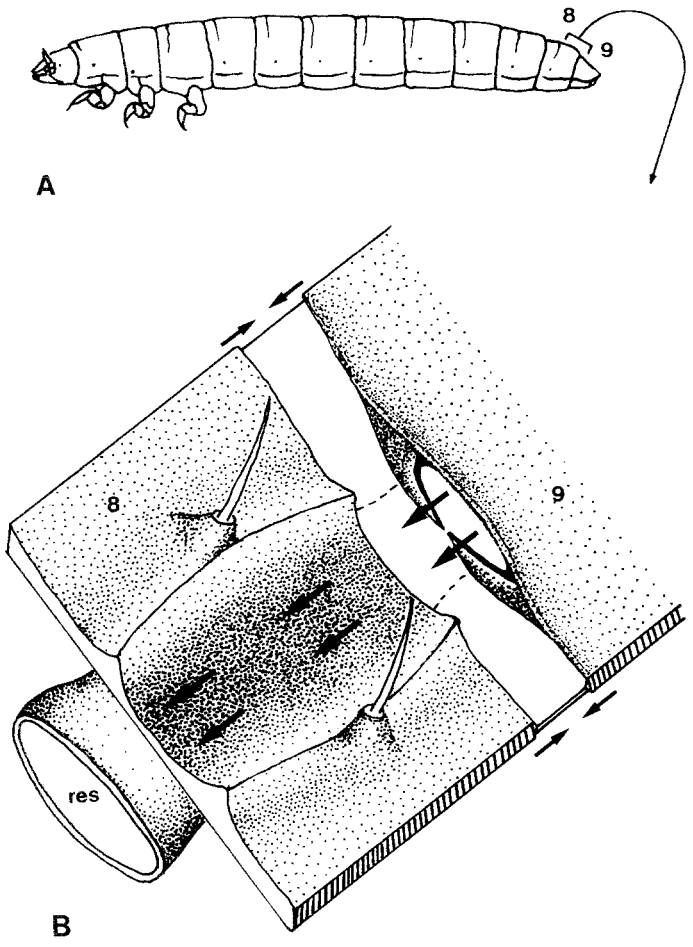

FIG. 4. Side view of a *Hypophloeus versipellis* larva (A). Schematic drawing (B, surface view) symbolizes pincer-like structures of reservoir opening at anterior border of tergite IX and troughlike surface of adjacent tergite VIII. Direction of anteriorly projected defensive secretion is indicated by parallel arrows. Opposed small arrows indicate movement of pincers when they are brought near to the depressed posterior border of tergite VIII. Interrupted lines below intersegmental membrane symbolize position of efferent duct of defensive gland reservoir (res: gland reservoir; 8, 9: tergites VIII and IX).

the main compound *p*-toluquinone (Figure 5). Apart from solutions of toluquinone, no tested Bolitocharini constituent or nature-identical mixture showed any significant topical irritancy, as indicated by the low number of three to seven contracting larvae per experiment. Only pure ethyl octadecanoate showed some

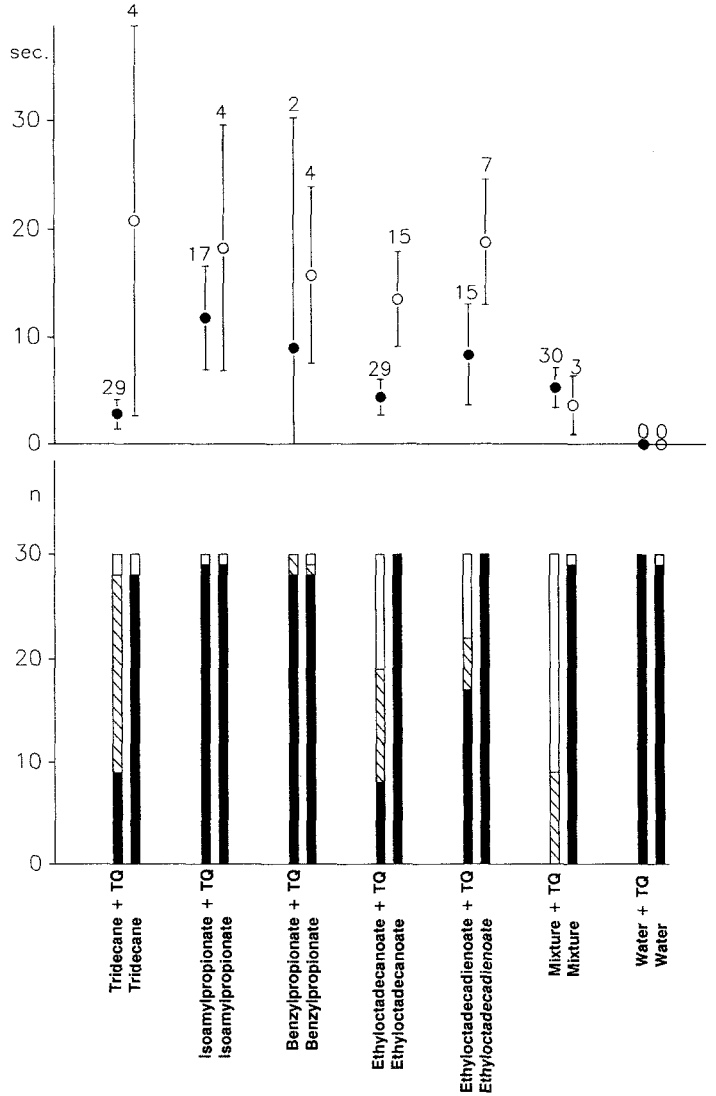

FIG. 5. Topical irritancy (top), mortality and development-delaying effect (bottom) of solvents from *Leptusa* secretion with (saturation) and without *p*-toluquinone. Top: Constriction time ($\bar{X} \pm$ SD) of larvae of *Calliphora vomitoria* previously topically treated with 2 μl of quinone solvents (solid mean symbol) and pure solvents (open mean symbol). Numbers indicate reacting larvae ($N = 30$ per test). Bottom: Intact pupae (black), damaged pupae (hatched), and dead larvae (white) four days after having been topically treated with 2 μl of fluids as indicated above. For composition of *Leptusa* mixture see Methods and Materials.

minor irritancy, with 15 positively reacting specimens and a medium contraction time of about 12 sec. Varying topical irritancies of quinoic defensive secretions actually correspond with the degree of penetration of quinone through the target's integument, into the body cavity of the fly larvae (Dettner and Grümmer, 1986; Dettner, 1991). As a consequence, all quinone-saturated *Leptusa* solvents exhibited an increased topical irritancy against *Calliphora* larvae as shown by increased numbers of contracting larvae and lower medium contraction times. When saturated with toluquinone, only tridecane, ethyl octadecanoate, and a mixture of 13 *Leptusa* solvents exhibited drastic irritant effects (number of contracting larvae: 29–30; medium contraction time below 5 sec). Therefore isoamyl propionate, benzyl propionate and ethyl linoleate are not effective quinone solvents and probably do not significantly contribute to the topical irritancy of the multicomponent defensive secretion.

These observations are confirmed or modified by recordings of mortalities or development-delaying effects on the targeted larvae. Four days afterwards, larvae previously treated with pure solvents had completely pupated, just like the water controls. Quinone-saturated ethyl linoleate shows a moderate topical activity, whereas quinoic tridecane or ethyl octadecanoate solutions produce drastic effects documented by lowered numbers of intact pupae along with damaged pupae and dead larvae. With increased numbers of dead larvae and damaged pupae, the synthetic quinone-containing *Leptusa* mixture showed the most pronounced irritant effect.

Topical Irritancy of Hypophloeus *Secretion.* The natural secretion of *Hypophloeus* larvae was topically tested against two unidentified species of Sciaridae larvae, which were found together with the beetle larvae under the same fragments of bark. Especially under moistened bark, these fly larvae aggregate in bark-beetle interstices. In the laboratory, it was observed that *Hypophloeus* larvae occasionally evacuated the defensive gland if they had mechanical contact with aggregations of jerking fly larvae and if the space available under the bark was limited.

Under more artificial conditions, vigorous touching of the gland reservoir opening of a *Hypophloeus* larva with a fly larva evoked a nearly complete depletion of gland reservoir. In this way, surface areas of the fly larvae were more or less topically treated with the secretion of the darkling beetle larvae. In many cases, a distinct contraction of the fly larvae was also evident.

As compared with the untreated control, topically treated fly larvae of both species always showed increased mortality rates. In the larger-sized yellow fly larvae (Figure 6 top), significantly increased mortalities ($^{**}P \leq 0.01$) were observed only between 50 and 96 hr after applying the defensive secretion. The smaller white fly larvae (Figure 6 bottom) even showed significantly ($^{*}P \leq 0.05$) increased mortalities directly after topical treatment with the secretion. After 50 hr, this effect was highly significant ($^{***}P \leq 0.001$). Due to increased

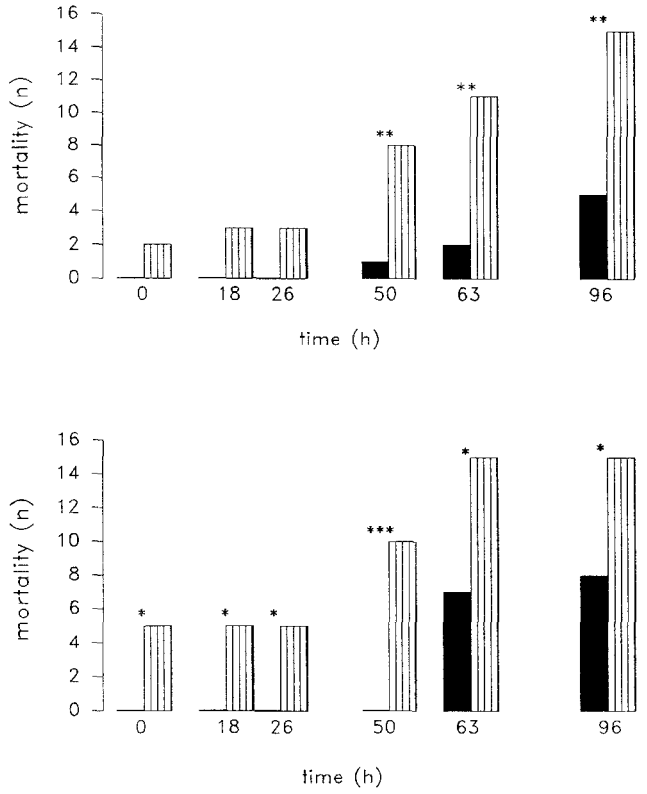

FIG. 6. Mortalities (number of dead larvae) of Sciaridae larvae (top: large yellow larvae; bottom: small white larvae), each of which had been topically treated with the defensive secretion of a *Hypophloeus* beetle larva. Mortalities at different times (0–96 hr) after topical treatment of the fly larvae are indicated as black (untreated control) and hatched (treated fly larvae) bars. Twenty larvae were used per experiment. Significance of differences between controls and topically treated groups as calculated by using Fisher's test (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

mortalities of untreated controls, the mortality differences at 63 and 96 hr only were significantly different (*).

DISCUSSION

Secretion Chemistry

In the contrast to chrysomelid beetles, in which the defensive secretions of larvae differ considerably from those of chemically protected adults (Dettner, 1987), the secretions of the two Aleocharinae and the tenebrionid larvae studied

here chemically resemble the abdominal gland constituents of corresponding adult rove beetles and darkling beetles (Tschinkel, 1975; Steidle and Dettner, 1993). This holds true not only for naphthoquinones but also for hydrocarbons or esters. Most compounds have been also detected in other arthropods (Blum, 1981). The more erratically distributed larval gland constituents include acetophenone (Ansteeg and Dettner, 1991), benzyl propionate, methyl hydroxybenzoate (Dettner, 1987), and naphthoquinones. The occurrence of naphthoquinone and its 6-alkyl derivatives seems very restricted in arthropods [darkling beetle genus *Argoporis* (Tschinkel, 1972) opilionids of the genus *Phalangium* (Wiemer et al., 1978)]. At the moment, it would be premature to discuss chemotaxonomic consequences with respect to larval secretions, since only two Aleocharinae species have been studied. However, it is striking that *Leptusa* and *Bolitochara* (Bolitocharini) dissolve their benzoquinone derivatives in ester or alkane solvents, which show considerable similarity between the two genera. *Hypophloeus* larvae not only possess morphologically different glands but also show considerable chemical discrepancies in synthesizing both benzoquinone and naphthoquinone derivatives and dissolving them in alkenes.

As to biological significance, these beetle larval defensive secretions act as topically active irritants based on their solid benzo- and naphthoquinones, which are dissolved with relatively inert solvents (Dettner, 1991; Peschke and Eisner, 1987). On the other hand, some compounds, such as methyl-*p*-hydroxybenzoate and also phenyl propionate or various benzo- and naphthoquinones, show antimicrobical effects. Acetophenone may act principally as a potent fumigant (Dettner et al., 1992), but its actual concentrations within larval secretions are extremely low and rule out this potential activity. Alkanes, alkenes, and long-chained esters exhibit no fumigancy against arthropods (Dettner et al., 1992), although possible fumigant activities of low-molecular-weight esters such as isoamyl acetate **(1)** and isoamyl propionate **(2)** cannot be ruled out. Highly effective fumigants may contribute to the overall defensive activity of the larval secretions because these beetle larvae inhabit small interstices under bark where volatile fumigants may be enriched to a certain extent (Dettner et al., 1992).

Morphology and Occurrence of Larval Defensive Gland in Aleocharinae

Various authors have recognized that many Aleocharinae larvae possess an unpaired exocrine gland associated with the eighth abdominal tergum. Moore (1978) stated that the presence/absence of this peculiar larval gland might be a first step toward a natural classification of this huge subfamily. In the meantime, gross morphology and distribution of this gland have been characterized in other aleocharine larvae (Ashe, 1981a, b, 1985, 1991; Ashe and Watrous, 1984; Ashe and Wheeler, 1988; Frank and Thomas, 1984). Until now, this apparently homologous larval gland of Aleocharinae has been identified in Myrmedoniini,

Drepanoxenini, Crotocini, Oxypodini, Athetini, Falagriini, Bolitocharini, Autaliini, Phytosini, and Oligotini (Frank and Thomas, 1984). Three morphological peculiarities of this larval gland have been noted: (1) Within Oligotini the colored reservoir represents an osmeterium that is everted on molestation (Moore et al., 1975). (2) Known representatives of Athetini, Oxypodini, Falagrini, Myrmedoniini, Drepanoxenini and Crotocini possess an eighth abdominal tergum in which the presence of an internal gland does not have a noticeable external manifestation (Frank and Thomas, 1984). (3) In Bolitocharini (except *Placusa*, now separated as Placusini), Autaliini (except *Euvira*, now: Placusini), and Phytosini, the eighth abdominal tergite bears a posterior process where the opening of the noneversible gland reservoir is located (Ashe, 1981a, 1985, 1991). As demonstrated for the genera *Bolitochara* and *Leptusa*, this reservoir is more or less darkened, and its wall consists of circular sclerotized ridges. Uniquely, posterior setae of the abdominal protuberance of most gyrophaenine larvae may be flattened, spatulate, or finally serrated apically to form a brushlike structure (Ashe, 1985). In view of the chemistry and topical irritancy of the bolitocharine secretion, it is now suggested that these peculiar brushlike setae serve for topically applying and distributing defensive secretions on small target organisms. Certainly this type of protuberant eighth tergite gland does not generate the silken coccoon (see Ashe, 1982) but represents a true defensive gland, as now evidenced by secretion analysis, bioassays and behavioral observations.

Unfortunately no glandular data from larval stages are available for the more primitive taxa Gymnusini, Deinopsini, and Myllaenini, which also lack the adult tergal gland (Steidle and Dettner, 1993). As observed in adult tergal glands from myrmecophilous and termitophilous Aleocharinae, a more or less complete reduction of the larval defensive gland of Aleocharinae may also be found in those larvae sharing the same habitat. This may be true for Trichopseniini, whose larvae apparently are devoid of an eighth tergite gland (Kistner and Howard, 1980). In accordance with Ashe (1991), it is suggested that the presence of an eighth tergite larval Aleocharinae gland represents a synapomorphy of advanced Aleocharinae.

Morphology of Tenebrionidae Larval Defensive Glands. Although no histological studies are available concerning glands from tenebrionid larvae, Doyen and Lawrence (1979) described a disk like structure situated in the membrane between tergites 8 and 9 that "appears to be attached to an internal reservoir." A comparable structure seems restricted to larvae of *Menimus* species (Gnathidini) (Watt, 1974), which belong to Hypophloeinae, as does the genus *Corticeus* (= *Hypophloeus*) (Doyen and Lawrence, 1979). It remains to be investigated whether this peculiar type of larval defensive gland is also present in other members of the so-called "Diaperine Lineage" of Doyen and Tschinkel (1982).

Bioassays. Topical efficiency of a defensive secretion depends mainly on the chemistry and quantity of the secretion and also on the size and species of

the target organism. On the other hand, only immediate damage to the targeted organisms after the application of the defensive secretion seems biologically useful. Therefore, the minute amounts of defensive secretions from *Hypophloeus* and bolitocharine larvae would be expected to be effective only on small target organisms that are the same size as the chemically defended beetle larvae or smaller. This is true especially of all early instars of Sciaridae fly larvae and also of Collembola, bark beetles, and their larvae. Other subcortical staphylinids or small-sized early pyrochroid and cerambycid larvae that were always found associated with *Leptusa* and *Hypophloeus* larvae therefore may be also targeted by the secretion. Targeted arthropods need not necessarily be predatory; irritation of the chemically defended beetle larvae can even occur after incidental mechanical contact within these bark interstices during the boring process, because in this habitat only limited space may be available. Although the application of 2-μl droplets of quinone mixtures to *Calliphora* larvae is certainly an artificial situation since *Leptusa* larvae exude only a fraction of this secretion, it does make it possible to check for the topical irritancy of single constituents of the secretions.

Small amounts of naphthoquinones (as little as 0.1 pg) have been identified as potent deterrents against the ant *Formica exsectoides*; toluquinone was less effective by about two orders of magnitude (Wiemer et al., 1978). This observation seems interesting, since chemically defended beetle larvae were sometimes found together with various ant species, especially if the bark area was very dry.

In the laboratory and in the field, subcortical insects may also suffer from various fungi and bacteria growing on the moist bark substrates. It seems highly probable that quinones and naphthoquinones along with aromatics such as benzyl propionate, methyl 4-hydroxybenzoate, or acetophenone may be also used as fungicides and bactericides. For example, this may be reflected in the lack of topical activity of the aromatic compound benzyl propionate. A further observation suggested that, under bark, benzo- and naphthoquinones might also act as effective fungicides: In the laboratory, several dead specimens of *Hypophloeus* larvae were completely covered by fungal hyphae like a cotton-wool ball. Only the clean abdominal tips of the larvae with their reservoir openings projected out of this ball. The antibiotic 6-alkylnaphthoquinones and other antimicrobials seem the most likely agents for this effect.

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REFERENCES

ANSTEEG, O., and DETTNER, K. 1991. Chemistry and possible biological significance of secretions from a gland discharging at the 5th abdominal sternit of adult caddisflies (Trichoptera). *Entomol. Gener.* 15:303–312.

ASHE, J.S. 1981a. Studies of the life history and habits of *Phanerota fasciata* Say (Coleoptera: Staphylindae: Aleocharinae) with notes on the mushroom as a habitat and descriptions of the immature stages. *Col. Bull.* 35:83–96.

ASHE, J.S. 1981b. Construction of pupal cells by larvae of Aleocharinae (Coleoptera: Staphylinidae). *Col. Bull.* 35:341–343.

ASHE, J.S. 1985. Structural features and phylogenetic relationships among larvae of genera of gyrophaenine staphylinids (Coleoptera: Staphylinidae: Aleocharinae). *Zoology* 30:1–60.

ASHE, J.S. 1991. The systematic position of Placusa Erichson and Euvira Sharp: The tribe Placusini described (Coleoptera: Staphylinidae: Aleocharinae). *Syst. Entomol.* 16:383–400.

ASHE, J.S., and WATROUS, L.E. 1984. Larval chaetotaxy of Aleocharinae (Staphylinidae) based on a description of *Atheta coriaria* Kraatz. *Col. Bull.* 38:165–179.

ASHE, J.S., and WHEELER, O.D. 1988. Revision of Tachiona sharp (Coleoptera: Staphylinidae: Aleocharinae) with a description of the larva of *T. latipennis*, new species, and a preliminary assessment of generic relationships. *J. N.Y. Entomol. Soc.* 96:176–199.

BLUM, M.S. 1981. Chemical Defenses of Arthropods. Academic, New York, 562 pp.

BOWIE, J.H., CAMERON, D.W., and WILLIAMS, D.H. 1965. Studies in mass spectrometry. 1. Mass spectra of substituted naphthoquinones. *J. Am. Chem. Soc.* 87:5094–5099.

BRASS, P. 1914. Das 10. Abdominalsegement der Käferlarven als Bewegungsorgan. *Zool. Jahrb. Abt. Syst. Ökol. Geogr. Tiere* 27:66–121.

DETTNER, K. 1987. Chemosystematics and evolution of beetle chemical defenses. *Annu. Rev. Entomol.* 32:17–48.

DETTNER, K. 1991. Solvent-dependent variability of effectiveness of quinone-defensive systems of Oxytelinae beetles (Coleoptera: Staphylinidae). *Entomol. Gener.* 15:275–292.

DETTNER, K., and GRÜMMER, R. 1986. Quasisynergism as evolutionary advance to increase repellency of beetle defensive secretions. *Z. Naturforsch.* 41c:493–496.

DETTNER, K., FETTKÖTHER, R., ANSTEEG, O., DEML, R., LIEPERT, C., PETERSEN, B., HASLINGER, E., and FRANCKE, W. 1992. Insecticidal fumigants from defensive glands of insects—a fumigant test with adults of *Drosophila melanogaster*. *J. Appl. Entomol.* 113:128–137.

DOYEN, J.T., and LAWRENCE, J.F. 1979. Relationships and higher classification of some Tenebrionidae and Zopheridae (Coleoptera). *Syst. Entomol.* 4:333–377.

DOYEN, J.T., and TSCHINKEL, W.R. 1982. Phenetic and cladistic relationships among tenebrionid beetles (Coleoptera). *Syst. Entomol.* 7:127–183.

FRANK, J.H., and THOMAS, M.C. 1984. Cocoon-spinning and the defensive function of the median gland in larvae of Aleocharinae (Coleoptera, Staphylinidae). A review. *Quaest. Entomol.* 20:7–23.

HÖLLDOBLER, B., MÖGLICH, M., and MASCHWITZ, U. 1982. Myrmecophilic relationship of *Pella* (Coleoptera: Staphylinidae) to *Lasius fuliginosus* (Hymenoptera: Formicidae). *Psyche* 88:347–374.

KISTNER, D.H., and HOWARD, R.W. 1980. First report of larval and pupal trichopseniinae: External morphology, taxonomy and behavior (Coleoptera: Staphylinidae). *Sociobiology* 5:3–20.

KLAUSNITZER, B. 1978. Ordnung Coleoptera (Larven). Dr. W. Junk, b.v. Publishers, The Hague, The Netherlands, pp. 223–248.

MOORE, I. 1978. Usefulness of larval osmeteria in determining natural classification in aleocharinae (Coleoptera: Staphylinidae). *Entomol. News* 89:245–246.

MOORE, I., LEGNER, E.F., and BADGLEY, M.E. 1975. Description of the developmental stages of the mite predator, *Oligota oviformis* Casey, with notes on the osmeterium and its glands (Coleoptera: Staphylinidae). *Psyche* 82:181–187.

PAULIAN, R. 1941. Les premiers états des staphylinoidea. Mem. Mus. Nat. Hist. Nat.: 15:168–175, 284–315.

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.

STEIDLE, J., and DETTNER, K. 1993. The tergal gland of adult Aleocharinae (Coleoptera: Staphylinidae). Morphology, chemistry and phylogenetic significance. *J. Syst. Entomol.*, In press.

TSCHINKEL, W.R. 1972. 6-Alkyl-1,4-naphthoquinones from the defensive secretion of the tenebrionid beetle, *Argoporis alutacea*. *J. Insect Physiol.* 18:711–722.

TSCHINKEL, W.R. 1975. A comparative study of the chemical defensive system of tenebrionid beetles: Chemistry of the secretions. *J. Insect Physiol.* 21:753–783.

TOPP, W. 1978. Bestimmungstabelle für die Larven der Staphylinidae. Ed. Klausnitzer, B. Ordnung Coleoptera (Larven). Dr. W. Junk, b.v. Publishers, The Hague, The Netherlands, pp. 304–334.

WATT, J.C. 1974. A revised subfamily classification of Tenebrionidae (Coleoptera). *N.Z. J. Zool.* 1:381–452.

WIEMER, D.F., HICKS, K., MEINWALD, J., and EISNER, T. 1978. Naphthoquinones in defensive secretion of an opilionid. *Experientia* 34:969–970.

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CARDENOLIDES FROM *Erysimum cheiranthoides*: FEEDING DETERRENTS TO *Pieris rapae* LARVAE

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Abstract—Larvae of the cabbage butterfly, *Pieris rapae*, refuse to feed on the wild mustard, *Erysimum cheiranthoides*, due to the presence of alcohol-extractable deterrents. The active components were extracted into *n*-BuOH, and this extract was separated into four fractions (I–IV) by reverse-phase HPLC. Fractions III and IV retained the feeding deterrent activity. The activity of fraction III was found to be due to the cardenolide diglycosides **1** and **2**, which were previously reported as oviposition deterrents for gravid *P. rapae* butterflies. Three active compounds were isolated from fraction IV by column chromatography on silica gel followed by reverse-phase HPLC. These compounds were identified as a monoglycoside, digitoxigenin 3-*O*-β-D-glucoside **(4)**, and two diglycosides, glucodigigulomethyloside **(5)** and glucodigifucoside **(6)**. An additional cardenolide isolated from fraction II was identified as cheirotoxin **(7)**. All compounds were identified by UV, NMR (^{1}H and ^{13}C), and mass spectrometry, as well as hydrolysis experiments. The feeding deterrent activity of these compounds was compared with that of related commercially available chemicals and other compounds isolated from *E. cheiranthoides*.

Key Words—*Pieris rapae*, *Erysimum cheiranthoides*, feeding deterrents, cardenolides, digitoxigenin 3-*O*-β-D-glucoside, glucodigigulomethyloside, glucodigifucoside, erychroside, cheirotoxin, strophanthidin glycosides, digitoxigenin glycosides, Lepidoptera, Pyridae.

INTRODUCTION

The cabbage butterfly, *Pieris rapae* L., is a specialist on crucifers (=Brassicaceae) and a few individual species in other plant families that are characterized

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by the presence of glucosinolates (Verschaffelt, 1911). However, despite the fact that almost all crucifers contain glucosinolates, not all of these plants are acceptable to *P. rapae*. Recent studies on the oviposition behavior of *P. rapae* have demonstrated the presence of oviposition deterrents in unacceptable plants (Renwick and Radke, 1985) that are effective even in plants that contain known stimulants to oviposition. (Renwick and Radke, 1987).

Oviposition deterrents of *Erysimum cheiranthoides* L. were characterized as cardenolides (Renwick et al., 1989) and identified as the diglycosides of strophanthidin, erysimoside **(1)**, and erychroside **(2)** (Sachdev-Gupta et al., 1990). Subsequent studies by Dimock et al. (1991) have shown that *P. rapae* larvae will not feed on *E. cheiranthoides*, and feeding deterrents responsible for this behavior were extractable into *n*-butanol. However, bioassay-guided HPLC of the active *n*-butanol extract revealed that the most effective feeding deterrent fraction did not contain the most active oviposition deterrents. Fraction III, which had some feeding deterrent activity, contained the previously identified cardenolides erysimoside **(1)** and erychroside **(2)**. However, the highly active fraction IV appeared to contain unrelated cardenolides.

In this paper we report on the isolation and characterization of three feeding deterrent cardenolides **(4–6)** from fraction IV. An additional cardenolide **(7)** from the inactive fraction II was also identified and bioassayed for activity. To determine the structure requirements for bioactivity, related cardenolides were also evaluated using a feeding deterrent bioassay.

METHODS AND MATERIALS

Extraction and Fractionation of Plant Material

E. cheiranthoides was grown in a greenhouse under the conditions described earlier (Renwick et al., 1989). Fresh foliage was boiled in ethanol (50 g/500 ml) for 5 min, cooled, homogenized in a Waring blender, and filtered through glass wool. After the removal of ethanol under reduced pressure, the extract was defatted with *n*-hexane. The defatted material was dissolved in water and extracted three times with equal volumes of *n*-BuOH. The *n*-BuOH fraction was concentrated under reduced pressure at approximately 50°C.

General Techniques and Instrumentation

Reverse-phase HPLC on a preparative level was performed on a Dynamax Macro HPLC column (25 × 2.1 cm, irregular 8-μm particles, Rainin Instrument Co.). The HPLC unit consisted of a Waters model U6K LC injector, a Waters 600 multisolvent delivery system, and a Waters Lambda-Max 481 LC spectrophotometer. The elution was monitored at 254 nm. Separation was achieved

with the following solvent gradient (gradient 1) at a constant flow rate of 15 ml/min.

Time	H_2O(%)	CH_3CN(%)	Curve
0	100	0	
10	80	20	linear
40	75	25	linear
45	0	100	linear

Flash column chromatography was carried out on silica gel (Woelm, 32–63 μm). For semipreparative HPLC, a Varian 5000 liquid chromatograph equipped with a Varian Micropak C-18 column (50 × 0.8 cm, irregular 10 μm particles) was used. Peaks were detected by their UV absorption at 215 nm. The following convex gradient of H_2O and CH_3CN (gradient 2) at a flow rate of 3.3 ml/min was used for the analysis:

Time	H_2O(%)	CH_3CN(%)
0	75	25
5	75	25
45	60	40
50	0	100

Thin-layer chromatography (TLC) of cardenolides was carried out on precoated silica gel plates (10 × 5 cm, 250 μm thickness, K6 silica gel, Whatman) in EtOAc–MeOH–H_2O (8:1:0.8, solvent system 1, for glycosides) or $CHCl_3$–MeOH (19:1, solvent system 2, for aglycones). Spots were visualized by spraying the plates with Kedde's reagent (Krebs et al., 1969), or with a 1% solution of ceric sulfate in 2 N H_2SO_4 followed by heating at 110°C for 30 min. Sugars were analyzed by chromatography on high-performance TLC cellulose plates. These chromatograms were developed in HCOOH–EtCOMe–*t*-BuOH–H_2O (15:30:40:15, solvent system 3). For visualization of spots, the plates were sprayed with aniline–phthalate reagent followed by heating at 110°C for 30 min (Stahl, 1969).

Gas chromatographic analysis of trimethylsilyl (TMS) derivatives of sugars was conducted on a Perkin Elmer model 3920 gas chromatograph equipped with a glass column (2 m × 2 mm) packed with 3% OV-101 supported on Chromosorb W-HP (mesh range 100–120), and a FID detector. The injector and detector temperatures were maintained at 280°C. The flow rate of the carrier gas, N_2, was kept at 40 ml/min. The initial temperature was kept constant at 100°C for 4 min and then increased to 305°C at 8°C/min.

Positive-ion FAB mass spectral analysis in a thioglycerol matrix was carried out on a Kratos MS-50 mass spectrometer and the molecules were bombarded with xenon atoms at 8 kV energy. High-resolution ^1H and ^{13}C NMR spectra in

pyridine-d_5 were recorded on a Varian XL-400 instrument. The solvent was used as an internal reference.

Isolation

The *n*-BuOH fraction was separated into four fractions (I–IV) by preparative reverse-phase HPLC [200 g leaf equivalents (gle)/injection] using gradient 1. Eluates were pooled between 23 and 27, 27 and 36 min, and 36 and 45 min, which corresponded to fractions II, III, and IV, respectively. These fractions were similar to fractions 2, 3, and 4 reported by Dimock et al. (1991). Further analysis of fraction III was accomplished by column chromatography on silica gel followed by reverse-phase HPLC as described previously (Sachdev-Gupta et al., 1990). Compounds **1, 2,** and **3** (Figures 1 and 2) were isolated from this fraction.

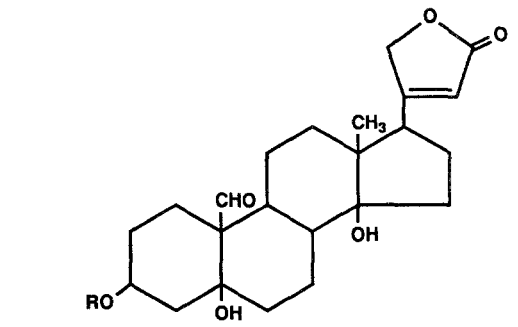
Fraction IV was subjected to flash chromatography on silica gel (Woelm, 32–63 μm). The column (2 g silica gel/100 gle fraction) was packed in EtOAc saturated with water [EtOAc (H_2O)]. The fraction, dissolved in MeOH, was adsorbed on a small amount of silica gel, dried under reduced pressure at ambient temperature, and then applied on the column. Fractions were eluted as follows: 1–5 (100 ml each) with EtOAc(H_2O)–MeOH (99.5:0.5), fractions 8–15 (75 ml each) with EtOAc(H_2O)–MeOH (99:1), fractions 16–20 (100 ml each) with EtOAc(H_2O)–MeOH (98:2), and fraction 21 with MeOH. The active fractions 3 and 4, 9–11, and 12–15 were further separated by HPLC on a C-18 column using gradient 2 to yield **4, 5,** and **6,** respectively (Figure 3). Repeated column chromatography of fraction II on silica gel (Woelm, 32–63 μm, 1.5 g/100 gle fraction) led to the isolation of **7.** Fractions containing **7** were eluted with EtOAc(H_2O)–12% MeOH.

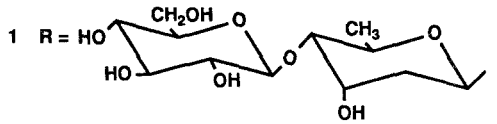
Hydrolysis of Pure Compounds

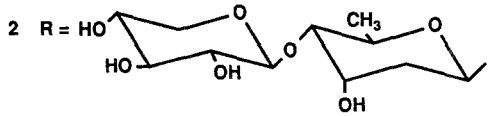
The compounds were subjected to hydrolysis under three different conditions as described below:

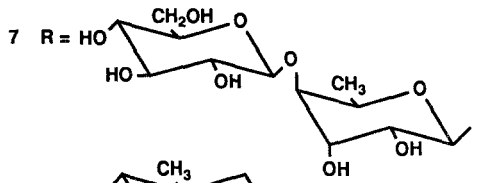
Enzyme-Catalyzed Hydrolysis. Each compound (2 mg) was dissolved in acetate buffer (pH = 5, 500 μl). After the addition of β-glucosidase enzyme (EC 3.2.1.2.1, from almonds, Sigma), the mixture was incubated for 24 hr. The hydrolysis products were separated into H_2O-soluble and MeOH-soluble fractions by solid-phase extraction using C-18 disposable extraction columns (J.T. Baker, spe columns, 3 ml, 40 μm particles). The MeOH-soluble fraction was analyzed for the aglycone and the H_2O-soluble fraction was analyzed for sugars by TLC in solvent system 3 and by GLC of the TMS derivative.

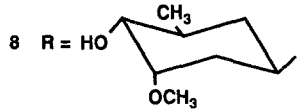
Mild Acid Hydrolysis. Each compound (2 mg) was treated with 0.05N HCl in 50% aqueous dioxane (500 μl). After heating for 45 min at ~50°C, the reaction mixture was diluted with water (500 μl), dioxane was removed under

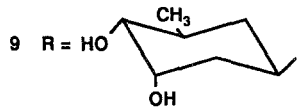












10 R = H

FIG. 1. Structures of strophanthidin derivatives.

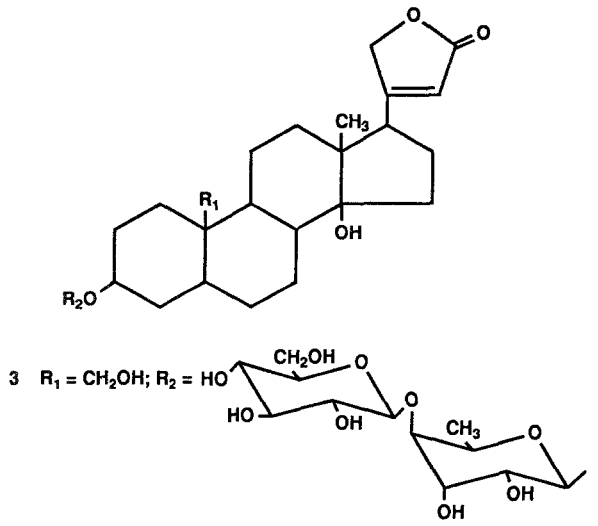


FIG. 2. Structure of erycordin.

reduced pressure, and the aqueous fraction was neutralized with Bio Rex[5] (CO_3^{2-} form) resin. The sugars present in this fraction were analyzed by TLC in solvent system 3 and by GLC of their TMS derivatives.

Hydrolysis with 2 N HCl. Each compound (2 mg) in 2 N HCl (in 50% aqueous dioxane, 500 μl) was heated at 80°C for 1.5 hr. The reaction mixture was then processed and analyzed as described in the preceding paragraph.

Preparation of Trimethylsilyl Derivatives of Sugars

In an airtight reaction vial, the aqueous fraction containing sugars was dissolved in Sil-Prep (Alltech Associates) silylating agent (100 μl) and stirred vigorously for 5 min. The mixture was heated at 80°C for 20 min. Analysis of the derivatives was done by GLC. TMS derivatives of standards, D-glucose, L-fucose (Sigma), and 6-deoxy-D-gulose (a gift from Dr. L.M. Lerner, SUNY, Brooklyn, New York) were prepared under similar conditions.

Bioassay

Binary choice feeding tests were conducted as described by Dimock et al. (1991) with some modifications. Leaf disks (177 mm^2) were cut with a cork borer from cabbage plants (var. Golden Acre) that were grown in a greenhouse with supplemental lighting provided by 400-W multivapor lamps with a 16:8 hr light–dark cycle and day–night temperatures of 23:17°C. Test compounds were applied to each side of leaf disks at concentrations of 1, 10, and 100 ng/

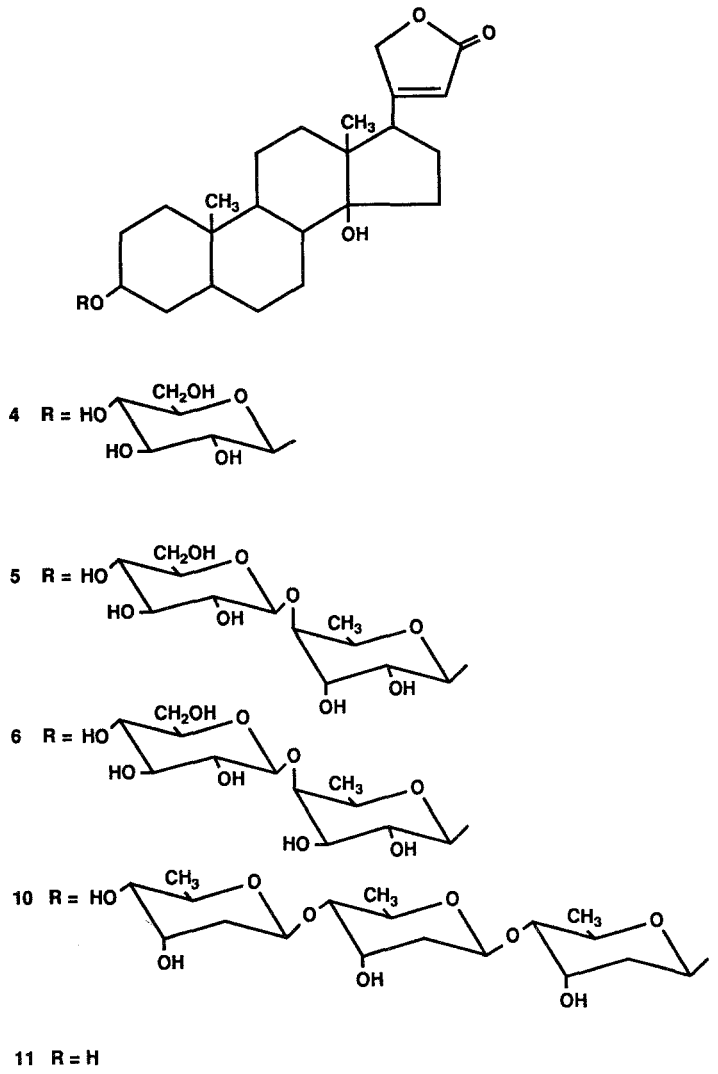


FIG. 3. Structures of digitoxigenin derivatives.

cm^2 in 10 μl of methanol using a Wheaton Step-Pette pipettor. After application to one side, the leaf disks were dried under an airstream before applying the solution to the second side. Control disks were treated with methanol alone. The disks were sandwiched between two 16-mm^2 disks of dental rubber dam for support and fastened with No. 3 insect pins. Six disks treated with the same compound and concentration, and six control disks were placed in each cup.

The disks were pinned approximately 5 mm above the surface in an alternating circle in a 250 ml waxed cup lined with paraffin wax on the bottom to support the pins. Wet filter paper was placed on the paraffin to add moisture. There were three to five replicates for each compound tested.

Fourth-instar larvae used for bioassays were obtained from a laboratory colony of *Pieris rapae* reared on cabbage as described in Renwick et al. (1989). Ten larvae were placed in the center of each cup. The cups were covered with translucent polystyrene lids and placed in a covered (40 × 27 × 9 cm) translucent polystyrene box that was lined with water-saturated paper towels to maintain high humidity. The boxes were placed in a growth chamber maintained at 25°C under fluorescent and incandescent lights for 6 hr, after which the larvae were removed and the box was refrigerated at 4°C overnight to prevent the leaf disks from drying.

The next morning the leaf disks were removed and the area consumed was measured in square millimeters using a Li-Corr model 3100 area meter. All disks were measured three times and the average was used to calculate the area consumed. Any disks that showed no sign of feeding were measured first, and the average area of all uneaten disks minus 16 mm^2 for the rubber dam was used to determine the total disk area exposed for feeding. The remaining disks were measured, and the average measurement of each was deducted from the total area available to yield the area consumed. A feeding deterrent index (FDI) was calculated using the total area consumed from all disks for each compound tested. FDI = $100(C - T)\ (C + T)$ where C represents the area consumed of control disks and T the area consumed of treated disks.

RESULTS AND DISCUSSION

Identification of Compound 4. Compound **4** was isolated from fractions 3 and 4 from the flash chromatography column by HPLC in gradient 2. This compound was detected as a major peak at R_t = 25.2 min, and TLC in solvent system 1 showed a pink spot (hR_f = 42.3), indicative of a cardenolide, after spraying the plate with Kedde's reagent. A negative response to the Keller-Killiani test (von Euw and Reichstein, 1948) indicated the absence of a 2-deoxy sugar, which is commonly found in many cardiac glycosides (Reichstein and Weiss, 1962).

Glycosides containing 2-deoxy sugars are easily hydrolyzed by mild acid hydrolysis (Reichstein and Weiss, 1962). Compound **4** remained unchanged under mild hydrolytic conditions, confirming the absence of a 2-deoxy sugar. When hydrolyzed with 2 N HCl, it yielded an aglycone, which was identified as digitoxigenin on the basis of TLC comparison (hR_f = 49.3, solvent system 2) with the standard compound. The aqueous fraction showed a single spot on

TLC (hR_f = 35, solvent system 3), which corresponded to D-glucose. GLC of the TMS derivative of the glycone showed the presence of two peaks at R_t = 17.1 and 18.3 min (corresponding to the α- and β-anomers), identical to those of trimethylsilyl glucose. Enzyme hydrolysis of **4** yielded the same two products, i.e., digitoxigenin and glucose. The glycosidic linkage in **4** could be cleaved with β-glucosidase, suggesting the nature of this linkage to be β. The above observations were further confirmed by FAB mass spectral data (Table 1). The J value (8 Hz) of the signal of the anomeric proton (Table 2) also indicated that

TABLE 1. FAB MASS SPECTRAL DATA OF **4**, **5**, AND **6**

Fragment ion	*m/z* value		
	4	**5**	**6**
$[M + Na]^+$	559	705	705
$[M + H]^+$	537	683	683
$[M\text{-}2H_2O\text{-}CH_3]^+$	485		
$[M\text{-}162]^+$	374	521	521
$[M\text{-}162\text{-}H_2O]^+$			488
$[M\text{-}162\text{-}2H_2O]^+$		485	485
$[M\text{-}162\text{-}H_2O\text{-}CH_3OH]^+$			471
$[Agl + H]^+$	375	375	375
$[Agl + H\text{-}H_2O]^+$	357	357	357
$[Agl + H\text{-}2H_2O]$	339	339	339

TABLE 2. ^{1}H NMR SPECTRAL DATA OF **4–6**[a]

Proton	**4**	**5**	**6**
17	2.77 m	2.79 m	2.77 m
18	0.80 s	0.79 s	0.80 s
19	1.0 s	1.0 s	1.0 s
21a	5.06 d (18)	5.03 d (18)	5.03 d (18)
21b	5.36 d (18)	5.35 d (18)	5.31 d (18)
22	6.13 s	6.12 s	6.12 s
1′	5.31 d (8)	5.30 d (9)	5.2 d (8)
6′		1.58 d (6)	1.62 d (6)
1″		4.59 d (7.5)	4.72 d (8)
Protons of the sugar unit(s) and H-3	4.04–4.61	3.85–4.52	3.76–4.51

[a]Chemical shifts are given in ppm and J values in parentheses are in Hz; s = singlet; d = doublet; m = multiplet.

a glucose moiety in its $^4C_1$ conformation was linked to the aglycone by a β-glycoside bond. The site of linkage of the two units was established by ^{13}C NMR spectroscopy. In comparison with ^{13}C NMR chemical shifts of C-2, C-3, C-4, and C-5 of digitoxigenin recorded in pyridine-d_5 (Ooi et al., 1985), **4** exhibited chemical shift changes for respective carbons by -1.5, 8.0, -3.4, and -0.04 ppm, respectively. These changes suggested that glucose was linked to digitoxigenin at the C-3 position (Robien et al., 1987). ^{13}C NMR data of **4** were identical to those of digitoxigenin-3-*O*-β-D-glucoside (Ooi et al., 1985; Cheung et al., 1981). This is the first reported isolation of digitoxigenin-3-*O*-β-D-glucoside from *E. cheiranthoides*.

In Table 3, we also report ^{13}C NMR data of **1–3.** Chemical shift assignments for these compounds were based on distortionless enhancement by polarization transfer (DEPT) (Doddrell et al., 1982) and heteronuclear chemical shift correlation (HETCOR) spectroscopy (Bax and Morris, 1981). These data were used in the ^{13}C chemical shift assignments of **5–7.**

Identification of Compound 5. Compound **5** was isolated from fractions 9–11. It was purified by reverse-phase HPLC (R_t = 28.0 min) using gradient 2. It showed a positive response to Kedde's test, a negative response to the Keller-Killiani test, and remained unchanged upon mild acid hydrolysis. These tests suggested that this compound was also a cardenolide lacking a 2-deoxy sugar. The FAB mass spectral data (Table 1, molecular weight 682) were indicative of a diglycoside. A loss of 180 mass units from the protonated parent ion at m/z = 683 suggested the outer sugar to be a hexose. An intense fragment ion at m/z = 375 corresponded to the protonated aglycone, suggesting that the inner sugar must have a molecular weight of 146 and should thus be a deoxy hexose.

Compound **5** was subjected to glucosidase enzyme hydrolysis. TLC of the MeOH-soluble fraction of the hydrolysate in solvent system 1 revealed the presence of unreacted **5** (hR_f = 31) and a hydrolyzed product (hR_f = 56.0) in a 2 : 1 ratio. The H_2O-soluble fraction, upon GLC analysis of its TMS derivative, revealed the presence of glucose (two peaks at R_t = 17.2 and 18.3 min). Acid hydrolysis of **5** with 2 N HCl yielded an aglycone, which was identified as digitoxigenin (co-TLC with a standard in solvent system 2, hR_f = 49.3). The glycone fraction gave two spots on TLC in solvent system 3 at hR_f = 35.0 and 65.0, corresponding to D-glucose and 6-deoxy-D-gulose, respectively. Their identities were further confirmed by GLC analysis of the TMS-derivatized glycone fraction. This resulted in two peaks at R_t = 13.1 and 13.8 min, corresponding to the R_ts of the two anomers of TMS derivatized 6-deoxy-D-gulose, and two other peaks at R_t = 17.2 and 18.3 min for the two anomers of TMS derivatized D-glucose. Thus, **5** consisted of an aglycone, digitoxigenin, 6-deoxy-D-gulose as the inner sugar, and β-linked D-glucose as the outer sugar. Further information regarding the structure of **5** was obtained by NMR studies. The

TABLE 3. ^{13}C NMR SPECTRAL DATA OF **1–7**[a]

Carbon	1	2	3	4	5	6	7
1	24.77	24.77	25.18	30.25	30.28	30.58	25.00
2	25.26	25.54	27.35[bb]	27.20[b]	27.22[b]	27.22[b]	26.02
3	74.96	74.88	74.95	74.10	74.20	74.52	73.56[b]
4	36.25	36.20	31.28	30.75	30.71	30.71	34.92
5	73.68	73.68	30.61	36.66	36.65	36.68	72.88[b]
6	36.96	36.92	27.71	27.08[b]	27.08[b]	27.08[b]	37.82
7	22.65	22.65	22.32[c]	21.53[c]	21.54[c]	21.54[c]	22.70
8	41.89	41.89	42.26	41.88	41.89	41.90	41.90
9	39.54	39.54	36.26	35.85	35.87	35.88	39.52
10	55.34	55.34	41.95	35.49	35.50	35.50	55.51
11	18.57	18.57	22.15[c]	21.99[c]	22.00[c]	22.02[c]	18.69
12	39.44	39.54	40.75	39.83	39.83	39.85	39.52
13	49.82	49.84	50.03	50.12	50.12	50.13	49.86
14	84.35	84.37	84.97	84.63	84.60	84.64	84.41
15	32.14	32.14	33.34	33.17	33.19	33.18	32.18
16	27.21	27.21	27.49[b]	27.29[b]	27.30[b]	27.31[b]	27.25
17	51.05	51.06	51.95	51.46	51.44	51.47	51.09
18	15.99	15.99	16.72	16.20	16.22	16.21	15.99
19	208.20	208.00	66.09	23.78	23.77	23.75	208.00
20	174.30[b]	174.47[b]	174.65[d]	176.07[d]	176.08[d]	176.08[d]	174.50[c]
21	73.99	73.99	74.05	73.73	73.71	73.75	73.71[b]
22	117.80	117.78	117.83	117.65	117.64	117.65	117.80
23	175.90[b]	175.72[b]	176.17[d]	174.57[d]	174.59[d]	174.60[d]	175.77[c]
1′	97.57	97.53	101.47	103.03	100.50	103.55	99.29
2′	38.99	39.12	69.79	75.39	69.50	71.55	69.38
3′	67.65	67.79	72.16	78.77[e]	72.11	76.29	71.88
4′	83.40	83.47	79.68	71.80	79.60	83.49	79.18
5′	69.08	69.09	69.39	78.54[e]	69.30	73.00	69.17
6′	18.67	18.61	17.74	62.90	17.68	17.70	17.16
1″	106.15	106.96	103.78		104.10	107.10	103.34
2″	75.13	75.00	75.11		75.08	75.59	74.83
2″	78.35	78.38	78.77		78.66[e]	78.70[e]	78.54
4″	71.49	71.03	70.70		70.71	70.53	70.08
5″	78.35	67.20	78.77		78.52[e]	78.58[e]	78.54
6″	62.55		63.32		63.43	62.82	63.05

[a]Spectra were recorded in pyridine-d_5; the chemical shifts are given in ppm.
[b–e]Values may be transposed within a vertical column.

nature of both glycosidic linkages was found to be β on the basis of the J values of signals of the anomeric protons (Table 2). Carbon chemical shifts of C-2, C-3, C-4, and C-5 (Table 3) suggested that the inner sugar, i.e., 6-deoxy-D-gulose was attached to the aglycone at C-3. ^{13}C NMR chemical shifts of the

sugar moieties of this compound were identical to those of **3** (Table 2), which revealed that the glucose unit was linked to 6-deoxy-D-gulose at C-4′. These data permitted characterization of **5** as glucodigigulomethyloside, which has been previously reported from seeds of *Cheiranthus allionii* hort (Cruciferae). (Makarevich et al., 1975).

Identification of Compound **6**. Compound **6** was isolated from fractions 12–15 by HPLC using gradient 2 (R_t = 29.3 min). It showed a single spot (hR_f = 28.8) using TLC with solvent system 1, and it gave a pink color, characteristic of cardenolides with Kedde's reagent. Absence of a 2-deoxy sugar was deduced on the basis of a negative response to the Keller-Killiani test and no change under mild acid hydrolysis conditions. The diglycosidic nature of **6** was evident from its molecular weight of 682 and additional FAB mass spectral data (Table 1). The fragmentation pattern suggested that **6** had an aglycone with a molecular weight of 374 and two sugars (molecular weights 180 and 146). It also indicated that the outer sugar was a hexose.

Compound **6** was incompletely hydrolyzed upon treatment with β-glucosidase enzyme. The MeOH-soluble fraction obtained after enzyme hydrolysis was analyzed by TLC in solvent system 1. This showed the presence of two spots at hR_f = 28.8 (compound **6**) and 70 (a partially hydrolyzed product) in the ratio of 1 : 1. Complete hydrolysis with 2 N HCl afforded digitoxigenin (hR_f = 49.3, TLC in solvent system 2), and two sugars at hR_f = 35 and 56 (TLC in solvent system 3) corresponding to D-glucose and D-fucose, respectively. The presence of these sugars was further confirmed by GLC analysis of their TMS derivatives (R_t = 14.2 and 14.6 min corresponding to the TMS derivatives of α- and β-anomers of fucose, and R_t = 17.2 and 18.4 min corresponding to the TMS derivatives of α- and β-anomers of D-glucose). Based on the above evidence, **6** was identified as a glucosyl fucoside of digitoxigenin. Sites of various linkages were confirmed by NMR data (Table 3). ^{13}C NMR data for the digitoxigenin moiety in **6** indicated C-3 to be the site of glycosidation. Carbon chemical shifts of the sugar units revealed C-4′ to C-1″ linkage between fucose and glucose. ^{1}H NMR data revealed a β-linkage at both glycosidic sites (Table 2). Thus **6** was identified as glucodigifucoside. This compound was first isolated from *Digitalis purpurea* (Okano, 1957). It has also been reported from *E. cheiranthoides*, *E. leptophyllum*, *E. suffruticosum*, and *E. marschallianum* (Makarevich, 1965), and *D. lanata* (Beale et al., 1988).

Identification of Compound **7**. Compound **7** (hR_f = 17.7 in solvent system 1) was found to be a cardenolide with a 2-deoxy sugar, as it gave a positive response to Kedde's test and a negative response to the Keller-Killiani test. Treatment with β-glucosidase enzyme furnished an aglycone (hR_f = 46.5 in solvent system 1) and a glycone identified as D-glucose, on the basis of TLC and GLC of its TMS derivative. Hydrolysis of **7** with 2 N HCl yielded an

aglycone identified as strophanthidin (co-TLC), and two sugars, identified as 6-deoxy-D-gulose and D-glucose (TLC, and GLC of their TMS derivatives).

Complete identification of **7** was achieved on the basis of its ^{13}C NMR data (Table 3). ^{13}C chemical shift assignments for the aglycone moiety of **7** were made on the basis of those for **1** and **2.** Similarly, assignment of chemical shifts of the carbons of the sugar units was made by comparing them with those of **3** and **5.** Thus, **7** was identified as cheirotoxin. Cheirotoxin has been previously reported from *Castilla elastica* (Moraceae) (Brauchli et al., 1961), two species of *Cheiranthus*, viz. *C. cheiri* (Cruciferae) (Moore et al., 1954) and *C. allionii* (Makarevich et al., 1974), and two species of *Erysimum*, viz. *E. cuspidatum* (Maslennikova et al., 1975) and *E. canescens* (Makarevich et al., 1980).

Bioassay. Compounds **1–12** were tested for their deterrent effect on feeding by *P. rapae* larvae. At the lowest concentration tested (1 ng/cm^2), all the compounds were inactive. At a concentration of 10 ng/cm^2 only digitoxin exhibited an FDI greater than 50%. At the highest concentration (100 ng/cm^2), all the cardenolides were deterrent. Among the strophanthidin derivatives tested at this concentration (Table 4) **2** and **8** had the highest FDIs. The glycosides **1** and **9** and their aglycone **(10)** were equally effective. This would suggest that substitution of glucosyldigitoxose or digitoxose alone on the aglycone, strophanthidin,

TABLE 4. FEEDING BY *pieris rapae* LARVAE ON CABBAGE LEAF DISKS TREATED WITH COMPOUNDS 1–12 AT 100 ng/cm^2

Compound	Total area consumed (cm^2)		Feeding deterrent index
	Treated	Control	
1. Erysimoside	32.6	145.5	63.4
2. Erychroside	20.4	163.4	77.8
3. Erycordin	83.1	224.2	45.9
4. Digitoxigenin 3-O-β-D-glucoside	3.7	113.5	93.7
5. Glucodigigulo methyloside	35.2	224.0	72.8
6. Glucodigifucoside	37.7	271.5	75.6
7. Cheirotoxin	89.2	154.4	26.8
8. Cymarin	45.5	248.7	69.1
9. Helveticoside	109.1	291.5	45.5
10. Strophanthidin	62.6	256.8	60.1
11. Digitoxin	29.3	276.1	80.1
12. Digitoxigenin	36.1	169.3	64.8

had no influence on its activity. All the digitoxigenin derivatives were active. Compound **4** appeared to be the strongest feeding deterrent, with an FDI of 93.7.

The results suggest that the refusal of *P. rapae* to feed on foliage of *E. cheiranthoides* may be explained by the combined effect of several cardenolides in this plant. However, the strong deterrent activity of certain individual cardenolides would indicate that any one of these would be sufficient to discourage feeding by this insect.

Distinct differences in the responses of larvae and adults of *P. rapae* to the cardenolides present in *E. cheiranthoides* are apparent. The ovipositing butterflies are most sensitive to the strophanthidin-based cardenolides, and some structural specificity has been demonstrated. The most active deterrents have the strophanthidin aglycone with substitution of the digitoxose sugar by a methyl group or another sugar at the 3 or 4 hydroxy group, e.g., **1, 2,** and **8** (Sachdev-Gupta et al., 1990). However, larvae of *P. rapae* appear to be sensitive to a wider range of deterrents. The digitoxin-based cardenolides, which have little or no activity as oviposition deterrents, are extremely active as feeding deterrents. The combination of different cardenolides in *E. cheiranthoides* thus offers two lines of defense against *P. rapae*, with different compounds responsible for deterring the adult and larval stages of the insect.

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REFERENCES

BAX, A., and MORRIS, G.A. 1981. An improved method for heteronuclear chemical shift correlation by two-dimensional NMR. *J. Magn. Reson.* 42:501–505.

BEALE, J.M., FLOSS, H.G., LEHMANN, T., and LUCKNER, M. 1988. Digitoxigenin-3β-*O*-[β-D-fucopranosyl-4-β-glucopyranoside], the main cardenolide of somatic embryos of *Digitalis lanata*. *Phytochemistry* 27:3143–3146.

BRAUCHLI, P., SCHINDLER, O., and REICHSTEIN, T. 1961. Die cardenolide von *Castilla elastica* Cerv. *Helv. Chim. Acta* 44:904–919.

CHEUNG, H.T.A., BROWN, L., BOUTAGY, J., and THOMAS, R. 1981. Cardenolide analogues. Part 12. ^{13}C NMR of semi-synthetic glycosides and side-chain modified genins. *J. Chem. Soc. Perkin Trans. I* 1981(6):1773–1778.

DIMOCK, M.B., RENWICK, J.A.A., RADKE, C.D., and SACHDEV-GUPTA, K. 1991. Chemical constituents of an unacceptable crucifer, *Erysimum cheiranthoides*, deter feeding by *Pieris rapae*. *J. Chem. Ecol.* 17:525–533.

DODDRELL, D.M., PEGG, D.T., and BENDALL, M.R. 1982. Distortionless enhancement of NMR signals by polarization transfer. *J. Magn. Reson.* 48:323–327.

KREBS, K.G., HEUSSER, D., and WIMMER, H. 1969. Spray reagents, pp. 854–911, *in* E. Stahl (ed.). Thin-Layer Chromatography (English translation). Springer-Verlag, Berlin.

MAKAREVICH, I.F. 1965. Presence of glucodigifucoside in *Erysimum cheiranthoides*. *Khim. Prir. Soedin*. No. 2:67–69 (English translation).

MAKAREVICH, I.F., KOLESNIKOV, D.D. and BELOKON, V.F. 1974. Cardiac glycosides of *Cheiranthus allionii*. *Khim. Prir. Soedin*. No. 5:616–618 (English translation).

MAKAREVICH, I.F., KOLESNIKOV, D.D., KOVALEV, I.P., GORDIENKO, V.G., and KABANOV, V.S. 1975. Cardiac glycosides of *Cheiranthus allioni*. *Khim. Prir. Soedin*. No. 6:778–781 (English translation).

MAKAREVICH, I.F., ZOLOT'KO, Z.S., and KOLESNIKOV, D.D. 1980. Extraction of strophanthidin and accompanying cardenolides from *Erysimum canescens* Roth. *Farm. Zh. (Kiev)* 6:33–35; *Chem. Abstr*. 94:180555g.

MASLENNIKOVA, V.A., UMAROVA, R.U., and ABUBAKIROV, N.K. 1975. Cardenolides of *Erysimum cuspidatum*. *Khim. Prir. Soedin*. No. 2:179–182 (English translation).

MOORE, J.A., TAMM, C., and REICHSTEIN, T. 1954. Glycosides of the wallflower, *Cheiranthus cheiri*. *Helv. Chim. Acta* 37:755–770.

OOI, Y., HASHIMOTO, T., MITSUO, N., and SATOH, T. 1985. Enzymic formation of β-alkyl glycosides by β-galactosidase from *Aspergillus oryzae* and its application to the synthesis of chemically unstable cardiac glycosides. *Chem. Pharm. Bull*. 33:1808-1814.

OKANO, A. 1957. Studies on the constituents of *Digitalis purpurea* L. VI. Glucodigifucoside, a new cardiotonic glycoside. *Chem. Pharm. Bull*. 5:272–276.

REICHSTEIN, T., and WEISS, E. 1962. The sugars of the cardiac glycosides. *Adv. Carb. Chem*. 17:65–120.

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.

ROBIEN, W., KOPP, B., SCHABLE, D., and SCHWARZ, H. 1987. Carbon-13 NMR spectroscopy of cardenolides and bufadienolides. *Prog. NMR Spectrosc*. 19:131–181.

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.

STAHL, E. (ed). 1969. Thin-Layer Chromatography. Springer-Verlag, New York, 1041 pp.

VERSCHAFFELT, E. 1911. The cause determining the selection of food in some herbivorous insects. *Proc. R. Acad. Amsterdam* 13:536–542.

VON EUW, J., and REICHSTEIN, T. 1948. Die Glykoside der Samen von *Strophanthus nicholsonii* Holm. Glykoside und Aglykone. *Helv. Chim. Acta* 31:883–892.

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ALLELOCHEMICALS FROM *Pilocarpus goudotianus* LEAVES[1]

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Abstract—The effect on germination, shoot, and root growth by bergapten, xanthotoxin, imperatorin, xanthyletin, xanthoxyletin, luvangetin, donatin and alloxanthoxyletol from *Pilocarpus goudotianus* leaves, on *Lactuca sativa* var. nigra seedlings has been evaluated. A structure-activity correlation is discussed based on the bioassay results. Furanocoumarins appear to be the most active compounds in comparison with pyrano- and simple coumarins. The presence of an oxygenated function at C-8 decreases the germination effect in furano- and pyranocoumarins, while C-5 substituents do not cause significant changes on the activity.

Key Words—*Pilocarpus goudotianus*; Rutaceae; coumarins; furanocoumarins; pyranocoumarins; allelopathy; *Lactuca sativa.*

INTRODUCTION

Pilocarpus goudotianus is a shrub growing in Colombia and Venezuela, a member of only 20 species that comprises the genus *Pilocarpus* (Rutaceae). In a previous publication (Amaro-Luis et al., 1990), we reported the isolation of new furano- and pyranocoumarins from the leaves extract of *Pilocarpus goudotianus*. Preliminary bioassays of the crude extracts showed them to be active on germination of *Lactuca sativa* seeds.

Coumarins are known to have a wide range of biological properties, such

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[1] Part 2 in the series: *Natural Product Models as Allelochemicals*. For Part 1 see: Macías *et al.* (1992).

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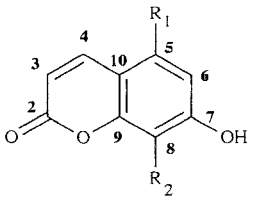
as anticoagulant (Arora and Mathur, 1963), cytostatic (Gosálvez et al., 1976; González et al., 1977a; Gawron and Glowniak, 1987), antibacterial and fungicidal (Jurd et al., 1971a,b) activities. Much attention has been paid to the photosensitizing effects on mammalian, bacterial, fungal, and viral cells caused by some furanocoumarins, effects which are attributed to photobinding of the coumarin to DNA (Kanne et al., 1982). Although various coumarins have long been known to exert stimulatory or inhibitory effects on seed germination and subsequent plant growth (Bennet and Bonner, 1953; Bose, 1958; Brown, 1981), few studies have addressed their possible allelochemical activity. The parent compound, coumarin, has been the most studied in this sense (Rodighiero, 1954). The inhibitory activity of coumarin on the germination of a number of crop and weed species such as cotton, prickly sida, redroot pigweed, etc., at a concentration of 10^{-3} M has been described (Williams and Hoagland, 1982). Simple coumarins such as umbelliferone and scopoletin were shown to inhibit Chinese cabbage root growth at 10^{-5}–10^{-7} M (Shimomura et al., 1982). Similar activities have been described for some furanocoumarins: psoralen, present in *Psoralea subacaulis* seeds, has been suggested to act as an allelopathic agent inhibiting germination and root length of competing species such as lettuce, tomato, cucumber, and radish (Baskin et al., 1967).

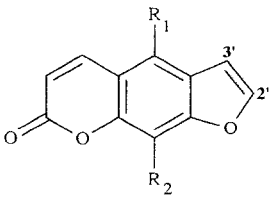
As a part of our research on bioactive natural products (coumarins, sesquiterpene lactones, lignans, phenolics, etc.) we are conducting a systematic study of their potential allelopathic activity (Macías et al., 1992). Thus, we are evaluating the regulatory effects of tested compounds on *Lactuca sativa* var. nigra (Leather and Einhellig, 1986).

In this paper we report the effect on *L. sativa* seed germination and root and shoot length of 10 different coumarins in aqueous solutions at concentrations of 10^{-4}–10^{-9} M. Simple coumarins 5,7-dihydroxycoumarin **(1)** and 7,8-dihydroxycoumarin **(2)**; furanocoumarins bergapten **(3)**, xanthotoxin **(4)** and imperatorin **(5)**; and the pyranocoumarins xanthyletin **(6)**, xanthoxyletin **(7)**, luvangetin **(8)**, donatin **(9)**, and alloxanthoxyletol **(10)** were tested (Figure 1).

METHODS AND MATERIALS

Tested Coumarins. 5,7-Dihydroxycoumarin **(1)** [mp 280–282°C, AcOEt, (Kaufman and Kelly, 1965) mp 280°C, water] was prepared from phloroglucinol and ethylpropiolate as described by Kaufman and Kelly (1965). 7,8-Dihydroxycoumarin **(2)** [mp 249–251°C, MeOH, (Shimomura et al., 1980) mp 253°C, EtOH] was obtained by Pechmann condensation of pyrogallol with malic acid (Sethna and Phadke, 1953). Bergapten **(3)** [mp 187–189°C, petrol/AcOEt, (González et al., 1977b) mp 188–189°C, EtOH], xanthotoxin **(4)** [mp 144–146°C, petrol/AcOEt, (González et al., 1977b) mp 145–146°C, benzene/





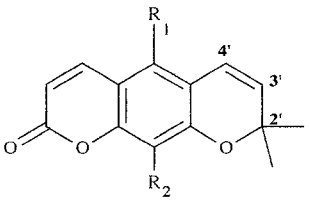
1 $R_1 = OH$ $R_2 = H$

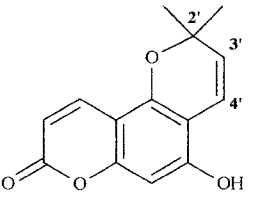
2 $R_1 = H$ $R_2 = OH$

3 $R_1 = OMe$ $R_2 = H$

4 $R_1 = H$ $R_2 = OMe$

5 $R_1 = H$ $R_2 = OCH_2CH{=}C(CH_3)_2$





6 $R_1 = R_2 = H$

7 $R_1 = OMe$ $R_2 = H$

8 $R_1 = H$ $R_2 = OMe$

9 $R_1 = H$ $R_2 = OCH_2CH{=}C(CH_3)_2$

10 =

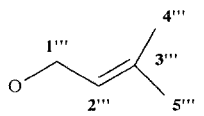


FIG. 1. Natural and synthetic coumarins tested for allelopathic activity.

AcOEt], imperatorin **(5)** [mp 98–100°C, petrol/AcOEt, (González et al., 1982) mp 102–103°C, benzene/AcOEt], xanthyletin **(6)** [mp 127–128°C, petrol/AcOEt, (González et al., 1977b) mp 128–130°C, petrol/AcOEt], xanthoxyletin **(7)** [mp 129–131°C, petrol/AcOEt, (Lassak and Pinhey, 1969) mp 131–132°C, AcOEt], luvangetin **(8)** [mp 106–107°C, petrol/AcOEt, (Agulló-Martínez et al., 1967) mp 106–107°C, benzene], donatin **(9)** (oil) and alloxanthoxyletol **(10)** (mp 217–219°C, dec. AcOEt) were isolated from *Pilocarpus goudotianus* leaves (Amaro-Luis et al., 1990).

Weight percentages (weight of isolated coumarins/weight of dry plant × 100) of coumarins isolated from *Pilocarpus goudotianus* leaves (Amaro-Luis et al., 1990) were the following: bergaptene **(3)**, 0.015%; xanthotoxin **(4)**, 0.02%;

imperatorin **(5)**, 1.8×10^{-3}%; xanthyletin **(6)**, 0.12% xanthoxyletin **(7)**, 1.4×10^{-3}%; luvangetin **(8)**, 6.1×10^{-3}%; donatin **(9)**, 5.9×10^{-3}%; alloxanthoxiletol **(10)**, 4.1×10^{-4}%.

Lettuce Seed Germination Bioassay. Seeds of lettuce, *Lactuca sativa* var. nigra, 1989 crop, were obtained from Estación Experimental Rancho de La Merced, Dirección General de Investigación y Extensión Agrarias, Junta de Andalucía, Jerez, Spain. All undersized and damaged seeds were discarded and the assay seeds were preselected for uniformity of size.

Bioassays were performed by germinating 25 lettuce seeds for five days (three for germination and two for root and shoot growth) in the dark at 25°C in 9-cm plastic Petri dishes containing a 10-cm sheet of Whatman No. 1 filter paper, and 7 ml of test or control solution. Test solutions, 10^{-4} M, were prepared using DMSO (1% v/v) as the initial solubilizing agent. Test solutions, 10^{-5}–10^{-9}M were obtained by diluting the previous solution. Studies with parallel deionized water controls and with the same DMSO concentration were also conducted. Four replicates of each treatment and of parallel controls were carried out. The number of seeds per replicate and the time and temperature of germination were chosen according to a number of preliminary experiments, varying the number of seeds, volume of test solution per dish, and the incubation period.

All the pH values were controlled before the bioassay and were between 6.5 and 7.0.

Statistical Treatment. The germination and root and shoot length values were tested by Student's t test; the differences between the experiment and the control were significant at value of $P = 0.01$ (Table 1).

RESULTS AND DISCUSSION

It has been reported that the presence of an alkylating α,β-unsaturated carbonyl function is related with its potential allelopathic activity (Fischer, 1986). Macías et al. (1992) reported that the presence of an α-methylene-γ-lactone group in sesquiterpene lactones is more related with the effects on seedling than on shoot and root lengths, where the effect of the conformational changes are crucial.

In this study we are evaluating some coumarins as a class of compounds with planar structures that contain an α,β-unsaturated δ-lactone moiety integrated in an aromatic system. We also include synthetic simple coumarins to compare them with the natural products and to establish a structure–activity correlation.

Table 1 and Figure 2 show that only at the highest concentration (10^{-4} M) do these compounds show a significant activity on germination and shoot and

TABLE 1. STATISTICAL RESULTS OF EFFECT OF SYNTHETIC COUMARINS **1** AND **2** AND NATURAL COUMARINS **3–10** ISOLATED FROM *Pilocarpus goudotianus* ON *L. sativa* VAR. NIGRA[a]

	Germination (%)						Radicle length (%)						Shoot length (%)					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Simple coumarins																		
5,7-Dihydroxycoumarin (**1**)	−24	+1	+8	+22	−2	+2	−5[b]	+15	+6[b]	+8[c]	+17	+13	0	+5[b]	+7[b]	+11[c]	+13	+1[b]
7,8-Dihydroxycoumarin (**2**)	−38	−1	+2	+13	−2	+8	−4[b]	+10[a]	+7[b]	+12	+20	+8[c]	−8[b]	+3[b]	+2[b]	+2[b]	+18	−13
Furanocoumarins																		
Bergaptene (**3**)	−87	−1	+3	−5	−6	0	−16[c]	+23	+3[b]	+17	+14	+10[c]	−32[c]	+16	−1[b]	+13	+3[b]	−11[c]
Xanthotoxin (**4**)	−82	+3	+1	+10	−5	+3	−39	+3[b]	+1[b]	+4[b]	+22	+12	−61	−4[b]	−4[b]	+15	+7[b]	+3[b]
Imperatorin (**5**)	−47	+5	−6	+9	−10	+9	+25	+21	+9[a]	+14	+20	+7[b]	−22[c]	+8[b]	+8[b]	+10	+8[b]	−9
Pyranocoumarins																		
Xanthyletin (**6**)	−78	−5	−2	+16	−3	−1	−5[b]	+3[b]	−1[b]	+2[b]	+20	+17	−29[c]	−6[b]	−5[b]	+3[b]	+15	+10
Xanthoxyletin (**7**)	−67	+1	−16	+14	−5	+7	+14[c]	+17	+4[b]	0	+11	+1[b]	−22[c]	−8[b]	−3[b]	−4[b]	−6[b]	−20
Luvangetin (**8**)	+16	−6	−4	+8	−12	−1	−56	+15	+10[c]	+10[c]	+5[b]	+22	−43	+15	+10[c]	+5[c]	+5[b]	+10[c]
Donatine (**9**)	−20	−8	−1	+10	−9	+1	+3[b]	+16	+1[b]	+4[b]	+13	+2[b]	−38	+7[b]	+8[b]	+13	+7[b]	−10[c]
Angular pyranocoumarins																		
Alloxanthoxiletol (**10**)	+25	+1	0	+20	−5	−17	+12[c]	+23	+6[b]	+9	+18	−17	+14[c]	+12	+11[c]	0	+20	+8[b]

[a]Values are expressed as percentages from the control and are significantly different with $P < 0.01$ for Student's t test.
[b]Values significantly different with $0.05 > P$.
[c]Values significantly different with $0.01 < P < 0.05$

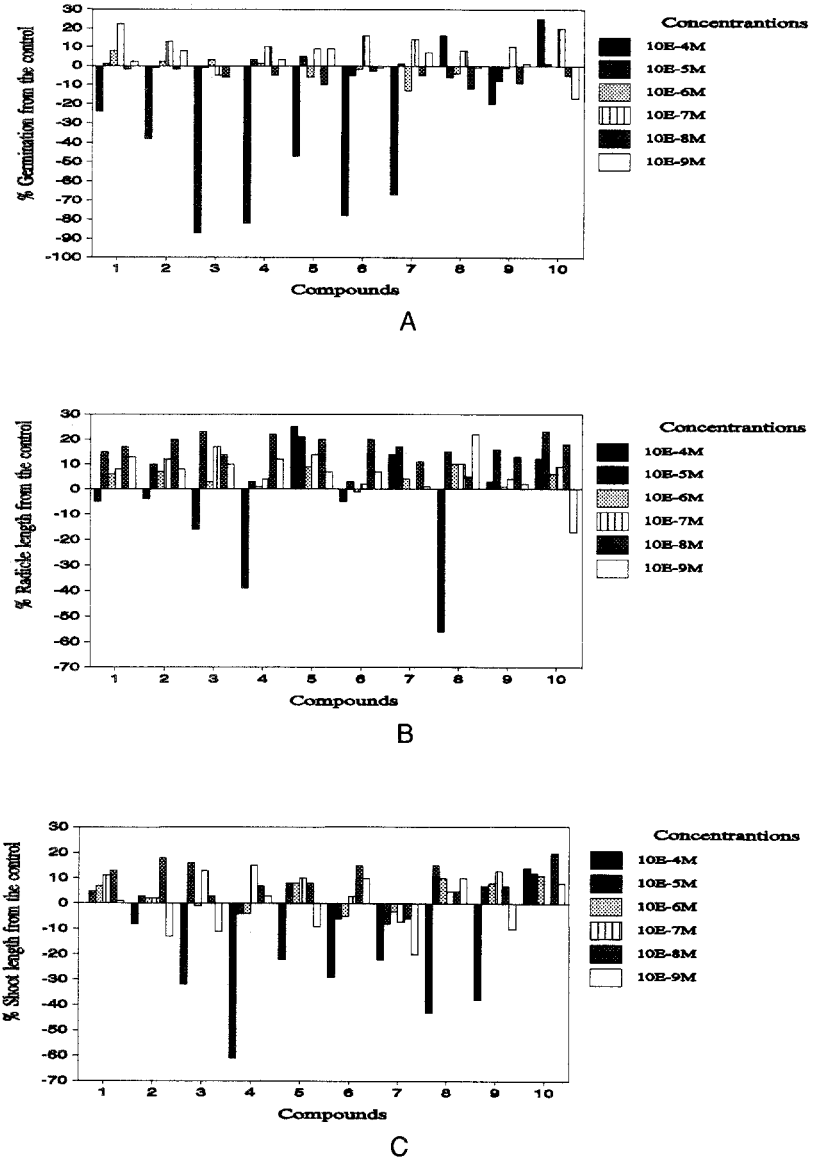


FIG. 2. Effects of coumarins 1–10 on the germination and growth of *L. sativa*. (A) Effect of coumarins 1–10 on the germination of *L. sativa*. (B) Effect of coumarins 1–10 on the radical length of *L. sativa*. (C) Effect of coumarins 1–10 on the shoot length of *L. sativa*.

similar phenolic compounds (Rodighiero, 1954; Williams and Hoagland, 1982; Inderjit and Dakshini, 1991).

The germination inhibitory effect of furanocoumarins usually is much more pronounced than those shown by pyranocoumarins and simple coumarins (Figure 2A). The activity displayed by pyranocoumarins **6** and **7** (−78% and −67%, respectively) is comparable to that shown by **3** and **4** (−87% and −82%), while **8** and **9**, with an oxygenated functionalization at C-8 have no significant effect (+16% and −20%). Therefore, the presence of a condensed furanic or pyranic ring appears to enhance the activity compared to simple coumarins. The presence of an oxygenated function at C-5 does not show significant changes on the activity, while functionalization at C-8 decreases the effect: imperatorin **(5)**, with an voluminous isoprenyl side chain at C-8 presents −47% of inhibition in comparison with **3** (−87%), and luvangetin **(8)** and donatin **(9)** (+16%, −20%), which are lower in comparison with **6** (−78%).

The case of alloxanthoxiletol **(10)** is rather different from the other compounds tested, perhaps due to a different geometry of the molecule, which implies a different possibility of access to the active site.

With respect to the root and shoot length parameters (Figure 2B and C, respectively), there is no significant effect, except for furano- and pyranocoumarins **4, 8,** and **9,** which are functionalizated at C-8. The presence of an oxygenated function at C-8 in these two families of coumarins could be related with the activity: the most active compounds are **4** and **8** for root length (−39%, −56%), and **4, 8,** and **9** for shoot length (−61%, −43%, −38%, respectively). The lack of a significant change on the profile of activity of these compounds could be attributed to the fact that the adoption of different spatial dispositions other than a planar conformation is not possible. Therefore, the activity could be more directly related to the presence of several oxygenated side chains at C-8 than with conformational changes. This fact is in agreement with the previous observations reported for sesquiterpene lactones (Macías et al., 1992), where the conformational changes are more related with root and shoot length profiles of activity than with germination effects.

Due to the high coumarin concentration (10^{-4} M) needed for the activity, the coumarins most likely could be responsible for the allelopathic behavior in those plants where they are present as major constituents. Low coumarin plant concentrations could be related with different bioactivities (antifungal, antifeedant, etc.), including plant growth regulation. Nevertheless, in this case, it is not possible to establish a direct correlation between the presence of coumarins and the allelopathic activity shown by the plant.

The crude extract, obtained by soaking dry leaves in water (3 : 1, water–dry plant weight) was shown to be active on *L. sativa* bioassays.

From the isolated coumarin percentages of *P. goudotianus*, the molar concentrations of these compounds in the 1 : 10 aqueous extract (obtained by diluting

the original aqueous extract with deionized water 1:10, 1:20, and 1:40) are: bergaptene **(3),** 2.3×10^{-5} M; xanthotoxin **(4),** 3.1×10^{-5} M; imperatorin **(5),** 2.2×10^{-6} M; xanthyletin **(6),** 1.7×10^{-4} M; xanthoxyletin **(7),** 1.8×10^{-6} M; luvangetin **(8),** 7.8×10^{-6} M; donatin **(9),** 6.3×10^{-6} M; alloxanthoxiletol **(10),** 5×10^{-7} M.

The concentrations of compounds **6, 4,** and **3** in the 1:10 aqueous extract are in the same range as those that were active in the bioassay. Thus, in this case, coumarins, and particularly compounds **6, 4,** and **3,** are very likely responsible for the allelopathic activity of *P. goudotianus* aqueous extract.

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REFERENCES

AGULLÓ-MARTÍNEZ, E., ESTÉVEZ-REYES, R., GONZÁLEZ, A.G., and RODRÍGUEZ-LUIS, F. 1967. Química de las Rutáceas VII. Cumarinas de las hojas de la *Ruta* spec., no. 46782. *An. Real Soc. Fis. Quim.* 63(B):197–204.

AMARO-LUIS, J.M., MASSANET, G.M., PANDO, E., RODRÍGUEZ-LUIS, F., and ZUBÍA, E. 1990. New coumarins from *Pilocarpus goudotianus. Planta Med.* 56:304–306.

ARORA, R.B., and MATHUR, C.N. 1963. Relation between structure and anticoagulant activity of coumarin derivatives. *Br. J. Pharmacol.* 20:29–35.

BASKIN, J.M., LUDLOW, C.J., HARRIS, T.M., and WOLF, F.T. 1967. Psoralen, an inhibitor in the seeds of *Psoralea subacaulis* (Leguminoseae). *Phytochemistry* 6:1209–1213.

BENNET, E.L., and BONNER, J. 1953. Isolation of plant growth inhibitor from *Thamnosa montana. Am. J. Bot.* 40:29–33.

BOSE, P.K. 1958. On some biochemical properties of natural coumarins *J. Ind. Chem. Soc.* 35:367–375.

BROWN, S.A. 1981. Coumarins, pp. 269–300, *in* E.E. Conn. (eds.). The Biochemistry of Plants, A Comprehensive Treatise. Academic Press. New York.

FISCHER, N.H. 1986. The function of mono and sesquiterpenes as plant germination and growth regulators, pp. 203–218, *in* A.R. Putnam and C.S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

GAWRON, A., and GLOWNIAK, K. 1987. Cytostatic activity of coumarins in vitro. *Planta Med.* 53:526–529.

GONZÁLEZ, A.G., DARIAS, V., ALONSO, G., BOADA, J.N., and RODRÍGUEZ-LUIS, F. 1977a. Cytostatic activity of some Canary Islands species of Rutaceae. *Planta Med.* 31:351–356.

GONZÁLEZ, A.G., DÍAZ, CHICO, E., LÓPEZ DORTA, H., LUIS, J.R., and RODRÍGUEZ-LUIS, F. 1977b. Nuevas fuentes de cumarinas naturales XXX. Componentes químicos de la *Ruta chalepensis* L. y del *Dictamnus hispánicus* Webb. *An. Quím.* 73:430–438.

GONZÁLEZ, A.G., LÓPEZ DORTA, H., MARTÍN-HIERRO, I., MEDINA, M.C., and RODRÍGUEZ-LUIS, F. 1982. Componentes de Umbelífera XXIII. Componentes de *Seseli webbiicossom. An. Quim.* 78(C):274–275.

GOSÁLVEZ, M., GARCÍA-CAÑERO, R., and BLANCO, M. 1976. A screening for selective anticancer agents among plants respiratory inhibitors. *Eur. J. Cancer* 12:1003–1009.

INDERJIT, and DAKSHINI, K.M.M. 1991. Hespejridin 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). *J. Chem. Ecol.* 17:1585–1591.

JURD, L., KING, A.D., JR., and MIHARA, K. 1971a. Antimicrobial properties of natural phenols III. Antimicrobial properties of umbelliferone derivatives. *Phytochemistry* 10:2965–2970.

JURD, L., CORSE, J., KING, A.D., JR., BAYNE, H., and MIHARA, K. 1971b. Antimicrobial properties of natural phenols IV. Antimicrobial properties of 6,7-dihydroxy-, 7,8-dihydroxy-, 6-hydroxy- and 8-hydroxycoumarins. *Phytochemistry* 10:2971–2974.

KANNE, D., STRAUB, K.M., HERAST, J.E., and RAPOPORT, H. 1982. Isolation and characterization of pyrimidine-psoralen-pyrimidine photodiadducts from DNA. *J. Am. Chem. Soc.* 104:6754–6764.

KAUFMAN, K.D., and KELLY, R.C. 1965. New synthesis of coumarins. *J. Heterocycl. Chem.* 2:91–92.

LASSAK, E.V., and PINHEY, J.T. 1969. Constituents of *Eriostemon trachyphyllus*. Structure of trachyphyllin, a new coumarin. *Aust. J. Chem.* 22:2175–2185.

LEATHER, P.R., and EINHELLIG, F.A. 1986. Bioassays in the study of allelopathy, pp. 133–145, *in* A.R. Putnam and C.S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

MACÍAS, F.A., GALINDO, J.C.G., and MASSANET, G.M. 1992. Potential allelopathic activity of several sesquiterpene lactone models. *Phytochemistry* 31:1969–1977.

RODIGHIERO, G. 1954. Influenza di furanocumarine naturali sulla germinazzione dei semi sullo sviluppo dei germogli e delle radici di lattuga. *G. Biochim.* 3:138–146.

SETHNA, S., and PHADKE, R. 1953. The Pechmann reaction *Org. React.* 7:1–58.

SHIMOMURA, H., SASHIDA, Y., and OHSHIMA, Y. 1980. The chemical components of *Artemisia apiacea* Hance. II. More coumarins from the flower heads. *Chem. Pharm. Bull.* 28:347–348.

SHIMOMURA, H., SASHIDA, Y., NAKATA, H., KAWASAKI, J., and ITO, Y. 1982. Plant growth regulators from *Heracleum lanatum*. *Phytochemistry* 21:2213–2215.

WILLIAMS, P.D., and HOAGLAND, R.E. 1982. The effect of natural occurring phenolic compounds on seed germination. *Weed. Sci.* 30:206–212.

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UNUSUAL PERIODICITY OF SEX PHEROMONE PRODUCTION IN THE LARGE BLACK CHAFER *Holotrichia parallela*

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Abstract—(*R*)-(−)-Linalool was identified as a minor component sex pheromone of the scarab beetle *Holotrichia parallela* (Coleoptera: Scarabaeidae). Field evaluations revealed that, although not attractive per se, (*R*)-(−)-linalool enhances the attractiveness of the major sex pheromone, L-isoleucine methyl ester (LIME). Analyses of the pheromone titers in the glands of field-collected females demonstrated the occurrence of peak levels of 48-hr ("circabidian") periodicity. The levels of LIME in the glands of 45-day-old virgin females increased over three times from the scoto- to the photophase of a calling day, but the amounts of (*R*)-(−)-linalool did not significantly change. Virgin females had in average two times more LIME and 3.6 times more (*R*)-(−)-linalool than the average amount found in the field-captured beetles throughout the season.

Key Words—*Holotrichia parallela*, large black chafer, scarab beetle, Coleoptera, Scarabaeidae, isoleucine methyl ester, linalool, sex pheromone, circabidian periodicity, pheromone titer.

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INTRODUCTION

Scarab beetles in the genus *Holotrichia* are important agricultural pests in many parts of the world, especially in Japan, China, and India. In Japan, the most notorious species in this genus is the large black chafer *H. parallela* (Motschulsky) (Coleoptera: Scarabaeidae), *ookurokogane* in Japanese, which was initially called *Lachnosterna morosa* Waterhouse (Anonymous, 1980) and later renamed (Anonymous, 1987).

Behavioral patterns of the beetle do not follow a circadian rhythm, but rather an unusual periodicity. Adults remain in the soil during daytime, coming to the surface every other evening soon after sunset. The rhythm of appearance was documented for four years in the field and also observed in the laboratory (Yoshioka and Yamasaki, 1983).

During evenings when they are active, adult females leave the soil and from higher places, such as leaves of host plants, display a calling behavior by extruding an abdominal gland (Figure 1) in a similar fashion to the calling of moths. Males, on the other hand, fly around searching for calling females. From an extract of these abdominal glands, the major sex pheromone has been isolated and identified as L-isoleucine methyl ester (Leal et al., 1992a). Recently, we were able to demonstrate that other scarabs possess two-component sex pheromone systems (Leal et al., 1993a,b), and this prompted us to further investigate the sex pheromone composition of the large scarab beetle with emphasis on minor components. As reported here, we found that *H. parallela* utilizes (*R*)-(−)-linalool as a minor component, which, although not attractive per se, significantly enhances the attractiveness of the major component. Furthermore, the production of the major sex pheromone, but not of the minor, follows the same periodicity as the appearance of the beetles in the field.

METHODS AND MATERIALS

Chromatographic and MS Analyses. GC analyses were performed on a Hewlett-Packard 5890 equipped with a DB-wax column (30 m × 0.25 mm; 0.25 μm) operated at 50°C for 1 min, programmed at 4°C/min to 180°C, held at this temperature for 1 min, programmed again at 10°C/min to 210°C and held at this temperature for 30 min [50(1)-180(1)/4-210(30)/10]. An HP-1 column (25 m × 0.2 mm; 0.33 μm) operated at 50(1)-100/4-230(10)/10 and a CP-Sil 19 CB column (25 m × 0.25 mm; 0.2 μm) operated at 70-110/4-250/10 were also used. Both internal and external standards were used for quantitative analyses. Enantiomeric resolution of linalool was obtained with a CP-cyclodextrin β-236-M-19 column (50 m × 0.25 mm; 0.25μm) operated at 100°C. Mass spectra were recorded on a Hewlett Packard 5891 mass selective detector using either HP-1 or DB-wax columns.

FIG. 1. Calling position of the large black chafer, *H. parallela*. An abdominal gland (indicated by an arrow) is exposed for the release of sex pheromone. (Bar = 1 cm)

Insects. Adults of *H. parallela* were either captured in the field before they were able to copulate or were raised in laboratory from eggs laid by field-collected mated females. They were separated by sex and kept in boxes containing soil and fresh mulberry leaves; rearing occurred at 25°C, 70% relative humidity, and 12:12 hr light–dark photoperiod. Laboratory-raised insects were kept in separate jars from the first larval stage; fresh carrots (renewed weekly) were provided as a source of food for the grubs.

Pheromone Collection, Isolation, and Quantitative Analyses. Abdominal tips of calling females were excised with forceps, cleaned, and then washed either with dichloromethane or ether. The crude extract was separated on a silica gel column (Wako C-200) by successively eluting with hexane–ether mixtures,

100:0, 95:5, 90:10, 80:20, 50:50, and 0:100. In order to perform quantitative analysis, either methyl caproate or nonadecane was dissolved in CH_2Cl_2 prior to extraction to serve as internal standards. This was done in various concentrations of the standards, but typically 100 μl of a 100 ng/μl solution of the internal standard was employed for extracting one gland. After 3 min, the gland was removed from the extract and 1 μl was injected into GC without any evaporation of the solvent.

In order to investigate the periodicity of pheromone production by *H. parallela*, female beetles were collected in the field during calling nights from August 3 to 11, 1992. The pheromone glands of five to six beetles were excised and extracted individually on the same night and their contents were quantified. On the following night, the pheromone titers of five to six beetles of the same group were individually measured after extraction at the same time they were calling on the previous night.

EAG. Electrophysiological responses of *H. parallela* antennae were recorded as previously described (Leal et al., 1992b).

Field Experiments. Evaluation of the pheromone system in the field was conducted at the National Institute of Sericultural and Entomological Science field in Tsukuba and at Chiba Prefectural Agricultural Experiment Station field in Chiba, Japan, in the summer of 1992. The traps used were either green funnel traps (Japan Tobacco Inc.), or water pan traps, either black or white (Sankei Chemical). L-Isoleucine methyl ester, synthesized as previously reported (Leal et al., 1992a), and (*R*)-(−)-linalool, provided by Fuji Flavor Co., were either incorporated into pellets made of a polyethylene–vinyl acetate copolymer or were applied to rubber septa (Daburu Kyappu No. 2, Araki Rubber Co. Ltd., Osaka). Traps were suspended with the pheromone dispenser at 1.5 m above the ground. Because of the short flight period of *H. parallela* in the field (less than 40 min/night), the effect of wind direction on trap catches (Cardé and Elkinton, 1984) was considered to be minimal and traps were therefore set in lines with at least two repetitions and intertrap distance of 10 m. They were not rerandomized during the same night, but between different test days. Capture data were transformed to log $(x + 1)$ and differences between means were tested for significance by ANOVA.

RESULTS AND DISCUSSION

Identification of the Minor Component. The major component of *H. parallela* sex pheromone has been previously isolated and identified by chromatographing the crude extract of female abdominal glands and monitoring the activity of the fractions with a field bioassay. Activity was demonstrated only in the ether fraction, where L-isoleucine methyl ester was identified (Leal et al., 1992a)

as the active component. However, the chromatographic analysis of the crude extract showed, in addition to the major sex pheromone constituent (R_t = 15.95 min, Figure 2A), other compounds (R_t = 32.75 – 44.27 min), which eluted in the hexane fraction (cuticular hydrocarbons), as well as a peak (R_t = 17.72 min), which also appeared in the ether–hexane 10:90 and 20:80 fractions. Not only the ether fraction, but also the fractions containing the peak at R_t = 17.72 min were EAG active.

A library search (Wiley) indicated that linalool was the best fit for the MS data of the EAG-active peak at R_t = 17.72 min (Figure 2B). Commercially available linalool gave the same retention times in DB-wax (R_t = 17.72 min), HP-1 (R_t = 14.91 min) and CP-Sil 19 CB (R_t = 7.67 min) columns. In addition, male antennae gave significant EAG responses to both synthetic L-isoleucine methyl ester and linalool.

Enantiomeric resolution of linalool was achieved on a chiral column (Figure 3). (*R*)-(−)- and (*S*)-(+)-linalool gave R_t = 34.3 and 34.7 min, respectively. By comparison of the retention times and separate coinjections with both enantiomers, the natural product was identified as (*R*)-(−)-linalool.

Field Evaluations. Catches of the large black chafer in traps having only the major sex pheromone as a lure were compared to those in traps baited with a mixture of L-isoleucine methyl ester and (*R*)-(−)-linalool in a 5:1 ratio. That ratio was the nominal average (5.1 ± 0.7) of the two compounds in the glands of beetles captured in the field at the beginning of the season (July 22). Due to

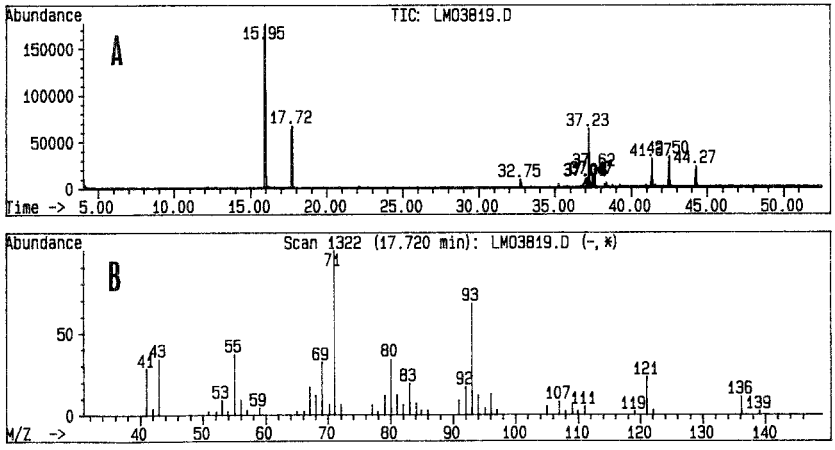

FIG. 2. (A) Reconstructed total ion monitor profile of the crude extract of abdominal glands of *H. parallela* adult females injected on a DB-wax capillary column. (B) MS of the peak at R_t = 17.72 min, later identified as (*R*)-(−)-linalool, a minor component sex pheromone.

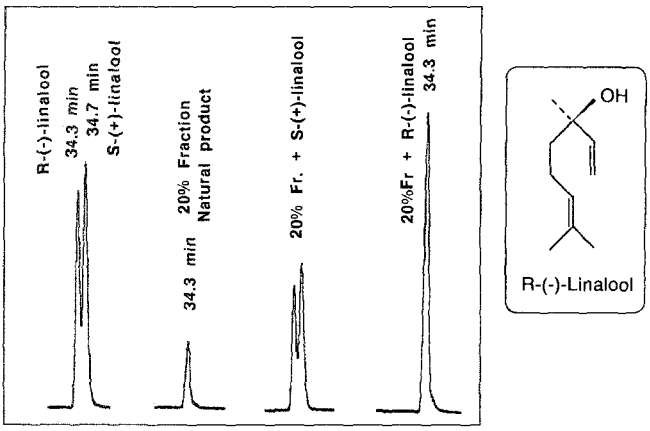

FIG. 3. Enantiomeric resolution of linalool on a chiral column, CP-cyclodextrin β-236-M-19, at 100°C. The natural product gave the same retention time as the *R* enantiomer. Coinjections of the minor sex pheromone with the two enantiomers confirmed that the natural product was (*R*)-(−)-linalool (displayed structure).

the short flight activity of the beetle in the field, many males are attracted at the same time to the pheromone-baited traps (Figure 4). Field tests conducted for 14 active nights from July 24 to August 19 revealed that the pheromone blend was significantly more attractive than the L-isoleucine methyl ester alone, which captured 29.4 ± 7.4 and 6.5 ± 2.2 beetles/trap and night, respectively.

The best catches were achieved when traps were baited with 100 mg of the lures. Due to the high volatility of LIME (bp_5 52–53°C), its release rate was very fast and, thus, lures were renewed before each set of experiments. Despite the "poaching" effect of the pheromone blend lures, traps baited only with L-isoleucine methyl ester were significantly more attractive than control traps. Catches in traps baited only with the major sex pheromone were much higher when the binary mixture was not evaluated in the same experiment, i.e., when the poaching effect was eliminated. On the other hand, (*R*)-(−)-linalool as a single-component lure was not significantly more attractive than control traps.

Pheromone Titer. Analyses of the gland contents of active virgin female beetles revealed that pheromone titers of 17-day-old beetles were below the detection limit, whereas 45-day-old females possessed in average 12.3 μg of L-isoleucine methyl ester and 1.7 μg of (*R*)-(−)-linalool. We therefore concluded from these results that pheromone production occurs rather late in the adult stage. However, the exact starting time of its production could not be determined. This observation is in accordance with the fact that in the beginning of the season, adults appear in the field for feeding and no calling females can

FIG. 4. Response of male *H. parallela* to a pheromone-baited funnel trap. Contrary to other scarabs, which are captured because the insects crash on the trap wings during searching flight, males of the large black chafer come very close to the pheromone source and fly around, probably looking for visual landing clues. By and large, they were caught when they did up-and-down searching flights very close to the lure and crashed inside the trap funnel.

be observed. By contrast, attraction of *Cyclocephala lurida* (Coleoptera: Scarabaeidae) adult males to grubs has been attributed to the occurrence of the (adult) sex pheromone in the larval stage as a noncommunicative volatile chemical (Haynes et al., 1992). It is unlikely that *H. parallela* would have L-isoleucine

methyl ester and (*R*)-(−)-linalool in the larval stage and lose the chemicals for sometime in the beginning of the adult stage.

The pheromone titers of groups of 45-day-old female beetles were compared during the scoto- and the photophases by extracting, in the morning, glands of half of the group that would be calling that night and extracting at night the glands of the others. Although the amounts of (*R*)-(−)-linalool did not significantly change between the two phases (Figure 5), the amounts of L-isoleucine methyl ester increased more than three times from the photo- to the scotophase. Contrary to this, the time of the day has been previously demonstrated to have no effect on pheromone release by the scarab beetle *Anomala cuprea* (Leal et al., 1993b), most probably because calling of the latter is not restricted to a certain short period of time. The amounts of pheromone in glands of virgin and field-captured females were also compared. Virgin females possessed nearly two times more L-isoleucine methyl ester and 3.6 times more (*R*)-(−)-linalool than the average of 101 beetles collected in the field throughout the season. However, the contents of the major sex pheromone in 5% and 10% of the field sample were 0.8 ± 0.5 and 1.6 ± 0.8 μg, respectively.

The periodicity of pheromone production was examined by measuring the pheromone contents in the glands of field-collected female beetles on the calling night and then on the following ones. Preliminary experiments revealed that the

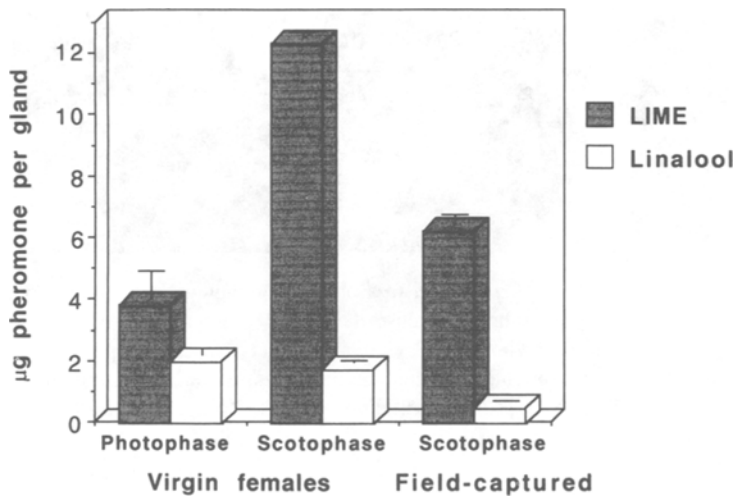

FIG. 5. Pheromone titer in the glands of 45-day-old virgin females during the photo- and the scotophase of a calling day. The amount of (*R*)-(−)-linalool remained nearly constant, but significantly more L-isoleucine methyl ester (LIME) was produced at the time of calling. In average, female beetles captured in the field throughout the season possessed in the scotophase less pheromone than virgin females.

pheromone titers of the major component were high on the first and third nights and low on the second and fourth nights. Careful examination of field-collected beetles for one week showed that the amounts of the major sex pheromone dramatically decreased in the night following the calling (Figure 6), but, on the other hand, the contents of (*R*)-(−)-linalool stayed nearly constant. Therefore, this clear-cut profile of the major sex pheromone titer reveals that pheromone production (at least of the major component) undergoes oscillation on a "circabidian" periodicity (Lat: *circa*, about + *bis*, twice + *dies*, day; exhibiting approximately 48-hr periodicity).

It would also be interesting to investigate this periodicity of pheromone production by directly collecting volatiles released during the calling behavior in order to determine whether this would give a profile similar to that obtained from gland extraction. However, the fact that the large black chafer does not display calling behavior when confined and the difficulty of collecting more than one time from the same single beetle in the field have been major obstacles to this experiment.

That female abdominal tips of some lepidopteran insects contained more

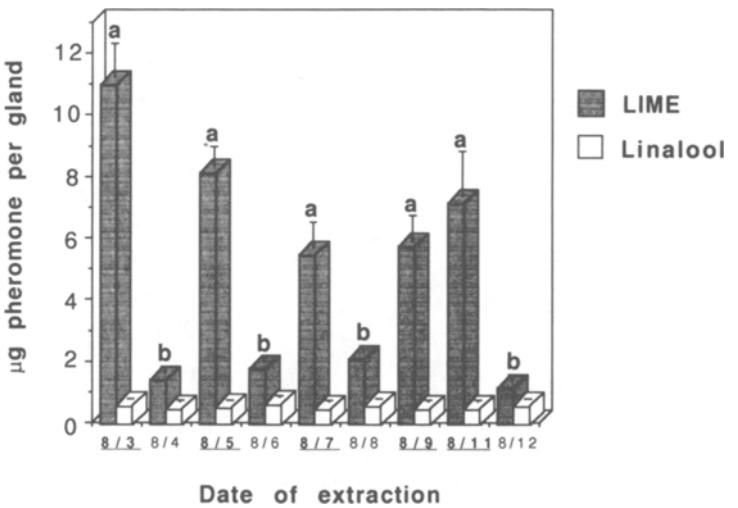

FIG. 6. Pheromone titer in *H. parallela* abdominal glands of field-captured females. Levels of L-isoleucine methyl ester (LIME) reached peaks during calling nights (dates in bold and underlined) and significantly decreased during the following nights (data for 8/10 missed). There were no significant differences for the amounts of (*R*)-(−)-linalool, which was kept nearly constant before and after calling as well as during the experiments. Columns labeled with the same letters are not significantly different (ANOVA, Sheffe *F* test at 5% level). Bars show 1 SE. These results revealed a "circabidian" rhythm of sex pheromone production, i.e., peak levels of pheromone titer appeared every other night.

pheromone during the photo- than the scotophase (when calling was at a maximum) has been considered to be due to the fast pheromonal release during the scotophase (Tamaki, 1985). It seems therefore reasonable to speculate that the pheromone titers of *H. parallela* during calling nights were underestimated because some release may have occurred before the insects were captured. Furthermore, the fact that the amount of L-isoleucine methyl ester in glands of virgin female beetles significantly increased from the photo- to the scotophase of a calling day (Figure 5) demonstrated that pheromone production starts again (or increases) sometime prior to the calling behavior.

Preliminary mark-and-recapture experiments revealed that the ability of male beetles to respond behaviorally to the (synthetic) sex pheromone system is very likely controlled also by a circabidian rhythm. Of 125 beetles released, 56.8% were recaptured on the same night (August 25), whereas the recapture decreased to 0.6% on the following night and increased again after 48 hr (6.4% in August 27). Catches of *H. parallela* males with pheromone-baited traps also showed peaks every other day. The daily average catches in two experiments were 170, 5, 39, 1, and 13; and 85, 1, 28, 1, and 21. That the captures in the peak days also decreased over time was due to the loss of the activity of the bait.

Further investigations are necessary to identify the endogenous and exogenous factors controlling this unusual periodicity of pheromone production and calling behavior by the large black chafer.

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REFERENCES

ANONYMOUS. 1980. Major insect and other pests of economic plants in Japan. Japan Plant Protection Assoc., Tokyo.

ANONYMOUS. 1987. Major insect and other pests of economic plants in Japan. The Japanese Society of Applied Entomology and Zoology (ed.). Japan Plant Protection Assoc., Tokyo.

CARDÉ, R.T., and ELKINTON, J.S. Field trapping with attractants: Methods and interpretation, pp. 111–112, *in* H.E. Hummel and T.A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, New York.

HAYNES, K.F., POTTER, D.A., and COLLINS, J.T. 1992. Attraction of male beetle to grubs: Evidence for evolution of a sex pheromone from larval odor. *J. Chem. Ecol.* 18: 1117–1124.

LEAL, W.S., MATSUYAMA, S., KUWAHARA, Y., WAKAMURA, S., and HASEGAWA, M. 1992a. An amino acid derivative as the sex pheromone of a scarab beetle. *Naturwissenschaften* 79:184–185.

LEAL, W.S., HASEGAWA, M., MOCHIZUKI, F., and YASUDA, T. 1992b. Behavioral and electrophysiological evidence of sex pheromone(s) in *Anomala schonfeldti* Ohaus (Coleoptera: Scarabaeidae). *Appl. Entomol. Zool.* 27:592–594.

LEAL, W.S., SAWADA, M., and HASEGAWA, M. 1993a. The scarab beetle *Anomala daimiana* utilizes a blend of two other *Anomala* spp. sex pheromones. *Naturwissenschaften* 80: In press.

LEAL, W.S., SAWADA, M., and HASEGAWA, M. 1993b. The scarab beetle *Anomala cuprea* utilizes sex pheromone of *Popillia japonica* as a minor component. *J. Chem. Ecol.* 19:1303–1313.

TAMAKI, Y. 1985. Sex pheromones, pp. 145–191, *in* G.A. Kerkut and L.I. Gilber (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 9. Pergamon Press, New York.

YOSHIOKA, K., and YAMASAKI, Y. 1983. Ecology of *Lachnosterna morosa* Waterhouse. I. Behavior of the time of appearance on the ground and oviposition of adult insects. *Jpn. J. Appl. Entomol. Zool.* 27:52–54.

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RESPONSE ON THE GROUND OF BARK BEETLE AND WEEVIL SPECIES COLONIZING CONIFER STUMPS AND ROOTS TO TERPENES AND ETHANOL

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Abstract—Responses of three *Hylastes* species, *Dryocoetes autographus*, and two *Hylobius* species to terpenes and ethanol were studied in field experiments on clear-cut forest sites in Sweden using baited ground traps. α-Pinene alone did not attract any of the six species. A terpene blend (spruce turpentine consisting mainly of α-pinene, β-pinene, and 3-carene) attracted *Hylastes cunicularius*, *H. brunneus*, and *Hylobius abietis* in some experiments, but not in others. The attractiveness of ethanol also varied; the only species consistently attracted was *H. abietis*. Baits containing both terpenes and ethanol, particularly the combination of spruce turpentine and ethanol, were attractive to all species except *Hylobius pinastri*. In *H. abietis*, the terpene plus ethanol/ethanol catch ratios increased during early summer. Seasonal differences in catch levels were observed in *H. cunicularius* and *H. abietis*. The addition of α-pinene reduced the attractiveness of the combination of spruce turpentine and ethanol to *H. cunicularius*, *H. opacus*, and *D. autographus*. The differences in response to the volatiles between species are probably related to differences in reproductive behavior and host preferences.

Key Words—*Hylastes cunicularius*, *Hylastes brunneus*, *Hylastes opacus*, *Dryocoetes autographus*, *Hylobius abietis*, *Hylobius pinastri*, α-pinene, terpenes, turpentine, ethanol, ground traps, Coleoptera, Scolytidae, Curculionidae.

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INTRODUCTION

Many coleopterans living in the inner bark and wood of conifers recognize suitable host material by its odor. Important volatiles mediating host recognition are monoterpenes, e.g., α-pinene, and degradation products such as ethanol (e.g., Borden, 1982; Fatzinger, 1985; Ikeda et al., 1980; Schroeder, 1988). Differences among insect species in their response to host volatiles may be related to differences among the volatiles released by the specific host substrates. For example, the ambrosia beetle *Trypodendron lineatum* Oliv., which breeds in conifer wood in a somewhat advanced stage of deterioration, is attracted during flight by ethanol, but not by α-pinene. In contrast, *Tomicus piniperda* L., which breeds in pine trees that have recently died, is strongly attracted by α-pinene, while high release rates of ethanol may decrease attraction to the host terpene (Schroeder and Lindelöw, 1989).

During the flight period, root-colonizing Coleoptera in the scolytid genus *Hylastes* and the weevil genus *Hylobius* search for suitable breeding substrate, which for most of the species is conifer stumps and roots that are dying or have recently died. The adults immigrate in large numbers into clear-felled areas. They are often abundant at rich sources of conifer volatiles such as fresh sawdust and chips, cut timber, and stacks of pulpwood. Flying *Hylastes cunicularius* Er. are attracted by stored spruce wood (Lindelöw et al., 1992) and by combinations of α-pinene and ethanol (Schroeder and Lindelöw, 1989). A combination of turpentine and ethanol strongly attracted flying *Hylastes salebrosus* Eichh., whereas the single compounds showed no or only a low attractiveness (Phillips, 1990).

After their flight period, the adult insects walk on the ground in search of reproductive substrate, which is usually hidden under the soil surface. Little is known about the orientation behavior of *Hylastes* species during this phase. They burrow deeply into the soil and can find breeding material at a depth of more than 50 cm (Stark, 1952; Hedqvist, 1961; Eidmann et al., 1977). *H. cunicularius* breeds mainly in the roots of spruce stumps and is rarely found in pine (Lekander et al., 1977). In laboratory experiments, this scolytid readily accepted both spruce and pine and had only a slight preference for spruce (Eidmann et al., 1991). The scolytid *Dryocoetes autographus* Ratz. is often found together with *H. cunicularius* and seems to have similar preferences regarding host material, but in contrast to the latter, *D. autographus* may also breed in spruce stems above ground level.

Host odors attract walking *Hylobius abietis* (L.) and induce the weevils to enter the soil in search of reproductive substrate (Nordlander et al., 1986); the combination of host odors and ethanol has a synergistic effect on attraction (Tilles et al., 1986; Nordlander, 1990). *Hylobius pinastri* (Gyllenhal) is also attracted to combinations of terpenes and ethanol. The response of *H. abietis*

to these substances appears to vary with the phases of the adult life cycle (Nordenhem and Eidmann, 1991). In the field, ground traps baited with α-pinene and ethanol attract not only *Hylobius* but also *Hylastes* and other beetles (Zumr and Stary, 1991).

The host orientation behavior of *Hylastes* species and pine weevils, particularly *H. abietis*, is of practical interest because of the damage these insects cause in reforestation areas. The adults feed on conifer seedlings and often cause high seedling mortality.

Trapping methods have been used widely for estimating the relative size of insect populations and damage risks. For instance, a method employing baited ground traps has been suggested for use in monitoring *H. abietis* (Nordlander, 1987). *H. cunicularius* can cause heavy losses of seedlings (Lindelöw, 1992), but no evaluations of damage risks have been published. The first estimates of *H. cunicularius* populations have been documented recently (Lindelöw, unpublished).

Earlier field experiments have shown that low numbers of *H. cunicularius* can be captured in ground traps baited with α-pinene and ethanol. If a more attractive blend could be found, it might be possible to develop an efficient method for estimating *H. cunicularius* populations. Spruce turpentine, a terpene blend consisting mainly of α-pinene, β-pinene, and 3-carene, was considered to be a promising candidate.

The present study had several aims: to evaluate the response of *Hylastes* and *Hylobius* species on the ground to α-pinene, spruce turpentine, and ethanol, used singly and in various combinations, and to establish if there are differences in response among species or seasonal differences within species.

METHODS AND MATERIALS

The responses of walking adults to volatiles were studied using ground traps on several sites during various periods in 1989 and 1990. Experimental sites were located in clear-felled areas in central Sweden. The ages (years elapsed) of the clear-fellings at the time of the experiment are denoted according to the definition used by Bejer-Petersen et al. (1962). Thus, the growing season immediately following cutting is referred to as year A; A + 1 refers to the following year, and so on. Experiments 1 and 6 were conducted on the same site; likewise, experiments 3 and 7 were on the same site. Site ages and trapping periods are given in Table 3 below.

The experiments were conducted using a randomized block design with 10 replicates. One treatment in each experiment consisted of unbaited traps. The minimum distance between traps was 4 m. The ground traps used were slightly conical with holes along the upper margin at ground-surface level. They were similar to the traps used by Nordlander (1987), but smaller (8 cm diameter, 12

cm height). Water containing a detergent in the bottom of the traps prevented escape.

The substances used were ethanol (96% technical), (−)-α-pinene (Fluka 97%, $[\alpha]_D^{20} - 42 \pm 3°$), and spruce turpentine (SCA) containing 440 g/liter α-pinene, 210 g/liter β-pinene, 76 g/liter 3-carene, and 13 g/liter unspecified minor components. The substances were released separately from open glass tubes containing a strip of filter paper. The approximate release rates were 300–400 μl/day for ethanol, 100 μl/day for α-pinene, and 100 μl/day for spruce turpentine.

The traps were inspected at intervals varying in length from a few days to two weeks. At each inspection, the insects were collected and the baits (chemicals and dispensers) were renewed. All bark beetles and pine weevils from experiments 1 and 2 were determined to species and counted in the laboratory. In the other experiments, only *H. cunicularius* and *H. abietis*, the species of prime interest, were counted. In the results, only data for species trapped in numbers larger than 10 in at least one of the treatments are presented.

Catches of *H. abietis* in unbaited traps generally are low, variable, and not very reliable as a basis for comparison among treatments. For that reason the relative catches of *H. abietis* over time in traps baited with ethanol and with combinations of terpene and ethanol in experiments 1 and 2 are expressed as catch ratios, i.e., number caught by combined treatment/number caught by ethanol-baited traps (Figure 2 below).

The statistical treatment comprised a nonparametric analysis of variance, according to Friedman, followed by Dunnet's test for differences between controls and treatments (Zar, 1984). The Wilcoxon signed-rank test was used to test for synergism between terpenes and ethanol, namely, whether the combined treatments caught significantly more insects than the sum of the two separate treatments with single substances. The level of significance was set at $P < 0.05$.

RESULTS

The experiments summarized in Tables 1 and 3 revealed significant differences in the catches of each species (except *H. pinastri* in experiment 2 and *H. cunicularius* in experiment 7) between one or several of the treatments and the untreated controls. All scolytid and weevil species were caught in significantly larger numbers in ethanol-baited traps than in unbaited control traps on the 1-year-old area in experiment 1 (Table 1). On the freshly clear-felled area in experiment 2, only *H. abietis* was caught in higher numbers in traps baited with ethanol than in unbaited traps. In experiments 1 and 2, traps baited with α-pinene or spruce turpentine alone did not catch significantly more insects than the

TABLE 1. NUMBER OF BEETLES CAUGHT IN GROUND TRAPS IN EXPERIMENT 1 (SITE AGE A + 1; 30 APRIL TO 29 JUNE 1990) AND EXPERIMENT 2 (SITE AGE A; 11 MAY TO 21 JUNE 1990)[a]

Species	Treatment							
	U	αP	St	Et	αP & Et	St & Et	St & Et & αP	St & Et & αP/St & Et[b]
Experiment 1								
Hylastes cunicularius	77	63	76	151[c]	207[c]	458[c]	211[c]	0.46**
H. opacus	31	8	14	76[c]	76[c]	236[c]	49	0.21**
H. brunneus	5	23	24[c]	32[c]	81[c]	144[c]	123[c]	0.85
Dryocoetes autographus	10	19	25	90[c]	304[c]	820[c]	352[c]	0.43**
Hylobius abietis	12	43	29	305[c]	507[c]	600[c]	476[c]	0.79
H. pinastri	8	10	8	69[c]	67[c]	44[c]	40[c]	0.91
Experiment 2								
H. cunicularius	27	28	24	20	71[c]	107[c]	57[c]	0.53*
H. opacus	3	5	8	14	33[c]	70[c]	18	0.26*
H. brunneus	1	3	12	7	31[c]	45[c]	35[c]	0.78
D. autographus	2	9	4	27	46[c]	100[c]	35[c]	0.35**
H. abietis	26	63	33	239[c]	426[c]	509[c]	439[c]	0.86*
H. pinastri	3	5	4	12	15	10	15	1.50

[a] Traps were baited with α-pinene (αP), spruce turpentine (St), or ethanol (Et) alone or in various combinations. Unbaited (U) traps were used as controls.

[b] Significant difference between St & Et & αP and St & Et; $*P < 0.05$, $**P < 0.01$, Wilcoxon signed-rank test.

[c] Significant difference between treatment and unbaited control traps; $P < 0.05$, Dunnet's test.

unbaited controls, with the exception of catches of *Hylastes brunneus* Er. with spruce turpentine in experiment 1.

In these two experiments, all species except *H. pinastri* in experiment 2 were caught in higher numbers by the combinations of α-pinene and ethanol or spruce turpentine and ethanol than in unbaited traps. The difference between the catch with the combination of α-pinene and ethanol and the sums of catches in α-pinene baited traps and ethanol baited traps was significant for *D. autographus* and *H. abietis* in experiment 1 (Table 2). In experiment 2 this was true only for *H. brunneus*. Traps baited with a combination of spruce turpentine and ethanol caught significantly higher numbers of *H. cunicularius*, *Hylastes opacus* Er., *H. brunneus*, and *D. autographus* than the sum of the two separate treatments. In experiment 2 this was also true for *H. abietis*.

The addition of α-pinene to the combination of spruce turpentine and ethanol significantly decreased the catches of *H. cunicularius*, *H. opacus*, and *D. autographus* as compared with the combination of spruce turpentine and ethanol in

TABLE 2. SUMS OF BEETLES CAUGHT IN GROUND TRAPS IN EXPERIMENTS 1 AND 2[a]

Species	Sum αP & Et	Com αP & Et	Sum St & Et	Com St & Et
Experiment 1				
Hylastes cunicularius	214	207	227	458[b]
H. opacus	84	76	90	230[b]
H. brunneus	55	81	56	144[b]
Dryocoetes autographus	109	304	115	820[b]
Hylobius abietis	348	507[b]	334	600
H. pinastri	79	67	77	44
Experiment 2				
H. cunicularius	48	71	44	107[b]
H. opacus	19	33	22	70[b]
H. brunneus	10	31[b]	19	45[b]
D. autographus	36	46	31	100[b]
H. abietis	302	426	272	509[b]
H. pinastri	17	15	16	10

[a]Sum = sum of separate catches with α-pinene (αP) or spruce turpentine (St) alone and ethanol (Et) alone. Com = catch with α-pinene or spruce turpentine and ethanol together in same trap.
[b]Significant difference between Sum and Com; $P < 0.05$; Wilcoxon signed-rank test.

experiments 1 and 2. Its addition did not affect *H. brunneus* or *H. pinastri* catches, and for *H. abietis* the reduction was significant only in experiment 2.

In experiment 1 on an A + 1 site, the numbers of *H. abietis* caught in traps baited with ethanol, with α-pinene and ethanol, and with spruce turpentine and ethanol decreased significantly as the trapping period progressed (Figure 1). No such decrease was observed on the fresh clear-cutting in experiment 2, nor did the numbers of *H. cunicularius* caught by these treatments show a tendency to decrease significantly during the trapping period in either of the experiments. For *H. abietis*, the catch ratios (αP & Et/Et and St & Et/Et) exceeded unity at all inspections in both experiments and increased towards the end of the trapping periods (Figure 2). The corresponding catch ratios for *H. cunicularius* varied in both experiments 1 and 2 but did not increase significantly towards the end of the trapping periods. Catches of *H. cunicularius* at the consecutive inspections in these experiments were generally higher with spruce turpentine and ethanol than with α-pinene and ethanol.

In the seven experiments covering various time periods (Table 3), catches of *H. cunicularius* and *H. abietis* were lower in the latter part of the season. No major temporal differences in the response of *H. cunicularius* to any of the treatments were observed, with the exception of a decrease over time in attraction to the combination of α-pinene and ethanol. The combination of spruce turpentine and ethanol was consistently most attractive. Terpenes (spruce tur-

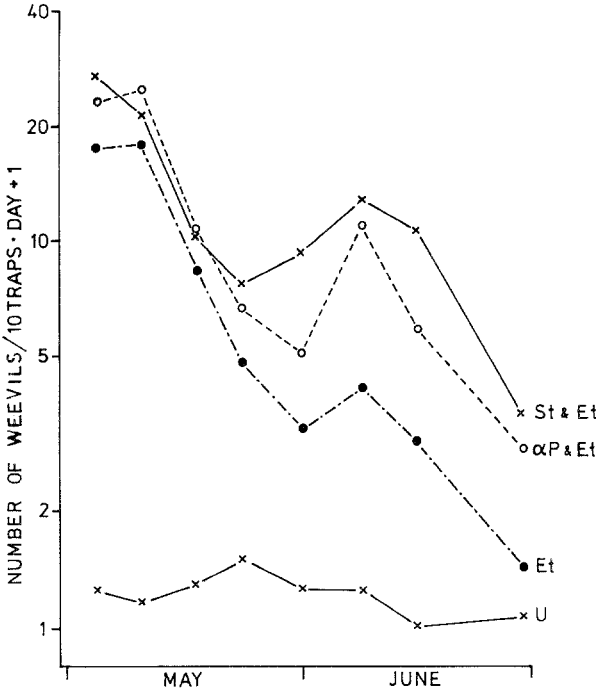

FIG. 1. Mean daily catches (1990) of *Hylobius abietis* in ground traps in four treatments of experiment 1. Treatments: U = unbaited control, Et = ethanol, αP & Et = α-pinene and ethanol, St & Et = spruce turpentine and ethanol. Ordinate: number of beetles/10 traps · day + 1, logarithmic scale.

pentine) caught significantly more *H. cunicularius* than unbaited traps in experiment 4, but not in any of the other experiments. *H. abietis* was also strongly attracted to the combinations of terpene and ethanol, and there was no major difference in this respect between spruce turpentine and α-pinene. In experiment 3, traps baited with spruce turpentine alone caught significantly more *H. abietis* than the unbaited traps.

DISCUSSION

In the present study, spruce turpentine occasionally attracted *H. cunicularius* (experiment 4), *H. brunneus* (experiment 1), and *H. abietis* (experiment 3), but none of the other three coleopteran species. α-Pinene did not significantly attract any of the species, but the catches of *H. abietis* with α-pinene in experiments 1 and 2 were two to three times higher than in unbaited controls. Attrac-

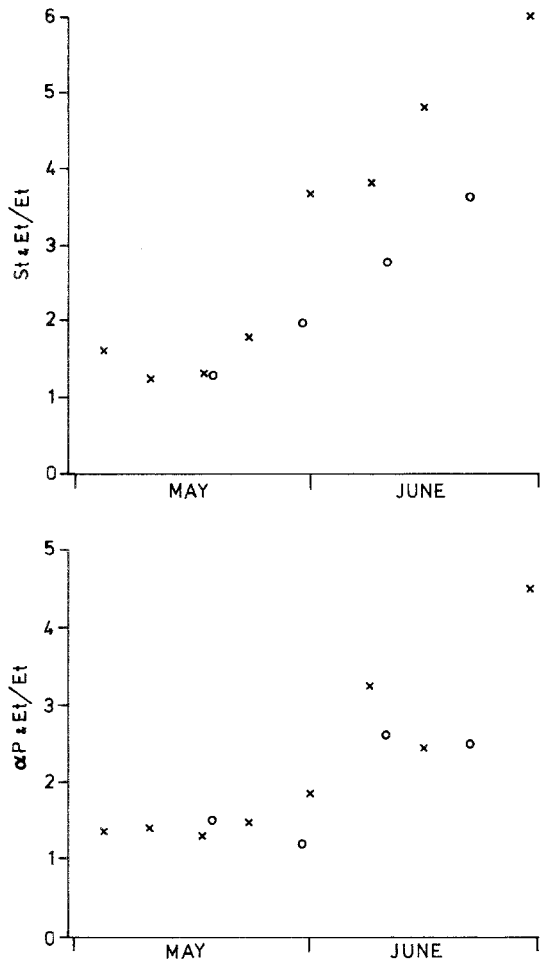

FIG. 2. Catches (1990) of *Hylobius abietis* in experiments 1 (×) and 2 (○) expressed as terpene and ethanol/ethanol catch ratios. Above: spruce turpentine and ethanol/ethanol catch ratio (St & Et/Et). Below: α-pinene and ethanol/ethanol catch ratio (αP & Et/Et).

tion of *H. abietis* and *H. pinastri* on the ground to α-pinene has been observed earlier (Nordlander, 1990). The attractiveness of ethanol varied among experiments, and only *H. abietis* was consistently attracted by ethanol. In earlier studies, traps baited with ethanol caught low numbers of *H. abietis* (Tilles et al., 1986), *H. radicis*, and *Pachylobius picivorus* (Hunt and Raffa, 1989). In contrast, the combinations of terpenes and ethanol were highly attractive, and the substances tended to act synergistically. Synergism between turpentine or stem section and ethanol was also found in the responses of *H. pales*, *H. radicis*,

TABLE 3. NUMBERS OF *Hylastes cunicularius* AND *Hylobius abietis* CAUGHT IN GROUND TRAPS BAITED WITH ETHANOL (Et), α-PINENE (αP), SPRUCE TURPENTINE (St), AND COMBINATIONS αP & Et AND St & Et[a]

Exp.	Site age	Trapping period	Treatment					
			U	αP	St	Et	αP & Et	St & Et
Hylastes cunicularius								
1	A + 1	Apr. 30–June 29, 1990	77	63	76	151[b]	207[b]	458[b]
2	A	May 11–June 21, 1990	27	28	24	20	71[b]	107[b]
3	A + 1	May 13–26, 1989	25	7	37		61[b]	
4	A + 2	May 31–June 13, 1989	15		58[b]		36	291[b]
5	A, A + 1	July 20–25, 1989	7		19	18		50[b]
6	A + 1	Sept. 1–19, 1990	20	3	21	15	6	68[b]
7	A + 1	Sept. 1–14, 1989	1	3	21	5	3	31
Hylobius abietis								
1	A + 1	Apr. 30–June 29, 1990	12	43	29	305[b]	507[b]	600[b]
2	A	May 11–June 21, 1990	26	63	33	239[b]	426[b]	509[b]
3	A + 1	May 13–26, 1989	9	8	36[b]		342[b]	
4	A + 2	May 31–June 13, 1989	0		3		31[b]	35[b]
5	A, A + 1	July 20–25, 1989	0		7	30[b]		92[b]

[a] Unbaited (U) traps used as controls. Age A refers to the first season after clear-felling according to Bejer-Petersen et al. (1962).
[b] Significant difference in catch between treatment and unbaited traps; $P < 0.05$, Dunnet's test.

and *P. picivorus*, while the single substances were not attractive (Raffa and Hunt, 1988; Hunt and Raffa, 1989).

The attraction of *H. cunicularius* and *D. autographus* to spruce turpentine and ethanol is in accordance with their preferred host substrate, which is spruce roots. On the other hand, both *H. brunneus* and *H. opacus*, living in pine roots (cf. Lekander et al., 1977) are also attracted to spruce turpentine and ethanol. The composition of the turpentine may reflect a common conifer odor, not specific enough to be recognized and accepted as spruce or pine by the beetles.

The addition of α-pinene to the combination of spruce turpentine and ethanol reduced attractiveness to *H. cunicularius*, *H. opacus*, and *D. autographus*, but not to *H. brunneus*. Apparently the species differ in their response to α-pinene. The reduced attractiveness suggests that the host orientation of these species is influenced by the amounts or proportions of the released volatiles. This is probably related to the host specificity of the species.

The lower attractiveness of the combination of α-pinene and ethanol to *H. cunicularius* in autumn suggests seasonal differences in response. Another sea-

sonal difference is the generally low catch of *H. cunicularius* and *H. abietis* at the end of July. Low catches of *H. abietis* with terpene and ethanol baits late in the season are in agreement with earlier studies (Nordenhem and Eidmann, 1991), which indicated that the prereproductive new generation in autumn is attracted by natural host material, but not by the combination of α-pinene and ethanol.

The gradual increase observed in the relative attractiveness to *H. abietis* of the combinations of terpenes and ethanol as compared with ethanol alone or, inversely, the decrease in relative attractiveness of ethanol, has not been demonstrated before. This change was observed on a freshly clear-cut area, comprising mostly young, newly immigrated, and reproductive weevils (cf. Nordenhem, 1989), as well as on a 1-year-old area comprising predominantly old, reproductive weevils that had immigrated the previous year. We doubt that this change in relative attractiveness has been caused by temperature-related differences in release rates during the trapping period, since the release rates of ethanol and α-pinene in ground traps were previously found to be similarly influenced by temperature (Nordlander, 1990). Instead, the change may reflect a short-term change of behavior in individual weevils. Alternatively, it could have been an indirect result of a numerical change in the proportions of weevil groups with different orientation behavior.

The combination of spruce turpentine and ethanol trapped about 3–20 times more *H. cunicularius* and about 20–50 times more *H. abietis* than unbaited traps. Thus, spruce turpentine and ethanol may prove useful in practical applications such as population estimates.

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REFERENCES

BEJER-PETERSEN, B., JUUTINEN, P., KANGAS, E., BAKKE, A., BUTOVITSCH, V., EIDMANN, H.H., HEQVIST, K.J., and LEKANDER, B. 1962. Studies on *Hylobius abietis* L. I. Development and life cycle in the Nordic countries. *Acta Entomol. Fenn.* 17:1–106.

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, 527 pp.

EIDMANN, H.H., LINDELÖW, Å., and SOLBRECK, B. 1977. [Black pine beetles in Swedish conifer plantations]. *Sver. Skogsvardsforb. Tidskr.* 75:499–508. (In Swedish with English summary.)

EIDMANN, H.H., KULA, E., and LINDELÖW, Å. 1991. Host recognition and aggregation behaviour of *Hylastes cunicularius* Erichson (Col., Scolytidae) in the laboratory. *J. Appl. Entomol.* 112:11–18.

FATZINGER, C.W. 1985. Attraction of the black turpentine beetle (Coleoptera: Scolytidae) and other forest coleoptera to turpentine-baited traps. *Environ. Entomol.* 14:768–775.

HEDQVIST, K.-J. 1961. Über das Vermögen von *Hylastes brunneus* Er, Brutplätze im Boden aufzusuchen. *Entomol. Tidskr.* 82:211–212.

HUNT, D.W.A., and RAFFA, K.F. 1989. Attraction of *Hylobius radicis* and *Pachylobius picivorus* (Coleoptera: Curculionidae) to ethanol and turpentine in pitfall traps. *Environ. Entomol.* 18:351–355.

IKEDA, T., ENDA, N., YAMANE, A., ODA, K., and TOYODA, T. 1980. Attractants for the japanese pine sawyer, *Monochamus alternans* Hope (Coleoptera: Cerambycidae). *Appl. Entomol. Zool.* 15:358–361.

LEKANDER, B., BEJER-PETERSEN, B., KANGAS, E., and BAKKE, A. 1977. The distribution of bark beetles in the Nordic countries. *Acta Entomol. Fenn.* 32:1–37.

LÍNDELÖW, Å. 1992. Seedling mortality caused by *Hylastes cunicularius* Er. (Coleoptera, Scolytidae) in Norway spruce plantations in northern Sweden. *Scand. J. For. Res.* 7:387–392.

LINDELÖW, Å. Monitoring populations of *Hylastes cunicularius* Erichson. using baited pitfall traps for forecasting subsequent spruce seedling mortality (Coleoptera; Scolytidae). Unpublished.

LINDELÖW, Å., RISBERG, B., and SJÖDIN, K. 1992. Attraction during flight of scolytids and other bark- and wood-dwelling beetles to volatiles from fresh and stored spruce wood. *Can. J. For. Res.* 22:224–228.

NORDENHEM, H. 1989. Age, sexual development, and seasonal occurrence of the pine weevil *Hylobius abietis* (L.). *J. Appl. Entomol.* 108:260–270.

NORDENHEM, H., and EIDMANN, H.H. 1991. Response of the pine weevil *Hylobius abietis* L. (Col., Curculionidae) to host volatiles in different phases of its adult life cycle. *J. Appl. Entomol.* 112:353–358.

NORDLANDER, G. 1987. A method for trapping *Hylobius abietis* (L.) with a standardized bait and its potential for forecasting seedling damage. *Scand. J. For. Res.* 2:199–213.

NORDLANDER, G. 1990. Limonene inhibits attraction to α-pinene in the pine weevils *Hylobius abietis* and *H. pinastri*. *J. Chem. Ecol.* 16:1307–1320.

NORDLANDER, G., EIDMANN, H.H., JACOBSSON, U., NORDENHEM, H., and SJÖDIN, K. 1986. Orientation of the pine weevil *Hylobius abietis* to underground sources of host volatiles. *Entomol. Exp. Appl.* 41:91–100.

PHILLIPS, T.W. 1990. Responses of *Hylastes salebrosus* to turpentine, ethanol, and pheromones of *Dendroctonus* (Coleoptera: Scolytidae). *Fla. Entomol.* 73:286–292.

RAFFA, K.F., and HUNT, D.W.A. 1988. Use of baited pitfall traps for monitoring pales weevil, *Hylobius pales* (Coleoptera: Curculionidae). *Great Lakes Entomol.* 21:123–125.

SCHROEDER, L.M. 1988. Attraction of the bark beetle *Tomicus piniperda* and some other bark- and wood-living beetles to the host volatiles α-pinene and ethanol. *Entomol. Exp. Appl.* 46:203–210.

SCHROEDER, L.M., and LINDELÖW, Å. 1989. Attraction of scolytids and associated beetles by different absolute amounts and proportions of α-pinene and ethanol. *J. Chem. Ecol.* 15:807–817.

STARK, V.N. 1952. Fauna SSSR, Vol. 31, Coleoptera: Ipidae. Moscow, 462 pp.

TILLES, D.A., SJÖDIN, K., NORDLANDER, G., and EIDMANN, H.H. 1986. Synergism between ethanol and conifer host volatiles as attractants for the pine weevil, *Hylobius abietis* (L.) (Coleoptera: Curculionidae). *J. Econ. Entomol.* 79:970–973.

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

ZUMR, V., and STARY, P. 1991. Effects of baited pitfall traps (*Hylobius abietis* L.) on non target forest insects. *J. Appl. Entomol.* 112:525–530.

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EXTRACTION, CHARACTERIZATION, AND BINDING ANALYSIS OF TWO PHEROMONALLY ACTIVE LIGANDS ASSOCIATED WITH MAJOR URINARY PROTEIN OF HOUSE MOUSE (*Mus musculus*)

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Abstract—Mouse urine contains substantial quantities of a family of proteins (MUPs) that are members of the lipocalycin family of proteins and that are potentially capable of binding hydrophobic molecules. We have used gas chromatography–mass spectrometry (GC-MS) to characterize two ligands associated with the MUPs, a thiazole and a brevicomin derivative. Previous work has suggested a role for these two ligands as androgen-dependent pheromones. In urine, nearly all of these ligands are protein bound and fractionation of MUPs on Mono-Q anion exchange chromatography indicated some specificity of ligand binding by the MUP subclasses.

Key Words—Mouse, *Mus musculus*, urine, olfaction, chemosignal, pheromone, protein.

INTRODUCTION

Nocturnal habits and dark living environments have led to the evolution of olfaction as a major method of communication in mice and other rodents. In mice the urine has been demonstrated as a source of olfactory signals affecting factors such as puberty onset in females (Vandenbergh et al., 1975) and inter-

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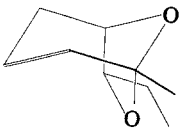
male aggression (Novotny et al., 1985). Evidence exists for the expression of genomic information in mouse urine in that inbred strains of mice differing in only small areas of the major histocompatibility complex (MHC) are able to differentiate between each other by olfaction (Yamazaki et al., 1979).

Mice, in common with other rodents, exhibit an obligate proteinuria, in the form of major urinary proteins (MUPs). This is a heterogeneous family of 19-kDa proteins exhibiting isoelectric points in the region of pH 4.2–4.7 (Duncan et al., 1988). MUPs can be separated into 15 distinct groups using isoelectric focusing (Clissold and Bishop, 1982), the levels of which show differences between both strains and sexes (Clissold et al., 1984). The source of these differences is thought to be either multiple allelic genes or differential rates of translation from mRNA.

Sequence and structural analysis have assigned MUP to the lipocalycin superfamily (Adams and Sawyer, 1990; Bocskei et al., 1991; Cavaggioni et al., 1987). The lipocalcin superfamily is characterized by a unique tertiary structure consisting of 8, 9, or 10 β-pleated sheets, commonly arranged in a cross-hatched formation, forming a central bowl or calyx. The calyx contains many hydrophobic residues, providing a suitable environment for binding or transport of hydrophobic ligands (North, 1991). In the context of a genome–odor link, a urinary protein belonging to an established family of binding/transport proteins could be potentially interesting. A further function of MUP may be to transmit olfactory signals pertaining to the environment. When certain radiolabeled xenobiotics, derived from industrial pesticides, are administered to mice and rats intraperitoneally, significant radioactivity is found in the urine associated with MUP and the analogous α2u protein (Larsen et al., 1990b). In rats a similar association follows oral dosing (Larsen et al., 1990a).

These two models for MUP function, transmission of both genomic and environmental information, would have opposing requirements for the binding specificity of the protein. In the genomic model, where information would be transmitted by differential translation of MUP subclasses, binding would have to be relatively specific and each signal ligand would be associated with a given subclass. By contrast, in the environmental model, a relatively nonspecific binding would be necessary, reflecting the wide range of environmental molecules or bacterial metabolites.

One further possible function of MUP is as a reservoir for pheromonal molecules that could be released gradually from the protein. This would have obvious advantages in a trail marking context. Male mice exhibit a greater proteinuria than females (Finlayson et al., 1963), and two pheromonally active molecules, 3,4-dehydro-*exo*-brevicomin (I) and 2-*sec*-butyl-4,5-dihydrothiazole (II) (Jemiło et al., 1985), are male specific (Schwende et al., 1986) (Scheme 1). Using established protein chemistry, headspace analysis, and solvent extraction of ligands associated with MUP, followed by gas chromatography and gas



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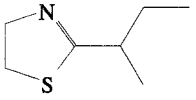

II

SCHEME. 1.

chromatography–mass spectrometry, we have addressed the questions of ligand binding specificity and relative proportions of bound and free ligands. The results provide strong evidence for MUP as a mediator in mouse olfactory communication.

METHODS AND MATERIALS

Animals and Urine Collection. Pooled urine was collected by bladder massage from male Balb-C mice. Mice were housed in groups of 8–16 in standard conditions.

Separation of MUP from Free Urinary Volatiles. Pooled urine was desalted on spun 5-ml Sephadex G-25 columns previously equilibrated in 20 mM HEPES buffer, pH 7.6, or 50 mM MES buffer, pH 5.0, prior to anion exchange chromatography. Aliquots were eluted (250 μl) using centrifugation at 200*g* for 1 min.

Solvent Extraction of Bound Ligands. Three volumes of desalted urine were added to two volumes of redistilled AnalaR grade chloroform (BDH Chemicals Ltd.) in a sealed glass vessel. Total volume was typically 0.5–1.0 ml in a 2-ml glass vial. This was then vortexed for 10 sec and stood for a further 60 min. Solvent and aqueous samples were then separated for screening. Hexane and dichloromethane extractions followed the same protocol with AnalaR grade solvents.

Gas Chromatography and Gas Chromatography–Mass Spectrometry. Solvent extracts were screened using a 25-m $\times$ 0.2-mm ID column coated with

Carbowax 20 M (0.2-μm film thickness) (Hewlett-Packard) fitted to a Hewlett-Packard 5890A GC equipped with an on-column injector. The column head was attached to 45 cm of 0.53-mm-ID deactivated silica retention-gap precolumn via a swagelock union and graphite ferrules. Detection of resolved molecules was achieved using a 5971A mass selective detector or flame ionization detector (FID). Data acquisition, tabulation, and analysis were controlled using MS-Chemstation software on an OpusVII PC (GC-MS) or a Hewlett-Packard 3393 integrator (GC-FID). All mass spectra were obtained by electron ionization at an ionization potential of 70 eV. Initial analysis of headspace and solvent samples used the GC-MS in the full-scan mode to obtain spectra of unknown compounds. Screening of column fractions to study binding specificity and the ratios of the bound and free ligands was performed by selected ion monitoring (SIM). The ions m/z 60 and 125 were monitored for 2-*sec*-butyl-4,5-dihydrothiazole and 3,4-dehydro-*exo*-brevicomin, respectively.

Anion Exchange Chromatography. Anion exchange chromatography was performed using an FPLC system (Pharmacia) fitted with a Mono-Q column (V_t = 1 ml). The column was equilibrated with 10 ml of 50 mM MES buffer, pH 5.0 prior to application of 0.5 ml of desalted urine in the same buffer. This contained typically between 5 and 7 mg of protein (primarily MUP) as estimated by UV spectroscopy at 280 nm [$E_{1\%}$ = 6.4 (Cavaggioni et al., 1990)]. Bound protein was eluted from the column using a linear salt gradient of 0–200 mM NaCl in 22 ml. Fractions (0.5 ml) were collected between elution volumes 19 and 36 ml inclusive. Fractions were chloroform extracted in the presence of an ethyl tridecanoate internal standard (4.2 μg/ml) and screened using GC.

Gel Filtration Chromatography. Gel filtration chromatography was performed on a Sephadex G-25 column (V_t = 15 ml) previously equilibrated with 60 ml of 20 mM HEPES buffer, pH 7.5. Whole urine (0.5 ml) was applied to the head of the column and eluted with 20 ml of buffer. Fractions (0.5 ml) were taken and extracted into chloroform as described above, in the presence of the same ethyl tridecanoate internal standard. Integrated GC peak areas were plotted as a function of fraction number.

Headspace Analysis. Headspace analysis essentially followed the technique of Novotny et al. (1974). A 1-ml sample of desalted denatured urinary protein (pH 7.5) was placed in a side-arm reaction vessel (SGE Ltd.) using a Pasteur pipet and purged for 3 hr at 100 ml/min with helium (BOC) prefiltered through a Tenax trap (Jones chromatography). Volatiles stripped in this manner were trapped and concentrated onto a 27-mg Tenax trap prepared in a splitless injector glass liner (Hewlett-Packard) plugged with silanized glass wool at either end. This trap had been preconditioned at 210°C in a stream of helium overnight.

Volatiles were desorbed from the trap by heating to 200°C in the splitless injection port of the gas chromatograph mentioned above. Volatiles desorbed in this manner were subsequently trapped onto a short length of 0.53-mm-ID deac-

tivated silica retention-gap precolumn cooled in Dry Ice. The retention-gap precolumn was coupled to the GC column using a swagelock union with graphite ferrules. These volatiles were chromatographed using a temperature gradient of 30–200°C at 5°/min on the previously mentioned Carbowax column.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE followed the method of Laemmli (1970) using a Tris chloride/Tris glycine discontinuous buffer system. Electrophoresis was run under nonreducing conditions in 20% acrylamide gels. Molecular mass determinations were made by comparison to a set of standard marker proteins (Bio-Rad).

RESULTS AND DISCUSSION

Preliminary observations in our laboratory established that a preparation of MUPs was odorless, but that after denaturation and extraction with a solvent such as chloroform, the organic phase acquired a strong "mousy" odor. This implied that the MUPs were capable of binding volatile molecules. MUPs, isolated from low-molecular-weight material by gel filtration and extracted with chloroform were analyzed by GC. The FID trace showed two major peaks (I and II) with similar retention times (6.2 and 6.7 min) in three different solvents: chloroform, dichloromethane, and hexane. The trace obtained from the chloroform extraction is shown in Figure 1. Further work using headspace analysis (Figure 2) again shows the presence of two major peaks (I and II), in addition to lesser concentrations of other material with shorter retention time.

These two major peaks were analyzed further by GC-MS. The spectrum

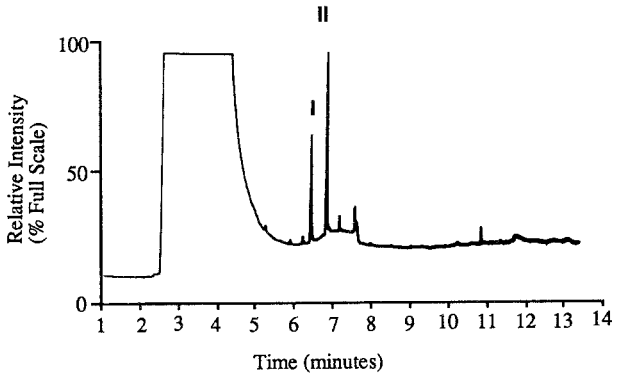

FIG. 1. Gas chromatogram obtained from chloroform extract of MUP. One microliter of chloroform extract was injected on-column onto a Carbowax 20M coated capillary column fitted with a retention gap. The GC temperature was maintained at 50°C for injection, held isothermally for 3 min and then raised to 200°C at 20°C/min.

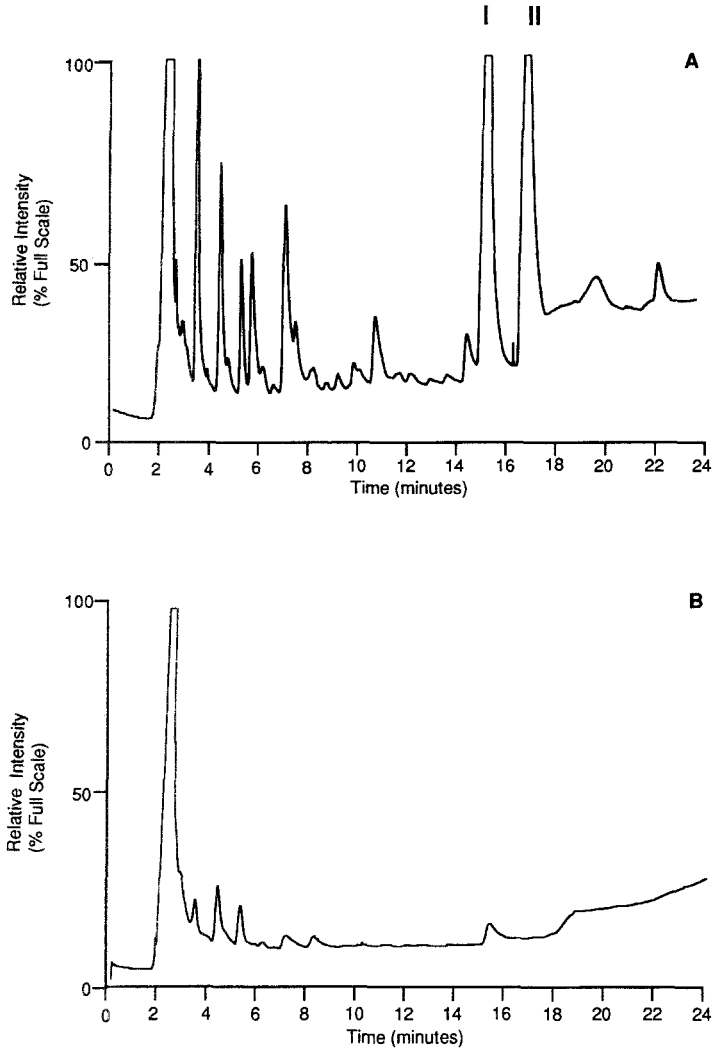

FIG. 2. Headspace analysis of MUP: 1 ml of MUP (A) was denatured by addition of an equal volume of 8 M guanidinium thiocyanate, and 1 ml of buffer (B) was treated in the same manner. Volatiles were sparged off with helium at 100 ml/min and trapped onto Tenax. Trapped volatiles were thermally desorbed and cryogenically focused onto the retention gap packed in Dry Ice. Subsequent chromatography was conducted isothermally for 3 minutes at 30°C followed by a temperature program of 30–200°C at 5°C/min.

from peak I shows major ions (with percent relative abundances given in parentheses) of *m/z* 41(19), 43(100), 53(10), 57(29), 81(16), 95(34), 111(31), and 125(18). The molecular ion ($M^{+\cdot}$) appears at *m/z* 154. The spectrum of peak II is more distinctive having major ions of *m/z* 45(11), 60(100), 115(59), and 128(8), with $M^{+\cdot}$ appearing at *m/z* 143 (mass spectra not shown).

Characterization of these two ligands was by comparison to published data on total urinary volatiles (Schwende et al., 1986). Peak I correlated with the spectrum from 3,4-dehydro-*exo*-brevicomin and peak II correlated with the spectrum of 2-*sec*-butyl-4,5-dihydrothiazole. Whole urine, analyzed in a similar manner also showed two major peaks with identical spectra. Both of these compounds are reported to be present at high levels in male urine and are dramatically reduced after castration (Schwende et al., 1986). Other components, present in much lower levels, have yet to be identified. However, these data indicate clearly a role for the MUPs in the binding of important chemosensory molecules.

Previous analysis of urinary volatiles has been based on headspace analysis of total mouse urine (Schwende et al., 1986). In this work, we have demonstrated a role for the MUPs in odorant binding. The partition of the 3,4-dehydro-*exo*-brevicomin and 2-*sec*-butyl-4,5-dihydrothiazole between the protein bound and the aqueous phases of freshly collected whole urine was discerned by gel filtration on Sephadex G-25 (Figure 3). The total elution profile was assessed by ultraviolet absorbance, and delineated the high-molecular-weight (>5-kDa) fraction, eluting at V_e = 5 ml and a broader peak of low-molecular-weight material eluting later at V_e = 10 ml. The protein nature of the first peak is confirmed by SDS-PAGE (inset). Each fraction was extracted with chloroform and analyzed by GC-MS in SIM mode as previously described. The elution profile shows that over 60% of the brevicomin and 98% of the thiazole are protein-bound in urine. This association is relatively stable, as the ligands remain bound under all of the protein-handling conditions noted in this text.

The MUPs are a complex family of proteins, and several alleles of the multigene family are expressed in different mouse strains (Clissold and Bishop, 1982). Partial fractionation of the MUPs can be achieved by chromatography of desalted MUP on the strong anion exchanger, Mono-Q (Figure 4). The profile consists of four major peaks followed by three minor peaks. SDS-PAGE analysis (inset) indicates the presence of similarly sized proteins (~18 kDa) in each major peak. The minor peaks are, however, slightly different. The first and the third contain a single species of approximately 21 kDa, whereas the second peak contains two species of approximately 18 and 21 kDa. In the absence of more detailed sequence data, these cannot be unequivocally identified as MUPs. Chloroform extracts of fractions derived from Mono-Q chromatography of desalted urine indicate that the 3,4-dehydro-*exo*-brevicomin and 2-*sec*-butyl-4,5-dihydrothiazole are associated with most of the MUP fractions, but that there is some

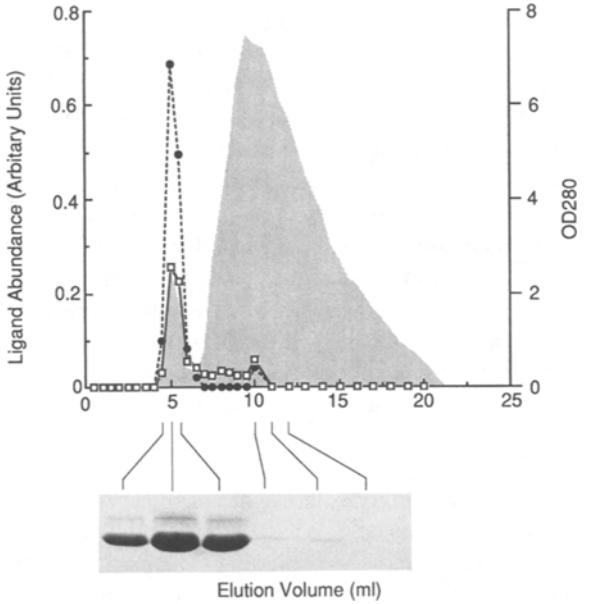

FIG. 3. Elution profile of whole mouse urine after gel filtration chromatography on Sephadex G-25: □ OD280, ---•--- thiazole, ——□—— brevicomin. Column bed volume = 15 ml. SDS-PAGE was conducted according to the method of Laemmli (1970) in 20% gels under nonreducing conditions.

specificity to the binding of these two ligands. A plot of 3,4-dehydro-*exo*-brevicomin expressed as a percentage of total extracted ligands (Figure 5) shows a degree of variation across the elution trace. Extracted brevicomin is associated with the second, third, and fourth major peaks, whereas extracted thiazole is associated with all peaks of the profile. The first major peak and all three minor peaks bind only thiazole. The maximum thiazole abundance occurs at V_e 23 ml (Figure 4), which does not coincide with a major protein or extracted brevicomin peak. This analysis provides evidence for specificity in ligand binding of the different MUPs.

If the function of this protein-bound reservoir is to prolong an olfactory message, the question of a mechanism for ligand release must be addressed. Ligands may be released by protein drying, but this mechanism would become ineffective in moist conditions, which would be significant in the mouse habitat. Alternatively, a protease such as the mouse-specific brush border and urinary protease meprin (Flannery et al., 1990) could be responsible. Finally, the relative proportions of released ligands could convey information. Proportions of 2-*sec*-butyl-4,5-dihydrothiazole and 3,4-dehydro-*exo*-brevicomin in the chloro-

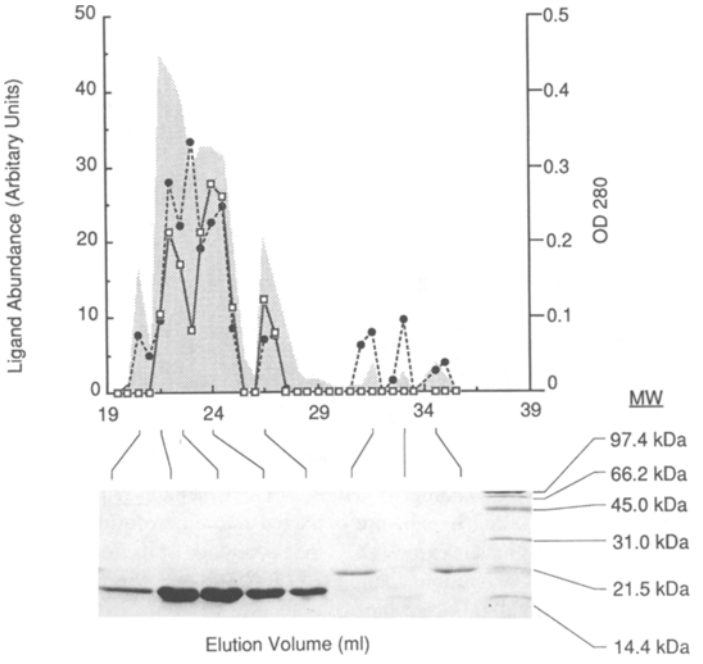

FIG. 4. Elution profile of MUP after anion exchange chromatography on Mono-Q: □ OD280, ---•--- thiazole, ——□—— brevicomin. SDS-PAGE followed the method of Laemmli (1970) in 20% gels run under nonreducing conditions.

form could reflect the physical nature of the extracting solvent. Headspace results (not shown) show the ratio of the two ligand peak areas to be about 5:1 in favor of thiazole. In the drying model, differential release could be achieved by overall levels of MUP subclasses; in the proteolysis model, by specificity of proteolytic cleavage.

The degree of binding specificity of 2-*sec*-butyl-4,5-dihydrothiazole and 3,4-dehydro-*exo*-brevicomin to MUP is at best small. 3,4-Dehydro-*exo*-brevicomin is slightly more specific than 2-*sec*-butyl-4,5-dihydrothiazole, which binds to all the MUP subclasses to some extent. This being the case, what purpose do the subclasses, which vary between sexes and strains, serve? The androgen-related nature of the two ligands (Jemilo et al., 1985) together with the differences in proteinuria (Finlayson et al., 1963) suggest that one function of MUP is the transmission of a message pertaining to male sexual viability. However, the inability of the ligands to elicit a response when presented alone or jointly in water suggests that some other factor needs to be present. Presumably this is, at least in part, the function of the other protein-associated ligands seen by

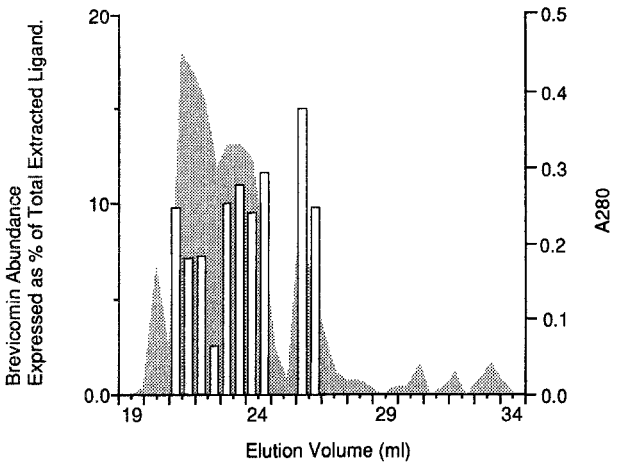

FIG. 5. Specificity of ligand binding by MUP. MUP is separated by anion exchange chromatography at pH 5.0 and ligands are extracted into chloroform as previously described. Brevicomin abundance is expressed as the percentage of the total extracted ligand and plotted as a function of elution volume. □ OD280, brevicomin abundance expressed as percent of total extracted ligand.

headspace analysis. Characterization of these ligands will establish other possible messages mediated or transmitted by MUP.

Finally, if a function of MUP is to transmit messages pertaining to diet or the environment, the low binding specificity demonstrated by 2-*sec*-butyl-4,5-dihydrothiazole and 3,4-dehydro-*exo*-brevicomin would be advantageous. Headspace analysis of the urines and MUPs of genetically identical mice, reared on different diets would go some way to testing this hypothesis. More recently, evidence has been documented showing that, by olfaction, rats can discriminate between the urines of genetically identical mice reared on different diets (Schellinck *et al.*, 1992). In this latter case a model is proposed involving a pool of metabolites created by the action of commensal gut bacteria, carried to the urine by class I MHC antigens.

While this manuscript was in preparation, two independent reports have been published demonstrating that MUPs bind one or both of the ligands reported here (Bacchini et al., 1992; Bocskei et al., 1992). While both papers have reported ligand association with the protein, no information was provided on the protein: free partition or specificity of MUP binding. Additionally, no mention is made of the possible function of such a protein association.

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REFERENCES

ADAMS, P., and SAWYER, L. 1990. Structure of rodent urinary proteins. *Biochem. Soc. Transac.* 18:936–937.

BACCHINI, A., GAETANI, E., and CAVAGGIONI, A. 1992. Pheromone binding proteins of the house mouse *Mus musculus*. *Experentia* 48:419–421.

BOCSKEI, Z., FINDLAY, J.B.C., NORTH, A.C.T., PHILLIPS, S.E.V., SOMERS, W.S., WRIGHT, C.E., LIONETTI, C., TIRINDELLI, R., and CAVAGGIONI, A. 1991. Crystallization of and preliminary X-ray data for the mouse major urinary protein and rat a-2u globulin. *J. Mol. Biol.* 218:699–701.

BOCSKEI, Z., GROOM, C.R., FLOWER, D.R., WRIGHT, C.E., PHILLIPS, S.E.V., CAVAGIONI, A., FINDLAY, J.B.C., and NORTH, A.C.T. 1992. Pheromone binding to two rodent urinary proteins revealed by X-ray crystallography. *Nature* 360:186–188.

CAVAGGIONI, A., SORBI, R.T., KEEN, J.N., PAPPIN, D.J.C., and FINDLAY, J.B.C. 1987. Sequence homology between pyrazine binding protein, major urinary protein and α-microglobulins. *FEBS. Lett.* 212(2):225–228.

CAVAGGIONI, A., FINDLAY, J.B.C., and TRINDELLI, R. 1990. Ligand binding characteristics of homologous rat and mouse urinary proteins and pyrazine binding protein of the calf. *Comp. Biochem. Physiol.* 96B(3):513–520.

CLISSOLD, P.M., and BISHOP, J.O. 1982. Variation in mouse major urinary protein genes and gene products within inbred lines. *Gene* 18:211–220.

CLISSOLD, P.M., HAINEY, S., and BISHOP, J.O. 1984. Messenger RNAs coding for MUPs are differentially induced by testosterone. *Biochem. Genet.* 22(3/4):379–387.

DUNCAN, R., MATTHAI, R., HUPPI, K., RODERICK, T., and POTTER, M. 1988. Genes that modify expression of major urinary proteins in mice. *Mol. Cell. Biol.* 8(7):2705–2712.

FLANNERY, A.V., DALZELL, G.N., and BEYNON, R.J. 1991. Proteolytic activity in mouse urine: Relationship to the kidney metallo-endopeptidase, meprin. *Biochim. Biophys. Acta.* 1041: 64–70.

FINLAYSON, J.S., POTTER, M., and RUNNER, R.C. 1963. Electrophoretic variation and sex dimorphism of the major urinary protein complex in inbred mice: A new genetic marker. *J. Natl. Cancer Inst.* 31:91–107.

JEMILO, B., ALBERTS, J., SOCHINSKI-WIGGINS, S., HARVEY, S., and NOVOTNY, M. 1985. Behavioral and endocrine responses of female mice to synthetic analogs of volatile compounds in male urine. *Anim. Behav.* 33:1114–1118.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680.

LARSEN, G.L., BAKKE, J.E., and HUWE, J.K. 1990a. Methylsulphone metabolites of *m*-dichlorobenzene as ligands for α2u-globulin in rat kidney and urine. *Xenobiotica* 20(1):7–17.

LARSEN, G.L., BERGMAN, A., and KLASSON-WEHLER, E. 1990b. A methylsulphonyl metabolite of a polychlorinated biphenyl can serve as a ligand for α2u-globulin in rat and major urinary protein in mice. *Xenobiotica* 20(12):1343–1352.

NORTH, A.C.T. 1991. Structural homology in ligand specific transport proteins. *Biochem. Soc. Symp.* 57:35–48.

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(7):333–338.

NOVOTNY, M., HARVEY, S., JEMILO, B., and ALBERTS, J. 1985. Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl. Acad. Sci. U.S.A.* 82:2059–2061.

SCHELLINEK, H.M., WEST, A.M., and BROWN, R.E. 1992. Rats can discriminate between the urine

odours of genetically identical mice maintained on different diets. *Physiol. Behav.* 51:1079–1082.

SCHWENDE, F.J., WEISLER, D., JORGENSON, J.W., CARMAC, M., and NOVOTNY, M. 1986. Urinary volatile constituents of the house mouse *Mus musculus* and their endocrine dependency. *J. Chem. Ecol.* 12(1):277–296.

VANDENBERGH, J.G., WHITSETT, J.M., and LOMBARDI, J.R. 1975. Partial isolation of a pheromone accelerating puberty in female mice. *J. Reprod. Fertil.* 43:515–523.

YAMAZAKI, K., YAMAGUCHI, M., BOYSE, E.A., and THOMAS, L. 1979. The MHC as a source of odours imparting individuality among mice, pp. 267–273, *in* Chemical Signals: Vertebrates and Aquatic Invertebrates. D. Muller-Schwarze and R.M. Silverstein (eds.). Plenum, New York.

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MEASUREMENT OF MONOTERPENE HYDROCARBON LEVELS IN VAPOR PHASE SURROUNDING SINGLE-LEAF PINYON (*Pinus monophylla* TORR. & FREM.: PINACEAE) UNDERSTORY LITTER

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Abstract—A headspace air-sampling experiment was performed in the laboratory to determine the identity and concentrations of monoterpene hydrocarbons that could be attained in the vapor phase surrounding single-leaf pinyon pine (*Pinus monophylla* Torr. & Frem.) understory litter using controlled air collection conditions at a simulated field temperature of 37.8°C. The total monoterpene hydrocarbon content in 21 sequential samples of air collected from a sealed glass carboy packed with 1.44 kg of single-leaf pinyon litter equivalent to a bulk density of 0.15 g/cm^3 averaged 3.56 ± 1.04 mg/liter. The monoterpenes α-pinene and camphene were present in the vapor phase at the highest concentrations, averaging 2.40 ± 0.64 and 0.68 ± 0.22 mg/liter, respectively. Myrcene, β-pinene, 3-carene, β-phellandrene, and γ-terpinene were all present at average concentrations below 0.30 mg/liter. The first two traps of the sequential air samples yielded the highest concentrations for the monoterpene hydrocarbons; however, the average total levels were relatively stable throughout the remaining 19 traps. Therefore, the data indicate that these hydrocarbons volatilize from the source pinyon litter and maintain an equilibrium in the vapor phase. Although this analysis was conducted using an artificial system, combined with results from our previous studies, mounting evidence indicates that monoterpene hydrocarbons present in the vapor phase of the single-leaf pinyon understory may be toxic to a variety of native plant species and thus further implicates allelopathy as a

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significant contributor to the observed patterning of associated vegetation in these forests.

Key Words—Allelopathy, phytotoxicity, vapor phase, monoterpene, single-leaf pinyon pine, *Pinus monophylla*, volatile hydrocarbons, pinyon litter, headspace analysis.

INTRODUCTION

Many observations have shown that either the living plant, decaying litter (Everett, 1987; Bradow and Connick, 1990) and/or soils (C.H. Muller, 1966, 1968, 1971) can potentially act as repositories for allelopathic monoterpene hydrocarbons. These compounds may volatilize from their source compartments and then become transported through the vapor phase to the affected "host" plant species. Many allelopathy studies have focused on describing the kinds and amounts of implicated phytotoxins present in the living source plant, decaying tissues comprising the litter, or retained in the soils surrounding a presumably affected area. The results from such analyses are normally used to perform in vitro bioassay experiments designed to identify bioactive constituents. Although such studies have documented the identity and levels of phytotoxic compounds present in these compartments, the actual amounts available in the natural environment for transport to the "target" organism have not been thoroughly investigated. Amounts of these bioactive compounds present in the vapor phase within the understory litter layer must be measured to establish the relationship between phytotoxicity and vapor phase concentration. Furthermore, the amount of time that concentrations must be maintained for toxic effects to be observed, along with specific modes of entrance and metabolism in "target" plants, requires further examination. Ultimately, dose–response relationships for growth inhibition of affected species could be determined by knowledge of the amounts and fate of phytotoxic hydrocarbons present in the surrounding vapor phase.

Our previous work has characterized the solvent-extractable monoterpene hydrocarbons in whole pinyon litter and foliage. The objectives of this study were to identify the monoterpenes present and their relative concentrations within the interstitial vapor phase surrounding single-leaf pinyon litter after volatilization from the source. The analysis was also designed to provide a preliminary indication of the vapor-phase equilibrium concentrations that could possibly be attained in nature during the typical hot and dry environmental conditions commonly encountered in these forests. The air-sampling data will also provide an indication of the rate of replacement to the vapor phase from the source litter material. Therefore, the experiments were performed using a completely closed system where both air volume and monoterpene concentration could be accurately determined.

Furthermore, preliminary data will be provided by these experiments that would be useful for determining appropriate laboratory bioassay conditions. These bioassay results, combined with knowledge of the monoterpene composition in the vapor phase of the carboy, could be highly indicative of whether or not toxicity to native pinyon understory "competitors" is possible in nature. In order to conclusively show the impact of allelopathy and its precise mechanisms in the single-leaf pinyon forest ecosystem, both the direction and design of future studies, including air sampling in the field and bioassay using the litter material itself as the source of allelopathins, are highly dependent on such preliminary analyses.

Admittedly, the results from controlled headspace analyses are artificial and any meaningful ecological interpretations are only suggestive. Many important questions must be answered in order to relate the results from such studies to "real world" ecological significance since natural systems are influenced by many interacting abiotic and biotic factors. For example, besides temperature, moisture, and wind conditions, the interstitial vapor-phase hydrocarbon levels in pine understory litter in either natural or artificial situations will be partially dependent on the age and depth of the litter layer, total amounts or bulk density, and composition (i.e., proportion of cones, bark, needles, etc.) and concentrations of essential oils in these various litter components. In the field, moisture, temperature, and other physical conditions are not constant, and fluctuations obviously can significantly affect volatilization and equilibria. These examples, of course, only represent a small number of the factors influencing vapor-phase equilibria in such systems. Ioffe and Vitenberg (1984) comprehensively review the techniques, design, and problems associated with headspace sampling experiments using gas chromatography.

Determination of the biochemistry of toxicity and the eventual identification of the genes responsible for resistance to allelopathins would have future beneficial results for agriculture (Putnam, 1983, 1985; Rice, 1984). For example, the development of new natural herbicides and food crop species that express genes to biosynthesize or exhibit resistance to certain allelochemicals could ultimately be developed with such knowledge (Marx, 1983).

METHODS AND MATERIALS

A large paper bag of dry single-leaf pinyon understory litter consisting of small branches, twigs, foliage, cones, seeds, and bark was collected in early October 1988 from the Virginia Mountains in western Nevada, T17N, R21E, sect. 6, se 1/4. The sample was removed from the top 5 cm of the litter layer and older material composing the dark "cakelike" decaying mat underneath was excluded. The age range of litter components from the time of tree abscission

was estimated to be variable, from recently deposited (days old) to not greater than 10 years. The bagged sample was immediately placed in a cold ice chest and brought back to the laboratory where the bag was quickly transferred to a −70°C freezer. A 9.5-liter Pyrex glass carboy was then fitted with a rubber stopper, which was lined with aluminum foil. Two glass tubes were fitted through the stoppers and sealed with a water-based glue. A vacuum gauge was attached to one of the protruding tubes; the other tube was connected with glass tubing to a 50-ml glass impinger. An air flowmeter was attached with a vacuum line to the impinger, which was connected to a vacuum pump through another length of tubing. A needle valve was placed in-line between the air flowmeter and the vacuum pump so that the air flow rate could be accurately controlled. A schematic diagram of the air-sampling system used to trap monoterpene vapors from the single-leaf pinyon litter is shown in Figure 1.

On the following day, the litter sample was removed from the −70°C freezer and 1.44 kg placed into the carboy, with frequent shaking to settle. Care was taken to avoid breaking and shattering the dry brittle litter material when loading it into the carboy. The final bulk density of litter inside the full glass chamber was 0.15 g/cm^3, which, although well within the range encountered naturally, is slightly lower than the average of 0.23 g/cm^3 reported for single-leaf pinyon forests by Everett and Thran (1992). After filling the carboy and sealing off the aluminum foil-lined stopper and glass tubes with a water-based glue, it was then placed in an oven and maintained at 37.8°C in the dark for one week. The carboy was removed from the oven and immediately connected to a vacuum pump. The impinger was filled with 30 ml of Fisher Optima-Grade hexane. Vacuum was then applied, with the air flow rate being adjusted to 150 ml/min. Air was sampled until the vacuum gauge indicated that exactly 0.5 atm

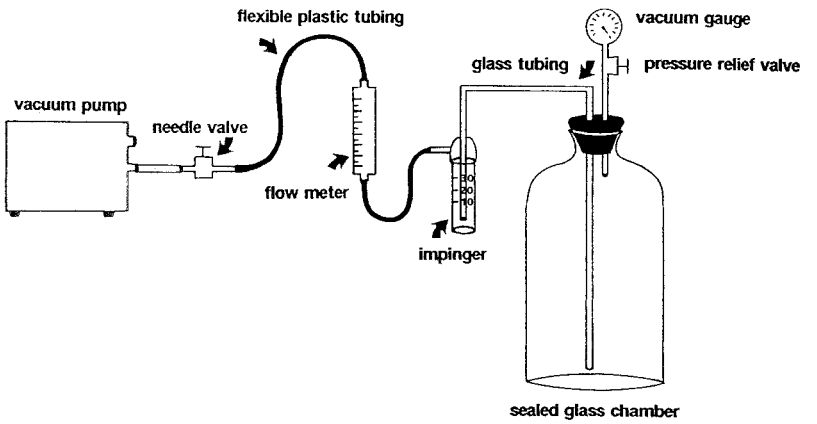

FIG. 1. Air-sampling apparatus with empty sealed glass chamber.

of air remained in the carboy. After the vacuum was shut off, fresh air was slowly allowed back into the system to reestablish atmospheric pressure. The contents of the impinger was transferred into glass containers, which were sealed and refrigerated. The carboy was disconnected from the vacuum pump and placed back in the 37.8°C oven for 1 hr. This procedure was repeated 21 times and the mean sampling time was about 55 min (RSD = 2.5%) with an average of approximately 8200 ml (RSD = 2.5%) of air being sampled during each run.

Each of the 21 samples in hexane were then evaporated to 3 ml under nitrogen while being kept cold in an ice bath. The samples were analyzed by injection of 2-μl aliquots onto a Varian 1700 gas chromatograph connected to a Hewlett-Packard 3393A reporting integrator. The gas chromatograph was temperature programmed from 55 to 155°C over 10 min. The monoterpenes were resolved on a Supelco (0.075 mm × 30 m) wide-bore glass capillary column with a 1.5-μm-thick methylsilicone coating. Concentrations of monoterpene hydrocarbons were determined using an external reference standard of α-pinene, since response factors for other monoterpene hydrocarbons were identical. Both individual and total monoterpenes were quantified, and the results are expressed as milligrams of monoterpene recovered per liter of air sampled. The identities of the individual monoterpenes present were confirmed by using a Finnigan 4023 GC-MS. The procedures and conditions for GC-MS analysis are the same as reported in Wilt et al. (1993), except that the samples were not cleaned up by passing through a silica gel column.

RESULTS

The 21 air samples were collected consecutively and collection took just over 42 hr to complete. Concentrations for the seven compounds and total monoterpenes present in the air samples are presented in Table 1. The compound occurring in the vapor phase at the highest concentrations was α-pinene. Camphene was present at the second highest concentration, while the other monoterpenes were present at much lower levels. Amounts of individual and total monoterpenes were highest in the first two traps and then fell to lower but generally consistent levels in the subsequent traps (RSD = 29.2%; Table 1). The monoterpenes identified in the samples were α-pinene, camphene, β-pinene, myrcene, limonene, β-phellandrene, 3-carene, and γ-terpinene. Limonene and β-phellandrene coeluted and were quantified as a single peak. Based on our previous work (Wilt et al., 1993) β-phellandrene was probably dominant in the source litter with smaller amounts of limonene. The limit of detection for these compounds on the analytical GC was about 10 ng.

TABLE 1. MONOTERPENE CONCENTRATIONS

	Compound (mg/liter air)[a]							
Trap	A	B	C	D	E	F	G	H
1	3.48	1.29	0.13	0.26	0.07	0.73	0.07	6.02
2	4.30	1.07	0.03	0.13	0.03	0.32	0.03	5.90
3	2.20	0.55	ND	0.43	0.10	0.27	0.01	3.30
4	2.24	0.48	0.01	ND	ND	0.16	ND	2.88
5	2.22	0.60	ND	ND	ND	0.27	ND	3.09
6	3.10	0.76	0.02	0.06	0.01	0.23	ND	4.18
7	2.48	0.61	0.001	0.01	ND	0.19	0.04	3.32
8	2.52	0.64	ND	0.06	ND	0.20	ND	3.43
9	2.14	0.55	ND	0.27	ND	0.19	ND	3.15
10	2.48	0.66	0.05	0.08	0.01	0.23	ND	3.46
11	2.05	0.55	0.01	0.11	ND	0.21	ND	2.92
12	1.70	0.48	0.01	0.04	0.02	0.20	0.01	2.46
13	1.74	0.48	ND	0.11	ND	0.18	ND	2.51
14	1.66	0.46	ND	ND	ND	ND	ND	2.12
15	1.95	0.55	ND	0.10	0.01	0.27	0.02	2.90
16	2.94	0.91	0.02	0.11	0.02	0.41	0.06	5.05
17	2.00	0.58	ND	0.22	ND	0.33	0.02	3.16
18	2.95	0.97	0.02	0.25	0.03	0.52	ND	4.73
19	2.47	0.74	0.02	0.41	ND	0.30	ND	3.94
20	1.81	0.53	0.01	0.22	ND	0.23	ND	2.79
21	2.07	0.93	0.01	0.37	ND	0.28	ND	3.36
Mean	2.40	0.67	0.03	0.17	0.03	0.29	0.03	3.56
SD	0.64	0.22	0.03	0.12	0.03	0.13	0.02	1.04
RSD	26.7	32.8	100	70.6	100	44.8	66.7	29.2

[a] A = α-pinene, B = camphene, C = β-pinene, D = myrcene, E = 3-carene, F = limonene/β-phellandrene, G = γ-terpinene, H = total monoterpene, SD = standard deviation, RSD = relative standard deviation, ND = not detected.

DISCUSSION

The air sampling results show that single-leaf pinyon litter is capable of liberating substantial amounts of monoterpene hydrocarbon vapors into the carboy at 37.8°C. During the course of the air sampling, the concentration of monoterpenes in the vapor phase remained relatively constant, indicating that the litter contains a significant reservoir of these compounds. The slightly higher levels of hydrocarbons in the first two traps are most likely due to the long initial oven equilibration period. Thus, as expected, when monoterpene hydrocarbons are removed from the vapor phase, continued volatilization of these compounds from the single-leaf pinyon litter occurs in order to maintain equilibrium (Ioffe and Vitenberg, 1984). The rates of replacement are likely tem-

perature dependent and, although an exact rate for this replacement was not determined, it appears to be relatively rapid since monoterpene concentrations in the carboy achieved 50% of maximum or greater values within 1 hr and were not considerably depleted over the course of the experiment. Because recovery efficiency was not examined in these experiments, all reported monoterpene concentrations must be considered "at least."

Since these air sampling experiments were performed using an artificial system and without determination of recovery efficiency, it is not possible to know whether the monoterpene concentrations found in the vapor phase of the sealed carboy can occur in nature. We feel that the one-week equilibration time before sampling is justified since temperatures within the pinyon litter layer can reach up to 54°C on hot sunny days and can remain near 38°C on warm summer nights (unpublished data). Similar vapor-phase equilibrium conditions as present within the sealed chamber could possibly occur in nature when combined with hot days, warm nights, and a deep litter layer with little or no atmospheric mixing. Therefore, it is probably safe to assume, given reasonable hydrocarbon recovery, that the equilibrium concentrations of monoterpenes in the vapor phase of the carboy represent the higher range of levels that may occur naturally. However, during very hot weather in the field, vapor-phase monoterpene equilibrium concentrations could conceivably surpass the levels present in the sealed carboy at 38°C due to both increased volatility and the higher saturation capacity of the surrounding atmosphere.

Although monoterpene levels in the single-leaf pinyon understory litter are quite variable, depending on the degree of weathering and stage of decay, our previous work (Wilt et al., 1988) showed that they are present in the upper layers at relatively high concentrations. The monoterpenes of the genus *Pinus* are biosynthesized within epithelial secretory cells, which form a thin layer (usually one cell layer thick) around schizogenously formed resin ducts (Esau, 1977). These resin ducts are present imbedded in virtually all parts of the tree, including needles, bark, roots, cones, bole, and stem wood (Esau, 1977; Fahn, 1979). The monoterpene hydrocarbons are deposited from the secretory cells into the lumina of the resin ducts and accumulate as an oily mixture (Fahn, 1979).

Loss of these hydrocarbons from pinyon litter likely occurs through gradual diffusion into the surrounding atmosphere. Monoterpene loss is also facilitated by other physical weathering processes, catabolism by fungi, bacteria, and consumption by invertebrates. Furthermore, it appears that exposure of the resin canals through decay may be an important part of the release process since the dark-colored pinyon needles forming a subsurface layer underneath the more recently accumulated litter contain relatively low amounts of monoterpenes compared to the levels present in the upper litter layers (Wilt et al., 1993). The relative contribution of abiotic versus biotic processes in the loss of monoterpene

hydrocarbons from single-leaf pinyon understory litter has not been determined. However, especially during hot and dry weather, high ambient temperatures (well over 38°C) may be reached within the pinyon understory, which would lead to rapid loss through volatilization. The general principles for evaporation rates of volatile hydrocarbons in petroleum oils, which are reviewed by Regnier and Scott (1975) and Stiver et al. (1989), can also be applied to monoterpene hydrocarbons. A thorough description of methods to predict volatility and loss rates, etc., of organic compounds from both aqueous and soil compartments is contained in Lyman et al. (1982).

Concentrations of monoterpenes present in the vapor phase surrounding pinyon litter are also likely to be correlated to their vapor pressures and boiling points, besides concentrations in source tissues. Such physical characteristics help to explain differences in relative amounts present in the vapor phase versus content of the source litter. Table 2 gives both vapor-pressure and boiling-point data for several monoterpene hydrocarbons. Since α-pinene is the major monoterpene present in single-leaf pinyon litter and has the lowest boiling point along with the highest vapor pressure at 38°C of the hydrocarbons we quantified, it was not surprising that it was present in the vapor phase at the greatest concentration. Furthermore, due to its relatively low boiling point, it was also not unusual for camphene to be present in the vapor phase at the second highest amounts.

The vapor pressure values for monoterpenes at 37.8°C presented in Table 2 were determined using temperatures for these compounds at 10 torr. Thus, the vapor pressure for camphene reported in Table 2 is lower than that for β-pinene, probably because camphene (mp = 51°C) was still solid at the temperature that yielded 10 torr of vapor pressure (47.2°C). Since camphene occurs

TABLE 2. VAPOR PRESSURES (37.8°C) AND BOILING POINTS (760 torr) OF EIGHT MONOTERPENE HYDROCARBONS

Compound	bp (°C)	vp (torr)
α-Pinene	156	10.09
Camphene	158–159	8.50_s[a]
β-Pinene	164	9.25
Myrcene	167	7.83
3-Carene	168	
α-Phellandrene	175–176	7.33
Limonene	178	7.76
γ-Terpinene	183	
Terpinolene	185	6.29

[a] $_s$ = vapor pressure of compound in solid state.

naturally in complex mixtures with many organic compounds, it can thus remain in a liquid state below its melting point (down to some lower temperature at which solidification occurs, of course). Therefore, although a value has not been determined, the relative vapor pressure of liquid camphene in such a mixture is likely to be appreciably higher than that of pure solid camphene at the same temperature. This helps to explain why camphene elutes before β-pinene on nonpolar GC column coatings and, furthermore, this may suggest why camphene is found in the vapor phase at a higher concentration than β-pinene, even though it is present in the source litter in much lower amounts. Camphene and α-pinene are the only two monoterpenes examined in this study that have boiling points under 160°C. All of the other monoterpenes we examined have boiling points above 160°C and were found in the vapor phase at much lower concentrations.

The slight vapor pressure anomaly between α-phellandrene and limonene is not readily explained; however, the difference in their vapor pressures may be within the experimental error for determining these values. Furthermore, β-pinene should have been present in the vapor phase at higher concentrations than myrcene because its vapor pressure is higher than myrcene's and was also found at higher levels in the source litter. Why myrcene occurred in the vapor phase at higher concentrations than β-pinene was not determined. The effect of a continuously lowering atmospheric pressure and temperature during the vacuum sampling on the evaporation characteristics of these compounds must also be considered in order to explain our results.

Muller (1971) has suggested that plants absorb volatile terpenoid toxins directly from the air and after contact with soils containing the deposited compounds. Thus, it is possible that the lipophilic components of soils or living plants may serve as repositories for the partitioning of nonpolar hydrocarbons (Muller, 1971). Notably, many grass, forb, and shrub species characteristic of single-leaf pinyon forests contain high lipid contents. For example, analysis of seed fat and oil content by Earle and Jones (1962), Jones and Earle (1966), and Barclay and Earle (1974) revealed the mean oil concentrations of seed for species in the Gramineae to be about 6% and ranging from 1 to 28%. These authors also reported the mean oil levels in other families containing species characteristic of the single-leaf pinyon understory: Compositae (27%), Cruciferae (28%), Rosaceae (21%), Chenopodiaceae (9%), Leguminosae (6.5%), and Polygonaceae (3%).

It is now generally accepted that phytotoxic monoterpenes can reach their "target" through the vapor phase after evaporation from the living or dead source plant material. Whether monoterpenes present in the vapor phase penetrate the seed coat directly and accumulate or interfere with lipophilic substances of susceptible seeds and/or seedlings remains to be examined. Possibly, the compounds are deposited onto the seed coat and surrounding soil media where they can be taken up indirectly (W.H. Muller, 1968). Furthermore, although

monoterpenes have low aqueous solubility, evidence suggests that they can also enter the plant during water imbibition (Halligan, 1976). In any case, an appropriately designed bioassay experiment would reveal if monoterpenes are absorbed by the living plant and would link toxicity to their presence, for example, exposing living seeds and seedlings to air containing radiolabeled monoterpene along with accurate descriptions of symptoms and measurements of the amounts reaching the target plant along with identification of breakdown products. Additionally, bioassay studies should be designed that also examine the influence of moisture stress on phytotoxicity.

Bioassays confirming that volatile organics emanating from allelopathic plants can cause toxic effects on other nearby plants have been developed and are now performed routinely (Putnam, 1983; Bradow and Connick, 1990). Previous work by our group (Wilt et al., 1988) found that mineral soils immediately beneath single-leaf pinyon litter contain amounts of monoterpene hydrocarbons at least 55 times lower than amounts in the litter layer directly above. This information, combined with bioassay experiments, suggests mineral soils of the single-leaf pinyon forest are not toxic to a variety of native understory grasses unless an overlying litter layer is present (Everett, 1987). Furthermore, native pinyon understory plant germination and growth is not stimulated by mechanical overstory removal alone, but is noted after the litter layer is removed by fire. These results are in direct agreement with the observations of C.H. Muller (1966, 1968) for the allelopathic control of herb growth in the fire cycle of the California chaparral.

CONCLUSIONS

Although this analysis was carried out in a controlled laboratory situation, our results confirm that monoterpene hydrocarbons characteristic of single-leaf pinyon litter are present in the surrounding vapor phase. The relative composition of monoterpenes in the vapor phase of the carboy only resembles litter in terms of α-pinene content since it is the major constituent of both. The remaining hydrocarbons exhibited a different compositional profile from litter extracts, which was not necessarily predictable from knowledge of litter concentrations and of vapor-pressure and boiling-point data alone. Furthermore, since the relative amounts of chiral monoterpene isomers in the vapor phase or the litter have not been determined, the actual rotational composition present in each compartment needs to be examined.

In conclusion, even though total amounts of monoterpenes collected in a natural situation may be somewhat different than collection from litter in a sealed chamber, it is nonetheless highly probable that the relative interstitial vapor-phase composition of single-leaf pinyon litter monoterpenes in nature closely

resembles the composition of the hydrocarbons sampled from the carboy given similar temperature and moisture conditions. However, future study of allelopathy in single-leaf pinyon ecosystems should involve a determination of the actual concentrations of bioactive monoterpenes under natural conditions. Further study of this system is also necessary in order to characterize biochemical toxicity mechanisms and define dose–response relationships for affected native plant species. The combined results from these investigations should indicate whether or not allelopathy can be a significant factor influencing vegetation growth and patterning in the single-leaf pinyon pine forest.

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REFERENCES

BARCLAY, A.S., and EARLE, F.R. 1974. Chemical analysis of seeds III: Oil and protein content of 1253 species. *Econ. Bot.* 28:178–236.

BRADOW, J.M., and CONNICK, W.J., JR. 1990. Volatile seed germination inhibitors from plant residues. *J. Chem. Ecol.* 16(3):645–666.

EARLE, F.R., and JONES, Q. 1962. Analysis of seed samples from 113 plant families. *Econ. Bot.* 16(4):221–250.

ESAU, K. 1977. Anatomy of Seed Plants, 2nd ed. John Wiley & Sons, Inc., New York, 550 pp.

EVERETT, R.L. 1987. Allelopathic effects of pinyon and juniper litter on emergence and growth of herbaceous species, pp. 62–67, *in* G.W. Frasier and R.A. Evans, (eds.). Proceedings, Symposium on Seed and Seedbed Ecology of Rangeland Plants. USDA-ARS, Tucson, Arizona, 21–23 April 1987.

EVERETT, R.L., and THRAN, D.F. 1992. Above-ground biomass and N, P and S capital in singleleaf pinyon woodlands. *J. Environ. Mngmt.* 34:137–147.

FAHN, 1979. Secretory Tissues in Plants. Academic Press, New York, 302 pp.

HALLIGAN, J.P. 1976. Toxicity of *Artemisia californica* to four associated herb species. *Am. Midlnd. Nat.* 95(2):406–421.

IOFFE, B.V., and VITENBERG, A.G. 1984. Head Space Analysis and Related Methods in Gas Chromatography. John Wiley & Sons, New York, 276 pp.

JONES, Q., and EARLE, F.R. 1966. Chemical analysis of seeds II: Oil and protein content of 759 species. *Econ. Bot.* 20(2):127–155.

LYMAN, W.J., REEHL, W.F., and ROSENBLATT, D.H. (eds.). 1982. Handbook of Physical Property Estimation Methods: Environmental Behavior of Organic Compounds. McGraw–Hill, New York, 977 pp.

MARX, J.L. 1983. Plants' resistance to herbicide pinpointed. *Science* 220:41–42.

MULLER, C.H. 1966. The role of chemical inhibition (allelopathy) in vegetational composition. *Bull. Torrey Bot. Club* 93:332–351.

MULLER, C.H. 1968. Allelopathic control of herb growth in the fire cycle of California chaparral. *Bull. Torrey Bot. Club* 95:225–231.

MULLER, C.H. 1971. Phytotoxins as plant variables, pp. 64–72, *in* Biochemical Interactions among Plants. National Academy of Sciences, Washington, D.C.

MULLER, W.H. 1968. Volatile growth inhibitors produced by *Salvia leucophylla*: Effect on seedling growth and respiration. *Bull. Torrey Bot. Club* 95:415–422.

PUTNAM, A.R. 1983. Allelopathic chemicals: Natures herbicides in action. *Chem. Eng. News* 61(14):34–45.

PUTNAM, A.R. 1985. Allelopathic research in agriculture: past highlights and potential, pp. 1–8, *in* A.C. Thompson (ed.). The Chemistry of Allelopathy. American Chemical Society, Washington, D.C.

RICE, E.L. 1984. Allelopathy, 2nd ed. Academic Press, New York, 400 pp.

REGNIER, Z.R., and SCOTT, B.F. 1975. Evaporation rates of oil compounds. *Environ. Sci. Technol.* 9(5):469–472.

STIVER, W., SHIU, W.Y., and MACKAY, D. 1989. Evaporation times and rates of specific hydrocarbons in oil spills. *Environ. Sci. Technol.* 23(1):101–105.

WILT, F.M., MILLER, G.C., and EVERETT, R.L. 1988. Monoterpene concentrations in litter and soil of singleleaf pinyon (*Pinus monophylla* Torr. & Frem.: Pinaceae) woodlands of the western great basin. *Great Basin Nat.* 48(2):228–231.

WILT, F.M., MILLER, G.C., EVERETT, R.L., and HACKETT, M. 1993. Monoterpene concentrations in fresh, senescent and decaying foliage of single-leaf pinyon (*Pinus monophylla* Torr. & Frem.: Pinaceae) from the western great basin. *J. Chem. Ecol.* 19(2):185–194.

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EFFECTS OF RESOURCE MANIPULATION ON THE CORRELATION BETWEEN TOTAL PHENOLICS AND ASTRINGENCY IN DOUGLAS-FIR

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Abstract—The correlation between total phenolics and tannin content within a species is often considered to be suitably strong to allow researchers to assume, with some degree of confidence, that levels of one will approximately parallel the other. However, the manipulation of resource availability could lead to disproportionate changes in total phenolics and tannins and/or in the specific monomers of which these fractions are composed, thus altering the correlation between these components. In order to test this hypothesis, we examined the correlation between foliar levels of total phenolics (as measured by the ferric chloride assay) and tannins (as measured by an astringency assay) in Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco) before and after the manipulation of nitrogen and water availability. Prior to manipulation of resources, the correlation between total phenolics and tannins was strong and highly significant ($r^2 = 0.869$; $P < 0.001$). This correlation was considerably weaker and not statistically significant following resource manipulation ($r^2 = 0.392$; $0.20 < P < 0.50$). These results demonstrate that manipulation of resource availability can alter the correlation between total phenolics and tannins in intraspecific comparisons. The causes underlying the observed degradation in the correlation between these measures (whether qualitative, quantitative, or both) are unknown and require further investigation.

Key Words—Phenolic compounds, tannins, astringency, total phenolics,

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intraspecific variation, resource availability, Douglas-fir, *Pseudotsuga menziesii*.

INTRODUCTION

Phenolic compounds can have important effects on a number of ecological processes, including herbivory (Coley et al., 1985; Feeny, 1976; Rhoades and Cates, 1976; Rosenthal and Janzen, 1979), allelopathy (Rice, 1984), and nutrient cycling in ecosystems (Rice and Pancholy, 1972, 1973, 1974; Gosz, 1981; Horner et al., 1988; Kuiters, 1990). Because of their putative importance in a number of ecological processes, phenolic compounds have received considerable attention in the ecological literature.

A number of different methods have been developed to quantify the phenolic and/or tannin content of plant tissues (reviewed in Bernays et al., 1990; Hagerman and Butler, 1989; Mole and Waterman, 1987a,b; Tempel, 1982). Among the most commonly used methods are those that ostensibly quantify total phenolics (e.g., Folin-Denis, ferric chloride assays), those that chemically assay tannin content (e.g., proanthocyanidin, vanillin assays), and those that measure the astringency of tannins (i.e., the ability of tannins to form complexes with proteins and other substrates with electronegative moieties; protein precipitation assays). However, each of these methods measures a different attribute of the mixture of compounds contained in a crude or partially purified extract.

In recent years, there has been concern over how well the results obtained from these various measures correlate with one another. In general, correlations between measures of total phenolics and measures of astringency have been shown to be poor, at least for interspecific comparisons (Martin and Martin, 1982; Mole and Waterman, 1987b). This lack of correlation in between-species comparisons has been attributed to qualitative differences in composition of phenolic monomers and/or the chemical structure of tannins. In contrast, since the specific compounds produced by individuals within a species should be qualitatively similar (if not identical), it has been assumed that correlations between total phenolics and tannins (in those species that produce both classes of compounds) may be suitably high to allow intraspecific comparisons (Martin and Martin, 1982; Mole and Waterman, 1987b).

However, intraspecific correlations between total phenolics and tannins could also be affected by differences in the relative concentrations of these fractions or in the relative monomeric composition of each of these fractions, differences that could be brought about by changes in resource availability. The phenolic composition within a species can be affected by the availability of light (Bryant, 1987; Larsson et al., 1986; Waring et al., 1985; Zangerl and Berenbaum, 1987), water (Dement and Mooney, 1974; Horner, 1988a,b, 1990), and nutrients (Bryant, 1987; Bryant et al., 1987; Larsson et al., 1986; Waring et

al., 1985; Wender, 1970). Furthermore, all of the individual components comprising the phenolic composition of a species are not affected similarly by changes in resource availability. That is, although there may be general, overall increases or decreases in response to a change in resource availability, specific components may be unaffected or may even change in a manner opposite the general trends (for example, see data in Larsson et al., 1986; Wender, 1970; Zangerl and Berenbaum, 1987; Zornoza and Esteban, 1984). If differences in resource availability lead to quantitatively disproportionate changes in the compounds comprising the phenolic and tannin contents of tissues, then the correlation between these measures could be affected. However, studies examining the effects of resource availability on the correlation between total phenolics and tannins within species are lacking. The purpose of this study was to examine the effects of treatment-induced differences in resource availability on the correlation between total phenolics and tannins within a species.

METHODS AND MATERIALS

Experimental Design. Ten 10-m^2 plots were established in even-aged stands of Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco) on Mt. Taylor, Cibola National Forest, western New Mexico, U.S.A. Plots were separated by a minimum of 10 m as a buffer. Within each plot, 10 trees (100 trees total) were permanently marked for use in foliage sampling.

In June of the following year, duplicate plots were randomly assigned to one of five treatments: control, irrigation, fertilization, carbohydrate amendment, or a combination of irrigation and carbohydrate amendment. Treatments were applied to the plot and to 2.5 m of the surrounding buffer on each edge of the plot. Control plots were unmanipulated. Irrigation was intended to approximately double the growing-season precipitation. The irrigation treatment consisted of weekly applications of 10 mm of water (taken from a nearby pond), which was distributed by pumping through a system of polyvinylchloride (PVC) lines leading to raised ("shrub") spray nozzles. Fertilization was intended to reduce the carbon–nitrogen (C/N) ratio of the forest floor to 20:1, thereby increasing rates of decomposition and nitrogen mineralization (Turner, 1977; Turner and Olson, 1976). Fertilization consisted of the even broadcast of ammonium sulfate in sufficient quantities to achieve the desired C/N ratios. The carbohydrate amendment was intended to roughly double the existing C/N ratio of the forest floor, which has been shown to decrease nitrogen mineralization rates, available nitrogen, and nitrogen content of plant tissues (Turner, 1977; Turner and Olson, 1976; White et al., 1988). This treatment consisted of the application of a 3:1 mixture of sawdust (slowly available carbohydrate) and sucrose (immediately available carbohydrate) in sufficient quantity to achieve the desired C/N

ratio. The combined irrigation plus carbohydrate amendment was a simple combination of the individual treatments. Treatment applications are described in more detail elsewhere (White et al., 1988).

Current foliage was sampled from throughout the canopy of each permanently marked tree during October of both years of the study. All tissue was placed on ice in cryogenic tubes immediately upon collection and placed in liquid nitrogen in the field. Tissues from throughout the canopy of each individual were bulked in the laboratory and stored at −80°C until analyzed.

Chemical Methods. Foliage (approximately 100 mg, 10 needles) was extracted as in Horner et al. (1987). Briefly, frozen tissue was ground in liquid nitrogen in a mortar and pestle, and the resulting powder was extracted with 50% aqueous methanol in a tissue homogenizer. The methanol was removed from the homogenate by shaking under a gentle stream of nitrogen at 55°C. The solution was brought to volume with distilled water and was filtered through a 0.45-μm nylon membrane filter.

Total phenolics were estimated on 1.0 ml of the sample extract using the method of Hagerman and Butler (1978), except that we used 4 ml of sodium dodecyl sulfate–triethanolamine, 1 ml $FeCl_3$, and 7 ml dH_2O. The absorbance at 510 nm was measured after 15 min using a reagent-only blank. Astringency (i.e., the ability to precipitate protein) was determined on 1.0 ml of the sample by the method of Martin and Martin (1983) as modified in Horner et al. (1987). Since gymnosperms produce only condensed tannins (Haslam, 1981), the reactivity of the sample extract was expressed relative to the reactivity of purified quebracho tannin (a condensed tannin), and values were expressed as quebracho equivalents (Qeq % dry wt). A moisture correction was determined by drying a subsample of foliage (approximately 200 mg) to constant weight at 60°C.

Statistical Analysis. The means of the 10 trees in each of the 10 plots were used to calculate correlation coefficients. Homogeneity of correlation coefficients from before and after treatment was determined by comparing Fisher's Z transformation of the correlation coefficients (Sokal and Rohlf, 1981; Zar, 1974). Percentage data were arcsine-transformed prior to analysis, and statistics were transformed back for presentation in figures.

RESULTS

The correlation between total phenolics and tannins in foliage prior to treatment was highly significant (Figure 1a). Furthermore, the coefficient of determination was high. Foliage collected after treatment application showed no significant correlation (Figure 1b). When these correlation coefficients were compared for homogeneity, we found that they were marginally different ($Z = 1.743$, $df = 8$, $0.05 < P < 0.10$).

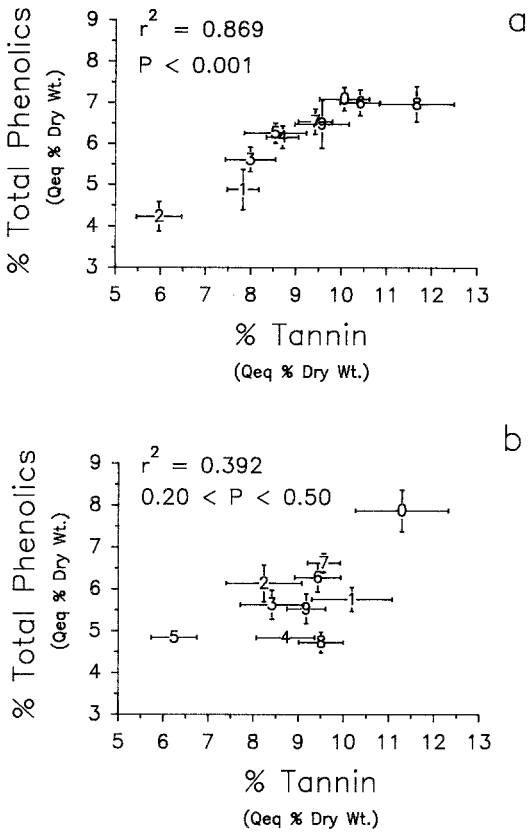

FIG. 1. Correlation between total phenolics and tannins in foliage of Douglas-fir (a) prior to and (b) after manipulation of resource availability. The mean of 10 trees per plot is indicated by a number representing the plot number. Horizontal and vertical lines represent 2 standard errors of the mean for tannins and total phenolics, respectively. Treatment assignments were as follows: control = 3 and 7; fertilized = 0 and 4; irrigated = 1 and 8; carbohydrate amended = 5 and 6; carbohydrate amendment + irrigation = 2 and 9.

DISCUSSION

Although assuming a correlation between total phenolics and astringency has been recognized as inaccurate when making interspecific comparisons (Martin and Martin, 1982; Mole and Waterman, 1987b), it has been supposed that this assumption may be justified when making intraspecific comparisons, where quantities of all components of the phenolic composition have been assumed to be affected comparably (Martin and Martin, 1982; Mole and Waterman, 1987b).

However, as Mole and Waterman (1987b, p. 155) point out, these intraspecific comparisons should take into consideration "factors such as plant age, growing conditions and plant part."

Our results are consistent with the assumption of a high correlation between total phenolics and tannins in unmanipulated stands. However, our data also demonstrate that the manipulation of resources can adversely affect the correlation between total phenolics and tannins: Following the manipulation of resources, the correlation between total phenolics and tannins was weakened (Figure 1b). Although the comparison of the correlation coefficients before and after treatment was only marginally different ($0.05 < P < 0.10$), determination of significant differences between correlation coefficients using Z transformations requires relatively large sample sizes (Sokal and Rohlf, 1981, pp. 587–591). Furthermore, the change in the coefficient of determination alone is sufficient to warrant caution in assuming a correlation between total phenolics and tannins following the manipulation of resources.

The changes in chemistry underlying the observed differences are unknown. Astringency can be affected by changes in the monomeric composition (Zucker, 1983), chain length (Beart et al., 1985; Goldstein and Swain, 1963; Oh and Hoff, 1979), and/or the absolute quantity of tannins. Similarly, the quantity of total phenolics (as determined by the ferric chloride assay) can be affected by patterns of substitution on the phenyl moiety (Hagerman and Butler, 1978, 1989) and the number of unsaturated bonds in the compounds (Grove and Pople, 1979), as well as by differences in the absolute quantity of phenolics present. Since measures of both astringency and total phenolics can be affected by a number of different attributes of the chemical composition, the specific chemical changes underlying the observed differences are unknown. Thus, while the observed differences could have been due to differential allocation among astringent and nonastringent phenolics, they could have been due as easily to qualitative changes in the particular monomers comprising these fractions, with no overall change in allocation. Further studies are needed to identify the cause of these differences.

It is noteworthy that, in several cases, trees in the replicate plots of a treatment responded differently to that treatment. For example, whereas irrigation caused an increase in both total phenolics and tannins in plot 1, this treatment caused a decrease in both components in the replicate plot (plot 8, Figure 1). Since the level of secondary metabolites may be affected not only by absolute levels of resources but also by relative resource availability (Bryant et al., 1983; Waring et al., 1985), the observed differences in response to treatment may have been due to unmeasured changes in relative resource availability.

Two comments on our methodology should be made. First, we utilized the ferric chloride method (Hagerman and Butler, 1978) for the estimation of total phenolics. Although this method is not as widely employed as the Folin-Denis

assay, correlations between results obtained from these two assays are relatively high (Waterman and Mole, 1987a). Therefore, we expect that results similar to ours may be obtained if the Folin-Denis assay had been used. Secondly, we utilized a plant that produces only condensed tannins. Since resource availability could influence the allocation between hydrolyzable and condensed tannins, a similar experiment on a species that produces both types of tannins could produce different results.

In summary, reasons for interest in the phenolic composition of plant tissues are manifold, ranging from effects on herbivory, allelopathy, and/or ecosystem-level processes to effects of resource availability on estimates of allocation patterns in plants. Measures of total phenolics have often been used as an index of the tannin content of the tissue (Martin and Martin, 1982). Our results suggest that a strong intraspecific correlation should not be assumed in studies involving the manipulation of resource availability. Further studies are needed in order to corroborate these results in other species and to elucidate the chemical changes resulting in the degradation of the correlation following resource manipulation. In addition, appropriately designed studies are needed that examine the effects of natural differences in resource availability among sites on the correlation between total phenolics and tannins within species.

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REFERENCES

BEART, J.E., LILLEY, T.H., and HASLAM, E. 1985. Plant polyphenols—secondary metabolism and chemical defence: Some observations. *Phytochemistry* 24:33–38.

BERNAYS, E.A., COOPER-DRIVER, G., and BILGENER, M. 1990. Herbivores and plant tannins. *Adv. Ecol. Res.* 19:263–302.

BRYANT, J.P. 1987. Feltleaf willow-snowshoe hare interactions: Plant carbon/nutrient balance and floodplain succession. *Ecology* 68:1319–1327.

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.

BRYANT, J.P., CHAPIN, F.S., III, REICHARDT, P.B., and CLAUSEN, T.P. 1987. Response of winter chemical defense in Alaska paper birch and green alder to manipulation of plant carbon/nutrient balance. *Oecologia* 72:510–514.

COLEY, P.D., BRYANT, J.P., and CHAPIN, F.S., III. 1985. Resource availability and plant antiherbivore defense. *Science* 230:895–899.

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.

FEENY, P. 1976. Plant apparency and chemical defense. *Recent Adv. Phytochem.* 10:1–40.

GOLDSTEIN, J.L., and SWAIN, T. 1963. Changes in tannins in ripening fruits. *Phytochemistry* 2:371–383.

GOSZ, J.R. 1981. Nitrogen cycling in coniferous ecosystems. *Ecol. Bull. (Stockholm)* 33:405–426.

GROVE, J.F., and POPLE, M. 1979. The identification and characterization of orthodihydric phenols. *Phytochemistry* 18:1071–1072.

HAGERMAN, A.E., and BUTLER, L.G. 1978. Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.* 26:809–812.

HAGERMAN, A.E., and BUTLER, L.G. 1989. Choosing appropriate methods and standards for assaying tannin. *J. Chem. Ecol.* 15:1795–1810.

HASLAM, E. 1981. Vegetable tannins, pp. 527–566, *in* E.E. Conn and P.K. Stumpf (eds.). The Biochemistry of Plants: A Comprehensive Treatise, Vol. 7. Secondary Plant Products. Academic Press, New York.

HORNER, J.D. 1988a. Astringency of Douglas-fir foliage in relation to phenology and xylem pressure potential. *J. Chem. Ecol.* 14:1227–1237.

HORNER, J.D. 1988b. Erratum: Astringency of Douglas-fir foliage in relation to phenology and xylem pressure potential. *J. Chem. Ecol.* 14:1977–1978.

HORNER, J.D. 1990. Nonlinear effects of water deficits on foliar tannin concentration. *Biochem. Syst. Ecol.* 18:211–213.

HORNER, J.D., CATES, R.G., and GOSZ, J.R. 1987. Tannin, nitrogen, and cell wall composition of green vs. senescent Douglas-fir foliage: Within- and between-stand differences in stands of unequal density. *Oecologia* 72:515–519.

HORNER, J.D., GOSZ, J.R., and CATES, R.G. 1988. The role of carbon-based plant secondary metabolites in decomposition in terrestrial ecosystems. *Am. Nat.* 132:869–883.

KUITERS, A.T. 1990. Role of phenolic substances from decomposing forest litter in plant-soil interactions. *Acta Bot. Neerl.* 39:329–348.

LARSSON, S., WIREN, A., LUNDGREN, L., and ERICSSON, T. 1986. Effects of light and nutrient stress on leaf phenolic chemistry in *Salix dasyclados* and susceptibility to *Galerucella lineola* (Coleoptera). *Oikos* 47:205–210.

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.

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.

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.

OH, H., and HOFF, J.E. 1979. Fractionation of grape tannins by affinity chromatography and partial characterisation of the fractions. *J. Food Sci.* 44:87–89.

RHOADES, D.F., and CATES, R.G. 1976. Toward a general theory of plant antiherbivore chemistry. *Recent Adv. Phytochem.* 10:168–213.

RICE, E.L. 1984. Allelopathy, 2nd ed. Academic Press, New York.

RICE, E.L., and PANCHOLY, S.K. 1972. Inhibition of nitrification by climax ecosystems. *Am. J. Bot.* 59:1033–1040.

RICE, E.L., and PANCHOLY, S.K. 1973. Inhibition of nitrification by climax ecosystems. II. Additional evidence and possible role of tannins. *Am. J. Bot.* 60:691–702.

RICE, E.L., and PANCHOLY, S.K. 1974. Inhibition of nitrification by climax ecosystems. III. Inhibitors other than tannins. *Am. J. Bot.* 61:1095–1103.

ROSENTHAL, G.A., and JANZEN, D.H. (eds.). 1979. Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, New York.

SOKAL, R.R., and ROHLF, F.J. 1981. Biometry, 2nd Ed. W.H. Freeman and Co., New York.

TEMPEL, A.S. 1982. Tannin-measuring techniques: A review. *J. Chem. Ecol.* 8:1289–1298.

TURNER, J. 1977. Effect of nitrogen availability on nitrogen cycling in a Douglas-fir stand. *For. Sci.* 23:307–316.

TURNER, J., and OLSON, P.R. 1976. Nitrogen relations in a Douglas-fir plantation. *Ann. Bot.* 40:1185–1193.

WARING, R.H., MCDONALD, A.J.S., LARSSON, S., ERICSSON, T., WIREN, A., ARWIDSSON, E., ERICSSON, A., and LOHAMMAR, T. 1985. Differences in chemical composition of plants grown at constant relative growth rates with stable mineral nutrition. *Oecologia* 66:157–160.

WENDER, S.H. 1970. Effects of some environmental stress factors on certain phenolic compounds in tobacco. *Recent Adv. Phytochem.* 3:1–29.

WHITE, C.S., GOSZ, J.R., HORNER, J.D., and MOORE, D.I. 1988. Seasonal, annual, and treatment-induced variation in available nitrogen pools and nitrogen-cycling processes in soils of two Douglas-fir stands. *Biol. Fertil. Soils* 6:93–99.

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.

ZAR, J.H. 1974. Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, New Jersey.

ZORNOZA, P., and ESTEBAN, R.M. 1984. Flavonoids content of tomato plants for the study of the nutritional status. *Plant Soil* 82:269–271.

ZUCKER, W.V. 1983. Tannins: Does structure determine function? An ecological perspective. *Am. Nat.* 121:335–365.

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DISTRIBUTION AND ANTIFEEDANT ASSOCIATIONS OF SESQUITERPENE LACTONES IN CULTIVATED SUNFLOWER (*Helianthus annuus* L.) ON WESTERN CORN ROOTWORM (*Diabrotica virgifera virgifera* LeConte)

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Abstract—Seven antifeedant sesquiterpene lactones (STLs), 4,5-dihydroniveusin A, argophyllin B, argophyllin A, 15-hydroxy-3-dehydrodesoxytifruticin, niveusin B, 1,2-anhydridoniveusin A, and an unidentified epoxide, in cultivated sunflower (*Helianthus annuus* L.) have been quantified by a high-performance thin-layer chromatography and UV-reflectance scanning densitometry analysis. Age-related expression of STL content in sunflower reveals a heretofore undescribed pattern in which nonpolar STLs such as 15-hydroxy-3-dehydrodesoxytifruticin predominate up to an age of three weeks, but are subsequently displaced by polar STLs, especially argophyllin A, through later foliar stages and anthesis. This leaf pattern of STL ontogeny is maintained in three widely different *H. annuus* cultivars (Giant Gray Stripe, Royal Hybrid 2141, Hybrid 7111), which in turn had similar total contents of STLs. Antifeedant activity for western corn rootworm was positively correlated with STL content, particularly with argophyllin A and its isomer argophyllin B, in respective tissue extracts. Enhanced amounts of highly antifeedant argophyllins, especially in newly grown leaf and floral tissues yielding sunflower progeny, strongly suggest that these epoxy-STLs are a chemical defense against insect herbivory.

Key Words—Antifeedant; Asteraceae, *Helianthus annuus* L., Chrysomelidae, *Diabrotica virgifera virgifera* LeConte, western corn rootworm, feeding deterrent, densitometry, HPTLC, sesquiterpene lactones.

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INTRODUCTION

Toxicological studies on sesquiterpene lactones (STLs) against insects have suggested their potential as tools in pest control (Picman, 1986; Duke, 1991; Gershenzon and Croteau, 1991; Mullin et al., 1991b). Generally STLs serve as feeding deterrents and growth inhibitory factors for insect and mammalian herbivores due to their bitter taste and associated chemical reactivities. Their α-methylene-γ-lactone and related conjugated moieties were proposed to elicit these biological effects as acceptors in a Michael reaction with nucleophilic groups such as sulfhydryl, hydroxyl, and amino functions in proteins (Mabry and Gill, 1979). More recently, germacranolide STLs of the argophyllin-type containing a 1,10-epoxide were found to be both antifeedant and neurotoxic to herbivorous beetles, eliciting excitability, hyperextension of the ovipositor, egg expulsion, and tarsal tetany following injection (Mullin et al., 1991a). The interesting chemical and biological activities of STLs and their potential agricultural application warrant their further study, particularly relative to their production as antiherbivore factors in plants.

Few studies have addressed the ontogenic expression of plant STLs relative to chemical defense against herbivores. Wisdom and Rodriguez (1983) followed seasonal variation of the STL, farinosin, and two chromenes in *Encelia farinosa*. Farinosin was more concentrated in younger tissues and appeared to explain deterrence, at least in part, against native populations of the chrysomelid *Trirhabda geminata*. In chicory, Rees and Harborne (1985) concluded that the STLs lactucin, lactupicrin, and 8-deoxylactucin, present uniformly throughout the growing season in root and leaf tissues, had an insect antifeedant role in combination with phenolics, particularly in actively growing plant tissues. Furthermore, Proksch et al. (1990) found a differential distribution of feeding deterrent phytochemicals for noctuids in *Ageratina adenophora*, wherein chromenes dominate in young plants but a δ-cadinene-type sesquiterpene and the phenolic chlorogenic acid were prevalent in upper leaves when plants aged. It was suggested that insects or other herbivores would be confronted by a varying set of antifeedant allelochemicals during development of *A. adenophora*, thus forming a multichemical obstacle to long-term adaptation and host utilization. These and other studies reveal an important developmentally changing role for STLs as an antiherbivore defense mechanism in the family Asteraceae, from which more than 90% of the known STLs have been reported (Seaman, 1982; Spring, 1989).

In the Giant Gray Stripe variety of cultivated sunflower, the major STLs are of the germacranolide skeletal type esterified with angelic acid and containing a five-membered α-methylene-γ-lactone ring (Figure 1). These sesquiterpene lactone angelates are strongly antifeedant (Mullin et al., 1991b) to western corn rootworm (WCR), a major corn pest of temperate North America. Comparison of the feeding deterrencies for WCR with approximate floral contents of STLs

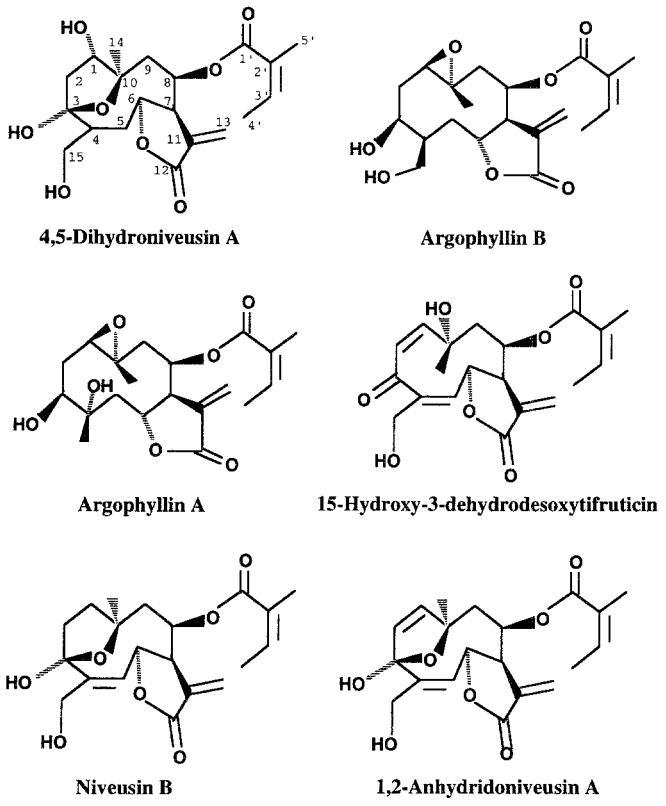

FIG. 1. Chemical structures of major STLs from cultivated sunflower.

and other active natural products isolated from cultivated sunflower (Mullin et al., 1991a), including diterpenes (e.g., kaur-16-en-18-oic acid, grandifloric acid, and its angelate) and flavonoids (e.g., nevadensin and 5-hydroxy-4,6,4′-trimethoxyaurone) and other phenolics (e.g., dicaffeoylquinic acids), established the sesquiterpenes as the most deterrent class of sunflower phytochemicals. However, a detailed study of the relationship among sunflower age, organ composition of STLs, and antifeedant activity for WCR was not undertaken. Presented here is the ontogeny of STL antifeedants for WCR in the cultivated sunflower using a quantitative STL analysis based on high-performance thin-layer chromatography (HPTLC) and UV-reflectance scanning densitometry and a rootworm consumption bioassay. The relationship found between the tissue and age distribution of STLs and their antirootworm effects has important implications for plant defense against herbivory.

METHODS AND MATERIALS

Plant and Insect Sources. Cultivated sunflower (*Helianthus annuus* L. var. Giant Gray Stripe) was planted in both the field and greenhouse in 1991. In addition, two other varieties (Royal Hybrid 2141 and the oil-seed Hybrid 7111, Agway Inc., Syracuse, New York) were planted in 1992 along with Giant Gray Stripe in four 20 ft × 32 ft blocks each at the Pennsylvania State University Russell E. Larson Field Research Lab. Leaves by node position and other aboveground plant organs were collected and grouped from representative growth stages of field grown sunflower, returned to the lab on ice, and then kept at below −30°C prior to analysis. Adult corn rootworms were collected from flowers of winter squash, *Cucurbita maxima* Duchesne var. Blue Hubbard at the same field site.

Fresh squash flowers for insect bioassay were grown under controlled temperature (approx. 26°C) and halide lamps (16:8 light–dark) in the greenhouse. Root samples were collected from greenhouse-grown sunflower due to the difficulty of collecting clean, intact roots from the field.

Reagents. Dimethylsulfoxide, naringenin, and *p*-dimethylaminobenzaldehyde were purchased from Sigma Chemical Co., St. Louis, Missouri. Silica gel 60 (0.063 to 0.200-mm particle size, 70–230 mesh ASTM), silica gel 60 F_{254} HPTLC plates (10 × 10-cm plate size, 0.1 mm thick), and regular silica gel 60 F_{254} TLC plates (20 × 20-cm plate size, 0.25 mm thick) were obtained through EM Separations (E. Merck), Gibbstown, New Jersey. Deuterochloroform ($CDCl_3$) and deuteroacetone (C_3D_6O) were from MSD Isotopes, Merck & Co., Inc., Rahway, New Jersey. All solvents and other reagents were analytical grade or better.

STL Extraction, Purification, and Identification. The sunflower sample (generally 10–70 g fresh weight) was surface extracted by gentle agitation for 5 min with ethyl acetate (300 ml), and then filtered on Whatman No. 2 paper. After concentrating this filtrate to minimal volume at room temperature using a rotary evaporator and water aspiration, the crude residue was dissolved in 2–3 ml of ethyl acetate and adsorbed onto 2.5 g of silica gel 60. After drying, the adsorbed silica gel sample was transferred to a sintered-glass filter (medium pore size, 15 ml), and washed in one step with 15 ml of chloroform followed by elution under mild aspiration of the bulk of the STLs in one step with 35 ml of ethyl acetate. The latter eluate (=STL sample) was concentrated to dryness and refrigerated until analysis (Chou and Mullin, 1993).

For STL purification and identification, the routine extraction procedure was scaled up to greater than 100 g fresh weight of leaf or disk flower sample, which was extracted with 600 ml ethyl acetate. The concentrated ethyl acetate residue from the batch silica gel clean-up was applied to a 1-cm-ID × 30-cm silica gel 60 column, and STLs eluted with 100 ml each of 100% chloroform,

chloroform–ethyl acetate (50:50), chloroform–ethyl acetate (20:80), and 100% ethyl acetate. Eluates were concentrated and applied to analytical TLC plates for further separation. The first eluate was discarded; the second eluate contained three major nonpolar STLs resolved by chloroform–ethyl acetate–acetonitrile (60:20:20) or chloroform–ethyl acetate (50:50); the third eluate contained two major argophyllin-type STLs resolved by chloroform–ethyl acetate–isopropanol (45:45:10); and the ethyl acetate eluate contained the argophyllins as well as two other STLs, which were not completely separated due to their similar polarities. Desired STL bands were scraped from the TLC plate, packed into a Pasteur pipet column plugged by cotton, and eluted with ethyl acetate. STL purity was determined via HPTLC (see below).

Isolated STLs were subjected to ^{1}H NMR and MS for structural identification, which generally indicated greater than 95% purity. ^{1}H NMR at 360 MHz on a Bruker WM-360 in $CDCl_3$, and MS, including electron impact (EI) on acetone solutions introduced by direct probe on a Kratos 950 or fast atom bombardment (FAB) in glycerol or 15-crown-5 on a Kratos MS-50 mass spectrometers, were obtained from the NMR and MS facilities, respectively, in the Department of Chemistry, Pennsylvania State University.

HPTLC and UV-Reflectance Scanning Densitometry Analysis. For quantitative analysis, 0.5 μl of the total STL sample (0.5 ml) in chloroform–ethyl acetate–isopropanol (20:40:40) was applied by capillary (Analtech, Newark, Delaware) onto the 1.0-cm mark of a 10 × 10-cm HPTLC plate. After drying for about 2 min at room temperature, the plate was developed using chloroform–ethyl acetate–isopropanol (45:45:10) in a closed tank (6 × 12 × 3.75 in.). UV-reflectance scanning densitometry was then performed on the air-dried plate at 220 nm using a Shimadzu CS-9000 dual wavelength flying-spot scanner, and STL content determined relative to standard curves of authentic compounds (Mullin et al., 1991a; Chou and Mullin, 1993). Content was corrected for the respective recovery of the internal standard, naringenin, added during the initial sample extraction stage. STL identity was based on cochromatography, the UV reflectance spectrum of the respective STL spot, and the color formed after spraying and warming the HPTLC plate with a solution of 0.5 g *p*-dimethylaminobenzaldehyde, 0.5 ml concentrated H_2SO_4, a few drops of glacial acetic acid, and 9.5 ml of ethanol (Picman et al., 1980).

Insect Bioassay for Feeding Deterrency. Antifeedant activity of STL samples on adult WCR was determined by a squash disk bioassay where relative consumption after 5, 24, and 48 hr of solvent- or compound-treated flower disks were measured. Chemicals were applied in 8 μl of a carrier solvent (methanol or acetone) with a Hamilton syringe repeating dispenser to the abaxial surface of 1.5-cm-diameter disks of a Blue Hubbard squash blossom. Two solvent-control and two treated disks were placed on a moist filter paper in a 2 × 10-cm Petri dish with four WCR per dish. Four replications, at least, were done

for each treatment. Consumption of disks was ranked visually from 0 (none) to 10 (total consumption) (Mullin et al., 1991a). Consumption ratios of treated to solvent control were then obtained. These ratios are arcsin square root transformed and means of at least four replicates at each dose tested for differences by one-way ANOVA (SAS/STAT User's Guide, 1990) or by a Student's *t* test, where appropriate.

RESULTS

STL Identity. A total of seven STLs were detected in cultivated sunflower, of which six were identified (Figure 1). All were germacran-6,12-olides with an 8-hydroxy group esterified with angelic acid. They are 4,5-dihydroniveusin A, argophyllin B, argophyllin A, 15-hydroxy-3-dehydrodesoxytifruticin, niveusin B, and 1,2-anhydridoniveusin A. This mixture of STLs was fully resolved by HPTLC on silica gel (Table 1) using chloroform–ethyl acetate–isopropanol (45:45:10), which was routinely used in analysis of sunflower samples.

Identity of these compounds was based on consistency of ^{1}H NMR, MS, and UV spectra (Chou, 1992) with those of previous works (Ohno and Mabry, 1980; Watanabe et al., 1982; Melek et al., 1985; Spring et al., 1982, 1989). Molecular ion peaks were observed for EI-MS of all STLs except for argophyllin

TABLE 1. HPTLC ANALYTICAL PARAMETERS FOR SESQUITERPENE LACTONES FROM *Helianthus annuus* L.

Compound	R_f[a] S1	S2	S3	S4	λ_{max} (nm)[b]	Color formation with *p*-DMAB[c]
4,5-Dihydroniveusin A	0.00	0.10	0.25	0.44	215	Magenta
Argophyllin B	0.06	0.18	0.37	0.55	212	Purple
Argophyllin A	0.07	0.19	0.44	0.62	220	Magenta
15-Hydroxy-3-dehydrodesoxytifruticin	0.12	0.30	0.57	0.72	235[d]	Brown
Niveusin B	0.17	0.37	0.64	0.73	218	Pink
1,2-Anhydridoniveusin A	0.24	0.44	0.69	0.75	210	Pink

[a] R_f values were measured on a 10 × 10-cm silica gel plate after development in solvent systems of S1, chloroform–ethyl acetate (50:50); S2, chloroform–ethyl acetate–acetonitrile (50:40:10); S3, chloroform–ethyl acetate–isopropanol (45:45:10); S4, chloroform–ethyl acetate–isopropanol (40:40:20).

[b] By UV reflectance densitometry.

[c] Spraying with acidic *p*-dimethylaminobenzaldehyde (*p*-DMAB) followed by warming.

[d] The two UV peaks (λ_{max} = 228, 255 nm; Mullin et al., 1991a) were not fully resolved by densitometry.

A, which required a FAB-MS in glycerol or 15-crown-5 in order for the molecular ion to be seen. All these STLs gave an m/z of 83 and 55, characteristic of the angelate and α,β-unsaturated lactone groups, respectively.

The seventh compound was partially characterized as an epoxy-STL angelate based on ^{1}H NMR decoupling experiments and δ values for key protons including the epoxide proton at δ 3.07 and two 13-H signals at δ 6.36 and 5.84. It represented about a 34% contaminant in the NMR spectrum of 4,5-dihydroniveusin A. Identity of this minor STL in sunflower will require further study. Due to the identical R_f values for this latter STL and 4,5-dihydroniveusin A, the two were quantified in combination.

Analysis of Sunflower STLs. Since the sunflower STLs were a family of germacranolide angelates of similar molecular weight with common UV absorbance characteristics (Table 1) exemplified by argophyllin A and 15-hydroxy-3-dehydrodesoxytifruticin, these more abundant compounds could be used for standard curve preparation. Figure 2 shows the standard curve of the epoxide, argophyllin A, which, in addition, was used to quantify the amounts of 4,5-

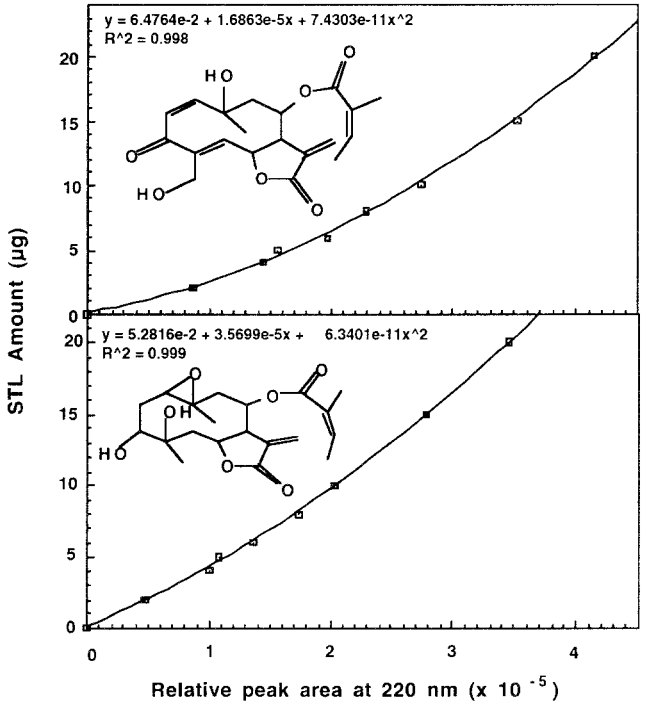

FIG. 2. Standard curves for 15-hydroxy-3-dehydrodesoxytifruticin (top) and argophyllin A (bottom) obtained by HPTLC and UV densitometry.

dihydroniveusin A, the unidentified epoxide, and argophyllin B, and also shows the standard curve of 15-hydroxy-3-dehydrodesoxytifruticin which, in turn, was used for quantification of niveusin B and 1,2-anhydridoniveusin A.

In order to get a better representative quantification, a percent recovery determination was done by including a dihydroflavone, naringenin (R_f = 0.80 in the routine solvent system S3, Table 1), as an internal standard, which was introduced during solvent extraction of the plant tissue. An average recovery of 74% was obtained, and all STL amounts calculated from standard curves were adjusted according to the respective recovery of naringenin. This internal standard could be distinguished from a STL 220-nm reflectance peak on the HPTLC plate by its additional peak at 290 nm.

Age Distribution of STLs in Three Varieties of Helianthus annuus. Total leaf expression of STLs in concomitantly grown Giant Gray Stripe, 7111, and 2141 hybrid sunflowers were remarkably similar (Figure 3). In all varieties, 15-hydroxy-3-dehydrodesoxytifruticin was the most abundant STL at three weeks of growth but was then dominated by argophyllin A in leaves of 9-week-old,

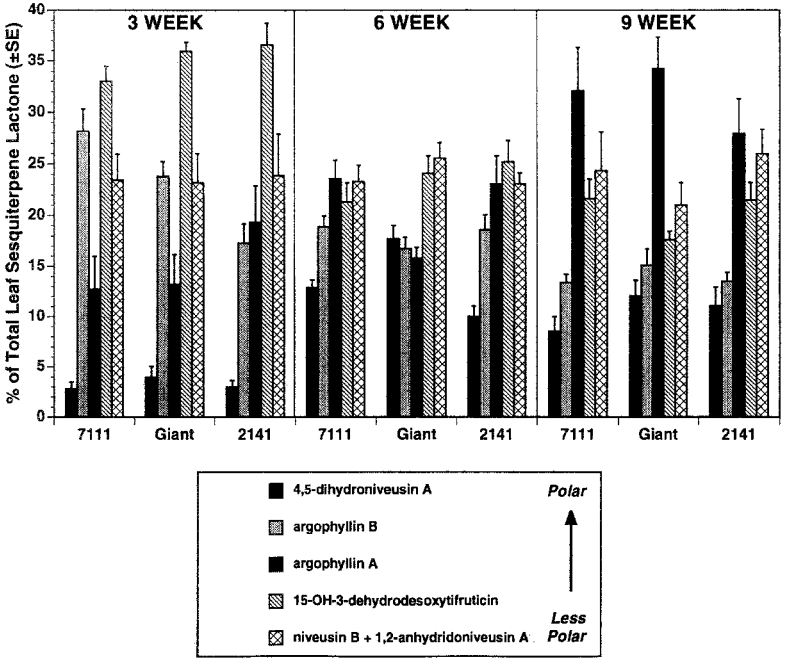

FIG. 3. Age expression of leaf sesquiterpene lactones in three varieties of cultivated sunflower (Giant Gray Stripe, Royal Hybrid 2141, Hybrid 7111); individual STL contents as a percentage of the total ± SE for two to three determinations each on three separate samples.

preanthesis plants. Only minor differences in STL patterns (e.g., relative content of argophyllin A and 4,5-dihydroniveusin A in Giant versus the other two hybrids) were found. In turn, total STL contents (micrograms per gram fresh tissue) were similar between sunflower cultivars (data not shown), and varied more depending on leaf size and age, ranging from 56 μg/g fresh tissue of STL in expanded, middle leaves of 9-week-old plants to 2700 μg/g in upper, new leaves of 3-week-old plants. The more polar argophyllins and 4,5-dihydroniveusin A prevailed in later-stage leaves, in contrast to leaves from young plants, which contained more nonpolar STLs including 15-hydroxy-3-dehydrodesoxytifruticin, niveusin B, and 1,2-anhydridoniveusin A. Argophyllin A content rose dramatically with plant age and dominated the STL profile in younger upper leaves, bracts, ray, and disk flowers of 10-week-old sunflower (Figure 4).

STL Content–Antifeedant Potency Relationships. Feeding deterrency towards adult WCR was determined using a dual choice test with compound-treated and control squash disks. Among pure STLs screened in this consumption bioassay (Table 2), argophyllin A was the most deterrent to feeding. Fur-

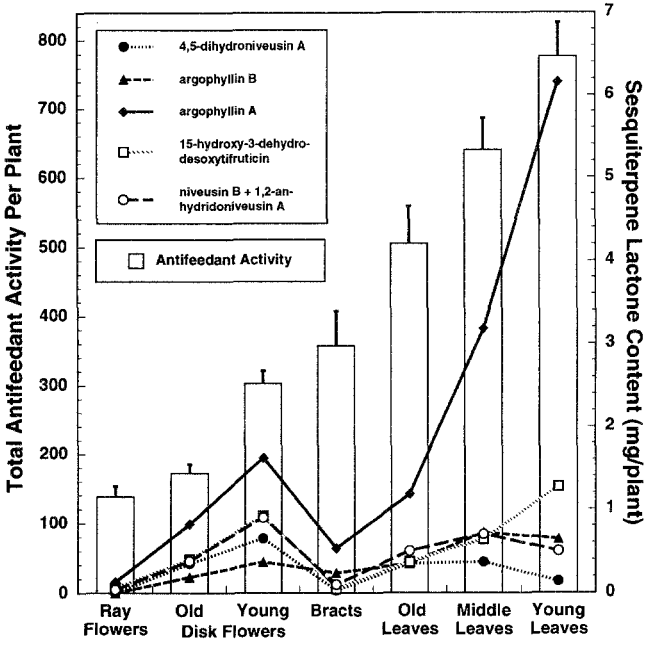

FIG. 4. Relationship between individual STL contents in fresh extracts of separated tissues from 10-week old Giant Gray Stripe sunflower and their respective WCR antifeedant activity on a per plant basis (total antifeedant activity = total extract amount ÷ amount in assay ÷ treated to control consumption ratio; mean ± SE for at least four replicates).

TABLE 2. FEEDING DETERRENCY OF STLS AND TISSUE EXTRACTS FROM SUNFLOWER ON ADULT WESTERN CORN ROOTWORM

Sample type[a] and compound or tissue name	Relative consumption (treated/control)[b]			
	40 μ/disk		80 μg/disk	
	24 hr	48 hr	24 hr	48 hr
STL				
4,5-Dihydroniveusin A[c]	0.58 ± 0.06	0.90 ± 0.11	0.39 ± 0.12	0.67 ± 0.08
Argophyllin B	0.52 ± 0.04	0.75 ± 0.05		
Argophyllin A	0.38 ± 0.15	0.53 ± 0.10		
15-Hydroxy-3-dehydro-desoxytifruticin	0.97 ± 0.16	0.90 ± 0.06	0.75 ± 0.08	0.82 ± 0.07
Niveusin B[c]	0.82 ± 0.04	0.88 ± 0.03	0.49 ± 0.04	0.56 ± 0.07
STL fraction from tissues[d]				
Ray flower			0.83 ± 0.09	0.97 ± 0.04
Bract			0.67 ± 0.09	0.85 ± 0.05
Disk flower			0.66 ± 0.08	0.84 ± 0.05
Leaf			0.52 ± 0.03	0.60 ± 0.03

[a] From Giant Gray Strip sunflower.
[b] Dual choice tests using 1.5-cm-diameter squash flower disks; mean ± SE for 4–16 replicates per dose. All samples had a significant antifeedant effect on WCR relative to a methanol control ($P < 0.05$ for at least one dose–time combination).
[c] 4,5-Dihydroniveusin A contained 30% of an unidentified epoxide STL; niveusin B contained 12% 1,2-anhydridoniveusin A.
[d] Ethyl acetate surface extracts of tissues were processed through the batchwise silica gel purification step to give STL-enriched residues as outlined in Methods and Materials.

thermore, polar STLs (argophyllin A, argophyllin B, 4,5-dihydroniveusin A, and the unidentified epoxide) were more antifeedant than the non-polar STLs (15-hydroxy-3-dehydrodesoxytifruticin, niveusin B, and 1,2-anhydridoniveusin A). Leaf tissues from Giant Gray Stripe sunflower contained the strongest activity among tissue STL extracts, followed by disk flower, floral bract, and ray flower (Table 2). Comparisons of antifeedant activity with the respective STL composition of selected tissue extracts from 10-week-old Giant sunflower indicate direct correlations, particularly with the argophyllin contents (Figure 4). Total antifeedant activity in these STL samples was strongly correlated with argophyllin A ($y = 0.00771x - 1.24$; $R^2 = 0.762$, $P = 0.0103$) and argophyllin B ($y = 0.000963x - 0.0391$; $R^2 = 0.829$, $P = 0.0044$) contents, but not with 15-hydroxy-3-dehydrodesoxytifruticin ($R^2 = 0.436$, $P = 0.106$), niveusin B ($R^2 = 0.167$, $P = 0.363$), or 4,5-dihydroniveusin A ($R^2 = 0.00084$, $P = 0.951$).

DISCUSSION

Common sunflower is rich in trichomal sesquiterpene lactone residues that are feeding deterrent and toxic for herbivores such as the WCR (Mullin et al., 1991a). While anti-insect STL defenses are generally known among this and other members of the genus *Helianthus* and the rest of the Asteraceae (Mabry and Gill, 1979; Picman, 1986; Isman et al., 1989; Gershenzon and Croteau, 1991; Mullin et al., 1991b), it is somewhat surprising that cultivated varieties of the sunflower have retained ecologically significant amounts of these antifeedants. Similar extracts from fresh flowers of wild *H. tuberosus* and *H. divaricatus* were equivalent or less active to WCR (Chou and Mullin, unpublished data).

Argophyllins A, B, niveusin B, and 4,5-dihydroniveusin A were detected both by us and by Melek et al. (1985) in Texas, whereas Spring et al. (1989) in Germany only found in *H. annuus* argophyllin B and niveusin B out of these four STLs. In turn, both Spring et al. and we found 15-hydroxy-3-dehydrodesoxytifruticin and 1,2-anhydridoniveusin A, which was unreported by Melek et al. (1985). Regarding these comparisons, some fundamental issues must be addressed. First, it is possible that alternative planting sites (Texas, Pennsylvania, and Germany) led to different STL patterns of synthesis. Second, even though the same species was employed, different varieties were used; var. Giganteus (Spring et al.), wild *H. annuus* L. (Melek et al.), and var. Giant Gray Stripe and two other cultivars (this work). Varietal differences in gene expression could alter STL patterns, particularly under diverse growth and site conditions as was found with the damsin-type STLs of *Ambrosia ambrosioides* (Seaman, 1982). Nevertheless, it is surprising that argophyllin A, the major STL detected in both wild (Melek et al., 1985) and cultivated sunflower (this study) went undetected in work of Spring et al. (1989).

A novel STL epoxide was detected for sunflower based on its ^{1}H NMR signals within 4,5-dihydroniveusin A samples, but its full identity remains unknown because of the small amount and the difficulty in separating it from the latter STL. Although it appears to be a germacran-6,12-olide angelate with a 1,10- or 4,5-epoxy group of higher polarity than argophyllin B, further work will be necessary for its characterization.

The rapid ethyl acetate surface extraction of sunflower followed immediately by batchwise silica gel adsorption and selective chloroform–ethyl acetate elution of the STLs gave very consistent phytochemical profiles between samples and replicates for sunflower tissues. During the extraction and isolation of STLs, reactive solvents such as methanol, ethanol, and acidic solvents were not used in order to avoid formation of chemical artifacts. Formation of methoxy analogs of native STLs can occur by simply using methanol as the extraction solvent (Gershenzon et al., 1984; Mullin et al., 1991a). No methoxy or ethoxy groups

were detected for STLs in this work, and all identified compounds were consistent with those published previously for cultivated sunflower. The six identified STLs are all comparatively stable in dry condition, and STL samples could be stored in a −20°C freezer for up to two months with no detectable degradation. This might be due to diterpenoid and flavonoid impurities, which could function as electron donators (antioxidants) for the STLs.

Reflectance densitometry at 220 nm allowed a representative measurement of all of the STLs encountered in sunflower due to similarity in UV absorbance at this wavelength (Table 1, Figure 2). Choice of the internal standard, naringenin, was based on its close chemical characters (three hydroxy groups, suitable polarity, and a UV_{max} at 220 nm) with sunflower STLs.

STLs in cultivated sunflower based on this HPTLC method are most abundant in leaves (average content of 445 μg/g fresh tissue), bracts (20 μg/g) and both ray (13 μg/g) and disk (343 μg/g) flowers, while lacking in roots, stems, cotyledons, pollen, and achenes (Chou, 1992; Chou and Mullin, 1993). Argophyllin A was the dominant STL in all new-growth tissues after four weeks. However, lower (older) leaves of sunflower throughout growth remained enriched in the nonpolar STLs 15-hydroxy-3-dehydrodesoxytifruticin, niveusin B, and 1,2-anhydridoniveusin A over that of the epoxide-diol argophyllins and 4,5-dihydroniveusin A. Large differences in STL profiles from leaves of three morphologically divergent *H. annuus* cultivars were not found, although Spring et al. (1985) observed a larger variation in content of two STLs among seven cultivars.

WCR was chosen here as the test insect because these STLs selectively deter this major corn pest from feeding on sunflowers (Mullin et al., 1991b). Of the six STLs isolated, argophyllin A is the most deterrent in the bitter squash disk bioassay (Table 2). Moreover, antifeedant activity on WCR within sunflower extracts correlates with their relative argophyllin A and/or B content (Figure 4). Argophyllin A is most abundant in the apical meristem and floral tissues most responsible for producing or protecting subsequent progeny. Taken together, these results strongly suggest that argophyllins have an important role in sunflower defense against herbivory by corn rootworm. These and other STLs present in wild *Helianthus* species have also been implicated as effective allelochemicals against a seed specialist, the sunflower moth, *Homoeosoma electellum* (Rossiter et al., 1986; Rogers et al., 1987).

Generally, nonpolar chemicals are more toxic than polar chemicals because they more easily dissolve into and penetrate through membrane structures. Surprisingly, the reverse was found here (Table 2), where the more polar STLs (argophyllin A, argophyllin B, 4,5-dihydroniveusin A, and the unidentified epoxide mixture) possess more antifeedant activity to WCR than nonpolar STLs (15-hydroxy-3-dehydrodesoxytifruticin, niveusin B, and 1,2-anhydridoniveusin A). Alternatively, more polarity and thus water solubility may be essential for

STL interaction with receptor sites on the gustatory sensilla of WCR. The epoxide function of argophyllin could be an important factor in determining feeding deterrency to WCR and other *Diabrotica*, and thus prove valuable as a lead in pesticide development for this major corn pest.

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REFERENCES

CHOU, J.-C. 1992. Phenologic and tissue distribution in cultivated sunflower of antifeedant sesquiterpene lactones for western corn rootworm. MS thesis. Pennsylvania State University, University Park, Pennsylvania.

CHOU, J.-C., and MULLIN, C.A. 1993. Phenologic and tissue distribution of sesquiterpene lactones in cultivated sunflower (*Helianthus annuus* L.). *J. Plant Physiol.* Accepted.

DUKE, S.O. 1991. Plant terpenoids as pesticides, pp. 269–296, *in* R.F. Keeler and A.T. Tu (eds.). Handbook of Natural Toxins: Toxicology of Plant and Fungal Compounds, Vol. 6. Marcel Dekker, New York.

GERSHENZON, J., and CROTEAU, R. 1991. Terpenoids, pp. 165–219, *in* G.A. Rosenthal, and M.R. Berenbaum (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites, 2nd ed., Vol. I. The Chemical Participants. Academic Press, New York.

GERSHENZON, J., MABRY, T.J., KORP, J.D., and BERNAL, I. 1984. Germacranolides from *Helianthus californicus*. *Phytochemistry* 23:2561–2571.

ISMAN, M.B., BRARD, N.L., NAWROT, J., and HARMATHA, J. 1989. Antifeedant and growth inhibitory effects of bakkenolide-A and other sesquiterpene lactones on the variegated cutworm, *Peridroma saucia* Hubner (Lep.; Noctuidae). *J. Appl. Entomol.* 107:524–529.

MABRY, T.J., and GILL, J.E. 1979. Sesquiterpene lactones and other terpenoids, pp. 501–537, *in* G.A. Rosenthal, and D.H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

MELEK, F.R., GAGE, D.A., GERSHENZON, J., and MABRY, T.J. 1985. Sesquiterpene lactone and diterpene constituents of *Helianthus annuus*. *Phytochemistry* 24:1537–1539.

MULLIN, C.A., ALFATAFTA, A.A., HARMAN, J.L., EVERETT, S.L., and SERINO, A.A. 1991a. Feeding and toxic effects of floral sesquiterpene lactones, diterpenes and phenolics from sunflower (*Helianthus annuus* L.) on western corn rootworm. *J. Agric. Food Chem.* 39:2293–2299.

MULLIN, C.A., ALFATAFTA, A.A., HARMAN, J.L., SERINO, A.A., and EVERETT, S.L. 1991b. Corn rootworm feeding on sunflower and other Compositae: Influence of floral terpenoid and phenolic factors, pp. 278–292, *in* P.A. Hedin (ed.). Naturally Occurring Pest Bioregulators. ACS Symposium Series No. 449. American Chemical Society, Washington, D.C.

OHNO, N., and MABRY, T.J. 1980. Sesquiterpene lactones and diterpene carboxylic acids in *Helianthus niveus* subspecies *canescens*. *Phytochemistry* 19:609–614.

PICMAN, A.K. 1986. Biological activities of sesquiterpene lactones. *Biochem. Syst. Ecol.* 14:255–281.

PICMAN, A.K., RANIERI, R.L., TOWERS, G.H.N., and LAM, J. 1980. Visualization reagents for sesquiterpene lactones and polyacetylenes on thin-layer chromatograms. *J. Chromatogr.* 189:187–198.

PROKSCH, P., WRAY, V., ISMAN, M.B., and RAHAUS, I. 1990. Ontogenetic variation of biologically active natural products in *Ageratina adenophora*. *Phytochemistry* 29:453–457.

REES, S.B., and HARBORNE, J.B. 1985. The role of sesquiterpene lactones and phenolics in the chemical defence of the chicory plant. *Phytochemistry* 24:2225–2231.

ROGERS, C.E., GERSHENZON, J., OHNO, N., MABRY, T.J., STIPANOVIC, R.D., and KREITNER, G.L. 1987. Terpenes of wild sunflowers (*Helianthus*): An effective mechanism against seed predation by larvae of the sunflower moth, *Homoeosoma electellum* (Lepidoptera: Pyralidae). *Environ. Entomol.* 16:586–592.

ROSSITER, M., GERSHENZON, J., and MABRY, T.J. 1986. Behavioral and growth responses of specialist herbivore, *Homoeosoma electellum*, to major terpenoid of its host, *Helianthus* spp. *J. Chem. Ecol.* 12:1505–1521.

SAS/STAT User's Guide. 1990. The ANOVA procedure, pp. 209–244, *in* ANOVA-FREQ. Version 6, 4th ed., Vol. 1. SAS Institute Inc., Cary, North Carolina.

SEAMAN, F.C. 1982. Sesquiterpene lactones as taxonomic characters in the Asteraceae. *Bot. Rev.* 48:121–592.

SPRING, O. 1989. Microsampling: An alternative approach using sesquiterpene lactones for systematics. *Biochem. Syst. Ecol.* 17:509–517.

SPRING, O., KUPKA, J., MAIER, B., and HAGER, A. 1982. Biological activities of sesquiterpene lactones from *Helianthus annuus*: Antimicrobial and cytotoxic properties; influence on DNA, RNA, and protein synthesis. *Z. Naturforsch.* 37c:1087–1091.

SPRING, O., PRIESTER, T., STRANSKY, H., and HAGER, A. 1985. Sesquiterpene lactones in sunflower seedlings: Distribution in the plant and occurrence in genetic varieties as determined by an isocratic HPLC technique. *J. Plant Physiol.* 120:321–329.

SPRING, O., BENZ, T., and ILG, M. 1989. Sesquiterpene lactones of the capitate glandular trichomes of *Helianthus annuus*. *Phytochemistry* 28:745–749.

WATANABE, K., OHNO, N., YOSHIOKA, H., GERSHENZON, J., and MABRY, T.J. 1982. Sesquiterpene lactones and diterpenoids from *Helianthus argophyllus*. *Phytochemistry* 21:709–713.

WISDOM, C.S., and RODRIGUEZ, E. 1983. Seasonal age-specific measurements of the sesquiterpene lactones and chromenes of *Encelia farinosa*. *Biochem. Syst. Ecol.* 11:345–352.

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FIELD EVALUATION OF *Anomala schonfeldti* OHAUS (COLEOPTERA: SCARABAEIDAE) SYNTHETIC SEX PHEROMONE

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Abstract—Synthetic 2-(*E*)-nonenol, previously identified as the sex pheromone of *Anomala schonfeldti* (Coleoptera: Scarabaeidae), is demonstrated to be very attractive to males in the field. Nevertheless, no significant differences were found between treatments with 1, 5, 10, and 20 mg dosages. Males of *A. schonfeldti* were more significantly attracted to traps at 30 cm high than at 90 cm. Although the observed behavior seemed to indicate a trend of more attraction to buried traps than those placed at 30 cm, there was no statistical difference between the two treatments. Pheromone-baited traps caught significantly more beetles than traps containing three virgin females. Over 70% of released beetles were recaptured in six traps surrounding the point of release and separated from each other by 50 m, suggesting a possible use of the pheromone (in combination with floral compounds) in mass trapping.

Key Words—*Anomala schonfeldti*, *Popillia japonica*, scarab beetle, Coleoptera, Scarabaeidae, 2-(*E*)-nonenol, sex pheromone, mark-and-recapture, field test, mass trapping.

INTRODUCTION

Throughout the world, larvae and beetles of the Scarabaeidae (Coleoptera) are pests of crops, pastures, turfgrass, and in some cases trees and fruits. Damage by these pests can be severe and, because most of their life is spent in the soil,

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scarabs have proven difficult to control. Although some are exotic, imported species, most pests are endemic species, often exploiting modified habitats (Jackson, 1992). In Japan, the area of amenity turf has dramatically increased in the past not only in golf courses, but also in sports fields for baseball, rugby, and soccer, as well as in parks, riverbanks, roads, and home gardens. This rapid increase has been pointed out as a cause of environmental destruction (by causing pollution with pesticides) and considered a serious social problem (Ochiai, 1991). Simultaneously, there has been a growing demand for environmentally sound methods of pest control as alternatives to minimize or replace conventional pesticides. These two factors have led the Chiba Prefectural Government to establish as a rule that no permission for the construction of new golf courses would be granted, unless the applicants "take the oath" (*seiyakusho*, in Japanese) that no insecticide will be used for pest management.

Therefore, new methods of control are highly sought. A cooperative project has been initiated in order to investigate the possibility of applying semiochemicals such as sex pheromones in integrated pest management (IPM) of turfgrass pests. In the course of this project, 2-(*E*)-nonenol was identified as the sex pheromone of *A. schonfeldti* (Leal et al., 1992a). In Japan this species is considered to be one of the most noxious May beetles for turfgrass (Hatsukade, 1991). We describe here the results of field tests with the synthetic sex pheromone, demonstrating its applicability for monitoring. Furthermore, mark-and-recapture experiments suggest that the pheromone may be useful for mass trapping.

METHODS AND MATERIALS

Controlled-Release Dispenser. The pheromone controlled-release dispensing system was prepared by Fuji Flavor Co. 2-(*E*)-Nonenol, synthesized as previously described (Leal et al., 1992a), was incorporated into pellets made of a polyethylene–vinyl acetate copolymer. Each pellet was 4–5 mm in diameter and contained either 1 or 5 mg of the pheromone.

Field Experiments. Evaluation of the pheromone in the field was carried out at the Chiba Prefectural Agricultural Experiment Station field in Chiba, Japan, from mid-June through late July, 1992. The traps used were either green funnel traps (JT, Japan Tobacco Inc.), or black water pan traps (Sankei, Sankei Chemical). The experiments were done in randomized blocks with an intertrap distance of 10 m. Traps were checked every day, the males removed, and the traps rerandomized within blocks. All treatments were replicated at least four times. Traps were suspended with the pheromone dispenser at 90 or 30 cm above the ground, or they were buried (JT traps only) so that the lip of the funnel was positioned at the surface.

Virgin females used in this study were mostly laboratory-raised, but some virgin female beetles were captured in the field at the beginning of the season. Females were placed (and renewed daily) in plastic bottles provided along with JT traps, in which small holes were opened. Capture data were transformed to log $(x + 1)$ and differences between means were tested for significance by ANOVA. Throughout this paper, treatments followed by the same letters are not significantly different at a 5% level in the Scheffe F test. Error bars in the figures show 1 SE.

Mark-and-Recapture. This test was conducted in a new turfgrass experimental field, where the population of *A. schonfeldti* seemed to be very small. Six JT traps baited with 2 mg of 2-(*E*)-nonenol were set on the ground (height of the pheromone device: 30 cm) in an hexagonal arrangement, separated from one another and from the center by ca. 50 m. Two light traps were set at ca. 15 m from the center. The direction of the wind (average 1.25 m/sec) at the peak of the flight time, 1800–1900 hr (JST), was ESE and ENE. Previously captured males were marked on the prothorax with a pink paint marker (Mitsubishi Empitsu) and released from the center of the circle before sunset. Recapture data were recorded for the traps described above, as well as for four traps placed at a height of 30 cm, and located 150 and 300 m away from the experimental area.

RESULTS AND DISCUSSION

Effect of Dosage on Catches. In order to determine the optimal dosage for capture of *A. schonfeldti*, catches by traps baited with 1, 5, 10, and 20 mg of 2-(*E*)-nonenol and set at a height of 90 cm from the ground were compared at the beginning of the flight season (June 16–18). Treatments of all four dosages of the synthetic sex pheromone captured significantly more beetles than unbaited traps (Figure 1), but there was no significant difference between dosages. Thus,

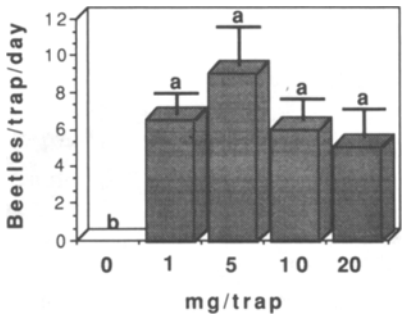

FIG. 1. Captures of *A. schonfeldti* males with different dosages of 2-(*E*)-nonenol.

although no optimal dosage could be determined, amounts of 1 mg or more of the pheromone can be recommended for monitoring applications. During these experiments, only a fraction of the large number of beetles attracted was captured in pheromone-baited traps, probably due to the height (90 cm) of the traps. Many of the beetles were observed crawling or flying just above the soil in regions close to the traps. We, therefore, compared the captures at different heights.

Effect of Traps Height on Captures. Catches were compared in experiments carried out from June 19 to 28, setting Sankei and JT traps at 90 or 30 cm above the ground. These results (Figure 2A) revealed that, although there were no significant differences between Sankei traps at the two heights, JT traps at 30 cm were significantly more attractive than those at 90 cm. Furthermore, there was no significant difference between the two types of traps at each height. Water pan traps were not used in further experiments because of these results and the fact that funnel traps are easier to manipulate in the field.

Catches by buried traps (0 cm) were not significantly different from those by traps placed at 30 cm (Figure 2B), although there seemed to be a trend to capture more beetles at the lower level. By contrast, traps for *Popillia japonica* (baited with food-type lures) have been demonstrated to be significantly more attractive at 56 or 112 cm than when the edges were at ground level (Ladd and Jurimas, 1972). Consequently, in later experiments with synthetic sex pheromone and food-type lures, traps were set at 56 cm (Klein et al., 1981). Nevertheless, traps baited with virgin female *P. japonica* captured significantly more beetles when positioned at 28 cm than at 0, 56, or 112 cm (Klein et al., 1972). This led to the conclusion that the type of lure may be of importance (Klein, 1981). These differences in heights for the optimal catches of these two scarabs, however, might also be due to their different mating behaviors. *A. schonfeldti* males emerge from the soil and appear on the ground just after sunset, where they crawl and fly at low level to locate females. They recognize (by the sex

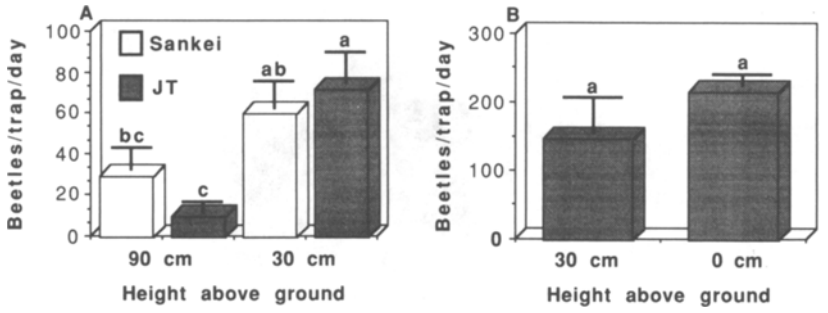

FIG. 2. Effect of the height of the traps on the catches of *A. schonfeldti* males.

pheromone) the place where female beetles are about to emerge and copulate even before the females have completely emerged. On the other hand, adults of *P. japonica* are pests of turfgrass and highland crops; thus, in natural conditions, males are adapted to respond to the calling of females either on the ground—where they form so-called "balls" (Ladd, 1970)—or in aerial parts of host plants where they also copulate.

Buried traps baited with 1 mg of 2-(*E*)-nonenol captured significantly more beetles (88.6 ± 51.1) than those baited with virgin female *A. schonfeldti* (15.6 ± 9.6). These findings suggested that *A. schonfeldti* may not utilize any minor components. However, two-component sex pheromone systems of other scarab beetles recently have been described (Leal et al., 1993a,b). The high attractiveness of single-compound pheromone systems may mask the occurrence of minor components in scarab beetle pheromones as seen with the large black chafer, *Holotrichia parallela*. When its major sex pheromone, L-isoleucine methyl ester (Leal et al., 1992b) was tested alone, although the results were not as striking as these, it seemed to be the sole component; but when it was mixed with (*R*)-(−)-linalool (which was not attractive by itself), the attractiveness significantly increased (Leal et al., 1993c). Therefore, further investigations will clarify whether *A. schonfeldti* utilizes any minor components.

Mark-and-Recapture. Preliminary experiments (July 9–13) gave a low recapture rate (28.6%) due to the small number of traps (4) set 50 m away from the releasing spot. However, later experiments (July 17–20) with a new design (Figure 3) showed that 66.4% of the 357 beetles released were recaptured during

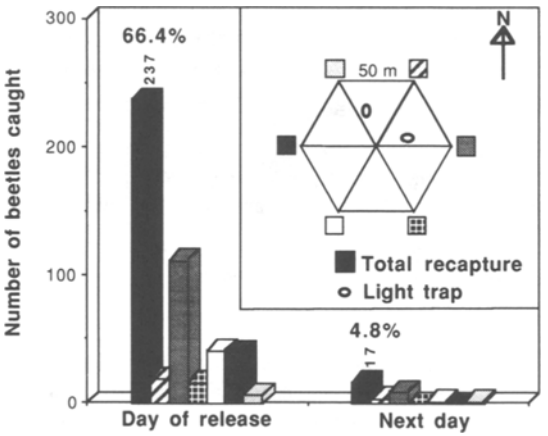

FIG. 3. Number of marked *A. schonfeldti* males recaptured out of 357 beetles released from the center of the hexagon formation of six JT traps. The first bar on the left of each group of data is the total recapture. Due to the direction of the wind, the trap on the east was by far the most attractive on the first night.

the same night and 4.8% the following night. Only one male was caught in one of the two light traps and a total of five individuals were recaptured in pheromone-baited traps located at least 150 m away from the releasing place. This high ability of *A. schonfeldti* to respond to the sex pheromone suggested that 2-(*E*)-nonenol may be useful for mass trapping of this turfgrass pest. This, coupled with the results of preliminary experiments (Leal et al., unpublished data) showing that floral compounds combined with the sex pheromone led to captures of both male and female beetles, justify future research on large-scale mass trapping.

The value of mass trapping in IPM is controversial. For example, the effect of mass trapping on insect populations and crop damage by the Japanese beetle has been considered questionable, since no report has documented the direct effect on crop protection (Bakke and Lie, 1989). Reliable data on crop protection can be achieved by mass trapping over large areas, where the sex pheromone cost is lower with *A. schonfeldti* than *P. japonica*. Furthermore, in marked contrast to the behavior of *P. japonica* adults, which feed on the foliage and/or fruits of at least 300 plant species (Villani et al., 1992), feeding is not essential to adults of *A. schonfeldti*. Therefore, the sex pheromone seems to play an even more significant role in the chemical ecology of the latter than in the former, which also relies on other semiochemicals for exploiting new food sources. After new host plants have been heavily colonized, Japanese beetle males found females quite easily, despite the fact that the females had already mated and were not emitting the sex attractant anymore (Klein, 1981).

In conclusion, 2-(*E*)-nonenol, which is very likely the only component of the pheromone system of *A. schonfeldti*, can be useful for monitoring populations of the beetle in the field by using funnel traps baited with at least 1 mg of the synthetic pheromone and set at 30 cm from the ground. The high attractiveness of the semiochemical suggests that it may be useful for mass trapping.

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REFERENCES

BAKKE, A., and LIE, R. 1989. Mass trapping, pp. 67–87, *in* A.R. Jutsum and R.F.S. Gordon (eds.). Insect Pheromones in Plant Protection. Wiley-Interscience, Chichester.

HATSUKADE, M. 1991. The biology and control of the important turfgrass insects in Japan. *Jpn. Pestic. Inf.* 59:10–12.

JACKSON, T.A. 1992. Scarabs—pests of the past or the future? pp. 1–10, *in* T.A. Jackson and T.R. Glare (eds.). Use of Pathogens in Scarab Pest Management. Intercept. Hampshire, England.

KLEIN, M. 1981. Trapping for suppression of Japanese beetle, pp. 183–190, *in* E.R. Mitchell (ed.). Management of Insect Pests with Semiochemicals. Plenum, New York.

KLEIN, M.G., LADD, T.L., JR., and LAWRENCE, K.O. 1972. The influence of height of exposure of virgin female Japanese beetle on captures of males. *Environ. Entomol.* 1:600–601.

KLEIN, M.G., TUMLINSON, J.H., LADD, T.L., and DOOLITTLE, R.E. 1981. Japanese beetle (Coleoptera: Scarabaeidae): Response to synthetic sex attractant plus phenethyl propionate: eugenol. *J. Chem. Ecol.* 7:1–7.

LADD, T.L. 1970. Sex attraction in the Japanese beetle. *J. Econ. Entomol.* 63:905–908.

LADD, T.L., and JURIMAS, J.P. 1972. Influence of trap height on captures of Japanese beetle. *J. Econ. Entomol.* 65:407–408.

LEAL, W.S., HASEGAWA, M., and SAWADA, M. 1992a. Identification of *Anomala schonfeldti* sex pheromone by high-resolution GC-behavior bioassay. *Naturwissenschaften* 79:518–519.

LEAL, W.S., MATSUYAMA, S., KUWAHARA, Y., WAKAMURA, S., and HASEGAWA, M. 1992b. An amino acid derivative as the sex pheromone of a scarab beetle. *Naturwissenschaften* 79:184–185.

LEAL, W.S., SAWADA, M., and HASEGAWA, M. 1993a. The scarab beetle *Anomala daimiana* utilizes a blend of two other *Anomala* spp. sex pheromones. *Naturwissenschaften* In press.

LEAL, W.S., SAWADA, M., and HASEGAWA, M. 1993b. The scarab beetle *Anomala cuprea* utilizes the sex pheromone of *Popillia japonica* as a minor component. *J. Chem. Ecol.* 19:1303–1313.

LEAL, W.S., SAWADA, M., MATSUYAMA, S., KUWAHARA, Y., and HASEGAWA, M. 1993c. Unusual periodicity of sex pheromone production in the large black chafer *Holotrichia parallela*. *J. Chem. Ecol.* 19:1381–1391.

OCHIAI, T. 1991. Present status of turfgrass and its management and associated problems in Japan. *Jpn. Pestic. Inf.* 59:3–5.

VILLANI, M.G., KRUEGER, S.R., and NYROP, J.P. 1992. Case study of the impact of the soil environment in insect/pathogen interactions: Scarabs in turfgrass, pp. 111–126, *in* T.R. Glare and T.A. Jackson (eds.). Use of Pathogens in Scarab Pest Management. Intercept, Hampshire, England.

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FUNCTIONAL SUBCASTE DISCRIMINATION (FORAGERS AND BROOD-TENDERS) IN THE ANT *Camponotus vagus* SCOP.: POLYMORPHISM OF CUTICULAR HYDROCARBON PATTERNS

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Abstract—In the ant *Camponotus vagus*, when selected foragers that had been earlier removed from the foraging arena and brood-tenders that had been earlier removed from the nest were placed together in a foraging arena, most of the brood-tenders and only a few of the selected foragers were carried back to the nest by nonselected foragers. We hypothesize that cuticular hydrocarbons serve as a cue that allows foragers to discriminate between members of their own subcaste and brood-tenders. It has been established that the proportions of certain hydrocarbons, which are the same regardless of the colony studied, vary from one worker subcaste to another and thus constitute a specific chemical signature. These hydrocarbons belong to a wide range of chemical families (alkanes, monomethylalkanes, and dimethylalkanes). The greatest differences between the two subcastes were observed on the thorax of workers. Principal component analyses performed on the hydrocarbons (or hydrocarbon combinations) corresponding to the 45 main peaks in the cuticular profiles of the head and thorax of brood-tenders and foragers of several colonies show that there exist quantitative differences between the various signatures that characterize the colony, the worker subcastes, and the various body parts within the same species, which can be classified in a hierarchy where the differences between worker subcastes are less pronounced than those between body parts or between colonies.

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Key Words—Ants, worker subcastes discrimination, polyethism, cuticular hydrocarbons.

INTRODUCTION

In many ant species, the members of a colony are able to recognize nestmates and to distinguish them from alien workers originating from other conspecific colonies. Alien discrimination results in aggressive, frequently fatal, encounters, resulting in closure of the colony. The signal underlying this process is chemical in nature [colony odor (Fielde, 1904) or colony signature]. We have established that in *Camponotus vagus* the chemical signature of each colony depends mainly on the relative proportions of some of the cuticular hydrocarbons (in particular dimethylalkanes) composing the specific mixture, which differ from one colony to another (Bonavita-Courgourdan et al., 1987a). The cuticular hydrocarbons have also been found to play a role in nestmate recognition in other ant species (Morel et al., 1988; Nowbahari et al., 1990). These substances are also involved in species recognition in termites (Howard et al., 1982; Bagnères et al., 1991).

In the complex system formed by an ant society, several interdependent subsystems coexist: the queen, the sexual winged males and females, the workers (which belong to various functional subcastes), and the brood at various stages of development. The functioning of the whole system (the colony) depends on interactions between these subsystems and between their individual members. The behavior of each individual furthermore depends strongly on the subsystem to which it belongs. Differences in the composition or proportions of the chemicals produced by individuals belonging to different subsystems may act as subsystem recognition signals, although other cues, such as tactile signals, may also be involved.

It has long been known that workers are able to recognize the queen (or queens) as being different from the other members of the society and that they are preferentially attracted to her (cf. Wilson, 1971; Hölldobler and Michener, 1980; Passera, 1984; Hölldobler and Wilson, 1990) or to a lure impregnated with queen extract in organic solvent (Stumper, 1956; Lofgren et al., 1983). Differences have correspondingly been observed between the proportions of some of the hydrocarbons in the cuticular patterns of queens and workers of some species, e.g., *Pseudomyrmex ferruginea* (Mintzer et al., 1987), *Camponotus vagus* (Bonavita-Courgourdan et al., 1990). Moreover, changes in the cuticle-borne chemical signature of the queen, depending on the stage of cyclic laying activity she has reached, may lead workers to modulate their behavior towards her (Bonavita-Cougourdan et al., 1990).

Evidence that workers are able to recognize larvae as such has been obtained in several species including *Solenopsis saevissima* (Glancey et al., 1970), *Solen-*

opsis invicta (Walsh and Tschinkel, 1974; Bigley and Vinson, 1975), *Atta cephalotes* (Robinson and Cherett, 1974), and *Myrmica rubra* (Brian, 1975). The distribution of larvae in the nest and the fact that they are fed differentially depending on their size or developmental stage imply the existence of a discrimination process as regards these characteristics (Le Masne, 1953; Markin, 1970). Correlatively, larvae have a specific cuticular signature composed of the same hydrocarbons as that of the other members of the colony, but the relative proportions of the main hydrocarbons are statistically different (Bonavita-Cougourdan et al., 1988). Moreover, as the larvae develop, the cuticular profile changes, the greatest quantitative increase being observed in the substances with high molecular weight (Bonavita-Cougourdan et al., 1990).

The existence of recognition and discrimination processes between members of the two main functional worker subcastes, i.e., foragers and brood-tenders, has not yet been demonstrated, however. An experimental model was sought for an ethological analysis of a possible recognition process between workers performing different tasks, probably at different ages. A suitable model was suggested to us during our observations on *Camponotus vagus* workers setting up of a colony in an experimental nest. The ant workers transported back to their nest individuals of all the types that are normally to be found in their nest, i.e., larvae, cocoons, nymphs, winged males and females, and queen (Hölldobler and Maschwitz, 1965 and personal observations).

The purpose of this study was: (1) to determine whether workers of one functional subcaste are able to distinguish workers of their own subcaste from those of another subcaste, and (2) to analyze and compare the cuticular hydrocarbons characteristic of the various body parts of workers belonging to the two main subcastes, i.e., foragers and brood-tenders. These differences might constitute a subcaste-specific chemical signature for mutual recognition and discrimination. Preliminary results on both points have already been reported (Bonavita- Courgourdan et al., 1987b). We also comparatively investigated the relative importance of the chemical signatures characterizing the society, the functional subcastes, and the various body parts.

METHODS AND MATERIALS

Experimental Model. Ethological tests and chemical analyses were performed on *Camponotus vagus* (subfamily of Formicinae), which form monogynous and very populous colonies in the trunks of fallen trees, and less commonly in tree stumps, in the south of France. Four colonies from three different sites were used. Each colony was queen-right and had several thousand workers.

Ethological tests and chemical analyses were performed in March on the

colony from the Var department (S1), in April on colonies from the departments of Bouches-du-Rhône (S2) and Alpes-Maritimes (S3), and in October on the colony (S4) from the Alpes-Maritimes department. The workers in S1, S2, and S3 were at least 5 months old at the time of testing; no new workers had yet emerged. The workers in S4 were sometimes younger at the time of testing than those in the above colonies.

Each colony was reared in a series of flat interconnecting nests. The upper surface of each was a sliding glass plate (covered by an opaque plate), which made it possible to remove brood-tenders. The nests were connected to a Perspex box (36 × 24 × 16 cm), which served as the foraging arena where food (honey and crickets) was provided.

Ethological Tests. At each ethological test (duration: 1 hr), five workers that had previously been removed from the foraging arena just after they had begun feeding on a honey-covered plate (called test foragers) and five workers that had been removed from the vicinity of the brood inside the nest (called test brood-tenders) were introduced simultaneously into the foraging arena where several dozen foragers were already present. Two hours before the test, immediately after their removal, the test workers from each subcaste were marked on the gaster with red or yellow silk paint; the colors were randomly assigned prior to each test. Before introducing the test workers into the foraging arena, the tube between the arena and the nest was closed in order to prevent the carrier forager and carried test forager or test brood-tender pairs from returning to the nest and to facilitate their removal.

Each series of experiments included 10 tests on each colony performed either once a day or every two days. All test foragers or test brood-tenders retrieved and brought back to the nest entrance were counted, regardless of whether they had been seized by one appendage and dragged or, more frequently, carried in the position characteristic of *Formicinae*. In *C. vagus*, as in other *Formicinae*, the carrier uses her mandibles to hold the mandibles of the carried worker, which is held with its dorsal side down and ventral side facing the carrier; the carried worker tucks in its legs (Le Masne, 1951; Möglich and Hölldobler, 1974).

The whole behavioral sequence of retrieving was observed in 22 cases (five in colony S1, five in S2, nine in S3, and three in S4).

Chemical Analysis. For each test, the carrier worker and the test brood-tender were collected separately and the cuticular composition of each was analyzed. Extracts were obtained by separately soaking the head and thorax of carriers and test brood-tenders, collected in two consecutive ethological tests, in 800 μl of pentane (Pestipur, 99%) for 10 min. A preliminary principal components analysis performed on the cuticular hydrocarbons corresponding to the 45 main peaks of the gas chromatography profile from the various body parts of five workers from one colony (from the Bouches-du-Rhône department) pre-

viously showed that the profiles of the head and abdomen were similar as regards the proportions of the hydrocarbons and differed from the thorax profiles in this respect: axis 1 (57.3% of the inertia) differentiated between thoraces on the one hand and heads and abdomens on the other hand; neither axes 2 (12.5%), 3 (7.5%), nor 4 (5.2% of the inertia) differentiated between workers' heads and abdomens. Consequently, the subcaste identification mark was painted on the abdomen and the substances from that body part were not analyzed. In each series of 10 ethological tests on each colony, 20 extracts were prepared, i.e., five from the heads and five from the thoraces of the carriers and likewise with the test workers. Eighty extracts in all were obtained; the number of animals used to obtain each extract varied from five to nine.

The extracts were dried under nitrogen to eliminate volatile compounds from exocrine glands. The resulting residue was redissolved in 50 μl of pentane. Two microliters of this solution were analyzed by chromatography using a Delsi 300 gas chromatograph with a flame ionization detector and equipped with a Chrompack capillary column CPSil5 WCOT (fused silicagel, 25 m $\times$ 0.22 mm). The injection mode was splitless (15 sec). A temperature program from 160 to 320°C at 3°/min was used. The carrier gas was helium. The chromatograph was coupled to an Enica 21 integrator. A standard alkane mixture from C_{20} to C_{36} was coinjected and the equivalent chain length (ECL) calculated. Most of the cuticular hydrocarbons obtained from different localities had been previously determined using mass spectrometry (Bonavita-Cougourdan et al., 1987b).

Data Processing. Multivariate principal components analysis on all four colonies combined (Addad software) and principal components analysis on each colony separately (Statgraphics and Unistat II softwares) were performed on the relative proportions corresponding to the 45 main peaks of the gas chromatography profile (which mainly included alkanes, monomethylalkanes, dimethylalkanes, and combinations of two of these products) from the heads and thoraces of carrier foragers and carried brood-tenders. In addition, further partial analyses were performed on the data obtained on each colony as regards the relative proportions of alkanes (six components), monomethylalkanes (19 or 20 components, depending on variations in the colony's specific chemical signature), or dimethylalkanes (10–13 components for the same reason) extracted from the heads and thoraces of the same insects. The relative proportions of the cuticular components were calculated on the basis of the corresponding peak surface given by the integrator coupled to the gas chromatograph and corrected using a flame ionization detector response coefficient as a function of the number of carbon atoms (Bagnères et al., 1990).

RESULTS

Behavior. When test foragers removed from the foraging arena containing numerous other workers and test brood-tenders taken from the brood inside the nest were introduced into the foraging arena, we observed that foragers present

in the arena carried most of the test brood-tenders back to the entrance of the nest within 1 hr. In the 40 tests performed (10 per colony), 73% (±6.1) of the 200 test brood-tenders were carried back to the entrance of the nest; whereas only 2.5% (±2.2) of the 200 test foragers were retrieved. In two of the four colonies, no test foragers were retrieved (Table 1).

Observation of worker carrying activities in the foraging arena (22 cases of return of test brood-tender ants back to the nest) showed that in all cases it was the future carrier that initiated the process. The future carrier repeatedly nibbled any part of the body of the test worker with its mandibles. The response to this stimulation was not always the same: either (1) the stimulated test worker could turn and face the future carrier so that the latter was able to seize it by its mandibles and lift it (18 cases); once it was off the ground, the test worker tucked its legs and antenna up and bent its abdomen forward against its own thorax; during the transport, the ventral side of the test worker was turned toward the ventral side of the carrier, or (2) when the stimulated test worker did not face the carrier the potential carrier could either simply give up or else grasp the test worker by a leg and drag it back to the nest (four cases), in some cases with the assistance of another worker.

Chemical Analysis. Multivariate principal component analysis using the 45 main peaks (from the peak corresponding to the alkane nC_{27}, peak 8) of the cuticular hydrocarbon profiles (Figure 1) of the heads (10 spectra for each colony) and thoraces (10 spectra for each colony) of the foragers and test brood-tenders collected during ethological tests were performed on the four colonies (i.e., 80 samples). The results showed (Figure 2) that axis 1 (40.6% of inertia) differentiated the worker heads from their thoraces, axis 2 (30.4% of inertia)

TABLE 1. RETURN OF TEST BROOD-TENDER ANTS AND TEST FORAGER ANTS CARRIED OR DRAGGED BY FORAGING WORKERS BACK TO NEST AFTER BEING SIMULTANEOUSLY INTRODUCED INTO FORAGING ARENA OF THEIR COLONY (5 WORKERS OF EACH FUNTIONAL SUBCASTE IN EACH TEST)

Colony	S1	S2	S3	S4	Σn[a]
Nn[b]	38	35	41	32	146
	76% ± 11.8	70% ± 12.7	82% ± 10.6	64% ± 13.3	73% ± 6.1
Fn[c]	0	2	0	3	5
	0%	4% ± 5.4	0%	6% ± 6.6	2.5% ± 2.2

[a] Σn: total number of test workers (brood-tenders and foragers) retrieved during the four experimental series (% ± SD); 200 test brood-tenders and 200 test foragers in all were introduced into the foraging arena of the four colonies.

[b] Nn: number of test brood-tenders retrieved during 10 tests (duration: 1 hr) carried out in the foraging arena of each of the four colonies S1-S4 (% ± SD).

[c] Fn: number of test foragers retrieved to the nest during the same tests (% ± SD).

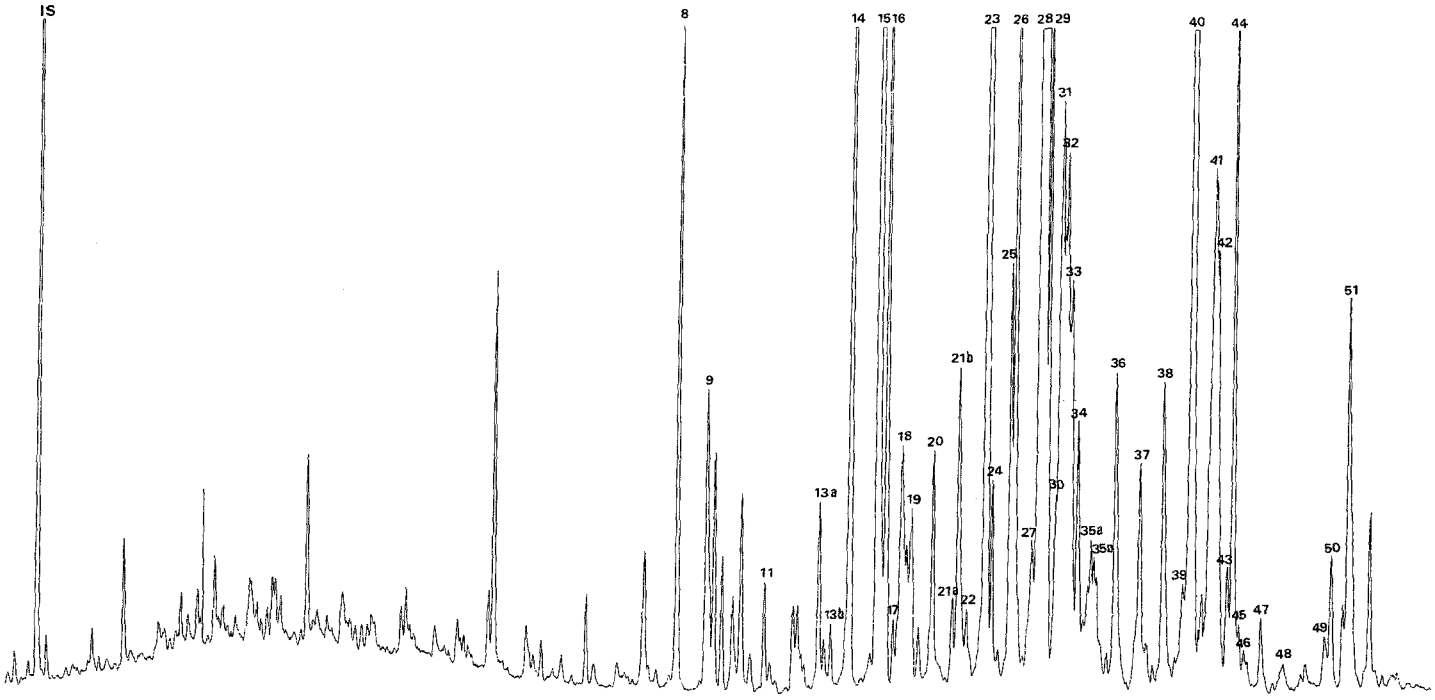


FIG. 1. Thoracic cuticular hydrocarbon profile of forager ant *Camponotus vagus*. The numbered peaks were those taken into account in the factorial analyses (Figures 2, 3, and 4). IS: internal standard (nC_{20}). Chemical analyses were performed on a fused silica capillary column with a temperature program from 160 to 320°C at 3°/min and the vector gas was helium; the injection mode was splitless (15 sec).

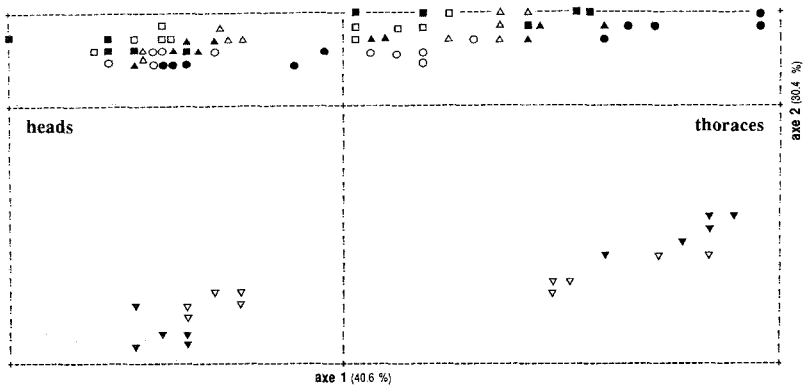


FIG. 2. Principal component analysis (Addad software) carried out on 45 normalized variables (the relative proportions of cuticular hydrocarbons numbered in Figure 1) recorded on the heads and thoraces of 10 groups of individuals (five from carried test brood-tender workers and five from carrying forager workers) belonging to each of the four colonies (40 groups of individuals in all). Cuticular extracts were obtained separately from the carriers and carried workers collected in two consecutive ethological tests. The number of animals used in each extract varied from five to nine. S1: black triangle, tip at the top, foragers; white triangle, tip at the top, test brood-tenders. S2: black triangle, tip down, foragers; white triangle, tip down, test brood-tenders. S3: black disk, foragers; white disk, test brood-tenders. S4: black square, foragers; white square, test brood-tenders. Heads and thoraces projected on the negative part and on the positive part of axis 1, respectively.

differentiated colony S2 from the other three colonies, and axis 3 (8.2% of inertia) differentiated foragers from test brood-tenders in the same colony at least as far as their thoraces were concerned.

The results of multivariate principal component analyses performed separately on each colony were generally homogeneous among the four colonies. The same hydrocarbons contributed most to the axes differentiating heads from thoraces and foragers from brood-tenders.

1. When analyses taking into account all the cuticular hydrocarbon families (i.e., alkanes, monomethylalkanes, and dimethylalkanes) were performed, axis 1 (in colony S1: 68.6%, S2: 78%, S3: 71%, S4: 61.9% of the inertia) differentiated heads from thoraces (Figure 3A). The hydrocarbons were distributed into two groups at each end of the axis (Figure 3B). The proportions of the lightest products (up to the methyls of C_{31}) were higher in the thorax than in the head, while the proportions of the heaviest products were higher in the head than the thorax. The products contributing largely to axis 1 in the four colonies were from different chemical families. In order of decreasing contribution, they were nC_{29} (peak 14), nC_{27} (peak 8), a dimethylalkane of C_{29} (peak 19), nC_{30}

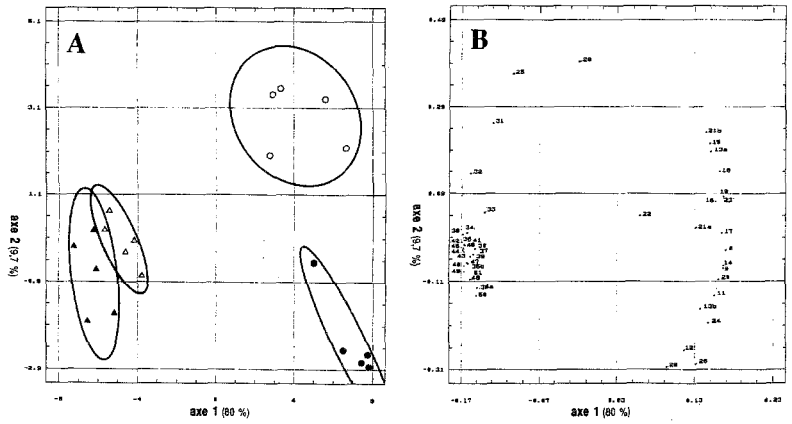


FIG. 3. Principal component analysis (Statgraphics software) carried out on 45 normalized variables (the relative proportions of cuticular hydrocarbons numbered in Figure 1) recorded on heads and thoraces of 10 groups of individuals belonging to colony S2: five from carried test brood-tenders and five from carrying foragers. Cuticular extracts were obtained from carrier and carried worker pairs collected separately in two consecutive ethological tests. The number of animals used in each extract varied from five to eight. (A) projection of individuals on the first two axes (87.7% of the inertia); (B) projection of cuticular hydrocarbons (the numbers corresponded to peaks of cuticular profile Figure 1) on the first two axes. Black triangles: heads of foragers; white triangles: heads of test brood-tenders. Black disks: thoraces of foragers; white disks: thoraces of test brood-tenders. Confidence ellipses (Unistat II software) were calculated with risk level 5%.

(peak 20), 4-meC_{30} (peak 23) projected onto axis 1 on the same part as the thoraces and, also in order of decreasing contribution, one or two components of the mixture: 2,19-dimeC_{33} + 3,21-dimeC_{33} + 4,23-dimeC_{33} (peak 42), 5,11-dimeC_{33} + 5,13-dimeC_{33} (peak 43), 11-meC_{33} + 13-meC_{33} (peak 40) and a unidentified product (peak 46) on the same part as the heads. Axis 1 in colony S2 also discriminated forager heads from test brood-tender heads.

Axis 2 (in colony S1: 13%, S2: 10%, S3: 13.3%, S4: 17.2%) consistently discriminated between the thoraces of foragers and those of test-brood-tenders. In colony S3, axis 2 also discriminated between the heads of the two worker subcastes. The products that contributed largely to axis 2 in all four colonies were 11-meC_{30} + 13-meC_{30} + 15-meC_{30} (peak 21b), 11,15-dimeC_{30} + 13,17-dimeC_{30} (peak 25), 11-meC_{29} + 13-meC_{29} + 15-meC_{29} (peak 15), which projected onto the same part of the axis as the test brood-tender thoraces, and 7-meC_{31} + 9-meC_{31} (peak 29), nC_{32} (peak 35a) and an unidentified product (peak 12) onto the same part as the forager thoraces. A small number of other products, some of which were identical in two or three colonies, also contributed strongly to this axis.

2. When principal components analyses were performed taking into account a single chemical family, i.e., either alkanes, monomethylalkanes, or dimethylalkanes, the overall results were similar. Axis 1 discriminated between heads and thoraces, while axis 2 discriminated between forager thoraces and test brood-tender thoraces.

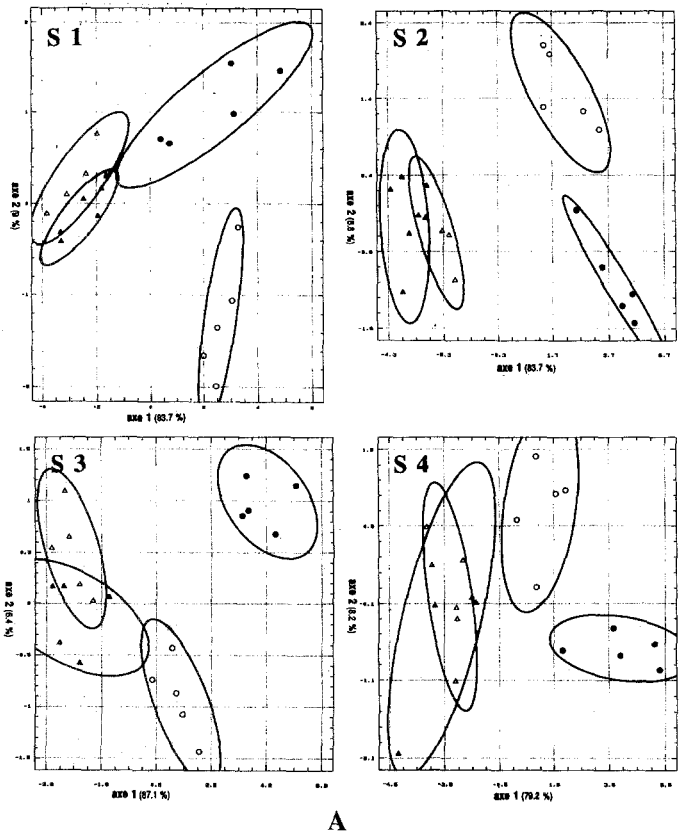
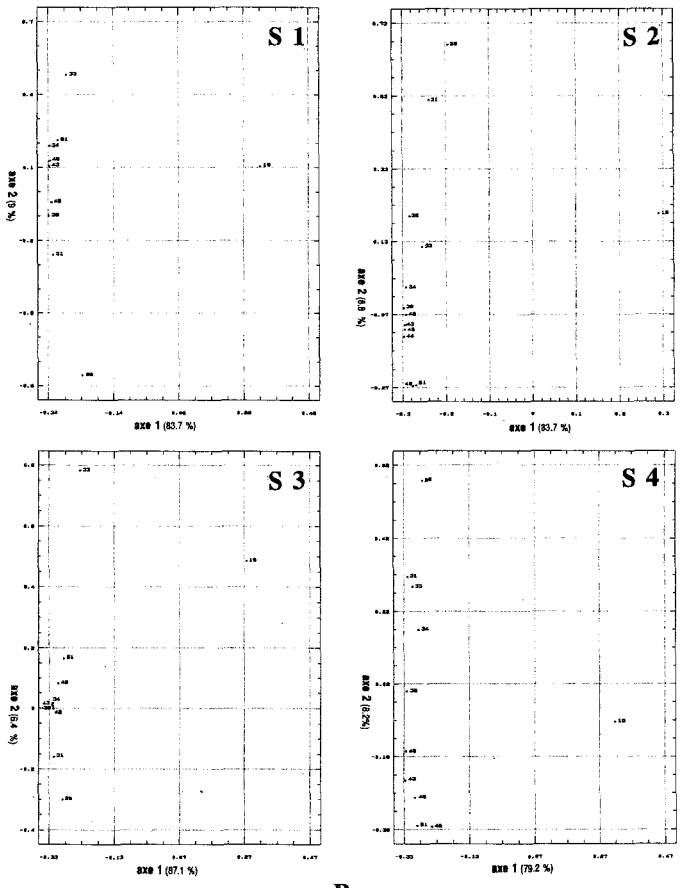


FIG. 4. Principal component analyses (Statgraphics software) carried out separately with each colony (S1, S2, S3, and S4) on relative proportions of cuticular dimethylalkanes from 10 groups of individuals: five groups of carried test brood-tenders and five groups of carrying foragers. The numbers of variables taken into account were: 10 in colony S1, 13 in colony S2, and 11 in colonies S3 and S4, depending on variations in the chemical signature specific to the colony. (A) Projection of individuals on the first two axes. Black triangles: heads of foragers; white triangles: heads of test brood-tenders. Black disks: thoraces of foragers; white disks: thoraces of test brood-tenders. Confidence ellipses (Unistat II software) were calculated with risk level 5%. (B) Projection of cuticular hydrocarbons on the first two axes. The numbers corresponded to peaks of cuticular profile Figure 1.

When only dimethylalkanes were taken into account (Figure 4A and B), the products contributing the most to axis 1 in the four colonies (83.7% of the inertia in colonies S1 and S2, 87.1% in colony S3, and 79.2% in colony S4) were a dimethylalkane of C_{29} (peak 19), which projected onto the same part of the axis as the thoraces, and a mixture of 5,11-dimeC_{33} and 5,13-dimeC_{33} (peak 43) and 11,20-dimeC_{32} (peak 38), corresponding to workers' heads (foragers and brood-tenders).

The dimethylalkanes contributing most to axis 2 in all the colonies (9% of the inertia in colony S1, 8.8% in colony S2, 6.4% in colony S3, and 8.2% in S4) were 11,15-dimeC_{30} + 13,17-dimeC_{30} (peak 25) and one or several components of the mixture 5,11-dimeC_{31} + 5,13-dimeC_{31} + 5,15-dimeC_{31} + 5,17-dimeC_{31} + 5,19-dimeC_{31} (peak 31), corresponding to the brood-tender thoraces,



B

FIG. 4. Continued

and a dimethylalkane of C_{35} (peak 51), corresponding to forager thoraces. A small number of products, some of which were identical in two or three colonies, also contributed strongly to this axis.

In all four colonies, when the analyses took alkanes into account, the products contributing the most to the part of axis 1 (75.5% of the inertia in colony S1, 90% in colony S2, 80% in colony S3, and 75.8% in colony S4) onto which the thoraces projected were as follows (in decreasing order): nC$_{29}$ (peak 14) and nC$_{27}$ (peak 8). The only alkane contributing strongly to the part of the axis onto which worker heads projected was nC$_{32}$ (peak 35a).

Axis 2 (18.8% of the inertia in colonies S1, 6.2% in S2, and 14% in S3) differentiated between the thoraces of foragers and brood-tenders (except in S4). Those contributing the most to the part of the axis onto which brood-tender thoraces projected were nC$_{27}$ (peak 8) and nC$_{29}$ (peak 14). The only alkane that contributed to the part of the axis on which foragers' thoraces projected was nC$_{32}$ (peak 35a).

When the analyses took monomethylalkanes into account, in three colonies (S1, S2, and S4) the results were similar to those obtained with alkanes and dimethylalkanes. Axis 1 (57.5% of the inertia in colony 1, 73% in colony S2, and 48.8% in S4) distinguished the heads from thoraces, while axis 2 (18.7% of inertia in colony S1, 12.3% in colony S2, and 17.3% in colony S4) discriminated between forager thoraces and test brood-tender thoraces.

The products contributing most to axis 1 were 4/2-meC$_{28}$ (peak 13a) and 4-meC$_{30}$ (peak 23), which corresponded to worker thoraces, and 11-meC$_{33}$ + 13-meC$_{33}$ (peak 40) and 15-meC$_{33}$ (peak 39), which corresponded to worker heads.

The products contributing most to axis 2 were 15-meC$_{30}$ (peak 21c), which corresponded to the test brood-tender thoraces, and 3-meC$_{30}$, which corresponded to the forager thoraces. A small number of other products, some of which were the same in two colonies, also contributed strongly to this axis.

CONCLUSION AND DISCUSSION

When test foragers and test brood-tenders were collected from the foraging arena and the inside of the nest, respectively, and shortly after placed together in the foraging arena of a colony of *Camponatus vagus*, we observed that foragers carried most of the test brood-tenders back to the nest while the test foragers were rarely retrieved.

This retrieving behavior probably involves a recognition process whereby these two subcastes are able to recognize each other. High-speed motion pictures will be needed to confirm our ethological observations and compare them to those obtained on *Camponotus socius* (Hölldobler, 1971) and *C. sericeus*

(Hölldobler et al., 1974). In these two species, the authors did not mention nibbling by the future carrier before the face-to-face position. This behavior nevertheless indicates that discrimination and recognition processes are involved.

A correlation was found to exist between the ethological and chemical data. The relative proportions of some cuticular hydrocarbons differed between the two functional subcastes. This was true when taking into account a single colony as well as several colonies belonging to populations from distant locations with very different cuticular spectra. The variations involved all the chemical families, i.e., alkanes, monomethylalkanes, and dimethylalkanes. Multivariate principal component analyses taking into account a single family showed that foragers could be discriminated from brood-tenders on the basis of alkanes alone, monomethylalkanes alone, or dimethylalkanes alone. The results of multivariate analyses using chromatographic data obtained from extracts of whole workers' bodies made it possible to differentiate between brood-tenders and foragers in *Camponotus vagus* (Bonavita-Cougourdan et al., 1990) and *C. floridanus* (Lavine et al., 1990).

It can therefore be said that each subcaste has a specific chemical signature. It should be pointed out, however, that the main differences in the proportions of cuticular hydrocarbons between foragers and brood-tenders were detected chiefly on the thorax. This finding does not rule out the possibility that these hydrocarbons may play a role in functional subcaste recognition. In fact, given the length of the ants' antennae and the fact that they are frequently thrown forward, olfactory messages from the thorax can be perceived by the future carrier when approaching the future carried worker.

On the basis of the multivariate principal components analyses, the chemical signatures characterizing the colony, the functional worker subcaste, or the various body parts of the workers within a given species could be classified hierarchically in decreasing order of importance. The first factorial axis discriminated between the heads and thoraces in all four colonies, whatever their geographical origin. The second axis discriminated between the colonies from different locations having very different chemical signatures. Factorial analyses showed that the chemical signatures of the functional worker subcastes were less significant. The possible biological role of the differences between the cuticular hydrocarbon spectra of different body parts remains to be elucidated.

In *Camponotus vagus* the most plentiful hydrocarbons are known to characterize the developmental stage (larvae or adult) and, in adults, to differentiate the workers and sexual winged males and females from the queen (Bonavita-Cougourdan et al., 1990). The chemical signatures specific to the colony, the functional worker subcastes, or the sexual males or females involve minor hydrocarbons. The answer to the question as to how these differences in hydrocarbon spectra are perceived is still a matter of conjecture. Recently, the existence of neuronal receptors sensitive to minor compounds in a sex pheromone mixture

was demonstrated in a lepidopteran species (Lucas and Renou, 1991). A mechanism of this kind may possibly operate in other insects.

The differences in the proportions of cuticular hydrocarbons from one subcaste to another may be age-dependent (Bonavita-Cougourdan et al., 1987b). Tasks carried out inside the nest, such as feeding larvae and more generally caring for the brood are performed by younger workers, while older workers forage for food outside the nest. This explanation may, however, be a misinterpretation, and the specialization observed here may actually depend on factors other than age, such as learning and rhythm of emergence of new workers (Deneubourg et al., 1987).

Many authors have shown the existence of a fairly flexible correlation between the main social functions served by workers during their life and structural and physiological factors, such as the level of ovarian development (*Formica*: Otto, 1958; Kneitz, 1970; Hohorst, 1972; Hansen and Viik, 1980; Ceusters et al., 1981; Billen, 1982; *Camponotus*: Traniello, 1977; *Neoponera*: Fresneau, 1984), the development of the fat body or the postpharyngeal and labial glands (*Formica*: Hansen and Viik, 1980; Ceusters et al., 1981), and the activity of various pheromone glands, e.g., the poison gland and Dufour's gland (*Myrmica*: Cammaerts-Tricot, 1974, 1975). Functional worker subcastes can also exhibit differences in the levels of circulating juvenile hormone (JH), as observed in honeybees (Fluri et al., 1982; Robinson, 1987; Robinson et al., 1989; Robinson et al., 1991; Huang et al., 1991). In the latter species differences in responsiveness to various environmental stimuli also have been noted between subcastes (Robinson, 1987).

In *Formica sanguinea*, adult carrying has been observed when the colony moved to a new nest (Möglich and Hölldobler, 1974). The carriers form a stabilized group of specialized workers with small ovaries, whereas the carried workers have well-developed ovaries. This relationship between the state of ovarian development and differences in carrying behavior led the authors to suggest that the carriers were "outside workers" (foragers) and most of the individuals being carried were "inside workers" (brood-tenders). However, at the onset of nest moving, some workers specialized in carrying are themselves carried to the new nest site by other workers belonging to the same functional group.

Differences in the proportions of cuticular hydrocarbons in workers of different subcastes are consistent with a possible role of cuticular hydrocarbons in subcaste recognition and may be used as subcaste recognition signals. Unfortunately this hypothesis cannot be checked with lures on which cuticular hydrocarbons of one subcaste are replaced by those of another, as we did in previous experiments (Bonavita-Cougourdan, 1987a) to investigate the relationships between different colonies in terms of intercolony aggressiveness and not the

retrieving behavior. The chemical treatment is lethal, and dead insects are not carried back to the nest.

In fact, other chemical (glandular) or behavioral factors may intervene in the recognition process. Differences possibly exist in the number or type (antennations, etc.) of behavioral acts directed by nurses or foragers toward the potential carriers. However, both the correlation found to exist here between the differential treatment of workers of the two subcastes and the characteristics of their cuticular hydrocarbons, and the role of cuticular hydrocarbons in nestmate recognition strongly suggest that these hydrocarbons may play a role in subcaste recognition.

It should also be noted that the opposite process, i.e., possible discrimination between the two subcastes by brood-tenders, was not investigated here.

The ability of foragers to distinguish between workers of their own subcaste and brood-tenders may serve an adaptive purpose during migration from one nest site to another (Möglich and Hölldobler, 1974), when young workers are carried from one nest to another in species with polycalic nests (Kneitz, 1964) and in the modulation of individual behavior as a function of their partners' characteristics, as in trophallactic behavior (Bonavita-Cougourdan and Morel, 1985). This discrimination ability may also play an adaptive role by regulating task assignment between workers in the same colony.

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REFERENCES

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., KILLIAN, A., CLÉMENT, J.L., and LANGE, C. 1991. Recognition among termites of the genus *Reticulitermes*: Evidence for a role for the cuticular hydrocarbons. *J. Chem. Ecol.* 17:2397–2420.

BIGLEY, W.S., and VINSON, S.B. 1975. Characterization of a brood pheromone isolated from the sexual brood of the imported fire ant, *Solenopsis invicta*. *Ann. Entomol. Soc. Am.* 68:301–304.

BILLEN, J. 1982. Ovariole development in workers of *Formica sanguinea* Latr. (Hym. Formicidae). *Insectes Soc.* 29:86–95.

BONAVITA-COUGOURDAN, A., and MOREL, L. 1985. Polyethism in social interactions in ants. *Behav. Proc.* 11:425–433.

BONAVITA-COUGOURDAN, A., CLÉMENT, J.L., and LANGE, C. 1987a. 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. 1987b. Sub-caste discrimination in the ant *Camponotus vagus* Scop., p. 475, *in* J. Eder and H. Rembold (eds.). Chemistry and Biology of Social Insects. Peperny, Munich.

BONAVITA-COUGOURDAN, A., CLÉMENT, J.L., and LANGE, C. 1988. Reconnaissance des larves chez

la Fourmi *Camponotus vagus* Scop. Phénotypes larvaires des spectres d'hydrocarbures cuticulaires. *C.R. Acad. Sci., Paris, Sér. D.* 306:299–305.

BONAVITA-COUGOURDAN, A., CLÉMENT, J.L., and POVÉDA, A. 1990. Les hydrocarbures et les processus de reconnaissance chez les Fourmis: Le code d'information complexe de *Camponotus vagus*. *Actes Coll. Insectes Soc.* 6:273–280.

BRIAN, M.V. 1975. Larval recognition by workers of the ant *Myrmica*. *Anim. Behav.* 23:745–756.

CAMMAERTS-TRICOT, M.C. 1974. Production and perception of attractive pheromones by differently aged workers of *Myrmica rubra* L., *Insectes Soc.* 21:235–248.

CAMMAERTS-TRICOT, M.C. 1975. Ontogenesis of the defense in the workers of *Myrmica rubra* L. *Anim. Behav.* 23:124–130.

CEUSTERS, R., BERTRANDS, H., PETIT, H., and VAN DE PERR, H. 1981. Polyéthisme chez *Formica polyctena* Först. et *Myrmica rubra* L. Stratification des ouvrières dans le nid selon leur état physiologique par rapport à un gradient de température. Colloque Sect. Fr. U.I.E.I.S., Toulouse, pp. 38–44.

DENEUBOURG, J.L., GOSS, S., PASTEELS, J.M., FRESNEAU, D., and LACHAUD, J.P. 1987. Self-organisation mechanisms in ant societies (II): Learning in foraging and division of labour, pp. 177–196, *in* J.M. Pasteels and J.L. Deneubourg (eds.). From Individual to Collective Behaviour in Social Insects. Birkhäuser, Basel.

FIELDE, A.M. 1904. Power of recognition among ants. *Biol. Bull.* 7:227–250.

FLURI, P., LÜSCHER, M., WILLE, H., and GERIG, L. 1982. Changes in weight of the pharyngeal gland and haemolymph titres of juvenile hormone, protein and vitellogenin in worker honey bees. *J. Insect Physiol.* 28:61–68.

FRESNEAU, D. 1984. Développement ovarien et statut social chez une fourmi primitive, *Neoponera obscuricornis* Emery. *Insectes Soc.* 31:387–402.

GLANCEY, B.M., STRINGER, C.E., CRAIG, C.H., BISHOP, P.M., and MARTIN, B.B. 1970. Pheromone may induce brood tending in the fire ant *Solenopsis saevissima*. *Nature* 226:863–864.

HANSEN, T.E., and VIIK, M.O. 1980. Seasonal changes in the content of reserve and cold-resistant substances in *Formica aquilonia*. *Zool. Zh.* 59:380–387.

HOHORST, B. 1972. Entwicklung und Ausbildung der Ovarien bei Arbeiterinnen von *Formica* (*Serviformica*) *rufibarbis* Fabricius. *Insectes Soc.* 19:398–402.

HÖLLDOBLER, B. 1971. Recruitment behavior in *Camponotus socius*. *Z. Vergl. Physiol.* 7:123–142.

HÖLLDOBLER, B., and MASCHWITZ, U. 1965. Die Hochzeitsschwarm der Rossameise *Camponotus herculeanus*. *Z. Vergl. Physiol.* 50:551–568.

HÖLLDOBLER, B., and MICHENER, C. 1980. Mechanisms of identification and discrimination in social Hymenoptera, pp. 35–56, *in* H. Markl (ed.). Evolution of Social Behavior. Verlag Chemie, Weinheim.

HÖLLDOBLER, B., and WILSON, E.O. 1990. The Ants. Springer, Berlin.

HÖLLDOBLER, B., MASCHWITZ, U., and MÖGLICH, M. 1974. Communication by tandem running in the ant *Camponotus sericeus*. *J. Comp. Physiol.* 90:105–112.

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.

HUANG, Z.Y., ROBINSON, G.E., TOBE, S.S., YAGI, K.J., STRAMBI, C., STRAMBI, A., and STAY, B. 1991. Hormonal regulation of behavioural development in the honey bee is based on changes in the rate of juvenile hormone biosynthesis. *J. Insect. Physiol.* 37:733–741.

KNEITZ, G. 1964. Saisonales Trageverhalten bei *Formica Polyctena* Foerst. *Insectes Soc.* 11:105–129.

KNEITZ, G. 1970. Jahreszeitliche Veränderungen der Ovariolenzustände in der Arbeiterinnenkaste des Waldameisenstaates von *Formica polyctena* Foerst. *Zool. Anz., Suppl.* 33:209–215.

LAVINE, B.K., VAN DER MEER, R.K., MOREL, L., GUNDERSON, R.W., HWA HAN, J., and STINE,

A. 1990. False color data imaging: A new pattern recognition technique for analyzing chromatographic profile data. *Microchem. J.* 41:288–215.

LE MASNE, G. 1951. Echanges de nourriture, trophallaxie et transports mutuels chez les Fourmis, pp. 1104–1119, *in* P.P. Grassé (ed.). Traité de Zoologie, 10, 2. Masson, Paris.

LE MASNE, G. 1953. Observations sur les relations entre le couvain et les adultes chez les Fourmis. *Ann. Sci. Nat., Zool. 11ème Sér.* 15:1–56.

LOFGREN, C.S., GLANCEY, B.M., GLOVER, A., ROCCA, J., and TUMLISON, J. 1983. Behavior of workers of *Solenopsis invicta* (Hymenoptera: Formicidae) to the queen recognition pheromone: Laboratory studies with an olfactometer and surrogate queens. *Ann. Entomol. Soc. Am.* 6:44–50.

LUCAS, P., and RENOU, M. 1991. A receptor neuron for Z-11 hexadecen-1-al: A minor pheromone component in *Mamestra suasa* (Lepidoptera, Noctuidae). *C.R. Acad. Sci. Sér. D, Paris* 312: 71–76.

MARKIN, G.P. 1970. Food distribution within laboratory colonies of the Argentine ant *Iridomyrmex humilis* (Mayr). *Insectes Soc.* 17:127–157.

MINTZER, A.C., WILLIAMS, H.J., and VINSON, S.B. 1987. Identity and variation of hexane soluble cuticular components produced by the acacia ant *Pseudomyrmex ferruginea. Comp. Biochem. Physiol.* 86B:27–30.

MÖGLICH, M., and HÖLLDOBLER, B. 1974. Social carrying behavior and division of labor during nest moving in ants. *Psyche* 81:219–236.

MOREL, L., VANDER MEER, R.K., and LAVINE, B.K. 1988. Ontogeny of nestmate recognition cues in the red carpenter ant (*Camponotus floridanus*): Behavioral and chemical evidence for the role of age and social experience. *Behav. Ecol. Sociobiol.* 22:175–183.

NOWBAHARI, E., LENOIR, A., CLÉMENT, J.L., LANGE, C., BAGNÈRES, 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.

OTTO, D. 1958. Über die Arbeitsteilung im Staate von *Formica rufa rufo-pratensis minor* Gössw. und ihre verhaltensphysiologischen Grundlagen: Ein Beitrag zur Biologie der Roten Waldameise. *Wiss. Abh. Dtsch. Akad. Landwirtstwiss. Berlin* 30:1–169.

PASSERA, L. 1984. L'organisation sociale des Fourmis. Privat, Toulouse.

ROBINSON, G.E. 1987. Regulation of honey bee age polyethism by juvenile hormone. *Behav. Ecol. Sociobiol.* 20:329–338.

ROBINSON, S.W., and CHERETT, J.M. 1974. Laboratory investigations to evaluate the possible use of brood-pheromones of the leaf-cutting ant *Atta cephalotes* L. as a component in an attractive bait. *Bull. Entomol. Res.* 63:519–529.

ROBINSON, G.E., PAGE, R.E., STRAMBI, C., and STRAMBI, A. 1989. Hormonal and genetic control of behavioral integration in honey bee colonies. *Science* 246:109–112.

ROBINSON, G.E., STRAMBI, C., STRAMBI, A., and FELDLAUFER, M.F. 1991. Comparison of juvenile hormone and ecdysteroid haemolymph titres in adult worker and queen honey bees (*Apis mellifera*). *J. Insect Physiol.* 37:929–935.

STUMPER, R. 1956. Sur les sécrétions des fourmis femelles. *C.R. Acad. Sci., Paris* 242:2487–2489.

TRANIELLO, J.F. 1977. Recruitment behavior, orientation and the organization of foraging in the carpenter ant *Camponotus pennsylvanicus* De Geer. *Behav. Ecol. Sociobiol.* 2:61–79.

WALSH, J.P., and TSCHINKEL, W.R. 1974. Brood recognition by contact pheromone in the red imported fire ant *Solenopsis invicta. Anim. Behav.* 22:695–704.

WILSON, E.O. 1971. The Insect Societies. Harvard University Press, Cambridge, Massachusetts.

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TRAIL AND ARENA MARKING BY CATERPILLARS OF *Archips Cerasivoranus* (LEPIDOPTERA: TORTRICIDAE)

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Abstract—The activity of *Archips cerasivoranus* caterpillars is largely limited to their colonial silk web and trails. Silk pulled directly from the spinnerets of caterpillars and wound onto paper strips to form artificial trails elicited locomotion from the larvae. Trails made from extracts of silk and silk glands also elicited locomotion. These and other observations reported here indicate that the caterpillars are responsive to a water-soluble pheromone that is a component of the silk strand. Marker pheromones appear not to be secreted from other regions of the body, as has been reported for some other trail-following caterpillars.

Key Words—*Archips cerasivoranus*, *Yponomeuta cagnagella*, trail following, silk, trail pheromone, Lepidoptera, Tortricidae.

INTRODUCTION

Archips cerasivoranus caterpillars live communally in choke cherry, *Prunus virginiana*, and other rosaceous species (Johnson and Lyon, 1988). The caterpillars are leaf tiers, and they spin copious quantities of silk to bind together leaves to form a shelter and foraging arena. The caterpillars largely stay within boundaries defined by the silk that envelops their shelter, venturing only short distances beyond the web to draw in new leaves. The colony usually finds sufficient leaves in a single contiguous patch to complete their larval development, but occasionally a patch is exhausted prematurely, forcing the caterpillars to move to a new location. During these migrations, the caterpillars depart individually or in small groups, covering the branches they follow with silk. The marked branches serve to guide successive waves of departing caterpillars

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along a common pathway, and enable the cohort to reassemble en masse at a new site.

Although the patch-restricted feeding of *A. cerasivoranus* is a common foraging pattern among social caterpillars (Fitzgerald and Peterson, 1988; Fitzgerald, 1993), the trail-following behavior of only a single patch-restricted caterpillar has been previously investigated (Roessingh, 1989, 1990). The study reported here was undertaken to add to our basic understanding of arena and trail marking in social caterpillars.

METHODS AND MATERIALS

Field observations were made in Cortland County, New York. Colonies for laboratory studies were collected from the field and maintained on potted *P. virginiana* trees.

A previous study (Fitzgerald and Edgerly, 1979) showed that strips of filter paper that *A. cerasivoranus* caterpillars had walked on elicited locomotion from conspecifics. It was not determined, however, whether the response was due to the silk deposited by the caterpillars or to a pheromone secreted from another site. To determine if silk, uncontaminated by contact with either the caterpillar's body or host substrate, stimulated locomotion, strands were wound directly from the spinnerets of caterpillars onto 30-mm-long × 3-mm-wide strips of filter paper. During this procedure, a caterpillar was restrained with a vacuum forceps. The end of the silk strand was grasped with a forceps, pulled from the spinneret and wound once or twice around a filter paper strip. The strip was inserted into a slotted dowel, which was then rotated by a motor to draw a long, continuous strand from the caterpillar (Figure 1). Strips so treated were used to form the stem and one arm of a Y maze. A blank strip was used as the other arm. The response of caterpillars to the two arms of the maze was determined by placing a third-instar caterpillar at the base of the stem, allowing it to walk to the choice point and recording the chosen arm. To prevent the caterpillars from leaving the maze, the three sections of the maze were elevated above the substrate with insect pins. To preclude a positional bias, the arrangement of the two arms was alternated for each replicate of a test. New sections were used for each caterpillar tested.

A second series of tests was conducted to determine if a soluble component of the trail elicited locomotion. To collect trails for these tests, 20 caterpillars were placed, one at a time, at the base of a vertically suspended strip of filter paper, 400 mm long × 3 mm wide. The caterpillars were allowed to walk to the top, then removed. The long strip was then cut into 30-mm-long sections. Some sections were washed in either hexanes or distilled water to extract soluble

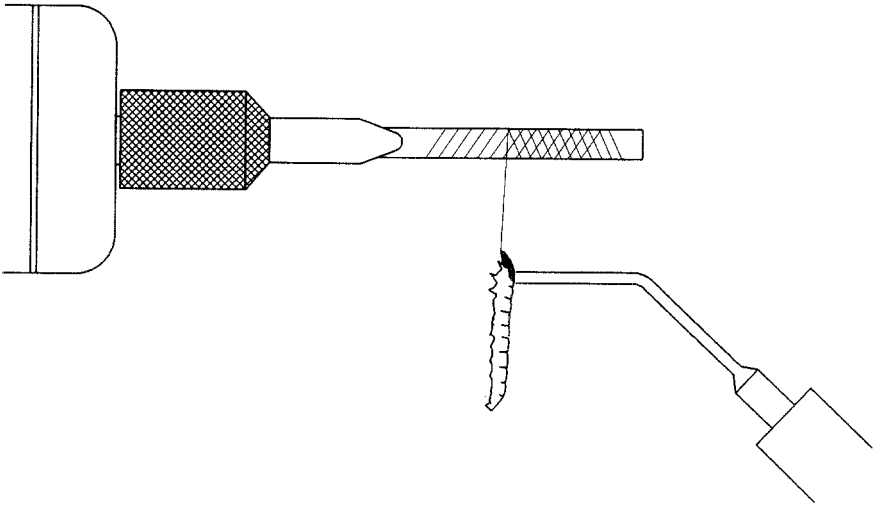

FIG. 1. Technique for obtaining silk from the spinneret of *Archips* caterpillars. See text for details.

components. After allowing them to dry, the extracted sections were paired with nonextracted sections to perform Y-maze tests as described above. For these tests, a nonextracted section, 30 mm long was used as the stem of the maze. All sections were used once, then discarded. To control for a potential affect of solvent residues, nonextracted sections were washed in hexanes or distilled water before they were used to collect trails.

Tests were also conducted to compare the rate at which caterpillars moved along 40-mm-long × 3-mm-wide sections of filter paper cut from longer strips that were previously traversed by groups of 20 caterpillars as described above. A third instar was placed at one end of a section and the rate (centimeters per second) at which it advanced recorded. Each caterpillar was allowed a maximum of 180 sec, and a test was terminated when a caterpillar reached the distant end of the section. The rate of travel on intact sections, sections extracted in hexanes or distilled water, and sections not previously walked on by caterpillars (blanks) was compared. In addition, the response of caterpillars to sections cut from longer strips previously walked on by groups of 20 caterpillars that had their spinnerets cauterized to prevent them from depositing silk was measured.

The possibility that caterpillars could distinguish new and old trails was investigated. Silk trails were collected on long paper strips as described above and allowed to age for 24 hr in the open in the laboratory. After 24 hr, fresh trails were collected and Y-maze tests conducted using aged trails and fresh trails (<1 hr old) as the arms of the maze. A section of aged trail was used as

the stem of the maze for the first 10 caterpillars tested and a section of the fresh trail for the second 10 caterpillars tested.

In another series of tests, the rate at which caterpillars moved along strips of filter paper treated with extracts prepared from silk, silk glands, or a leaf fragment/fecal pellet mixture was recorded. To prepare the silk extracts, 30 mg of silk were collected from laboratory colonies, cleaned of debris and extracted, first in 0.5 ml of hexanes, then in 0.5 ml of distilled water. Since the silk contained traces of fecal material and leaf fragments, the debris collected while cleaning the silk was extracted in 0.5 ml of methanol and the extract used as a control. To prepare gland extract, the silk glands of 20 fifth-instar caterpillars were removed and ground in 0.5 ml of methanol. The material was then centrifuged and the clear supernatant used to treat strips. All strips used in these tests were 20 mm long $\times$ 3 mm wide and were wetted with 10 μl of extract. The rate at which caterpillars moved along the strips was recorded as described above. In preliminary tests, caterpillars showed no response to strips of filter paper treated with only the solvents. All statistical analyses are from Sokal and Rohlf (1969).

RESULTS

In the field, *Archips* moths oviposited on the mainstem of choke cherry trees near to the ground, but the larvae constructed their shelters in the uppermost branches of the tree. Seven egg masses were located shortly after eclosion and the distance between them and the first feeding site measured. In all cases, the caterpillars initiated leaf-tying at the top of the tree, an average 101.4 $\pm$ 15.6 cm (SE) from the egg mass. One tree had two egg masses, but the caterpillars coalesced into a single composite colony. Another tree with a single egg mass had two distinct feeding sites, indicating that the colony had fragmented while moving from the base of the tree to the top. In all other cases, trees had a single egg mass and the aggregate established a single feeding site. The trees that the caterpillars colonized branched considerably, providing many alternative pathways larvae might have followed when moving from the egg mass to feeding sites. The cohesiveness of colonies indicates that newly eclosed and unfed caterpillars mark and follow trails as they move from the egg mass to the top of the tree.

Observations of field and laboratory colonies showed that caterpillars largely confined their activity to the vicinity of the shelter following their initial migration from the egg mass to the top of the tree. In contrast to the readiness with which they moved over surfaces covered with silk, caterpillars in all instars advanced hesitantly and turned back repeatedly when they moved onto previ-

ously unmarked substrate. A group of hungry third-instar caterpillars videotaped as they moved over a horizontal branch from their shelter in search of leaves moved forward singly or in small groups at the average rate of only 0.32 ± 0.02 cm/min, taking approximately 80 min to traverse the 25-cm length of the branch (Figure 2).

In all 10 separate Y-maze tests, caterpillars chose arms of the maze onto which silk had been wound directly from spinnerets over blank sections, indicating that a component or components of silk elicit trail following. When sections previously traversed by 20 caterpillars were extracted, their effectiveness was significantly reduced. In Y-maze tests, caterpillars preferred trail sections previously marked by conspecifics to similar sections extracted in hexanes 16:4 ($P < 0.01$, χ^2 test) and to those extracted in distilled water 18:2 ($P < 0.01$), indicating that soluble components of the trail promote a trail-following response.

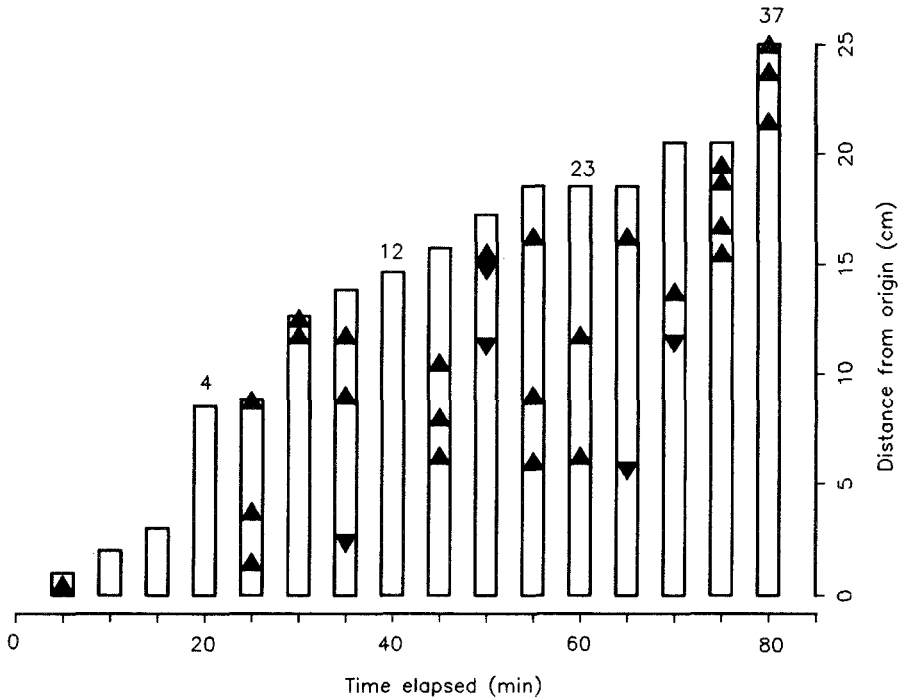

FIG. 2. Rate of movement of a colony of third-instar *Archips cerasivoranus* caterpillars from their web onto a previously unmarked branch. Arrows indicate position and direction of travel of individual caterpillars on the branch during discrete observations made every 5 min. Numbers at top indicate total number of caterpillars that moved from their web onto the branch to that point in time.

As shown in Table 1, trails of intact caterpillars extracted in water were less effective in eliciting trail following than were those extracted in hexanes, indicating that the water-soluble component of the trail is the more important. In contrast to their response to intact trail sections, caterpillars turned back repeatedly and advanced slowly over the extracted sections. Although wetting of *Archips*' silk causes it to shrink markedly in length (Fitzgerald et al., 1991), *Archips*' trails placed briefly in water-saturated air to induce contraction of the silk were no less effective in eliciting trail following than trails with intact silk. This indicates that a water-induced physical change in the strands does not reduce the effectiveness of water-washed trails. Table 1 also shows that sections cut from strips previously traversed by caterpillars with sealed spinnerets were no more effective in eliciting trail following than blank control sections, indicating that markers are not secreted from parts of the body other than the spinneret.

There was no significant difference in the trail type selected when caterpillars were allowed to choose between aged and new trails in two-choice tests. In the first test, five of 10 caterpillars chose the new trails over the old. In the second test, six of 10 caterpillars chose the new trail. These tests show that silk trails are long lived and lack ephemeral components that might otherwise allow the caterpillars to distinguish short-term differences in trail age.

The response of caterpillars to strips of filter paper treated with extracts prepared from silk, silk glands, and debris is shown in Figure 3. These data show that a component or components of silk or silk glands extracted with polar solvents elicit comparable trail-following responses. The caterpillars showed little response to sections marked with extracts of debris and an inconsistent response to hexane extracts. A Wilcoxon-Mann-Whitney test showed that caterpillars followed trails prepared from the debris significantly more slowly than

TABLE 1. RATE OF TRAVEL OF THIRD-INSTAR *A. cerasivoranus* CATERPILLARS ON PAPER STRIPS

Treatment	*N*	Travel, cm/sec (mean ± SE)[a]
Strips crossed by intact caterpillars		
Nonextracted	10	0.34 ± 0.057a
Hexane extracted	10	0.25 ± 0.044a
Water extracted	10	0.09 ± 0.048b
Strips crossed by caterpillars with sealed spinnerets	10	0.01 ± 0.003b
Blank strips	10	0.01 ± 0.011b

[a]Treatments followed by different letter are significantly different ($P < 0.05$, Wilcoxon-Mann-Whitney test).

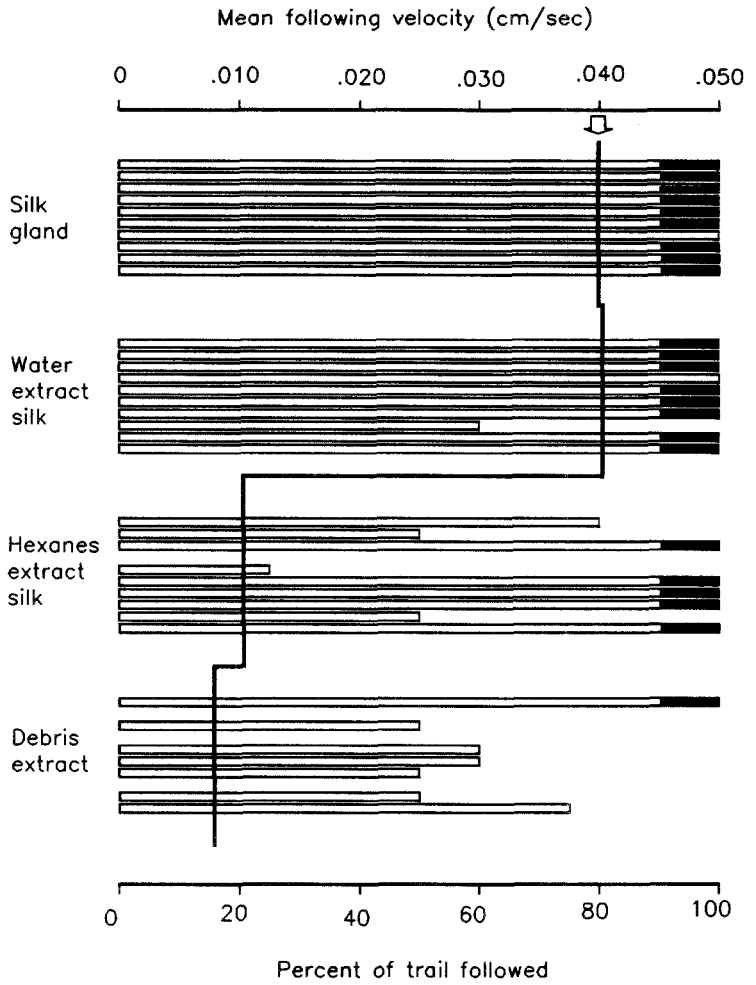

FIG. 3. Movement of third-instar *Archips cerasivoranus* caterpillars on paper strips marked with various extracts. Bars indicate percentage of trail followed during 180-sec tests by an individual caterpillar. Filled tip of bar indicates that the caterpillar turned back after reaching the end of the marked section of the strip. Line indicates average rate of travel for treatment group.

those prepared from silk or gland extract ($P < 0.05$). There was no significant difference in the rate of movement on debris-extract trails and trails prepared from hexane extracts. In all cases the caterpillars showed marked reluctance to advance over the strips prepared from extracts compared to their response to

intact trails. Comparison of these data with those presented above shows that extracts are, at best, only about half as effective in eliciting trail following as extracted silk strands and an order of magnitude less effective than intact trails. Possible reasons for this are discussed below.

DISCUSSION

The use of pheromones to mark trails or foraging arenas appears to be widespread among caterpillars, but only a handful of species have been studied to date (Fitzgerald and Peterson, 1988; Fitzgerald, 1993). The source of caterpillar pheromones has been identified or tentatively identified in only six cases, including that reported here. In tent caterpillars (*Malacosoma*), a trail and recruitment marker is secreted from the sternum at the tip of the abdomen (Fitzgerald, 1976; Fitzgerald and Edgerly, 1982; Peterson, 1988). Although they cover their trails with a dense layer of silk, silk in itself does not elicit trail following in tent caterpillars (Fitzgerald and Edgerly, 1979; Fitzgerald and Costa, 1986), but the caterpillars readily follow artificial trails consisting solely of synthetic pheromone (Peterson and Fitzgerald, 1991). The European birch tent caterpillar, *Eriogaster lanestris*, has also been shown to use a trail pheromone (Weyh and Maschwitz, 1978). The source of its pheromone has not been determined, but the gregarious caterpillar is related to *Malacosoma* and the larvae may also secrete the pheromone independently of their silk. In all other cases reported, the pheromone has been shown to be a component of the silk.

Silk spinning is a ubiquitous habit among caterpillars, and it is reasonable to suspect that chemical components of silk would serve as pheromones. Weyh and Maschwitz (1982) showed that silk trails of the swallowtail caterpillar, *Iphiclides podalirius*, were markedly less effective in eliciting trail following after they were rinsed in acetone. Roessingh (1990), however, correctly pointed out that some critical physical property of the trail might be altered by the extractive process and that this, rather than extraction of a pheromone, could account for the loss of its effectiveness. Less equivocal evidence for the existence of a silk-borne pheromone was provided by Roessingh (1990) for *Yponomeuta cagnagella* and by Capinera (1980) for the range caterpillar *Hemileuca oliviae*. These researchers demonstrated that not only was activity lost when trails were washed, but they also showed that artificial trails made with extracts of the silk trail elicited trail following. Although none of these researchers tested silk pulled directly from the spinneret to preclude the possibility that the pheromonal component of the silk trail was actually a contaminant from another part of the body, Roessingh (1990) showed that extracts of the abdomen, head, and prothoracic organ failed to yield a pheromone. Furthermore, both Capinera (1980) and

Roessingh (1990) demonstrated that artificial trails made from extracts of the silk glands elicited trail following in the species they studied.

Roessingh (1990) found that trails of *Y. cagnagella* extracted in hexanes were less affected by the process than were trails washed in water. Thus, his results are similar to those reported here for *A. cerasivoranus*: the major chemical stimulus for trail following in both species appears to be a water-soluble component of silk. *Y. cagnagella* has been established in North America (Hoebeke, 1987) and colonies are common in Cortland County. Following the publication of Roessingh's (1990) study, I carried out Y-maze studies, involving 10 caterpillars of each species, to assess the response of *A. cerasivoranus* and *Y. cagnagella* to each other's silk trails. In all but one case, both species selected their own trails over those of the other species, indicating that the caterpillars are not responding to common components of caterpillar silk. Furthermore, Roessingh (1989, 1990) found that while-*Y. cagnagella* showed no preference for its own trails over those of five congeneric species, it did prefer its trails to those of the European lackey moth caterpillar, *Malacosoma neustrium*. Fitzgerald and Edgerly (1979) also showed that when allowed to choose between their trail and the trails of the forest and eastern tent caterpillars (*M. disstria* and *M. americanum*), *A. cerasivoranus* larvae selected their own trails. Thus, while Roessingh's studies of *Yponomeuta* and previous studies of *Malacosoma* (Crump et al., 1987) show that congeners may respond to the same trail stimuli, more distantly related species of caterpillars appear to respond to different stimuli.

The results of the present study show that trails prepared from silk or gland extracts are markedly less effective in eliciting trail following than intact trails. Capinera (1980) and Roessingh (1990) reported similar results for the silk and gland extracts of the species they studied. There are several possible reasons for this discrepancy. Although the sensory basis for trail following has not been determined for either *Hemileuca* or *Archips*, the caterpillars are likely to employ contact chemoreceptors and sense only the surface deposit, as has been shown for *Yponomeuta* and *Malacosoma* (Roessingh et al., 1988). Thus, dilution due to absorption by the card substrates to which the extracts were applied might account for part of the observed loss in effectiveness. Roessingh (1990), however, used Y mazes constructed from nonporous metal and still found marked reduction in the response to extracts as compared to intact trails, even when the extracts were applied at rates nearly 10 times greater than those used in the study reported here. Another possibility is that extraction was inefficient or caused alteration of the pheromone. Finally, for these caterpillars, both the physical and chemical properties of silk may be required to elicit the full trail-following response. Roessingh (1989) showed that the head of *Y. cagnagella* bears mechanoreceptive setae and that the caterpillar is responsive to the physical properties of its silk. When allowed to choose between a bare substrate and one

covered with the silk of other species of caterpillars or even cotton fibers, *Y. cagnagella* caterpillars choose the covered substrates. In the present study, *A. cerasivoranus* caterpillars also followed extracted silk trails, although much less readily than intact trails. Extracted silk trails, however, can not be restored to their former effectiveness simply by reapplying extracts to them.

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REFERENCES

CAPINERA, J.L. 1980. A trail pheromone from the silk produced by larvae of the range caterpillar *Hemileuca oliviae* (Lepidoptera: Saturniidae) and observations on aggregation behavior. *J. Chem. Ecol.* 3:655–644.

CRUMP, D., SILVERSTEIN, R.M., WILLIAMS, H.J., and FITZGERALD, T.D. 1987. Identification of the trail pheromone of the 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* 194:961–993.

FITZGERALD, T.D. 1993. Sociality in caterpillars, pp. 372–403, *in* N.E. Stamp and T.M. Casey (eds.). Caterpillars: Ecological and Evolutionary Constraints on Foraging. Chapman Hall, New York.

FITZGERALD, T.D., and COSTA, J.T. 1986. Trail-based communication and foraging behavior of young colonies of the forest tent caterpillar *Malacosoma disstria* Hubn. (Lepidoptera: Lasiocampidae). *Ann. Entomol. Soc. Am.* 79:999–1007.

FITZGERALD, T.D., and EDGERLY, J.S. 1979. Specificity of trail markers of forest and eastern tent caterpillars. *J. Chem. Ecol.* 5:564–574.

FITZGERALD, T.D., and EDGERLY, J.S. 1982. Site of secretion of the trail marker of the eastern tent caterpillar. *J. Chem. Ecol.* 8:31–39.

FITZGERALD, T.D., and PETERSON, S.C. 1983. Elective recruitment communication by the eastern tent caterpillar (*Malacosoma americanum*). *Anim. Behav.* 31:417–442.

FITZGERALD, T.D., and PETERSON, S.C. 1988. Cooperative foraging and communication in caterpillars. *Bioscience* 38:20–25.

FITZGERALD, T.D., CLARK, K.L., VANDERPOOL, R., and PHILIPS, C. 1991. Leaf shelter-building caterpillars harness forces generated by axial retraction of stretched and wetted silk. *J. Insect Behav.* 4:21–32.

HOEBEKE, R.E. 1987. *Yponomeuta cagnagella* (Lepidoptera: Yponomeutidae): A palearctic ermine moth in the United States, with notes on its recognition, seasonal history, and habits. *Ann. Entomol. Soc. Am.* 80:462–467.

JOHNSON, W.T., and LYON, H.H. 1976. Insects that Feed on Trees and Shrubs. Comstock Publishing, Cornell University Press, Ithaca, New York.

PETERSON, S.C. 1988. Chemical trail marking and following by caterpillar of *Malacosoma neustria*. *J. Chem. Ecol.* 14:815–824.

PETERSON, S.C., and FITZGERALD, T.D. 1991. Chemoorientation of eastern tent caterpillars to trail pheromone 5β-cholestane-3,24-dione. *J. Chem. Ecol.* 17(10):1963–1972.

ROESSINGH, P. 1989. The trail following behaviour of *Yponomeuta cagnagellus*. *Entomol. Exp. Appl.* 51:49–57.

ROESSINGH, P. 1990. Chemical trail marker from silk of *Yponomeuta cagnagellus*. *J. Chem. Ecol.* 16:2203–2216.

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.

SOKAL, R.R., and ROHLF, F.J. 1969. Biometry. W.H. Freeman, San Francisco.

WEYH, R., and MASCHWITZ, U. 1978. Trail substance in larvae of *Eriogaster lanestris* L. *Naturwissenshaften* 65:64.

WEYH, R., and MASCHWITZ, U. 1982. Individual trail marking by larvae of the scarce swallowtail *Iphiclides podalirius* L. (Lepidoptera: Papilionidae). *Oecologia* 52:415–416.

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PHENOLIC COMPOUNDS FROM MALE CASTOREUM OF THE NORTH AMERICAN BEAVER, *Castor canadensis*

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Abstract—North American beaver (*Castor canadensis*) mark their territories with castoreum, the contents of their castor sacs. In their territories, beaver respond with scent marking to experimental scent marks consisting of castoreum, or selected single components. In part, the unique odor of castoreum is due to large amounts of phenolic compounds. Purified phenolic components were analyzed by GC, GC-MS, and NMR; identifications were confirmed by comparing the spectra of synthetic phenols with those of the isolated phenols. Of the 15 phenols reported elsewhere, only five were confirmed in our analysis; the other 10 phenolic compounds are either absent or are not volatile enough to be detected by our methods. In addition, 10 phenolic compounds have been identified in this study that were not reported in the previous papers concerning the constitution of castoreum.

Key Words—*Castor canadensis*, beaver castor sac, phenolic compounds, coinjection, fractionation, synthesis, identification, territory marking.

INTRODUCTION

Castoreum is a strong smelling brown paste contained in the castor sacs of both the Old and New World beaver (*Castor fiber* and *Castor canadensis*, respectively). Used since the time of ancient Greece for medicinal purposes, the behavioral role of castoreum in the ecology and sociobiology of the North American beaver has received renewed attention (Hodgdon, 1978; Bollinger, 1980; Svend-

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sen, 1980; Müller-Schwarze and Heckman, 1980; Bollinger et al., 1983). Beaver place mud piles at the banks of their ponds and apply castoreum to these "scent mounds." These scent marks have territorial significance, as shown in the following two types of experiments. In occupied territories, resident adult beaver will destroy a strange scent mark (Müller-Schwarze and Heckman, 1980; Svendsen, 1980). At vacant beaver sites, experimental scenting with castoreum reduces the probability that new beaver will immigrate and occupy a given site (Welsh and Müller-Schwarze, 1989).

By building dams, lodges, and canals, by flooding, and by clear-cutting, beaver arguably invest more heavily in habitat modification than any other nonhuman mammal. They are strongly territorial and defend this investment yearround. Scent communication is very important in beaver; they lack long-distance acoustic communication and, as a primarily nocturnal species, cannot rely on visual communication.

Castor canadensis is an ideal model mammalian species for bioassaying pheromones because chemical cues can be presented in the field to an intact family unit in their natural context. The animals can be observed directly before and during dusk and are present on the same site throughout the scent-marking season.

The constitution of castoreum has been investigated several times. Lederer (1946, 1949) first identified 15 phenols (including salicylaldehyde and *p*-hydroxyacetophenone) and about 25 other compounds from castoreum. It is not clear which of the two beaver species Lederer worked with. He mentioned "Canadian beaver (*Castor fiber*)." The castoreum was most likely from North American beaver, as it was traded commercially at that time, while the European beaver was rare. Valenta and Khaleque (1959) determined the structure of castoramine and Valenta et al. (1960) separated *cis*-cyclohexane-1,2-diol from the beaver castor sac. Other nitrogen-containing compounds were identified by Maurer and Ohloff (1976). Many C_{15}–C_{22} carboxylic acids and their waxy esters from the anal gland secretion were characterized by Grønneberg (1978) and Grønneberg and Lie (1984) using thin-layer chromatography (TLC), gas chromatography (GC), and mass spectroscopy (MS). No further work concerning phenols in castoreum has been reported.

We recently reinvestigated the volatile phenolic compounds extracted from male castoreum. The extracted compounds were isolated by packed column GC fractionation and analyzed by capillary GC, gas chromatography–mass spectrometry (GC-MS), capillary GC coinjection, nuclear magnetic resonance (NMR) spectrometry, and by comparing the spectra of the unknown components with those of authentic samples. Using the above-mentioned methods, 10 previously unreported phenols were identified from castoreum.

METHODS AND MATERIALS

Chemicals. The solvents used for extraction were reagent grade. Solvents used for capillary GC were HPLC grade. Dry toluene was distilled immediately before use. Anhydrous ether was used from a freshly opened bottle. All reagents were purchased from Aldrich Chemical Company.

Instrumentation. Analytical capillary GC was performed on a Varian 3700 gas chromatograph equipped with a splitless injector, fused silica column, and a flame ionization detector (FID). Standard capillary GC conditions for all identification work used the following temperature program: 40°C (3 min), 3°C/min, 210°C (30 min). Carrier and makeup gases for capillary GC work were helium. Capillary GC analyses were carried out on one of three columns: column A, DB-1 column, 30 m × 0.25 mm; column B, FFAP column, 50 m × 0.25 mm; or column C, DB-WAX column, 60 m × 0.25 mm.

Capillary GC coinjection was conducted by taking 0.5 ml of a fraction (5–10 ng/μl) and adding 1 μg of a standard to it; 2 μl of this mixture were injected onto the capillary GC. For those unknown compounds that were identified only by GC-MS and coinjection (i.e., no NMR data), the samples were coinjected with the same standards on two or more capillary GC columns (always at least one polar column and one nonpolar column were used).

Semipreparative-scale packed-column GC fractionations were performed on a Varian 2700 aerograph instrument. All GC fractions were collected in glass capillary tubes (30 cm × 3 mm) using a Brownlee-Silverstein thermal gradient collector (Brownlee and Silverstein, 1968). The purity of the isolated fractions was checked by capillary GC on column A. The pure fractions were then characterized by GC-MS and NMR. The following packed columns were used to purify the crude extract: column D, OV-101 (5%) on Chromosorb W, 3 m × 10 mm OD; column E, Carbowax-20 M (4%) on Chromosorb G, 6, 3, 2, and 1 m × 6 mm OD. The length of column used was determined by the volatility of the particular components in the fraction; for less volatile components, a shorter column was used.

For low-boiling fractions that were collected using column D, the following temperature program was used: 75°C (0 min), 2°C/min, 210°C (10 min). For high-boiling fractions using column D, a different program was used: 100°C (0 min), 4°C/min, 230°C (5 min). All GC collections using column E were collected using the following conditions: 100°C (0 min), 8°C/min, 220°C (10 min).

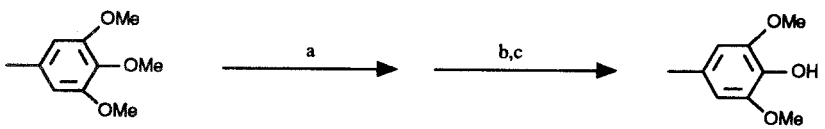
Mass spectra were obtained using a Finnigan 4500 automated gas chromatography/EI-CI mass spectrometer system (GC-MS). All spectra were obtained at 70 eV. Proton NMR (^{1}H) spectra were obtained using one of the following instruments: a Varian EM-360 (60 MHz), a Varian XL-100 (100

MHz), or a General Electric GN-500 (500 MHz). All spectra were run in $CDCl_3$ using the residual $CHCl_3$ protons as an internal reference. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were obtained using the General Electric GN-500 instrument at 125 MHz in $CDCl_3$. All chemical shifts are given in parts per million relative to tetramethylsilane.

Isolation and Identification. Sixteen frozen male castor sacs (970 g) were blended in a Waring Laboratory Blender for 2 min in methylene chloride (500 ml). The homogenate was filtered through a large Büchner funnel and the solvent was removed by fractional distillation through a Vigreux column (17.5 cm). The residue was treated with dilute sulfuric acid (200 ml of 2 N) under cooling with an ice bath and extracted with ether (3 × 200 ml) to isolate the neutral, acidic, and phenolic materials. The ether extract was washed with saturated sodium bicarbonate (3 × 150 ml) to remove the acidic compounds. The ether solution was then treated with enough sodium hydroxide to bring the pH to 12 (ca. 200 ml of 20%). The alkaline extract containing the phenols was neutralized with dilute hydrochloric acid (ca. 300 ml of 2 N) and extracted with ether (3 × 100 ml). The final ether solution was dried over sodium sulfate, filtered, and the solvent removed by distillation.

Synthetic Work. 2,6-Dimethoxy-4-methylphenol, which was identified and not commercially available, was synthesized. Enough material was made not only to confirm our identification, but also to enable us to carry out field work.

2,6-Dimethoxy-4-methylphenol (Scheme 1). This method is based on that of Hurd and Winberg (1942) and Jackman et al. (1975) for the selective demethylation of tri-*O*-methylpyrogallol. The methyl Grignard reagent was prepared from methyl iodide (15.8 g, 111 mmol) and magnesium turnings (2.68 g, 110 mmol) in dry ether (50 ml). A solution of 3,4,5-trimethoxytoluene (5.0 g, 27 mmol) in dry toluene (30 ml) was added with stirring to the Grignard reagent and the ether was removed by distillation (toluene was continuously added during the distillation to maintain a constant volume until the boiling point of the distillate reached 100°C). The mixture was heated to reflux for 18 hr, cooled, and acidified with dilute hydrochloric acid. The organic phase was treated with sodium hydroxide (10%). The alkaline solution was neutralized with hydrochloric acid (6 N) and extracted with ether. The phenol **2** was purified by distillation (101–102°C/0.75 mmHg) to yield 2.2 g (12%) of a reddish solid.



1 **2**

SCHEME. 1. (a) CH_3I, Mg, Et_2O; (b) toluene, reflux 18 hr; (c) NaOH, HCl, 12%.

^{1}H NMR (500 MHz, $CDCl_3$): δ 2.28 (s, 1H, ArH), 3.83 (s, 6H, $2OCH_3$), 5.37 (bs, 1H, ArOH), 6.36 (s, 2H, ArH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 27.1, 56.2, 105.5, 128.5, 132.2, 146.5.

RESULTS AND DISCUSSION

We identified fifteen volatile phenols in beaver castoreum. They are shown in Figure 1. Table 1 summarizes the methods used for identification of these phenols. Among these 15 compounds, 10 phenol structures were suggested by preliminary GC-MS study and confirmed by coinjection of authentic standards on nonpolar column A and either polar column B or C with the exception of catechol, whose retention time on a polar column is very long. Nine phenols were isolated on packed GC columns. Table 2 lists the retention times of these phenols. The structure of 2,6-dimethoxy-4-methylphenol was confirmed by synthesis because this compound is not commercially available. The ^{1}H and ^{13}C

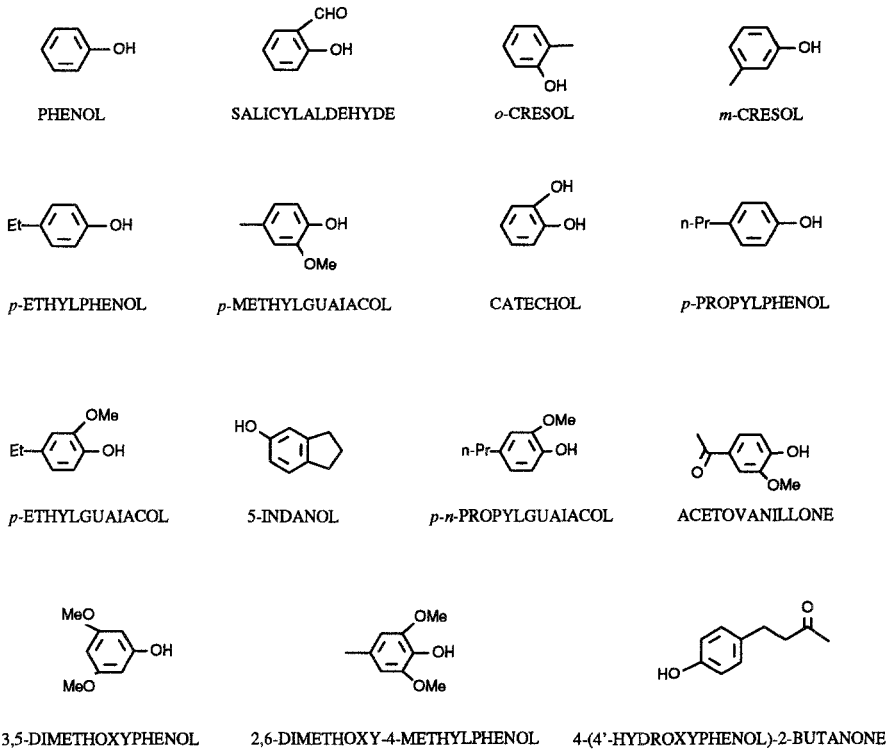


FIG. 1. Phenols from castoreum of beaver (*Castor canadensis*).

TABLE I. METHODS USED FOR CHARACTERIZATION OF PHENOLS FROM CASTOREUM

Name	Retention times (min)			Confirmed by		
	DB-1	DB-Wax	FFAP	GC-MS	^{1}H NMR	Synthesis
Phenol	8.60				X	
Salicylaldehyde	9.94	61.7	35.6	X		
o-Cresol	11.4	51.8		X		
m-Cresol	12.5	55.2		X		
p-Ethylphenol	16.6	53.1		X		
p-Methylguaiacol	17.0	50.2		X	X	
Catechol	18.7			X		
p-Propylphenol	20.9				X	
p-Ethylguaiacol	21.0				X	
5-Indanol	24.1	70.6		X		
p-*n*-Propylguaiacol	24.5				X	
Acetovanillone	29.1		70.0	X		
3,5-Dimethoxyphenol	29.3		85.5	X		
2,6-Dimethoxy-4-methylphenol	28.4				X	X
4-(4′-Hydroxyphenol)-2-butanone	33.3		97.7	X		

TABLE 2. RETENTION TIMES OF PHENOLS FROM CASTOREUM ON PACKED COLUMNS USED FOR ISOLATION

Name	Retention time (min)	
	Column D[a]	Column E[b]
Phenol	24.8	20.7
o-Cresol	30.0	
m-Cresol	32.3	
p-Ethylphenol	38.6	31.6
p-Methylguaiacol	42.5	
p-Propylphenol	47.1	
p-Ethyguaiacol	47.6	39.1
p-*n*-Propylguaiacol	53.4	36.3
2,6-Dimethoxy-4-methylphenol	59.2	53.3

[a]Column D: OV-101 (5%) on chromosorb W, 3 m × 6 mm OD; 75°C (0 min), 2°C/min, 210°C (10 min).
[b]Column E: Carbowax-20 M (4%) on chromosorb G, 3, 2, or 1 m × 6 mm OD; 100°C (0 min), 4°C/min, 230°C (5 min).

NMR data of the synthetic compound are in agreement with our isolated compounds. None of the compounds found in this study are new compounds to the chemical literature; thus no further characterization of these compounds was carried out after we were sure of their identity.

Of these 15 compounds, only five had been reported previously as being constituents of castoreum. Surprisingly, of the 15 phenols reported by Lederer as being constituents of castoreum, only five phenols common to both studies were found: salicylaldehyde, pyrocatechol, 4-ethylphenol, 4-propylphenol, and 4-ethylguaiacol. The other 10 phenols reported by Lederer were not found in our phenolic fractions. The seven phenols, hydroquinone, 4-methoxyphenol, 4-methylcatechol, 4-ethylcatechol, 4-allylphenol, *p*-hydroxyacetophenone, and betuligenol may be absent or only present in trace amounts. These differences may be due to the fact that Lederer may have worked with castoreum from the Eurasian beaver (*Castor fiber*). From the sensitivity of the capillary GC and from the total volume of sample, the minimum detectable level of our method of analysis can be deduced. It is about 5 μg/g a gland. The other three phenols reported by Lederer, 2,4′-dihydroxydiphenylmethane, 2′,3′-dihydroxydibenz-2-pyrone, and 4,4′-dihydroxydiphenic acid dilactone, may be present in our sample; however, they are not volatile enough to be detected by our methods. Indeed, in our fractionation work, about one twenty-fifth of the total phenol fraction was a brown viscous substance, some of which accumulated in the head

of the packed column. Because our focus is on the volatiles from castoreum, no further isolation work was undertaken on the high-boiling components.

Our purpose for investigating the phenolic constituents of beaver castoreum is to determine what role these and other compounds (i.e., neutrals and basics) from beaver scent mounds play in chemical communication and behavior. Table 3 gives the gland equivalents (GE) of these phenols; the quantity refers to the amount of each individual phenol per castor gland. The determination of GE was as follows: the most abundant phenol, *p*-ethylguaiacol, was isolated and weighed directly. The gland equivalents of the remaining phenols were obtained by comparing the GC peak areas of the compound to that of *p*-ethylguaiacol. We are now routinely analyzing individual scent mounds and castoreum from individual beaver. Now that a large number of compounds have been identified, we can search for patterns that, for example, are specific to sex, age, class, or season. Revealing such patterns would enable us to answer behavioral, ecological, and evolutionary questions. The results of ongoing field bioassays of natural and synthetic castoreum samples will be reported elsewhere.

The presence of these phenols in castoreum might result from the "recycling" of tree compounds: many are known as secondary plant compounds (or plant defense compounds) from the tree species on which the beaver feeds. Lederer (1949) had remarked on the possible correlation between diet and castoreum composition. The beaver probably sequesters these phenolics and a number of oxygenated monoterpenes (to be reported in a subsequent paper), some

TABLE 3. GLAND EQUIVALENT OF PHENOLS FROM CASTOREUM

Name	Gland equivalent (μg)
Phenol	0.72
Salicylaldehyde	0.32
o-Cresol	0.76
m-Cresol	1.17
p-Ethylphenol	2.24
p-Methylguaiacol	2.59
Catechol	0.64
p-Propylphenol	2.09
p-Ethylguaiacol	28.1
5-Indanol	0.34
p-*n*-Propylguaiacol	0.64
Acetovanillone	0.93
3,5-Dimethoxyphenol	0.18
2,6-Dimethoxy-4-methylphenol	3.22
4-(4′-Hydroxyphenol)-2-butanone	5.41

possibly after modification in the castoreum. Castoreum, in turn, is used in chemical communication, specifically to demarcate a territory.

Field bioassay shows that beaver use several of these phenolics as signal compounds. Applied to artificial mud piles individually or in mixture on the banks of beaver ponds, 4-ethylphenol, 4-methylguaiacol, 1,2-dihydroxybenzene, 4-propylguaiacol, and 4-ethylguaiacol released significant sniffing responses in beaver. In addition, all but the last one attracted beaver out of the water to the scent mound, and the first three even triggered scent marking.

The results reported here now allow us to measure relative amounts of the phenolics in castoreum. We have analyzed fresh samples of castoreum "milked" from live-trapped, immobilized beaver. The data are being analyzed for sex, age, and seasonal differences.

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REFERENCES

ALLEN, C.F.H., and GATES, J.W., JR. 1955. *o*-Eugenol. *Org. Synth. Coll.* 3:418–419.

AUGUSTINE, R.L. 1965. Catalytic Hydrogenation. Marcel Dekker, New York, p. 57.

BOLLINGER, K.S. 1980. Scent marking behavior of beaver (*Castor canadensis*). MS thesis. University of Massachusetts, Amherst, 186 pp.

BOLLINGER, K.S., HODGDON, H.E., and KENNELLY, J.J. 1983. Factors affecting weight and volume of castor and anal glands of beaver (*Castor canadensis*). *Acta Zool. Fenn.* 174:115–116.

BROWNLEE, R.G., and SILVERSTEIN, R.M. 1968. A micro-preparative gas chromatography and a modified carbon skeleton determinator. *Anal. Chem.* 40:2077–2080.

GRØNNEBERG, T.Ø. 1978. Analysis of a wax ester fraction from the anal gland secretion of beaver (*Castor fiber*) by chemical ionization mass spectrometry. *Chem. Scr.* 13:56–58.

GRØNNEBERG, T.Ø., and LIE, T. 1984. Lipids of the anal gland secretion of beaver (*Castor fiber*). *Chem. Scr.* 24:100–103.

HODGDON, H.E. 1978. Social dynamics and behavior within an unexploited beaver (*Castor canadensis*) population. PhD thesis. University of Massachusetts, Amherst, 292 pp.

HURD, C.D., and WINBERG, H.E. 1942. The behavior of pyrogallol trimethyl ether and 3,4,5-trimethoxynitrile toward Grignard reagents. *J. Am. Chem. Soc.* 64:2085–2086.

JACKMAN, D.A., SARGENT, M.V., and ELIX, J.A. 1975. Structure of the lichen depsidone pannarin. *J. Chem. Soc. Perkin Trans. I* 18:1971–1985.

LEDERER, E. 1946. Chemistry and biochemistry of the scent glands of the beaver (*Castor fiber*). *Nature* 157:231–232.

LEDERER, E. 1949. Chemistry and biochemistry of some mammalian secretions and excretions. *J. Chem. Soc. (London)* 1949:2115–2125.

LEDERER, E. 1949. Castoreum and ambergris. Chemical and biochemical constituents. *Perf. Essent. Oil Rec.* 40:353–359.

MAURER, B., and OHLOFF, G. 1976. Zur Kenntnis der stickstoffhaltigen Inhaltsstoffe von Castoreum. *Helv. Chim. Acta* 59:1169–1185.

MÜLLER-SCHWARZE, D., and HECKMAN, S. 1980. The social role of scent marking in beaver (*Castor canadensis*). *J. Chem. Ecol.* 6:81–95.

MÜLLER-SCHWARZE, D., MOREHOUSE, L., CORRADI, R., CHENG-HUA ZHAO, and SILVERSTEIN, R.M. 1986. Odor images: Responses of beaver to castoreum fractions, Vol. 4. pp. 561–570, in D. Duvall, D. Müller-Schwarze, and R.M. Silverstein (eds.). Chemical Signals in Vertebrates, Plenum, New York.

SVENDSEN, G.E. 1980. Patterns of scent-mounding in a population of beaver (*Castor canadensis*). *J. Chem. Ecol.* 6:133–148.

VALENTA, Z., and KHALEQUE, A. 1959. The structure of castoramine. *Tetrahedron Lett.* 12:1–5.

VALENTA, Z., KHALEQUE, A., and RASHID, M.H. 1960. *cis*-Cyclohexane-1,2-diol in the beaver gland. *Experientia* 17:130.

WELSH, R.G., and MÜLLER-SCHWARZE, D. 1989. Experimental habitat scenting inhibits colonization by beaver, *Castor canadensis*. *J. Chem. Ecol.* 3:887–893.